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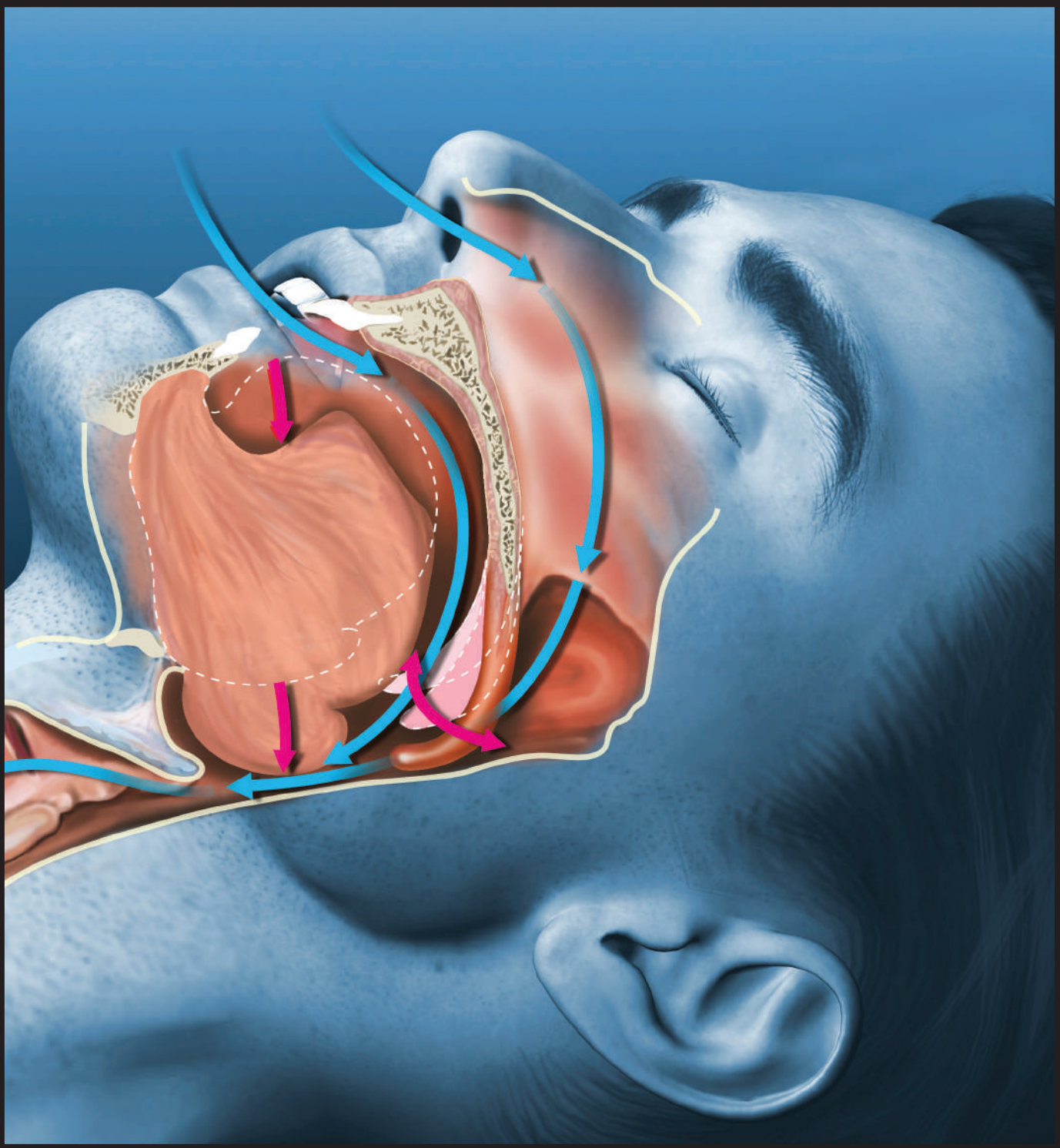

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EDITORIAL

OVERVIEW

SCIENCE

The following are online-only papers that are available as part of Issue 54(1) online.

ON THE COVER: The American Academy of Sleep Medicine estimates that as many as 25 million adults in the United States suffer from obstructive sleep apnea (OSA), a disorder characterized by repetitive interruptions in breathing during sleep. OSA is associated with hypertension, heart disease, type 2 diabetes, stroke, and depression. It has long been known that obesity is a risk factor for OSA, and increasing rates of obesity over the past several decades correlate with the greater incidence of OSA. In this issue of *Laboratory Medicine*, Fadaei and colleagues explore the relationship between angiopoietin-like 3 (ANGPTL3), a hepatokine that has a role in lipid metabolism, and C1q/TNF-related protein 9 (CTRP9), an adiponectin paralog that helps regulate glucose metabolism. The authors suggest that elevated levels of these 2 proteins may contribute to the increased risk for type 2 diabetes and cardiovascular disease in patients with OSA.

EDITORIAL

COP27 Climate Change Conference: Urgent Action Needed for Africa and the World

Wealthy nations must step up support for Africa and vulnerable countries in addressing past, present and future impacts of climate change†

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†This Editorial is being published simultaneously in multiple journals. For the full list of journals see: https://www.bmj.com/content/full-listauthors-and-signatories-climate-emergency-editorial-october-2022

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The 2022 report of the Intergovernmental Panel on Climate Change (IPCC) paints a dark picture of the future of life on earth, characterised by ecosystem collapse, species extinction, and climate hazards such as heatwaves and floods.[1] These are all linked to physical and mental health problems, with direct and indirect consequences of increased morbidity and mortality. To avoid these catastrophic health effects across all regions of the globe, there is broad agreement—as 231 health journals argued together in 2021—that the rise in global temperature must be limited to less than 1.5°C compared with pre-industrial levels.

While the Paris Agreement of 2015 outlines a global action framework that incorporates providing climate finance to developing countries, this support has yet to materialise.[2] COP27 is the fifth Conference of the Parties (COP) to be organised in Africa since its inception in 1995. Ahead of this meeting, we—as health journal editors from across the continent—call for urgent action to ensure it is the COP that finally delivers climate justice for Africa and vulnerable countries. This is essential not just for the health of those countries, but for the health of the whole world.

Africa has Suffered Disproportionately Although it has Done Little to Cause the Crisis

The climate crisis has had an impact on the environmental and social determinants of health across Africa, leading to devastating health effects.[3] Impacts on health can result directly from environmental shocks and indirectly through socially mediated effects..[4] Climate change-related risks in Africa include flooding, drought, heatwaves, reduced food production, and reduced labour productivity.[5]

Droughts in sub-Saharan Africa have tripled between 1970-1979 and 2010-2019.[6] In 2018, devastating cyclones impacted 2.2 million people in Malawi, Mozambique, and Zimbabwe.[6] In west and central Africa, severe flooding resulted in mortality and forced migration from loss of shelter, cultivated land, and livestock.[7] Changes in vector ecology brought about by floods and damage to environmental hygiene have led to increases in diseases across sub-Saharan Africa, with rises in malaria, dengue fever, Lassa fever, Rift Valley fever, Lyme disease, Ebola virus, West Nile virus, and other infections.[8,9] Rising sea levels reduce water quality, leading to water-borne diseases, including diarrhoeal diseases, a leading cause of mortality in Africa.[8] Extreme weather damages water and food supply, increasing food insecurity and malnutrition, which causes 1.7 million deaths annually in Africa.[10] According to the Food and Agriculture Organization of the United Nations, malnutrition has increased by almost 50% since 2012, owing to the central role agriculture plays in African economies.[11] Environmental shocks and their knock-on

effects also cause severe harm to mental health.[12] In all, it is estimated that the climate crisis has destroyed a fifth of the gross domestic product (GDP) of the countries most vulnerable to climate shocks.[13]

The damage to Africa should be of supreme concern to all nations. This is partly for moral reasons. It is highly unjust that the most impacted nations have contributed the least to global cumulative emissions, which are driving the climate crisis and its increasingly severe effects. North America and Europe have contributed 62% of carbon dioxide emissions since the Industrial Revolution, whereas Africa has contributed only 3%.[14]

The Fight Against the Climate Crisis Needs All Hands on Deck

Yet it is not just for moral reasons that all nations should be concerned for Africa. The acute and chronic impacts of the climate crisis create problems like poverty, infectious disease, forced migration, and conflict that spread through globalised systems.[6,15] These knock-on impacts affect all nations. COVID-19 served as a wake-up call to these global dynamics and it is no coincidence that health professionals have been active in identifying and responding to the consequences of growing systemic risks to health. But the lessons of the COVID-19 pandemic should not be limited to pandemic risk.[16,17] Instead, it is imperative that the suffering of frontline nations, including those in Africa, be the core consideration at COP27: in an interconnected world, leaving countries to the mercy of environmental shocks creates instability that has severe consequences for all nations.

The primary focus of climate summits remains to rapidly reduce emissions so that global temperature rises are kept to below 1.5°C. This will limit the harm. But, for Africa and other vulnerable regions, this harm is already severe. Achieving the promised target of providing $100bn of climate finance a year is now globally critical if we are to forestall the systemic risks of leaving societies in crisis. This can be done by ensuring these resources focus on increasing resilience to the existing and inevitable future impacts of the climate crisis, as well as on supporting vulnerable nations to reduce their greenhouse gas emissions: a parity of esteem between adaptation and mitigation. These resources should come through grants not loans, and be urgently scaled up before the current review period of 2025. They must put health system resilience at the forefront, as the compounding crises caused by the climate crisis often manifest in acute health problems. Financing adaptation will be more cost effective than relying on disaster relief.

Some progress has been made on adaptation in Africa and around the world, including early warning systems and infrastructure to defend against extremes. But frontline nations are not compensated for impacts from a crisis they did not cause. This is not only unfair, but also drives the spiral of global destabilisation, as nations pour money into responding to disasters, but can no longer afford to pay for greater resilience or to reduce the root problem through emissions reductions. A financing facility for loss and damage must now be introduced, providing additional resources beyond those given for mitigation and adaptation. This must go beyond the failures of COP26 where the suggestion of such a facility was downgraded to "a dialogue."[18]

The climate crisis is a product of global inaction and comes at great cost, not only to disproportionately impacted African countries, but to the whole world. Africa is united with other frontline regions in urging wealthy nations to finally step up, if for no other reason than that the crises in Africa will sooner rather than later spread and engulf all corners of the globe, by which time it may be too late to effectively respond. If so far, they have failed to be persuaded by moral arguments, then hopefully their self-interest will now prevail.

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REFERENCES

1. IPCC. *Climate Change 2022: Impacts, Adaptation and Vulnerability*. Working Group II Contribution to the IPCC Sixth Assessment Report. IPCC; 2022.
2. UN. *The Paris Agreement: United Nations*. UN; 2022. https://www.un.org/en/climatechange/paris-agreement. Accessed September 12, 2022.
3. Kaddu, JB, Gebru B, Kibaya P, Munabi IG. *Climate Change and Health in Sub-Saharan Africa: The Case of Uganda*. Climate Investment Funds; 2020.
4. WHO. *Strengthening Health Resilience to Climate Change*. WHO; 2016.
5. Trisos CH, Adelekan IO, Totin E, et al. Africa. In: Climate Change 2022: Impacts, Adaptation, and Vulnerability. Working Group II Contribution to the IPCC Sixth Assessment Report. IPCC; 2022. https://www.ipcc.ch/report/ar6/wg2/. Accessed September 26, 2022.
6. World Bank. *Climate Change Adaptation and Economic Transformation in Sub-Saharan Africa*. World Bank; 2021.
7. Opoku SK, Leal Filho W, Hubert F, Adejumo O. Climate change and health preparedness in Africa: Analysing trends in six African countries. *Int J Environ Res Public Health.* 2021;18(9):4672. doi:10.3390/ijerph18094672.
8. Evans M, Munslow B. Climate change, health, and conflict in Africa's arc of instability. *Perspect Public Health.* 2021;141(6):338–341. doi:10.1177/17579139211058299.
9. Stawicki, SP, Papadimos TJ, Galwankar SC, Miller AC, Firstenberg MS. Reflections on climate change and public health in Africa in an era of global pandemic. In: *Contemporary Developments and Perspectives in International Health Security*. Vol. 2. Intechopen; 2021.
10. Climate change and health in Africa: Issues and options. African Climate Policy Centre; 2013. https://archive.uneca.org/sites/default/files/PublicationFiles/policy_brief_12_climate_change_and_health_in_africa_issues_and_options.pdf. Accessed September 12, 2022.
11. Climate change is an increasing threat to Africa 2020. https://unfccc.int/news/climate-change-is-an-increasing-threat[-to-africa. Accessed September 12, 2022.

12. Atwoli L, Muhia J, Merali Z. Mental health and climate change in Africa. *BJPsych Int.* 2022:1–4. https://www.cambridge.org/core/journals/bjpsych-international/article/mental-health-and-climate-change-in-africa/65A414598BA1D620F4208A9177EED94B. Accessed September 26, 2022.
13. Vulnerable Twenty Group. *Climate Vulnerable Economies Loss Report*. Vulnerable Twenty Group; 2020.
14. Ritchie H. Who has contributed most to global CO2 emissions? Our World in Data. https://ourworldindata.org/contributed-most-global-co2. Accessed September 12, 2022.
15. Bilotta N, Botti F. *Paving the Way for Greener Central Banks. Current Trends and Future Developments Around the Globe*. Edizioni Nuova Cultura for Istituto Affari Internazionali (IAI); 2022.
16. WHO. *COP26 Special Report on Climate Change and Health: The Health Argument for Climate Action*. WHO; 2021.
17. Al-Mandhari A, Al-Yousfi A, Malkawi M, El-Adawy M. "Our planet, our health": saving lives, promoting health and attaining well-being by protecting the planet – the Eastern Mediterranean perspectives. *East Mediterr Health J.* 2022;28(4):247–248. https://doi.org/10.26719/2022.28.4.247. Accessed September 26, 2022.
18. Evans S, Gabbatiss J, McSweeney R, et al. COP26: Key outcomes agreed at the UN climate talks in Glasgow. Carbon Brief [Internet]. 2021. https://www.carbonbrief.org/cop26-key-outcomes-agreed-at-the-un-climate-talks-in-glasgow/. Accessed September 12, 2022.

OVERVIEW

Blood Donors with Thalassemic Trait, Glucose-6-Phosphate Dehydrogenase Deficiency Trait, and Sickle Cell Trait and Their Blood Products: Current Status and Future Perspective

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Keywords: donor, thalassemia, G6PD, sickle cell, microparticle, blood product

Abbreviations: G6PD, glucose-6-phosphate dehydrogenase; SCT, sickle cell trait; RBC, red blood cells; PC, platelet concentrate; FFP, fresh frozen plasma; CPP, cryoprecipitate; Hb, hemoglobin; HbH, hemoglobin H; HbE, hemoglobin E; MCV, mean corpuscular volume; RBCs, red blood cells; CBC, complete blood count; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; PLT, platelets; MP, microparticle; RMP, red blood cell-derived microparticle; PMP, platelet-derived microparticle; LMP, leukocyte-derived microparticle; EMP, endothelial-derived microparticle; PS, phosphatidylserine; ROS, reactive oxygen species; 2,3-DPG, 2,3-bisphosphoglycerate; HbS, sickle hemoglobin.

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ABSTRACT

The use of blood products for different medical purposes has increased in recent years. To meet increasing demand, some blood centers allow volunteer donors with thalassemic trait, glucose-6-phosphate dehydrogenase deficiency (G6PD) trait, and sickle cell trait (SCT) to donate blood if their hemoglobin values fall within acceptable ranges and show no signs of hemolysis. Currently, there are no standard guidelines or policies regarding the use or management of blood products obtained from these donors. However, in recent years, there has been advanced research on eligible donors who have these underlying conditions. In this review, we summarize the current knowledge from in vitro and in vivo studies regarding donor characteristics, changes in physical and biochemical parameters in blood products during processing and storage, and posttransfusion efficacy of blood products. In addition, we discuss some unresolved issues concerning blood products from thalassemic trait, G6PD-deficiency trait, and SCT donors.

Blood components are essential treatments for several diseases, including bleeding disorders and cancers and for patients with massive blood loss due to surgery or injury.[1,2] Typically, there are 4 types of transfusable blood products: red blood cells, platelet concentrate (PC), fresh frozen plasma (FFP), and cryoprecipitate (CPP).[3] As recommended by international guidelines, transfusion laboratories prepare blood products from healthy volunteer donors who are allowed to donate blood after assessment of their health status and medical history and completion of a questionnaire about health and risks.[4] Individuals with glucose-6-phosphate dehydrogenase (G6PD) deficiency trait, thalassemic trait, and sickle cell trait (SCT) are eligible to donate blood if their hemoglobin levels are within the ranges recommended by the international guideline and they show no signs of hemolysis. Research shows the prevalence of thalassemia traits and G6PD deficiency trait in Thai blood donors to be 21.1% and 7.7%, respectively.[5] In Malaysia, screening blood samples in blood centers showed that 16.25% of donors carried thalassemic trait, with 38.5% having α-thalassemia/hemoglobin E (HbE) and 30.8% having β-thalassemia.[6] An epidemiological study conducted in Saudi Arabia reported that 2% and 0.78% of blood donors had SCT and G6PD-deficiency trait, respectively.[7] Taken together, these findings indicate the potential effects on routine blood banking of blood donors with underlying thalassemic trait, G6PD deficiency trait, and SCT. Given the heterogeneity of blood donors, awareness of this issue by medical professionals working in blood centers is important to provide safe, adapted blood products for patients and to maintain a pool of volunteer donors.

Recent years have seen significant advances in research on blood donors and blood products obtained from eligible volunteer donors with underlying conditions. Here, we have summarized recent data on blood products prepared from eligible donors carrying G6PD-deficency trait, SCT, and thalassemic trait. In this review, we outline the physical and biochemical changes in blood products obtained from healthy volunteers and donors with thalassemic trait, G6PD deficiency trait, and SCT. We then discuss the areas in which research is still needed to improve our knowledge regarding blood donors with these underlying conditions.

Thalassemia is a heterogeneous group of genetic disorders characterized by absent or decreased production of the α- or β-chains of hemoglobin.[8–10] Silent carriers of α-thalassemia show Hb values and mean corpuscular volume (MCV) values like those within the range used to indicate healthy individuals. β-thalassemia has varying degrees of

severity, including (1) severe anemia, which requires blood transfusion to maintain Hb levels; (2) intermedia, which shows mild or moderate anemia; and (3) asymptomatic, which consists of mild hypochromic microcytic anemia without clinical complications.[11] G6PD deficiency is another genetic disorder that occurs in many regions of the world.[12,13] This disease is characterized by decreased or absent activity of the G6PD enzyme, which is an important antioxidant system in RBCs. G6PD deficiency also varies in its degree of severity from severe deficiency, with G6PD activity <1%, to normal to G6PD activity 60%–150%.[14] Sickle cell disease, an inherited autosomal disorder, is characterized by the presence of crescent-shape RBCs in the peripheral blood circulation.[15] However, heterozygous sickle cell disease shows no sign of the previously described pathologies. Given the varying severity of these diseases, individuals with the thalassemic trait, G6PD-deficiency trait, or SCT who show no signs or symptoms of anemia or hemolysis are allowed to donate blood for transfusion in some countries that have a high prevalence of these diseases.[6,16–18]

In transfusion laboratories, the preparation of blood products requires several processes.[3,19] After the volunteer donors give informed consent and pass the Hb screening test, whole blood units are collected into bags containing anticoagulants. Following that, whole blood units are centrifuged many times to prepare RBCs and buffy coat-removed PC. Next, the remaining plasma is prepared as FFP and CPP. Blood units can also be subjected to leukoreduction by filtration to minimize adverse reactions in their recipients. After preparation, the blood products might be stored and their quality maintained and sent to recipients upon request. Several changes have been reported during the collection and processing of blood units obtained from donors with thalassemic trait, G6PD deficiency trait, and SCT.

Hematological Parameters of Donors with Thalassemic Trait, G6PD Deficiency Trait, and SCT

A previous study evaluated complete blood counts (CBCs) in donors with thalassemic trait and found that Hb, MCV, and mean corpuscular hemoglobin (MCH) levels were reduced.[5] Noulsri et al[20] compared CBC values in 70 thalassemic trait donors and 255 healthy volunteer donors and found lower levels of RBC, Hb, MCV, and MCH values in thalassemic trait donors than in nonthalassemic donors. Hb typing analysis characterized these thalassemic donors as α-thalassemia trait, HbE, β-thalassemia trait, hemoglobin constant spring, and other Hb variants. Although there were differences in CBC values between donors with and without thalassemia, the Hb levels in thalassemic donors were within the acceptable range for donating blood as recommended by the guidelines. In addition, evaluation of the RBC indices in eligible volunteer blood donors in Malaysia showed that 8.9% of donors with normal Hb levels had thalassemia and 36.7% of those with suspected thalassemia or other underlying conditions had MCH levels of less than 27 pg.[21]

Regarding donors with G6PD-deficiency trait, examination of their RBC indices found no significant difference between them and individuals with normal G6PD activity.[5] Furthermore, evaluation of RBC parameters in Italian donors showed that their hematological parameters were within normal ranges.[22] However, the G6PD-deficiency trait donors had higher MCV values but lower RBC counts, Hb levels, and MCHC values when healthy volunteers and G6PD-deficiency trait donors were compared. A later study by Noulsri et al[23] evaluated 49 G6PD-deficient donors and found that among the CBC parameters, only their MCV levels were significantly increased compared to the controls. An investigation of hematological profiles in 6 G6PD-deficient volunteers revealed higher MCV levels when compared to controls.[24] This discrepancy might be explained by variations in the pathophysiology and severity of G6PD deficiency, as previously documented.[12]

For donors with SCT, evaluation of hematochemical and biochemical properties in donor blood found that RBC indices in SCT donors were similar to those in individuals without sickle cells.[25] In addition, another study by Alabdulaali et al[7] examined 1150 blood samples from donors with SCT and G6PD deficiency and found that their Hb levels, RBC counts, MCV, MCH, and RBC distribution width (RDW) values were within normal ranges.

Alterations in Blood Products Obtained from Thalassemic Trait, G6PD-Deficiency Trait, and SCT Donors

Morphological and Physical Changes

Morphological transformation of RBCs and platelets (PLTs) has been observed during the processing of blood products. In physiological terms, RBCs have a biconcave structure that is highly deformable and has an abundant surface area. These properties help RBCs reform while traveling through various sizes of capillaries and endothelial slits in the red pulp of the spleen. During storage with preservative solution, the discocyte RBCs in PRBCs are time-dependently transformed into echinocytes, spheroechinocytes, and spherocytes.[26,27]

Regarding PLTs in PC units, resting PLTs are discoid shaped and have an average diameter of 1.5–3 μm. When activated or in the later days of storage, PLTs change shape to a spherical form to release secretory granules, which results in decreased mean platelet volume. Changes in PLT shape have also been reported in PC units during storage under blood bank conditions (22°C ± 2°C).[3] A cross-sectional study of the platelet-rich plasma PC, buffy coat PC, and apheresis PC during storage found that apheresis PC had higher morphological scores but lower soluble P-selectin levels than other PLT products.[28] These morphological shifts are important because they can lead to the release of cell-derived microparticles (MPs), the levels of which are related to the quality of blood products.[29,30] MPs are heterogeneous groups of small vesicles with an average size of 0.1 to 1 μm that are released into the circulation when the cells are under stress, in apoptosis, or activated by any of several agonists. The released pool of extracellular vesicles might also contain small exosomes (<0.15 μm) or apoptotic bodies (>1 μm).[31–33] MPs are derived from several cells, including RBCs (called RMPs), leukocytes (called LMPs), platelets (called PMPs), and vascular endothelial cells (called EMPs). The formation and release of MPs are regulated by various cellular processes and are initiated by an influx of calcium (Ca^{2+}) into the cells' cytoplasm. The increased intracellular Ca^{2+} levels further activate calpain, a protease sensitive to Ca^{2+}, which results in the cleavage of actin filaments and remodeling of the cytoskeleton. In addition, elevated intracellular Ca^{2+} induces changes in the phospholipids expressed on the surfaces of cell membranes. Under normal physiological circumstances, the asymmetrical distribution of phospholipid is regulated by flippase, floppase, and scramblase. Floppase enables the lipid to move into the inner leaflet of the membrane, and floppase inactivation results in the increased expression of phosphatidylserine (PS) on the outer leaflet of the membrane. Released MPs also carry biochemical

compounds and molecules from their original cells, which can help characterize their origin. Furthermore, the shed MPs also exhibit procoagulant activity due to the externalization of PS and the expression of tissue factor.[34,35] These MP characteristics support the finding that transfusion of blood products containing high MP levels is associated with posttransfusion complications in recipients, including thrombosis and transfusion-related acute lung injury.[36,37] Regarding elevated MPs in blood products, several studies have found that donor variability, product processing, and storage duration contribute to changes in their levels.[38–40] Alterations in MPs have also been reported during the preparation and storage of blood products obtained from donors with thalassemic trait, G6PD deficiency trait, and SCT.

One study used flow cytometry to determine the MP levels in blood products prepared from 70 thalassemic trait donors.[20] It reported that MP concentrations in unprocessed whole blood units obtained from thalassemic trait donors were lower than in those obtained from healthy volunteer donors. In addition, both donor groups demonstrated increased MP concentrations in the RBC units and PCs. A recent study examined the effects of storage duration on RMP levels in RBC units from thalassemic trait and nonthalassemic donors.[41] In RBC units stored for 38 days, it found that both groups had similar RMP concentrations and a trend of positive association between RMP concentration and the duration of storage. Collectively, these data suggest that the thalassemic trait status of eligible blood donors has little influence on MP levels during the processing and storage of blood products.

Regarding G6PD-deficiency, Nantakomol et al[42] analyzed circulating plasma MPs in individuals with varying G6PD activity. Their results demonstrated that G6PD-deficient individuals had higher MP levels than those with normal G6PD activity and that the concentration of MPs was negatively correlated with G6PD activity. A subsequent study by Noulsri et al[23] determined MP concentrations in 49 volunteer blood donors with G6PD deficiency. The results found that RBC units from G6PD-deficient donors had higher concentrations of RMP than those from healthy volunteers. However, G6PD-deficient donors had lower concentrations than healthy donors of the following: PMP in unprocessed whole blood and PC, LMPs in unprocessed whole blood and plasma, and total MPs in PC. This indicates the influence of donor G6PD status on changes in RMPs during the processing of blood products. A morphological examination also demonstrated a lower number of sphero-echinocytes and other types of irreversibly modified RBCs in RBC units from G6PD-deficient donors compared to control from days 28–42 of storage.[24] Additionally, the percentage of PS-exposed RBCs in G6PD-deficient donors was greater than in controls. In addition to RBC units, examination of FFP obtained from G6PD-deficient donors found more PS-exposing MPs with high procoagulant activity than in FFP obtained from controls with normal G6PD activity.[43]

Focusing on SCT trait donors, several changes were found in blood units during the first week of storage, including declines in total white blood cell and RBC counts, as well as Hb and hematocrit levels.[25] A review report by Schuetz et al[45] has suggested that temperature, pH, osmolality, types of anticoagulants used, and storage duration all contributed to changes in the quality of blood units received from donors with SCT after leukoreduction by filtration.[44] Another study aimed to determine the effect of leukofiltration on RBC units obtained from donors with the SCT. Its results found high residual white blood cell counts in about 30% of RBC units with prolonged and slow flow-filtration times. It also found postfiltration hemolysis in RBC units with high residual white blood cell counts, suggesting the potential effect of leukofiltration on the quality of blood products obtained from donors with the SCT. A later in vitro study by Stroncek et al[46] suggested that citrate anticoagulant and low O_2 saturation induced polymerization of sickle hemoglobin (HbS) as a result of failure of leukoreduction in blood units prepared from donors with SCT. An enormous amount of research focusing on problems with leukocyte filtration in donors with SCT has attempted to resolve this issue. One study evaluated the use of citrate anticoagulant added to blood collection instruments to minimize occlusion by leukofiltration in RBC units obtained from individuals with the SCT.[47] The results showed that RBC units with citrate had better filtration performance, with RBC recovery of 85% and residual white blood cell counts of less than 5×10^6 after storage at 1°C to 6°C for 24 h. Another study aimed to assess the efficacy of a blood collection device including citrate anticoagulant in reducing the detrimental effects of acidic and hyperosmotic citrate anticoagulant solutions on the leukoreduction and storage of SCT donor whole blood units.[48] In line with that study, another study reported that adding air to the bags improved the filtration efficiency of RBC units obtained from donors with the SCT.[49] Collectively, these findings indicate that leukoreduction filters are ineffective with RBCs from donors with the SCT.

Biochemical Changes

Alterations in biochemical parameters have been documented during the preparation and storage of RBC units from donors with thalassemia traits, G6PD-deficiency trait, and the SCT. A recent study by Anastasiadi et al[50] examined the roles of proteasomes in stored RBC units obtained from donors with the β-thalassemia trait. The results demonstrated the time-dependent translocation and activation status of proteasomes in these RBC units. The study also documented enriched kinase and metabolic enzymes and differentially expressed arginase-1, piezo-1, and phospholipid scramblase. Tzounakas et al[51] examined nitrogen-related metabolism in RBCs obtained from eligible thalassemic donors. Their proteomic analysis results showed upregulation of myosin proteoforms, arginase-1, heat shock proteins, and protein kinases, but downregulation of nitrogen-related transporters. The study also documented an association between MP release and stress hemolysis and proteome control modulation in RBC membranes during storage.

Regarding metabolic changes in RBCs obtained from G6PD-deficient donors, an in vitro study by Reisz et al[52] demonstrated significant changes in fumarate, sulfur metabolism, glutathione homeostasis, and other antioxidant components in G6PD-deficient RBCs when compared to normal G6PD RBCs during storage of RBC units for 42 days. Peters et al[53] used flow cytometry to examine G6PD activity in RBC units during storage and identified a decrease in the mean fluorescent intensity of the intercellular G6PD activity after day 3 of storage and a gradual decline in the level of G6PD activity during storage. Another study performed proteomics and metabolomics analyses on stored RBCs obtained from G6PD-deficient donors.[24] The stored G6PD-deficient RBC units demonstrated levels of endogenous reactive oxygen species (ROS) comparable to those in the controls but significantly higher in G6PD-deficient RBCs treated with diamide or tert-BHP during storage. In addition, proteomic analysis of stored G6PD-deficient RBCs demonstrated higher levels of peroxiredoxin-1 and -2, superoxide dismutase, heat shock proteins, and other proteasome compounds. A further study examined several markers related to oxidative stress in stored RBCs collected from G6PD-deficient individuals and found that stimulation

with oxidants during the storage period increased ROS generation significantly compared to that in control RBCs with normal G6PD activity.[54] Furthermore, analysis of metabolites in the glycolytic and pentose phosphate pathways showed that G6PD-normal and G6PD-deficient RBCs did not differ in levels of glucose and fructose 1,6-bisphosphate during storage.[55] However, G6PD-deficient RBCs had increased glycolysis and high levels of 2,3-bisphosphoglycerate (2,3-DPG) and pyruvate throughout the storage period. Besides RBC products, an assessment of FFP units obtained from G6PD-deficient volunteers demonstrated alterations in several pathways involving riboflavin, purine, and glycerolipid/glycerophospholipid metabolisms.[43] That study also found that G6PD-deficient FFPs had excesses of diacylglycerols, glycerophosphoinositol, aconitate, and ornithine but deficiencies in riboflavin, flavin mononucleotide, adenine, and arginine compared to the controls.

Recent studies of sickle cell disease have explored the biochemical processes involving rigidity of sickle RBCs to improve the recovery of transfused RBCs in recipients. The mechanisms of RBC sickling have been suggested as being associated with water influx and increased permeability of K^+, Na^+, and Ca^{2+}, which result in overhydrated RBCs and decreased deformability.[56] One study that used laser optical tweezers to assess the elasticity of stored RBCs obtained from individuals with SCT found that the elasticity of stored RBCs was higher than in the control groups on days 1, 14, and 21 of storage and that the most rigidity was demonstrated on days 28 and 35.[57] Further study that examined the quality of RBCs during storage under blood bank conditions reported increased 2,3-DPG levels and low osmotic fragility.[58] The potential role of 2,3-DPG in the deformability of RBCs was supported by Suzuki et al,[59] who found an association between 2,3-DPG augmentation and decreased RBC deformability. In addition, a study by Poillon et al[60] that evaluated the anti-sickling effect of 2,3-DPG depletion on sickle RBCs found that activation of 2,3-DPG phosphatase and reduction of physiologic medium and isosmotic CO_2/bicarbonate-buffered saline in pH 7.0 reduce the polymer fraction of glycolate-treated sickle RBCs and the sickling of RBCs by 32%–63% and 46%–95%, respectively. Changes of ATP in RBCs from SCT donors has also been documented in RBC units subjected to 2500 cGy of gamma irradiation, but this phenomenon has also observed in RBC units prepared from donors with normal Hb types.[61]

Studies Related to the Efficacy of Blood Units

Several in vivo and clinical studies have investigated the transfusion efficacy of blood products prepared from G6PD-deficient, thalassemic trait, and SCT donors. These investigations have addressed several issues related to adverse reactions, posttransfusion recovery, and changes in parameters that decrease the posttransfusion survival of the transfused cells.

One longitudinal in vivo study compared the hemolytic reactions in patients transfused with G6PD-deficient and normal RBCs.[62] The results found significantly increased serum bilirubin and lactate dehydrogenase in the recipients of G6PD-deficient RBCs than in G6PD-normal RBCs 24 hours after transfusion. However, Huang et al[63] transfused G6PD-deficient RBCs into recipients and then examined blood counts, bilirubin, and heptoglobin. They found no significant differences in these parameters between recipients transfused with G6PD-deficient and G6PD-normal RBCs. A recent study investigating whether transfusion with G6PD-deficient RBCs affects transfusion outcome found that in children with sickle cells, Hb clearance was positively correlated with severely G6PD-deficient units and RBC alloimmunization but negatively correlated with recipient splenectomy.[64] Interestingly, the study demonstrated that higher Hb clearance was associated with higher levels of HbS and reticulocyte counts, implying a potentially shortened posttransfusion survival of G6PD-deficient RBCs. A recent study that addressed the effect of storage on the quality of RBCs obtained from G6PD-deficient donors reported poorer RBC recovery 24 hours after transfusion in recipients who had received G6PD-deficient RBCs than in those who had received G6PD-normal RBCs.[55] In addition, G6PD-deficient RBCs showed increased glycolysis and impaired glutathione and purine oxidation during storage when compared to G6PD-normal RBCs, suggesting an effect of storage on the quality of RBC units from G6PD-deficient donors. This assumption is supported by a study by Tzounakas et al,[65] who sought to determine whether storage and donor G6PD status affect RBC proteasome networks. Proteasome activities in the stored RBCs were shown to be greater in the RBC cytosol of G6PD units than in that of controls. Furthermore, the proteasome profiles correlated significantly with the G6PD metabolome and other markers of quality in RBC units.

Studies related to blood products from thalassemic donors provide limited data on transfusion efficacy. However, Pornprasert et al[66] demonstrated that blood transfusions from donors with HbE interfered with the diagnosis of thalassemia by producing conflicting results regarding Hb typing, CBC indices, and follow-up medical history. Anastasiadi and colleagues[67] conducted in vitro investigations on human β-thalassemia trait and control RBCs after reconstitution in plasma from thalassemia patients and healthy controls. They found lower hemolysis and procoagulant extracellular vesicles levels but higher proteasome activity in the reconstituted samples of β-thalassemia trait RBCs compared to control. Moreover, they found higher posttransfusion recovery of β-thalassemia trait RBCs than control in xenobiotic animal models of transfusion.

Regarding SCT, patients who received blood units from donors with SCT reported no adverse reactions in one study,[68] although a prior study had suggested that transfusion from donors with SCT might contribute to vaso-occlusion during surgery or prolonged hypoxemia.[69] A mouse-model study of the effects of RBC storage on stability and posttransfusion recovery found that the survival of human hemoglobin AS RBCs transfused into the mouse model was lower than that of transfused hemoglobin AA RBCs when cells were kept in cold storage,[70] suggesting the effects of storage on the quality of RBCs obtained from donors with SCT. Furthermore, another study that evaluated the use of an automated cell processing system to cryopreserve and deglycerolize RBC units from SCT donors found that deglycerolization resulted in RBC recovery rates of 43%–76.5%, whereas recovery rates for control RBCs were greater than 80%.[71]

Future Perspective

Although studies have improved the knowledge regarding donors who have underlying conditions and the blood products they donate, several issues require further clarification and warrant future investigation. First, further research is needed to address the health status of regular donors who have underlying conditions. Although the Hb levels of volunteer donors with thalassemic trait, G6PD-deficiency trait, and SCT may be acceptable for blood donation, the influence of this regular blood

donation on body iron storage and other iron metabolism parameters warrants more exploration. More studies in this area could be important for donor recruitment and retention. Second, focusing on the various morphological and biochemical changes previously documented and understanding the molecular mechanisms related to these changes will be useful for developing preservation strategies to prevent deleterious effects during the processing and storage of blood products. Third, it has been suggested that the G6PD status of donors might increase MP levels in blood products. Further studies should be conducted to address the clinical significance of blood products with high MP levels. The knowledge gained from these investigations will be useful for therapeutic applications, such as how the efficacy of these blood products for the treatment of active bleeding compares to that for prophylaxis purposes. A novel approach might be needed to inhibit MP release in blood products during preparation and storage, thereby minimizing posttransfusion reactions. Fourth, studies should evaluate the effects of various filtration procedures or novel filtration devices on the blood units obtained using them. This is due to the possibility that passing blood through transfusion devices might stress the cells and decrease the quality of the blood products prepared from this blood. Fifth, additional research should be conducted to investigate the storability parameters of blood products obtained from these donors during storage in blood bank conditions. Finally, the interference of transfused blood with Hb analysis emphasizes that (1) hemoglobin typing should be performed in individuals who have been transfusion-free for at least 3 months, and (2) thalassemia screening of donors could be applied in areas where the disease is prevalent. In this context, research data will support the use of blood products collected from eligible volunteers with thalassemic trait, G6PD deficiency trait, and SCT in clinical practice to benefit both donors and recipients.

Conclusion

A review of the literature reveals some advanced research on thalassemic trait, G6PD-deficiency trait, and SCT donors and their blood products. However, further studies are needed to investigate clinical utility, and several issues remain to be resolved in practical applications. Knowledge gained from in vitro and in vivo studies will increase the understanding of differences in blood units during processing, storage, and filtration and enable development of novel strategic management to inform revision of policy regarding donor recruitment and retention, improve transfusion efficacy, and minimize posttransfusion reactions.

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REFERENCES

1. Yaddanapudi S, Yaddanapudi L. Indications for blood and blood product transfusion. *Indian J Anaesth*. 2014;58(5):538–542.
2. Shorthouse J, Wilson S. Use of blood components in clinical practice. *Nurs Stand*. 2019;34(11):76–82.
3. Basu D, Kulkarni R. Overview of blood components and their preparation. *Indian J Anaesth*. 2014;58(5):529–537.
4. WHO Guidelines Approved by the Guidelines Review Committee. *Blood Donor Selection: Guidelines on Assessing Donor Suitability for Blood Donation*. Geneva, Switzerland: World Health Organization; 2012.
5. Kittisares K, Palasuwan D, Noulsri E, Palasuwan A. Thalassemia trait and G6PD deficiency in Thai blood donors. *Transfus Apher Sci*. 2019;58(2):201–206.
6. Rosline H, Ahmed SA, Al-Joudi FS, Rapiaah M, Naing NN, Adam NA. Thalassemia among blood donors at the Hospital Universiti Sains Malaysia. *Southeast Asian J Trop Med Public Health*. 2006;37(3):549–552.
7. Alabdulaali MK, Alayed KM, Alshaikh AF, Almashhadani SA. Prevalence of glucose-6-phosphate dehydrogenase deficiency and sickle cell trait among blood donors in Riyadh. *Asian J Transfus Sci*. 2010;4(1):31–33.
8. Lee JS, Cho SI, Park SS, Seong MW. Molecular basis and diagnosis of thalassemia. *Blood Res.* 2021;56(S1):S39–s43.
9. Fibach E, Rachmilewitz EA. Pathophysiology and treatment of patients with beta-thalassemia—an update. *F1000Res.* 2017;6:2156.
10. Muncie HL, Jr., Campbell J. Alpha and beta thalassemia. *Am Fam Physician*. 2009;80(4):339–344.
11. Ben Salah N, Bou-Fakhredin R, Mellouli F, Taher AT. Revisiting beta thalassemia intermedia: past, present, and future prospects. *Hematology*. 2017;22(10):607–616.
12. Luzzatto L, Ally M, Notaro R. Glucose-6-phosphate dehydrogenase deficiency. *Blood*. 2020;136(11):1225–1240.
13. Belfield KD, Tichy EM. Review and drug therapy implications of glucose-6-phosphate dehydrogenase deficiency. *Am J Health Syst Pharm*. 2018;75(3):97–104.
14. Cunningham AD, Mochly-Rosen D. Structural analysis of clinically relevant pathogenic G6PD variants reveals the importance of tetramerization for G6PD activity. *Matters (Zur)*. 2017. doi: 10.19185/matters.201705000008.
15. Lonergan GJ, Cline DB, Abbondanzo SL. Sickle cell anemia. *Radiographics*. 2001;21(4):971–994.
16. Adu P, Simpong DL, Takyi G, Ephraim RK. Glucose-6-phosphate dehydrogenase deficiency and sickle cell trait among prospective blood donors: a cross-sectional study in Berekum, Ghana. *Adv Hematol*. 2016;2016:7302912.
17. Antwi-Baffour S, Asare RO, Adjei JK, Kyeremeh R, Adjei DN. Prevalence of hemoglobin S trait among blood donors: a cross-sectional study. *BMC Res Notes*. 2015;8:583. doi:10.1186/s13104-015-1583-0.
18. Nuinoon M, Kruachan K, Sengking W, Horpet D, Sungyuan U. Thalassemia and hemoglobin E in southern Thai blood donors. *Adv Hematol*. 2014;2014:932306. doi: 10.1155/2016/7302912.
19. Devine DV, Serrano K. Preparation of blood products for transfusion: is there a best method? *Biologicals*. 2012;40(3):187–190.
20. Noulsri E, Lerdwana S, Palasuwan D, Palasuwan A. Cell-derived microparticles in blood products from thalassemic blood donors. *Lab Med*. 2021;52(2):150–157.
21. Riahi S, Mei IL, Idris FB, George E, Noor SM. Evaluation of red blood cell indices related disorders among eligible blood donors at the Universiti Putra Malaysia (UPM). *Southeast Asian J Trop Med Public Health*. 2015;46(5):911–917.
22. Maffi D, Pasquino MT, Mandarino L, et al. Glucose-6-phosphate dehydrogenase deficiency in Italian blood donors: prevalence and molecular defect characterization. *Vox Sang*. 2014;106(3):227–233.
23. Noulsri E, Lerdwana S, Palasuwan D, Palasuwan A. Cell-derived microparticles in blood products from blood donors deficient in glucose-6-phosphate dehydrogenase. *Lab Med*. 2021;52(6):528–535.
24. Tzounakas VL, Kriebardis AG, Georgatzakou HT, et al. Glucose 6-phosphate dehydrogenase deficient subjects may be better "storers" than donors of red blood cells. *Free Radic Biol Med*. 2016;96:152–165.

25. Aninagyei E, Doku ET, Adu P, Egyir-Yawson A, Acheampong DO. Storage related haematological and biochemical changes in *Plasmodium falciparum* infected and sickle cell trait donor blood. *BMC Hematol*. 2018;18:30. doi: 10.1186/s12878-018-0128-x.

26. Barshtein G, Pajic-Lijakovic I, Gural A. Deformability of stored red blood cells. *Front Physiol*. 2021;12:722896. doi: 10.3389/fphys.2021.722896.

27. Putter JS, Seghatchian J. Cumulative erythrocyte damage in blood storage and relevance to massive transfusions: selective insights into serial morphological and biochemical findings. *Blood Transfus*. 2017;15(4):348–356.

28. Jain A, Marwaha N, Sharma RR, Kaur J, Thakur M, Dhawan HK. Serial changes in morphology and biochemical markers in platelet preparations with storage. *Asian J Transfus Sci*. 2015;9(1):41–47.

29. Kriebardis A, Antonelou M, Stamoulis K, Papassideri I. Cell-derived microparticles in stored blood products: innocent-bystanders or effective mediators of post-transfusion reactions? *Blood Transfus.* 2012;10(suppl 2):s25–s38.

30. Saas P, Angelot F, Bardiaux L, Seilles E, Garnache-Ottou F, Perruche S. Phosphatidylserine-expressing cell by-products in transfusion: a pro-inflammatory or an anti-inflammatory effect? *Transfus Clin Biol*. 2012;19(3):90–97.

31. Lee Y, El Andaloussi S, Wood MJ. Exosomes and microvesicles: extracellular vesicles for genetic information transfer and gene therapy. *Hum Mol Genet.* 2012;21(R1):R125–134.

32. Raposo G, Stoorvogel W. Extracellular vesicles: exosomes, microvesicles, and friends. *J Cell Biol*. 2013;200(4):373–383.

33. Battistelli M, Falcieri E. Apoptotic bodies: particular extracellular vesicles involved in intercellular communication. *Biology (Basel)*. 2020;20;9(1):21. doi: 10.3390/biology9010021.

34. Nielsen T, Kristensen AF, Pedersen S, Christiansen G, Kristensen SR. Investigation of procoagulant activity in extracellular vesicles isolated by differential ultracentrifugation. *J Extracell Vesicles*. 2018;7(1):1454777.

35. Nomura S, Shimizu M. Clinical significance of procoagulant microparticles. *J Intensive Care*. 2015;3(1):2.

36. Burnouf T, Chou ML, Goubran H, Cognasse F, Garraud O, Seghatchian J. An overview of the role of microparticles/microvesicles in blood components: are they clinically beneficial or harmful? *Transfus Apher Sci*. 2015;53(2):137–145.

37. Noulsri E. Quantitation of cell-derived microparticles in blood products and its potential applications in transfusion laboratories. *Lab Med*. 2020;51(5):452–459.

38. Noulsri E, Palasuwan A. Effects of donor age, donor sex, blood-component processing, and storage on cell-derived microparticle concentrations in routine blood-component preparation. *Transfus Apher Sci*. 2018;57(4):587–592.

39. Noulsri E, Udomwinijsilp P, Lerdwana S, Chongkolwatana V, Permpikul P. Differences in levels of platelet-derived microparticles in platelet components prepared using the platelet rich plasma, buffy coat, and apheresis procedures. *Transfus Apher Sci*. 2017;56(2):135–140.

40. Noulsri E, Lerdwana S, Palasuwan D, Palasuwan A. Quantitation of phosphatidylserine-exposing platelets in platelet concentrate prepared in routine blood transfusion laboratory. *Transfus Apher Sci*. 2020;59(1):102598.

41. Noulsri E, Lerdwana S, Palasuwan D, Palasuwan A. Storage duration and red blood cell-derived microparticles in packed red blood cells obtained from donors with thalassemia. *Lab Med*. 2022;53(3):302–306.

42. Nantakomol D, Palasuwan A, Chaowanathikhom M, Soogarun S, Imwong M. Red cell and platelet-derived microparticles are increased in G6PD-deficient subjects. *Eur J Haematol*. 2012;89(5):423–429.

43. Tzounakas VL, Gevi F, Georgatzakou HT, et al. Redox status, procoagulant activity, and metabolome of fresh frozen plasma in glucose 6-phosphate dehydrogenase deficiency. *Front Med (Lausanne)*. 2018;5(5):16. doi: 10.3389/fmed.2018.00016.

44. Schuetz AN, Hillyer KL, Roback JD, Hillyer CD. Leukoreduction filtration of blood with sickle cell trait. *Transfus Med Rev*. 2004;18(3):168–176.

45. Amar KO, Bourdonné O, Bruneau S, Sellami F, Richard P. Assessment of leucoreduction of sickle cell trait blood: quality of the filtered product. *Blood Transfus.* 2014;12(suppl 1):s193–s198.

46. Stroncek DF, Rainer T, Sharon V, et al. Sickle Hb polymerization in RBC components from donors with sickle cell trait prevents effective WBC reduction by filtration. *Transfusion*. 2002;42(11):1466–1472.

47. Bryant BJ, Bianchi M, Wesley RA, Stroncek DF, Leitman SF. Leukoreduction filtration of whole-blood units from sickle trait donors: effects of a metered citrate anticoagulant system. *Transfusion*. 2007;47(12):2233–2241.

48. Stroncek DF, Byrne KM, Noguchi CT, Schechter AN, Leitman SF. Increasing hemoglobin oxygen saturation levels in sickle trait donor whole blood prevents hemoglobin S polymerization and allows effective white blood cell reduction by filtration. *Transfusion*. 2004;44(9):1293–1299.

49. Byrne KM, Leitman SF, Schechter AN, Stroncek DF. Increasing oxygen tension improves filtration of sickle trait donor blood. *Br J Haematol*. 2003;122(4):678–681.

50. Anastasiadi AT, Tzounakas VL, Arvaniti VZ, et al. Red blood cell proteasome in beta-thalassemia trait: topology of activity and networking in blood bank conditions. *Membranes (Basel)*. 2021;17;11(9):716. doi: 10.3390/membranes11090716.

51. Tzounakas VL, Anastasiadi AT, Stefanoni D, et al. Beta thalassemia minor is a beneficial determinant of red blood cell storage lesion. *Haematologica*. 2022;107(1):112–125.

52. Reisz JA, Tzounakas VL, Nemkov T, et al. Metabolic linkage and correlations to storage capacity in erythrocytes from glucose 6-phosphate dehydrogenase-deficient donors. *Front Med*. 2017;4:248.

53. Peters AL, van Bruggen R, de Korte D, Van Noorden CJ, Vlaar AP. Glucose-6-phosphate dehydrogenase activity decreases during storage of leukoreduced red blood cells. *Transfusion*. Feb 2016;56(2):427–432.

54. Tzounakas VL, Kriebardis AG, Georgatzakou HT, et al. Data on how several physiological parameters of stored red blood cells are similar in glucose 6-phosphate dehydrogenase deficient and sufficient donors. *Data Brief*. 2016;8:618–627.

55. Francis RO, D'Alessandro A, Eisenberger A, et al. Donor glucose-6-phosphate dehydrogenase deficiency decreases blood quality for transfusion. *J Clin Invest*. 2020;130(5):2270–2285.

56. Barabino GA, Platt MO, Kaul DK. Sickle cell biomechanics. *Annu Rev Biomed Eng*. 2010;12:345–367.

57. Brandão MM, Saad ST, Cezar CL, Fontes A, Costa FF, Barjas-Castro ML. Elastic properties of stored red blood cells from sickle trait donor units. *Vox Sang*. 2003;85(3):213–215.

58. Ould Amar AK, Kérob-Bauchet B, Robert P, et al. Assessment of qualitative functional parameters of stored red blood cells from donors with sickle cell trait (AS) or with heterozygote (AC) status. *Transfus Clin Biol*. 1996;3(4):225–233.

59. Suzuki Y, Nakajima T, Shiga T, Maeda N. Influence of 2,3-diphosphoglycerate on the deformability of human erythrocytes. *Biochim Biophys Acta*. 1990;1029(1):85–90.

60. Poillon WN, Kim BC, Labotka RJ, Hicks CU, Kark JA. Antisickling effects of 2,3-diphosphoglycerate depletion. *Blood*. 1995;85(11):3289–3296.

61. Jin YS, Anderson G, Mintz PD. Effects of gamma irradiation on red cells from donors with sickle cell trait. *Transfusion*. 1997;37(8):804–808.

62. Shalev O, Manny N, Sharon R. Posttransfusional hemolysis in recipients of glucose-6-phosphate dehydrogenase-deficient erythrocytes. *Vox Sang*. 1993;64(2):94–98.

63. Huang CS, Sung YC, Huang MJ, Yang CS, Shei WS, Tang TK. Content of reduced glutathione and consequences in recipients of

glucose-6-phosphate dehydrogenase deficient red blood cells. *Am J Hematol*. 1998;57(3):187–192.

64. Sagiv E, Fasano RM, Luban NLC, et al. Glucose-6-phosphate-dehydrogenase deficient red blood cell units are associated with decreased posttransfusion red blood cell survival in children with sickle cell disease. *Am J Hematol*. 2018;93(5):630–634.
65. Tzounakas VL, Dzieciatkowska M, Anastasiadi AT, et al. Red cell proteasome modulation by storage, redox metabolism and transfusion. *Blood Transfus*. 2022;20(1):27–39.
66. Pornprasert S, Jaiping K. Blood transfusion from a Hb E trait donor can affect β-thalassemia diagnosis. *Hemoglobin*. 2014;38(4):295–298.
67. Anastasiadi AT, Paronis EC, Arvaniti VZ, et al. The post-storage performance of RBCs from beta-thalassemia trait donors is related to their storability profile. *Int J Mol Sci*. 2021;22(22):12281. doi: 10.3390/ijms222212281.
68. Kaufman M, Steier W, Applewhaite F, Ruggiero S, Ginsberg V. Sickle-cell trait in blood donors. *Am J Med Sci*. 1965;249:56–61.
69. Piccin A, Fleming P, Eakins E, McGovern E, Smith OP, McMahon C. Sickle cell disease and dental treatment. *J Ir Dent Assoc*. 2008;54(2):75–79.
70. Osei-Hwedieh DO, Kanias T, Croix CS, et al. Sickle cell trait increases red blood cell storage hemolysis and post-transfusion clearance in mice. *EBioMedicine*. 2016;11:239–248.
71. Ackley RJ, Lee-Stroka AH, Bryant BJ, Stroncek DF, Byrne KM. Cryopreserving and deglycerolizing sickle cell trait red blood cell components using an automated cell-processing system. *Immunohematology*. 2008;24(3):107–112.

Essential Thrombocythemia and Post-Essential Thrombocythemia Myelofibrosis: Updates on Diagnosis, Clinical Aspects, and Management

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Abbreviations: ET, essential thrombocythemia; MF, myelofibrosis; AML, acute myeloid leukemia; MDS, myelodysplastic syndromes; pre-MF, prefibrotic MF; AVWS, acquired von Willebrand syndrome; ASA, acetylsalicylic acid; IPSS, International Prognostic Score System; DIPSS, Dynamic International Prognostic Score System; PMF, primary MF; SMF, secondary MF; SAL, Study Alliance Leukemia; allo-HSCT, allogeneic hematopoietic stem cell transplantation; OS, overall survival; EBMT, European Society for Blood and Bone Marrow Transplantation; PFS, progression-free survival; GVHD, graft-versus-host disease; MYSEC-PM, Myelofibrosis Secondary to PV and ET-Prognostic Model; MTSS, MF transplant scoring system; LDH, lactate dehydrogenase; WBC, white blood cell count.

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ABSTRACT

Although several decades have passed since the description of myeloproliferative neoplasms (MPN), many aspects of their pathophysiology have not been elucidated. In this review, we discuss the mutational landscape of patients with essential thrombocythemia (ET), prognostic scores and salient pathology, and clinical points. We discuss also the diagnostic challenges of differentiating ET from prefibrotic MF.

We then focus on post-essential thrombocythemia myelofibrosis (post-ET MF), a rare subset of MPN that is usually studied in conjunction with post-polycythemia vera MF. The transition of ET to post-ET MF is not well studied on a molecular level, and we present available data. Patients with secondary MF could benefit from allogenic hematopoietic stem cell transplantation, and we present available data focusing on post-ET MF.

Introduction

Essential thrombocytosis (ET) is a rare hematological malignancy with a risk to evolve to secondary myelofibrosis (post-ET MF) or to transform to acute myeloid leukemia (AML). Management of post-ET MF can be challenging, as thrombocytosis may coexist with anemia and other manifestations of MF. In the literature, post-ET MF and post-polycythemia vera (PV) MF are often grouped together. In this mini-review, we will discuss pertinent aspects of ET and post-ET MF and provide insights into management.

Biology and Diagnosis of ET: A Closer Look

ET is a rare hematological condition that falls into the myeloproliferative disorders spectrum. Diagnostic criteria require presence of 4 major criteria: (1) platelet count ≥450 × 10^9/L; (2) megakaryocytic proliferation and morphologic changes; (3) exclusion of BCR-ABL1-positive chronic myeloid leukemia, PV, primary MF (PMF), or other myeloid neoplasms; and (4) *JAK2, CALR,* or *MPL* mutation; or, in the absence of the fourth major criteria, the first 3 major criteria and the presence of a clonal marker or exclusion of other hematological conditions that may present with thrombocytosis, and exclusion of reactive processes that can manifest with thrombocytosis.[1,2] A clonal marker such as mutations that affect genes including *JAK2*, *CALR*, *C-MPL* or other mutations/ chromosomal aberrations can be useful for the diagnosis. The *JAK2 V617F* is the most common mutation, with some reports estimating 50% to 60% of World Health Organization (WHO)-defined cases,[3] followed by *CALR* in ~25% of the patients,[4] and *C-MPL*, the most rare of the 3 in the context of ET, occurring in 3% of patients.[3] Co-occurrence of *JAK2* and *CALR* mutations has been described but it is exceedingly rare.[5] It should be noted that the sensitivity of detection of mutations varies between techniques,[6] and frequency of mutations can vary significantly between studies. For example, a recent meta-analysis[7] indicated that *JAK2 V617F* was reported to occur in 31% to 72% of ET patients, whereas C-MPL between ~1% and 12 % of patients. In the same meta-analysis,[7] the *CALR* mutation was noted to be present in ~12% to

50 % of ET patients. The *CALR* mutation repertoire can be more diverse than the more defined ones affecting *JAK2* and *MPL* genes. A database looking into the *CALR* mutations in the context of ET is freely available and can be helpful.[8] Notably, different types of *CALR* mutations (type I vs type II) may affect outcomes of patients diagnosed with ET.[9] Some of the cases of myeloproliferative neoplasms (MPN) that are labeled triple-negative may harbor mutations at a level below the detection limit of commonly used techniques.[6] Alternatively, triple-negative ET may harbor noncanonical mutations of the key driver genes.[10] Nevertheless, in clinical practice, other myeloid mutations can be detected apart from *JAK2 V617/CALR/MPL* mutations in ET patients with panel testing of genes frequently mutated in myeloid neoplasms.[11,12] The impact of those mutations is still being explored and will be discussed below.

Familial myeloproliferative neoplasms are rare, but information is accumulating over time.[13,14] Interestingly, *ATG2B* and *GSKI* mutations were reported to confer propensity to develop ET.[13] Case-control and cohort studies have explored the association of environmental factors and development of myeloproliferative neoplasms. For the development of ET, living in houses made of certain volcanic material, exposure to benzene, and obesity were identified as risk factors.[14,15]

Thrombocytosis in peripheral blood is the major finding in ET, whereas white blood cell count (WBC) and hemoglobin are usually normal. The platelets vary in size from very small to very large. A bone marrow biopsy should be performed on all patients with suspicion of ET to maximize diagnostic yield. Marrow cellularity is normal in most cases, and most significant abnormalities affect the megakaryocytic lineage, including marked proliferation of megakaryocytes with many large forms displaying hypersegmented nuclei and mature cytoplasm (**FIGURES 1A** and **B**). Reticulin fibers are often normal or minimally increased in the majority of the cases (**FIGURE 1C**).

A study looked at megakaryocytic morphology and bone marrow characteristics in patients with ET harboring or not harboring the *JAK2 V617F* mutation.[16] Megakaryocytes were more numerous in patients with the *JAK2 V617F* mutation. There were no differences in the localization, emperipolesis, cytoplasmatic diameter, or nuclear to cytoplasm ratio.[16]

A distinctive feature of megakaryopoiesis is the ability of megakaryocytes to increase their size and DNA content through a unique process for hemopoietic cells called endomitosis.[17,18] In this process, megakaryocytes undergo repeated cycles of aborted mitosis, and DNA content can increase to 128N with a concomitant increase in cell size. Ultimately, megakaryocytes form elongated cytoplasmic appendages termed proplatelets that release platelets.[17] The megakaryocytic modal ploidy in healthy individuals is 16N. Studies have reported that in reactive thrombocytosis, mean ploidy closely resembles that of healthy individuals.[19] However, in patients with ET the mean ploidy is increased; in 1 study, the mean ploidy was 32N, with almost half of the megakaryocytes having a ploidy greater or equal to 32N.[19] Megakaryocytic ploidy was also elevated in PV similar to ET. Although ploidy could have a role in differentiating ET from reactive thrombocytosis, practically this is difficult to evaluate. Clinical laboratories do not routinely perform nuclear stains, isolating megakaryocytes can be laborious, and immunofluorescent panels may not include megakaryocytic markers. Moreover, some reports have been conflicting,[20] underscoring the inherent difficulty of studying megakaryocytes.

Chromosomal abnormalities in ET are uncommon (~5% to 7% of patients).[21–23] Chromosomal aberrations are not specific and may include abnormalities of chromosome 1, 2, 4, inv(9), +8, +9, complex karyotype and del(5).[21,22] Notably, patients with del(5q) and *JAK2 V617F* mutation can pose the diagnostic dilemma of differentiating ET from myelodysplastic syndromes (MDS), as cytopenias may be mild or not be present and platelets could be increased.[24]

The features seen in ET can be encountered in other MPN and nonclonal hematologic disorders. Therefore, patients presenting with thrombocythemia require a thorough evaluation considering other conditions included in differential diagnosis. It is important to differentiate ET from the closely mimicking prefibrotic MF (pre-MF).[25] Pre-MF was initially incorporated in the WHO 2008 classification of myeloid neoplasms, but it was recognized as a distinct entity in the 2017 classification.[1,25] The distinction has meaningful impact and can affect management and prognosis.[26,27] In pre-MF, the megakaryocytes are increased but tend to form clusters, and morphologically, nuclei may exhibit bizarre, highly atypical morphology.[28] Increased age-adjusted bone marrow cellularity and granulocytic hyperplasia in addition to megakaryocytic changes are common features in pre-MF.

The WBC and platelet count has been reported to be higher in patients with pre-MF compared to ET.[25,26,29] However, these differences individually cannot practically differentiate the 2 entities in clinical practice. Splenomegaly is more frequently present in patients with pre-MF than those with ET. Mathematical formulas that do not dependent on marrow morphology (eg, Bergamo algorithm[30]) have been published in the context of pre-MF. These formulas[27,30] include variables such as hemoglobin, spleen size, lactate dehydrogenase (LDH), and WBC and may aid in diagnostic dilemmas between ET and pre-MF.

A question at hand is whether mutations could help discriminate between pre-MF and ET. Recently a study from China examined the mutational burden and laboratory findings of 72 patients with ET and an equal number of patients diagnosed as pre-MF.[31] The study found that pre-MF patients harbored more frequent high molecular risk mutations than those diagnosed with ET (19% vs 4%). In particular, *ASXL1* was found in 17% of pre-MF patients compared to 3% of ET patients. Regarding the classical driver mutations, *JAK2 V617F* and *CALR* allelic burden were higher in pre-MF participants (median, 0.41 and 0.46, respectively) compared to participants with ET (median, 0.25 and 0.42, respectively). A study from Japan also examined the mutational status of 13 pre-MF patients compared with 87 patients with ET.[32] The *JAK2 V617F* mutational burden was higher in pre-MF patients but did not reach statistical significance (42.6 vs 31.4 %; $P = .09$).

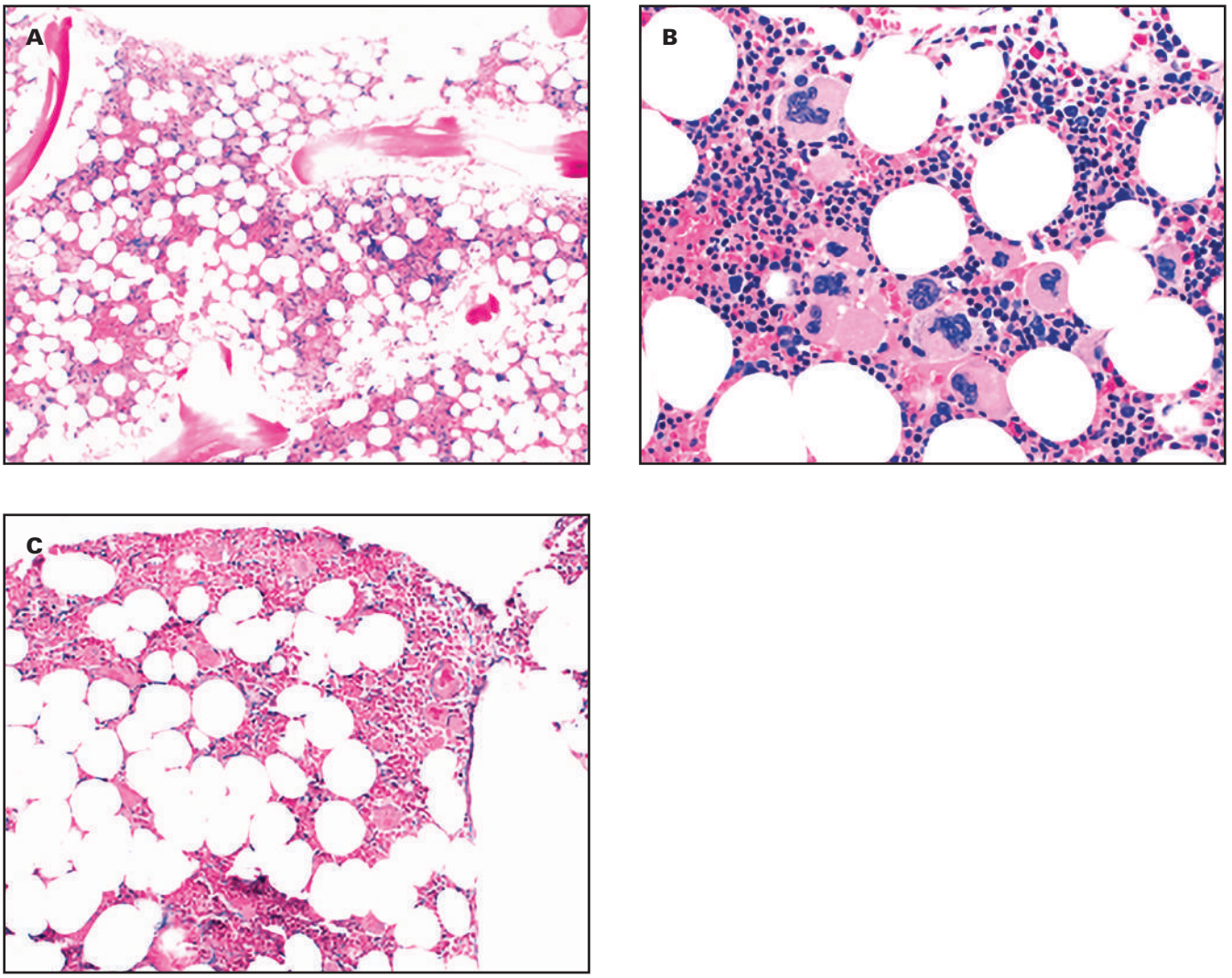
Iron deficiency can promote thrombocytosis,[33–35] and recently a putative mechanism has been uncovered. In addition, an inflammatory state can lead to increase in platelets.[36] Obtaining inflammatory markers and studies to assess iron level might be useful.

Finally, The ET may transition to PV and vice versa, but details and frequency of such transitions are scarce.[37]

ET Clinical Presentation

Patients with ET tend to be symptomatic at presentation (46%–63%) according to some studies.[38] In addition, a common complaint is fatigue, and arthralgias, night sweats, and pruritus are reported frequently.[39] Some experts use the MPN symptom assessment score to define disease burden to incorporate JAK2 inhibitors in the management of ET.[40]

FIGURE 1. **ET bone marrow morphology. A, A normocellular marrow with increased number of megakaryocytes with overall normal appearing erythroid and myeloid lineages. B, Megakaryocytes vary in size with some significantly large ones with hyperlobated nuclei without bizarre forms. C, No reticulin fibrosis is seen on reticulin stain.**



Generally, the use of JAK inhibitors for MPN-related symptomatology in ET in our experience is quite uncommon. Younger patients (<40 years old) present with higher platelet counts and palpable splenomegaly compared to older patients.[41]

ET and Platelet Function

Patients afflicted by ET may develop bleeding diathesis from the degradation of high molecular weight von Willebrand multimers. This condition is described in the literature as acquired von Willebrand syndrome (AVWS) secondary to ET. Patients with ET present more often with thrombosis rather than bleeding[38] and arterial events are more prevalent than venous.[38] A correlation between platelet counts and thrombosis has not been firmly established[42] and prognostic/stratification scores do not use platelet counts as variables.[42–44] Pregnant ET patients may be challenging to manage. In a small retrospective study of 24 pregnancies, 80% were diagnosed with AVWS.[45] Of note, none of the patients had a presenting platelet count of more than 1 million.

ET Prevalence

The prevalence of ET has not been accurately determined, with estimates ranging between 11 and 57 per 100,000.[46] It afflicts women twice as often as men.[47] ET is not common in persons less than 40 years old; in a Mayo Clinic registry capturing MPN, such patients comprised 7%.[41] Similarly, in a Korean registry, young ET patients represented 12% of patients.[48]

ET and Thrombosis

The international prognostic score of thrombosis in ET has undergone refinements over time.[44,49] Thrombotic risk is estimated to be 6% to 10% per year and bleeding to be ~1% per patient-year. The reported incidence of major thrombotic events at diagnosis can range from ~10% to 35% for ET with similar incidence in the follow-up period.[50,51]

Patients with ET and *JAK2 V617F* mutation have greater thrombotic risk than *JAK2* wild-type ET patients.[52] In a study using samples from 3 studies, 414 patients with *JAK2* V617F and 362 patients with wild-type *JAK2* were analyzed.[52] The venous thrombotic events were higher

in mutated *JAK2 V617F* ET patients prior to diagnosis compared to those with wild-type *JAK2* gene. Notably, patients with *JAK2* mutation had a higher arterial thrombosis when treated with acetylsalicylic acid (ASA) and anagrelide compared to ASA and hydroxyurea. The anagrelide mechanism of action involves mainly interfering with megakaryocyte physiology,[53] and it is unclear why more arterial events were noted in that study.

Retrospective studies have demonstrated that there is overall benefit for the use of ASA in combination with cytoreduction in high-risk ET patients[54] despite increased bleeding risk. Until very recently, there have been no randomized trials for antiplatelet treatment as prophylaxis for ET patients.[54] A randomized study indicated that twice daily ASA can offer a more pronounced inhibition of platelet reactivity, but the impact of such intervention to thrombotic events remains unknown.[54] Thrombotic events are not common in younger patients (<40 years old); arterial events predominate compared to venous.[48]

ET and Bone Marrow Fibrosis

The fibrotic burden in ET is typically low at diagnosis[55] but increases during the transition to post-ET MF. The impact of treatments for ET in the fibrotic burden have not been studied extensively. In a series with a small number of patients with ET, the use of anagrelide resulted in increase in reticulin deposition (5 out 16 patients).[55] The number of megakaryocytes in the bone marrow of patients with ET did not decrease significantly after 2 years of treatment. Although the exact mechanism of action of anagrelide remains elusive, it appears to inhibit polyploidization that correlates with platelet production.[55]

ET Prognosis

Patients with a diagnosis of ET have a life expectancy that broadly resembles that of the general population, although a study from Mayo Clinic cast doubt on this notion. For patients less than 60 years old, the median survival approaches 33 years. Younger patients (<40 years old) have a median overall survival of 35 years.[41] Overall, ET is considered to have the best outcomes among Philadelphia chromosome-negative MPN. Patients are at risk for thrombosis, bleeding, and progression to secondary MF (SMF) or MDS/AML.

There have been various scores regarding prognostication and different characteristics of ET have been studied. The impact of chromosomal abnormalities in ET has not been firmly established, with perhaps more ominous significance attributed to chromosomal aberrations that evolve during the course of the disease[56,57] The significance of splenomegaly in the setting of ET is unclear. In a single institution study,[58] 238 patients with ET were followed for a median of ~46 months and ~15% had splenomegaly. The presence of splenomegaly in ET was associated with higher risk for death and worse survival.[58] The small number of patients and the retrospective nature of the study limits generalization of this finding.

Mutational analysis has gained attention, initially focusing on *JAK2*, *CALR*, *and c-MPL* mutations. The caveat is that these mutations are not present in all ET patients. In recent years, molecular panels employing next-generation sequencing have revealed mutations in other genes.

Approximately half of the patients with ET may harbor mutations in addition to *CALR/MPL/JAK2 V617F*.[11,59] Studies have demonstrated that the mutations that are commonly seen in MDS are infrequent in ET. For example, *SRSF2*, *U2AF1*, *RUNX1,* and *ZRSR2* are reported to occur in less than 5% of patients, and the impact on ET patients appears to be detrimental.[60] Mutations affecting *TP53* are reported in a minority of ET patients and have a detrimental role.[61] Other mutations associated with progression to MF or transformation to acute myeloid leukemia include *IDH2*, *EZH2*, *SF3B1*, and *SH2B3* mutations.[62] In addition, increased numbers of mutations were detected in post-ET MF and secondary MDS/AML, suggesting accumulation of mutations during disease progression.[12]

In a study involving a small number of Chinese patients harboring *ASXL1* mutations (n = 14), a propensity for thrombotic events was noted.[63] The majority of the *ASXL1*-mutated ET patients also harbored the *JAK2 V617F* mutation.[63] It is unclear whether the *ASXL1* mutation further predisposes patients to a thrombotic event.

Some studies have estimated that the risk of progression to leukemia is ~2% to 5%, and post-ET MF up to ~11%, for a 15-year time period,[64] although others have reported a higher proportion of patients with progression. A possible explanation for the discrepancies, especially in older studies, may be the grouping of ET and pre-MF patients together.[64]

In a study from the Mayo Clinic, the median survival was 18.9 years for patients afflicted with ET.[65] Notably, survival in ET patients was not affected by mutations in *JAK2* gene. No difference between mutations affecting *CALR* and *JAK2* genes has been reported by others.[66] The median overall survival for patients with *CALR* or *JAK2* mutations was estimated at ~19 years. A contemporary Korean study revealed a rate of progression to SMF of 2.8% at 8 years,[67] whereas in older studies, the risk was ~4% at 10 years.[65] A study using strict criteria to differentiate ET from pre-MF determined the 15-year cumulative incidence to be 9.3%.[68]

Progression to AML is rare in patients with ET; risk factors include age above 60 years old, thrombocytosis (>1,000,000), leukocytosis (>15 × 10^9/L), and *TP53* and *EZH2* mutations.[69] A report from a South Korean population revealed a cumulative incidence of 3.6%, whereas a cohort from the Mayo Clinic revealed a cumulative incidence of 3.8%; similar results were reported by older studies, ~1% at 10 years.[65] Applying strict criteria, the incidence of transformation was determined to be as low as 2% (15-year cumulative incidence).[68,69]

Recently, a prognostic score was formulated using data from patients in the US and Italy. The score, called mutation-enhanced international prognostic systems for ET and PV, uses more mutational data apart from driver mutations.[70] Spliceosome mutations such as those affecting *U2AF1*, *SRSF*, *SF3B1*, as well as mutations affecting *TP53*, had significant impact in prognosis.[70] ET patients without adverse mutations had a median overall survival of ~20 years, or double that of patients harboring such mutations. Progression to leukemia and MF was significantly increased in patients with adverse mutations (19% vs 3% and 39% vs 14%, respectively). The score also considered age above 60 years and male gender as risk factors. High-risk patients had a median overall survival of 8.3 years compared to a median overall survival of 34.4 years in patients with low risk (ie, 60 years and younger; absence of adverse mutations).[70]

Prognostic schemas do not consider treatments that may be used, especially for high-risk ET patients. Concerns have been raised for risk of progression to AML or post-ET MF secondary to treatments. Data for hydroxyurea and anagrelide have not been conclusively associated with increased risk of disease progression.[71]

In a large study, the use of anagrelide did not lead to excess transformation to SMF.[72] However, this was true only when patients were diagnosed based on WHO criteria, thus excluding patients who may have pre-MF. In another study from Japan with 53 high-risk ET patients, 7.5 % developed MF.[73] The exposure adjustment incidence was 2.66 per 100 patient-years.[73] Another multi-institutional retrospective study included 150 patients treated with anagrelide, and approximately 10% developed SMF.[74] In this study, the median time of anagrelide initiation to SMF was 5.7 years; progression was noted as early as 20 months.[74] Notably, not all patients were diagnosed with ET based on WHO-based criteria in that particular study.

In a report encompassing MPN patients, those with ET had the lowest risk of progression to MDS/AML.[75] Twenty-five percent of the MPN patients who progressed to MDS or AML did not have exposure to alkylating agents, radioactive phosphorus, or hydroxyurea.[75] Also, in this study, the patients with ET had a trend for better survival after development of MDS or AML than patients with antecedent PV or PMF, although it was not statistically significant.

In a cohort of Chinese patients, the probability of transformation to post-ET MF was ~10% in a decade.[38] A minority of patients developed AML, although the majority of patients were exposed to hydroxyurea.[38] Risk factors for leukemic transformation were exposure to melphalan and MF.[38]

Diagnosis of Post-ET MF

The criteria for post-ET MF require development of fibrosis (grade 2–3 on a 0–3 scale) in a documented WHO-defined ET, and 2 of the additional criteria including anemia, leukoerythroblastosis, elevated LDH, new or worsening splenomegaly, and constitutional symptoms. Bone marrow biopsy reveals increased reticulin fibrosis that can be accompanied by collagen deposition. The factors leading to increased fibrotic burden are not well understood.

Notably, the criteria to progression do not specify a decrease in platelets. It is imperative that other conditions that can manifest with fibrosis should be considered and excluded.[76]

Post-ET MF Prevalence

Based on data derived from 2 large health plans,[46] the age-adjusted prevalence of post-ET MF was estimated to be 0.462 to 1.076 per 100,000, whereas the age-adjusted incidence was 0.215 to 0.362 per 100,000.[46] Different studies have provided estimates that tend to vary depending on the geographic region and methodology.[14,47]

A large registry[77] from 16 institutions that contained 333 patients with post-ET MF indicated a median age of 64 years, with a median time to progression to SMF of 10.3 years. The median platelets were 379,000 and the range was 40,000 to 1,900,000. It is notable that the patients with post-ET MF did not retain thrombocytosis.

Molecular Aspects of Post-ET MF

The literature regarding changes in post-ET MF molecular aspects is scarce,[78,79] although PMF is known to harbor more mutations than PV and ET. In addition, based on a large registry, only 3% of patients with SMF were negative for *CALR/MPL/JAK2* mutations.[80] Prognostic scores have been proposed, such as Myelofibrosis Secondary to PV and ET-Prognostic Model (MYSEC-PM) that incorporate some molecular aspects of the disease (eg, *CALR* mutation) but do not consider others, such as spliceosome mutations or *TP53* status. In a study using next-generation sequencing analysis to detect mutations in patients with MPN (including patients with ET and post-ET MF), 8 patients had samples available from both the ET/PV and SMF courses of their disease.[81] Patients with SMF and RNA splicing mutations, *EZH2*, chromatin modifier genes, and *TP53* mutations had a worse overall survival (OS) in univariate analysis.[81] In a study of patients with post ET/PV MF, including those who progressed to secondary AML, *TP53* mutations were more common compared to patients who progressed to AML from PMF.[82]

Prognostic Scores on Post-ET MF

In the setting of post-ET MF, prognostic scores such as the International Prognostic Score System (IPSS), Dynamic International Prognostic Score System (DIPSS), or DIPSS-plus do not retain their value compared to PMF. The reasons for this discrepancy are not well understood. One of the scores that has been demonstrated to have prognostic value is the MYSEC-PM.[77] This score was developed by analyzing data from 685 patients for whom driver mutation status was available, from multiple institutions spanning over 2 decades. Three hundred thirty-three patients had post-ET MF, with 70% of them exposed to cytoreductive therapy.[77] Median age was 64 years with median time to progression to SMF of 10.3 years. Patients had a median Hgb of 10.7 g/dL and median platelets of 379,000. Notably, some patients presented with marked thrombocytopenia whereas others presented with platelets approaching 2,000,000. The median palpable spleen size was 4 cm, but spleen size could measure up to 27 cm. The scoring system is based on age, allocating points for presence of anemia (Hgb less than 11 g/dL), presence of thrombocytopenia (platelets less than 150,000), presence of circulating blasts (at a cutoff ≥3%), presence of constitutional symptoms, and mutational status of *CALR* gene (unmutated genotype has a negative impact on the score). A patient can fall into 4 risk groups (low, intermediate-1, intermediate-2, high). The score has been independently validated by data derived from the Spanish registry of MF.[80] Two hundred sixty-two patients with post-ET MF and 121 patients with post-PV MF were included.[80] Compared to IPSS, the MYSEC-PM reclassified more patients to a lower risk group classification.[80] A potential limitation of MYSEC-PM is that age has a key role in the scoring schema. Moreover, MYSEC-PM does not contain provisions for the impact of high-risk mutations such as spliceosome mutations. In addition, it does not factor in the interval of progression between ET to post-ET MF, karyotype, and performance status. In addition, the patient age is a continuous variable; a hypothetical patient with mild thrombocytopenia and anemia, circulating blasts, and *CALR* wild type will fall into intermediate-1 risk group (median survival, 9.3 years) if the age is 59 years. The same hypothetical patient will fall into intermediate risk -2 group (median survival, 4.5 years) if the age is 60 years. Moreover, the MYSEC-PM included patients that were not treated uniformly, further complicating applicability of the score in individual patient prediction. In a multicenter European study, including 421 patients with either primary or SMF treated with ruxolitinib, 69 had post-ET MF.[83] In this study, the MYSEC-PM outperformed the DIPSS score. Importantly, in this study, post-ET MF patients had higher platelet count but decreased hemoglobin[83] when compared to SMF evolved from PV.

The MYSEC-PM has not been validated during disease progression (eg, it is not a dynamic score). However, one study suggested that it may retain its value in different stages of the disease.[83]

Patients with SMF and unfavorable cytogenetics (defined as aberrations other than 13q– and 20q–) had a dismal prognosis in a retrospective study of the Mayo Clinic.[84] Notably, the study included a small number of patients (11 had unfavorable cytogenetics) and both post-PV and post-ET were included.

Splenic Extramedullary Hemopoiesis in Post-ET MF

Extramedullary hematopoiesis is a compensatory response that is secondary to insufficient bone marrow function and migration of hematopoietic stem cells in other organs.[85] The mechanisms leading to splenic extramedullary hematopoiesis remain a topic of research and studies are scarce.[86,87] Studies have demonstrated that morphology and immunohistochemistry can reveal differences between a Philadelphia chromosome-negative MPN and reactive conditions in the spleen tissue.[86] Hemopoiesis in the spleen can have a diffuse and nodular pattern, with at least one study reporting, in addition, a mixed picture.[86]The prognostic impact of spleen histological findings remains unclear. Furthermore, data regarding the importance of granulocytic predominance in the spleen as a prognostic marker are conflicting.

Thrombosis in Post-ET-MF

Data specifically for post-ET MF thrombosis and mitigation strategies are lacking. In the data on 330 patients with post-ET MF used for the MYSEC-PM score, the incidence of thrombosis per 100 patient-years was 2.4 (1.6–3.4).[77] In the German Study Alliance Leukemia (SAL)-MPN registry,[88] 19 patients with post-ET MF were included. Eight of them experienced a thrombotic event; the odds ratio was 1.46 for experiencing a thromboembolic event.

Bleeding in Post-ET MF

Information regarding bleeding in post-ET MF is limited, especially in the periprocedural period. Limited reports from ET exist[89] but do not provide information regarding a consensus approach; rather, they highlight the risks associated with ET and surgery, especially in patients with malignancy. High platelet counts can be associated with bleeding through a mechanism involving development of AVWS.[90] Plateletpheresis, although rarely employed, can lead to rapid lowering of platelets especially in combination with agents such as hydroxyurea.[90] In the German SAL-MPN registry,[88] bleeding events were rare, with odds for a significant bleeding event being 0.6 for post-ET MF; it was the second-lowest odds ratio in the different subgroups, with first being 0.34 for ET.

Treatment Options

JAK2 Inhibitors

The JAK2 inhibitors ruxolitinib and fedratinib have been approved for patients with primary or secondary MF. Approximately 23% of patients in a randomized clinical trial of ruxolitinib vs best available therapy in the context of MF had post-ET MF.[91] Spleen size reduction and symptom burden were independent of the MF subtype.[91] In addition, JAK2 mutation burden was decreased irrespective of the subtype of MF.

The use of ruxolitinib[92] in combination with other agents is an area of great interest, with several clinical trials in progress. Because the outcomes of post-ET MF are typically not reported separately, it is difficult to draw firm conclusions. In a study[93] of 53 patients, hydroxyurea was added to ruxolitinib for 12 patients with post-ET MF. At the initiation of ruxolitinib, the median platelet count for the whole cohort was 190,000 (range 55,000–900,000) with similar median and range at the initiation of hydoxyurea. At 48 weeks (n = 33), the median platelets decreased to 158,000 (range 78,000–523,000). Grade 3/4 thrombocytopenia was reported in 13% of patients. The median dose of hydroxyurea was 1000 mg daily.

Fedratinib is approved for patients with MF, and the pivotal studies included patients with post-ET MF. At week 24, approximately half of the patients with post-ET MF had at least a 35% spleen reduction at a dose range of 400 to 500 mg daily[94] compared to none in the placebo group.

Interferons

Interferons act with a pleiotropic mechanism in MPN that includes induction of apoptosis of hemopoietic progenitor cells and modulation of the immune system.[95,96] Interferons have been studied most extensively in ET and PV in the context of MPN. Few studies of interferon use have been published in the context of PMF or SMF, with more encouraging data for patients that fall into the low/intermediate-1 risk group. In a study of interferon[96] use in patients with PMF or SMF, 2 patients (7%) had post-ET PV. Results are not reported separately, but overall 37% were noted to achieve complete remission or partial remission.

Allogeneic Hematopoietic Stem Cell Transplantation for Post-ET MF

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is the only potential curative therapy for MF, post-PV MF, and post-ET MF, and those who are transplant-eligible do benefit from allo-HSCT but not without risk. Allo-HSCT can induce molecular remission and resolution for bone marrow fibrosis.[97,98] Available data on allo-HSCT for MF include both primary and secondary MF, without differential approaches according to subtype, restricting a precise interpretation of the results; furthermore, limited molecular data and the lack of data on JAK inhibition in the older retrospective reports/series makes the use of this data in the current era difficult. No randomized data regarding the impact of allo-HSCT in post-ET MF compared to other treatment modalities such as JAK-inhibitors is available. A recent publication of a large multicenter cohort of patients treated with (551 patients) and without (1377 patients) allo-HSCT was analyzed, showing the long-term overall survival advantage of allo-HSCT in patients with DIPSS Intermediate-1 or higher risk at the expense of early transplant-related mortality.[99]

A retrospective study from the European Society for Blood and Bone Marrow Transplantation (EBMT) analyzed outcomes of patients with PMF and SMF (including post-ET MF) that received allo-HSCT between 1995 and 2014.[100] The study did not differentiate post-ET from post-PV MF. The cohort included 2459 patients from 178 transplant centers across 15 countries. A total of 17% of the patients (n = 421) had SMF. In multivariate analysis for OS, SMF was an adverse factor. There was also a trend for worse disease-free survival, but it did not reach statistical

significance (P = .071). The relapse risk was not affected by the type of MF. Moreover, in multivariate analysis for excess mortality between 2 and 10 years from transplant for patients that were relapse-free 2 years after allo-HSCT (n = 1055), SMF was associated with inferior survival compared to PMF.[100]

The EBMT also reported the outcomes of patients with advanced PV/ET who received allo-HSCT between 1994 and 2010.[101] The total cohort included 250 patients with initial diagnosis of ET (n = 130) and PV (n = 120) who underwent allo-HSCT due to progression to MF (n = 193) or AML (n = 57). The 3-year overall survival rate and relapse incidence was 55% and 32%, respectively. The OS was significantly longer in younger patients (<55 years, 65% vs 47%) and in patients with SMF than AML (62% vs 28%).[101] The overall 3-year cumulative incidence of nonrelapse mortality was 28%, with the most frequent causes of death being relapse/progression, infections, and graft-versus-host disease (GVHD), but it was higher in older patients (>55 years, 35% vs 20%), in those transplanted from an unrelated donor rather than a related donor (34% vs 18%), and in patients with a diagnosis of AML compared to SMF (29% vs 27%). The limitations of this study include the heterogeneity of the data in terms of patient characteristics (age, comorbidities, etc), nonstandardized conditioning regimens, GVHD prophylaxis strategies, lack of cytogenetic data, and short follow-up. Nonetheless, this study confirmed that allo-HSCT is potentially curative for advanced PV/ET.[101]

In another cohort of patients that received allo-HSCT for PV (n = 42) or ET (n = 75) between 1990 and 2007,[102] 39% (n = 29) of the patients with ET had progressed to MF by the time of the allo-HSCT. The 5-year survival and progression-free survival (PFS) for patients with ET was 55% and 47%. The two main causes of death for the ET patients were organ toxicity (50%) and GVHD (22%). Transformation status did not affect engraftment- or transplant-related mortality; however, acute GVHD grade ≥2 and chronic GVHD rates at 1 and 5 years were higher in the transformed population. Relapse rates at 1 and 5 years were lower in the transformed patients. The limitations of this retrospective study include a small number of patients, analyzing post ET/PV MF together, lack of uniform preparative protocols/GVHD prophylaxis, and lack of some key parameters such as cytogenetics.[102]

A study from the Fred Hutchinson Cancer Research Center published in 2007 included patients with post-ET (n = 18) and post-PV MF (n = 12).[103] Patients with SMF had better OS compared to PMF and other conditions included in the study (eg, MF with other hematological conditions). Notably, lower platelet counts were found to be associated with mortality. The study did not report data for post ET MF separately.[103]

There are several prognostic scoring systems used in clinical practice, primarily developed in patients with PMF, that are used to predict survival and for transplant decision-making. Based on the older classifications such as DIPSS and DIPSS plus, patients with at least intermediate-2 risk were recommended to proceed with allo-HSCT given dismal outcomes.[104] However, the ability of these scoring systems is suboptimal to predict survival in SMF patients and doesn't account for the patient mutational burden or the impact of JAK2 inhibitors. Hernandez-Boluda et al[105] used data from the Spanish Registry of Myelofibrosis to compare the performance of IPSS, DIPSS, and DIPSS-plus for their discriminatory power to predict survival. This contemporary cohort included 544 patients with PMF and SMF including 133 patients with post-ET MF. The median survival of post-ET MF patients was 9.65 years and not significantly different from the patients with PMF (9.46 years), suggesting that the original MPN, either PMF or post-ET MF, had no influence on survival in this cohort with comparable prognostic factors between PMF and SMF.[105]

The MYSEC-PM and DIPSS scores were used in 83 patients with post-ET MF undergoing allo-HSCT.[106] The median follow-up for post-ET MF patients was 38 months, with a median time to transplant of 124 months. The median age was 57 years, with MYSEC-PM intermediate-2/high patients comprising ~33%. Approximately 61% of the patients had DIPSS score of at least intermediate-2. A minority of patients had triple negative (2%) and *JAK2/MPL* mutations were present in 71% with the rest having the *CALR* mutation. The study showed that MYSEC-PM could allow a more accurate prediction of post-transplant survival than DIPSS but only modestly.[106]

The impact of prognostic scores on predicting post allo-HSCT outcomes in patients with post-ET MF is not firmly established. A study from Europe introduced the molecular MF transplant scoring system (MTSS),[107] which is an integrated clinical-molecular prognostic model to predict outcomes post allo-HSCT in MF, including patients with SMF (55 patients had post-ET MF). The variables that were predictors of outcome in the model included age ≥57 years, Karnofsky performance status <90%, platelet count $<150 \times 10^9$/L, leukocyte count $>25 \times 10^9$/L, mismatched unrelated donor, presence of the *ASXL1* mutation, and a non-*CALR/MPL* genotype.[107] The MTSS enables a 4-level risk stratification including low, intermediate, high, and very high risk. The corresponding 5-year OS according to each risk group was 90% (low), 77% (intermediate), 50% (high), and 34% (very high risk). This model is applicable to post-ET MF patients, improving personalization of therapy.[107]

Management of MF with ruxolitinib prior to transplantation has become the standard of care for most patients with PMF or SMF to improve general performance status and to decrease splenomegaly.[108] The use of ruxolitinib for SMF has been reported both pre- and post-allo-HSCT[108,109] but the studies have not differentiated between post-ET and post-PV MF. Ruxolitinib withdrawal syndrome is a serious and life-threatening immune syndrome that can occur if ruxolitinib is abruptly discontinued.[110] Data from a small French study suggests that ruxolitinib withdrawal can be severe among allo-HSCT recipients; therefore, consideration of continuation of ruxolitinib throughout the transplant process should be given, especially for those patients receiving and therapeutically responding to ruxolitinib prior to allo-HSCT.[111] A recent prospective phase 1 open-label study (NTC02917096) including 18 older and high-risk patients with primary and secondary MF has evaluated the safety and feasibility of peritransplant use of ruxolitinib.[112] Two dose levels of ruxolitinib were studied (5 and 10 mg twice daily). Conditioning for this cohort was fludarabine/melphalan and GVHD prophylaxis consisting of tacrolimus and sirolimus. The graft source was mobilized peripheral blood stem cells from 8/8 human leukocyte antigens (HLA)-matched donors (related and unrelated). After a median follow up of 22.6 months, the 1-year OS and PFS were 77% and 71%, respectively. The incidence of acute GVHD grade 3 to 4 was 11% and the 1-year cumulative incidence of chronic GVHD was 42%. The incidence of infection was similar to what is expected in the allo-HSCT population. The peritransplant use of ruxolitinib was not associated with delayed engraftment or engraftment failure. The maximum tolerated dose was determined to be 10 mg twice a day. In summary, peritransplant use of ruxolitinib was safe and well tolerated in this cohort of patients PMF and SMF.[112]

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Conflicts of Interest

Dr Papadantonakis has received indirect (paid to institution) funding from AbbVie, Gilead, Takeda, and ImmunoGen. He also has received honoraria and participated in advisory board for CTI Biopharma. Dr Castaneda Puglianini reports personal fees from Celgene Corporation and personal fees from Adaptive Biotechnologies Corporation outside the submitted work. Dr Peker has served as an advisory board member and received honoraria from AbbVie, Seattle Genetics, Blueprint Medicine, and Lilly Oncology.

REFERENCES

1. Arber DA, Orazi A, Hasserjian R, et al. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. *Blood.* 2016;127:2391.
2. Barbui T, Thiele J, Gisslinger H, et al. The 2016 WHO classification and diagnostic criteria for myeloproliferative neoplasms: document summary and in-depth discussion. *Blood Cancer J.* 2018;8:15.
3. Tefferi A, Vainchenker W. Myeloproliferative neoplasms: molecular pathophysiology, essential clinical understanding, and treatment strategies. *J Clin Oncol.* 2011;29:573–582.
4. Klampfl T, Gisslinger H, Harutyunyan AS, et al. Somatic mutations of calreticulin in myeloproliferative neoplasms. *N Engl J Med.* 2013;369:2379–2390.
5. Al Assaf C, Van Obbergh F, Billiet J, et al. Analysis of phenotype and outcome in essential thrombocythemia with CALR or JAK2 mutations. *Haematologica.* 2015;100:893–897.
6. Chatterjee T, Ahuja A. Summary and review of the abstracts on Philadelphia-negative myeloproliferative neoplasms presented at Haematocon 2017. *Indian J Hematol Blood Transfus.* 2018;34:227–232.
7. Mejía-Ochoa M, Acevedo Toro PA, Cardona-Arias JA. Systematization of analytical studies of polycythemia vera, essential thrombocythemia and primary myelofibrosis, and a meta-analysis of the frequency of JAK2, CALR and MPL mutations: 2000–2018. *BMC Cancer.* 2019;19:590.
8. El Jahrani N, Cretin G, de Brevern AG. CALR-ETdb, the database of calreticulin variants diversity in essential thrombocythemia. *Platelets.* 2021;00:1–11.
9. Pietra D, Rumi E, Ferretti VV, et al. Differential clinical effects of different mutation subtypes in CALR-mutant myeloproliferative neoplasms. *Leukemia.* 2016;30:431–438.
10. Jang MA, Choi CW. Recent insights regarding the molecular basis of myeloproliferative neoplasms. *Korean J Intern Med.* 2020;35:1–11.
11. Tefferi A, Lasho TL, Guglielmelli P, et al. Targeted deep sequencing in polycythemia vera and essential thrombocythemia. *Blood Adv.* 2016;1:21–30.
12. Alduaij W, McNamara CJ, Schuh A, et al. Clinical utility of next-generation sequencing in the management of myeloproliferative neoplasms: a single-center experience. *HemaSphere.* 2018;2:e441–e449.
13. Rumi E, Cazzola M. Advances in understanding the pathogenesis of familial myeloproliferative neoplasms. *Br J Haematol.* 2017;178:689–698.
14. McMullin MF, Anderson LA. Aetiology of myeloproliferative neoplasms. *Cancers (Basel).* 2020;12:1–11.
15. Anderson LA, Duncombe AS, Hughes M, et al. Environmental, lifestyle, and familial/ethnic factors associated with myeloproliferative neoplasms. *Am J Hematol.* 2012;87:175–182.
16. Vytrva N, Stacher E, Regitnig P, et al. Megakaryocytic morphology and clinical parameters in essential thrombocythemia, polycythemia vera, and primary myelofibrosis with and without JAK2 V617F. *Arch Pathol Lab Med.* 2014;138:1203–1209.
17. Papadantonakis N, Makitalo M, McCrann D, et al. Direct visualization of the endomitotic cell cycle in living megakaryocytes: differential patterns in low and high ploidy cells. *Cell Cycle.* 2008;7:2352–2356.
18. Papadantonakis N, Ravid K. Development of megakaryocytes. In: *Molecular Basis of Hematopoiesis*. New York, NY: Springer; 2009:95–126.
19. Jacobsson S, Carneskog J, Ridell B, et al. Flow cytometric analysis of megakaryocyte ploidy in chronic myeloproliferative disorders and reactive thrombocytosis. *Eur J Haematol.* 2009;56:287–292.
20. Tomer A, Friese P, Conklin R, et al. Flow cytometric analysis of megakaryocytes from patients with abnormal platelet counts. *Blood.* 1989;74:594–601.
21. Hussein K, Van Dyke DL, Tefferi A. Conventional cytogenetics in myelofibrosis: literature review and discussion. *Eur J Haematol.* 2009;82:329–338.
22. Sever M, Kantarjian H, Pierce S, et al. Cytogenetic abnormalities in essential thrombocythemia at presentation and transformation. *Int J Hematol.* 2009;90:522–525.
23. Gangat N, Tefferi A, Thanarajasingam G, et al. Cytogenetic abnormalities in essential thrombocythemia: prevalence and prognostic significance. *Eur J Haematol.* 2009;83:17–21.
24. Sangiorgio VFI, Geyer JT, Margolskee E, et al. Myeloid neoplasms with isolated del(5q) and JAK2 V617F mutation: a "grey zone" combination of myelodysplastic and myeloproliferative features? *Haematologica.* 2020;105:E276–E279.
25. Curto-Garcia N, Ianotto JC, Harrison CN. What is pre-fibrotic myelofibrosis and how should it be managed in 2018? *Br J Haematol.* 2018;183:23–34.
26. Rumi E, Boveri E, Bellini M, et al. Clinical course and outcome of essential thrombocythemia and prefibrotic myelofibrosis according to the revised WHO 2016 diagnostic criteria. *Oncotarget.* 2017;8:101735–101744.
27. Schalling M, Gleiss A, Gisslinger B, et al. Essential thrombocythemia vs. pre-fibrotic/early primary myelofibrosis: discrimination by laboratory and clinical data. *Blood Cancer J.* 2017;7. doi: 10.1038/s41408-017-0006-y.
28. Fujiwara H. Histological evaluation of myeloproliferative neoplasms. *J Clin Exp Hematop.* 2018;58:45–50.
29. Palandri F, Latagliata R, Polverelli N, et al. Mutations and long-term outcome of 217 young patients with essential thrombocythemia or early primary myelofibrosis. *Leukemia.* 2015;29:1344–1349.
30. Carobbio A, Finazzi G, Thiele J, et al. Blood tests may predict early primary myelofibrosis in patients presenting with essential thrombocythemia. *Am J Hematol.* 2012;87:203–204.
31. Cheng C, Lai JWY, Yung Y, et al. Mutational spectrum and prognosis in Chinese patients with prefibrotic primary myelofibrosis. *eJHaem.* 2022;3:184–190.
32. Edahiro Y, Araki M, Inano T, et al. Clinical and molecular features of patients with prefibrotic primary myelofibrosis previously diagnosed as having essential thrombocythemia in Japan. *Eur J Haematol.* 2019;102:516–520.
33. Nagai T, Komatsu N, Sakata Y, et al. Iron deficiency anemia with marked thrombocytosis complicated by central retinal vein occlusion. *Intern Med.* 2005;44:1090–1092.
34. Xavier-Ferrucio J, Scanlon V, Li X, et al. Low iron promotes megakaryocytic commitment of megakaryocytic-erythroid progenitors in humans and mice. *Blood.* 2019;134:1547–1557.
35. Brissot E, Troadec MB, Loréal O, Brissot P. Iron and platelets: a subtle, under-recognized relationship. *Am J Hematol.* 2021;96:1008–1016.

36. Jimenez K, Khare V, Evstatiev R, et al. Increased expression of HIF2α during iron deficiency-associated megakaryocytic differentiation. *J Thromb Haemost.* 2015;13:1113–1127.
37. Birgegård G. Advances and challenges in the management of essential thrombocythemia. *Ther Adv Hematol.* 2015;6:142–156.
38. Chim CS, Kwong YL, Lie AKW, et al. Long-term outcome of 231 patients with essential thrombocythemia: prognostic factors for thrombosis, bleeding, myelofibrosis, and leukemia. *Arch Intern Med.* 2005;165:2651–2658.
39. Mesa RA, Niblack J, Wadleigh M, et al. The burden of fatigue and quality of life in myeloproliferative disorders (MPDs): an international internet-based survey of 1179 MPD patients. *Cancer.* 2007;109:68–76.
40. Scherber RM, Geyer HL, Mesa RA. Incorporating symptomatic assessment in therapy choice for patients with myeloproliferative neoplasms. In: Mesa RA, Harrison C, eds. *Managing Myeloproliferative Neoplasms.* Cambridge, UK: Cambridge University Press; 2016:45–55.
41. Szuber N, Vallapureddy RR, Penna D, et al. Myeloproliferative neoplasms in the young: Mayo Clinic experience with 361 patients age 40 years or younger. *Am J Hematol.* 2018;93:1474–1484.
42. Galvez C, Stein BL. Thrombocytosis and thrombosis: is there really a correlation? *Curr Hematol Malig Rep.* 2020;15:261–267.
43. Mancuso S, Accurso V, Santoro M, et al. The essential thrombocythemia, thrombotic risk stratification, and cardiovascular risk factors. *Adv Hematol.* 2020;2020:1–5.
44. Barbui T, Vannucchi AM, Buxhofer-Ausch V, et al. Practice-relevant revision of IPSET-thrombosis based on 1019 patients with WHO-defined essential thrombocythemia. *Blood Cancer J.* 2015;5:e369.
45. Rottenstreich A, Kleinstern G, Amsalem H, Kalish Y. The course of acquired von Willebrand syndrome during pregnancy among patients with essential thrombocytosis. *J Thromb Thrombolysis.* 2018;46:304–309.
46. Mehta J, Wang H, Iqbal SU, Mesa R. Epidemiology of myeloproliferative neoplasms in the United States. *Leuk Lymphoma.* 2014;55:595–600.
47. Titmarsh GJ, Duncombe AS, Mcmullin MF, et al. How common are myeloproliferative neoplasms? A systematic review and meta-analysis. *Am J Hematol.* 2014;89:581–587.
48. Choi HS, Hong J, Hwang SM, et al. Evaluation of the need for cytoreduction and its potential carcinogenicity in children and young adults with myeloproliferative neoplasms. *Ann Hematol.* 2021;100:2567–2574.
49. Grinfeld J. Prognostic models in the myeloproliferative neoplasms. *Blood Rev.* 2020;42:100713.
50. Elliott MA, Tefferi A. Thrombosis and haemorrhage in polycythaemia vera and essential thrombocythaemia. *Br J Haematol.* 2005;128:275–290.
51. Arachchillage DRJ, Laffan M. Pathogenesis and management of thrombotic disease in myeloproliferative neoplasms. *Semin Thromb Hemost.* 2019;45:604–611.
52. Campbell PJ, Scott LM, Buck G, et al. Definition of subtypes of essential thrombocythaemia and relation to polycythaemia vera based on JAK2 V617F mutation status: a prospective study. *Lancet.* 2005;366:1945–1953.
53. Sarma A, McLornan DP, Harrison CN. Spotlight on anagrelide hydrochloride for the treatment of essential thrombocythemia. *Orphan Drugs Res Rev.* 2017;7:11–23.
54. Alvarez-Larrán A, Besses C. Antiplatelet therapy in the management of myeloproliferative neoplasms. *Curr Hematol Malig Rep.* 2014;9:319–323.
55. Hultdin M, Sundström G, Wahlin A, et al. Progression of bone marrow fibrosis in patients with essential thrombocythemia and polycythemia vera during anagrelide treatment. *Med Oncol.* 2007;24:63–70.
56. Suppiah R, Shah J, Kalaycio M. Clinical significance of cytogenetics in myeloproliferative disorders. *Lab Med.* 2007;38:109–115.
57. Lanzarone G, Olivi M. The prognostic role of cytogenetics analysis in Philadelphia negative myeloproliferative neoplasms. *Medicina (Kaunas).* 2021;57:813.
58. Accurso V, Santoro M, Raso S, et al. Splenomegaly impacts prognosis in essential thrombocythemia and polycythemia vera: a single center study. *Hematol Rep.* 2019;11:95–97.
59. Barbui T, Thiele J, Ferrari A, et al. The new WHO classification for essential thrombocythemia calls for revision of available evidences. *Blood Cancer J.* 2020;10:20–25.
60. Skov V. Next generation sequencing in MPNs. Lessons from the past and prospects for use as predictors of prognosis and treatment responses. *Cancers (Basel).* 2020;12:2194.
61. Ayres-Silva JP, Bonamino MH, Gouveia ME, et al. Genetic alterations in essential thrombocythemia progression to acute myeloid leukemia: a case series and review of the literature. *Front Oncol.* 2018;8. doi: 10.3389/fonc.2018.00032.
62. Tefferi A, Vannucchi AM. Genetic risk assessment in myeloproliferative neoplasms. *Mayo Clin Proc.* 2017;92:1283–1290.
63. Nie YB, Sun M, He CK, et al. ASXL1 mutations in Chinese patients with essential thrombocythemia. *Exp Ther Med.* 2018;15:4149–4156.
64. Cerquozzi S, Tefferi A. Blast transformation and fibrotic progression in polycythemia vera and essential thrombocythemia: a literature review of incidence and risk factors. *Blood Cancer J.* 2015;5:e366–e366.
65. Wolanskyj AP, Schwager SM, McClure RF, et al. Essential thrombocythemia beyond the first decade: life expectancy, long-term complication rates, and prognostic factors. *Mayo Clin Proc.* 2006;81:159–166.
66. Rumi E, Pietra D, Ferretti V, et al. JAK2 or CALR mutation status defines subtypes of essential thrombocythemia with substantially different clinical course and outcomes. *Blood.* 2014;123:1544–1551.
67. Hong J, Lee JH, Byun JM, et al. Risk of disease transformation and second primary solid tumors in patients with myeloproliferative neoplasms. *Blood Adv.* 2019;3:3700–3708.
68. Barbui T, Thiele J, Passamonti F, et al. Survival and disease progression in essential thrombocythemia are significantly influenced by accurate morphologic diagnosis: an international study. *J Clin Oncol.* 2011;29:3179–3184.
69. Yogarajah M, Tefferi A. Leukemic transformation in myeloproliferative neoplasms: a literature review on risk, characteristics, and outcome. *Mayo Clin Proc.* 2017;92:1118–1128.
70. Tefferi A, Guglielmelli P, Lasho TL, et al. Mutation-enhanced international prognostic systems for essential thrombocythaemia and polycythaemia vera. *Br J Haematol.* 2020;189:291–302.
71. Björkholm M, Hultcrantz M, Derolf, R. Leukemic transformation in myeloproliferative neoplasms: therapy-related or unrelated? *Best Pract Res Clin Haematol.* 2014;27:141–153.
72. Birgegård G, Besses C, Griesshammer M, et al. Treatment of essential thrombocythemia in Europe: a prospective long-term observational study of 3649 high-risk patients in the evaluation of anagrelide efficacy and long-term safety study. *Haematologica.* 2018;103:51–60.
73. Kanakura Y, Shirasugi Y, Yamaguchi H, et al. A phase 3b, multicenter, open-label extension study of the long-term safety of anagrelide in Japanese adults with essential thrombocythemia. *Int J Hematol.* 2018;108:491–498.
74. Mazzucconi MG, Baldacci E, Latagliata R, et al. Anagrelide in essential thrombocythemia (ET): results from 150 patients over 25 years by the "Ph1-negative Myeloproliferative Neoplasms Latium Group". *Eur J Haematol.* 2020;105:335–343.
75. Björkholm M, Derolf, R, Hultcrantz M, et al. Treatment-related risk factors for transformation to acute myeloid leukemia and myelodysplastic syndromes in myeloproliferative neoplasms. *J Clin Oncol.* 2011;29:24102415.
76. Orazi A. Histopathology in the diagnosis and classification of acute myeloid leukemia, myelodysplastic syndromes, and myelodysplastic/myeloproliferative diseases. *Pathobiology.* 2007;74:97–114.

77. Passamonti F, Giorgino T, Mora B, et al. A clinical-molecular prognostic model to predict survival in patients with post polycythemia vera and post essential thrombocythemia myelofibrosis. *Leukemia.* 2017;31:2726–2731.
78. Masarova L, Bose P, Daver N, et al. Patients with post-essential thrombocythemia and post-polycythemia vera differ from patients with primary myelofibrosis. *Leuk Res.* 2017;59:110–116.
79. Rotunno G, Pacilli A, Artusi V, et al. Epidemiology and clinical relevance of mutations in postpolycythemia vera and postessential thrombocythemia myelofibrosis: A study on 359 patients of the AGIMM group. *Am J Hematol.* 2016;91:681–686.
80. Hernández-Boluda J-CC, Pereira A, Correa J-GG, et al. Performance of the myelofibrosis secondary to PV and ET-prognostic model (MYSEC-PM) in a series of 262 patients from the Spanish registry of myelofibrosis. *Leukemia.* 2018;32:553–555.
81. Wanquet A, Courtier F, Guille A, et al. Mutation patterns in essential thrombocythemia, polycythemia vera and secondary myelofibrosis. *Leuk Lymphoma.* 2019;60:1289–1293.
82. Courtier F, Carbuccia N, Garnier S, et al. Genomic analysis of myeloproliferative neoplasms in chronic and acute phases. *Haematologica.* 2017;102:e11–e14.
83. Palandri F, Palumbo GA, Iurlo A, et al. Differences in presenting features, outcome and prognostic models in patients with primary myelofibrosis and post-polycythemia vera and/or post-essential thrombocythemia myelofibrosis treated with ruxolitinib: new perspective of the MYSEC-PM in a large multicenter study. *Semin Hematol.* 2018;55:248–255.
84. Dingli D, Schwager SM, Mesa RA, et al. Presence of unfavorable cytogenetic abnormalities is the strongest predictor of poor survival in secondary myelofibrosis. *Cancer.* 2006;106:1985–1989.
85. Tremblay D, Schwartz M, Bakst R, et al. Modern management of splenomegaly in patients with myelofibrosis. *Ann Hematol.* 2020;99:1441–1451.
86. O'Malley DP, Kim YS, Perkins SL, et al. Morphologic and immunohistochemical evaluation of splenic hematopoietic proliferations in neoplastic and benign disorders. *Mod Pathol.* 2005;18:1550–1561.
87. Prakash S, Hoffman R, Barouk S, et al. Splenic extramedullary hematopoietic proliferation in Philadelphia chromosome-negative myeloproliferative neoplasms: heterogeneous morphology and cytological composition. *Mod Pathol.* 2012;25:815–827.
88. Kaifie A, Kirschner M, Wolf D, et al. Bleeding, thrombosis, and anticoagulation in myeloproliferative neoplasms (MPN): analysis from the German SAL-MPN-registry. *J Hematol Oncol.* 2016;9. doi: 10.1186/s13045-016-0242-9.
89. Zhu Y, Jiang H, Chen Z, et al. Abdominal surgery in patients with essential thrombocythemia. *Medicine (Baltimore).* 2017;96:e8856.
90. Boddu P, Falchi L, Hosing C, et al. The role of thrombocytapheresis in the contemporary management of hyperthrombocytosis in myeloproliferative neoplasms: a case-based review. *Leuk Res.* 2017;58:14–22.
91. Verstovsek S, Mesa RA, Gotlib J, et al. A double-blind, placebo-controlled trial of ruxolitinib for myelofibrosis. *N Engl J Med.* 2012;366:799–807.
92. Li Y, Zhu S, Liu W, et al. Ruxolitinib-based combinations in the treatment of myelofibrosis: worth looking forward to. *Ann Hematol.* 2020;99:1161–1176.
93. Breccia M, Luciano L, Pugliese N, et al. Efficacy and safety of ruxolitinib and hydroxyurea combination in patients with hyperproliferative myelofibrosis. *Ann Hematol.* 2019;98:1933–1936.
94. Pardanani A, Harrison C, Cortes JE, et al. Safety and efficacy of fedratinib in patients with primary or secondary myelofibrosis: a randomized clinical trial. *JAMA Oncol.* 2015;1:643–651.
95. How J, Hobbs G. Use of interferon alfa in the treatment of myeloproliferative neoplasms: perspectives and review of the literature. *Cancers (Basel).* 2020;12:1–13.
96. Silver RT, Barel AC, Lascu E, et al. The effect of initial molecular profile on response to recombinant interferon-α (rIFNα) treatment in early myelofibrosis. *Cancer.* 2017;123:2680–2687.
97. Wolschke C, Badbaran A, Zabelina T, et al. Impact of molecular residual disease post allografting in myelofibrosis patients. *Bone Marrow Transplant.* 2017;52:1526–1529.
98. Thiele J, Kvasnicka HM, Dietrich H, et al. Dynamics of bone marrow changes in patients with chronic idiopathic myelofibrosis following allogeneic stem cell transplantation. *Histol Histopathol.* 2005;20:879–889.
99. Gowin K, Ballen K, Ahn KW, et al. Survival following allogeneic transplant in patients with myelofibrosis. *Blood Adv.* 2020;4:1966–1973.
100. Robin M, De Wreede LC, Wolschke C, et al. Long-term outcome after allogeneic hematopoietic cell transplantation for myelofibrosis. *Haematologica.* 2019;104:1782–1788.
101. Lussana F, Rambaldi A, Finazzi MC, et al. Allogeneic hematopoietic stem cell transplantation in patients with polycythemia vera or essential thrombocythemia transformed to myelofibrosis or acute myeloid leukemia: a report from the MPN subcommittee of the Chronic Malignancies Working Party of the European Group for Blood and Bone Marrow Transplantation. *Haematologica.* 2014;99:916–921.
102. Ballen KK, Woolfrey AE, Zhu X, et al. Allogeneic hematopoietic cell transplantation for advanced polycythemia vera and essential thrombocythemia. *Biol Blood Marrow Transplant.* 2012;18:1446–1454.
103. Kerbauy DMB, Gooley TA, Sale GE, et al. Hematopoietic cell transplantation as curative therapy for idiopathic myelofibrosis, advanced polycythemia vera, and essential thrombocythemia. *Biol Blood Marrow Transplant.* 2007;13:355–365.
104. Salit RB, Deeg HJ. Transplant decisions in patients with myelofibrosis: should mutations be the judge? *Biol Blood Marrow Transplant.* 2018;24:649–658.
105. Hernández-Boluda JC, Pereira A, Correa JG, et al. Prognostic risk models for transplant decision-making in myelofibrosis. *Ann Hematol.* 2018;97:813–820.
106. Gagelmann N, Eikema D-JJ, de Wreede LC, et al. Comparison of dynamic international prognostic scoring system and MYelofibrosis SECondary to PV and ET prognostic model for prediction of outcome in polycythemia vera and essential thrombocythemia myelofibrosis after allogeneic stem cell transplantation. *Biol Blood Marrow Transplant.* 2019;25:e204–e208.
107. Gagelmann N, Ditschkowski M, Bogdanov R, et al. Comprehensive clinical-molecular transplant scoring system for myelofibrosis undergoing stem cell transplantation. *Blood.* 2019;133:2233–2242.
108. Salit RB, Scott BL, Stevens EA, et al. Pre-hematopoietic cell transplant ruxolitinib in patients with primary and secondary myelofibrosis. *Bone Marrow Transplant.* 2020;55:70–76.
109. Zhang L, Yang F, Feng S. Allogeneic hematopoietic stem-cell transplantation for myelofibrosis. *Ther Adv Hematol.* 2020;11:204062072090600.
110. Tefferi A, Pardanani A. Serious adverse events during ruxolitinib treatment discontinuation in patients with myelofibrosis. *Mayo Clin Proc.* 2011;86:1188–1191.
111. Robin M, Francois S, Huynh A, et al. Ruxolitinib before allogeneic hematopoietic stem cell transplantation (HSCT) in patients with myelofibrosis: a preliminary descriptive report of the JAK ALLO study, a phase II trial sponsored by GOELAMS-FIM in collaboration with the SFGMTC. *Blood.* 2013;122:306–306.
112. Ali H, Tsai N-C, Synold T, et al. Peritransplant ruxolitinib administration is safe and effective in patients with myelofibrosis: a pilot open-label study. *Blood Adv.* 2021. doi: 10.1182/bloodadvances.2021005035.

Association between Methylene-Tetrahydrofolate Reductase C677T Polymorphism and Human Immunodeficiency Virus Type 1 Infection in Morocco

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Abbreviations: $CD4^+$, cluster of differentiation 4; *MTHFR*, methylenetetrahydrofolate reductase; 5-MeTHF, 5-methyltetrahydrofolate; Hcy, homocysteine; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; PLWH, people living with HIV-1; CHU, University Hospital Center; OD, odds ratio; CI, confidence interval; ANOVA, analysis of variance; ART, antiretroviral therapy; BMI, body mass index; SNV, single nucleotide variant; BP, base pair.

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ABSTRACT

Human immunodeficiency virus type 1 (HIV-1) infection varies substantially among individuals. One of the factors influencing viral infection is genetic variability. Methylenetetrahydrofolate reductase (*MTHFR*) C677T polymorphism is a genetic factor that has been correlated with different types of pathologies, including HIV-1. The *MTHFR* gene encodes the MTHFR enzyme, an essential factor in the folate metabolic pathway and in maintaining circulating folate and methionine at constant levels, thus preventing the homocysteine accumulation. Several studies have shown the role of folate on $CD4^+$ T lymphocyte count among HIV-1 subjects. In this case-control study we aimed to determine the association between the *MTHFR* C677T polymorphism and HIV-1 infection susceptibility, AIDS development, and therapeutic outcome among Moroccans. The C677T polymorphism was genotyped by polymerase chain reaction followed by fragment length polymorphism digestion in 214 participants living with HIV-1 and 318 healthy controls. The results of the study revealed no statistically significant association between *MTHFR* C677T polymorphism and HIV-1 infection ($P > .05$). After dividing HIV-1 subjects according to their AIDS status, no significant difference was observed between C677T polymorphism and AIDS development ($P > .05$). Furthermore, regarding the treatment response outcome, as measured by HIV-1 RNA viral load and $CD4^+$ T cell counts, no statistically significant association was found with *MTHFR* C677T polymorphism. We conclude that, in the genetic context of the Moroccan population, *MTHFR* C677T polymorphism does not affect HIV-1 infection susceptibility, AIDS development, or response to treatment. However, more studies should be done to investigate both genetic and nutritional aspects for more conclusive results.

Since its first isolation in 1983,[1,2] human immunodeficiency virus type 1 (HIV-1) has emerged widely in the world.[3] Genetic variability[4,5] and sociodemographic[6] and behavioral factors[7] are the main causes of this ongoing pandemic. Globally, statistics from the Joint United Nations Programme on HIV/AIDS showed that approximately 1.5 million (1.0 million–2.0 million) people became newly infected and 680,000 (480,000–1.0 million) people died from AIDS-related illnesses at the end of 2020. Meanwhile, there are up to 37.7 million (30.2 million–45.1 million) people currently living with HIV infection worldwide.[3] In Morocco, an estimated 22,000 (19,000–24,000) individuals were living with HIV/AIDS in 2020.[8]

The HIV-1 infection progresses through several stages, including acute primary infection,[9,10] the asymptomatic stage, and the symptomatic stage called AIDS.[11] Disease development is closely related to many environmental factors[12,13] such as nutrient intake, since micronutrients have been shown to be essential for maintaining proper immunologic functions.[14] It has been shown that nutrition is significantly associated with mortality and morbidity among HIV^+/AIDS patients[15] Moreover, micronutrient supplementation has revealed several clinical benefits to the patient, including improvement in immune reconstitution and

reduction in mitochondrial toxicity.[16] Vitamin B_{12} and folate deficiencies have been found in a larger number of HIV-infected subjects.[17] They are associated with a host of neurological and hematological abnormalities, neurocognitive dysfunction, and other related disorders.[18] In fact, folic acid deficiency depresses the cell-mediated immunity response and vitamin B_{12} deficiency impairs neutrophil function. Some studies have shown that folate deficiency induces a decrease in the proliferation of cluster of differentiation 4 ($CD4^+$) lymphocytes and cellular apoptosis, which explains low resistance to infection and the rapid progression to the AIDS stage.[19] The absorption of folic acid appears to be significantly impaired in HIV infection, regardless of the stage of the disease.[20] The supplementation in folate increases the number of $CD4^+$ T cells[21] and restores the proliferation of lymphocyte cells in HIV-infected subjects.[19]

In this regard, the role of methylenetetrahydrofolate reductase (MTHFR), a crucial element of folate metabolism, has been demonstrated. It catalyzes the irreversible reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate (5-MeTHF), which is necessary for the remethylation of homocysteine (Hcy) to methionine.[22] This conversion is catalyzed by methionine synthase, which is found in human tissues where vitamin B_{12} is used as a cofactor.[23] Normal MTHFR activity helps preserve methionine and folate in the bloodstream at constant levels, preventing Hcy accumulation.[24]

As with many metabolic cycles in humans, the remethylation and transsulfuration of methionine are strongly influenced by polymorphisms in several of its effector enzymes. Polymorphisms in the gene encoding for *MTHFR* could underlie its enzymatic activity.[25] A common polymorphism in *MTHFR*, C677T (rs1801133), which is a substitution of cytosine by thymine at nucleotide 677 of the *MTHFR* gene (677C > T), has been the most widely studied. It is translated into the protein sequence by the substitution of an alanine by a valine on the 222 codons.[26,27] This mutation steers to the formation of a reduced activity enzyme variant (thermolabile variant) causing a reduction in folic activity and a decrease in the body's ability to transform Hcy to methionine.[27,28]

Although several studies have investigated the *MTHFR* C677T polymorphism and its relationship to serum folate and vitamin B_{12} levels among HIV-1 subjects, no study has described the association between this C677T polymorphism and susceptibility to HIV-1 infection, AIDS progression, and therapeutic outcome. This study addresses these aspects among Moroccan HIV-1–infected individuals.

Materials and Methods

Study Population

In this present case-control study, a total of 532 Moroccan individuals were recruited. The cohort consisted of 214 persons living with HIV-1 (PLWH) and 318 healthy controls. The controls were HIV-negative healthy individuals. Diagnosis of HIV-1 infection was established based on the presence of anti-HIV antibodies by using enzyme-linked immunosorbent assay and Western blot methods. The HIV-1 individuals were stratified into 2 groups according to their AIDS stage based on the Center for Disease Control and Prevention 1993 classification.[29] A total of 100 participants were at the clinical latency period and 148 were at the AIDS stage. The age for subjects in the clinical latency period ranged from 19 to 60 years, for the participants with AIDS from 18 to 62 years, and for controls, ranged from 22 to 84 years. The main inclusion criterion adopted for our study population (cases and controls) was the age of the subjects, which had to be >18 years. Moreover, the controls selected in this study had to be individuals who do not suffer from any chronic disease or bacterial, parasitic, or viral infection. All HIV-1 subjects were followed at the Infectious Disease Center, CHU Ibn Rochd of Casablanca from September 2002 to July 2015. Sociodemographic parameters and clinical data were extracted in detail from the department clinical database, Nadis.[30] The healthy controls were recruited by the Pasteur Institute of Casablanca (Morocco). All study participants provided informed consent and the study was approved by the ethics committee for biomedical research at Mohammed V University at Rabat, Morocco, with the given registration number N°24/18.

Isolation of Human Genomic Material and C677T Polymorphism Genotyping

The DNA samples were prepared from peripheral blood samples collected on EDTA-containing tubes. All individual's DNA was extracted using the standard method of phenol-chloroform extraction[44] Genotyping of C677T *MTHFR* polymorphism was performed by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. The C/T polymorphism was evaluated using a primer pair (forward 5'TGAAGGAGAAGGTGTCTGCGGGA3'and reverse 5'AGGACGGTG GCGTGAGAGTG3').[27] Conventional PCR amplification was performed using a 2720 Thermal Cycler (Applied Biosystems) in a final volume of 25 µL containing 5 µL 5× PCR buffer, 2 µL forward primer, 2 µL reverse primer (10 µmol/L), 0.25 µL MyTaq DNA polymerase (Bioline), and 20 ng genomic DNA; nuclease-free water was added to achieve the final volume. The thermal cycling conditions were an initial denaturation of 95°C for 4 min followed by 35 regular cycles (95°C 30 s, 64°C 30 s, and 72°C 30 s) and a final extension of 72°C for 10 min. The PCR products were then digested overnight at 37°C with the restriction enzyme HinfI (Thermo Fisher) according to the manufacturer's protocol. The digested PCR-RFLP products were analyzed on an agarose gel of 3%. The C allele coding for C677T was represented by an undigested fragment of 198 bp and a T allele by 2 fragments of 175 and 23 bp.

Statistical Analysis

Continuous variables were recapitulated as mean ± SD or as median and interquartile range, and categorical variables as frequency and percentage. SPSS software package version 21.0 (IBM) and GraphPad PRISM version 7.00 (GraphPad Software) were used for statistical analysis. The distribution of different genotypes in the total population was tested for Hardy-Weinberg equilibrium. χ^2 tests were used to determine the differences between the frequencies of genotypes and alleles among the different groups.

Crude odds ratios (ORs) with their 95% confidence intervals (CIs) were applied to assess the strength of association between C677T polymorphism and AIDS development. The adjusted ORs were calculated with the corresponding 95% CIs. A $P < .05$ was considered statistically significant. The Mann-Whitney *U* test, ordinary 1-way analysis of variance (ANOVA), and χ^2 tests were used when appropriate. All tests were 2-sided.

Results

Clinicobiological Characteristics

In this case-control study, we enrolled 318 healthy individuals (131 males and 187 females) identified here as controls and 214 PLWH

(94 males and 120 females) identified as cases. The median age of HIV-1 cases and their matched controls was 35 years and 53 years, respectively. Among the 214 PLWH, 148 patients had reached the AIDS stage and 66 were asymptomatic (non-AIDS patients). The baseline characteristics of HIV-1 subjects according to demographic, clinical, and viral status are shown in **TABLE 1**. No statistical difference was observed for age, sex, antiretroviral therapy (ART), and viral load levels between the AIDS and non-AIDS groups ($P > .05$). However, the non-AIDS group exhibited significantly higher levels of $CD4^+$ T count cells ($P < .0001$). Weight and body mass index (BMI) were also significantly elevated in the non-AIDS group.

TABLE 1. Demographic and Clinical Characteristics of the Study Subjects[a]

Characteristic	AIDS (n = 148)	Non-AIDS (n = 66)	*P* Value
Age (y)	36 (18–62)	32 (19–60)	.2349
Sex (F/M)	81/67	39/27	.5536
Weight (kg)	60 (30–112)	70 (48–118)	<.0001
Size (cm)	165 (143–190)	166 (143–187)	.7314
BMI (kg/m^2)	37.5 (17.65–67.47)	42.42 (27.91–72.84)	<.0001
ART	133 (89.86)	60 (90.91)	.8130
$CD4^+$ T count (cell/μL)	145 (2.5–1 679)	506.2 (57–2 520)	<.0001
Viral load (log_{10} copies/mL)	5.10 (1.30–6.90)	4.34 (1.30–6.56)	.0279

ART, antiretroviral therapy; BMI, body mass index.
[a]*Values are presented as mean (range), number (%), or mean.*

Association of the C677T Polymorphism with HIV-1 Infection and AIDS Development

The *MTHFR* C677T single nucleotide variant (SNV) genotyping in cases and controls showed that the allele and genotype distributions respected the Hardy-Weinberg equilibrium in both groups ($P > .05$) (**TABLE 2**).

The impact of the C677T variant on HIV-1 infection outcome is displayed in **TABLE 2**. In healthy subjects, 144 (45.28%) out of 318 were homozygous for the wild type C/C, 139 (43.71%) were heterozygous C/T, and 35 (11.01%) were homozygous for T/T, with a T allele frequency of 32.87% and a C allele frequency of 67.14%.

In the HIV-1 $^+$ group, the homozygous C/C genotype was 46.26% and T/T genotype was 7.94%. There were no significant differences in the distribution of the genotype frequencies between the 2 groups ($P > .05$). When the C/C genotype was used as the reference group, none of the C/T and T/T genotypes were associated with HIV-1 infection (OR, 1.026, 95% CI = .713–1.475 and OR 0.706, 95% CI, 0.375–1.331; respectively). Frequency differences of the C677T alleles were also not significant in PLWH compared to healthy controls ($P > .05$).

To investigate whether the C677T SNV is associated with clinical status, we subdivided our PLWH group into AIDS and non-AIDS subjects. Our data showed that no significant differences were observed between those 2 groups ($P > .05$). The results of the C677T genotypes and alleles distributions in AIDS and non-AIDS subjects are shown in **TABLE 3**.

Association between the C677T Polymorphism and $CD4^+$ T-Cell Counts and HIV-1 RNA Viral Load

To investigate the effect of the C677T genotypes on ART outcome, $CD4^+$ cell count levels and HIV-1 RNA viral load were analyzed before and after ART among 214 PLWH. The statistical data revealed no significant associations between the genotypes of C677T SNV and $CD4^+$ T-cell levels before and after ART ($P > .05$) (**FIGURE 1A** and **1B**, respectively). Likewise, the analysis of the HIV-1-RNA viral loads in HIV-1 seropositive individuals indicated no significant association with the C677T genotypes at baseline and after ART ($P > .05$) (**FIGURE 1C** and **1D**, respectively).

Discussion

The accumulation of data on the high correlation between B-complex vitamins and genetic variants that alter Hcy metabolism in HIV-1 carriers sparked our interest in studying the involvement of *MTHFR* genotyping, as it is closely linked to folate metabolism in the methionine remethylation cycle. The C677T is a major missense polymorphism in *MTHFR* that confers enzyme thermolability at 46°C and a decrease in its specific activity by approximately 35%.[31] This decrease in the activity of the enzyme in TT genotype carriers is responsible for significantly elevated serum Hcy levels.[32] The differences observed in the C677T frequencies can be attributed to randomness, since the genotypic distribution of the *MTHFR* C677T polymorphism has been shown to be strongly associated with ethnicity.[33–35] However, in HIV-1 individuals, most studies on this topic were on both vitamin B_{12} and folate levels, whereas few have focused on its genetic aspect. This provides information only on the nutritional profile altered by the infection outcome with a possible effect of ART response. Thereby, we proposed to examine the *MTHFR* C677T polymorphism and its impact on HIV-1 infection susceptibility, AIDS development, and treatment response outcomes among a Moroccan population.

The aim of this population-based case-control study was to investigate the possible role of *MTHFR* C677T polymorphism as a risk factor for HIV-1 infection susceptibility and the development of AIDS in a cohort of Moroccan individuals. Result analysis showed no significant association between C677T polymorphism and susceptibility to HIV-1 infection. These results are consistent with the only published similar study, carried out in HIV-1 Brazilian subjects.[36] Our finding was also comparable with that of ElDeeb et al,[37] who reported no statistically significance differences in the distributions of *MTHFR* C677T genotypes and hepatitis C virus genotype 4 infection. Furthermore, genotype and allelic frequency distributions showed no significant association between C677T polymorphism and AIDS development. Likewise, similar results in which no correlation has been demonstrated between *MTHFR* C677T genotypes and other viral infections have been published.[33,38] However, *MTHFR* genotype TT × CT/CC polymorphism showed an association with the degree of steatosis and fibrosis among subjects infected with hepatitis C virus genotype non-1 ($P < .05$).[35] Likewise, it has been shown that *MTHFR* C677T polymorphism contributed to the development of fibrosis in patients with chronic hepatitis C virus infection.[39] Jiao et al[40] also showed that the TT genotype and T allele of *MTHFR* C677T may significatively confer a protective effect on disease progression to hepatocellular carcinoma in HBV-infected individuals. Moreover, Botezatu et al[41] showed that the T allele of *MTHFR* C677T polymorphism was statistically associated with cervical dysplastic lesions in human papilloma virus–positive infection. In the light of these observations, our results may be explained by the fact that there is always a difference between a predictor variable and an outcome variable that depends on the sample size, the actual magnitude of that association in the target

TABLE 2. Susceptibility Study by Comparing C677T-*MTHFR* Genotypes/Alleles between Cases and Controls[a]

Genotype/Allele	PLWH (n = 214)	Control Subjects (n = 318)	OR (95% CI)	χ^2	*P* Value
CC	99 (46.26)	144 (45.28)	1 (reference)	—	—
CT	98 (45.79)	139 (43.71)	1.026 (0.713–1.475)	0.02	.892
TT	17 (7.94)	35 (11 .01)	0.706 (0.375–1.331)	1.16	.281
Dominance model	99/115	144/174	0.961 (0.679–1.361)	0.05	.824
Recessive model	197/17	283/35	1.433 (0.781–2.631)	1.36	.243
C	0.692 ± 0.0215	0.671 ± 0.0187	1 (reference)	—	—
T	0.308 ± 0.0215	0.329 ± 0.0187	0.911 (0.700–1.186)	0.48	.489
HWE	$\chi^2 = 1.156$; $P = .338$	$\chi^2 = 0.028$; $P = .899$			

CI, confidence interval; HWE, Hardy-Weinberg equilibrium; OR, odds ratio; PLWH, people living with HIV-1.
[a]Values are presented as number (%) or mean ± SD.

TABLE 3. Disease Progression Study and Distribution of C677T *MTHFR* Polymorphism Genotypes among People Living with HIV-1 Group (AIDS vs Non-AIDS)[a]

Genotype/Allele	AIDS Subjects (n = 148)	Non-AIDS Subjects (n = 66)	OR (95% CI)	χ^2	*P* Value
CC	69 (46.62)	30 (45.45)	1 (reference)	—	—
CT	67 (45.27)	31 (46.97)	0.940 (0.514–1.719)	0.04	.840
TT	12 (8.11)	5 (7.58)	1.043 (0.338–3.223)	0.01	.941
Dominance model	69/79	30/36	0.954 (0.533–1.708)	0.03	.874
Recessive model	136/12	61/5	0.929 (0.314–2.752)	0.02	.894
C	0.693 ± 0.0260	0.689 ± 0.0383	1 (reference)	—	—
T	0.307 ± 0.0260	0.311 ± 0.0383	0.985 (0.632–1.535)	0	.948
HWE	$\chi^2 = 0.589$; $P = .563$	$\chi^2 = 0.618$; $P = .570$			

CI, confidence interval; HWE, Hardy-Weinberg equilibrium; OR, odds ratio.
[a]Values are presented as number (%) or mean ± SD.

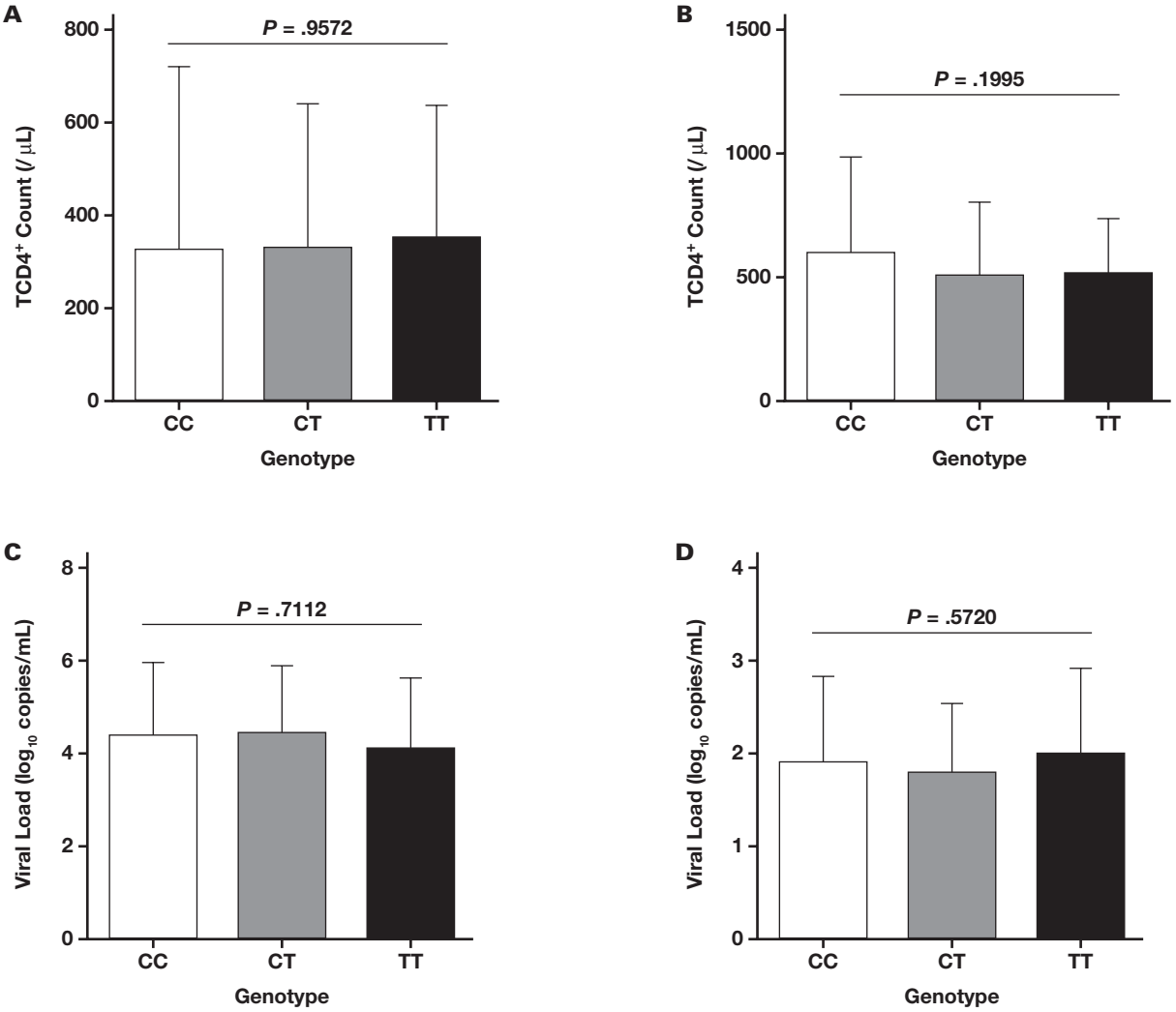
population, and other factors. In fact, no association could be found when there actually is an association (type II error). This may occur if the investigator fails to reject a null hypothesis that is actually false in the population.[42]

Despite the effectiveness of ART against HIV-1, AIDS remains one of the most significant and challenging infectious diseases worldwide.[43] The efficacy of ART is highly variable between individuals, which highlights the importance of the role of genetics in the response to treatments.[44,45] Very little data on the effect of ART on the metabolism of Hcy are available. Hence the interest of this work, which aimed to investigate for the first time the impact of the *MTHFR* C677T polymorphism, a key factor in this metabolism, on the response to treatment in Moroccan HIV-1–infected subjects. Based on our results, the observed differences in $CD4^+$ T cell levels and HIV-1 viral load values before and after treatment showed no significant correlation with the distribution of C677T genotype frequencies. These results are in accordance with a previous study that indicated that ART was not associated with variations in serum folate and B_{12} levels among HIV-1–infected subjects.[36] In addition, no association was found between either the *MTHFR* C677T polymorphism and the outcome of treatment with PEGylated interferon α plus ribavirin in hepatocellular carcinoma patients[46] or between hyper Hcy and length of exposure to ART or protease inhibitors.[47] So far, no study has been able to prove the link between this polymorphism and treatment response in infectious diseases.

The absence of data on serum folate of participants should be considered, since the *MTHFR* C677T polymorphism is associated with low folate levels, which lead to changes in the DNA methylation pattern of host cells as well as in the HIV-1 genome, causing changes in gene expression that could lead to infection persistence. On the other hand, DNA integrity may be compromised, increasing the possibility of integration of HIV-1 genetic material into host DNA, an essential step in the retroviral replication cycle, which will cause a high HIV-1 production and release of many new copies invading other cells[48,49] and thus the progression of AIDS and the host's ability to respond to ART. Moreover, lack of information about other factors must be considered, like sexual behavior, multiparity, smoking and drinking habits, the presence of other sexually transmitted diseases, and other genetic factors.

Further studies are needed to carry out more in-depth investigations of *MTHFR* genetic aspects and their role in treatment response outcomes among those infected with HIV-1 and the increased life expectancy of such persons. The results found in our study could have been due to the sample size and confounding variables not analyzed in this cohort. Other studies should focus on the combination between the genetic aspect and the increase or decrease of serum vitamin B_{12} and folate levels among the HIV-1–infected population. They should also focus on other *MTHFR* polymorphisms.

FIGURE 1. Association study between *MTHFR* C677T polymorphism and response to antiretroviral therapy (ART) in HIV-1 subjects. A, Comparison of C677T genotypes frequencies and CD4⁺ T-cell counts in people living with HIV-1 (PLWH) before ART. B, Comparison of C677T genotypes frequencies and CD4⁺ T-cell counts in PLWH after ART. C, Comparison of C677T genotypes frequencies and HIV-1 RNA viral load in PLWH before ART. D, Comparison of C677T genotypes frequencies and HIV-1 RNA viral load in PLWH after ART. All data are presented as mean with median. Statistical tests were performed using Mann-Whitney *U* test and analysis of variance.



Conclusion

This work focused on investigating the effect of *MTHFR* C677T genotypes in the HIV-1–infected Moroccan population, which could be considered as a key factor in the pathophysiology of AIDS in these individuals. This relationship has never been explored. We hypothesized that there may be a relationship between the *MTHFR* C677T polymorphism and susceptibility of HIV-1 infection, AIDS development, and treatment response. However, the results showed no association. Our results must be interpreted within the limitations of cross-sectional studies; that is, the small sample size and heterogeneity between studies with respect to allele frequencies and penetrance in different ethnicities, in addition to variations in age, lifestyle behaviors, and medication usage. Moreover, since HIV-1 is a disease that remains poorly understood, a multicenter study investigating factors relating to both genetics and nutrition considering the possible differences in folate consumption among participants is recommended. This could then explain the discrepancies among different populations for this very precise study. Further studies in Moroccan and other populations are needed to reassess HIV-1 infection susceptibility, AIDS development, and treatment outcome response in the HIV-1–infected population to improve survival. In this preliminary study, our data suggest that the *MTHFR* C677T polymorphism does not affect the HIV-1 infection.

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REFERENCES

1. Barre-Sinoussi F, Chermann JC, Rey F, et al. Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). *Science.* 1983;220(4599):868–871. doi:10.1126/science.6189183.

2. Gallo RC, Sarin PS, Gelmann EP, et al. Isolation of human T-cell leukemia virus in acquired immune deficiency syndrome (AIDS). *Science.* 1983;220(4599):865–867.
3. Global HIV & AIDS Statistics — 2020 Fact Sheet. https://www.unaids.org/en/resources/fact-sheet#:~:text=GLOBAL%20HIV%20STATISTICS&text=37.7%20million%20%5B30.2%20million%E2%80%9345.1,AIDS%2Drelated%20illnesses%20in%202020.
4. Blanco M, Machado LY, Díaz H, Ruiz N, Romay D, Silva E. HIV-1 genetic variability in Cuba and implications for transmission and clinical progression. *MEDICC Rev.* 2015;17(4):25–31.
5. Abidi SH, Shahid A, Lakhani LS, et al. HIV-1 progression links with viral genetic variability and subtype, and patient's HLA type: analysis of a Nairobi-Kenyan cohort. *Med Microbiol Immunol.* 2014;203(1):57–63.
6. Hudelson C, Cluver L. Factors associated with adherence to antiretroviral therapy among adolescents living with HIV/AIDS in low- and middle-income countries: a systematic review. *AIDS Care.* 2015;27(7):805–816.
7. Rosińska M, Sierosławski J, Wiessing L. High regional variability of HIV, HCV and injecting risks among people who inject drugs in Poland: comparing a cross-sectional bio-behavioural study with case-based surveillance. *BMC Infect Dis.* 2015;21(15):83. PMID: 25879904.
8. *Maroc*. Morocco: UNAIDS; 2020. https://www.unaids.org/fr/regionscountries/countries/morocco.
9. Clark SJ, Saag MS, Decker WD, et al. High titers of cytopathic virus in plasma of patients with symptomatic primary HIV-1 infection. *N Engl J Med.* 1991;324(14):954–960.
10. Daar ES, Moudgil T, Meyer RD, Ho DD. Transient high levels of viremia in patients with primary human immunodeficiency virus type 1 infection. *N Engl J Med.* 1991;324(14):961–964.
11. Buchbinder SP, Katz MH, Hessol NA, O'Malley PM, Holmberg SD. Long-term HIV-1 infection without immunologic progression. *AIDS.* 1994;8(8):1123–1128.
12. Nichols L, Tchounwou PB, Mena L, Sarpong D. The effects of environmental factors on persons living with HIV/AIDS. *Int J Environ Res Public Health.* 2009;6(7):2041–2054.
13. Alfsen A. Environmental factors in HIV/AIDS epidemic development: new perspectives for gender equity and global protection against HIV transmission. *Ann N Y Acad Sci.* 2004;1023(1):164–174.
14. Cunningham-Rundles S, McNeeley DF, Moon A. Mechanisms of nutrient modulation of the immune response. *J Allergy Clin Immunol.* 2005;115(6):1119–1128.
15. Suttajit M. Advances in nutrition support for quality of life in HIV+/AIDS. *Asia Pac J Clin Nutr J*. 2007;16(suppl 1):318-322. PMID: 17392127.
16. Drain PK, Kupka R, Mugusi F, Fawzi WW. Micronutrients in HIV-positive persons receiving highly active antiretroviral therapy. *Am J Clin Nutr.* 2007;85(2):333–345.
17. Tilkian SM, Lefevre G. Altered folate metabolism in early HIV infection. *JAMA.* 1988;259(21):3128–3129.
18. Adhikari PMR, Chowta MN, Ramapuram JT, Rao S, Udupa K, Acharya SD. Prevalence of vitamin B12 and folic acid deficiency in HIV-positive patients and its association with neuropsychiatric symptoms and immunological response. *Indian J Sex Transm Dis AIDS.* 2016;37(2):178–184.
19. Courtemanche C, Elson-Schwab I, Mashiyama ST, Kerry N, Ames BN. Folate deficiency inhibits the proliferation of primary human CD8+ T lymphocytes in vitro. *J Immunol.* 2004;173(5):3186–3192.
20. Revell P, O'Doherty MJ, Tang A, Savidge GF. Folic acid absorption in patients infected with the human immunodeficiency virus. *J Intern Med.* 1991;230(3):227–231.
21. Adhikari PM, Chowta MN, Ramapuram JT, Rao SB, Udupa K, Acharya SD. Effect of vitamin B12 and folic acid supplementation on neuropsychiatric symptoms and immune response in HIV-positive patients. *J Neurosci Rural Pract*. 2016;7(3):362–367.
22. Miner SES, Evrovski J, Cole DEC. Clinical chemistry and molecular biology of homocysteine metabolism: an update. *Clin Biochem.* 1997;30(3):189–201.
23. Selhub J. Homocysteine metabolism. *Annu Rev Nutr.* 1999;19(1):217–246.
24. Park W-C, Chang J-H. Clinical implications of methylenetetrahydrofolate reductase mutations and plasma homocysteine levels in patients with thromboembolic occlusion. *Vasc Specialist Int.* 2014;30(4):113–119.
25. Dell'Edera D, L'Episcopia A, Simone F, Lupo MG, Epifania AA, Allegretti A. Methylenetetrahydrofolate reductase gene C677T and A1298C polymorphisms and susceptibility to recurrent pregnancy loss. *Biomed Rep.* 2018;8(2):172–175.
26. Goyette P, Christensen B, Rosenblatt DS, Rozen R. Severe and mild mutations in cis for the methylenetetrahydrofolate reductase (MTHFR) gene, and description of five novel mutations in MTHFR. *Am J Hum Genet.* 1996;59(6):1268–1275.
27. Frosst P, Blom HJ, Milos R, et al. van den Heuvel LP. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. *Nat Genet.* 1995;10(1):111–113.
28. van der Put NM, van den Heuvel LP, Steegers-Theunissen RP, et al. Decreased methylene tetrahydrofolate reductase activity due to the 677C-->T mutation in families with spina bifida offspring. *J Mol Med.* 1996;74(11):691–694.
29. Anon. 1993 revised classification system for HIV infection and expanded surveillance case definition for AIDS among adolescents and adults. *MMWR Morb Mortal Wkly Rep.* 1992;41(RR-17):1–19.
30. Pugliese P, Cuzin L, Enel P, et al. NADIS 2000, development of an electronic medical record for patients infected by HIV, HBV and HCV [in French]. *Presse Med.* 2003;32(7):299–303.
31. Kang SS, Zhou J, Wong PW, Kowalisyn J, Strokosch G. Intermediate homocysteinemia: a thermolabile variant of methylenetetrahydrofolate reductase. *Am J Hum Genet.* 1988;43(4):414–421.
32. Brattström L, Wilcken DE. Homocysteine and cardiovascular disease: cause or effect? *Am J Clin Nutr.* 2000;72(2):315–323.
33. Saffroy R. The MTHFR 677C > T polymorphism is associated with an increased risk of hepatocellular carcinoma in patients with alcoholic cirrhosis. *Carcinogenesis.* 2004;25(8):1443–1448.
34. Naqvi H, Ahmad MK, Hussain SR, Gupta S, Waseem M, Mahdi AA. Retrospective case-control study of correlation between MTHFR gene and OSCC risk in North India. *Clin Oral Investig.* 2017;21(6):1929–1934.
35. Siqueira ER, Oliveira CP, Muniz MT, Silva F, Pereira LM, Carrilho FJ. Methylenetetrahydrofolate reductase (MTHFR) C677T polymorphism and high plasma homocysteine in chronic hepatitis C (CHC) infected patients from the Northeast of Brazil. *Nutr J.* 2011;10:86.
36. Malavazi I, Abrão EP, Mikawa AY, Tagliavini SA, Costa P. Avaliação do polimorfismo no gene da metilenotetrahidrofolato redutase e concentração de folato e vitamina B12 em pacientes portadores do HIV-1 em tratamento com anti-retrovirais. *Rev Soc Bras Med Trop.* 2004;37(6):469–475.
37. ElDeeb MK, Ghazal AA, Metwally DE, Elghlied LA. Possible roles of methylenetetrahydrofolate reductase polymorphism and folate status in patients with early hepatitis C virus genotype 4. *Arab J Gastroenterol.* 2021;22(2):121–126.
38. Qiao K, Zhang S, Trieu C, et al. Genetic polymorphism of MTHFR C677T influences susceptibility to HBV-related hepatocellular carcinoma in a Chinese population: a case-control study. *Clin Lab.* 2017;63(4):787-795.
39. Altintas E, Altintas ZM, Sezgin O, et al. Polymorphisms in methylenetetrahydrofolate reductase gene: their impact on liver steatosis and fibrosis of chronic hepatitis C patients. *Open J Gastroenterol.* 2014;4(2):73–80.
40. Jiao X, Luo Y, Yang B, et al. The MTHFR C677T mutation is not a risk factor recognized for HBV-related HCC in a population with a high prevalence of this genetic marker. *Infect Genet Evol.* 2017;49:66–72.

41. Botezatu A, Socolov D, Iancu IV, et al. Methylenetetrahydrofolate reductase (MTHFR) polymorphisms and promoter methylation in cervical oncogenic lesions and cancer. *J Cell Mol Med.* 2013;17(4):543–549.
42. Banerjee A, Chitnis UB, Jadhav SL, Bhawalkar JS, Chaudhury S. Hypothesis testing, type I and type II errors. *Ind Psychiatry J.* 2009;18(2):127.
43. Bao L, Salomon JA, Brown T, Raftery AE, Hogan DR. Modelling national HIV/AIDS epidemics: revised approach in the UNAIDS estimation and projection package 2011. *Sex Transm Infect.* 2012;88(suppl 2):i3–i10.
44. Michaud V, Bar-Magen T, Turgeon J, Flockhart D, Desta Z, Wainberg MA. The dual role of pharmacogenetics in HIV treatment: mutations and polymorphisms regulating antiretroviral drug resistance and disposition. *Pharmacol Rev.* 2012;64(3):803–833.
45. Kaslow RA, Carrington M, Apple R, et al. Influence of combinations of human major histocompatibility complex genes on the course of HIV-1 infection. *Nat Med.* 1996;2(4):405–411.
46. Borgia G, Gentile I, Fortunato G, et al. Homocysteine levels and sustained virological response to pegylated-interferon alpha2b plus ribavirin therapy for chronic hepatitis C: a prospective study. *Liver Int.* 2009;29(2):248–252.
47. Uccelli MC, Torti C, Lapadula G, et al. Influence of folate serum concentration on plasma homocysteine levels in HIV-positive patients exposed to protease inhibitors undergoing HAART. *Ann Nutr Metab.* 2006;50(3):247–252.
48. Brown PO. Integration of retroviral DNA. In: Swanstrom R, Vogt PK, eds. *Retroviruses. Current Topics in Microbiology and Immunology*. Berlin: Springer; 1990:19–48.
49. Coffin JM, Hughes SH, Varmus HE, eds. *Retroviruses*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 1997.

Culturing Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) for Diagnosis and Genome Sequencing

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Keywords: re-positive, SARS-CoV-2, virus culturing, viral load, diagnosis, sequencing

Abbreviations: SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; TCID50, 50% tissue culture infectious dose; CT, cycle threshold; ORF, open reading frame; RT-PCR, real-time polymerase chain reaction; Vero E6, African green monkey kidney epithelial cell; cDNA, complementary deoxyribonucleic acid.

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ABSTRACT

Objective: The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) nucleic acid detection "re-positive" phenomenon is encountered clinically. The accuracy of a viral nucleic acid test is crucial to prevent reintroduction of the virus into the community. This study evaluated the effect of virus culturing on increasing the sensitivity and specificity of real-time polymerase chain reaction (RT-PCR) detection and viral genomic sequencing.

Methods: A series of tenfold dilutions of a SARS-CoV-2 viral stock were conducted and cultured for either 24 or 48 hours. The viral load of cultured samples was determined by RT-PCR. The cultured and nonc-ultured samples of 1x 50% tissue culture infectious dose (TCID50) were sequenced using metagenomic next-generation sequencing. The depth and coverage of SARS-CoV-2 genome were measured.

Results: The lowest viral load detectable in a sample with RT-PCR was 0.01 TCID50. After a 24-h culture, the viral ORF 1ab and *N*-gene cycle threshold (CT) values were reduced by 4.4 points and 1 point, respectively. One TCID50 viral load of post 24-h culture revealed the sequence depth reached an average of 752 reads, compared with 0.15 in the nonculture; furthermore, the coverage was 99.99% while 6.42% in the nonculture.

Conclusion: These results indicate that virus culturing can significantly increase the viral load, which can increase the certainty of true-positive detection of the viral nucleic acids, and improve the quality of virus genomic sequencing.

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the virus that causes coronavirus disease 2019 (COVID-19), has become a global threat to public health. In the early stages of the COVID-19 pandemic, SARS-CoV-2 was detected in human samples by next-generation sequencing (NGS), electron microscopy, and cell culture.[1] The full genome sequence of the virus (29870 bp, excluding the poly [A] tail), was reported on January 10, 2020. Several laboratories began to develop molecular detection tools that targeted open reading frame (ORF) 1ab, the RNA-dependent RNA polymerase *N* gene, and the E regions of the viral spike genes.[2-4] SARS-CoV-2 can be rapidly identified owing to advances in virus detection techniques, including real-time polymerase chain reaction (RT-PCR), reverse transcription loop-mediated isothermal amplification, and RT-PCR. RT-PCR is a widely used technique for diagnosing SARS-CoV-2 infection, and several nucleic acid test kits have been commercialized for clinical use.[4]

At present, the Omicron variant of SARS-CoV-2, generating BA 1.1, BA 2, and BA 3, have been identified in more than 100 countries and regions worldwide.[5,6] The N501Y, H655Y, N679K, and P681H mutations have been shown to have a higher rate of transmission[7] than other variants. Given the continuous emergence of new variants, subtype analysis is a critical part of SARS-CoV-2 diagnosis. RT-PCR is used to detect the presence of SARS-CoV-2 viral RNA, and genome sequencing could be meaningful for finding novel mutations and identifying the subtypes.

Several studies have shown that discharged COVID-19 patients can retest positive for SARS-CoV-2 after a period of time. The phenomenon of these "re-positive" SARS-CoV-2 PCR cases has been attributed to various mechanisms. First, the virus might not have been completely eliminated in these recovered patients. In the initial test after discharge, the viral load may have been below the RT-PCR detection threshold, leading to a

false negative. The second possibility is that although the live virus was completely killed by the immune system, degraded viral nucleic acid in the airways was still able to be detected, and these viral RNA fragments may have been transported from the lower respiratory tract to the nose/oropharynx region. Third, because the re-positive detection of viral nucleic acid often occurs after discharge, the possibility of re-infection and secondary infection cannot be ignored. Clarifying these 3 possibilities is crucial for COVID-19 pandemic control. In addition, RT-PCR carries some risk of yielding a false-negative result when there is a low viral load in a sample. A low viral load can be related to (1) the source of the collected sample, (2) the conditions of the sample collection and transport, and (3) the sensitivity of the RT-PCR kit.[8] To address these problems, the virus culturing technique, a gold standard for the investigation of viruses, was assessed. By culturing virus from recovered patients, it becomes possible to distinguish live virus from noninfectious viral RNA. Furthermore, viral cultures could provide sufficient amounts of viral nucleic acid for RT-PCR to avoid false-negative results and to determine the virus subtypes through genetic sequencing. In this study, we evaluated the effectiveness of virus culturing with Vero E6 cells on the detection limit of RT-PCR and genetic sequencing of SARS-CoV-2.

Methods

Virus Dilution

A SARS-CoV-2 sample (Genebank accession No. MT123290.1) was obtained from a clinical isolate. Using the Reed–Muench method, the viral titer was determined by the 50% tissue culture infectious dose (TCID50) according to the cytopathic effect. A SARS-CoV-2 virus stock (10^7 TCID50/mL) was prepared in phosphate-buffered saline (PBS) and diluted to 10, 1, 0.1, 0.01, and 0.001 TCID50/mL in a biosafety III laboratory.

Isolation and Culture

A SARS-CoV-2 dilution series (10, 1, 0.1, 0.01, 0.001 TCID50/mL) was cultured respectively, for 24 and 48 h in African green monkey kidney epithelial (Vero E6) cells in triplicate (n = 3). The cells were cultured at 37°C and 5% CO_2 in infection medium, which was Dulbecco's Modified Eagle Medium (Gibco) supplemented with 2% fetal bovine serum and 100 U/mL mycillin.

To construct the RNA library for metagenomics next-generation sequencing, we cultured the 1 TCID50 titer of the virus in Vero E6 cells for 24 h. Three study groups were established: (1) negative control (NC): only Vero E6 cells; (2) nonculture (VC0, virus titer: 1 TCID50); and (3) 24 h culture (VC24, virus titer: 1 TCID50). Before sequencing, Vero E6 cells were added to the nonculture group to make the sequencing background consistent with that of the cultured group.

RNA Purification and RT-PCR Detection of SARS-CoV-2

In a biosafety III laboratory, total RNA was extracted from 100 μL samples of the 3 groups (NC, VC0, VC24) using the Vazyme FastPure Viral DNA/RNA Mini Kit (Vazyme) following the manufacturer's instructions. The RT-PCR assay was performed using the 2019-nCoV Nucleic Acid Detection Kit (fluorescence PCR) (Daan Gene), which is commercially available and widely used clinically in China and detected by the 7500 Real-Time PCR System (Fisher Scientific). A positive detection was made when signals for both nucleic acid targets (ORF 1ab and *N*) were within the valid range (CT ≤ 40).

Library Preparation, Sequencing, and Analysis

For each sample, total RNA was quantified using the Qubit RNA HS Assay Kit (Thermo Fisher Scientific). First-strand complementary deoxyribonucleic acid (cDNA) was synthesized with the HiScriptIII 1st Strand cDNA Synthesis Kit in the presence of specific reagents to ensure that only RNA was used as a template. Double-stranded cDNA was synthesized using DNA Polymerase I Klenow Fragment exo (Vazyme), dNTP mix (Thermo Fisher), and Random Hexamer Primer (Thermo Fisher); the products were purified with Agencourt AMPure XP beads (Beckman). Next, the DNA libraries were prepared using the TruePrep DNA Library Prep Kit V2 for Illumina (Vazyme). The concentration of the DNA libraries was measured by Qubit. Finally, the sequencing was carried out on an Illumina NextSeq 500 system with 75 cycles and using the associated reagent kit.

For each library, low-quality reads, adaptor sequences, noncomplex reads, and duplicated reads were removed using Fastp (v0.22.0). The remaining reads were mapped against the SARS-CoV-2 genome (accession No. NC_045512.2) with Bowtie2 (v2.3.5.1). Samtools (v1.13) was used to extract aligned reads for genome sequence assembly. The abundance level of SARS-CoV-2 was estimated by mapping reads against the corresponding genome and was measured as the read count using Bedtools (v2.3.5.1). A SARS-CoV-2 genome coverage plot of the 3 samples (genome coverage >80% and average depth >100×) was generated by an R script within the Gviz (v1.34.1) package. Aligned reads of the 3 samples were assembled using SPAdes (v3.15.3) to retrieve their full-length individual genome sequence with a single parameter named "isolate." The NGS data for the nonculture and culture samples were uploaded to the Gene Expression Omnibus (accession No. GSE189731).

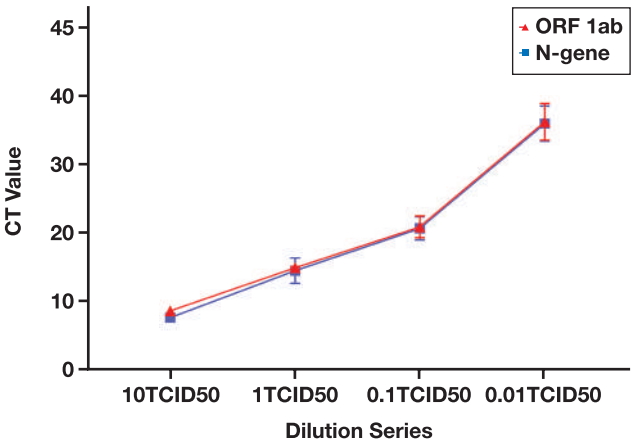
Statistical Analysis

Statistical analysis was performed using GraphPad Prism 7.0 software. The mean and standard deviation of the cycle threshold (CT) values of ORF 1ab and *N* of SARS-CoV-2 were calculated.

Results

A low viral load in a sample may be the main reason for false-negative viral nucleic acid testing results initially, prior to re-positive detection. To test this hypothesis, we constructed a dilution series (10, 1, 0.1, 0.01, and

FIGURE 1. Cycle threshold (CT) values of the SARS-CoV-2 dilution series.



0.001 TCID50 titers) of the SAR-CoV-2 culture to determine the lowest viral load detectable by the RT-PCR kit. The lowest viral load that the kit could detect was 0.01 TCID50, which was weakly positive (**FIGURE 1**, **TABLE 1**). To evaluate the effect of virus culturing on the sensitivity of the RT-PCR method, we cultured the virus with initial concentrations of 10, 1, 0.1, 0.01, and 0.001 TCID50 for 24 h or 48 h, followed by RT-PCR detection. The virus with initial 0.001 TCID50 titer could not be effectively amplified (**FIGURE 2**, **TABLE 2**). However, when the virus with initial 0.01 TCID50 titer was cultured for 24 h, the virus ORF 1ab CT value decreased by 4.4, and the virus *N*-gene CT value reduced by 1. After 24 h of culture, the virus titers of 0.1 TCID50 and above showed an average decrease of greater than 9 for the ORF 1ab CT value and an average decrease of 2 for the virus *N*-gene CT value. These results indicate that if the virus titer of a specimen is as low as 0.01 TCID50, then after culturing the detection sensitivity may be dramatically increased.

TABLE 1. Average RT-PCR CT Values of SARS-CoV-2 at Serial Dilutions

Probe	CT Value (Mean ± SD, n = 3)				
	10TCID50	1TCID50	0.1TCID50	0.01TCID50	0.001TCID50
ORF 1ab	24.34 ± 0.10	27.47 ± 0.08	30.45 ± 0.78	38.1 ± 1.33	Negative
N-gene	23.84 ± 0.17	27.26 ± 0.92	30.34 ± 0.84	37.95 ± 1.28	Negative

CT, cycle threshold; RT-PCR, real-time polymerase chain reaction; TCID50, 50% tissue culture infectious dose.

FIGURE 2. The relationship between real-time polymerase chain reaction cycle threshold (CT) values of ORF1ab/*N*-gene and different SARS-CoV-2 culturing conditions. A, 10TCID50. B, 1TCID50. C, 0.1TCID50. D, 0.01TCID50. TCID50, 50% tissue culture infectious dose.

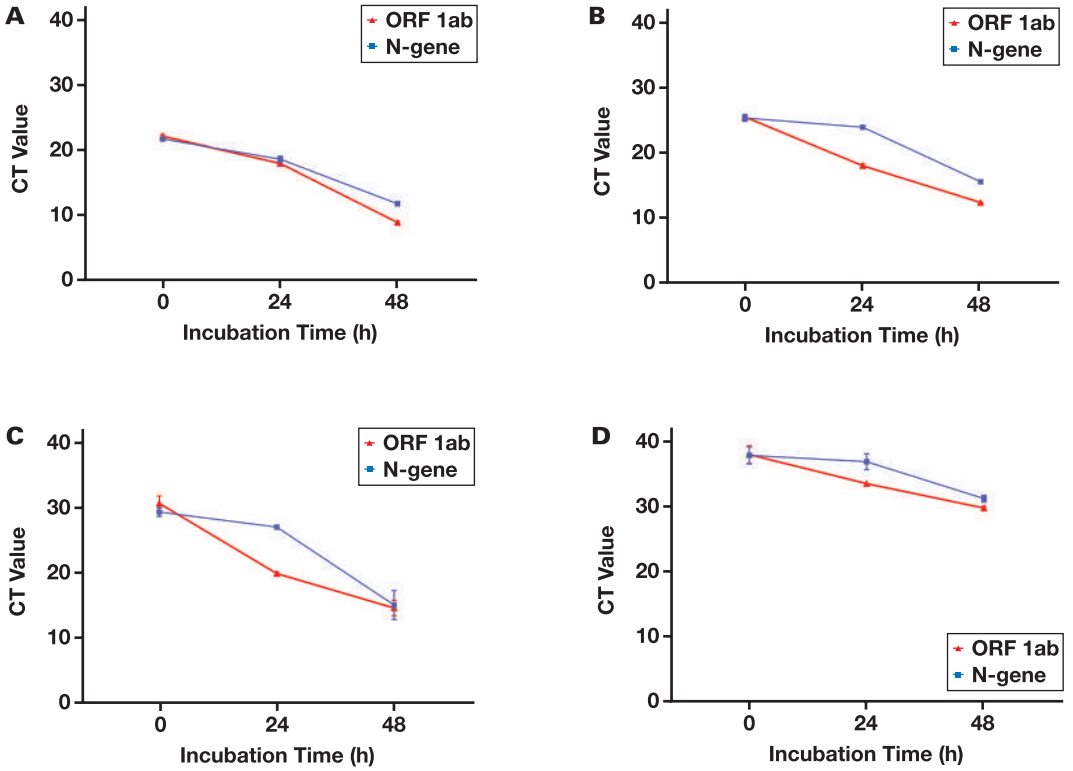


TABLE 2. Average RT-PCR CT Values of SARS-CoV-2 After Culturing for 24 h and 48 h with Different Initial Viral Concentrations

Virus titer	Probe	CT Value (Mean ± SD, n = 3)		
		0 h	24 h	48 h
10TCID50	ORF 1ab	22.01 ± 0.29	13.25 ± 0.27	9.0 ± 0.15
	N-gene	21.59 ± 0.37	18.65 ± 0.47	11.85 ± 0.13
1TCID50	ORF 1ab	25.42 ± 0.20	17.96 ± 0.27	12.32 ± 0.04
	N-gene	25.28 ± 0.52	23.85 ± 0.30	15.51 ± 0.04
0.1TCID50	ORF 1ab	30.61 ± 1.15	19.87 ± 0.23	14.58 ± 1.18
	N-gene	29.29 ± 0.65	26.99 ± 0.11	15.04 ± 2.21
0.01 TCID50	ORF 1ab	38.1 ± 1.33	33.63 ± 0.25	29.9 ± 0.30
	N-gene	37.95 ± 1.28	36.99 ± 1.22	31.34 ± 0.52
0.001 TCID50	ORF 1ab	Negative	Negative	Negative
	N-gene	Negative	Negative	Negative

CT, cycle threshold; RT-PCR, real-time polymerase chain reaction; TCID50, 50% tissue culture infectious dose.

Genome sequencing has been applied to identify SARS-CoV-2 variants in re-positive cases. However, virus genomic sequencing requires a high viral load. Because the noncultured viral loads in a specimen at concentrations of 0.01 or 0.1 TCID50 were extremely low, they could not provide enough material for sequencing. Therefore, our study used the 1 TCID50 noncultured samples and 24 h cultured samples with initial 1TCID50 viral concentration for NGS. The sequencing results of the 24-h culture were significantly improved over those of the noncultured sample, reaching an average of 752 reads, while the nonculture one had only 0.15 reads (**TABLE 3**). Furthermore, the coverage of the 24-h culture sample was 99.99%, while that of the nonculture one was only 6.42% (**FIGURE 3**). These results indicate that the 24-h culturing, with initial 1 TCID50 viral loading, could render NGS to achieve good results for mutation and subtype analyses.

Discussion

The phenomenon of recovered patients showing a re-positive SARS-CoV-2 nucleic acid detection has been widely reported. Zhang et al[9] reported the clinical and laboratory characteristics of 7 patients in Guangdong, China, who were readmitted owing to re-positive PCR detection. Four patients tested positive from rectal swabs, 2 tested positive from throat swabs, and 1 patient tested positive after both throat and rectal swabs positive.[9] A 72-year-old woman from South Korea tested re-positive by RT-PCR 6 days after 2 negative results.[10] In Switzerland, 2 older women with underlying heart diseases had positive nasopharyngeal swab detection results 18 and 21 days after 2 consecutive negative results.[11] If re-positive patients are not isolated, SARS-CoV-2 could potentially spread in the community. Therefore, it is important to identify the nature of the re-positivity, including the infectivity and virus subtype.

TABLE 3. Coverage and Mean Depth of Sequencing for Cultured and Noncultured Samples

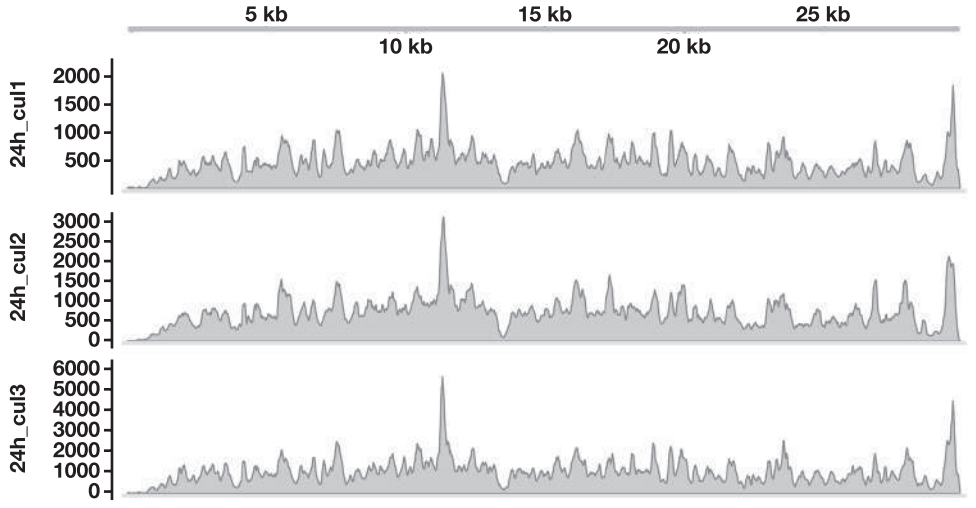
Sample	Coverage (%)	Mean Depth
24 h culture	99.9911	752.031
Nonculture	6.419637	0.147666
Negative control	0	0

The cause of re-positivity needs to be clarified. There are several reasons for a re-positive detection, such as an originally false-negative RT-PCR results, reactivation of the virus, persistent infection, or a new infection with another strain.[12]

Low viral loads are thought to be the main cause of false-negative results. Additionally, the viral load in a sample is affected by the method and location of sample collection. As found in this study, a 0.01 TCID50 virus titer was the minimum threshold that could be detected by this RT-PCR kit. Thus, a viral load below this level may lead to false-negative results when using this RT-PCR kit. A systematic review found that different RT-PCR kits have different sensitivities, ranging from 71% to 98%.[13] Therefore, the minimum virus titer necessary for detection depends on the performance of individual kits. Moreover, RT-PCR cannot distinguish between infectious and noninfectious viral RNA.[11] Our results showed that as long as the virus titer was above 0.01 TCID50, an incubation of at least 24 h greatly increased the viral load. Virus culturing not only increases the viral load but also helps to differentiate whether live virus is present in a sample; this process can increase the sensitivity and specificity of viral nucleic acid detection. Regarding virus reactivation, findings have been limited. One study found that some individuals could be virus carriers even after they recovered.[14] However, Arevalo-Rodriguez et al[13] found that most re-positive cases were asymptomatic and had low viral loads, which suggests that re-positivity is attributed to a low viral load rather than to reactivation. The possibility of persistent infection with the same subtype is also low, and re-positivity is more likely related to prolonged viral shedding.[15] As the virus is still evolving, it is possible to become infected with another variant. To identify subsequent infections, the virus could be cultured as in this study to increase the depth and coverage of sequencing, providing the possibility for high-quality NGS results. To prevent community transmission of live virus from re-positive cases, we recommend that samples from these patients should be isolated and cultured before RT-PCR and high-throughput sequencing to increase the detection rate and analyze the virus subtype.

In the future, we will conduct a cohort study of re-positive cases to evaluate the effectiveness of virus culturing for diagnosis.

FIGURE 3. Coverage of the SARS-CoV-2 genome. 24 h_cul1–3, 24-h culture of SARS-CoV-2 with 3 replicates.



Conclusion

Virus culturing is beneficial for diagnosing SARS-CoV-2 infection and identifying the subtype in re-positive cases.

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Disclosure

Hua Guo was employed by Baso Diagnostics.

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Data Availability

The datasets generated and analyzed during the current study are available in the Gene Expression Omnibus (accession No. GSE189731).

REFERENCES

1. Zhu N, Zhang D, Wang W, et al. A novel coronavirus from patients with pneumonia in China, 2019. *N Engl J Med*. 2020;382(8):727–733.
2. Corman VM, Landt O, Kaiser M, et al. Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. *Euro Surveill.* 2020;25(3):2000045.
3. Chen L, Liu W, Zhang Q, et al. RNA based mNGS approach identifies a novel human coronavirus from two individual pneumonia cases in 2019 Wuhan outbreak. *Emerging Microbes Infect.* 2020;9(1):313–319.
4. Chu D, Pan Y, Cheng S, et al. Molecular diagnosis of a novel coronavirus (2019-nCoV) causing an outbreak of pneumonia. *Clin Chem*. 2020;66(4):549–555.
5. Meo SA, Meo AS, Al-Jassir FF, Klonoff DC. Omicron SARS-CoV-2 new variant: global prevalence and biological and clinical characteristics. *Eur Rev Med Pharmacol Sci.* 2021;25(24):8012–8018.
6. Thakur V, Ratho RK. OMICRON (B.1.1.529): a new SARS-CoV-2 variant of concern mounting worldwide fear. *J Med Virol.* 2022;94(5):1821–1824.
7. Kimura I, Kosugi Y, Wu J, et al. The SARS-CoV-2 Lambda variant exhibits enhanced infectivity and immune resistance. *Cell Reports*. 2022;38(2):110218.
8. Xu K, Cai H, Shen Y, et al. Management of COVID-19: the Zhejiang experience [in Chinese]. *Zhejiang Da Xue Xue Bao Yi Xue Ban.* 2020;49(2):147–157.
9. Zhang B, Liu S, Dong Y, et al. Positive rectal swabs in young patients recovered from coronavirus disease 2019 (COVID-19). *J Infect.* 2020;81(2):e49–e52.
10. Chae KJ, Jin GY, Lee CS, Lee HB, Lee JH, Kwon KS. Positive conversion of COVID-19 after two consecutive negative RT-PCR results: a role of low-dose CT. *Eur J Radiol.* 2020;129:109122.
11. Ravioli S, Ochsner H, Lindner G. Reactivation of COVID-19 pneumonia: a report of two cases. *J Infect.* 2020;81(2):e72–e73.
12. Osman AA, Al Daajani MM, Alsahafi AJ. Re-positive coronavirus disease 2019 PCR test: could it be a reinfection? *New Microbes New Infect.* 2020;37:100748.
13. Arevalo-Rodriguez I, Buitrago-Garcia D, Simancas-Racines D, et al. False-negative results of initial RT-PCR assays for COVID-19: a systematic review. *PLoS One.* 2020;15(12):e0242958.
14. Lan L, Xu D, Ye G, et al. Positive RT-PCR test results in patients recovered from COVID-19. *JAMA*. 2020;323(15):1502–1503.
15. Xu K, Chen Y, Yuan J, et al. Factors associated with prolonged viral RNA shedding in patients with COVID-19. *Clin Infect Dis*. 2020;71(15):799–806.

Association of *Catalase* Gene Polymorphisms with Idiopathic Nephrotic Syndrome in a Chinese Pediatric Population

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Keywords: catalase, idiopathic nephrotic syndrome, polymorphisms, susceptibility, single-nucleotide variants, children

Abbreviations: CAT, catalase; INS, idiopathic nephrotic syndrome; SNVs, single-nucleotide variants; SSNS, steroid-sensitive nephrotic syndrome; SDNS, steroid-dependent nephrotic syndrome; SRNS, steroid-resistant nephrotic syndrome; ROS, reactive oxygen species; PCR, polymerase chain reaction; FSGS, focal segmental glomerulosclerosis; SD, standard deviation; OR, odds ratio; CI, confidence interval.

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ABSTRACT

Objective: Our aim was to investigate the association between gene polymorphisms in *catalase* (*CAT*), a well-known oxidative stress regulator, and susceptibility to idiopathic nephrotic syndrome (INS) or responses to steroid therapy in a Chinese pediatric population.

Methods: We analyzed 3 *CAT* single-nucleotide polymorphisms (SNVs; rs7943316, rs769217, and rs12270780) using multi-polymerase chain reaction combined with next-generation sequencing in 183 INS patients and 100 healthy controls.

Results: For the allele and genotype frequencies of the *CAT* SNVs, no significant differences were observed between INS patients and controls. Patients with C allele of *CAT* rs769217 had a higher risk of developing steroid-dependent nephrotic syndrome than the steroid-sensitive nephrotic syndrome patients ($P = 0.018$; odds ratio = 1.76).

Conclusion: Our data suggests that genetic variations in *CAT* were unlikely to confer susceptibility to INS in Chinese children, whereas the C allele of the *CAT* rs769217 polymorphism showed a strong association with steroid-dependent responses in Chinese INS children.

Idiopathic nephrotic syndrome (INS) is the most common glomerular disease in children, characterized by massive proteinuria, hypoalbuminemia, edema, and hyperlipidemia.[1,2] The incidence of INS has been reported to be 1.15 to 16.9 children per 100,000, depending on ethnicity and region,[3] and in China the average annual number of new cases is 28,000 to 56,000.[4] At this time, corticosteroids are still the mainstay of treatment for INS. According to the different responses to steroid therapy, INS is divided into steroid-sensitive nephrotic syndrome (SSNS), steroid-dependent nephrotic syndrome (SDNS), and steroid-resistant nephrotic syndrome (SRNS).[5] Most children respond well to steroids, but a number of patients relapse frequently or develop dependence or resistance to steroids at a later stage, requiring repeated treatment or replacement therapy, leading to a long course of disease.[3,6] Considering the potentially serious adverse effects of steroids and the urgency of taking appropriate strategies, it is particularly important to predict and evaluate the efficacy of steroids in the treatment of INS.

The exact cause of INS remains unclear, but the pathogenic mechanisms are thought to involve cellular oxidative stress, inflammatory responses, and immune dysregulation.[7] The production of free radicals has been suggested to affect the permeability of the glomerular capillary wall,[8,9] and the excessive production of reactive oxygen species (ROS) leads to cell damage through lipid peroxidation, which is one pathogenesis of progressive renal injury.[10,11] Catalase (CAT) is a crucial intracellular antioxidant enzyme and a member of the scavenging system of free radicals and ROS. It can protect cells from the damage caused by excessive formation of ROS and prevent the accumulation of hydrogen peroxide formed in the process of oxygen transport, thus reducing oxidative stress to a considerable extent.[9,12] Decreased serum CAT activity has been reported in adult NS patients.[9] Moreover, the CAT activity in peripheral blood lymphocytes of pediatric INS patients also showed a marked reduction.[7] These results indicate that CAT might play a role in the pathophysiology of INS through regulation of oxidative stress.

In addition, genetic risk has been reported as an important factor influencing the disease incidences and responses to steroid treatment of INS.[13] For example, the single-nucleotide variants (SNVs) in *HLA-DQA1* and *HLA-DQB1* genes were closely related to SSNS.[3,7,14] Mutations in key podocyte genes such as *NPHS1*, *NPHS2*, *LAMB2*, or *WT1* might play a role in the pathogenesis of SRNS or focal segmental glomerulosclerosis (FSGS).[13,15] Mitochondrial gene mutations associated with coenzyme

Q10 biosynthesis led to defects in 1% of familial SRNS cases.[15] The *MDR1* gene polymorphism showed a correlation with steroid resistance in SRNS children.[16] Nevertheless, to our knowledge, there have been no studies on the linkage between *CAT* gene polymorphisms and INS, particularly in ethnically diverse populations. Therefore, the present study was carried out to explore the relationship of *CAT* gene SNVs with the susceptibility and the response to steroid treatment of INS in Chinese children, so as to estimate the population-specific predictive effect of *CAT* genetic variation on INS.

Methods

Study Subjects

We recruited a total of 283 Chinese child participants, including 183 patients with INS and 100 healthy controls. All patients were diagnosed and hospitalized at the Children's Hospital, Zhejiang University School of Medicine. The diagnosis of INS was confirmed according to the following general criteria: the appearance of edema, urinary protein excretion ≥50 mg/kg/d or ≥40 mg/m^2/h, morning urinary protein to creatinine ratio ≥2.0 (mg/mg), serum albumin ≤2.5 g/dL, and the disease of unknown causes.[2,17] Children with active infection or any systemic diseases or other autoimmune disorders were excluded from this study. All subjects with INS received the standard steroid treatments and were divided into 3 subgroups in line with their clinical responses to steroids, namely SSNS, SRNS, and SDNS groups. SSNS was defined as patients whose proteinuria turned negative within 4 weeks when treated with prednisone at a dose of 2 mg/kg/d or 60 mg/m^2/d. SRNS was considered if the patients whose proteinuria was still positive for more than 4 weeks were treated with sufficient prednisone (2 mg/kg/d or 60 mg/m^2/d). The diagnosis of SDNS was made for patients who were sensitive to steroid but relapsed within 2 weeks after 2 consecutive dosage reductions or the withdrawal of steroid.[5]

During the same period, 100 unrelated healthy controls with no history of any inflammatory, systemic, or autoimmune diseases were chosen from the children who underwent routine physical examination in the health checkup center of the hospital. All subjects were of Chinese Han ethnicity. Their demographic and clinical information, including age and gender, were collected and are described in **TABLE 1**. The research protocol was conducted in accordance with the principles of the Helsinki Declaration and approved by the Ethics Committee of the Children's Hospital of Zhejiang University School of Medicine (2020-IRB-057).

Genomic DNA Extraction

A peripheral blood sample from each subject was drawn and collected in an EDTA tube. DNA was extracted from 200 μL anticoagulant whole blood by Biospin Genomic DNA Purification Kit (BIOER technology, #BSC06S1) according to the manufacturer's instructions. The DNA purity and concentration were detected using Nanodrop 1000c spectrophotometer (Thermo Scientific). The integrity of DNA was evaluated by agarose gel electrophoresis (1.0%). The samples meet the requirements were kept at −20°C until genotype assessment and then at −80°C for long-term storage.

Selection of SNVs and Genotyping

Three SNVs, rs7943316 (c.−89C>T), rs769217 (c.1167C>T), and rs12270780 (c.66 + 85G>A), in the *CAT* gene were selected. Genotyping analysis was done by Shanghai BioWing Applied Biotechnology on the high-throughput genotyping platform, Illumina X-10, using multiplex polymerase chain reaction (PCR) combined with next-generation sequencing.[18] The primers for *CAT* rs7943316 were 5′- CAATCAGAAGGCAGTCCTCC -3′ (forward) and 5′- CTCCTTCCAGTGCTGCATC -3′ (reverse). The primers for *CAT* rs769217 were 5′- TTATATGTTACTGCCCCTAGTCAG -3′ (forward) and 5′- CAACGTCTTTAGGCCTACCC -3′ (reverse). The primers for *CAT* rs12270780 were 5′- AAGGTCCGTTTAGAAAGCGG -3 ′ (forward) and 5′- AAAAGTGCAAAATTCTGAAGCAAC -3′ (reverse).

Statistical Analysis

The software of Statistical Package for Social Science (SPSS) revision 17.0 for windows (IBM) was used for all statistical analysis. The Hardy-Weinberg equilibrium for each SNV polymorphism was examined by the goodness-of-fit χ^2 test. Continuous variables were expressed as mean ± standard deviation (SD), and Student *t*-test or Wilcoxon rank-sum test was used for comparison between every 2 groups. The categorical variables were expressed as percentage or ratio; χ^2tests were applied for differences of gender. The frequencies of allele and genotype were compared between every 2 groups using the χ^2 test. The unconditional logistic regression analysis was used to calculate the odds ratio (OR) and 95% confidence intervals (CIs) between the different groups for estimating the relative risk. All *P* values were bilateral, and $P < .05$ was considered statistically significant.

Results

Patient Characteristics

The demographic and clinical characteristics of the 183 INS patients and 100 healthy controls included in this study are given in **TABLE 1**. The median age ± SD at disease onset of all patients was 5.5 ± 1.2 years, with a male-to-female ratio of 1.95. The children in the control group had a mean age of 4.9 ± 1.9 years and consisted of more males than females, with a ratio of 1.94. No significant difference in mean age and gender was found between case and control patients. Among the cases, 89 (48.6%) were confirmed as SSNS, 73 (39.9%) were SDNS, and 21 (11.5%) were SRNS. There were no significant differences in gender and average age among the SSNS, SDNS and SRNS groups.

Gene Polymorphisms in INS Patients and Healthy Children

The allele and genotype frequencies of the 3 *CAT* SNVs in INS cases and controls are shown in **FIGURE 1**. There was no significant correlation between rs7943316, rs769217, and rs12270780 in *CAT* gene and INS susceptibility (all $P > .05$).

Gene Polymorphisms in Patients with SSNS and SDNS

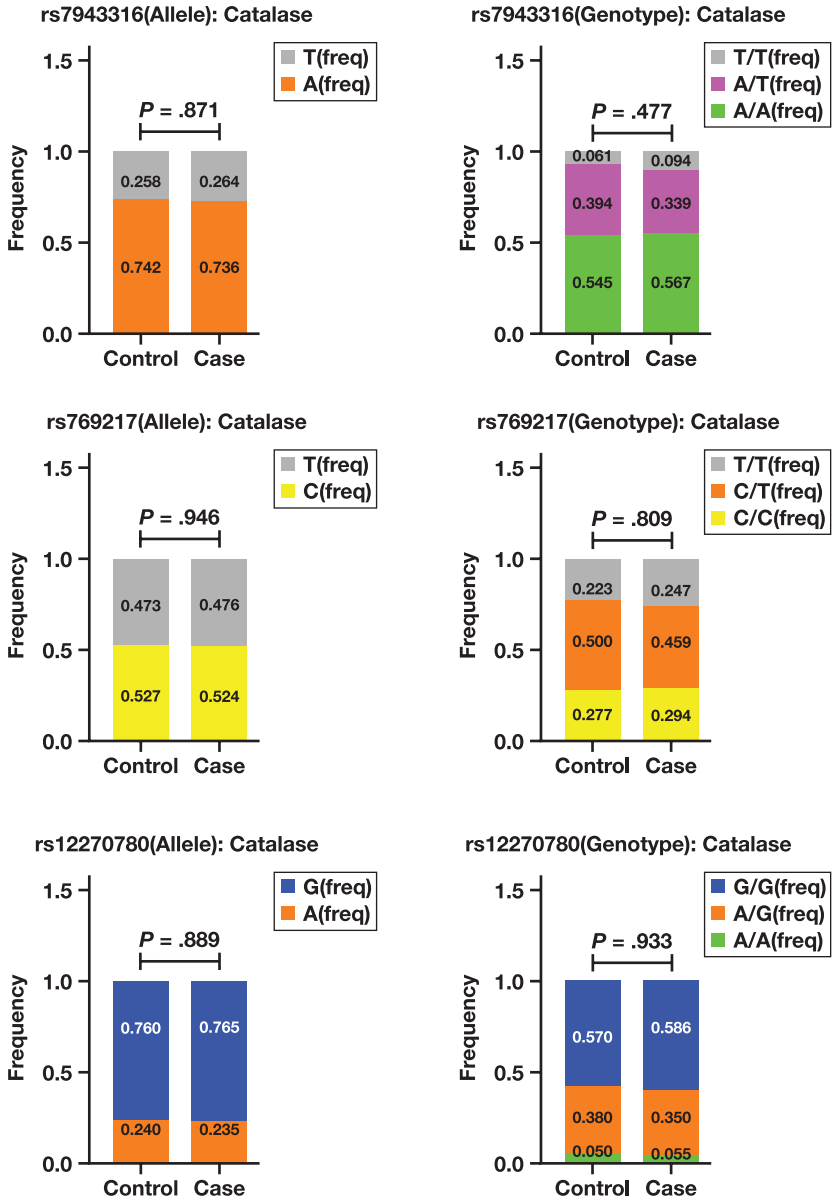
The allele and genotype frequencies of the 3 polymorphic sites of the *CAT* gene in SSNS and SDNS patients are presented in **FIGURE 2**. Patients with C allele of *CAT* rs769217 (Asp389=) had a significantly higher risk of SDNS (59.3% of SDNS vs 45.7% of SSNS; OR 1.76, 95% CI 1.10–2.82; $P = .018$). Nevertheless, the allele frequencies of the other 2 SNVs (rs7943316 and rs12270780) and the genotype distribution of all 3 SNVs displayed no significant difference between the SSNS and the SDNS groups.

TABLE 1. The Main Demographics and Clinical Characteristics of INS Patients and Controls

Characteristics	Number	Sex		*P* Value	Age, y	*P* Value
		Male, No. (%)	Female, No. (%)		Mean ± SD	
Controls	100	66 (66.00)	34 (34.00)	.57	4.9 ± 1.9	.16
INS patients	183	121 (66.12)	62 (33.88)		5.5 ± 1.2	
SSNS	89	63 (70.79)	26 (29.21)	.66	5.6 ± 0.9	.72
SDNS	73	48 (65.75)	25 (34.25)		5.3 ± 1.0	
SRNS	21	13 (61.90)	8 (38.10)		5.6 ± 1.1	

INS, idiopathic nephrotic syndrome; SD, standard deviation; SDNS, steroid-dependent nephrotic syndrome; SRNS, steroid-resistant nephrotic syndrome; SSNS, steroid-sensitive nephrotic syndrome.

FIGURE 1. ***CAT*** **gene polymorphisms in patients with idiopathic nephrotic syndrome (INS) and healthy children.**



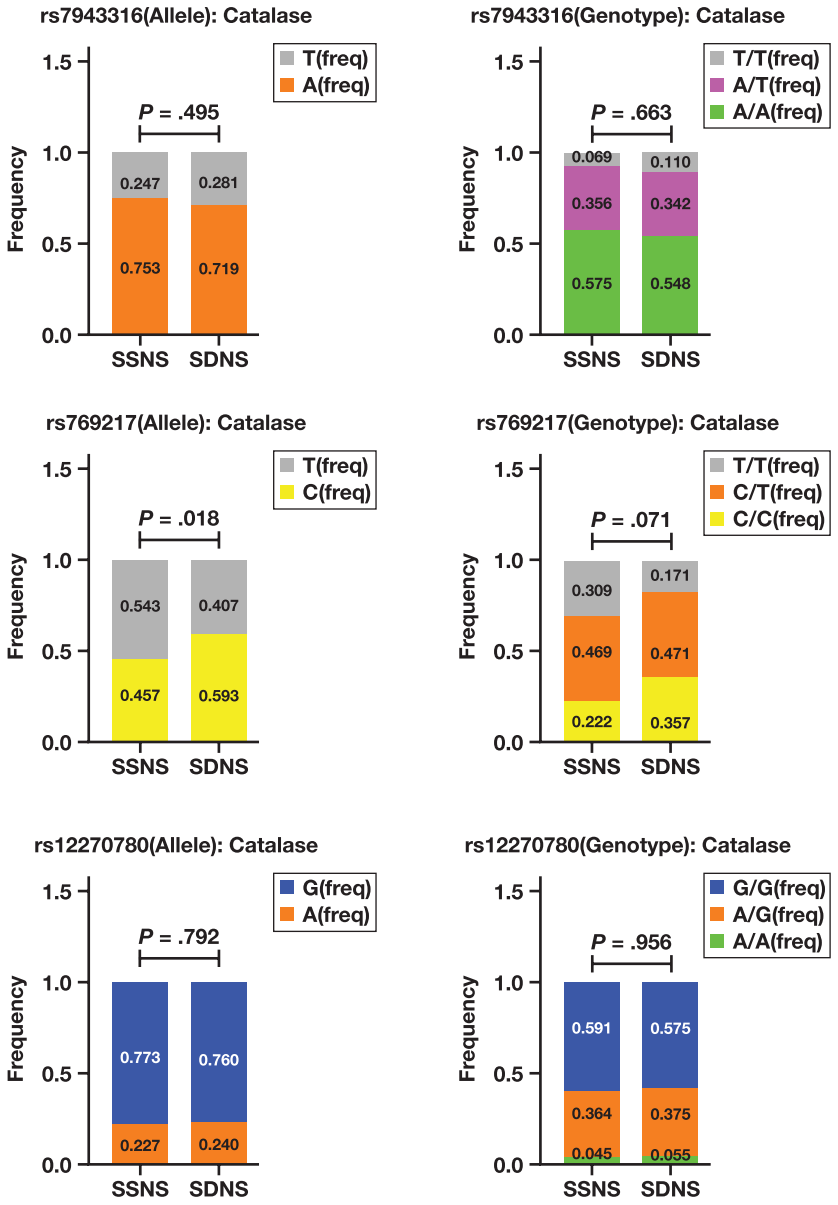
Gene Polymorphisms in Patients with SSNS and SRNS

As shown in **FIGURE 3**, no significant differences in the allele and genotype frequencies of the 3 *CAT* SNVs (rs7943316, rs769217, and rs12270780) were observed between SSNS patients and SRNS patients.

Discussion

Oxidative stress and gene mutations have been reported to play a vital role in the onset and development of INS, as well as in the response to glucocorticoid therapy in INS patients.[2,19–21] Many studies indicate

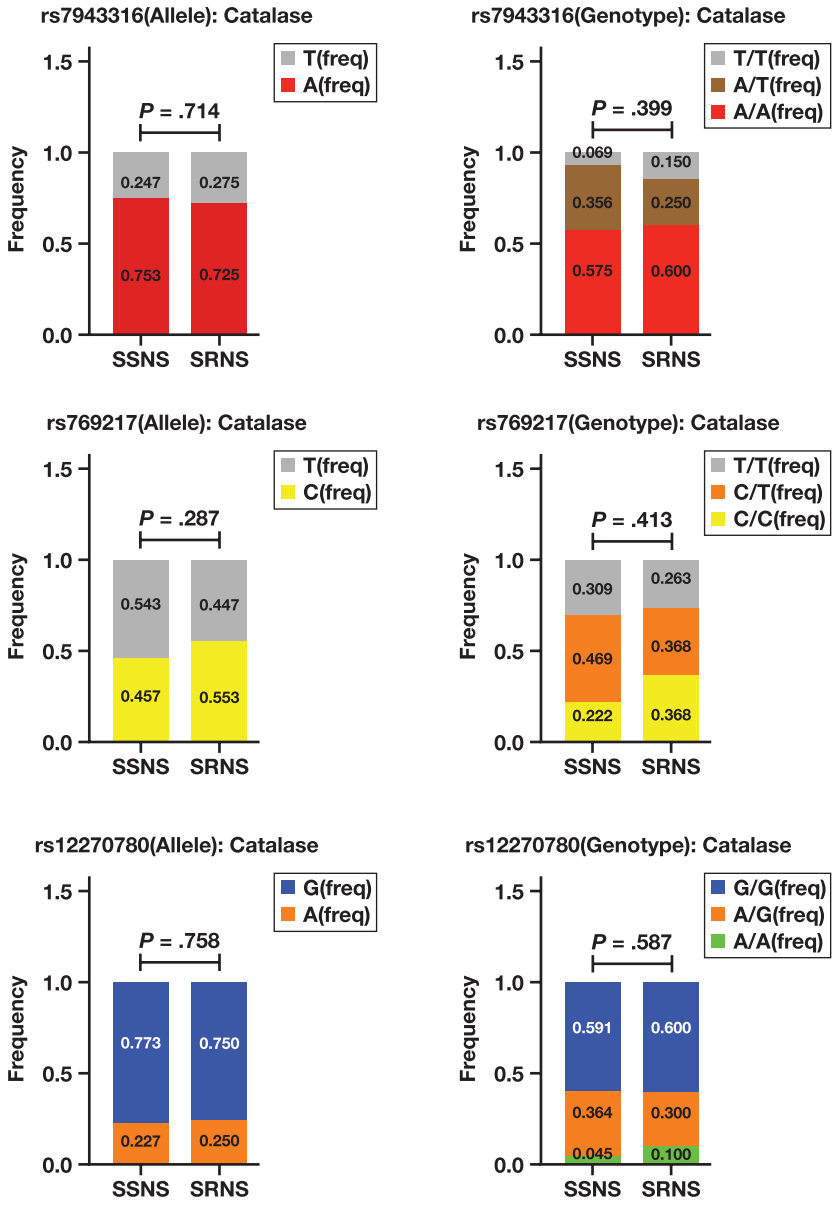
FIGURE 2. ***CAT*** **gene polymorphisms in patients with steroid-sensitive nephrotic syndrome (SSNS) and steroid-dependent nephrotic syndrome (SDNS).**



that the genetic variation of antioxidants is related to oxidative stress-related kidney disorders. For example, Mohammedi et al[22] showed that superoxide dismutase2 allelic variations were associated with the susceptibility and the progression of diabetic nephropathy. These results are consistent with a major role of antioxidant enzymes in protecting against oxidative stress.[22] Another investigation presented a correlation between gene polymorphisms of myeloperoxidase and the incidence of glomerulonephritis.[23] Moreover, the *CAT* rs769217 polymorphism was found to be associated with hospital morbidity of oxidative stress-related acute kidney injury among a Turkish population.[24] Nevertheless, research to date on polymorphisms of the ROS/ROS inhibition system regulator are rarely focused on their relevance with INS pathogenesis. In a survey in 2017, Sugimoto et al[25] pointed out that the polymorphisms of prohibitin-2, a modulator of oxidative stress reaction, might decrease the tolerance of glomerular epithelial cells to ROS and result in local high ROS exposure, increasing permeability of the glomerular filtration barrier to bring about severe proteinuria. In addition, the activities of CAT in serum and peripheral blood lymphocytes have been demonstrated to be decreased in patients with nephrotic syndrome.[7,9] In this study, we analyzed the distribution of genotypes of *CAT* SNVs in children with INS and controls. The results showed that all 3 SNVs in the *CAT* gene were not significantly associated with susceptibility to INS, indicating genetic variants in *CAT* might not be predictors for the risk of INS; however, these results may be due to the relatively small sample size and ethnic origin of the subjects.

For INS, response to corticosteroids is the best indicator of disease outcome. Ongoing research attempts to combine genetic and clinical data to clarify the relationship between gene polymorphism of antioxidant enzyme CAT and steroid efficacy and to provide baseline data

FIGURE 3. ***CAT*** **gene polymorphisms in patients with steroid-sensitive nephrotic syndrome (SSNS) and steroid-resistant nephrotic syndrome (SRNS).**



for medical prediction. Our results revealed that children with INS carrying the C allele of *CAT* rs769217 had a higher risk of recurrence of symptoms after reducing or discontinuing corticosteroid. Thus, the *CAT* rs769217 polymorphism might predict whether patients with INS would relapse frequently and could have implications for early identification of better therapeutic approaches. Also, in the genotyping analysis of *CAT* SNVs, we found that the proportion of TT genotype of rs769217 was low in patients with SDNS, but there was no statistical difference (P = .071). However, the genotypic distribution of *CAT* should be observed in a larger sample size before a conclusion is made.

As reported, 10% to 20% of the INS patients exhibited steroid-resistant responses.[6] FSGS is the most common diagnosis in children with ineffective corticosteroids, and it is also an important cause of renal failure.[2] Therefore, it is necessary to carry out genetic testing in children with SRNS to make etiological diagnosis, appropriate therapy recommendations, and prognosis prediction. Current literature has indicated that approximately one-third of patients with SRNS have a genetic background[26] and thus far, more than 50 SRNS-related monogenic causes have been identified, including *NPSH1*, *NPSH2*, *ACTN4*, *TRCP6*, *LAMB2*, *ITGB4*, *WT1*, *LMX1B*, *CoQ2*, *CoQ6*, *NUP93*, *NUP107*, *ANKFY1*, *SMARCAL1*, etc.[21,26] Furthermore, genotyping of paraoxonase1, a serum enzyme with antioxidant properties bound to high-density lipoproteins, has been demonstrated to contribute to the early prediction of FSGS in Kuwaiti Arab children with INS.[27] In the current investigation, we examined the difference in *CAT* SNVs polymorphisms between SRNS and SSNS populations. However, the results suggested no correlation to steroid-resistant response with *CAT* genotypes in pediatric patients with INS, implying that *CAT* polymorphisms might not confer the risk of SRNS.

In conclusion, the lack of an association of *CAT* gene polymorphisms with the risk of INS in our research implies that genetic variation in *CAT* is unlikely to confer the susceptibility to INS in Chinese children. The C-allele of *CAT* rs769217 might be used to predict a high risk of steroid-dependent responses in Chinese children with INS. In the future, genetic research in cross-ethnic populations with a larger sample size are needed to further explore the linkage between genetic variation in *CAT* and INS. Prospective work on alterations in expression levels and protein function caused by changes associated with the *CAT* gene should also be carried out.

Acknowledgments

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REFERENCES

1. Downie ML, Gallibois C, Parekh RS, et al. Nephrotic syndrome in infants and children: pathophysiology and management. *Paediatr Int Child Health.* 2017;37(4):248–258. doi:10.1080/20469047.2017.1374003.
2. Wang CS, Greenbaum LA. Nephrotic syndrome. *Pediatr Clin North Am.* 2019;66(1):73–85. doi:10.1016/j.pcl.2018.08.006.
3. Noone DG, Iijima K, Parekh R. Idiopathic nephrotic syndrome in children. *Lancet* 2018;392(10141):61–74. doi:10.1016/S0140-6736(18)30536-1.
4. Ye Q, Mao JH. Immunologic pathogenesis of idiopathic nephrotic syndrome in children: the present and future. *Zhonghua Er Ke Za Zhi.* 2020;58(9):705–707.
5. Subspecialty Group of Renal Diseases, the Society of Pediatrics, Chinese Medical Association. Evidence-based guideline on diagnosis and treatment of steroid-sensitive, relapsing/steroid-dependent nephrotic syndrome in children (2016). *Zhonghua Er Ke Za Zhi.* 2017;55:729–734.
6. Stone H, Magella B, Bennett MR. The search for biomarkers to aid in diagnosis, differentiation, and prognosis of childhood idiopathic nephrotic syndrome. *Front Pediatr.* 2019;7:404. doi:10.3389/fped.2019.00404.
7. Al-Eisa A, Dhaunsi GS. NOX-mediated impairment of PDGF-induced DNA synthesis in peripheral blood lymphocytes of children with idiopathic nephrotic syndrome. *Pediatr Res.* 2017;82(4):629–633. doi:10.1038/pr.2017.122.
8. Zima T, Tesar V, Stípek S, et al. The influence of cyclosporin on lipid peroxidation and superoxide dismutase in adriamycin nephropathy in rats. *Nephron.* 1997;75(4):464–468. doi:10.1159/000189586.
9. Begenik H, Soyoral YU, Erkoc R, et al. Serum malondialdehyde levels, myeloperoxidase and catalase activities in patients with nephrotic syndrome. *Redox Rep.* 2013;18(3):107–112. doi:10.1179/1351000213Y.0000000048.
10. Mishra OP, Gupta AK, Prasad R, et al. Antioxidant status of children with idiopathic nephrotic syndrome. *Pediatr Nephrol.* 2011;26(2):251–256. doi:10.1007/s00467-010-1696-6.
11. Kinra S, Rath B, Kabi BC. Indirect quantification of lipid peroxidation in steroid responsive nephrotic syndrome. *Arch Dis Child.* 2000;82(1):76–78. doi:10.1136/adc.82.1.76.
12. Nandi A, Yan LJ, Jana CK, et al. Role of catalase in oxidative stress- and age-associated degenerative diseases. *Oxid Med Cell Longev.* 2019;2019:9613090. doi:10.1155/2019/9613090.
13. Hinkes BG, Mucha B, Vlangos CN, et al. Nephrotic syndrome in the first year of life: two thirds of cases are caused by mutations in 4 genes (NPHS1, NPHS2, WT1, and LAMB2). *Pediatrics*. 2007;119(4):e907–919.
14. Gbadegesin RA, Adeyemo A, Webb NJ. et al. HLA-DQA1 and PLCG2 are candidate risk loci for childhood-onset steroid-sensitive nephrotic syndrome. *J Am Soc Nephrol*. 2015;26(7):1701–1710.
15. Sadowski CE, Lovric S, Ashraf S, et al. A single-gene cause in 29.5% of cases of steroid-resistant nephrotic syndrome. *J Am Soc Nephrol*. 2015;26(6):1279–1289.
16. Han SS, Xu YQ, Lu Y, et al. A PRISMA-compliant meta-analysis of MDR1 polymorphisms and idiopathic nephrotic syndrome: susceptibility and steroid responsiveness. *Medicine (Baltimore).* 2017;96(24):e7191. doi:10.1097/MD.0000000000007191.
17. Lombel RM, Gipson DS, Hodson EM. Kidney disease: improving global outcomes. Treatment of steroid-sensitive nephrotic syndrome: new guidelines from KDIGO. *Pediatr Nephrol.* 2013;28(3):415–426. doi:10.1007/s00467-012-2310-x.
18. Chen K, Zhou YX, Li K, et al. A novel three-round multiplex PCR for SNP genotyping with next generation sequencing. *Anal Bioanal Chem.* 2016;408(16):4371–4377. doi:10.1007/s00216-016-9536-6.
19. Bakr A, Abul Hassan S, Shoker M, et al. Oxidant stress in primary nephrotic syndrome: does it modulate the response to corticosteroids? *Pediatr Nephrol.* 2009;24(12):2375–2380. doi:10.1007/s00467-009-1246-2.
20. Habriel Ruslie R, Rina Ramayani O, Darmadi D, et al. Oxidative stress markers in initial therapy and remission of nephrotic syndrome and serum malondialdehyde level predictor from routine laboratory test. *Med Glas (Zenica).* 2021;18(1):90–95. doi:10.17392/1192-21.
21. Rheault MN, Gbadegesin RA. The genetics of nephrotic syndrome. *J Pediatr Genet.* 2016;5(1):15–24. doi:10.1055/s-0035-1557109.
22. Mohammedi K, Bellili-Muñoz N, Driss F, et al. Manganese superoxide dismutase (SOD2) polymorphisms, plasma advanced oxidation protein products (AOPP) concentration and risk of kidney complications in subjects with type 1 diabetes. *PLoS One.* 2014;9(5):e96916. doi:10.1371/journal.pone.0096916.
23. Winterbourn CC, Vissers MC, Kettle AJ. Myeloperoxidase. *Curr Opin Hematol.* 2000;7(1):53–58. doi:10.1097/00062752-200001000-00010.
24. Kidir V, Uz E, Yigit A, et al. Manganese superoxide dismutase, glutathione peroxidase and catalase gene polymorphisms and clinical outcomes in acute kidney injury. *Ren Fail.* 2016;38(3):372–377. doi:10.3109/0886022X.2015.1135468.
25. Sugimoto K, Miyazawa T, Miyazaki K, et al. Minimal change nephrotic syndrome and prohibitin-2 gene polymorphism. *Clin Exp Nephrol.* 2017;21(4):665–670. doi:10.1007/s10157-016-1325-1.
26. Bensimhon AR, Williams AE, Gbadegesin RA. Treatment of steroid-resistant nephrotic syndrome in the genomic era. *Pediatr Nephrol.* 2019;34(11):2279–2293. doi:10.1007/s00467-018-4093-1.
27. Al-Eisa AA, Sukumaran VJ, Haider MZ. Paraoxnase1 gene polymorphism in childhood idiopathic nephrotic syndrome. *Nephron.* 2016;132(2):137–143. doi:10.1159/000442998.

Assessment of the Stability of Midregional Proadrenomedullin in Different Biological Matrices

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Keywords: midregional-proadrenomedullin, biological matrices, serum, EDTA plasma, heparin plasma, assay stability

Abbreviations: MR-proADM, midregional proadrenomedullin; ADM, adrenomedullin; PTV, Policlinico Tor Vergata University Hospital; CBM, Campus Bio-Medico University Hospital.

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ABSTRACT

Midregional proadrenomedullin (MR-proADM) has been shown to play a key role in endothelial dysfunction, with increased levels helping to prevent early stages of organ dysfunction. Recent clinical evidence has demonstrated MR-proADM to be a helpful biomarker to identify disease severity in patients with sepsis as well as pneumonia. This biomarker is helpful at triage in emergency departments to assess risk level of patients. The aim of this study is to evaluate the stability of MR-proADM in different biological matrices. The results, obtained by Bland-Altman and scatter plot analyses, demonstrate that deviation of MR-proADM concentration in serum compared to EDTA plasma unequivocally shows that serum should not be used as a sample matrix. Instead, the excellent correlation of heparin plasma vs EDTA plasma samples shows that heparin plasma can be used without reservation in clinical routine and emergency samples.

The rapid measurement of blood biomarkers is crucial to make early and accurate clinical decisions. The measurement of a panel of different biomarkers from a single blood sample can facilitate faster results and reduce the laboratory time required to prepare different matrices for testing.

One peptide gaining great interest is adrenomedullin (ADM), which has been shown to play a key role in microcirculation and microvascular dysfunction, with increased levels helping to prevent local tissue hypoxia and the early stages of organ dysfunction.[1–3] However, the reliable measurement of ADM is complicated by a number of issues, such as a short half-life, rapid metabolism, low concentration, rapid degradation by proteases, and binding to complement factor H.[4] Accordingly, ADM levels typically can be underestimated. The measurement of midregional proadrenomedullin (MR-proADM) provides a solution to these problems.[5]

MR-proADM is a fragment of 48 amino acids that splits from the proADM molecule in a ratio of 1:1 with ADM and proportionally represents the level and activity of ADM. Its biological inactivity means that it is not involved in the binding to vessel walls and surfaces found with ADM. Its longer half-life of several hours and biological inactivity result in a more accurate estimation of plasma concentration level than using ADM.[4] Recent clinical evidence has shown it to accurately identify disease severity in patients with sepsis[6,7] as well as patients with pneumonia[8] and urinary tract infections.[9] Such findings can help guide treatment strategies and patient disposition decisions.[10–12]

At present, however, the B·R·A·H·M·S MR-proADM sandwich immunoassay is only recommended for use in EDTA blood samples, thus limiting its potential adoption into daily clinical routine. Nevertheless, MR-proADM matrix expansion is beginning to gain interest, with 2 recent studies showing similar serum MR-proADM concentrations or cutoffs in a healthy population of 102 blood donors[13] and 79 noncardiac surgery patients,[14] to preestablished concentration ranges found in EDTA plasma elsewhere for healthy blood donors[15] or mortality prediction.[6] However, a number of limitations could be observed in this comparison, including the use of only 1 blood sample per patient, allowing no direct concentration comparisons to be made between matrices within the same patient. Furthermore, the use of only healthy blood donors did not consider the potential effects of different disease conditions on MR-proADM concentrations among different matrices. A direct comparison was made in a limited set of healthy blood donors[16] that showed a >30% decrease in serum compared to EDTA plasma. However, part of the determinations were around or below the assay limits, leading to great imprecision in the measurement.

Therefore, this technical evaluation aims to test the preanalytic stability of MR-proADM in serum and heparin plasma compared to the recommended EDTA plasma in freshly collected blood samples of the

TABLE 1. Agreement and Correlation of Serum and Heparin Plasma in Comparison to EDTA Plasma

	EDTA vs Serum	EDTA vs Heparin Plasma
Median bias	–21.3% (–25.9 to –15.0)	–2.6% (0.3 to 4.2)
Spearman correlation	0.97	1.00
Passing-Bablok		
Intercept (CI)	–0.03 (–0.09 to 0.02)	–0.00 (–0.02 to 0.01)
Slope (CI)	0.83 (0.76 to 0.90)	0.98 (0.96 to 1.00)
Agreement at 0.87 nmol/L		
Positive (>0.87 nmol/L)	78%	100%
Negative (≤0.87 nmol/L)	100%	100%
Total	87%	100%
Agreement at 1.50 nmol/L		
Positive (>1.50 nmol/L)	78%	100%
Negative (<1.50 nmol/L)	100%	100%
Total	93%	100%

same patient, both before and after centrifugation. This serves to make an assessment of whether heparin plasma and/or serum could be used reliably for measuring MR-proADM concentration in clinical routine, thus potentially decreasing the number of blood samples taken from a patient for diagnostic testing.

Material and Methods

Study Design

Samples were collected at the Policlinico Tor Vergata University Hospital (PTV) and the Campus Bio-Medico University Hospital (CBM). The local ethics committee approved the study design (R.S. 116/18), and participating patients gave written consent.

Samples were collected in the morning during routine blood draws, using BD Vacutainers without gel for EDTA plasma and serum (CBM) or BD Vacutainers without gel for EDTA plasma and lithium heparin (PTV). All samples were centrifuged within 90 minutes after collection. First

FIGURE 1. Bland-Altman plot of serum vs EDTA plasma (A) and heparin plasma vs EDTA plasma (B).

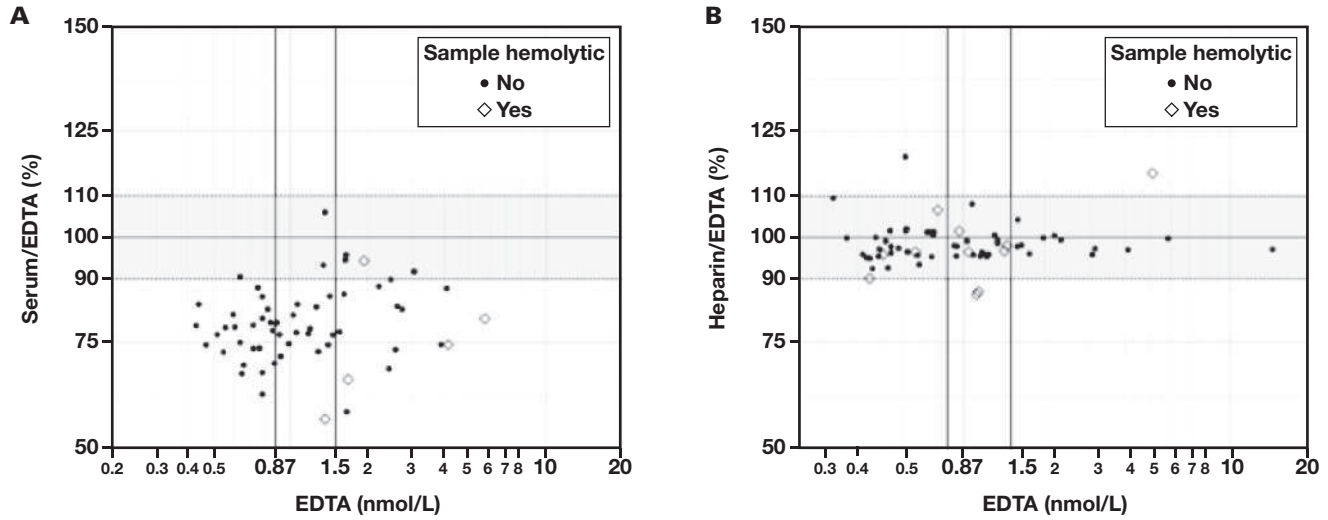


FIGURE 2. Scatterplot of serum vs EDTA plasma (A) and heparin plasma vs EDTA plasma (B).

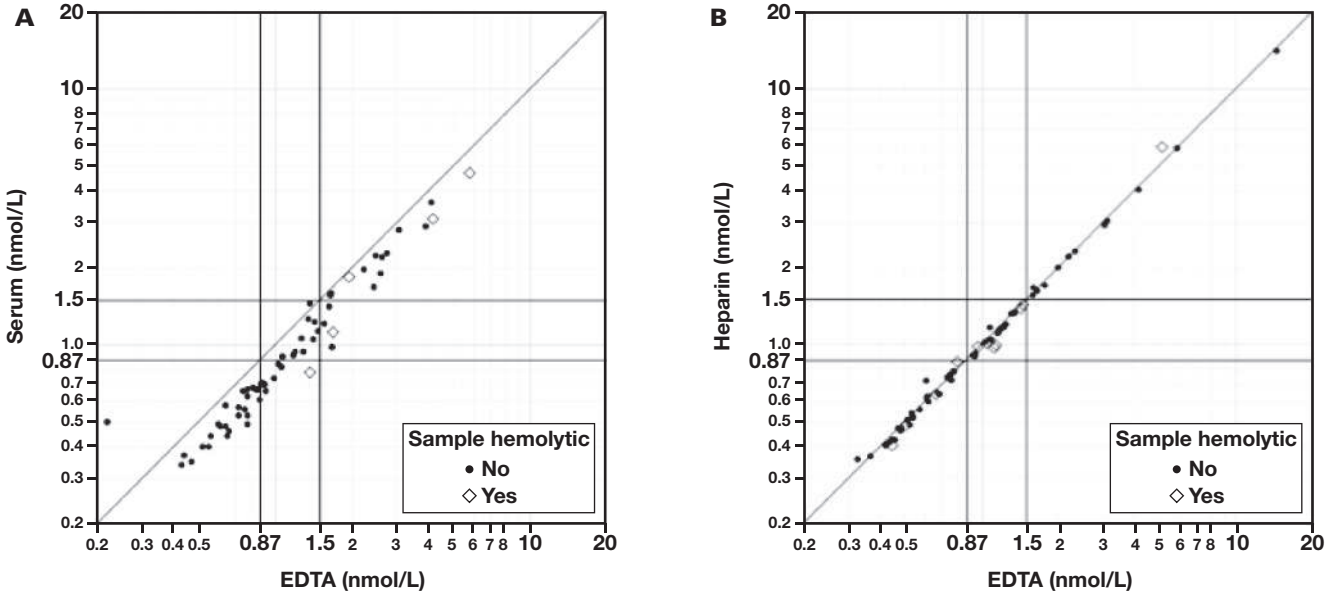
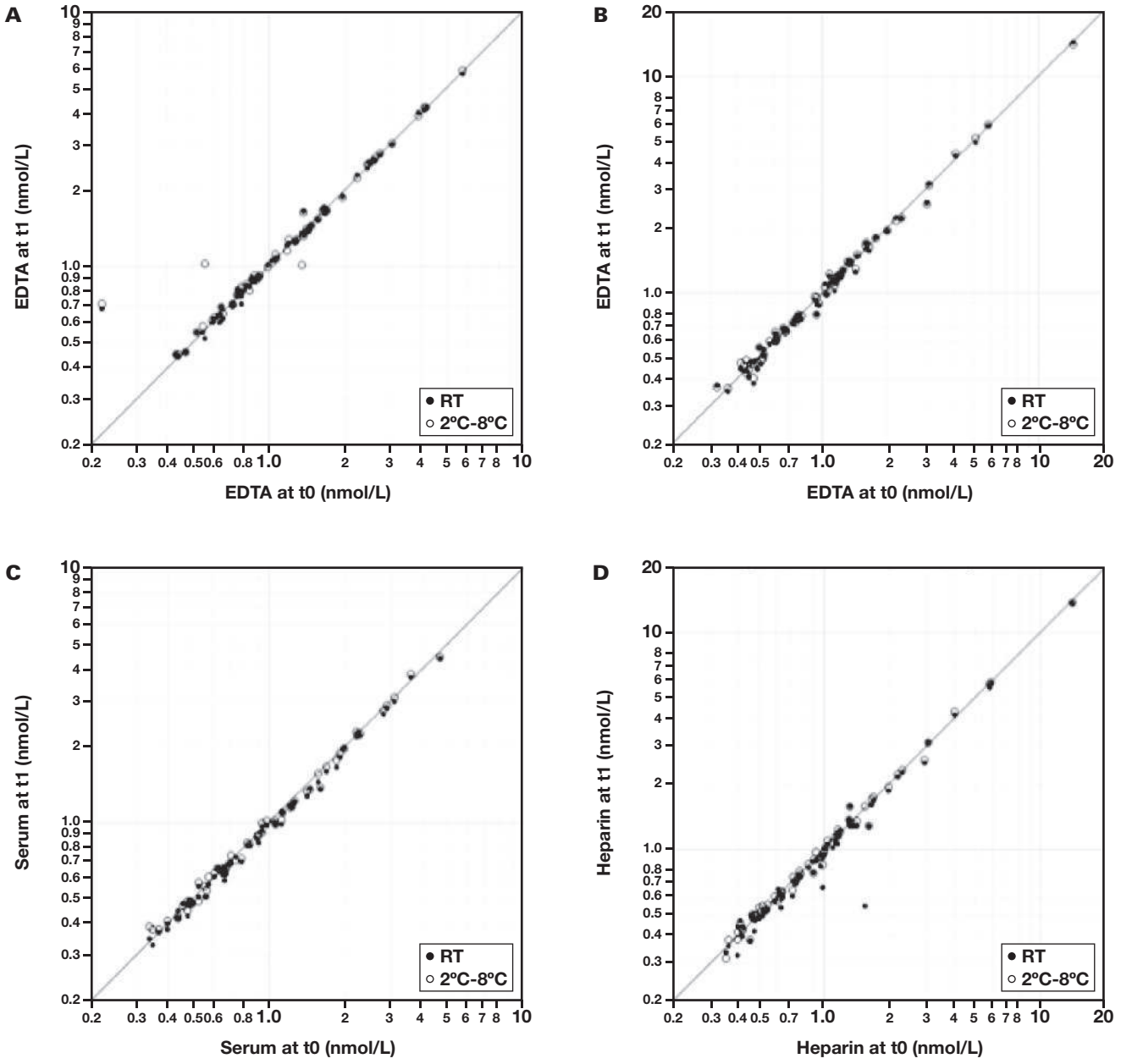


FIGURE 3. Six-hour stability of MR-proADM in EDTA plasma from the 2 sites. A and C, Samples collected at Campus Bio-Medico University Hospital. B and D, Samples collected at Policlinico Tor Vergata University Hospital.



measurement in duplicate (2 aliquots) started after 45 minutes, after which 1 aliquot of each matrix was stored at either room temperature or at 2°C–8°C for 6 hours, after which MR-proADM was measured again.

Measurements

MR-proADM was measured using the B·R·A·H·M·S MR-proADM KRYPTOR on a KRYPTOR Compact Plus. The assay has a direct measuring range from 0.05 to 10 nmol/L. The allowed sample type is EDTA plasma. The limit of quantification has been estimated in accordance with Clinical and Laboratory Standards Institute guideline EP 17-A to be 0.23 nmol/L. The maximum intra-assay coefficient of variation (CV) was estimated to be 10.8% for low concentrations. The reference range for healthy subjects (based on 144 measurements) was median 0.38 nmol/L and 97.5 percentile at 0.55 nmol/L.

Statistics

The MR-proADM values obtained in the different matrices were compared with Spearman's correlation, Passing and Bablok regression, Bland-Altman plots, and percentage agreement at 2 clinically relevant

TABLE 2. Correlation Between Measurement on Fresh Sample and After 6-Hour Storage

	Storage at RT	Storage at 2°C–8°C
EDTA plasma (at CBM)		
Median bias (IQR)	0% (–2.2 to 2.0)	0.6% (–1.5 to 3.4)
Passing-Bablok		
Intercept (CI)	0.00 (–0.01 to 0.03)	0.01 (–0.00 to 0.04)
Slope (CI)	1.00 (0.98 to 1.01)	1.00 (0.97 to 1.01)
Serum (at CBM)		
Median bias (IQR)	–4.0% (-–6.7 to –1.0)	–2.1% (–4.7 to 2.1)
Passing-Bablok		
Intercept	0.02 (–0.00 to 0.03)	0.02 (–0.01 to 0.03)
Slope	0.94 (0.93 to 0.97)	0.96 (0.94 to 0.99)
EDTA plasma (at PTV)		
Median bias (IQR)	–0.4% (–3.9 to 2.7)	0.4% (–3.7 to 3.2)
Passing-Bablok		
Intercept (CI)	0.00 (–0.00 to 0.00)	–0.01 (–0.03 to 0.02)
Slope (CI)	1.00 (0.97 to 1.03)	1.01 (0.97 to 1.03)
Heparin plasma (at PTV)		
Median bias (IQR)	–3.5% (–6.0 to 0.2)	0% (–3.7 to 3.3)
Passing-Bablok		
Intercept (CI)	–0.00 (–0.03 to 9.02)	0.01 (–0.02 to 0.02)
Slope (CI)	0.97 (0.93 to 1.00)	0.99 (0.97 to 1.02)

CBM, Campus Bio-Medico University Hospital; CI, confidence interval; IQR, interquartile range; PTV, Policlinico Tor Vergata University Hospital; RT, room temperature.

cut-offs, namely 0.87 nmol/L[11,17–20] and 1.5 nmol/L.[11,17,21,22] Mean bias with interquartile range was calculated.

The statistical analysis was carried out with Analyse-It and JMP from SAS.

Results

EDTA Plasma vs Serum

Serum and EDTA plasma was collected from 61 patients (40 female/21 male; mean age, 69 years). Seven patients received anticoagulation, and in 5 patients at least 1 sample was visibly hemolytic.

The main results are shown in **TABLE 1**. Although the Spearman correlation between serum and EDTA plasma was good (0.97), the mean bias (–2.3%) and the Passing and Bablok slope (0.83) indicate a systematic deviation that can be seen in the Bland-Altman and scatter plots (**FIGURES 1** and **2**, respectively). This is also reflected in the poor agreement at clinically relevant cut-offs (0.87 and 1.5 nmol/L). Although hemolytic samples can give imprecise results, there was no indication that this influenced the results (data not shown). Also, the use of anticoagulants did not influence the result (data not shown)

EDTA Plasma vs Heparin Plasma

EDTA plasma and heparin plasma was collected from 63 patients (33 female/30 male; mean age, 62 years). Seven patients received anticoagulant therapy, and in 11 patients at least 1 sample was visibly hemolytic. The main results are shown in **TABLE 1**. Spearman correlation between heparin plasma and EDTA plasma was excellent (1.00), and the low mean bias (2.6%) and the Passing and Bablok slope (0.98) also indicate an excellent correlation. The Bland-Altman and scatter plots (**FIGURES 1** and **2**, respectively) show no signs of concentration-dependent deviation. This is also reflected in the excellent agreement at clinically relevant cut-offs (0.87 and 1.5 nmol/L). Although hemolytic samples can give imprecise results, there was no indication that this influenced the results. Also, the use of anticoagulants did not influence the result (data not shown).

Stability at Room Temperature and at 2°C–8°C

All samples were tested for 6-hour stability after centrifugation at room temperature and at 2°C–8°C. Comparison was done for heparin plasma, serum, and EDTA plasma (separate for the 2 centers) (**FIGURES 3**). When compared to their original measurement at t0, all matrices showed good correlation reflected in the Passing-Bablok slope and the low median bias (**TABLE 2**). No apparent difference in stability was seen between storage at room temperature and at 2°C–8°C.

Discussion

This study shows for the first time the concentration of MR-proADM in serum and plasma (EDTA and heparin) in matched samples collected from routine clinical patients. The clear deviation of MR-proADM concentration in serum compared to EDTA plasma unequivocally shows that serum should not be used as the sample matrix. Heparin plasma, however, showed only minimal bias compared to EDTA plasma. The excellent correlation and the agreement at clinically relevant cut-offs show that the use of heparin plasma can be used without reservation in clinical routine and emergency samples.

We were not able to reproduce the findings in serum by Lorubbio et al,[13] who found a similar 95th percentile for studies using serum samples from heathy blood donors and studies in EDTA plasma. However, cohort-specific age and body mass index distribution might have masked the differences, as there was a significant correlation of MR-proADM concentration with both.[13,15,16] In addition to healthy blood donors,[16] we have now confirmed the breakdown of MR-proADM in the serum of in-hospital patients. The lower bias in patients compared to healthy blood donors (21% vs >30%) probably relates to the higher concentration in patients; that is, all values were well above the functional assay sensitivity.

Despite the difference in MR-proADM in serum compared to EDTA plasma, after clotting and sample preparation, the concentration was stable in all 3 matrices for at least 6 hours independent of storage temperature, meaning that the breakdown of MR-proADM in serum took place during the coagulation phase. Although in serum, proteases and cofactors are activated during coagulation, in plasma, they are mainly inhibited by the use of anticoagulants, such as EDTA (metallo- and Ca2+-dependent proteases) and heparin (thrombin, factor Xa).[23] Indeed, the total proteolytic activity was shown previously to be the highest in serum and citrate plasma followed by heparin plasma and EDTA plasma,[24] with each matrix having its own specific mix of active peptidases recognizing specific amino acid motifs. Therefore, some peptides might be broken down in serum and EDTA or heparin plasma, whereas others

are only broken down in one of them, depending on their amino acid composition.

Conclusions

This study shows that, in addition to the available EDTA plasma, heparin plasma can be used to measure MR-proADM levels. Values are completely comparable; the clinical cut-offs established in EDTA plasma[10,11] can also be used on values obtained from heparin plasma. This may potentially decrease the number of blood samples taken from a patient for diagnostic testing and increase the implementation of this biomarker.

Another collateral but no less important aspect is the possibility of using heparin samples for emergency room analysis. In fact, the use of lithium or sodium heparin samples is reserved for the assay of biomarkers for emergency tests (such as myocardial biomarkers). In this regard, the use of the same sample for different biomarkers could reduce the amount of blood drawn from the patient, and additional tests, like MR-proADM, could be very helpful to facilitate the clinical diagnostic and/or prognostic pathway. The use of this biomarker in different settings has been evaluated and it could be advantageous in clinical practice. In the emergency setting, its value has been demonstrated in sepsis diagnosis and prognosis as well as in bacterial or viral infection.[10,25–28] Very recently, in COVID-19 patients, MR-proADM was also evaluated as a helpful marker of endothelial damage, disease severity, and mortality.[29–31]

Acknowledgments

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REFERENCES

1. Muller-Redetzky HC, Will D, Hellwig K, et al. Mechanical ventilation drives pneumococcal pneumonia into lung injury and sepsis in mice: protection by adrenomedullin. *Crit Care.* 2014;18(2):R73.
2. Temmesfeld-Wollbruck B, Brell B, David I, et al. Adrenomedullin reduces vascular hyperpermeability and improves survival in rat septic shock. *Intensive Care Med.* 2007;33(4):703–710. doi:10.1007/s00134-007-0561-y.
3. Vallet B. Endothelial cell dysfunction and abnormal tissue perfusion. *Crit Care Med.* 2002;30(5 suppl):S229–S234. doi:10.1097/00003246-200205001-00010.
4. Struck J, Tao C, Morgenthaler NG, et al. Identification of an adrenomedullin precursor fragment in plasma of sepsis patients. *Peptides.* 2004;25(8):1369–1372. doi:10.1016/j.peptides.2004.06.019.
5. Caruhel P, Mazier C, Kunde J, et al. Homogeneous time-resolved fluoroimmunoassay for the measurement of midregional proadrenomedullin in plasma on the fully automated system B.R.A.H.M.S KRYPTOR. *Clin Biochem.* 2009;42(7-8):725–728.
6. Andaluz-Ojeda D, Nguyen HB, Meunier-Beillard N, et al. Superior accuracy of mid-regional proadrenomedullin for mortality prediction in sepsis with varying levels of illness severity. *Ann Intensive Care.* 2017;7(1):15.
7. Bernal-Morell E, Garcia-Villalba E, et al. Usefulness of midregional pro-adrenomedullin as a marker of organ damage and predictor of mortality in patients with sepsis. *J Infect.* 2018;76(3):249–257.
8. Legramante JM, Mastropasqua M, Susi B, et al. Prognostic performance of MR-pro-adrenomedullin in patients with community acquired pneumonia in the emergency department compared to clinical severity scores PSI and CURB. *PLoS One.* 2017;12(11):e0187702.
9. van der Starre WE, Zunder SM, Vollaard AM, et al. Prognostic value of pro-adrenomedullin, procalcitonin and C-reactive protein in predicting outcome of febrile urinary tract infection. *Clin Microbiol Infect.* 2014;20(10):1048–154.
10. Elke G, Bloos F, Wilson DC, et al. The use of mid-regional proadrenomedullin to identify disease severity and treatment response to sepsis—a secondary analysis of a large randomised controlled trial. *Crit Care.* 2018;22(1):79.
11. Saeed K, Wilson DC, Bloos F, et al. The early identification of disease progression in patients with suspected infection presenting to the emergency department: a multi-centre derivation and validation study. *Crit Care.* 2019;23(1):40.
12. Stalenhoef JE, van Nieuwkoop C, Wilson DC, et al. Biomarker guided triage can reduce hospitalization rate in community acquired febrile urinary tract infection. *J Infect.* 2018;77(1):18–24.
13. Lorubbio M, Conti AA, Ognibene A. Midregional pro-adrenomedullin (MR-ProADM) reference values in serum. *Clin Biochem.* 2018;53:173–174.
14. Golubovic M, Jankovic R, Sokolovic D, et al. Preoperative midregional proadrenomedulin and high sensitivity troponin T predict perioperative cardiovascular events in non-cardiac surgery. *Med Princ Pract.* 2018;27(3):278–284. doi:10.1159/000488197.
15. Krintus M, Kozinski M, Braga F, et al. Plasma midregional proadrenomedullin (MR-proADM) concentrations and their biological determinants in a reference population. *Clin Chem Lab Med.* 2018;56(7):1161–1168.
16. Morgenthaler NG, Struck J, Alonso C, et al. Measurement of midregional proadrenomedullin in plasma with an immunoluminometric assay. *Clin Chem.* 2005;51(10):1823–1829.
17. Albrich WC, Dusemund F, Ruegger K, et al. Enhancement of CURB65 score with proadrenomedullin (CURB65-A) for outcome prediction in lower respiratory tract infections: derivation of a clinical algorithm. *BMC Infect Dis.* 2011;11:112.
18. Brusse-Keizer M, Zuur-Telgen M, van der Palen J, et al. Adrenomedullin optimises mortality prediction in COPD patients. *Respir Med.* 2015;109(6):734–742.
19. Citgez E, Zuur-Telgen M, van der Palen J, et al. Stable-state midrange proadrenomedullin is associated with severe exacerbations in COPD. *Chest.* 2018;154(1):51–57.
20. Gonzalez Del Castillo J, Clemente-Callejo C, Llopis F, et al. Midregional proadrenomedullin safely reduces hospitalization in a low severity cohort with infections in the ED: a randomized controlled multi-centre interventional pilot study. *Eur J Intern Med.* 2021;88:104–113.
21. Heining L, Giesa C, Ewig S. MR-proANP, MR-proADM, and PCT in patients presenting with acute dyspnea in a medical emergency unit. *Lung.* 2016;194(2):185–191.
22. Renaud B, Schuetz P, Claessens Y-E, et al. Proadrenomedullin improves risk of early admission to ICU score for predicting early severe community-acquired pneumonia. *Chest.* 2012;142(6):1447–1454.
23. Bottger R, Hoffmann R, Knappe D. Differential stability of therapeutic peptides with different proteolytic cleavage sites in blood, plasma and serum. *PLoS One.* 2017;12(6):e0178943.
24. Jambunathan K, Galande AK. Sample collection in clinical proteomics—proteolytic activity profile of serum and plasma. *Proteomics Clin Appl.* 2014;8(5-6):299–307.
25. Angeletti S, Spoto S, Fogolari M, et al. Diagnostic and prognostic role of procalcitonin (PCT) and MR-pro-Adrenomedullin (MR-proADM) in bacterial infections. *APMIS.* 2015;123(9):740–748.
26. Spoto S, Cella E, de Cesaris M, et al. Procalcitonin and MR-proadrenomedullin combination with SOFA and qSOFA scores for sepsis diagnosis and prognosis: a diagnostic algorithm. *Shock.* 2018;50(1):44–52.
27. Spoto S, Fogolari M, De Florio L, et al. Procalcitonin and MR-proadrenomedullin combination in the etiological diagnosis and prognosis of sepsis and septic shock. *Microb Pathog.* 2019;137:103763.

28. Spoto S, Legramante JM, Minieri M, et al. How biomarkers can improve pneumonia diagnosis and prognosis: procalcitonin and mid-regional-pro-adrenomedullin. *Biomark Med.* 2020;14(7):549–562.
29. Lo Sasso B, Gambino CM, Scichilone M, et al. Clinical utility of midregional proadrenomedullin in patients with COVID-19. *Lab Med.* 2021;52(5):493–498.
30. Minieri M, Di Lecce VN, Lia MS, et al. Role of MR-proADM in the risk stratification of COVID-19 patients assessed at the triage of the emergency department. *Crit Care.* 2021;25(1):407.
31. Spoto S, Agrò FE, Sambuco F, et al. High value of mid-regional proadrenomedullin in COVID-19: a marker of widespread endothelial damage, disease severity, and mortality. *J Med Virol.* 2021;93(5):2820–2827.

Serum Ferritin Has Limited Prognostic Value on Mortality Risk in Patients with Decompensated Cirrhosis: A Propensity Score Matching Analysis

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Keywords: ferritin, propensity score matching analysis, liver cirrhosis, prognosis, MELD, mortality

Abbreviations: PSM, propensity score matching; MELD, model for end-stage liver disease; HCC, hepatocellular carcinoma; CTP, Child-Turcotte-Pugh class; TJMUGH, Tianjin Medical University General Hospital; EGVB, esophagogastric variceal bleeding; PT-INR, prothrombin-international normalized ratio; WBC, white blood cell count; IQR, interquartile range; HR, hazard ratio; CI, confidence interval; TBIL, total bilirubin

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ABSTRACT

Objective: The prognostic value of serum ferritin remains elusive in the literature. We aimed to examine the association between serum ferritin and mortality risk in cirrhosis.

Methods: A total of 257 cirrhotic patients were recruited. The cut-off of serum ferritin was determined by X-tile. The Cox regression and Kaplan-Meier method were used. A 1:1 propensity score matching (PSM) was performed to diminish the impacts of selection bias and possible confounders.

Results: The difference regarding mortality was mostly significant for serum ferritin >158 ng/mL. Before PSM, serum ferritin >158 ng/mL was an independent predictor of mortality. However, the clinical relevance of high ferritin level for prognostication was blunted after PSM (survival rate: 86.8% vs 96.3%, $P = .078$). Cox regression indicated that model for end-stage liver disease remains only independent risk factor of 180-day mortality after PSM.

Conclusion: Serum ferritin may not serve as an independent prognostic indicator of mortality risk in decompensated cirrhotic patients.

Ferritin represents the principal protein for iron storage, and the liver is the main storage site of ferritin.[1] Ferritin structurally consists of 24 subunits constructing a sphere containing 4500 iron atoms, which are deposited as hydroxyl-phosphates. The iron is stored and occasionally exported back to the circulation via ferroportin in response to iron deficiency after being detoxified in the ferritin.[2] Moreover, it has been suggested that ferritin contributes to iron transport by sequestering iron when not required and releasing it when in demand.[3] Mounting evidence indicates that patients with liver diseases present higher levels of serum ferritin because necro-inflammation of liver tissues and release of ferritin from injured hepatocytes or a response to macrophage activation.[4,5] Notably, ferritin can serve as both a cytoprotective agent and a pathogenic factor. The production of ferritin, referring to an acute phase protein, is stimulated by several inflammatory cytokines such as tumor necrosis factor-α and interleukin-1β.[6] Under oxidative stress, the expression of ferritin is upregulated due to nuclear factor-κB and is essential for its antioxidant properties.[7] Ferritin also regulates the inflammatory and immune response by detoxifying iron, whereas free iron potentiates oxidative stress, lipid peroxidation, and some novel forms of regulated cell death such as ferroptosis.[8]

High serum ferritin appears to be a significant prognostic biomarker that has been associated with poor outcomes in advanced chronic liver diseases and plays a nefarious role in some pathological entities, countering its cytoprotective and antioxidant activities.[9–12] The interpretation of ferritin levels in the context of cirrhosis is complicated due to inconsistent findings in the existing literature. Decades ago, Walker and colleagues[13] showed that high serum ferritin can predict 180-day and 1-year waiting list mortality in patients with cirrhosis independent of a model for end-stage liver disease (MELD) or the presence of hepatocellular carcinoma (HCC). Another study enrolling 318 patients with decompensated cirrhosis also indicated that increased levels of

serum ferritin correlate with liver disease severity and are associated with short-term liver-related death independent of MELD.[14] However, a study including a large-scale cohort of 1079 patients found limited value of serum ferritin as a prognostic indicator for pre- or post-liver transplant survival.[15] The authors claimed that the association between ferritin and dire outcomes is compromised in established liver prognostic models, namely MELD and Child-Turcotte-Pugh class (CTP). Recently, Fallet et al[16] showed that both low and high serum ferritin levels are associated with lower survival rates.

As described above, several factors in relation to disease severity may affect the prognostic value of serum ferritin and, as such, exposed and unexposed patients (ie, those with high vs low levels of serum ferritin) tend to differ in many clinical and biochemical characteristics. This imbalance makes fair comparisons between distinctly exposed patients challenging, where randomization can circumvent this issue by assigning any "confounder" naturally balanced among groups. In response to the inherent issues of observational studies, propensity score matching (PSM) analysis has been increasingly used in the medical field.[17–19] PSM aims to mimic randomization; therefore, we used this analytical technique to optimize the variable similarity among patients with cirrhosis with different levels of serum ferritin. On the other hand, a new method designated as X-tile is the method of choice to generate the cut-off of serum ferritin in relation to short-term mortality in a study population. This graphical representation can provide insight into the biological nature of a biomarker and its relationship with outcomes by defining substantial exposed subpopulations, where cut-off selection is complicated by time-dependent evaluation of outcomes.[20]

Here, we used the X-tile method to determine a cut-off of serum ferritin for 180-day mortality in hospitalized patients with cirrhosis and hypothesize that serum ferritin may be independently associated with survival probability testing by the PSM analytical technique.

Methods

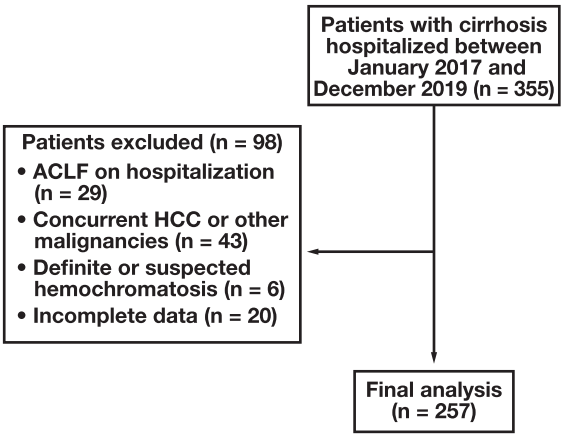
Study Population

A total of 355 patients with decompensated cirrhosis were recruited from the Department of Gastroenterology and Hepatology, Tianjin Medical University General Hospital (TJMUGH) between January 2017 and December 2019. The exclusion criteria were as follows: (1) acute-on-chronic liver failure on hospitalization; (2) concurrent HCC or other malignancies; (3) definite or suspected diagnosis of hemochromatosis; (4) incomplete clinical, laboratory, or follow-up data. Finally, 257 patients with cirrhosis were included (**FIGURE 1**). All patients were followed until death or for a minimum of 180 days. We ascertained cirrhosis through medical history, laboratory results, imaging data, gastroscopy findings, and liver biopsy results. The presence of decompensated episodes were recorded, including ascites, esophagogastric variceal bleeding (EGVB), hepatic encephalopathy, and infection. The study was performed adherent to the Declaration of Helsinki and approved by the Ethics Committee of TJMUGH. Informed consent was obtained from all participants.

Clinical and Biological Data Acquisition

Baseline clinical and biological data, including age, gender, etiology, presence of decompensated episodes, serum ferritin levels, hepatic function tests, complete blood cell counts, prothrombin-international normalized ratio (PT-INR), electrolytes, and liver-oriented prognostic scores (CTP class and MELD) were retrieved within 48 hours of hospitalization. All chemical analysis was performed on a Beckman Coulter AU5800/UniCel DxI800 Access autoanalyzer according to the manufacturer's instructions. The primary outcome of interest is patient death within 180 days. All tests were measured in our routine laboratory unit in TJMUGH following standardized protocol.

FIGURE 1. A flowchart of the study population. ACLF, acute-on-chronic liver failure; HCC, hepatocellular carcinoma.



X-tile Analysis

We selected the X-tile program to provide a single and global assessment of every possible way to divide the population into high- and low-level marker expression (serum ferritin in the current study). In addition, the X-tile analysis can provide a rigorous statistical estimation by dividing the study cohort into training and validation subsets to determine the best *P* value when it is unavailable for these training and validation cohorts.[20,21] X-tile makes a separate list of "censored" and "uncensored" observations in terms of follow-up time aimed at generating training and validation cohorts. Patients were included in training and validation sets by selecting every other patient from the list of participants. This analytical technique not only normalizes the baseline survival curve but also prevents the possibility of obtaining distinct *P* values due to reanalyzing the same data.

PSM Analysis

Because patients in the current study were allocated in terms of serum ferritin instead of a random selection, potential confounding impact and selection bias may diminish the reliability of retrieved results. One purpose of this study was to delineate whether serum ferritin can serve as a reliable and independent biomarker to identify patients at high mortality risk. Therefore, PSM analysis was conducted to simulate random allocation and to minimize these confounding effects. Using a logistic regression model, the propensity scores were calculated according to baseline characteristics, including MELD, hemoglobin, platelets, white blood cell count (WBC), sodium, albumin, and the presence of EGVB. A 1:1 matching was employed using 0.1 caliper. Data for 106 patients (53 pairs) were used in the final analysis.

Statistical Analysis

Data are presented as mean ± standard deviation, median (interquartile range, IQR), or number (n). We compared the continuous

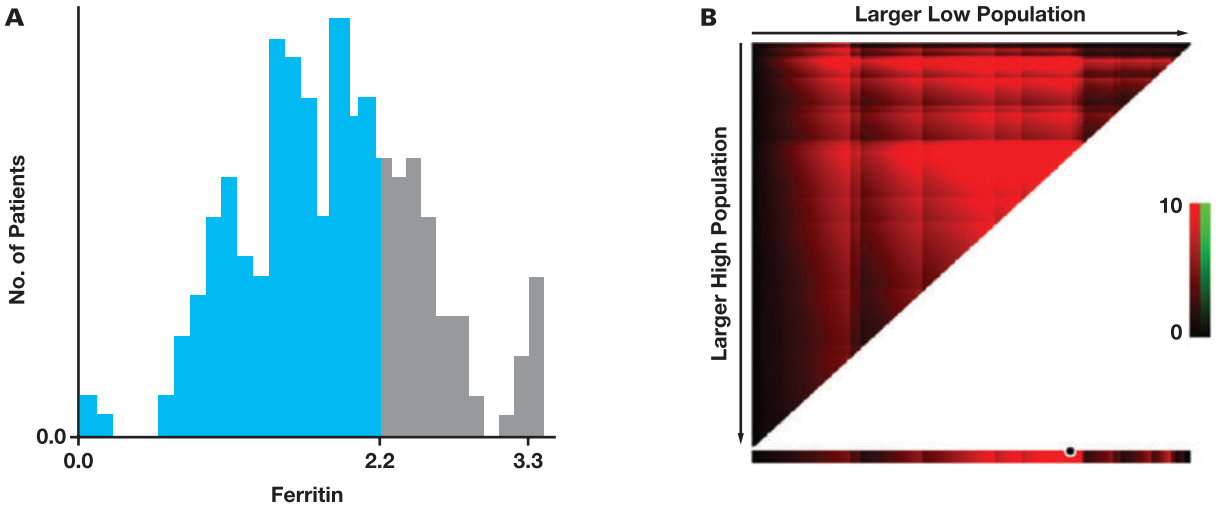
TABLE 1. Baseline Characteristics of the Study Cohort Stratified by Serum Ferritin[a]

Characteristic	Total (n = 257)	Serum Ferritin (ng/mL)		*P* Value
		≤158 (n = 183)	>158 (n = 74)	
Age (y)	63 (57, 69)	63 (57, 68)	62 (55.8, 70.3)	.6113
Gender (male:female)	128: 129	85: 98	43: 31	.0996
Outcome				.0003
Dead	17 (6.7%)	5 (2.7%)	12 (16.2%)	
Alive	240 (93.3%)	178 (97.3%)	62 (83.8%)	
CTP class				<.0001
A	90 (35.0%)	82 (45%)	8 (10%)	
B	128 (49.8%)	86 (47%)	42 (57%)	
C	39 (15.2%)	15 (8%)	24 (33%)	
MELD	9 (6, 12)	9 (5, 11)	11 (7.5, 17)	<.0001
Hemoglobin (g/L)	89 (71, 110)	83 (66, 101)	104 (85, 122)	<.0001
Platelet ($\times 10^9$/L)	82 (51, 114)	78 (49, 108)	94.5 (61.8, 123.3)	.0346
WBC ($\times 10^9$/L)	3.5 (2.4, 5.0)	3.3 (2.3, 4.4)	4.6 (3.2, 6.5)	<.0001
PT-INR	1.3 (1.2, 1.4)	1.3 (1.2, 1.4)	1.5 (1.2, 1.6)	<.0001
Sodium (mmol/L)	140 (138, 142)	141 (138, 142)	140 (137, 142)	.0452
Potassium (mmol/L)	3.9 (3.5, 4.1)	3.9 (3.6, 4.1)	3.8 (3.5, 4.1)	.8394
Creatinine (mmol/L)	59 (50, 72)	59 (47, 73)	59 (52.5, 72)	.4366
TBIL (μmol/L)	21.7 (13.9, 37.8)	18.1 (12.7, 27.3)	41 (22.6, 112.5)	<.0001
Albumin (g/L)	28.9 ± 5.9	30.1 ± 5.8	25.9 ± 5.0	<.0001
Ascites				.3244
Present	151 (58.8)	104 (58%)	47 (63.5%)	
Absent	106 (41.2%)	79 (42%)	27 (36.5%)	
HE				.1375
Present	21 (8.2%)	12 (6.6%)	9 (12.2%)	
Absent	236 (91.8%)	171 (93.4%)	65 (87.8%)	
EGVB				<.0001
Present	178 (69.3%)	140 (76.5%)	38 (51%)	
Absent	79 (30.7%)	43 (23.5%)	36 (49%)	
Infection				.1492
Present	30 (11.7%)	18 (10%)	12 (16.2%)	
Absent	227 (88.3%)	165 (90%)	62 (83.8%)	
Etiology				.1119
Alcohol	55 (21.4%)	32 (17.5%)	23 (31.1%)	
HBV/HCV	72 (28%)	55 (30%)	17 (23%)	
Autoimmune/cholestasis	75 (29.2%)	56 (30.6%)	19 (25.7%)	
Cryptogenic/other	55 (21.4%)	40 (21.9%)	15 (20.2%)	

CTP, Child-Turcotte-Pugh; EGVB, esophagogastric variceal bleeding; HE, hepatic encephalopathy; MELD, model for end-stage liver disease; PT-INR, prothrombin-international normalized ratio; TBIL, total bilirubin; WBC, white blood cell count.
[a]*Values are presented as the mean ± standard deviation, median (interquartile range), or number of patients (%).*

data by using the independent Student *t*-test or the Mann-Whitney *U* test for groups without normal distribution. Likewise, we compared the categorical variables by using χ^2 test or Fisher exact test, as appropriate. The correlation was determined according to Pearson correlation coefficient (*r*) and serum ferritin was presented in its logarithmic transformation. Multivariate analyses performed by the Cox proportional hazard model with a backward elimination procedure were used to identify independent risk factors of 180-day mortality. Hazard ratio (HR) and 95% confidence interval (CI) were obtained. The survival rates were calculated by using the Kaplan-Meier method and compared to detect statistically significant differences by using the log-rank test. A *P* value <.05 was regarded as statistically significant. All procedures were carried out by using SPSS 23.0 (IBM).

FIGURE 2. Analysis of serum ferritin using X-tile software. A, Prognostic significance of serum ferritin in patients with cirrhosis was obtained by using the statistical algorithm in X-tile to calculate the most efficient cut-off. B, X-tile plots of the value of serum ferritin. The data on the horizontal ordinate increase from the left to the right, defined as the larger low population. The data on the vertical ordinate decrease from the top to the bottom, defined as the larger high population.



Results

Characteristics of Patients

The baseline clinical characteristics and biological data of recruited patients are shown in **TABLE 1**. A total of 257 patients with cirrhosis with a median age of 63 years (IQR: 57, 69 years) were enrolled in the study. The etiologies of the cirrhosis were autoimmune/cholestasis in 75, hepatitis B virus or hepatitis C virus infection in 72, alcoholism in 55, and cryptogenic/others in 55 participants. The cirrhosis-associated complications included EGVB in 178, ascites in 151, infection in 30, and hepatic encephalopathy in 21 patients. Among these patients, 90 were stratified as CTP class A, 128 as CTP class B, and 39 as CTP class C. The median MELD score on hospitalization was 9 (IQR: 6, 12 points); 6.6% (n = 17) of the cohort died within the 180-day follow-up period. The 180-day mortality of choice was adherent to prior investigation and consideration regarding statistical validity and data integrity. The reasons of death were all attributed to cirrhosis-associated episodes, including liver failure (n = 7), severe infection (n = 4), EGVB (n = 4), and hepatic encephalopathy (n = 2). X-tile analysis was performed to determine optimal serum ferritin cut-off in terms of 180-day mortality in the entire cohort. In detail, histogram analysis showed that serum ferritin levels represent a diffused continuous distribution with no discernable subpopulations (**FIGURE 2A**). Using the histogram as a guide, X-tile identified the optimal division of the cohort into 2 populations (≤158 and >158 ng/mL), and the red color in the plot in **FIGURE 2B** illustrates that patients with higher levels of serum ferritin exhibited poorer survival. As a result, the threshold of serum ferritin >158 ng/mL (shown as a black circle on the rectangular X-tile plot) was most significant in distinguishing dire outcomes in patients with cirrhosis.

These participants were divided into 2 groups based on serum ferritin level. Of these, 183 (71.2%) patients were in the group with serum ferritin ≤158 ng/mL and 74 (28.8%) were in the group with serum ferritin >158 ng/mL. There were significant differences in CTP class, MELD, hemoglobin, platelet, WBC, sodium, total bilirubin (TBIL), albumin, and the presence of EGVB. Of note, patients in the serum ferritin >158 ng/mL group were mostly CTP class C and had higher MELD, higher hemoglobin, higher platelets, higher WBC, lower sodium, higher TBIL, lower albumin, and less presence of EGVB than the ≤158 ng/mL group patients.

Independent Prognostication of 180-Day Mortality

After examination of risk factors with significant differences by using univariate analysis, ascites ($P = .015$), infection ($P = .026$), TBIL ($P < .001$), WBC ($P = .003$), sodium ($P < .001$), albumin ($P = .002$), creatinine ($P = .005$), MELD ($P < .001$), CTP class ($P < .001$), and serum ferritin fitted into a multivariate Cox regression model (**TABLE 2**). Given that both CTP class and MELD are regarded as conventional mainstays for predicting mortality in the context of cirrhosis, we established 2 models to detect whether serum ferritin serves as a risk factor of 180-day mortality independently. In model 1, the HRs were significantly higher for decreased sodium ($P < .001$), more severe MELD ($P < .001$), and serum ferritin > 158 ng/mL ($P = .001$). In model 2, the HRs were significantly higher for increased WBC ($P = .032$), decreased sodium ($P = .004$), increased creatinine ($P = .020$), CTP class ($P < .001$), and serum ferritin >158 ng/mL ($P < .001$). Additionally, patients with serum ferritin >158 ng/mL had significantly higher 180-day mortality rate than patients with serum ferritin ≤158 ng/mL (16.2% vs 2.7%, $P = .003$, **FIGURE 3**)

The Correlation Between Serum Ferritin and Other Laboratory Parameters

It has been suggested that increased serum ferritin may indicate either iron overload or increased inflammatory action.[22] Therefore, we investigated the correlation between serum ferritin and 2 relevant variables, namely WBC and hemoglobin (see Supplemental Figure 1). As expected, serum ferritin levels positively correlated with both WBC ($r = 0.397$, $P < .0001$) and hemoglobin ($r = 0.307$, $P < .0001$) with statistical significance.

TABLE 2. Univariate and Multivariate Analysis of 180-Day Mortality in Patients with Cirrhosis Before PSM

	Univariate Analysis		Multivariate Analysis Model 1[a]		Multivariate Analysis Model 2[b]	
	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value
Age	0.991 (0.946, 1.039)	.706				
Gender						
Male	1.125 (0.42, 3.014)	.815				
Female	Ref					
Ascites						
Present	12.44 (1.624, 95.354)	.015				
Absent	Ref					
HE						
Present	2.643 (0.695, 10.056)	.154				
Absent	Ref					
Infection						
Present	3.583 (1.166, 11.01)	.026				
Absent	Ref					
EGVB						
Present	0.473 (0.175, 1.274)	.139				
Absent	Ref					
Hemoglobin	0.998 (0.979, 1.017)	.813				
TBIL	0.137 (0.051, 0.365)	<.001				
WBC	1.181 (1.056, 1.32)	.003			1.206 (1.016,1.431)	.032
Sodium	0.801 (0.727, 0.882)	<.001	0.818 (0.737, 0.907)	<.001	0.831 (0.731, 0.943)	.004
Potassium	1.844 (0.978, 3.475)	.058				
Platelet	1.001 (0.994, 1.01)	.714				
Albumin	0.855 (0.773, 0.945)	.002				
Creatinine	1.008 (1.003, 1.014)	.005			1.011 (1.002, 1.021)	.02
MELD	1.207 (1.108, 1.316)	<.001	1.243 (1.101, 1.403)	<.001		
CTP class	2.018 (1.498, 2.719)	<.001			2.165 (1.427, 3.285)	<.001
Serum ferritin, ng/mL						
≤158	Ref					
>158	5.151 (1.829, 14.506)	.002	12.963 (2.973, 56.524)	.001	55.725 (7.51, 413.49)	<.001

CI, confidence interval; CTP, Child-Turcotte-Pugh; EGVB, esophagogastric variceal bleeding; HE, hepatic encephalopathy; HR, hazard ratio; MELD, model for end-stage liver disease; PSM, propensity matching analysis; TBIL, total bilirubin; WBC, white blood cell count.
[a]Model 1: ascites, albumin, MELD, serum ferritin, sodium, infection.
[b]Model 2: CTP class, creatinine, serum ferritin, sodium, WBC.

The Prognostic Value of Serum Ferritin after PSM

Because a wide array of variables were imbalanced between the serum ferritin ≤158 ng/mL and serum ferritin >158 ng/mL patients, we adopted a 1:1 ratio PSM to reduce potential confounding effects. In the PSM analysis, we matched 53 patients from the ferritin ≤158 ng/mL group with 53 patients with serum ferritin >158 ng/mL (n = 53) by using the nearest neighbor algorithm. The clinical characteristics and biological data were well balanced and evenly distributed between these 2 groups (all $P > .05$, **TABLE 3**). Intriguingly, the Kaplan-Meier survival curve showed no statistically significant difference in 180-day survival rate between patients with serum ferritin >158 ng/mL and those with serum ferritin ≤158 ng/mL (86.8% vs 96.3%, $P = .078$, **FIGURE 4**). In addition, multivariate Cox analysis also indicated that serum ferritin >158 ng/mL is not an independent predictor of 180-day mortality (HR = 3.22, 95% CI 0.669–15.500, $P = .145$, **TABLE 4**) after PSM.

Discussion

The predictive utility of serum ferritin in the context of liver cirrhosis is still in debate. The majority of previous publications only performed univariate and multivariate regression analyses, wherein overfitting is common because of covariate selection.[13,14,23] In this regard, even multiple analyses may lead to relatively unreliable findings. Given this, we performed PSM, for the first time, to investigate the prognostic value of serum ferritin on 180-day mortality in a retrospective cohort with

FIGURE 3. Kaplan-Meier analysis of 180-day mortality in patients with cirrhosis before propensity score matching. The mortality rate was higher in patients with serum ferritin ≤158 ng/mL than patients with ferritin >158 ng/mL. *P* < .001.

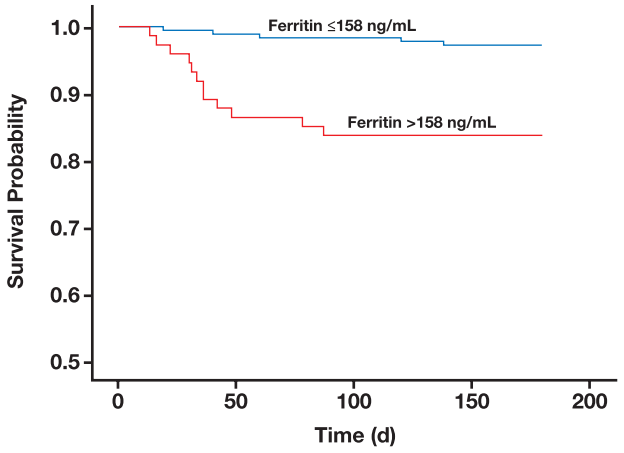
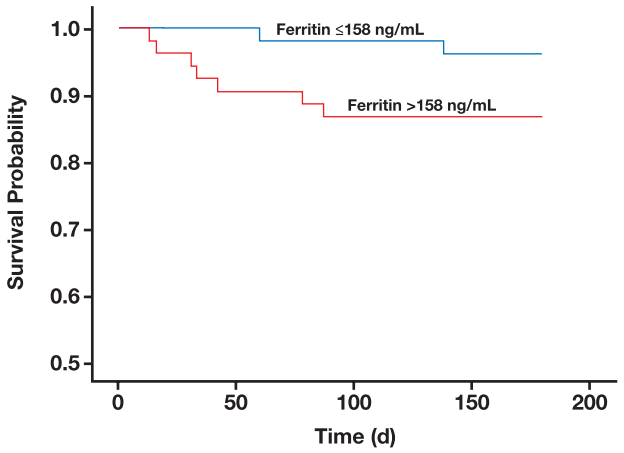


FIGURE 4. Kaplan-Meier analysis of 180-day mortality in patients with cirrhosis after propensity score matching. No significant difference of 180-day mortality rate was found between patients with serum ferritin ≤158 ng/mL and >158 ng/mL. *P* = .078.



a large number of participants. We found that high serum ferritin level may not predict mortality in the context of cirrhosis.

The reason why the prognostic value of serum ferritin has been repeatedly explored in the field of hepatology is probably due to the simplicity and cost-effectiveness in routine practice. Moreover, it is tempting to consider that survival may be increased by effectively managing and optimizing iron dysregulation, which is common in cirrhosis, because serum ferritin is a sensitive biomarker of iron homeostasis.[10,24] Likewise, ferritin acts as an acute phase reactant in response to inflammatory action and thus facilitates the progression of advanced-stage liver diseases such as fibrosis, cirrhosis, and HCC.[11] On the other hand, it has been demonstrated that ferritin can suppress intensive iron overload and bears anti-inflammatory, immunomodulatory, and hepatoprotective effects.[25] Collectively, the pleiotropic functions of ferritin make the interpretation of its clinical utility even more complicated. Another concern that should be addressed is whether ferritin can be independently associated with other traditional scoring systems (eg, CTP class or MELD) by adding more pathophysiological information or whether it is just an indicator of underlying liver disease severity. Keeping this in mind, we looked into the practical relevance of serum ferritin to mortality risk by using PSM analysis. It has been widely proved that characteristics of PSM render it an overall appealing method when compared with traditional regression analyses. PSM is more likely to obtain a similar distribution of baseline variables among exposed and unexposed participants, which closely mimics what would be expected in randomized control trials.[26,27] Traditional analysis of observational studies is limited in that the observed differences may result from both varying patient characteristics as well as differences in relation to exposure (eg, serum ferritin in our population), making it difficult to distinguish the true impact of exposed factors. Indeed, as showed in **TABLE 1**, several parameters, such as WBC, hemoglobin, presence of EGVB, and MELD markedly differed between groups with low and high serum ferritin levels. In our study, use of PSM resulted in adequate balance in groups with and without high serum ferritin levels across all baseline variables, as described in **TABLE 3**. However, the prognostic usefulness of serum ferritin decreased when the aforesaid parameters were balanced. As shown in **TABLE 4**, MELD remained the sole independent predictor of 180-day mortality. Given these results, we believe that the greater reduction of confounding impact afforded by PSM increases the probability of obtaining a more valid estimation of the relationship between exposure and outcomes.

We herein acknowledge several limitations of the current research. First, taking into account its retrospective nature, there may exist unobserved confounders not included in the propensity matching analysis. Serum iron, transferrin, and hepcidin can reflect iron homeostasis and hematopoiesis and have been shown to associate with various outcomes in liver diseases. Therefore, our group is now conducting seminal studies to explore the prognostic value of other iron-related biomarkers. Second, this research was unable to internally validate our results by using separate cohort samples but rather used X-tile software to detect the prerequisite serum ferritin cut-off. This was a deliberate choice, because the graphical representation of X-tile may result in insight into the feature of a biomarker and the evaluation of multiple cut-offs via cross-validation. Also, X-tile has been widely used in the medical field.[28,29] Last, there were only 17 deaths in the entire cohort, which renders the research underpowered.

In conclusion, our results indicate that serum ferritin may not serve as a prognostic indicator of mortality independently in patients with decompensated cirrhosis.

Supplementary Data

Supplemental figures and tables can be found in the online version of this article at www.labmedicine.com

Acknowledgments

We acknowledge all the nurses who took part in this research.

TABLE 3. Baseline Characteristics in the PSM Cohort[a]

Characteristic	Total (n = 106)	Serum Ferritin (ng/mL)		*P* Value
		≤158 (n = 53)	>158 (n = 53)	
Age (y)	61.5 (55, 69)	61 (54.5, 68)	62 (56.5, 72)	.3277
Gender (male:female)	51: 55	20: 33	31: 22	.0514
Outcome				.1607
Dead	9 (8.5%)	2 (3.7%)	7 (13.2%)	
Alive	97 (91.5%)	51 (96.3%)	46 (86.8%)	
CTP class				.1358
A	22 (23.5%)	15 (28.3%)	7 (13.2%)	
B	57 (53%)	27 (50.9%)	30 (56.6%)	
C	27 (23.5%)	11 (20.7%)	16 (30.2%)	
MELD score	10 (7.8, 12.3)	10 (8, 11)	10 (6, 13)	.1187
Hemoglobin (g/L)	98.5 (79.8, 117)	98 (71.5, 116.5)	101 (81.5, 117.5)	.3685
Platelet ($\times 10^9$/L)	82.5 (60,114.5)	83 (60, 117)	88 (60.5, 112.5)	.8122
WBC ($\times 10^9$/L)	4.0 (2.9, 5.7)	3.7 (2.7, 4.8)	4.5 (3.0, 6.3)	.0734
PT-INR	1.3 (1.2, 1.5)	1.3 (1.2, 1.4)	1.3 (1.2, 1.6)	.1868
Sodium (mmol/L)	140 (137, 142)	140 (137, 142)	140 (136.5, 142)	.3400
Potassium (mmol/L)	3.7 (3.5, 4.1)	3.6 (3.4, 4)	3.8 (3.6, 4.2)	.0834
Creatinine (mmol/L)	56.5 (47, 66)	54 (45, 61.5)	59 (51, 72)	.0791
TBIL (μmol/L)	27.9 (16.2, 49.9)	23.9 (15.1, 38)	37.5 (20, 81.2)	.0521
Albumin (g/L)	27 (23, 30)	27 (23, 30.5)	26 (24, 29)	.4884
Ascites				>.9999
Present	69 (65.1%)	33 (62.3%)	36 (67.9%)	
Absent	37 (34.9%)	20 (37.7%)	17 (32.1%)	
HE				>.9999
Present	8 (7.5%)	4 (7.5%)	4 (7.5%)	
Absent	98 (92.5%)	49 (92.5%)	49 (92.5%)	
EGVB				>.9999
Present	66 (62.3%)	33 (62.3%)	33 (62.3%)	
Absent	40 (37.7%)	20 (37.7%)	20 (37.7%)	
Infection				.7752
Present	14 (13.2%)	6 (11.3%)	8 (15.1%)	
Absent	92 (86.8%)	47 (88.7%)	45 (84.9%)	
Etiology				.1295
Alcohol	23 (21.7%)	7 (13.2%)	16 (30.2%)	
HBV/HCV	30 (28.3%)	18 (34%)	12 (22.6%)	
Autoimmune/cholestasis	35 (33%)	20 (37.7%)	15 (28.3%)	
Others	18 (17%)	8 (15.1%)	10 (18.9%)	

CTP, Child-Turcotte-Pugh; EGVB, esophagogastric variceal bleeding; HE, hepatic encephalopathy; MELD, model for end-stage liver disease; PSM, propensity score matching; PT-INR, prothrombin-international normalized ratio; TBIL, total bilirubin; WBC, white blood cell count.
[a]*Values are presented as the median (interquartile range) or number of patients (%).*

REFERENCES

1. Harrison PM, Arosio P. The ferritins: molecular properties, iron storage function and cellular regulation. *Biochim Biophys Acta.* 1996;1275(3):161–203. doi:10.1016/0005-2728(96)00022-9.
2. Sakamoto S, Kawabata H, Masuda T, et al. H-Ferritin is preferentially incorporated by human erythroid cells through transferrin receptor 1 in a threshold-dependent manner. *PLoS One.* 2015;10(10):e0139915.
3. Anderson GJ, Frazer DM. Current understanding of iron homeostasis. *Am J Clin Nutr.* 2017;106(suppl 6):1559S–1566S.
4. Adams P. Management of elevated serum ferritin levels. *Gastroenterol Hepatol (N Y).* 2008;4(5):333–334.
5. Dev S, Babitt JL. Overview of iron metabolism in health and disease. *Hemodial Int.* 2017;21(suppl 1):S6–S20.
6. Tran TN, Eubanks SK, Schaffer KJ, et al. Secretion of ferritin by rat hepatoma cells and its regulation by inflammatory cytokines and iron. *Blood.* 1997;90(12):4979–4986.
7. Pham CG, Bubici C, Zazzeroni F, et al. Ferritin heavy chain upregulation by NF-kappaB inhibits TNFalpha-induced apoptosis by suppressing reactive oxygen species. *Cell.* 2004;119(4):529–542.

TABLE 4. Univariate and Multivariate Analysis of 180-Day All-Cause Mortality in Patients With Cirrhosis After PSM

Variables	Univariate Analysis			Multivariate Analysis		
	HR	95% CI	*P* Value	HR	95% CI	*P* Value
Age	0.989	0.927, 1.055	.737			
Gender						
Male	1.359	0.365, 5.060	.648			
Female	Ref					
Ascites						
Present	4.009	0.525, 31.981	.191			
Absent	Ref					
HE						
Present	1.875	0.235, 14.991	.553			
Absent	Ref					
Infection						
Present	3.864	0.966, 15.449	.271			
Absent	Ref					
EGVB						
Present	0.717	0.193, 2.671	.620			
Absent	Ref					
Hemoglobin	0.994	0.968, 1.021	.646			
TBIL	1.005	1.000, 1.010	.916			
WBC	1.041	0.909, 1.192	.566			
Sodium	0.887	0.835, 0.943	.043	0.910	0.824, 1.005	.062
Potassium	3.827	0.317, 2.681	.882			
Platelet	0.999	0.988, 1.010	.825			
Albumin	0.910	0.795, 1.041	.169			
Creatinine	1.011	0.998, 1.024	.126			
MELD	1.113	1.002, 1.236	.046	1.112	1.002, 1.236	.048
CTP class	1.300	0.890, 1.899	.175			
Serum ferritin, ng/mL						
≤158	Ref					
>158	3.22	0.669, 15.500	.145			

CI, confidence interval; CTP, Child-Turcotte-Pugh; EGVB, esophagogastric variceal bleeding; HE, hepatic encephalopathy; HR, hazard ratio; MELD, model for end-stage liver disease; PSM, propensity matching analysis; TBIL, total bilirubin; WBC, white blood cell count.

8. Mao L, Zhao T, Song Y, et al. The emerging role of ferroptosis in non-cancer liver diseases: hype or increasing hope? *Cell Death Dis.* 2020;11(7):518.
9. Czaja AJ. Review article: iron disturbances in chronic liver diseases other than haemochromatosis—pathogenic, prognostic, and therapeutic implications. *Aliment Pharmacol Ther.* 2019;49(6):681–701.
10. Tornai D, Antal-Szalmas P, Tornai T, et al. Abnormal ferritin levels predict development of poor outcomes in cirrhotic outpatients: a cohort study. *BMC Gastroenterol.* 2021;21(1):94.
11. Hagstrom H, Nasr P, Bottai M, et al. Elevated serum ferritin is associated with increased mortality in non-alcoholic fatty liver disease after 16 years of follow-up. *Liver Int.* 2016;36(11):1688–1695.
12. Ripoll C, Keitel F, Hollenbach M, et al. Serum ferritin in patients with cirrhosis is associated with markers of liver insufficiency and circulatory dysfunction, but not of portal hypertension. *J Clin Gastroenterol.* 2015;49(9):784–789.
13. Walker NM, Stuart KA, Ryan RJ, et al. Serum ferritin concentration predicts mortality in patients awaiting liver transplantation. *Hepatology.* 2010;51(5):1683–1691.
14. Maiwall R, Kumar S, Chaudhary AK, et al. Serum ferritin predicts early mortality in patients with decompensated cirrhosis. *J Hepatol.* 2014;61(1):43–50.
15. Al-Freah MA, Kriese S, Foxton MR, et al. The association of pretransplant ferritin level with waiting list and post-transplant survival. Does ferritin actually predict outcome? *Transpl Int.* 2013;26(11):1070–1079.
16. Fallet E, Rayar M, Landrieux A, et al. Iron metabolism imbalance at the time of listing increases overall and infectious mortality after liver transplantation. *World J Gastroenterol.* 2020;26(16):1938–1949.
17. Lonjon G, Porcher R, Ergina P, et al. Potential pitfalls of reporting and bias in observational studies with propensity score analysis assessing a surgical procedure: a methodological systematic review. *Ann Surg.* 2017;265(5):901–909.
18. Austin PC. Primer on statistical interpretation or methods report card on propensity-score matching in the cardiology literature from 2004 to 2006: a systematic review. *Circ Cardiovasc Qual Outcomes.* 2008;1(1):62–67.
19. Yao XI, Wang X, Speicher PJ, et al. Reporting and guidelines in propensity score analysis: a systematic review of cancer and cancer surgical studies. *J Natl Cancer Inst.* 2017;109(8):djw323.
20. Camp RL, Dolled-Filhart M, Rimm DL. X-tile: a new bio-informatics tool for biomarker assessment and outcome-based cut-point optimization. *Clin Cancer Res.* 2004;10(21):7252–7259.

21. Deng Y, Fan X, Ran Y, et al. Prognostic impact of neutrophil-to-lymphocyte ratio in cirrhosis: a propensity score matching analysis with a prespecified cut-point. *Liver Int.* 2019;39(11):2153–2163.
22. Chiou B, Connor JR. Emerging and dynamic biomedical uses of ferritin. *Pharmaceuticals (Basel).* 2018;11(4):124.
23. Oikonomou T, Goulis I, Soulaidopoulos S, et al. High serum ferritin is associated with worse outcome of patients with decompensated cirrhosis. *Ann Gastroenterol* 2017;30(2):217–224.
24. Gkamprela E, Deutsch M, Pectasides D. Iron deficiency anemia in chronic liver disease: etiopathogenesis, diagnosis and treatment. *Ann Gastroenterol.* 2017;30(4):405–413.
25. Kernan KF, Carcillo JA. Hyperferritinemia and inflammation. *Int Immunol.* 2017;29(9):401–409.
26. Dahabreh IJ, Sheldrick RC, Paulus JK, et al. Do observational studies using propensity score methods agree with randomized trials? A systematic comparison of studies on acute coronary syndromes. *Eur Heart J.* 2012;33(15):1893–1901.
27. Kuss O, Legler T, Borgermann J. Treatments effects from randomized trials and propensity score analyses were similar in similar populations in an example from cardiac surgery. *J Clin Epidemiol.* 2011;64(10):1076–1084.
28. Hou L, Deng Y, Fan X, et al. A sex-stratified prognostic nomogram incorporating body compositions for long-term mortality in cirrhosis. *JPEN J Parenter Enteral Nutr.* 2021;45(2):403–413.
29. Wang X, Feng H, Hui Y, et al. A predictive nomogram incorporating gait speed for all-cause mortality in hospitalized cirrhotics. *Postgrad Med.* 2021;133(6):680–687.

Evaluation of RT-LAMP Assay for Rapid Detection of SARS-CoV-2

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Keywords: COVID-19, severe acute respiratory syndrome coronavirus 2, LAMP assay, COVID-19 diagnostic testing, meta-analysis, SARS-CoV-2

Abbreviations: SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; COVID-19, coronavirus disease 2019; RT-qPCR, reverse transcription–quantitative polymerase chain reaction; LAMP, loop-mediated isothermal amplification; RT-LAMP, reverse transcription loop-mediated isothermal amplification; PRISMA-DTA, Preferred Reporting Items for a Systematic Review and Meta-Analysis of Diagnostic Test Accuracy Studies; NGS, next-generation sequencing; TPs, true positives; FPs, false positives; FNs, false negatives; TNs, true negatives; QUADAS-2, quality assessment of the diagnostic accuracy studies; SEN, sensitivity; SPC, specificity; PLR, positive likelihood ratio; NLR, negative likelihood ratio; DOR, diagnostic odds ratio; SROC, summary receiver operating characteristic; CT, cycle threshold; I^2, inconsistency index; POCT, point-of-care testing; AI-LAMP, artificial intelligence–assisted rapid detection of color changes associated with the loop-mediated isothermal amplification reaction; OP, oropharynx swab; RT-LAMP-LFB, reverse transcription loop-mediated isothermal amplification lateral flow biosensor; NA, nonapplicable; P, pharyngeal swab (includes oropharynx swab and pharyngeal swab); NP, nasopharyngeal swab; N1-STOP-LAMP, N1 gene single-tube Optigene loop-mediated isothermal amplification assay; NGS, next-generation sequencing; RT-PCR, reverse transcription polymerase chain reaction

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ABSTRACT

Objective: To evaluate the accuracy of the reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay for rapid detection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in community or primary-care settings.

Method: We systematically searched the Web of Science, Embase, PubMed, and Cochrane Library databases. We conducted quality evaluation using ReviewManager software (version 5.0). We then used MetaDisc software (version 1.4) and Stata software (version 12.0) to build forest plots, along with a Deeks funnel plot and a bivariate boxplot for analysis.

Result: Overall, the sensitivity, specificity, and diagnostic odds ratio were 0.79, 0.97, and 328.18, respectively. The sensitivity for the subgroup with RNA extraction appeared to be higher, at 0.88 (0.86–0.90), compared to the subgroup without RNA extraction, at 0.50 (0.45–0.55), with no significant difference in specificity.

Conclusion: RT-LAMP assay exhibited high specificity regarding current SARS-CoV-2 infection. However, its overall sensitivity was relatively moderate. Extracting RNA was found to be beneficial in improving sensitivity.

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative agent of coronavirus disease 2019 (COVID-19), poses the most severe threat to global public health in the past 100 years.[1] Currently, the reference standard for diagnosing COVID-19 is reverse transcription–quantitative polymerase chain reaction (RT-qPCR).[2] It enables the detection of viral RNA at very low levels, and is highly sensitive and especially reliable. However, although PCR-based tests can diagnose COVID-19 within a short period,[3] they still have a variety of limitations, such as the complex experimental conditions that are required, the demand for skilled technical personnel, and the high cost.[4]

Loop-mediated isothermal amplification (LAMP) is a technique that can perform highly specific, efficient, and rapid amplification of

DNA under isothermal conditions. It has been used to detect viruses, bacteria, and fungi.[5] Over the years, reverse transcription loop-mediated isothermal amplification (RT-LAMP) has been developed to detect infectious diseases caused by RNA viruses.[6] The convenience, sensitivity, and low cost of this technology make RT-LAMP a promising candidate for rapid screening for COVID-19.[7]

Many factors contribute to the molecular detection of SARS-CoV-2, including the temporal variations of viral loads, the selection and handling of specimens, adequacy of RNA purification, and selection of the extraction-free assays or RNA extraction kit,[8,9] all of which can lead to false-positive and false-negative results. False-negative results in patients with COVID-19 are particularly harmful because they can result in delayed treatment and increased risk of transmission in severely ill patients.[10] Therefore, it is necessary to evaluate the diagnostic accuracy of the RT-LAMP assay for detecting SARS-COV-2 and the factors that affect the diagnostic accuracy, which is the purpose of this meta-analysis.

The primary outcomes were the overall sensitivity and specificity of the RT-LAMP assay. The secondary outcomes were specific sensitivity and specificity in the subgroup based on study or participant characteristics, including the need for RNA extraction, specimen type, and viral load.

Methods

Protocol and Registration

A protocol was predetermined in PROSPERO, for which the registration number was CRD42020212489 (https://www.crd.york.ac.uk/prospero/display_record.php?ID=CRD42020212489). This research was conducted following Preferred Reporting Items for a Systematic Review and Meta-Analysis of Diagnostic Test Accuracy Studies (PRISMA-DTA).[11]

Search Strategy

The investigators systematically searched the Embase, PubMed, Cochrane Library, and Web of Science databases, with the keywords COVID-19 (or SARS-CoV-2) and RT-LAMP assay (or LAMP or loop-mediated isothermal amplification) for studies before September 21, 2020, with no restrictions on geography or language.

Selection Criteria

The investigators systematically sifted through all the articles based on preestablished screening criteria. The inclusion criteria were as follows: specimens from the hospital or routine COVID-19 screening station, any test based on the RT-LAMP technology for detecting SARS-CoV-2 with results available within 2 hours of specimen collection, viral culture or RT-qPCR or RT-PCR (not quantitative) or next-generation sequencing (NGS) as the reference standard. The exclusion criteria were as follows: review articles, editorials, case reports, comments, and letters; small-scale studies with fewer than 10 specimens or participants; nonhuman specimens; and preprints from the medRxiv and bioRxiv servers.

Data Extraction

The 2 review authors extracted the data independently and resolved the differences through dialogue. A third review author was consulted when necessary. We collected information on the country; study design; specimen type; index test; RNA extraction or lack thereof; viral load (cycle threshold); and reference testing and true positives (TP), false positives (FP), false negatives (FN), and true negatives (TN).

Assessment of Methodological Quality

The 2 researchers independently reviewed the methodological quality of the included articles, based on the quality assessment of the diagnostic accuracy studies (QUADAS-2), recommended by the Cochrane Collaboration. Any disagreements were resolved through discussion, and a third review author could be consulted if necessary. RevMan systematic review software helped us in collecting and putting together relevant data, and in building appropriate figures.

Statistical Analysis and Data Synthesis

Using Meta-DiSc software, version 1.4,[12] we calculated the sensitivity (SEN), specificity (SPE), positive likelihood ratio (PLR), negative likelihood ratio (NLR), and diagnostic odds ratio (DOR) with corresponding 95% CIs. Also, the area under the summary receiver operating characteristic (SROC) curve was used to evaluate the overall accuracy. Whereas studies presented only sensitivity estimates, we fitted univariate-random effects logistic regression models using Stata software, version 12.0 (StataCorp). A bivariate boxplot was constructed to perform heterogeneity testing. A Deeks funnel plot was constructed to evaluate potential publication bias visually.

Subgroup Analysis

Subgroup analysis was adopted to explore the potential sources of heterogeneity. Subgroup analysis was performed according to whether RNA extraction was needed (group A), specimen type (group B), and viral load (group C). Further, to synthesize the effects of RNA extraction and viral load, we performed a subgroup analysis of RNA extraction in high- and low-viral-load groups, respectively.

Results

Search Results

According to the selection criteria, a total of 258 publications were retrieved. After eliminating duplicates, 135 articles were left. After screening the abstracts, 73 articles were left. After reviewing the complete text, 51 articles were excluded. The exclusion reasons were shown in **FIGURE 1**. Finally, 22 qualified articles were included.

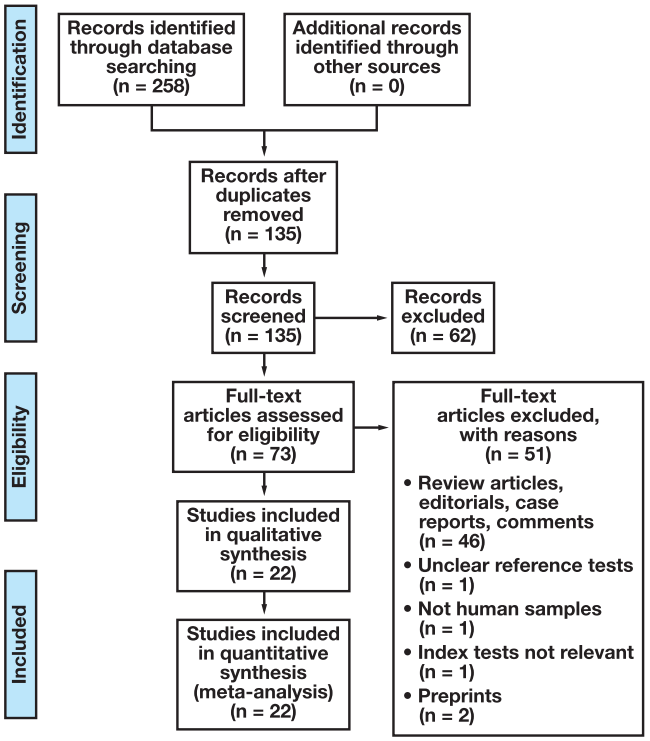
Characteristics of the Included Studies

We extracted 30 fourfold tables from the 22 included articles. Taking cycle threshold (CT) 30 as the cutoff value of the high and low viral loads group, there were 11 high and 10 low viral load fourfold tables. The characteristics of the studies included in the articles are shown in **TABLE 1**.[13–34]

Methodological Quality

The overall quality of the 22 included studies is shown in **FIGURE 2**. In the patient selection domain, 7 studies (31.8%) were at high risk of bias, considering some studies were case control studies. A total of 9 studies (40.9%) were rated as "high risk" in the index test domain because many studies were usually unclear on whether the index tests were interpreted without knowledge of the results of the reference standard. In total, 3

FIGURE 1. Flow diagram of study identification and inclusion.



studies (13.6%) were rated as "high risk" in the reference standard domain, and no study was judged to be a high bias risk in the flow and timing domain.

Threshold Effect Analysis

As can be observed in the SROC curve (**FIGURE 3A**), there was no "shoulder-arm" distribution. Also, the Spearman correlation coefficient was −0.346 (<0.6), and the *P* value was .06 (>.05), thus not statistically significant. Therefore, we concluded that there was no threshold effect in the included studies.

Overall Accuracy of RT-LAMP Assay

To determine the diagnostic accuracy of the RT-LAMP assay, we used a random effects model in our research. As shown in **FIGURES 3A** and **3B**, area under the curve (AUC) = 0.9863, the Q index = 0.9499 (SE = 0.0161), and the DOR was 328.18 (95% CI, 113.22–951.26). As shown in **FIGURES 3C** and **3D**, the SEN was 79% (95% CI, 77%–81%), and the SPE was 97% (95% CI, 96%–97%). As shown in **FIGURES 3E** and **3F**, the PLR was 36.13 (95% CI, 11.40–114.51), and the NLR was 0.13 (95% CI, 0.08–0.22), which indicates that RT-LAMP had relatively moderate sensitivity and high specificity in detecting the presence of SARS-CoV-2.

Heterogeneity Analysis of the Nonthreshold Effect

Quantitative indicators of heterogeneity were judged by the inconsistency index (I^2), which was automatically generated by the Meta-DiSc software. The I^2 was interpreted as follows: 0%–40%, might not be important; 30%–60%, moderate heterogeneity; 50%–90%, substantial heterogeneity; 75%–100%, considerable heterogeneity.[35] As shown in **FIGURE 3**, high heterogeneity was detected across studies: DOR (I^2 = 86.6%), SEN (I^2 = 93.9%), SPE (I^2 = 92.8%), PLR (I^2 = 95.5%), and NLR (I^2 = 96.1%).

Subgroup Analysis

Subgroup analysis was performed according to whether RNA extraction was needed (group A), specimen type (group B), and viral load (group C). The results of subgroup meta-analyses were summarized in **TABLE 2**. According to the bivariate boxplot (**FIGURE 4A**), there were 4 sets of data outside the circle.

Group A (regardless of whether RNA extraction was needed): With RNA extraction, the sensitivity was 88% (95% CI, 86%–90%; I^2 = 87.3%), and the specificity was 97% (95% CI, 96%–97%; I^2 = 94.5%). Without RNA extraction, the sensitivity was 50% (95% CI, 45%–55%; I^2 = 92.4%), and the specificity was 97% (95% CI, 95%–98%; I^2 = 69.6%).

Group B (by specimen type in the subgroup with RNA extraction): in the specimen with the pharyngeal swab, the sensitivity was 76% (95% CI, 74%–79%; I^2 = 94.9%), and the specificity was 96% (95% CI, 95%–97%; I^2 = 94.3%). In other specimen types, sensitivity was 86% (95% CI, 82%–90%; I^2 = 85.5%), and the specificity was 99% (95% CI, 98%–100%; I^2 = 45.5%).

Group C (by viral load): For the high viral load subgroup, the total sensitivity was 97% (95% CI, 95%–99%; I^2 = 76.1%), and the sensitivity with RNA extraction was 100% (95% CI, 99%–100%; I^2 = 0.0%); however, the sensitivity without RNA extraction was 85% (95% CI, 73%–97%; I^2 = 88.9%). For the low viral load subgroup, the total sensitivity was 37% (95% CI, 19%–55%; I^2 = 3.4%), and the sensitivity with RNA extraction was 51% (95% CI, 22%–81%; I^2 = 93.8%); however, the sensitivity without RNA extraction was 9% (95% CI, 4%–14%; I^2 = 0.0%).

Publication Bias

We used a Deeks funnel plot to assess the presence of any potential publication bias visually. Despite the fact that some points were not distributed symmetrically, as shown in **FIGURE 4B**, the *P* value of the Deeks test was .57, which indicated that there was no significant bias in this study. If the test results were $P < .01$, the publication bias test results were significant.

Discussion

The focus of our research was to evaluate the value of the RT-LAMP assay in the diagnosis of COVID-19. Before subgroup analysis, the SEN, SPE, PLR, NLR, and DOR were 0.79, 0.97, 36.13, 0.13, and 328.18, respectively. The SROC AUC was 0.9863 (close to 1.000), which indicated the moderate sensitivity and high specificity of this technique in identifying COVID-19. To some extent, our study supported the conclusions of Mustafa Hellou et al,[36] who reported that RT-LAMP or isothermal assays had a sensitivity of 84.2% (95% CI, 75.5%–90.5%) and specificity of 97.7% (95% CI, 92.8%–99.3%) in detecting SARS-CoV-2.

Also, we performed a subgroup analysis to investigate the sources of heterogeneity of the included articles. When subgroup analysis was performed directly based on RNA extraction, specimen type, and viral load, the heterogeneity of sensitivity was slightly reduced but still high. We discovered that the heterogeneity of sensitivity was significantly reduced to 0 when RNA was extracted with high viral load and not extracted with low viral load. The pooled sensitivity for the subgroup with RNA extraction appeared to be higher, at 88% (95% CI, 86%–90%), compared with the subgroup without RNA extraction, at 50% (95% CI, 45%–55%), with little change in the specificity. These findings indicated that extracted RNA might affect the sensitivity of the test. Pan et al[37] also reported that extraction-free techniques are easier to perform and are more amenable

TABLE 1. Characteristics of the Studies Included

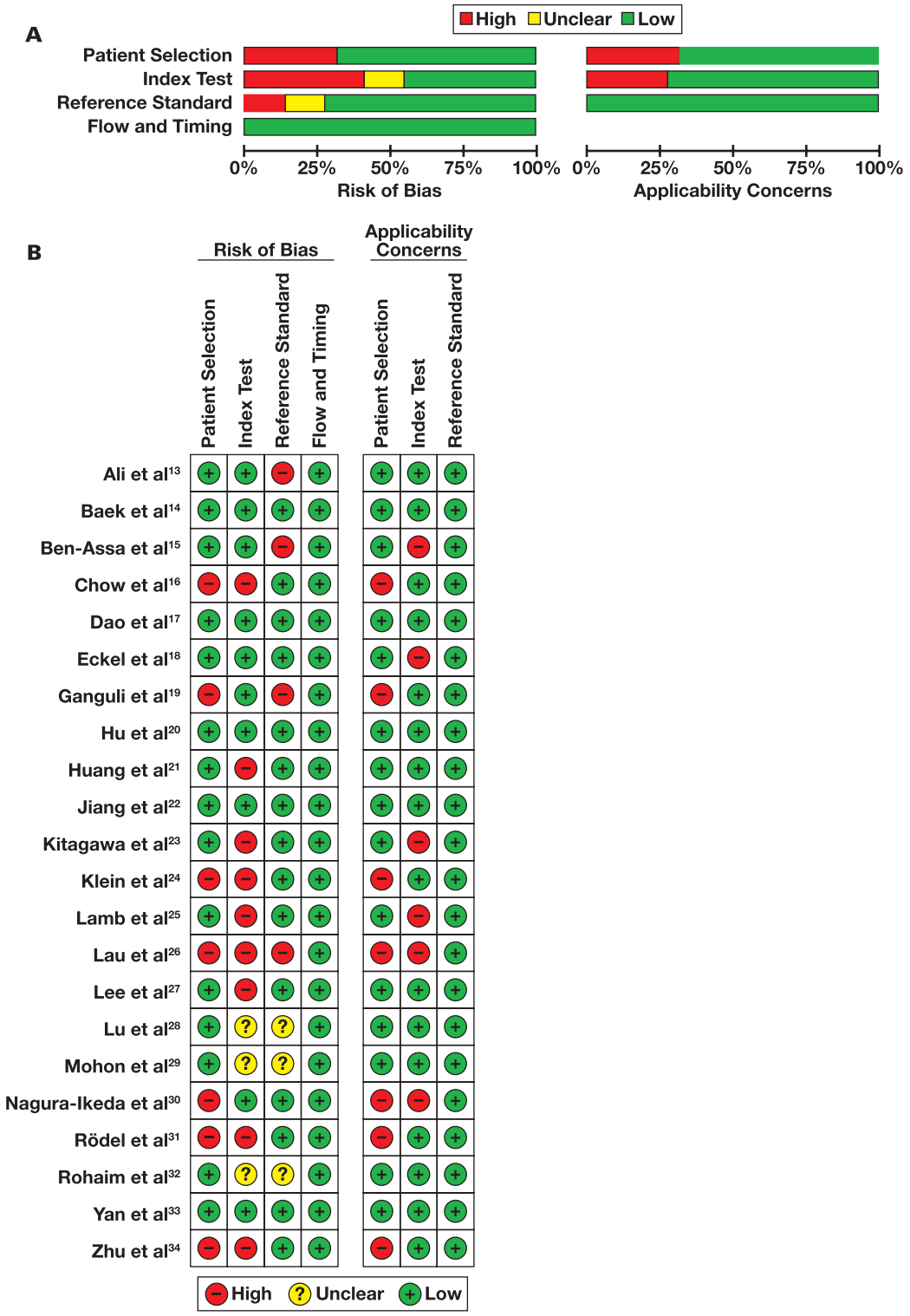
Author	Year	Study Design	Country	Specimen Type	Index Test	RNA Extraction	CT	Reference Standard	Result			
									TP	FP	FN	TN
Zhu et al[34]	2020	Prospective	China	OP	RT-LAMP-LFB	Yes	NA	RT-qPCR	33	0	0	96
Yan et al[33]	2020	Prospective	China	Other	RT-LAMP	Yes	NA	RT-qPCR	58	0	0	72
Dao Thi et al[17]	2020	Prospective	Germany	P	RT-LAMP	Yes	0–40	RT-qPCR	83	2	34	649
Dao Thi et al[17]	2020	Prospective	Germany	P	Hot swab RT-LAMP	No	0–40	RT-qPCR	60	1	68	214
Dao Thi et al[17]	2020	Prospective	Germany	P	Direct swab RT-LAMP	No	0–40	RT-qPCR	26	9	56	144
Rohaim et al[32]	2020	Prospective	England	NP	AI-LAMP	Yes	NA	RT-qPCR	61	65	9	64
Rödel et al[31]	2020	Prospective	Germany	Other	Variplex RT-LAMP	No	NA	RT-qPCR	29	0	9	35
Nagura-Ikeda et al[30]	2020	Prospective	Japan	Other	RT-LAMP	Yes	NA	RT-qPCR	73	0	30	0
Mohon et al[29]	2020	Prospective	Canada	NP	RT-LAMP	Yes	NA	RT-PCR	65	0	1	58
Lu et al[28]	2020	Prospective	China	P	RT-LAMP	Yes	NA	RT-qPCR	34	2	2	18
Lee et al[27]	2020	Prospective	Australia	NP	N1-STOP-LAMP	Yes	NA	RT-qPCR	93	0	14	50
Lau et al[26]	2020	Prospective	Malaysia	NP	RT-LAMP	Yes	NA	RT-qPCR	47	0	0	42
Lamb et al[25]	2020	Prospective	United States	Other	RT-LAMP	Yes	NA	RT-qPCR	19	2	1	18
Lamb et al[25]	2020	Prospective	United States	NP	RT-LAMP	No	0–40	RT-qPCR	4	0	6	10
Klein et al[24]	2020	Prospective	Germany	P	Colorimetric RT-LAMP	Yes	0–40	RT-qPCR	33	0	19	29
Klein et al[24]	2020	Prospective	Germany	P	Fluorescent RT-LAMP	Yes	0–40	RT-qPCR	41	0	11	29
Kitagawa et al[23]	2020	Prospective	Japan	NP	RT-LAMP	Yes	25.31–36.08	RT-qPCR	30	2	0	44
Jiang et al[22]	2020	Prospective	China	Other	RT-LAMP	Yes	0–40	RT-qPCR	32	0	3	133
Jiang et al[22]	2020	Prospective	China	Other	RT-LAMP	Yes	0–40	RT-qPCR	10	1	2	79
Huang et al[21]	2020	Prospective	China	P	RT-LAMP	No	0–36	RT-qPCR	8	0	0	8
Hu et al[20]	2020	Prospective	China	NP	RT-LAMP	Yes	NA	NGS	31	3	4	291
Hu et al[20]	2020	Prospective	China	Other	RT-LAMP	Yes	NA	NGS	41	1	5	105
Ganguli et al[19]	2020	Prospective	United States	NP	RT-LAMP	No	20.00–30.00	RT-PCR	10	0	0	10
Eckel et al[18]	2020	Prospective	Germany	P	Variplex LAMP	No	NA	RT-PCR	8	7	39	55
Chow et al[16]	2020	Prospective	China	NP	RT-LAMP	Yes	NA	RT-qPCR	95	0	1	143
Chow et al[16]	2021	Prospective	China	Other	RT-LAMP	Yes	NA	RT-qPCR	65	0	2	143
Chow et al[16]	2022	Prospective	China	P	RT-LAMP	Yes	NA	RT-qPCR	59	0	1	143
Ben-Assa et al[15]	2020	Prospective	Israel	P	RT-LAMP	No	0–35	RT-qPCR	42	1	10	30
Baek et al[14]	2020	Prospective	South Korea	NP	RT-LAMP	Yes	21.11–32.76	RT-qPCR	14	2	0	138
Ali et al[13]	2020	Prospective	South Korea	NP	RT-LAMP-coupled CRISPR-Cas12	Yes	0–40	RT-qPCR	17	0	3	4

AI-LAMP, artificial intelligence–assisted rapid detection of color changes associated with the loop-mediated isothermal amplification reaction; CT, cycle threshold; FN, false negative; FP, false positive; LAMP, loop-mediated isothermal amplification; NA, nonapplicable; NGS, next-generation sequencing; NP, nasopharyngeal swab; N1-STOP-LAMP, N1 gene single-tube Optigene loop-mediated isothermal amplification assay; OP, oropharynx swab; P, pharyngeal swab (includes oropharynx swab and pharyngeal swab); RT-LAMP, reverse transcription loop-mediated isothermal amplification; RT-LAMP-LFB, reverse transcription loop-mediated isothermal amplification lateral flow biosensor; RT-PCR, reverse transcription polymerase chain reaction; RT-qPCR, reverse transcription quantitative polymerase chain reaction; TN, true negative; TP, true positive.

for point-of-care testing (POCT), but the release of RNA by preheating the specimen may reduce the sensitivity of detection of specimens containing low viral load. The average sensitivity of the non-RNA extraction group was 50% and varied from 45%–55%. The reasons for the significant difference between the studies are unclear and may be due to characteristics of the studies themselves. More data are needed to conclude that not extracting RNA will certainly affect the detection accuracy.

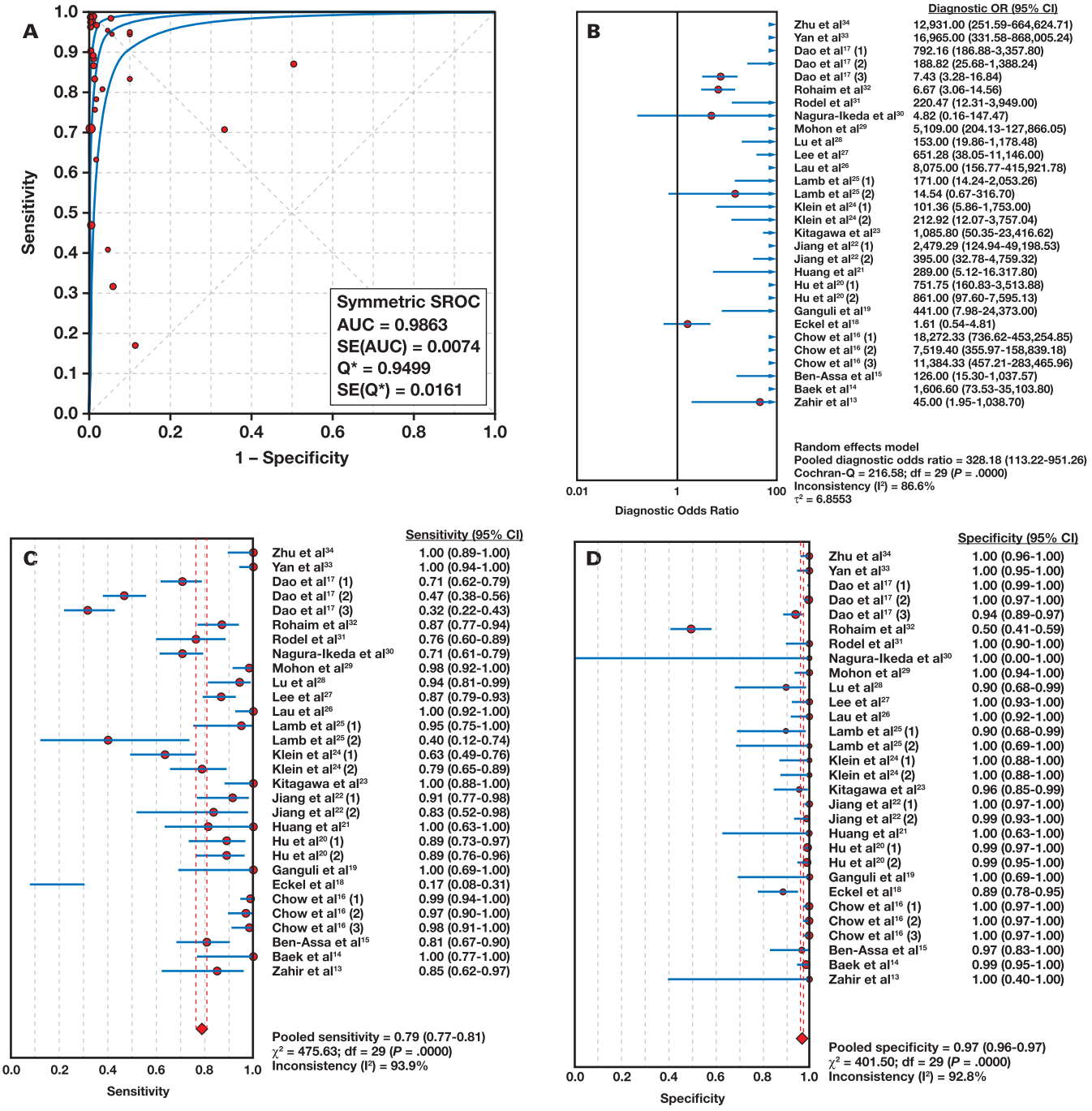
In addition, we performed a subgroup analysis on the specimen collection types. We found no significant differences in sensitivity or specificity between the subgroup of pharyngeal swabs and the subgroup of the other specimen collection types, indicating that the specimen collection type is not the cause of heterogeneity. We then conducted subgroup analysis by viral load. The CT threshold for high viral loads was 30 or less.[38] On average, the sensitivity of low viral loads was very poor, at 37% (95% CI, 19%–55%), compared with that of the high viral load group, at 97% (95% CI, 95%–99%), with little change in the specificity. We observed significant differences in sensitivity according to viral load and suspected that differences in the distribution of specimens with high and low viral load between studies may have affected overall accuracy estimates. However, the findings of 2 studies remained high sensitivity, with regard to low viral loads. By analyzing the research design and test method of each of those manuscripts, we speculated that the findings might be related to the small positive specimen size[22] and the combined test method.[13] More data are needed to determine whether test performance can be repeated at low viral loads.

FIGURE 2. Quality levels of the studies. A, Overall quality assessment of the included studies. B, Quality assessment of the individual studies.



Along with the aforementioned 3 sources, some other sources of heterogeneity are still being considered, such as patient symptom severity, time from symptom onset to index test, the target gene of the LAMP assay, and whether other detection methods and reference standards are being used. Only a few studies have reported patient clinical background information. Nagura-Ikeda et al[30] divided the specimens according to the time from symptom appearance to detection into early phase, late phase, and no specific time (asymptomatic), and the respective sensitivities in these categories were 85.2%, 44.4%, and 60.0%.

FIGURE 3. **Forest plots for reverse transcription loop-mediated isothermal amplification assay for coronavirus disease 2019 of the summary receiver operating characteristic (SROC) curve (A), diagnostic odds ratio (B), sensitivity (C), specificity (D), positive likelihood ratio (E), and negative likelihood ratio (F). AUC, area under the curve.**



Viral load varies depending on the time of infection and severity of symptoms, contributing to false-negative results in asymptomatic patients.[39] Due to lack of relevant reporting, the LAMP assay detection effect may be different in different prevalence settings. As to the target gene for the LAMP assay, some studies only use the N, RdRP, or M gene as the target gene; others combined 1 of those genes with 2 or more genes, among which the N gene was used as the target gene in most studies. Corman et al[40] suggest that the N gene is one of the best targets for high-sensitivity detection of SARS-CoV-2. Because there were few studies on other genes as the target gene, we did not conduct subgroup analysis in this regard.

For the studies that combined another detection method with the primary one studied, one using AI-LAMP (artificial intelligence–assisted rapid detection of color changes associated with the LAMP reaction) has detected the SARS-CoV-2 RNA in specimens that had tested negative via qRT-PCR,[32] the other using coupled CRISPR-Cas12 maintained high

FIGURE 3. (cont)

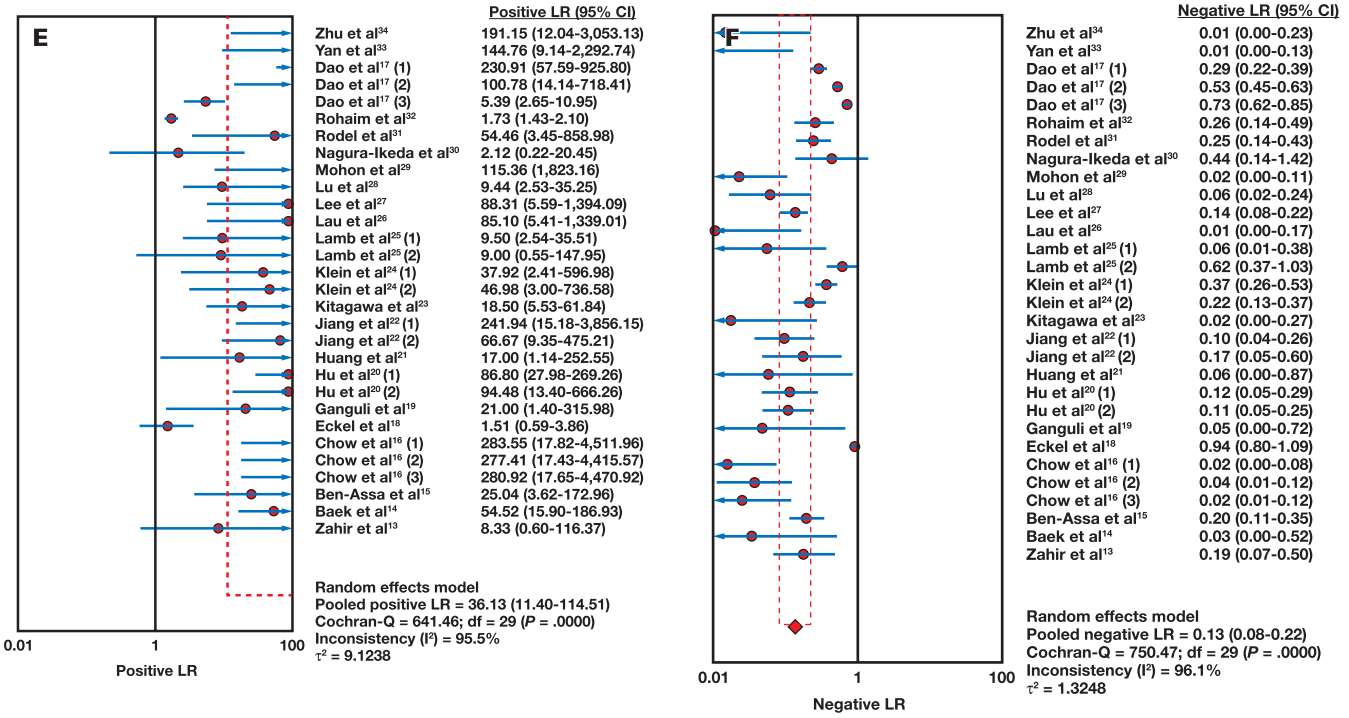
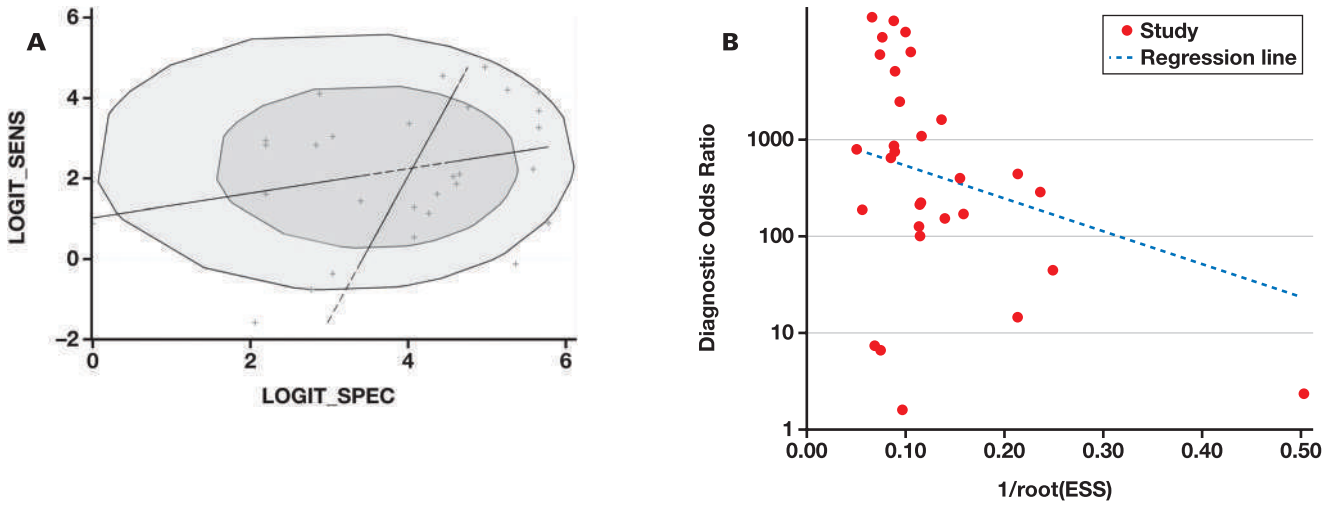


FIGURE 4. **Testing for reverse transcription loop-mediated isothermal amplification assay. A, Bivariate boxplot to perform heterogeneity testing. B, Deeks funnel plot asymmetry test to assess publication bias.**



accuracy in detecting low viral load.[13] Except for the studies by Hu et al,[20] all studies used RT-qPCR or RT-PCR only as the reference standard for diagnosing SARS-CoV-2 infection.

This study has several limitations. The weaknesses of the review primarily reflect the shortcomings in the primary studies and their reporting. Many studies omitted descriptions of the participants and the key aspects of the study design and execution. We grouped the viral loads only for studies that provided the CT threshold. We acknowledged that using CT values as a surrogate for viral load is inappropriate, when we discussed viral load in the setting of symptomatic and asymptomatic infection. Further prospective and comparative evaluation of RT-LAMP tests, especially in clinically relevant settings, is urgently needed.

Conclusion

In this article, RT-LAMP assay exhibited high specificity with regards to SARS-CoV-2 infection. However, its overall sensitivity was relatively moderate. Moreover, extracting RNA was found to be beneficial in improving sensitivity. It showed high sensitivity in high viral load

TABLE 2. Results of Subgroup Analysis

Subgroup Analysis	No. of Studies	Sensitivity (95% CI)	I^2	Specificity (95% CI)	I^2
Group A					
With RNA extraction	22	0.88 (0.86–0.90)	87.3%	0.97 (0.96–0.97)	94.5%
Without RNA extraction	8	0.50 (0.45–0.55)	92.4%	0.97 (0.95–0.98)	69.6%
Group B					
Pharyngeal swab	22	0.76 (0.74–0.79)	94.9%	0.96 (0.95–0.97)	94.3%
Other	8	0.86 (0.82–0.90)	85.5%	0.99 (0.98–1.00)	45.5%
Group C					
High viral load					
Total	11	0.97 (0.95–0.99)	76.1%	NA	NA
With RNA extraction	6	1.00 (0.99–1.00)	0	NA	NA
Without RNA extraction	5	0.85 (0.73–0.97)	88.9%	NA	NA
Low viral load					
Total	10	0.37 (0.19–0.55)	93.4%	NA	NA
With RNA extraction	6	0.51 (0.22–0.81)	93.8%	NA	NA
Without RNA extraction	4	0.09 (0.04–0.14)	0%	NA	NA

NA, not available.

specimens, and it may be used for rapid detection and separation of the greatest number of patients with positive results. However, more clinical data are needed to support these conclusions.

REFERENCES

1. Wang W, Xu Y, Gao R, et al. Detection of SARS-CoV-2 in different types of clinical specimens. *JAMA*. 2020;323(18):1843–1844.
2. Shen M, Zhou Y, Ye J, et al. Recent advances and perspectives of nucleic acid detection for coronavirus. *J Pharm Anal*. 2020;10(2):97–101.
3. Buella Parivallal P, Prasanna Kumar MK, Vasanthakumar KC, Thavuroollah FF, Manoharan Y, Muthu S. LAMP assay: could it be a boon for the molecular diagnosis of COVID-19? *Indian J Clin Biochem*. 2020;35(4):506–507.
4. Esbin MN, Whitney ON, Chong S, Maurer A, Darzacq X, Tjian R. Overcoming the bottleneck to widespread testing: a rapid review of nucleic acid testing approaches for COVID-19 detection. *RNA*. 2020;26(7):771–783.
5. Wong Y-P, Othman S, Lau Y-L, Radu S, Chee H-Y. Loop-mediated isothermal amplification (LAMP): a versatile technique for detection of micro-organisms. *J Appl Microbiol*. 2018;124(3):626–643.
6. Silva S, Pardee K, Pena L. Loop-mediated isothermal amplification (LAMP) for the diagnosis of Zika virus: a review. *Viruses*. 2019;12(1):19.
7. Augustine R, Hasan A, Das S, et al. Loop-mediated isothermal amplification (LAMP): a rapid, sensitive, specific, and cost-effective point-of-care test for coronaviruses in the context of COVID-19 pandemic. *Biology*. 2020;9(8):182.
8. Feng W, Newbigging AM, Le C, et al. Molecular diagnosis of COVID-19: challenges and research needs. *Anal Chem*. 2020;92(15):10196–10209.
9. Lalli MA, Langmade SJ, Chen X, et al. Rapid and extraction-free detection of SARS-CoV-2 from saliva by colorimetric reverse-transcription loop-mediated isothermal amplification. *Clin Chem*. 2021;67(2):415–424.
10. Ji T, Liu Z, Wang G, et al. Detection of COVID-19: a review of the current literature and future perspectives. *Biosens Bioelectron*. 2020;166:112455.
11. McInnes MDF, Moher D, Thombs BD, McGrath TA, Bossuyt PM; and the PRISMA-DTA Group. Preferred reporting items for a systematic review and meta-analysis of diagnostic test accuracy studies: the PRISMA-DTA statement. *JAMA*. 2018;319(4):388–396.
12. Zamora J, Abraira V, Muriel A, Khan K, Coomarasamy A. Meta-DiSc: a software for meta-analysis of test accuracy data. *BMC Med Res Methodol*. 2006;6:31.
13. Ali Z, Aman R, Mahas A, et al. iSCAN: an RT-LAMP-coupled CRISPR-Cas12 module for rapid, sensitive detection of SARS-CoV-2. *Virus Res*. 2020;288:198129.
14. Baek YH, Um J, Antigua KJC, et al. Development of a reverse transcription-loop-mediated isothermal amplification as a rapid early-detection method for novel SARS-CoV-2. *Emerg Microbes Infect*. 2020;9(1):998–1007.
15. Ben-Assa N, Naddaf R, Gefen T, et al. Direct on-the-spot detection of SARS-CoV-2 in patients. *Exp Biol Med (Maywood)*. 2020;245(14):1187–1193.
16. Chow FW-N, Chan TT-Y, Tam AR, et al. A rapid, simple, inexpensive, and mobile colorimetric assay COVID-19-LAMP for mass on-site screening of COVID-19. *Int J Mol Sci*. 2020;21(15):5380.
17. Dao Thi VL, Herbst K, Boerner K, et al. A colorimetric RT-LAMP assay and LAMP-sequencing for detecting SARS-CoV-2 RNA in clinical samples. *Sci Transl Med*. 2020;12(556):eabc7075.
18. Eckel F, Küsters F, Drossel B, Konert M, Mattes H, Schopf S. Variplex™ test system fails to reliably detect SARS-CoV-2 directly from respiratory samples without RNA extraction. *Eur J Clin Microbiol Infect Dis*. 2020;39(12):1–5.
19. Ganguli A, Mostafa A, Berger J, et al. Rapid isothermal amplification and portable detection system for SARS-CoV-2. *bioRxiv*. 2020;117(37):22727–22735.
20. Hu X, Deng Q, Li J, et al. Development and clinical application of a rapid and sensitive loop-mediated isothermal amplification test for SARS-CoV-2 infection. *mSphere*. 2020;5(4):e00808-20.
21. Huang WE, Lim B, Hsu C-C, et al. RT-LAMP for rapid diagnosis of coronavirus SARS-CoV-2. *Microb Biotechnol.* 2020;13(4):950–961.
22. Jiang M, Pan W, Arasthfer A, et al. Development and validation of a rapid, single-step reverse transcriptase loop-mediated isothermal amplification (RT-LAMP) system potentially to be used for reliable and high-throughput screening of COVID-19. *Front Cell Infect Microbiol*. 2020;10:331.

23. Kitagawa Y, Orihara Y, Kawamura R, et al. Evaluation of rapid diagnosis of novel coronavirus disease (COVID-19) using loop-mediated isothermal amplification. *J Clin Virol*. 2020;129:104446.
24. Klein S, Müller TG, Khalid D, et al. SARS-CoV-2 RNA extraction using magnetic beads for rapid large-scale testing by RT-qPCR and RT-LAMP. *Viruses*. 2020;12(8):863.
25. Lamb LE, Bartolone SN, Ward E, Chancellor MB. Rapid detection of novel coronavirus/Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) by reverse transcription-loop-mediated isothermal amplification. *PLoS One*. 2020;15(6):e0234682.
26. Lau YL, Ismail I, Mustapa NI, et al. Real-time reverse transcription loop-mediated isothermal amplification for rapid detection of SARS-CoV-2. *PeerJ*. 2020;8:e9278.
27. Lee JYH, Best N, McAuley J, et al. Validation of a single-step, single-tube reverse transcription loop-mediated isothermal amplification assay for rapid detection of SARS-CoV-2 RNA. *J Med Microbiol*. 2020;69(9):1169–1178.
28. Lu R, Wu X, Wan Z, Li Y, Jin X, Zhang C. A novel reverse transcription loop-mediated isothermal amplification method for rapid detection of SARS-CoV-2. *Int J Mol Sci*. 2020;21(8):2826.
29. Mohon AN, Oberding L, Hundt J, et al. Optimization and clinical validation of dual-target RT-LAMP for SARS-CoV-2. *J Virol Methods*. 2020;286:113972.
30. Nagura-Ikeda M, Imai K, Tabata S, et al. Clinical evaluation of self-collected saliva by quantitative reverse transcription-PCR (RT-qPCR), direct RT-qPCR, reverse transcription–loop-mediated isothermal amplification, and a rapid antigen test to diagnose COVID-19. *J Clin Microbiol*. 2020;58(9):e01438-20.
31. Rödel J, Egerer R, Suleyman A, et al. Use of the Variplex™ SARS-CoV-2 RT-LAMP as a rapid molecular assay to complement RT-PCR for COVID-19 diagnosis. *J Clin Virol*. 2020;132:104616.
32. Rohaim MA, Clayton E, Sahin I, et al. Artificial intelligence-assisted loop mediated isothermal amplification (AI-LAMP) for rapid detection of SARS-CoV-2. *Viruses*. 2020;12(9):972.
33. Yan C, Cui J, Huang L, et al. Rapid and visual detection of 2019 novel coronavirus (SARS-CoV-2) by a reverse transcription loop-mediated isothermal amplification assay. *Clin Microbiol Infect*. 2020;26(6):773–779.
34. Zhu X, Wang X, Han L, et al. Multiplex reverse transcription loop-mediated isothermal amplification combined with nanoparticle-based lateral flow biosensor for the diagnosis of COVID-19. *Biosens Bioelectron*. 2020;166:112437.
35. Deeks JJ, Higgins JPT, Altman DG. *Analysing Data and Undertaking Meta-Analyses*. Cochrane Statistical Methods Group; 2020.
36. Mustafa Hellou M, Górska A, Mazzaferri F, et al. Nucleic acid amplification tests on respiratory samples for the diagnosis of coronavirus infections: a systematic review and meta-analysis. *Clin Microbiol Infect*. 2021;27(3):341–351.
37. Pan Y, Long L, Zhang D, et al. Potential false-negative nucleic acid testing results for Severe Acute Respiratory Syndrome Coronavirus 2 from thermal inactivation of samples with low viral loads. *Clin Chem*. 2020;66(6):794–801.
38. Dinnes J, Deeks JJ, Adriano A, et al. Rapid, point-of-care antigen and molecular-based tests for diagnosis of SARS-CoV-2 infection. *Cochrane Database Syst Rev*. 2020;8:CD013705.
39. Zou L, Ruan F, Huang M, et al. SARS-CoV-2 viral load in upper respiratory specimens of infected patients. *N Engl J Med*. 2020;382(12):1177–1179.
40. Corman VM, Landt O, Kaiser M, et al. Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. *Euro Surveill*. 2020;25(3):2000045.

Application of the Single-Molecule Real-Time Technology (SMRT) for Identification of HKαα Thalassemia Allele

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Abbreviations: SMRT, single-molecule real-time technology; HKαα, the Hong Kongαα; gap-PCR, gap-polymerase chain reaction; CCS, circular consensus sequencing; SNV, single nucleotide variant; indels, insertions and deletions; RDB, reverse dot blot hybridization; MLPA, multiplex ligation-dependent probe amplification.

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ABSTRACT

Objective: Single-molecule real-time technology (SMRT) is a sequencing technology using the DNA polymerases and fluorescently tagged nucleotides to accurately sequence DNA strands. The purpose of this study was to evaluate the detection accuracy of SMRT for identification of the Hong Kongαα (HKαα) thalassemia allele.

Methods: We conducted a blinded study of 33 samples of known HKαα alleles. These alleles were detected using SMRT to evaluate accuracy.

Results: We conducted a blinded study of 33 known HKαα samples and found all HKαα variants detected by SMRT to be concordant with those independently assigned by gap-polymerase chain reaction (gap-PCR), reverse dot blot hybridization, and 2-round nested PCR. In addition, SMRT detected 2 β-thalassemia variants that were missed by conventional techniques.

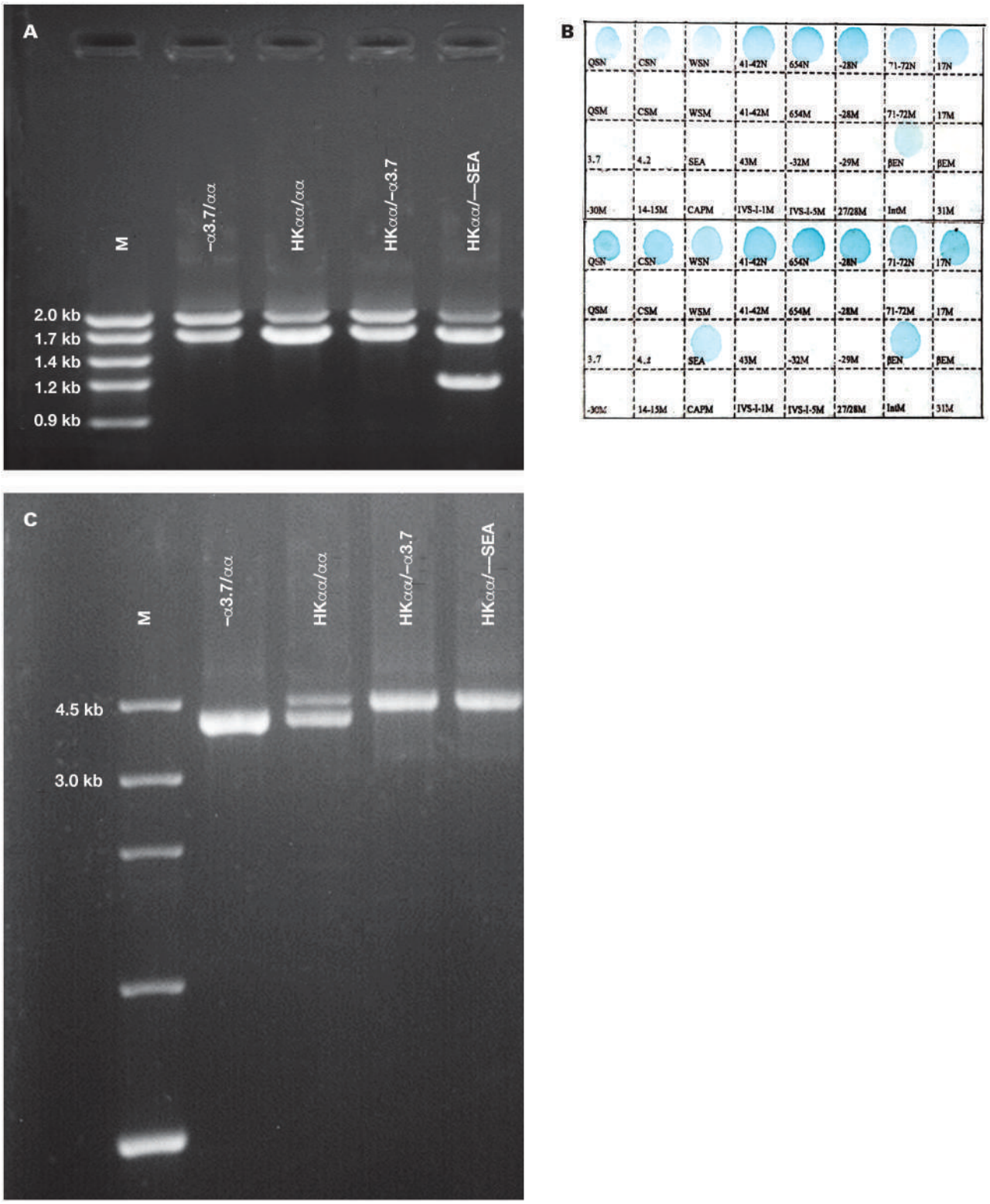
Conclusion: The results demonstrate that SMRT offers a higher detection accuracy of thalassemia rare and new loci. It is an efficient, reliable, and broad-spectrum test that can be widely used for thalassemia screening in the clinic.

Thalassemia is an inherited hematologic disorder characterized by a decrease in or absence of hemoglobin chain synthesis. It is one of the most commonly occurring monogenic disorders, with clinical severity varying from almost asymptomatic to lethal hemolysis.[1–3] Conventional polymerase chain reaction (PCR) kits currently used in the clinic for the diagnosis of thalassemia mainly include 4 α-globin gene deletions, 3 α-globin point mutations of the α-thalassemia allele, and 17-point mutations of the β-thalassemia allele, which are far from sufficient for the detection of other rare and new variant loci. Such a limited detection scope may lead to inaccurate or missed diagnoses of thalassemia.

The HKαα allele is a rearrangement crossover of the α-globin gene cluster containing both $-\alpha^{3.7}$ and $\alpha\alpha\alpha^{anti4.2}$ crossover junctions.[4] The rate of the HKαα carriers was 0.07%~0.33% and 2.27%~8.81% in $-\alpha^{3.7}$ positive samples.[5–8] Previous studies on the hematologic phenotype of HKαα/αα carriers showed that erythrocyte and hemoglobin electrophoresis parameters were not significantly different from those of normal subjects and present a normal phenotype. In addition, the hematologic parameters of $--^{SEA}$/HKαα showed obvious characteristics of α-thalassemia, which are similar to $--^{SEA}$/αα in manifestations without significant hematological differences.[4–10] The detection area of the conventional thalassemia gene kit does not include $\alpha\alpha\alpha^{anti4.2}$ variations, and the results of gap-polymerase chain reaction (gap-PCR) indicating a $-\alpha^{3.7}$/αα condition may actually be $-\alpha^{3.7}$/αα, $-\alpha^{3.7}$/HKαα, or HKαα/αα. Similarly, a result of $--^{SEA}$/HKαα may be misdiagnosed as HbH, which may lead to inaccurate genetic counseling and unnecessary invasive prenatal diagnosis. Hence, there is an urgent need for the development and application of newer methods to accurately diagnose rare and novel thalassemia alleles like HKαα.

In recent years, single-molecule real-time technology (SMRT) has been proven to be of potential clinical value in the detection of thalassemia. PacBio SMRT uses a dumbbell library structure to sequence the insert for multiple times and takes the advantage of circular consensus sequencing (CCS) to achieve high-fidelity sequencing for single

FIGURE 1. **Result of the HKαα allele by gap-polymerase chain reaction (PCR), reverse dot blot hybridization (RDB), and nested PCR. A, Electrophoretogram of the gap-PCR. B, Result of the RDB with $-\alpha^{37}/\alpha\alpha$ and $-\alpha^{37}$, $--^{SEA}$ and normal α2 alleles. C, Electrophoretogram of the nested PCR.**



nucleotide variants (SNVs) and small insertions and deletions (indels). With long-read sequencing, SMRT can detect structural variations and distinguish cis- and trans-configuration for 2 or more variants. Full-length sequencing of HBA1/2 and HBB genes associated with thalassemia using SMRT provided complete variation information of the 2 alleles.[11] Further, SMRT increased the positive detection rate of the thalassemia gene by 4.9% to 9.91% compared to the conventional genetic techniques.[12,13] This has helped confirm that SMRT is a scalable,

TABLE 1. The 33 Samples Carrying the HKαα Allele Detected by Gap-PCR, RDB, and 2-Round Nested PCR

Samples	Sex	Age (y)	RBC ($x10^{12}$/L)	Hb (g/L)	MCV (fL)	MCH (pg)	HbA2 (%)	α Genotype	β Genotype
1[a]	F	26	4.30	125	78.4	29.1	2.7	HKαα/αα	β^N/β^N
2[a]	F	30	4.05	120	83.5	29.6	2.7	HKαα/αα	β^N/β^N
3	F	25	4.20	118	76.7	25.6	2.4	HKαα/αα	β^N/β^N
4	M	28	5.10	140	83.1	28.8	2.6	HKαα/αα	β^N/β^N
5	M	3	—	—	—	—	2.9	HKαα/αα	β^N/β^N
6[a]	F	24	3.27	90	84.4	27.5	2.2	HKαα/αα	β^N/β^N
7[a]	F	31	4.34	104	75.3	24	2.3	HKαα/αα	β^N/β^N
8	M	28	5.34	159	84.8	29.8	2.6	HKαα/αα	β^N/β^N
9	F	27	4.36	119	80	27.3	2.4	HKαα/αα	β^N/β^N
10[a]	F	32	4.57	113	70.9	24.7	2.5	HKαα/αα	β^N/β^N
11[a]	F	30	3.50	103	88	29.4	2.6	HKαα/αα	β^N/β^N
12	M	42	5.11	139	82.2	27.9	2.9	HKαα/αα	β^N/β^N
13[a]	F	22	4.1	108	75.8	25.8	2.5	HKαα/αα	β^N/β^N
14[a]	F	31	4.15	123	81.2	29.6	2.7	HKαα/αα	β^N/β^N
15[a]	F	27	4.61	105	74.4	22.8	2.1	HKαα/αα	β^N/β^N
16[a]	F	34	4.68	131	81.6	28	3	HKαα/αα	β^N/β^N
17[a]	F	29	4.61	133.1	79.4	28.9	2.9	HKαα/αα	β^N/β^N
18[a]	F	27	4.28	116	80.4	27.1	2.6	HKαα/αα	β^N/β^N
19[a]	F	25	3.58	108	89.4	30.2	2.4	HKαα/αα	β^N/β^N
20[a]	F	26	4.30	120	82	27.3	2.6	HKαα/αα	β^N/β^N
21	F	28	3.65	114	89.6	31.2	2.6	HKαα/αα	β^N/β^N
22	M	28	4.79	141	93.1	29.4	2.6	HKαα/αα	β^N/β^N
23	M	23	5.29	147	92.2	29.1	2.4	HKαα/αα	β^N/β^N
24	F	47	4.21	93	75.3	22.1	2.5	HKαα/αα	β^N/β^N
25[a]	F	29	4.95	125	78.8	25.3	2.6	HKαα/αα	β^N/β^N
26[a]	F	26	5.56	107	59.4	19.2	5.8	HKαα/αα	$\beta^{Codons\ 41/42}/\beta^N$
27	M	3	—	—	—	—	—	HKαα/αα	$\beta^{Codons\ 41/42}/\beta^N$
28	F	28	4.17	95	72	23.9	4.8	HKαα/αα	$\beta^{Codons\ 41/42}/\beta^N$
29[a]	F	43	4.35	90	63.2	20.7	5.1	HKαα/αα	$\beta^{IVS\text{-}II\text{-}654}/\beta^N$
30[a]	F	26	4.70	125	80.4	26.6	3.1	HKαα/$-\alpha^{3.7}$	β^N/β^N
31	M	4	5.00	123	80.2	27.3	2.9	HKαα/$-\alpha^{3.7}$	β^N/β^N
32	F	27	4.80	119	66.4	30.1	2.4	HKαα/$--^{SEA}$	β^N/β^N
33[a]	F	28	5.82	120	65.8	20.6	2.3	HKαα/$--^{SEA}$	β^N/β^N

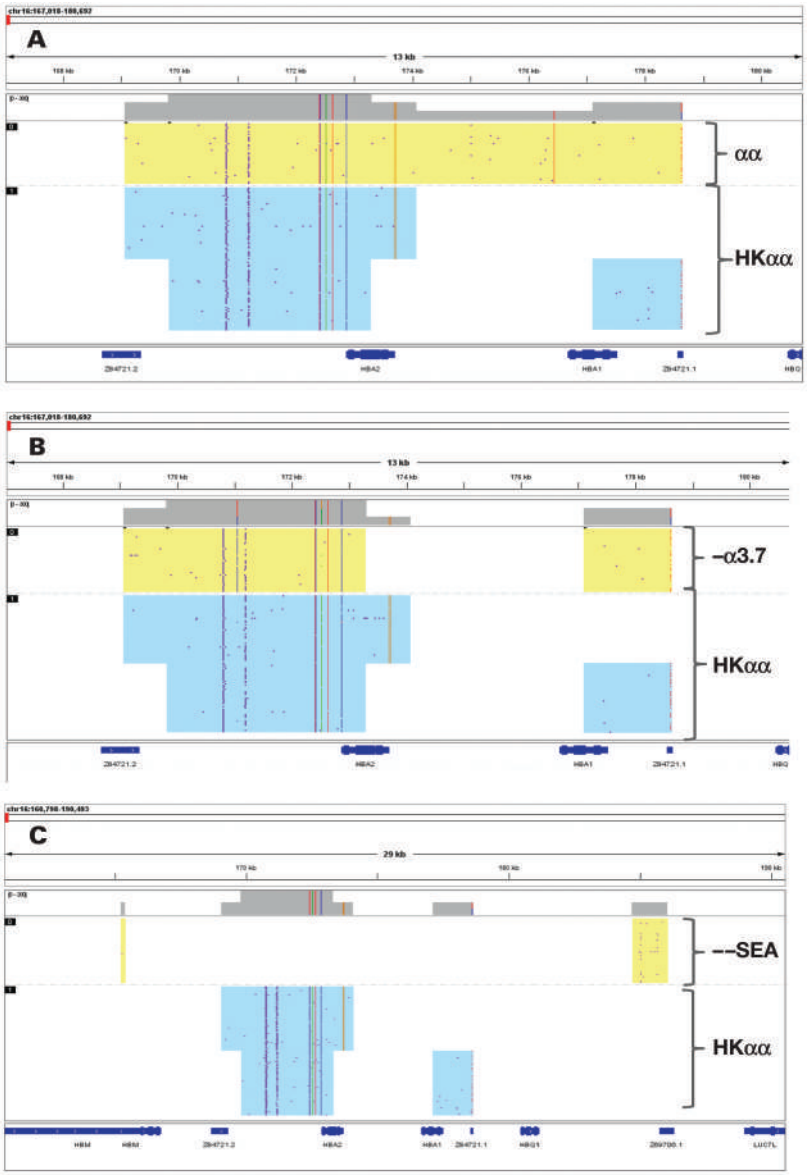
codons 41/42, HBB:c.126_129delCTTT; Hb, hemoglobin; IVS-II-654, HBB: c.316-197C4T; MCH, mean corpuscular hemoglobin; MCV, mean corpuscular volume; N, normal; PCR, polymerase chain reaction; RBC, red blood cell count; RDB, reverse dot blot hybridization; —, no data available.
[a]The hematology data and hemoglobin analysis were tested during pregnancy.

TABLE 2. The HKαα Allele Identified by SMRT and Conventional Genetic Diagnosis Techniques

Genotype Identified by SMRT		Genotype Identified by Conventional Techniques		
α-Thalassemia Genotypes	β-Thalassemia Genotypes	α-Thalassemia Genotypes	β-Thalassemia Genotypes	No. of Samples
HKαα/αα	β^N/β^N	HKαα/αα	β^N/β^N	24
HKαα/αα	HBB:c.316-197C > T Hete	HKαα/αα	HBB:c.316-197C > T Hete	1
HKαα/αα	HBB:c.126_129delCTTT Hete	HKαα/αα	HBB:c.126_129delCTTT Hete	3
HKαα/αα	lHBB:c.341T > A Hete	HKαα/αα	β^N/β^N	1
$-\alpha^{3.7}$/HKαα	β^N/β^N	$-\alpha^{3.7}$/HKαα	β^N/β^N	2
$--^{SEA}$/HKαα	β^N/β^N	$--^{SEA}$/HKαα	β^N/β^N	1
$--^{SEA}$/HKαα	HBB:c.316-45G > C Hete	$--^{SEA}$/HKαα	β^N/β^N	1

SMRT, single-molecule real-time technology.

FIGURE 2. **Molecular plots for the identification of α-thalassemia allele using single-molecule real-time technology. Graphs presented of samples with genotypes. A, HKαα/αα. B, –α^{37}/HKαα. C, --^{SEA}/HKαα alleles.**



accurate, and cost-effective method for genotyping. Recently, an increasing number of false-negative and false-positive results obtained due to the limitations of traditional diagnostic techniques have been uncovered using SMRT.[12,13] Given the enhanced accuracy of SMRT compared to conventional techniques, we conducted a blinded study of 33 samples of known HKαα alleles. These alleles were detected using SMRT, and the results illustrated the efficiency, reliability, and broad-spectrum applicability of SMRT in the diagnosis of common and rare variants of thalassemia in the clinical setting.

Materials and Methods

Ethical Statement

The study was approved by the Clinical Ethics Committee of the Fujian Provincial Maternity and Children's Hospital. All procedures were performed in accordance with the Declaration of Helsinki and international and national guidelines for human studies. Written informed consent was obtained from all participants following a detailed description of the purpose of the study.

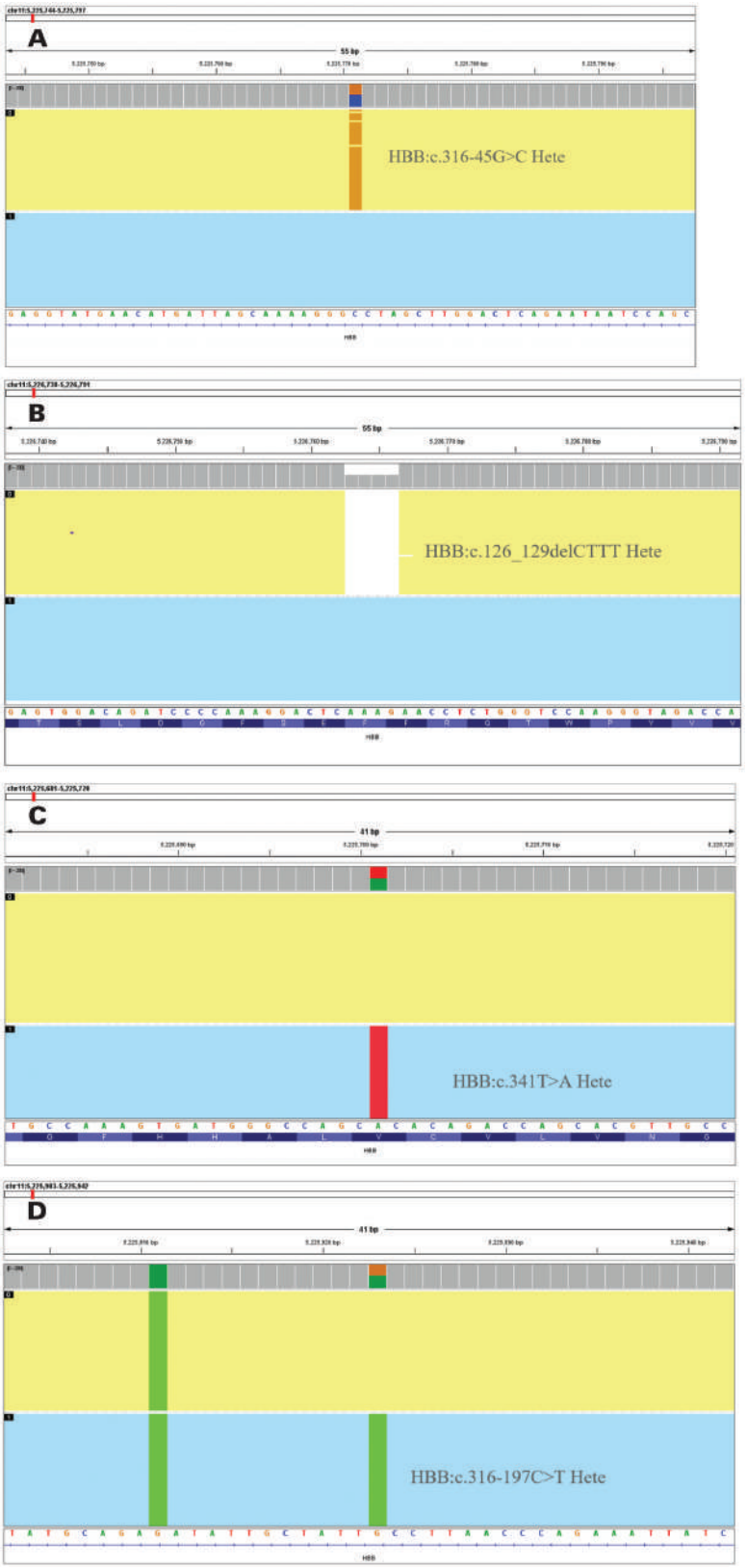
Sample Collection

A total of 33 samples of the HKαα allele were collected from February 2016 to December 2019 from the Fujian Provincial Maternity and Children's Hospital, Fuzhou, China. The study was approved by the Clinical Ethics Committee of the Fujian Provincial Maternity and Children's Hospital. Following a blind approach, all samples of the HKαα allele were detected by SMRT, and the detection accuracy was determined by comparing it to that obtained post gap-PCR, reverse dot blot hybridization (RDB), and 2-round nested PCR, as described previously.[7]

Gene Amplification and DNA Libraries

Genomic DNA was amplified by PCR using primers covering the majority of known structural variations, SNVs, and indels in the *HBA1*,

FIGURE 3. **Molecular plots for the identification of β-thalassemia allele using single-molecule real-time technology. Graphs presented are of samples with genotypes. A, HBB:c.316-45G > C Hete. B, HBB:c.126_129delCTTT Hete. C, HBB:c.341T > A Hete. D, HBB:c.316-197C > T Hete.**



HBA2, and *HBB* genes. Barcoded adaptors were added to the PCR products using a 1-step end-repair and ligation reaction to construct the prelibraries. Prelibraries were then pooled together with equal mass and converted to the SMRT bell library using Sequel Binding and Internal Ctrl Kit 3.0 (Pacific Biosciences). The CCS mode of the Sequel II platform (Pacific Biosciences) was used to sequence the SMRT bell library. Subreads were processed to CCS reads using CCS software (Pacific Biosciences) and debarcoded using LIMA in the PB Bioconda package (Pacific Biosciences). After alignment of the debarcoded CCS reads to genome build hg38 using PBMN2 (Pacific Biosciences), structural variations were identified according to HbVar, Ithanet, and LOVD databases; and SNVs and indels were identified using FreeBayes1.3.4 (https://www.geneious.com/plugins/freebayes; Biomatters).

Results

Sample Identification and Genotyping Data

A total of 33 HKαα allele samples were sequenced and verified using the conventional gap-PCR, RDB, and 2-round nested PCR. Out of the 33 samples, a total of 26 were cases of HKαα/αα compound normal β-thalassemia genotypes, 1 case was HKαα/αα compound HBB:c.316-197C > T Hete, 3 cases were HKαα/αα compound HBB: c.126_129delCTTT Hete, 2 cases were $-\alpha^{3.7}$/HKαα compound normal β-thalassemia genotypes, 1 case was $--^{SEA}$/HKαα compound normal β-thalassemia genotypes, and 1 case was $--^{SEA}$/HKαα compound HBB:c.316-45G > C Hete (**FIGURE 1**). Among the 33 HKαα allele carriers, 9 cases had normal hematological data (**TABLE 1**).

Using SMRT for Detection of the HKαα Allele

Among the 33 cases of HKαα allele identified using SMRT, 24 cases were HKαα/αα compound normal β-thalassemia genotypes, 1 case was HKαα/αα compound HBB:c.316-197C > T Hete, 3 cases were HKαα/αα compound HBB:c.126_129delCTTT Hete, 2 cases were $-\alpha^{3.7}$/HKαα compound normal β-thalassemia genotypes, 1 case was $--^{SEA}$/HKαα compound normal β-thalassemia genotypes, and 1 case was $--^{SEA}$/HKαα compound HBB:c.316-45G > C Hete. All of the cases of HKαα allele identified by SMRT were consistent with the conventional techniques. Further, the positive detection rate of SMRT was 100%. Notably, 2 of the detections made using SMRT differed from the conventional techniques. These were HKαα/αα compound HBB:c.341T > A Hete and $--^{SEA}$/HKαα compound HBB:c.316-45G > C Hete, whereas the results of conventional techniques were HKαα/αα combined normal β-thalassemia genotypes and $--^{SEA}$/HKαα compound normal β-thalassemia genotypes (**TABLE 2**, **FIGURES 2** and **3**).

Discussion

The HKαα allele arises due to a rearrangement in the α-globin gene cluster and contains both the $-\alpha^{3.7}$ and $\alpha\alpha\alpha^{anti4.2}$ crossover.[4] Some of the samples diagnosed as $-\alpha^{3.7}$/αα by the conventional techniques, like gap-PCR, were actually HKαα carriers, which needs to be further confirmed by 2-round nested PCR or other techniques.[5,7,8,10,14,15] Conventional genetic diagnosis techniques can only detect target loci; they cannot detect rare and new loci directly, and the methods can be time-consuming.

Currently, there is no gold standard for detecting HKαα allele genotype(s). The most common molecular diagnostic method used is the 2-round nested PCR.[4–7] Two-round nested PCR is an economical and effective method to detect HKαα alleles; however, it cannot identify other genotypes, and there is a high probability of cross-contamination during the second PCR amplification. Other technologies for detecting HKαα alleles include quantitative PCR, real-time PCR-based multicolor melting curve analysis, and multiplex ligation-dependent probe amplification (MLPA). MLPA is used to determine the copy number variation of genes, but it cannot determine whether the deletions and duplications are located on the same chromosome. Further, the results of MLPA are incorrect when balanced translocations of chromosomes coexist.[8,16] Therefore, when HKαα positive results are suspected by these techniques, verification by another technique, such as Sanger sequencing, is needed to ensure accuracy.

SMRT technology can be used to test point mutations, structural variations, and triplet and fusion gene mutations without extraction or interrupting the DNA. The technique directly reads full-length gene sequences and conducts a comprehensive and accurate genetic testing for thalassemia, avoiding misjudgments caused by conventional genetic methods for diagnoses.[17–19] For rare types of thalassemia that cannot be detected using conventional genetic technologies, SMRT can improve the diagnostic rate, which is highly valued in clinical applications.[11–13]

In this study, all cases of the HKαα allele identified by SMRT were consistent with the conventional techniques. Among the 33 HKαα allele carriers, 9 cases had normal hematological data, which were likely to be neglected in the clinic. Importantly, 2 abnormalities were detected in β-thalassemia (HBB: C.341T > A Hete and HBB: C.316-45G > C Hete) that were missed by the conventional techniques. HBB: C.341T > A Hete and HBB: C.316-45G > C Hete are considered rare β-hemoglobin variants. The c.341T > A heterozygote is a silent carrier, with c.341T > A Hete having a normal hematological phenotype and a normal reference range of HbA2 and HbF.[12,20–22] Zhong et al[23] hypothesized the rare variant c.316-45G > C Hete to be more likely a polymorphism. An in silico analysis verified it as a benign sequence variant without thalassemic effect. The phenotype of c.316-45G > C Hete combined with other types of α+ thalassemia may be a case of silent or mild α-thalassemia.[13]

Conclusion

The SMRT technique showing a high detection accuracy and reproducibility that can detect not only common thalassemia variants but also variants of rare and new loci. It therefore has an important potential clinical application for thalassemia carrier screening in clinic.

Acknowledgments

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REFERENCES

1. Muncie HL Jr, Campbell J. Alpha and beta thalassemia. *Am Fam Physician.* 2009;80(4):339–344.
2. Munkongdee T, Chen P, Winichagoon P, Fucharoen S, Paiboonsukwong K. Update in laboratory diagnosis of thalassemia. *Front Mol Biosci.* 2020;7:74.

3. Lee JS, Cho SI, Park SS, Seong MW. Molecular basis and diagnosis of thalassemia. *Blood Res.* 2021;56(S1):S39–S43.
4. Wang W, Chan AY, Chan LC, Ma ES, Chong SS. Unusual rearrangement of the alpha-globin gene cluster containing both the -alpha3.7 and alphaalphaalphaanti-4.2 crossover junctions: clinical diagnostic implications and possible mechanisms. *Clin Chem.* 2005;51(11):2167–2170.
5. Shang X, Li Q, Cai R, Huang J, Wei X, Xu X. Molecular characterization and clinical presentation of HKαα and anti-HKαα alleles in southern Chinese subjects. *Clin Genet.* 2013;83(5):472–476.
6. Wu MY, Li J, Li SC, Li Y, Li DZ. Frequencies of HKαα and anti-HKαα alleles in Chinese carriers of silent deletional α-thalassemia. *Hemoglobin.* 2015;39(6):407–411.
7. Zhang M, Huang H, Chen M, et al. Frequencies and hematological manifestations of the HKαα allele in southern Chinese population. *Int J Clin Exp Pathol.* 2019;12(8):3058–3062.
8. Chen DM, Ma S, Tang XL, Yang JY, Yang ZL. Diagnosis of the accurate genotype of HKαα carriers in patients with thalassemia using multiplex ligation-dependent probe amplification combined with nested polymerase chain reaction. *Chin Med J.* 2020;133(10):1175–1181.
9. Li Z, Cai S, Rong K, Song G, Li Y, Guo R. The first compound heterozygosity for HKalpha alpha allele and Southeast Asian deletion allele. *Clin Biochem.* 2007;40(5-6):407–410.
10. Zhong LY, Wang F, Chen PS, et al. Analysis of genotype and hematological phenotype of 14 patients with coinheritance HKαα and South-East deletion thalassemia [in Chinese]. *Zhonghua Yi Xue Za Zhi.* 2018;98(2):117–121.
11. Xu L, Mao A, Liu H, et al. Long-molecule sequencing: a new approach for identification of clinically significant DNA variants in α-thalassemia and β-thalassemia carriers. *J Mol Diagn.* 2020;22(8):1087–1095.
12. Liang Q, Gu W, Chen P, et al. A more universal approach to comprehensive analysis of thalassemia alleles (CATSA). *J Mol Diagn.* 2021;23(9):1195–1204.
13. Luo S, Chen X, Zeng D, et al. The value of single-molecule real-time technology in the diagnosis of rare thalassemia variants and analysis of phenotype-genotype correlation. *J Hum Genet.* 2021;67(4):1–13.
14. Huang Q, Wang X, Tang N, Yan T, Chen P, Li Q. Simultaneous genotyping of α-thalassemia deletional and nondeletional mutations by real-time PCR-based multicolor melting curve analysis. *J Mol Diagn.* 2017;19(4):567–574.
15. Long J, Liu E. The carriage rates of αααanti3.7, αααanti4.2, and HKαα in the population of Guangxi, China measured using a rapid detection qPCR system to determine CNV in the α-globin gene cluster. *Gene.* 2021;768:145296.
16. Xie XM, Wu MY, Li DZ. Evidence of selection for the α-globin gene deletions and triplications in a Southern Chinese population. *Hemoglobin.* 2015;39(6):442–444.
17. Roberts RJ, Carneiro MO, Schatz MC. The advantages of SMRT sequencing. *Genome Biol.* 2013;14(7):405.
18. Hestand MS, Ameur A. The versatility of SMRT sequencing. *Genes.* 2019;10(1):24.
19. Xiao T, Zhou W. The third generation sequencing: the advanced approach to genetic diseases. *Transl Pediatr.* 2020;9(2):163–173.
20. Viprakasit V, Tachavanich K, Suwantol L, Pung-Amritt P, Chinchang W, Tanphaichitr VS. Allele related mutation specific-polymerase chain reaction for rapid diagnosis of Hb New York (beta 113 (G15) Val–>Glu, beta(CD113 GTG–>GAG)). *J Med Assoc Thai.* 2002;85 Suppl 2:S558–S563.
21. Lou JW, Wang T, Liu YH, et al. Prevalence and molecular characterization of structural hemoglobin variants in the Dongguan region of Guangdong province, southern China. *Hemoglobin.* 2014;38(4):282–286.
22. Chaibunruang A, Singha K, Srivorakun H, Fucharoen G, Fucharoen S. Molecular characteristics of Hb New York [β113(G15)Val→Glu, HBB: c.341T>A] in Thailand. *Hemoglobin.* 2018;42(1):11–15.
23. Zhong L, Wang Y, Lin W, et al. Prenatal genetic counseling in a Chinese pregnant woman with rare thalassemia: a case report. *Front Genet.* 2021;12:670168.

Pourbiax Diagrams as an Aid to Understanding the Impact of Acid/Base Disturbance on Blood Glucose Point-of-Care Testing

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Keywords: diabetes mellitus, PQQ-dependent glucose dehydrogenase, capillary blood glucose, clinical chemistry, chemistry, basic science

Abbreviations: POCT, point-of-care testing; PQQ, pyrroloquinoline quinone.

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ABSTRACT

Objective: Assays based on redox reactions that involve proton transfer are vulnerable to artifactual findings in metabolic acidosis/alkalosis. We evaluated the impact of pH on the measurement of blood glucose by the glucose dehydrogenase/pyrroloquinoline quinone system used in point-of-care-testing.

Methods: We applied a series of thermodynamic equations to adjust the Gibbs energy for the pyrroloquinoline quinone couple. This adjusts values taken under standard conditions to those more closely resembling the physiological state.

Results: Under standard conditions, the pyrroloquinoline quinone couple has E^{o} = –0.125 V whereas adjustment to the physiological state (pH 7.40, ionic strength 0.15 mol/L, and temperature 310.15°K) yields $E^{o\prime}$ = –0.166 V. This corresponds to an uncertainty in blood glucose determination of approximately 0.13 mmol/L.

Conclusion: We have demonstrated that the impact of pH on blood glucose determination by the glucose dehydrogenase/pyrroloquinoline quinone system (under physiologically relevant conditions of ionic strength and temperature) is not clinically significant.

Derangements in acid-base chemistry are one of the most common metabolic disturbances encountered in clinical practice. Although the consequences of metabolic acidosis/alkalosis are well understood, some of the subtle effects on redox-dependent processes are overlooked. This could be of clinical significance, as alterations in redox behavior due to acidosis/alkalosis have the potential to affect many clinical chemistry determinations, such as blood glucose determination by point-of-care testing (POCT).

Most POCT blood glucose assays use a sequence of reactions that are dependent on redox processes. For example, Roche's Accu-Chek uses an immobilized glucose dehydrogenase/pyrroloquinoline quinone (PPQ) system to mediate the reactions:

$$\beta\text{-}D\text{-Glucose} + O_2 \rightarrow \beta\text{-}D\text{-Glucono-1, 5-lactone} + 2H^+ + 2e^- \quad (1a)$$

$$PQQ + 2H^+ + 2e^- \rightleftharpoons PQQH_2 \quad (1b)$$

The reduced $PQQH_2$ is then reoxidized by an electron transfer mediator,[1] such as 2 molar equivalents of ferrocyanide:

$$2[Fe(CN)_6]^{3-} + 2e^- \rightleftharpoons 2[Fe(CN)_6]^{4-} \quad (2)$$

The ferricyanide is re-reduced at a gold working electrode that produces a current in accordance with the Cottrell equation. It follows that the current is proportional to glucose concentration, and with appropriate calibration will produce a result in units of concentration.[2] As equation 1a involves a 2-electron, 2-proton exchange, Le Châtelier's principle dictates that this process will be dependent on pH, with acidic conditions favoring the reverse process and a subsequent underestimation of blood glucose.

The feasibility of redox reactions is ultimately governed by the change in Gibbs energy, ΔG, which in turn is dependent on ionic strength and temperature, as well as pH. However, this is rarely accounted for in the medical biochemistry literature; instead, values are expressed under standard laboratory conditions (dilute aqueous solution at zero ionic strength and a temperature of 298°K). Generally, the ionic strength of plasma does not vary considerably, even with extensive fluid resuscitation, and it maintains a value close to 0.15 mol/L.[3] Likewise, aside from exposure to extreme environmental conditions, body temperature rarely varies much in thermodynamic terms, even in malignant hyperthermia

or sepsis. In contrast to these, however, relatively large changes in hydrogen ion concentration can occur in vivo and so, of the factors affecting redox processes, this is the most notable. For the purposes of this work, we assume that the maximum physiological pH range compatible with life is 7.0–8.0, a convenient interval consistent with case reports in the literature.[4,5]

We sought to provide a physiochemical explanation for the experimental observation that blood pH affects the accuracy of blood glucose determination by POCT. To make our findings more accessible, we present our analysis using a Pourbiax diagram, which provides a clear summary of the relationship between transformed thermodynamic properties and pH. We also assessed the theoretical impact on blood glucose measurement by the glucose dehydrogenase/PQQ system.

Methods

We recall that reversible reactions such as equation 1b are described by an equilibrium constant, K, which is in turn related to a change in Gibbs energy. For the PQQ/$PQQH_2$ couple this is

$$K = \frac{a(PQQH_2)}{a(PQQ) \cdot a(H^+)^2} = 5.93 \times 10^{-5} \quad (3)$$

$$\Delta G = -RT\ln K = 24.12\ kJ \cdot mol^{-1} \quad (4)$$

where R = 8.314 × 10^{-3} $kJ{\cdot}K^{-1}{\cdot}mol^{-1}$ and T is the temperature in Kelvin. The change in Gibbs energy in equation 4 relates to measurements at 25°C (298.15°K) in dilute aqueous solution of zero ionic strength, conditions not consistent with those found in vivo. Therefore, the first adjustment to account for physiological conditions is a correction for the nominal ionic strength of plasma (I = 0.15 mol/L), which is obtained via the extended Debye-Hückel equation.[6]

$$\Delta G(I) = \Delta G^o - \frac{\alpha z^2 I^{1/2}}{1 + BI^{1/2}} \quad (5)$$

where the Debye-Hückel constant α = 1.20732 $kg^{½}{\cdot}mol^{-½}$ at 37°C (310.15°K), z is the charge of the species, and B = 1.6 $L^{½}{\cdot}mol^{-½}$. Likewise, the Gibbs energy in equation 4 must be adjusted to compensate for T = 310.15°K (ie, body temperature). If we assume that standard enthalpies and entropies of formation are independent of temperature (which we must, as the heat capacities for these species are unknown), we can adjust ΔG for temperature using:

$$\Delta G(T) = \left(\frac{310.15}{298.15}\right)\Delta G^o + \left(1 + \frac{310.15}{298.15}\right)\Delta H^o \quad (6)$$

where we take ΔH^o as −69.74 $kJ{\cdot}mol^{-1}$ (estimated by a group additivity method) for equation 1b. The final adjustment that must be made for physiological conditions is pH. Alberty[7] demonstrated that ΔG for a species varies with pH according to the relationship:

$$\Delta G(pH) = \Delta G^o - N\left[RT\ln\left(10^{-pH}\right)\right] \quad (7)$$

where N is the number of hydrogen atoms in the species (N = 8 for $PQQH_2$). Finally, the adjusted reduction potential, $E^{o\prime}$, is evaluated as

$$E^{o\prime} = -\frac{\Delta G}{nF} \quad (8)$$

where n is the number of electrons transferred and F is the Faraday constant, 96.5 $kJ{\cdot}mol^{-1}{\cdot}V^{-1}$.

Results

Application of equations 5–8 to the reduction of the PQQ couple yields $E^{o\prime}$ = −0.166 V at pH 7.4, I = 0.15 mol/L and T = 310.15 K, whereas the corresponding value for typical experimental conditions (pH 7.0, I = 0 mol/L and T = 298.15°K) is E^o = −0.125 V. The variation of the reduction potential with pH under these two sets of conditions is shown as a Pourbiax diagram in **FIGURE 1**. We see that in both cases a lower pH favors formation of PQQ, whereas at higher pH the reduced $PQQH_2$ form is favored. The theoretical impact of pH on POCT blood glucose determination is shown in **TABLE 1**. In these data, we assume a sample has a known blood glucose of 5.0 mmol/L and that the effect of pH on the ratio of PQQ/$PQQH_2$ is directly proportional to the reported glucose concentration. In so doing, we show that at lower pH glucose is underestimated by approximately 0.1 mmol/L, whereas at higher pH it is overestimated by about 0.15 mmol/L; this gives a corresponding overall uncertainty of 0.13 mmol/L.

Discussion

Accurate modeling of in vitro diagnostic reactions is possible through consideration of the fundamental thermodynamics of the processes involved. The results presented in **FIGURE 1** take the form of a Pourbiax diagram, first proposed by Marcel Pourbaix in the 1930s as a visual means of predicting how the reduction of a species changes with pH.[8] From this diagram, we see that correction for physiological conditions shifts the reduction potential for PQQ by approximately 13 mV, which seems relatively insignificant. When this result is carried through to the determination of blood glucose by a POCT device using the glucose dehydrogenase/PQQ system, we see that the impact is clinically insignificant and within the accepted accuracy of the assay. However, our evaluation does not consider the additive impact of an abnormal hematocrit

FIGURE 1. Pourbiax diagram for reduction of pyrroloquinoline quinone (PPQ) under standard conditions (dashed line) and physiological conditions (solid line). To the left of the vertical line at pH 7.40 the oxidized form (PPQ) is favored whereas to the right of the vertical line, the reduced ($PPQH_2$) is favored.

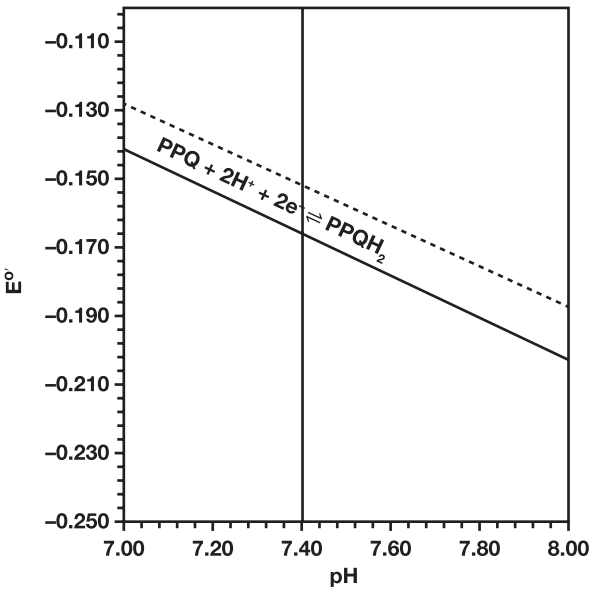

TABLE 1. Theoretical Impact of pH on Glucose Measurement by a System Employing the Glucose Dehydrogenase/ Pyrroloquinoline Quinone System[a]

pH	K	K′	Glucose (mmol/L)
7.00	3.4×10^7	1.3×10^7	4.87
7.20	1.4×10^7	5.1×10^6	4.94
7.40	5.8×10^6	2.0×10^6	5.00
7.60	2.4×10^6	8.1×10^5	5.06
7.80	9.8×10^5	3.2×10^5	5.12
8.00	4.0×10^5	1.3×10^5	5.18

[a]*Note the thermodynamic equilibrium constant is dimensionless.*

plus metabolic acidosis/alkalosis, the former of which is known to cause inaccuracy in POCT measurements.

Tang et al[9] experimentally demonstrated that at normal blood glucose levels, pH did not significantly affect glucose measurements. However, when glucose levels were high, a low pH decreased the reported glucose level, and conversely, a high pH overestimated glucose levels. This is of significance in the context of a hyperosmolar hyperglycemic state, where high glucose, high hematocrit, and potential lactic acidosis (eg, as in sepsis) will make POCT glucose determination unreliable.[10] This evaluation highlights the need for careful interpretation of blood glucose results obtained at the bedside and, more generally, the impact of pH on redox process is of clinical diagnostic interest.

Supplementary Data

Supplemental figures and tables can be found in the online version of this article at www.labmedicine.com.

REFERENCES

1. Kulys J, Tetianec L, Bratkovskaja I. Pyrroloquinoline quinone-dependent carbohydrate dehydrogenase: activity enhancement and the role of artificial electron acceptors. *Biotech J.* 2010;5(8):822–828.
2. Cha KH, Qin Y, Meyerhoff ME. Origin of low detection limit and high selectivity of Roche Accu-Chek test strips that enables measurement of tear glucose levels. *Electroanalysis* 2015;27(3):670–676.
3. Covington AK, Robinson RA. References standards for the electrometric determination, with ion-selective electrodes, of potassium and calcium in blood serum. *Anal Chim Acta*. 1975;78(1):219–222.
4. Tugrul S, Telci L, Yildirim A, Yanar H, Cakar N. Case report of severe metabolic alkalosis: life-compatible new level. *J Trauma*. 2010;68(3):61–63.
5. Heller I, Halevy J, Cohen S, Theodor E. Significant metabolic acidosis induced by acetazolamide: not a rare complication. *Arch Intern Med*. 1985;145(10):1815–1817.
6. Goldberg N, Tewari YB. Thermodynamics of the disproportionation of adenosine 5′-diphosphate to adenosine 5′-triphosphate and adenosine 5′-monophosphate: I, equilibrium model. *Biophys Chem*. 1991;40(3):241–261.
7. Alberty RA. Standard apparent reduction potentials for biochemical half reactions as a function of pH and ionic strength. *Arch Biochem Biophys*. 2001;289(1):94–109.
8. Pesterfield LL, Maddox JB, Crocker MS, Schweitzer GK. Pourbiax (E-pH-M) diagrams in three dimensions. *J Chem Ed.* 2012;89(7):891–899.
9. Tang Z, Du X, Louie RF, Kost GJ. Effects of pH on glucose measurements with handheld glucose meters and a portable glucose analyzer for point-of-care-testing. *Arch Pathol Lab Med*. 2000;124(4):577–582.
10. Patel M. Emergencies in diabetes—diabetic ketoacidosis and hyperosmolar hyperglycaemia state. *Medicine* 2021;49(2):106–109.

A Meta-Analysis on the Association of Colibactin-Producing *pks+ Escherichia coli* with the Development of Colorectal Cancer

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Keywords: colorectal cancer, colon neoplasms, pathogenicity islands, polyketide synthase, *Escherichia coli*, meta-analysis

Abbreviations: CRC, colorectal cancer; *pks*, polyketide synthase; NOS, Newcastle-Ottawa Scale; OR, odds ratio; CI, confidence interval; NRPS-PKS, nonribosomal peptide synthase-polyketide synthase; SBS, single base substitution; ID, insertions and deletions; HGF, hepatocyte growth factor; FGF, fibroblast growth factor; GM-CSF, granulocyte-macrophage colony-stimulating factor.

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ABSTRACT

Objective: Previous studies on the association between *pks*$^+$ *Escherichia coli* and colorectal cancer (CRC) demonstrated conflicting results. Hence, we performed a meta-analysis to obtain more precise estimates.

Methods: Related literature was obtained from PubMed, ScienceDirect, Google Scholar, and Cochrane Library. Data were then extracted, summarized, and subjected to analysis using Review Manager 5.4 by computing for the pooled odds ratios at the 95% confidence interval.

Results: Overall analysis showed that individuals carrying *pks*$^+$ *E coli* had a greater risk of developing CRC. Subgroup analysis further showed that individuals from Western countries carrying *pks*$^+$ *E coli* and individuals with *pks*$^+$ *E coli* in their tissue samples had increased risk of developing CRC.

Conclusion: Results of this meta-analysis suggest that individuals with *pks*$^+$ *E coli* have a greater risk of developing CRC. However, more studies are needed to confirm our claims.

Colorectal cancer (CRC) is the third-most diagnosed (10%) and second-most deadly (9.4%) cancer in the world. In 2020 alone, more than 1.9 million CRC cases and more than 930,000 CRC-related deaths were reported. CRC incidence and mortality rates are mainly higher in males.[1] Furthermore, the incidence and mortality trends of CRC are increasing in developing countries rather than following the decreasing trends found in developed countries.[2]

Studies have shown that the risk of acquiring CRC increases with an unhealthy diet, alcohol consumption, obesity, and physical inactivity. A diet mainly composed of processed meat and high levels of fat with an inadequate intake of fruits, vegetables, and whole grains further increases this risk.[1,3] Other major risk factors include a family history of CRC, polyps in the colon or rectum, and inflammatory bowel disease.[4] Interestingly, evidence has shown that the body's innate intestinal microbiome is also a significant contributor to CRC development.[3,5,6]

The gut microbiota consists of microorganisms such as bacteria under the *Bacteroides*, *Bifidobacterium*, *Lactobacillus*, *Eubacterium*, *Peptococcus*, *Fusobacterium*, *Clostridium*, and *Escherichia* genera.[7,8] These microorganisms form a symbiotic relationship with the host, wherein they function to metabolize nutrients, provide and maintain the structural integrity of the gut mucosal barrier, and protect the host against pathogenic microorganisms.[9] An imbalance in the normal composition of intestinal microflora results in gut dysbiosis, which may involve a loss of beneficial microbial signal, loss of overall microbial diversity, or pathobiont expansion.[10]

Dysbiotic states, which alter the host's physiological functions, have often been associated with extraintestinal diseases.[11] Strains of *Escherichia coli*, mainly those belonging to the B2 phylogroup, are the predominantly isolated bacteria from the colon.[12–15] The virulence of most *E coli* B2 strains has been attributed to the polyketide synthase (*pks*) genomic island. This *pks* island encodes nonribosomal peptide

synthases, polyketide synthases, and hybrid peptide-polyketide synthases, which synthesize a genotoxic compound called colibactin. Production of this toxin then induces double-stranded DNA breakage and activates the DNA damage checkpoint pathway, ultimately leading to cell death.[16]

Among the members of the Enterobacteriaceae family, colibactin-producing *E coli* B2 strains have been prevalent in CRC tissue and stool samples.[17–19] Studies have found that *pks*$^+$ *E coli* persisted in the colon, inducing colon inflammation, epithelial damage, and cell proliferation in vivo.[15,20] In animal models, *pks*$^+$ *E coli* enhanced tumor growth in infected mice,[17,21] suggesting its potential role in colon carcinogenesis. However, previous studies on the association between *pks*$^+$ *E coli* and CRC demonstrated conflicting results.[17,19,22,23] Hence, this meta-analysis was conducted to obtain more precise estimates and determine whether *pks*$^+$ *E coli* is associated with CRC development.

Materials and Methods

Literature Search Strategy

Related literature that reported the association of *pks*$^+$ *E coli* with colorectal cancer was obtained from four databases, namely PubMed, ScienceDirect, Google Scholar, and Cochrane Library, as of October 18, 2021. The Medical Subject Heading (MeSH) terms used for the literature search were divided into 3 components: *pks* (eg, genomic island, pathogenicity island, colibactin, *clbA*, *clbB*, *clbN*, *clbQ*, and *clbS*); *E coli* (eg, *Escherichia coli*); and colorectal cancer (eg, colon cancer, colon adenocarcinoma, colon carcinoma, rectal cancer, malignant colon, and malignant colorectal tumor). There were no restrictions applied on the publication date of the studies.

Study Assessment and Eligibility Criteria

The first screening was done to determine whether the titles and abstracts of the search studies qualified for full-text evaluation. Irrelevant and duplicate studies were excluded immediately. Relevant articles that passed the first screening were assessed further based on the following inclusion criteria: biological samples (eg, tissue or stool) were from patients with confirmed CRC (case) and cancer-free individuals (control); *pks*$^+$ *E coli* or colibactin genes were present in the biological samples of CRC patients, and studies were either a case-control or cohort type. Meanwhile, studies conducted on animal models, review articles, editorials, communication, book chapters, clinical trials, indices, short reports, preprints, conference abstracts, case reports, and case studies were excluded from this meta-analysis. Studies that were not written in or translated into English were also excluded. Additional eligible articles were identified, screened, and assessed from the references cited in the previously selected studies.

Data Extraction

Data were extracted from each publication that met the inclusion criteria. For this review, the following were obtained: first author's last name, year of publication, country where the study was conducted, biological sample analyzed from the CRC patients and control patients, control group criteria, method for CRC identification/diagnosis, method for genotyping, the total number of *pks*$^+$ CRC patients, the total number of *pks*$^+$ control individuals, the total number of *pks*$^-$ CRC patients, and the total number of *pks*$^-$ control individuals. The total number of participants was also computed by combining the number of CRC patients and control individuals.

All the mentioned data were independently extracted by M.E.G. and P.O.L. and they agreed on all the items. R.E.T. and P.M.A. verified the included data and resolved any disagreements. Data obtained were then tabulated and organized. The number and percentage of CRC patients and control groups with *pks*$^+$ and *pks*$^-$ were computed.

Quality Assessment of the Eligible Studies

The Newcastle-Ottawa Scale (NOS) assessment was used to check the methodological quality of the eligible studies. The selected studies were judged based on 3 parameters: selection (maximum of 4 points), comparability (maximum of 1 point), and exposure (maximum of 3 points). The rating system has total scores ranging from 0 (worst) to 8 (best) points. Studies with an accumulated score of ≤4 points were regarded as low-quality studies, 5 to 6 points as moderate-quality studies, and ≥7 points were regarded as high-quality studies.[24]

Meta-Analysis Protocol

Statistical analysis was carried out using Review Manager 5.4.1 (Copenhagen: Nordic Cochrane Centre, The Cochrane Collaboration, 2020) and Meta-Essentials.[25] The protocol used for this meta-analysis was based on the procedure of Pabalan et al[26–28] with some modifications. The association of colibactin-producing *pks*$^+$ *E coli* with CRC was estimated using the generated pooled odds ratios (OR) and 95% confidence interval (CI), which were analyzed using either a fixed- or random-effects model. The model used was dependent on the heterogeneity of the data, which was based on the computed *P* value and I^2 statistics. An I^2 value of 0% to 40% indicated that the data were homogenous; 30% to 60% indicated moderate heterogeneity; 50% to 90% indicated substantial heterogeneity; and 75% to 100% indicated considerable heterogeneity. A random-effects model was used if heterogeneity was present, and a fixed-effects model was used if it was absent. All *P* values for the ORs and 95% CI were 2-sided with a significance threshold of <.05. On the other hand, the *P* value for heterogeneity testing was set at <.10 due to the low power of the test.[29]

Sensitivity analysis was used to determine the robustness of the overall summary effects wherein each study's influence on the pooled ORs was examined by the systematic removal of one study at a time.

Results

Search Result

In this meta-analysis, 48,910 publications were obtained from PubMed, ScienceDirect, Google Scholar, and Cochrane Library (see **FIGURE 1** for the summary of the literature search).

After an in-depth review of the title and abstract and removing duplicate studies, only 452 articles were further evaluated. From this number, 433 were excluded for the following reasons: 231 were unrelated or irrelevant to the study, 200 had insufficient data or information regarding the topic, and 21 did not have a full text available. Based on the inclusion and exclusion criteria above, only 19 publications qualified for full-text evaluation. Among the 19 articles, 7 were excluded because their samples involved random participants, patients with other diseases (eg, recurrent *Clostridium difficile*), or CRC cells treated with *pks*$^+$ *E coli*. Only 12 articles were included in this study, namely those of Arthur et al,[17] Bonnet et al,[30]

TABLE 1. Characteristics of the Included Studies

First Author	Year of Publication	Country	Sample	Molecular Method	Source of Control	CRC		Control		Newcastle-Ottawa Scale
						pks$^+$	*pks*$^-$	*pks*$^+$	*pks*$^-$	
Piciocchi	2021	Italy	Fresh tissue	Real-time qPCR	Disease-free individuals	14	54	15	108	8
Eklöf	2017	Sweden	Fresh stool	Real-time qPCR	Patients with no neoplastic findings	22	12	17	53	8
Raisch	2014	France	Fresh tissue	(ERIC)-PCR, (RAPD)-PCR	Patients with diverticulosis	23	6	15	9	8
Prorok-Hamon	2013	United Kingdom	Fresh tissue	PCR	Sporadic polyps or irritable bowel syndrome	11	2	10	22	8
Bonnet	2013	France	FFPE tissue	PCR	Patients with diverticulosis	21	13	22	20	7
Buc	2013	France	Fresh tissue	(ERIC)-PCR, (RAPD)-PCR	Patients with diverticulosis	21	6	17	25	6
Arthur	2012	United States	Tissue[a]	Not indicated	Non-CRC individuals	14	5	7	19	5
Villariba-Tolentino	2021	Philippines	FFPE tissue	Real-time qPCR	Benign tissue	12	27	32	11	7
Iyadorai	2020	Malaysia	Fresh tissue	PCR	No previous history of IBD or CRC, or any form of cancer	8	1	40	45	7
Shimpoh	2017	Japan	FFPE tissue	Real-time qPCR	Non-CRC individuals	15	12	20	14	6
Viljoen	2015	South Africa	Fresh-frozen tissue	Real-time qPCR	Adjacent normal mucosa of the same CRC patient	12	13	42	42	6
Gómez-Moreno	2019	Puerto Rico & United States	Stool	Real-time qPCR	Healthy individuals without prior history of colorectal neoplasia	6	2	17	21	7

CRC, colorectal cancer; (ERIC)-PCR, enterobacterial repetitive intergenic consensus PCR; FFPE, formalin-fixed paraffin-embedded; IBD, inflammatory bowel disease; qPCR, quantitative polymerase chain reaction; (RAPD)-PCR, randomly amplified polymorphic DNA PCR.
[a]Not indicated in the paper whether tissues analyzed were fresh frozen or FFPE.

Buc et al,[18] Eklöf et al,[22] Gómez-Moreno et al,[31] Iyadorai et al,[7] Piciocchi et al,[32] Prorok-Hamon et al,[19] Raisch et al,[15] Shimpoh et al,[33] Viljoen et al,[34] and Villariba-Tolentino et al.[23]

Characteristics of the Included Studies

TABLE 1 summarizes the characteristics of the included studies. Of the 12 studies included, only 1 was a cohort study and the rest were case-control studies. Furthermore, 3 were conducted in France, 2 in the United States, and 1 each in Japan, Italy, Iran, Malaysia, Philippines, Sweden, Puerto Rico, South Africa, and the United Kingdom. The articles were published between 2013 and 2021, of which 5 were recent publications (2020–2021). The NOS scoring indicated that the included studies were of high quality with a mean and standard deviation of 6.92 ± 1.00 and a median of 7.0.

Overall and Subgroup Analysis for the Association of *pks*$^+$ *E coli* with Colorectal Cancer

Summary of Overall Events

Twelve studies were included in the overall analysis for the association between *pks*$^+$ *E coli* and CRC. A random-effects model (**FIGURE 2**) exhibited significant association (OR: 2.27; 95% CI: 1.13–4.57; P^A = .02) and a high degree of heterogeneity (I^2 = 79%, P^H < .00001). Results of the pooled ORs signify that individuals carrying *pks*$^+$ *E coli* had a greater risk of developing CRC than individuals without *pks*$^+$ *E coli*.

Outlier Analysis Outcomes

Heterogeneity was found to be significant among the included studies (I^2 = 79%, P^H < .00001). Galbraith plot analysis (**FIGURE 3**) determined 4 studies as the sources of heterogeneity, those of Arthur et al,[17] Eklöf et al,[22] Prorok-Hamon et al,[19] and Villariba-Tolentino et al.[23] The omission of these outlier studies in the overall analysis resulted in a significant loss in the heterogeneity (I^2 = 36%, P^H = .14). A fixed-effects model (**FIGURE 4**) of the pooled ORs after outlier analysis still indicated that carriers of *pks*$^+$ *E coli* had a greater risk of developing CRC (OR: 1.86; 95% CI: 1.29–2.68; P^A = .0009) than individuals without *pks*$^+$ *E coli*. Outcomes from this comparison were found to be robust, indicating the stability of our findings (data not shown).

Post-Outlier Subgroup Analysis

Subgroup analysis was also conducted by stratifying the studies based on the geographical region where the studies were conducted (Western vs Eastern countries) and the type of sample (tissue vs stool samples) collected from the participants. Stratification showed that the risk for CRC development among participants from Western countries (**FIGURE 5**) (OR: 2.32; 95% CI: 1.47–3.68; P^A = .0003) and individuals tested using tissue samples (**FIGURE 6**) (OR: 2.23; 95% CI: 1.39–3.60; P^A = .0010) have similar significant results as the overall findings.

Discussion

CRC is the third-most diagnosed cancer and the second-leading cause of cancer-related deaths worldwide.[1,35] Thus, early detection and prevention approaches have been investigated for the past few years. CRC development has been previously associated with several factors, including unhealthy diet, alcohol consumption, obesity, physical inactivity, family history, and inflammatory bowel disease.[3,36–39]

FIGURE 1. **PRISMA flow diagram for the selection of included studies.**

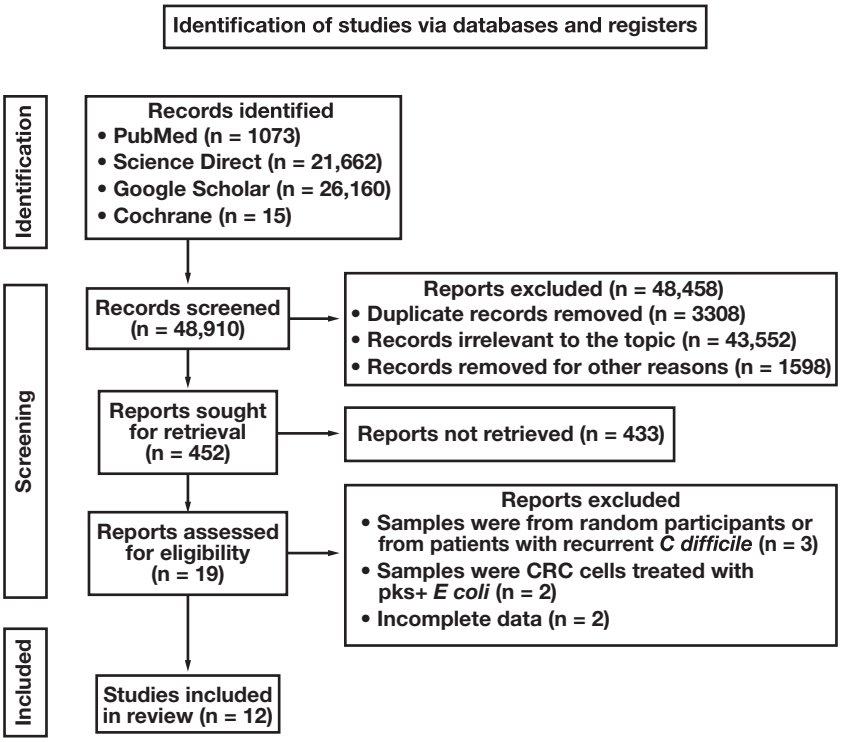
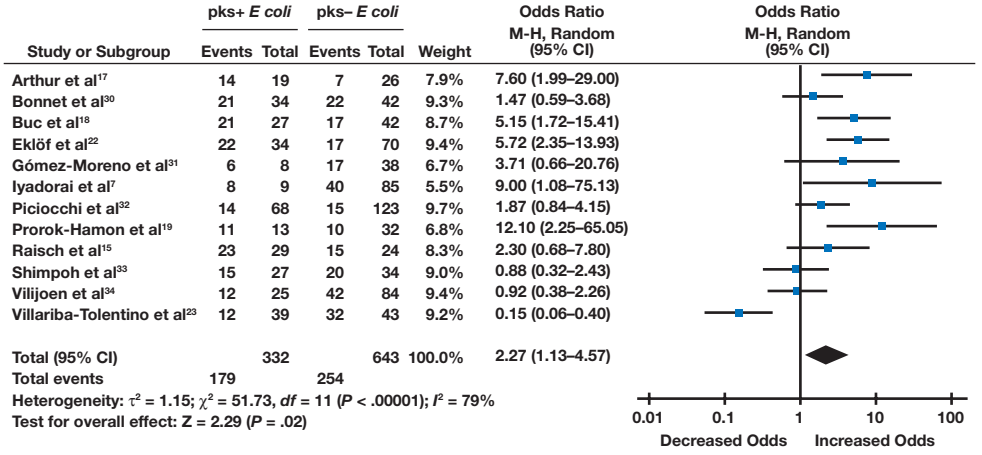


FIGURE 2. **Overall analysis of the association of *pks*$^+$ *Escherichia coli* with colorectal cancer. CI, confidence interval; *df*, degrees of freedom.**



The gut microbiota can affect host cells by producing metabolites and toxins. *E coli* is an opportunistic pathogen associated with various intestinal infections, such as diarrhea, cholecystitis, ulcerative colitis, and Crohn's disease.[40,41] It has also been suggested that *E coli* is among the bacterial agents capable of promoting carcinogenesis through its *pks* pathogenicity island, which induces DNA damage in host cells.[16,42] Hence, this study determined whether *pks*$^+$ *E coli* is associated with CRC development through a meta-analysis of studies published between 2013 and 2021.

The findings of this meta-analysis provide significant evidence that the presence of *pks*$^+$ *E coli* is associated with an increased risk of developing CRC. Previous studies have found that *pks*$^+$ *E coli* was predominant in CRC patients compared to healthy controls.[7,15,17,18,33] Subgroup analysis also showed that individuals with *pks*$^+$ *E coli* in their tissue samples are more likely to develop CRC.[15,18,30,32] This agrees with studies that showed higher expression of *pks*$^+$ *E coli* strains in tissue samples of patients diagnosed with CRC.[17,21,43]

Gut dysbiosis of the intestinal mucosa observed in CRC patients has been attributed to *E coli* B2 strains.[11] Notably, these adherent *E coli* strains have been found to harbor a *pks* genomic island.[12–15] This 54-kilobase pathogenicity island consists of a *clb* gene cluster (*clb*A to *clb*S) that encodes a large multi-domain, hybrid nonribosomal peptide synthase-polyketide synthase (NRPS-PKS). The NRPS-PKS gene cluster is responsible for synthesizing a genotoxin called colibactin, which is implicated in the virulence of *pks*$^+$ *E coli* in the human gut.[16]

FIGURE 3. Identification of outlier studies using Galbraith plot analysis. A, Initial Galbraith plot analysis showing the studies of Arthur, Eklöf, Prorok-Hamon, and Villariba-Tolentino as outliers. B, After removal of the studies of Arthur et al,[17] Eklöf et al,[22] Prorok-Hamon et al,[19] and Villariba-Tolentino et al.[23] All remaining studies are within the 95% confidence interval of the Galbraith plot.

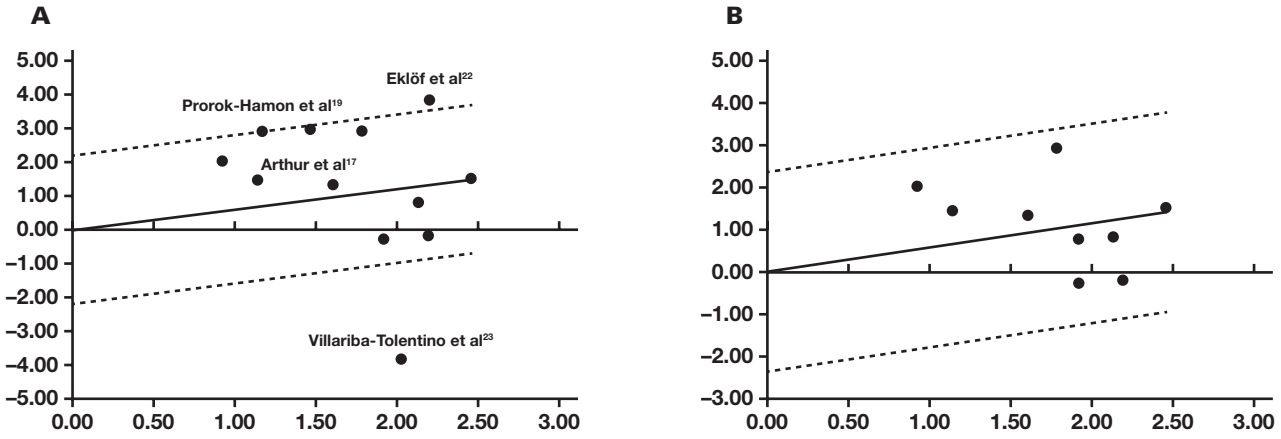


FIGURE 4. Forrest plot analysis for the post-outlier outcomes of the association of *pks*$^+$ *Escherichia coli* with colorectal cancer. CI, confidence interval; *df*, degrees of freedom.

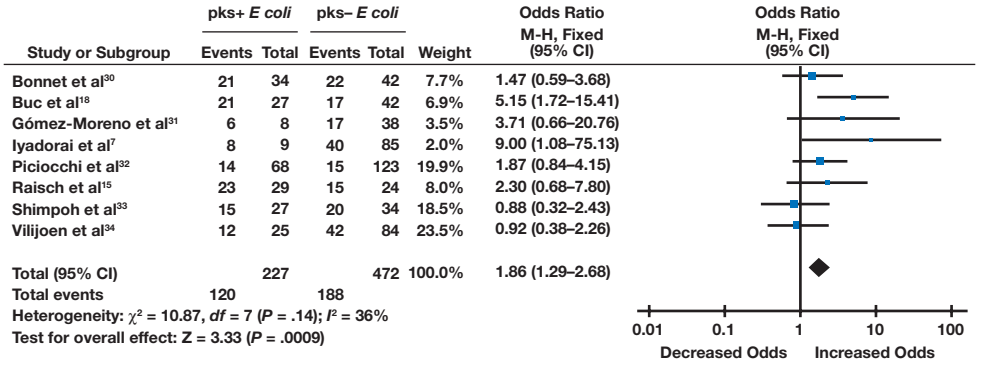
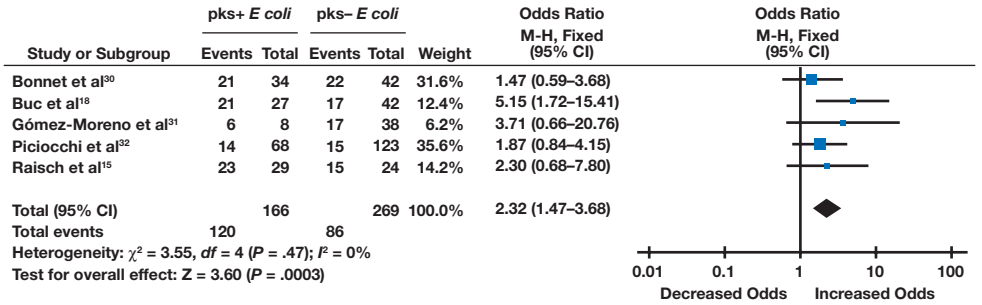


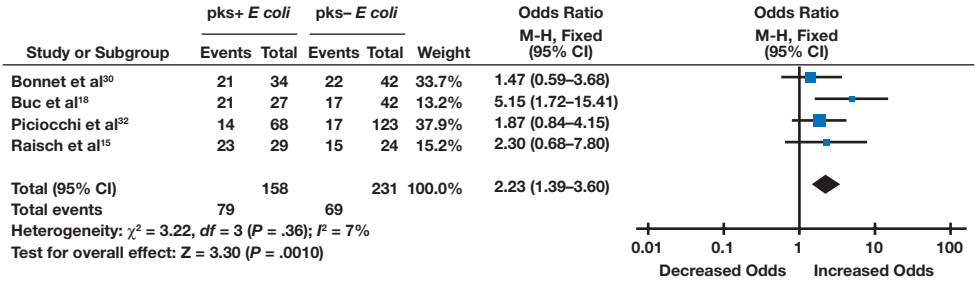
FIGURE 5. Forrest plot analysis for the post-outlier outcomes (Western studies) of the association of *pks*$^+$ *Escherichia coli* with colorectal cancer. CI, confidence interval; *df*, degrees of freedom.



Colibactin is among the cyclomodulins capable of modulating cell differentiation, apoptosis, and proliferation.[44] It was initially found that direct cell contact of *pks*$^+$ *E coli* with eukaryotic cells was necessary for colibactin to cause double-strand DNA breaks and activate the DNA damage checkpoint pathway,[16,45,46] which are hallmarks of cancer development.[47,48] Studies have since further explored the nature of these DNA lesions and other cellular mechanisms involved in tumor growth associated with colonization by *pks*$^+$ *E coli*.

After *pks*$^+$ *E coli* exposure, active colibactin alkylates DNA through covalent modifications that increase the reactivity of cyclopropane toward adenine residues in DNA. Through alkylation, colibactin generates interstrand cross-links, adenine adducts, and double-stranded DNA breaks in the nucleus.[49,50] Misrepair of these DNA lesions causes alterations in tumor suppressor genes and oncogenes, which promote tumor progression in CRC.[51,52] Furthermore, this DNA damage by *pks*$^+$ *E coli* influences mitotic aberrations in epithelial cells, such as chromosome

FIGURE 6. **Forrest plot analysis for the post-outlier outcomes (tissue samples) of the association of *pks*$^+$ *Escherichia coli* with colorectal cancer. CI, confidence interval; *df*, degrees of freedom.**



rings, aneuploidy, polyploidy, and anaphase lags and bridges,[41] which facilitate tumorigenesis.

The tumorigenic activity of colibactin is also demonstrated by its ability to induce single base substitutions (SBS), mainly T > N substitutions, and small insertions and deletions (ID), particularly single T deletions at T homopolymers in organoids. Interestingly, this distinct SBS and ID mutational signature of *pks*$^+$ *E coli* was predominantly detected in CRC genomes from 2 independent cohorts, further presenting an association between colibactin and CRC development.[16,42]

Additionally, the accumulation of DNA damage leads to cellular senescence, which contributes to oncogenesis through a senescence-associated secretory phenotype that can alter the cellular microenvironment.[53,54] Following senescence of *pks*$^+$ *E coli*-infected intestinal epithelial cells, there was an increased expression of growth factors, namely hepatocyte growth factor (HGF), fibroblast growth factor (FGF), and granulocyte-macrophage colony-stimulating factor (GM-CSF).[21] The *pks*$^+$ *E coli*-induced expression of these growth factors promotes tumor invasiveness and progression in CRC. The HGF has been established as a key determinant of CRC progression, as aberrant activation of this growth factor promotes the progression and metastasis of CRC tumors.[55,56] The HGF also facilitates cell motility, allowing cancer cells to be detached from the primary tumor to invade nearby tissue.[57] The GM-CSF may promote the metastasis of CRC by stimulating the secretion of vascular endothelial growth factor,[58] a key mediator of angiogenesis in cancer,[59] and increasing the invasiveness of CRC cells by inducing an epithelial-to-mesenchymal transition.[60,61] Similarly, increased expression of FGF was found to influence proliferation, migration, and malignancy of CRC cells[38,62]

Our analysis also found that individuals from Western countries carrying *pks*$^+$ *E coli* were more likely to develop CRC. Center and colleagues[63] found that CRC incidence is highest in Europe, North America, and Oceania, whereas it is lowest in South and Central Asia and Africa. Cases from Italy, France, Puerto Rico, and the United States (**TABLE 1**) have been studied for patterns and trends in the overall CRC incidence over the most recent 10-year period.[64]

Various epidemiological studies have suggested that CRC incidence tends to be higher in high-income countries due to the prevailing Western lifestyle.[63,64] The Western diet comprises a diet rich in red and processed meats, high in saturated fats, refined carbohydrates, and low in fruits, vegetables, seafood, poultry, and whole grains.[65] It has been observed that this type of diet favors the emergence of *E coli* in the ileal, cecal, and colonic mucosa.[66]

An abnormal proportion of adherent-invasive *E coli* has been recovered from the digestive mucosa of mice under a high fat/high sugar diet compared to mice under a conventional diet.[66] A recent study showed that exposure to a high-fat diet could impair mitochondrial function in the intestinal epithelium and disrupt the anaerobic environment of the colonic lumen, which drives the expansion of *E coli*.[67] Possible mechanistic factors in the relationship between red meat consumption and CRC include N-nitroso compounds, heterocyclic amines, polycyclic aromatic hydrocarbons, heme iron in red meat, polyunsaturated fatty acids, bile acids, nonhuman sialic acid, and infectious agents.[68–70] These mechanistic factors affect the bioavailability of host-derived nitrate (NO_3^-), and an array of molecules, which can also fuel the anaerobic respiration of *pks*$^+$ *E coli* by acting as the terminal electron acceptor.[71–76]

Overall, this meta-analysis suggests that *pks*$^+$ *E coli* is associated with an increased risk of developing CRC. However, care should be taken when interpreting the results of this study given its limitations: (1) a wide range of years of publication and patient sample sizes across individual studies; (2) inconsistent case and control specimen used across the different studies; (3) only 2 types of biological samples (tissue and stool sample) were included; (4) lack of representation of other countries and race where CRC is prevalent; and (5) other factors attributed to the association of *pks*$^+$ *E coli* with CRC were not examined. Given these limitations, the findings of this meta-analysis should be treated with caution when used in the clinical setting. On the other hand, these limitations are balanced by the study's strengths: (1) homogenous post-outlier outcomes, (2) consistency of significant comparisons, and (3) robustness of comparisons.

Conclusion

To our knowledge, this is the first meta-analysis to explore the association between *pks*$^+$ *E coli* and CRC development. Generally, this meta-analysis demonstrated that individuals with *pks*$^+$ *E coli* have an increased risk of developing CRC. As such, *pks*$^+$ *E coli* may help distinguish healthy from CRC individuals and evaluate the risk of developing CRC. Moreover, *pks*$^+$ *E coli* can be used as a predictive biomarker for detecting early-stage CRC, especially in high-risk patients. Further studies involving a wider range of geographical populations may help better understand the increased prevalence of *pks*$^+$ *E coli* among individuals in Western countries. Noninvasive biological samples, such as stool samples, may also be further studied for the presence of *pks*$^+$ *E coli* and their potential in assessing a person's risk of developing CRC.

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REFERENCES

1. Bray F, Ferlay J, Soerjomataram I, et al. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin.* 2018;68(6):394–424. doi:10.3322/caac.21492.
2. Wong MCS, Huang J, Lok V, et al. Differences in incidence and mortality trends of colorectal cancer worldwide based on sex, age, and anatomic location. *Clin Gastroenterol Hepatol.* 2021;19(5):955–966. e61. doi:10.1016/j.cgh.2020.02.026.
3. Sánchez-Alcoholado L, Ramos-Molina B, Otero A, et al. The role of the gut microbiome in colorectal cancer development and therapy response. *Cancers (Basel).* 2020;12(6):1406.
4. Kim ER, Chang DK. Colorectal cancer in inflammatory bowel disease: the risk, pathogenesis, prevention and diagnosis. *World J Gastroenterol.* 2014;20(29):9872–9881. doi:10.3748/wjg.v20.i29.9872.
5. Cheng Y, Ling Z, Li L. The intestinal microbiota and colorectal cancer. *Front Immunol.* 2020;11:615056.
6. Wong SH, Yu J. Gut microbiota in colorectal cancer: mechanisms of action and clinical applications. *Nat Rev Gastroenterol Hepatol.* 2019;16(11):690–704. doi:10.1038/s41575-019-0209-8.
7. Iyadorai T, Mariappan V, Vellasamy KM, et al. Prevalence and association of pks+ *Escherichia coli* with colorectal cancer in patients at the University Malaya Medical Centre, Malaysia Thevambiga. *PLoS One.* 2020;15(1):e0228217.
8. Rinninella E, Raoul P, Cintoni M, et al. What is the healthy gut microbiota composition? A changing ecosystem across age, environment, diet, and diseases. *Microorganisms.* 2019;7(1):14.
9. Jandhyala SM, Talukdar R, Subramanyam C, et al. Role of the normal gut microbiota. *World J Gastroenterol.* 2015;21(29):8836–8847.
10. Martinez JE, Kahana DD, Ghuman S, et al. Unhealthy lifestyle and gut dysbiosis: a better understanding of the effects of poor diet and nicotine on the intestinal microbiome. *Front Endocrinol (Lausanne).* 2021;12:667066.
11. Carding S, Verbeke K, Vipond DT, et al. Dysbiosis of the gut microbiota in disease. *Microb Ecol Heal Dis.* 2015;26:26191. doi:10.3402/mehd.v26.26191.
12. Fang X, Monk JM, Mih N, et al. *Escherichia coli* B2 strains prevalent in inflammatory bowel disease patients have distinct metabolic capabilities that enable colonization of intestinal mucosa. *BMC Syst Biol.* 2018;12(1):1–10.
13. Kotlowski R, Bernstein CN, Sepehri S, et al. High prevalence of *Escherichia coli* belonging to the B2+D phylogenetic group in inflammatory bowel disease. *Gut* 2007;56(5):669–675.
14. Mirsepasi-Lauridsen HC, Halkjaer SI, Mortensen EM, et al. Extraintestinal pathogenic *Escherichia coli* are associated with intestinal inflammation in patients with ulcerative colitis. *Sci Rep.* 2016;6:1–10.
15. Raisch J, Buc E, Bonnet M, et al. Colon cancer-associated B2 *Escherichia coli* colonize gut mucosa and promote cell proliferation. *World J Gastroenterol.* 2014;20(21):6560–6572.
16. Nougayrède JP, Homburg S, Taieb F, et al. *Escherichia coli* induces DNA double-strand breaks in eukaryotic cells. *Science (1979).* 2006;313(5788):848–851.
17. Arthur JC, Perez-chanona E, Mühlbauer M, et al. Intestinal inflammation targets cancer-inducing activity of the microbiota. *Science (1979).* 2012;338(6103):120–123.
18. Buc E, Dubois D, Sauvanet P, et al. High prevalence of mucosa-associated *E. coli* producing cyclomodulin and genotoxin in colon cancer. *PLoS One.* 2013;8(2):e56964.
19. Prorok-Hamon M, Friswell MK, Alswied A, et al. Colonic mucosa-associated diffusely adherent afaC+ *Escherichia coli* expressing lpfA and pks are increased in inflammatory bowel disease and colon cancer. *Gut.* 2013;63(5):761–770.
20. Payros D, Secher T, Boury M, et al. Maternally acquired genotoxic *Escherichia coli* alters offspring's intestinal homeostasis. *Gut Microbes.* 2014;5(3):313–325.
21. Cougnoux A, Dalmasso G, Martinez R, et al. Bacterial genotoxin colibactin promotes colon tumour growth by inducing a senescence-associated secretory phenotype. *Gut.* 2014;63(12):1932–1942.
22. Eklöf V, Löfgren-Burström A, Zingmark C, et al. Cancer-associated fecal microbial markers in colorectal cancer detection. *Int J Cancer.* 2017;141(12):2528–2536.
23. Villariba-Tolentino C, Cariño AM, Notarte KI, et al. pks + *Escherichia coli* more prevalent in benign than malignant colorectal tumors. *Mol Biol Rep.* 2021;48(7):5451–5458.
24. Wells G, Shea B, O'Connell D et al. The Newcastle-Ottawa Scale (NOS) for assessing the quality of nonrandomised studies in meta-analyses. Ottawa Hospital Research Institute; 2014. https://www.ohri.ca//programs/clinical_epidemiology/oxford.asp
25. Suurmond R, van Rhee H, Hak T. Introduction, comparison, and validation of Meta-Essentials: a free and simple tool for meta-analysis. *Res Synth Methods.* 2017;8(4):537–553.
26. Pabalan N, Pineda MR, Jarjanazi H, et al. Association of the +331G/A progesterone receptor gene (PgR) polymorphism with risk of endometrial cancer in Caucasian women: a meta-analysis. *Arch Gynecol Obstet.* 2014;291(1):115–122.
27. Pabalan N, Singh N, Pineda MR, et al. Meta-analysis of the association between PTPN11 G/A polymorphism at intron 3 with risk of gastric atrophy among East Asians. *J Gastrointest Cancer.* 2014;45(3):319–324.
28. Pabalan N, Singian E, Tabangay L, et al. Associations of the A66G methionine synthase reductase polymorphism in colorectal cancer: a systematic review and meta-analysis. *Biomark Cancer.* 2015;7(1):21–28.
29. Higgins JPT, Thompson SG. Quantifying heterogeneity in a meta-analysis. *Stat Med.* 2002;21(11):1539–1558.
30. Bonnet M, Buc E, Sauvanet P, et al. Colonization of the human gut by *E. coli* and colorectal cancer risk. *Clin Cancer Res.* 2013;20(4):859–867.
31. Gómez-Moreno R, González-Pons M, Soto-Salgado M, et al. The presence of gut microbial genes encoding bacterial genotoxins or pro-inflammatory factors in stool samples from individuals with colorectal neoplasia. *Diseases.* 2019;7(1):16.
32. Piciocchi A, Germinario EAP, Garcia Etxebarria K, et al. Association of polygenic risk score and bacterial toxins at screening colonoscopy with colorectal cancer progression: a multicenter case-control study. *Toxins (Basel).* 2021;13:(8):569. doi:10.3390/toxins13080569.
33. Shimpoh T, Hirata Y, Ihara S, et al. Prevalence of pks-positive *Escherichia coli* in Japanese patients with or without colorectal cancer. *Gut Pathog.* 2017;9:1-8. doi:10.1186/s13099-017-0185-x.
34. Viljoen KS, Dakshinamurthy A, Goldberg P, et al. Quantitative profiling of colorectal cancer-associated bacteria reveals associations between *Fusobacterium* spp., enterotoxigenic *Bacteroides fragilis* (ETBF) and clinicopathological features of colorectal cancer. *PLoS One.* 2015;10(3):1–21.
35. World Health Organization. World fact sheets cancers. *Globocan 2020.* 2020;419:1–2.
36. Gagnière J, Raisch J, Veziant J, et al. Gut microbiota imbalance and colorectal cancer. *World J Gastroenterol.* 2016;22(2):501–518.
37. Montalban-Arques A, Scharl M. Intestinal microbiota and colorectal carcinoma: implications for pathogenesis, diagnosis, and therapy. *EBioMedicine.* 2019;48:648–655.
38. Patel A, Williams N, Azharian S, et al. PWE-341 The potential role of fibroblast growth factor 7 (fgf- 7) in field cancerisation of colon cancer. *Gut.* 2015;64:A359–A360.
39. Yang J, McDowell A, Kim EK, et al. Development of a colorectal cancer diagnostic model and dietary risk assessment through gut microbiome analysis. *Exp Mol Med.* 2019;51(10):1–15. doi:10.1038/s12276-019-0313-4.
40. Liu J, Yan Q, Luo F, et al. Acute cholecystitis associated with infection of Enterobacteriaceae from gut microbiota. *Clin Microbiol Infect.* 2015;21(9):851.e1–851.e9.

41. Mirsepasi-Lauridsen HC, Vallance BA, Krogfelt KA, et al. *Escherichia coli* pathobionts associated with inflammatory bowel disease. *Clin Microbiol Rev.* 2019;32(2):e00060–e00018.
42. Pleguezuelos-Manzano C, Puschhof J, Rosendahl Huber A, et al. Mutational signature in colorectal cancer caused by genotoxic pks + *E. coli*. *Nature.* 2020;580(7802):269–273.
43. Kim KS, Kim JT, Lee SJ, et al. Overexpression and clinical significance of carcinoembryonic antigen-related cell adhesion molecule 6 in colorectal cancer. *Clin Chim Acta.* 2013;415:12–19.
44. El-Aouar Filho RA, Nicolas A, De Paula Castro TL, et al. Heterogeneous family of cyclomodulins: Smart weapons that allow bacteria to hijack the eukaryotic cell cycle and promote infections. *Front Cell Infect Microbiol.* 2017;7:1–17.
45. Bossuet-Greif N, Vignard J, Taieb F, et al. The colibactin genotoxin generates DNA interstrand cross-links in infected cells. *mBio.* 2018;9(2):e02393–e02317.
46. Reuter C, Alzheimer M, Walles H, et al. An adherent mucus layer attenuates the genotoxic effect of colibactin. *Cell Microbiol.* 2018;20(2):e12812.
47. Alhmoud JF, Woolley JF, Moustafa AA, et al. DNA damage/ repair management in cancers and. *Cancers (Basel).* 2020;12(4):1–22.
48. Turgeon MO, Perry NJS, Poulogiannis G. DNA damage, repair, and cancer metabolism. *Front Oncol.* 2018;8:1–8. doi:10.3389/fonc.2018.00015.
49. Wernke KM, Xue M, Tirla A, et al. Structure and bioactivity of colibactin. *Bioorg Med Chem Lett.* 2020;30(15):127280.
50. Wilson MR, Jiang Y, Villalta PW, et al. The human gut bacterial genotoxin colibactin alkylates DNA. *Science (1979).* 2019;363(6428):1–15. doi:10.1126/science.aar7785.
51. Armaghany T, Wilson JD, Chu Q, et al. Genetic alterations in colorectal cancer in older patients. *Manag Color Cancers Older People.* 2012;5(1):19–27.
52. Grady WM, Carethers JM. Genomic and epigenetic instability in colorectal cancer pathogenesis. *Gastroenterology.* 2008;135(4):1079–1099.
53. Yang J, Liu M, Hong D, et al. The paradoxical role of cellular senescence in cancer. *Front Cell Dev Biol.* 2021;9:722205.
54. Zeng S, Shen WH, Liu L. Senescence and cancer. *Cancer Transl Med.* 2018;4(3):70–74.
55. Huang CY, Zhou QY, Hu Y, et al. Hepatocyte growth factor is a prognostic marker in patients with colorectal cancer: a meta-analysis. *Oncotarget.* 2017;8(14):23459–23469.
56. Yao JF, Li XJ, Yan LK et al. Role of HGF/c-Met in the treatment of colorectal cancer with liver metastasis. *J Biochem Mol Toxicol.* 2019;33(6):e22316.
57. Jibiki N, Saito N, Kameoka S, et al. Clinical significance of fibroblast growth factor (FGF) expression in colorectal cancer. *Int Surg.* 2014;99(5):493–499.
58. Chen G, Han G, Shen B, et al. GM-CSF facilitates the development of inflammation-associated colorectal carcinoma. *Oncoimmunology.* 2014;3(1):2–3.
59. Carmeliet P. VEGF as a key mediator of angiogenesis in cancer. *Oncology.* 2005;69(3):4–10.
60. Chen Y, Zhao Z, Chen Y, et al. An epithelial-to-mesenchymal transition-inducing potential of granulocyte macrophage colony-stimulating factor in colon cancer. *Sci Rep.* 2017;7(1):1–12.
61. Ribatti D, Tamma R, Annese T. Epithelial-mesenchymal transition in cancer: a historical overview. *Transl Oncol.* 2020;13(6):100773.
62. Duan T, Zhou D, Yao Y, et al. The association of aberrant expression of FGF1 and mTOR-S6K1 in colorectal cancer. *Front Oncol.* 2021;11:1–12.
63. Center MM, Jemal A, Smith RA, et al. Worldwide variations in colorectal cancer. *Dis Colon Rectum.* 2010;53(6):1099.
64. Arnold M, Sierra MS, Laversanne M, et al. Global patterns and trends in colorectal cancer incidence and mortality. *Gut.* 2017;66(4):683–691.
65. Bishehsari F, Mahdavinia M, Vacca M, et al. Epidemiological transition of colorectal cancer in developing countries: environmental factors, molecular pathways, and opportunities for prevention. *World J Gastroenterol.* 2014;20(20):6055–6072.
66. Agus A, Denizot J, Thévenot J, et al. Western diet induces a shift in microbiota composition enhancing susceptibility to adherent-invasive E. coli infection and intestinal inflammation. *Sci Rep.* 2016;6:1–14. doi:10.1038/srep19032.
67. Lee JY, Cevallos SA, Byndloss MX, et al. High-fat diet and antibiotics cooperatively impair mitochondrial bioenergetics to trigger dysbiosis that exacerbates pre-inflammatory bowel disease. *Cell Host Microbe.* 2020;28(2):273–284.e6.
68. Aykan NF. Red meat and colorectal cancer. *Oncol Rev.* 2015;9(1):38–44.
69. Chan DSM, Lau R, Aune D, et al. Red and processed meat and colorectal cancer incidence: meta-analysis of prospective studies. *PLoS One.* 2011;6(6):e20456.
70. Cross AJ, Ferrucci LM, Risch A, et al. A large prospective study of meat consumption and colorectal cancer risk: an investigation of potential mechanisms underlying this association. *Cancer Res.* 2010;70(6):2406–2414.
71. Byndloss MX, Olsan EE, Rivera-chávez F, et al. Microbiota-activated PPAR-γ-signaling inhibits dysbiotic Enterobacteriaceae expansion. *Science (1979).* 2017;357(6351):570–575.
72. Khademian M, Imlay JA. *Escherichia coli* cytochrome c peroxidase is a respiratory oxidase that enables the use of hydrogen peroxide as a terminal electron acceptor. *Proc Natl Acad Sci USA.* 2017;114(33):E6922–E6931.
73. Miller BM, Liou MJ, Zhang LF, et al. Anaerobic respiration of NOX1-derived hydrogen peroxide licenses bacterial growth at the colonic surface. *Cell Host Microbe.* 2020;28(6):789–797.
74. Spees AM, Wangdi T, Lopez CA, et al. Streptomycin-induced inflammation enhances *Escherichia coli* gut colonization through nitrate respiration. *MBio.* 2013;4(4):e00430–e00413.
75. Winter SE, Winter MG, Xavier MN, et al. Host-derived nitrate boosts growth of E. coli in the inflamed gut Sebastian. *Science (1979).* 2013;339(6120):708–711.
76. Zhu W, Miyata N, Winter MG, et al. Editing of the gut microbiota reduces carcinogenesis in mouse models of colitis-associated colorectal cancer. *J Exp Med.* 2019;216(10):2378–2393.

Increased Levels of ANGPTL3 and CTRP9 in Patients With Obstructive Sleep Apnea and Their Relation to Insulin Resistance and Lipid Metabolism and Markers of Endothelial Dysfunction

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Keywords: adipokine, hepatokine, endothelial dysfunction, dyslipidemia, lipoprotein lipase, adhesion molecule

Abbreviations: OSA, obstructive sleep apnea; ANGPTL3, angiopoietin-like protein 3; CTRP9, C1q/TNF-related protein 9; ICAM-1, intercellular adhesion molecule 1; VCAM-1, vascular cell adhesion protein 1; T2DM, type 2 diabetes mellitus; CVD, cardiovascular disease; LPL, lipoprotein lipase; HDL, high-density lipoprotein; AMPK, 5' adenosine monophosphate-activated protein kinase; CAD, coronary artery disease; PSG, polysomnography; AHI, apnea-hypopnea index; BMI, body mass index; FBG, fasting blood glucose; TC, total cholesterol; TG, triglyceride; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; SD, standard deviation; IQR, interquartile range; DBP, diastolic blood pressure; SBP, systolic blood pressure; SpO_2, saturation of peripheral oxygen; HOMA-IR, homeostasis model assessment of insulin resistance; ROC, receiver operating characteristic; CV, coefficient of variation.

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ABSTRACT

Objective: Obstructive sleep apnea (OSA) has a close relation with obesity and perturbation in adipokines and hepatokines, which are linked to OSA consequences such as insulin resistance, dyslipidemia, and endothelial dysfunction. This study aimed to assess the relation of C1q/TNF-related protein 9 (CTRP9) and angiopoietin-like protein 3 (ANGPTL3) with OSA and biochemical measurements.

Methods: Serum levels of ANGPTL3, CTRP9, adiponectin, leptin, intercellular adhesion molecule 1 (ICAM-1), and vascular cell adhesion protein 1 (VCAM-1) were determined in 74 OSA patients and 27 controls using enzyme-linked immunosorbent assay kits.

Results: Levels of ANGPTL3, CTRP9, leptin, ICAM-1, and VCAM-1 were increased in the patients compared to the controls, whereas adiponectin levels decreased. ANGPTL3 had a positive correlation with total cholesterol, triglyceride, low-density lipoprotein cholesterol, ICAM-1, and VCAM-1 and was inversely correlated with leptin. CTRP9 showed a positive correlation with body mass index, insulin resistance, ICAM-1, and VCAM-1.

Conclusion: The results indicated the relation of ANGLTP3 and CTRP9 with OSA and its complications, which suggested a possible role for these factors in the consequences of OSA.

Obstructive sleep apnea (OSA) is a common sleep disorder worldwide and can be caused by partial or complete occlusion of the upper airways during sleep due to anatomical changes.[1,2] Nonmodifiable risk factors for OSA include genetics, sex, and age, whereas obesity is a prominent modifiable risk factor.[3] People diagnosed with OSA are at increased risk of developing type 2 diabetes (T2DM) and atherosclerotic cardiovascular disease through disruption of multiple pathways including inflammation, oxidative stress, insulin resistance, and endothelial dysfunction.[4,5]

Factors released from adipose tissue (adipokines), liver (hepatokines), and skeletal muscle (myokines) modulate metabolic and pathological processes such as glucose and lipid metabolism, insulin sensitivity, and endothelial function.[6] Perturbations of adipokine and hepatokine functions in patients with OSA are associated with the severity of OSA, insulin resistance, and cardiovascular disease (CVD).[7,8]

Angiopoietin-like protein 3 (ANGPTL3) is a hepatokine that inhibits hydrolysis of phospholipids and triglycerides by regulating endothelial lipoprotein lipase (LPL). LPL regulates the hydrolysis of triacylglycerol harvested from lipoproteins. The serum clearance of triglyceride-rich lipoproteins is disrupted during OSA[9] and the secretion of fatty acids for tissue use is negatively affected by hypoxia.[10] Promotion and decreases in LPL activity due to mutations can reduce and raise the risk of CVDs, respectively, by altering blood triglyceride levels.[6,11] Among the various factors that can affect LPL metabolism, ANGPTLs (such as ANGPTL3, 4, and 8) are of particular importance, as ANGPTL3 regulates fatty

metabolism by inhibiting LPL activity and hydrolyzing triglycerides.[12–14] Decreases in serum levels of ANGPTL3 can reduce cholesterol and very-low-density lipoprotein and high-density lipoprotein (HDL) levels.[12,15,16] Moreover, ANGPTL3 has an important role in glucose and lipid metabolism by affecting insulin sensitivity.[11,12,14] Although there are little data on the relation of OSA with ANGPTL3, a study reported lower levels of ANGPTL3 in OSA patients with coronary artery disease (CAD) than in controls.[17] Moreover, serum levels of ANGPTL4 and ANGPTL8 are elevated in patients with OSA, suggesting a role for these hepatokines in lipid metabolism.[18] C1q/TNF-related protein (CTRP) is a newly discovered adipokine that has been suggested to compensate for the low expression of adiponectin.[19] CTRP9 has high structural homology with adiponectin levels and has favorable effects on insulin sensitivity, inflammation, and endothelial function.[20] CTRP9 increases 5' adenosine monophosphate-activated protein kinase (AMPK) activity and expression of fatty acid oxidation enzymes[21] and has protective effects against the proliferative and chemotactic activity of vascular smooth muscle cells.[22] Several clinical studies reported perturbations of serum concentrations of CTRP9 in patients with diabetes mellitus, CAD, and nonalcoholic fatty liver disease.[23,24] Although there is no report on the levels of CTRP9 in OSA compared to healthy controls, a study by Li et al[17] indicated lower CTRP9 levels in patients with a combination of CAD and moderate/severe OSA compared with CAD patients with no or mild OSA.

Changes in serum levels of ANGPTL3 and CTRP9 are poorly studied in patients with OSA. In this study, we measured serum levels of ANGPTL3 and CTRP9 and related their changes to alterations in adiponectin, leptin, and makers of endothelium dysfunction in patients with OSA.

Materials and Methods

Study Population

A total of 101 patients from the sleep clinic of Farabi Hospital in Kermanshah, Iran, were enrolled in this study. Exclusion criteria included having diabetes (according to the criteria of the American Diabetes Association), and a history of cardiovascular disease, cancer, and autoimmune diseases. All members of the studied population signed written informed consent. The study was in accordance with the Declaration of Helsinki and was approved by the ethics committee of the Kermanshah University of Medical Sciences (IR.KUMS.REC.1398.411).

All participants underwent a polysomnography (PSG) test to confirm a diagnosis of OSA, as previously described.[25,26] Briefly, overnight PSG was performed for all participants using SOMNOscreen plus (SOMNOmedics), where continuous PSG recordings were obtained for 7 hours. We used the American Academy of Sleep Medicine (AASM 2012) guidelines to classify hypopnea as a reduction of ≥30% in airflow accompanied by a decrease in oxygen desaturation index of ≥3% or arousal, and a complete cessation of breathing (lasting for ≥10 seconds) as apnea. The apnea-hypopnea index (AHI) was defined as the mean number of apneas and hypopneas per hour. An AHI score of AHI ≥5 events/hour of sleep was used to confirm OSA diagnosis; patients were categorized into 3 group-based on their AHI scores: mild: 5≤ AHI <15, moderate: 15≤ AHI <30, and severe: AHI ≥30.

Blood Collection and Anthropometric and Laboratory Parameters

After the PSG test was performed, each participant provided a signed consent form, and a fasting blood sample of 5 mL was collected. The serum was immediately separated from the blood sample and stored in a freezer at −70°C for later use. A standard sphygmomanometer was used to measure blood pressure after patients were in a resting position for 15 minutes. Height and weight were measured to calculate body mass index (BMI) using the formula: weight (kg)/height (m^2). A spectrophotometric assay with a commercial auto-analyzer was used to determine serum levels of fasting blood glucose (FBG), triglyceride (TG), total cholesterol (TC), and low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C).

Serum Levels of Adipokines and Adhesion Molecules

An enzyme-linked immunosorbent assay (ELISA) kit (Aviscera Bioscience) was used to measure serum levels of ANGPTL3. Intra-assay and inter-assay coefficients of variation were 3.1%–5.7% and 6.2%–9.8%, respectively. CTRP9 serum levels were measured using an ELISA kit (USCN Life Science) with an intra-assay CV of 3.4% and an interassay coefficient of variation (CV) of 4.3%. Serum levels of adiponectin were determined using an ELISA kit (Adipogen) with intra- and interassay variations of 4.6% and 4.4%, respectively. An ELISA kit was used to measure leptin (R&D Systems), intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion protein 1 (VCAM-1) (Quantikine, R&D Systems), and insulin (Monobind) according to the manufacturer's instructions. The intra- and interassay (CV) of insulin, leptin, VCAM-1, and ICAM-1 were <5.6%, <5.9%, <6.5%, and <7%, respectively. Moreover, minimum detectable range for ANGPTL3, CTRP9, leptin, adiponectin, ICAM-1, VCAM-1 were 7.8 pg/mL, 1.32 ng/mL, 7.8 pg/mL, 100 pg/mL, 0.096 ng/mL, and 0.6 ng/mL, respectively.

Statistical Analysis

SPSS software (version 20.0; SPSS) was used to analyze the raw data. The distribution of variables was checked using the Kolmogorov–Smirnov test, and normally distributed data were shown with mean ± standard deviation (SD) and nonnormal distributed data were shown using median and interquartile range (IQR). A χ^2 test was used to determine differences in quantitative data. A Student *t*-test or Mann–Whitney *U* test was used to identify mean differences between 2 groups, according to the normality of the data. The 1-way ANOVA or Kruskal–Wallis test were used to identify differences when comparing multiple groups, according to the normality test. The correlation of adipokines with quantitative data was determined by a Pearson correlation test. Statistical power calculation was 0.80 and a $P < .05$ was considered statistically significant.

Results

The Characteristics of the Study Participants

The characteristics of the study participants are shown in **TABLE 1.** The 2 study groups (control and OSA) were matched for age, sex, and BMI. Diastolic blood pressure (DBP) ($P = .024$) and systolic blood pressure (SBP) ($P = .017$) were elevated in OSA patients compared to the control group. The AHI was greater in OSA participants than in control patients ($P < .001$), and the average saturation of peripheral oxygen (SpO_2) was

TABLE 1. Demographic Characteristics, Sleep Index, and Biochemical Measurements

Variables	Control (n = 27)	OSA (n = 74)	*P* Value
	Mean ± SD	Mean ± SD	
Age (y)	45.63 ± 9.15	45.97 ± 12.75	.899
BMI (kg/m^2)	26.1 (24.37–29.27)	26.7 (25–27.9)	.548
Sex (male)	14 (51.9%)	45 (63.4%)	.298
SBP (mm Hg)	112.5 (110–120)	120 (115–125)	.017
DBP (mm Hg)	70 (70–80)	80 (70–80)	.024
AHI (events/h)	2.25 (1.17–2.97)	18.9 (9–37.3)	<.001
Average SpO_2 (%)	95 (93–95)	93 (91–94)	.004
FBG (mg/dL)	93.65 ± 12.33	95.69 ± 11.44	.375
Insulin (μU/mL)	3.54 ± 1.89	5.61 ± 3.93	.022
HOMA-IR	0.737 (0.57–1.17)	1.217 (0.52–1.64)	.019
TC (mg/dL)	160.73 ± 34.39	161.29 ± 45.69	.954
TG (mg/dL)	110.5 (89.35–138.65)	121 (103–172)	.029
LDL-C (mg/dL)	94.15 ± 25.98	97.55 ± 33.18	.633
HDL-C (mg/dL)	46.47 ± 8.04	43.07 ± 9.71	.109

AHI, apnea-hypopnea index; BMI, body mass index; DBP, diastolic blood pressure; FBG, fasting blood glucose; HDL-C, high-density lipoprotein cholesterol; HOMA-IR, homeostasis model assessment of insulin resistance; LDL-C, low-density lipoprotein cholesterol; OSA, obstructive sleep apnea; SBP, systolic blood pressure; SD, standard deviation; SpO_2, saturation of peripheral oxygen; TC, total cholesterol; TG, triglyceride.

reduced in OSA patients compared to controls (P = .004). Serum insulin (P = .022) and homeostasis model assessment of insulin resistance (HOMA-IR) values (P = .019) were greater in patients with OSA, whereas FBS was similar in the 2 groups (P = .375). Values for serum TC, LDL-C, and HDL-C were similar in the 2 groups, whereas TG levels were higher in patients with OSA (P = .029).

Serum Levels of Adipokines, Hepatokine, and Adhesion Molecules

Circulating levels of adiponectin were lower in patients with OSA (3.87 [3.10, 4.95] μg/mL) than patients in the control group (6.14 [3.31, 8.28] μg/mL, P < .001) (**FIGURE 1A**), whereas leptin levels were increased in patients with OSA (10.26 ± 2.82 pg/mL) compared to patients in the control group (7.98 ± 1.97 pg/mL, P < .001) **(FIGURE 1B)**. Moreover, ANGPTL3 levels were higher in the OSA group (245.14 ± 73.14 pg/mL) than in the control group (130.49 ± 43.61 pg/mL, P < .001) **(FIGURE 1C)**. Similarly, serum levels of CTRP9 were increased in patients with OSA (198.1 [142.9, 269.3] ng/mL) compared to control patients (147 [121, 177] ng/ml, P < .001) (**FIGURE 1D**). Furthermore, serum levels of ICAM-1 (295.46 ± 85.78 ng/mL vs 198.11 ± 48.65 ng/mL) and VCAM-1 (510.49 ± 135.43 ng/mL vs 320.74 ± 108.28 ng/mL) were higher in the OSA group compared to controls (P < .001 for both) **(FIGURE 1E** and **F)**. An ANCOVA analysis was used to eliminate the influence of age, sex, and BMI on adipokine levels and the results remained significant. In addition, the possible impact of the lipid profile (TG, TC, LDL-C, and HDL-C) was adjusted on the serum levels of adipokines and hepatokine and all the results remained significant.

Serum levels of adipokines were examined according to the severity of OSA **(TABLE 2).** The levels of adiponectin were lower in patients with severe OSA than those with mild and moderate OSA. Although there were no changes in ANGPTL3, leptin, CTRP9, ICAM-1, and VCAM-1 levels according to OSA severity, serum levels of leptin increased in the severe group compared to controls, and CTRP9 increased in moderate and severe groups compared to controls. Moreover, ANGPTL3, ICAM-1, and VCAM-1 were elevated in all patient groups compared to controls.

A binary logistic regression analysis was performed to test the association of adipokines and ANGPTL3 with the risk of OSA. Adiponectin levels were inversely related to the risk of OSA, whereas ANGPTL3 and leptin levels were positively associated with the risk of OSA. Adjusting for the possible impact of covariates (age, BMI, and sex) on these associations did not alter these associations (**TABLE 3**).

FIGURE 1. Serum levels of adipokines, ANGPTL3 and adhesion molecules. A, Adiponectin concentrations in OSA and control groups. B, Leptin concentrations in OSA and control group. C, ANGPTL3 concentrations in OSA and control groups. D, CTRP9 concentrations in OSA and control groups. E, ICAM-1 concentration in OSA and control groups. F, VCAM-1 concentration in OSA and control groups. *P < .001. ANGPTL3, angiopoietin-like protein 3; CTRP9, C1q/TNF-related protein; ICAM-1, intercellular adhesion molecule 1; OSA, obstructive sleep apnea; VCAM-1, vascular cell adhesion protein 1.

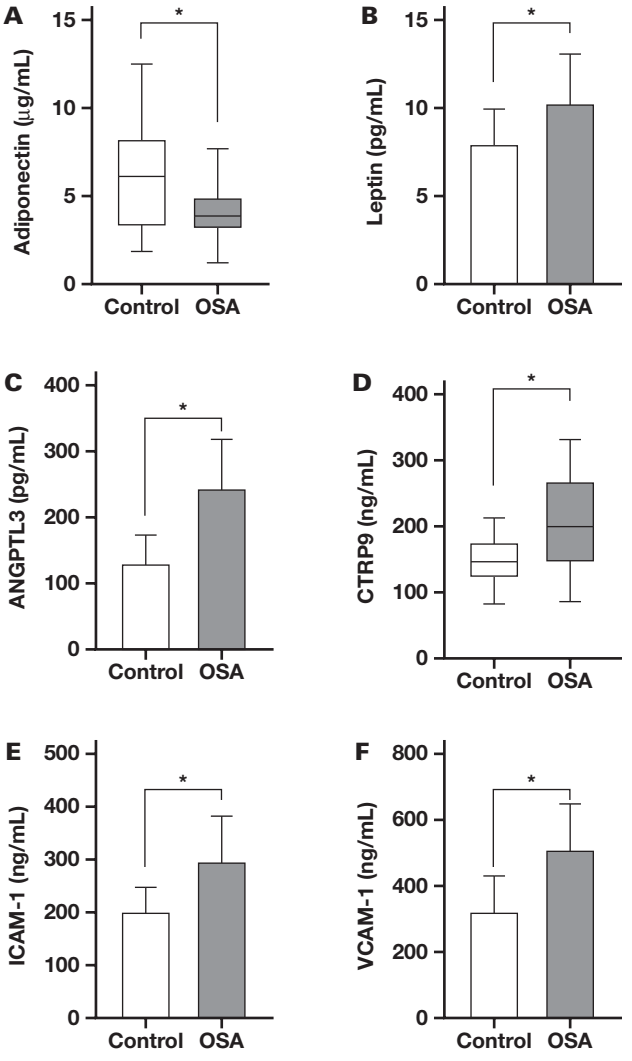


TABLE 2. Serum Levels of Adipokines, Hepatokine, and Adhesion Molecules According to the Severity of OSA

	Control (n = 27)	Mild (n = 27)	Moderate (n = 22)	Severe (n = 25)	*P* Value
Adiponectin (µg/mL)	6.14 (3.31, 8.28)	4.2 (3.28, 5.57)[a,c]	4.21 (3.74, 4.98)[a,d]	2.28 (2.01, 4.12)[b,d]	<.001
Leptin (pg/mL)	7.98 ± 1.97	9.65 ± 3.10	9.71 ± 2.73	11.40 ± 2.37[b,d]	<.001
ANGPTL3 (pg/mL)	130.49 ± 43.61	239.49 ± 82.44[b,d]	243.73 ± 63.06[b,d]	251.76 ± 76.46[b,d]	<.001
CTRP9 (ng/mL)	147 (121, 177	184 (130.2, 269.8)	197.8 (149.8, 271)[b,c]	202.3 (162.7, 274.7)[b,d]	.001
ICAM-1 (ng/mL)	198.11 ± 48.65	262.73 ± 75.88[b,c]	308.92 ± 86.64[b,d]	311.46 ± 88.4[b,d]	<.001
VCAM-1 (ng/mL)	320.74 ± 108.28	471.27 ± 111.95[b,d]	546.4 ± 155.06[b,d]	509.04 ± 128.13[b,d]	<.001

ANGPTL3, angiopoietin-like protein 3; CTRP9, C1q/TNF-related protein; ICAM-1, intercellular adhesion molecule 1; OSA, obstructive sleep apnea; VCAM-1, vascular cell adhesion protein 1.
[a]Compared with severe group.
[b]Compared with control.
[c]P < .05.
[d]P < .01.

TABLE 3. Association of Adipokines and Hepatokine with Risk of OSA Present[a]

	Models	OR	95% CI	*P* Value
Adiponectin	Crude	0.682	0.545, 0.853	.001
	Adjusted	0.686	0.548, 0.859	.001
Leptin	Crude	1.137	1.055, 1.225	.001
	Adjusted	1.152	1.061, 1.250	.001
ANGPTL3	Crude	1.033	1.019, 1.047	<.001
	Adjusted	1.034	1.020, 1.049	<.001
CTRP9	Crude	1.019	1.008, 1.030	.001
	Adjusted	1.022	1.010, 1.034	<.001

ANGPTL3, angiopoietin-like protein 3; CTRP9, C1q/TNF-related protein; OSA, obstructive sleep apnea.
[a]Data were adjusted for age, sex, and body mass index.

The ability of ANGPTL3 and CTRP9 to distinguish patients with OSA from control patients was determined using receiver operating characteristic (ROC) curve analysis **(FIGURE 2).** It was possible to use serum levels of ANGPTL3 (AUC [95%CI]: 0.918 [0.866, 0.970], cut-off value: 190.41, sensitivity: 80%, and specificity: 94%, $P < .001$) and CTRP9 (AUC [95%CI]: 0.744 [0.645, 0.842], cut-off value: 160.17, sensitivity: 67%, and specificity: 66%, $P < .001$) to distinguish patients in the OSA group from those in the control group **(FIGURE 2).**

Correlation of ANGPTL3, Adiponectin, Leptin, and CTRP9 with Anthropometric and Biochemical Data

Serum levels of adiponectin were inversely correlated with AHI and average SpO_2 in the OSA group **(TABLE 4).** Leptin levels were inversely correlated with SBP in the control group and positively correlated with BMI in the control and OSA groups, and inversely correlated with ANGPTL3 levels in the OSA group. There was an inverse correlation of ANGPTL3 with TC, TG, and LDL-C and a positive correlation with SBP in the OSA group. Serum levels of CTRP9 were positively correlated with BMI, neck circumference, insulin, HOMA-IR, ICAM-1, and VCAM-1 in the OSA group **(TABLE 4).**

Discussion

This study reports that serum levels of ANGPTL3, CTRP9, and leptin were increased in patients with OSA, whereas adiponectin levels were decreased. The finding of increases in ANGPTL3 in patients with OSA is in accordance with other studies reporting increases in ANGPTL3 in patients with OSA and CAD[17] and of ANGPTL4 and ANGPTL8 in patients with OSA.[18] ANGPTL3 and ANGPTL4 regulate lipid metabolism by modulating LPL hydrolysis, and ANGPTL3 acts in concert with ANGPTL8 to regulate LPL activity.[27,28] Serum levels of ANGPTL3 were positively correlated with TG in our study, which is likely due to the inhibitory effects of ANGPTL3 on LPL, as also reported in patients with T2DM.[17] The association of TG levels with CAD risk[29,30] and with extracellular adhesion markers (ICAM and VCAM) of endothelial dysfunction[31,32] has previously been reported.

On the other hand, leptin levels were increased in patients with OSA and were negatively correlated with ANGPTL3. A previous study reported that leptin reduces serum ANGPTL3 levels and decreases the expression of ANGPTL3 in liver cells.[27] Findings of our study confirm these reports that leptin negatively regulates ANGPTL3 levels. Genetic defects in ANGPTL3 regulation reduce TC and LDL-C levels.[28] The findings indicate that ANGPTL3 levels were positively correlated with serum levels of TC and LDL-C. ANGPTL3 affects lipoprotein metabolism by regulating the effects of LPL and endothelial LPL, which leads to increases in TC, LDL-C, and TG levels.[33] These findings for ANGPTL3 might link it to dyslipidemia in OSA patients, and future studies are needed to dissect possible underlying mechanisms.

A novel finding of the present study is that CTRP9 levels are increased in patients with OSA. Another study by Li et al[34] reported that CTRP9 levels were decreased in patients with a combination of CAD and moderate or severe OSA compared to subjects with no or mild OSA. The differences between the present study and that of Li et al[34] are likely due to the inclusion of patients with CAD in the OSA group in the latter study. Levels of CTRP9 were also correlated with insulin levels in patients with or without obesity[35] and with insulin resistance in patients with T2DM and CAD.[35,36]

Obesity and changes in neck circumference contribute to pharyngeal airway narrowing in OSA.[5] Li et al[34] reported an independent correlation between BMI and CTRP9 in individuals with OSA. Our study indicates that serum levels of CTRP9 were positively correlated with insulin levels and HOMA-IR and also with BMI and neck circumference, confirming a previous report of an association between CTRP9 and BMI.[35] However, a study by Moradi et al[37] also reported a correlation between CTRPR9 and insulin resistance in patients with T2DM but not in patients with CAD, suggesting that the correlation between BMI and CTRP9 is dependent on

FIGURE 2. Receiver operating characteristic analysis for testing the ability of CTRP9 (A) and ANGPTL3 (B) to discriminate patients in the obstructive sleep apnea control groups.

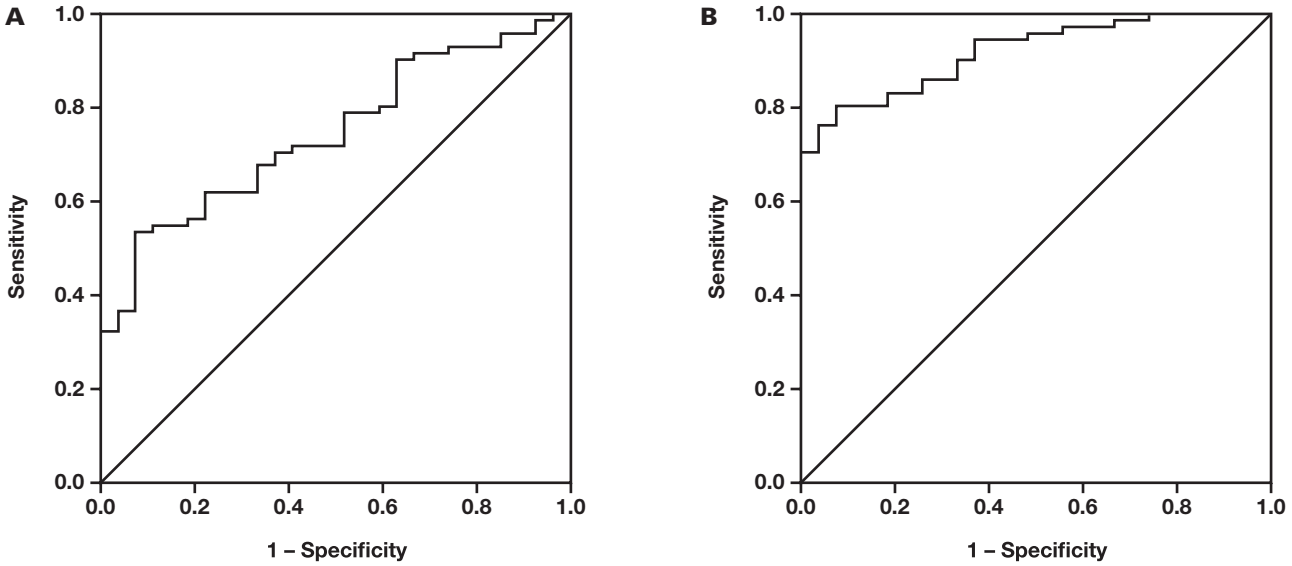


TABLE 4. Correlation of Adiponectin, Leptin, ANGPTL3, and CTRP9 with Anthropometric and Biochemical Variables

	Adiponectin		Leptin		ANGPTL3		CTRP9	
	Control (n = 27)	OSA (n = 74)	Control (n = 27)	OSA (n = 74)	Control (n = 27)	OSA (n = 74)	Control (n = 27)	OSA (n = 74)
CTRP9	–0.050	–0.058	0.227	–0.033	–0.210	0.003	1	1
Leptin	0.174	–0.157	1	1	-0.246	–0.343[b]	–0.210	0.003
ANGPTL3	–0.042	–0.090	–0.246	–0.343[b]	1	1	–0.050	–0.058
Age	0.345	–0.208	–0.289	–0.014	0.119	0.111	–0.227	–0.053
BMI	0.389[a]	–0.187	0.369[a]	0.394[b]	–0.277	0.156	0.260	0.412[a]
Neck circumference	0.332	–0.119	–0.242	0.077	–0.196	0.011	–0.113	0.254[a]
SBP	0.155	–0.130	–0.406[a]	0.010	–0.325	0.238[a]	0.099	0.187
DBP	0.188	–0.033	–0.323	–0.001	–0.213	0.055	0.145	0.120
AHI	0.054	–0.432[b]	0.056	0.222	0.131	0.082	0.001	0.070
Average SpO_2	–0.079	0.319[b]	0.315	–0.121	0.071	0.088	–0.053	–0.052
FBS	–0.008	–0.133	–0.233	0.035	–0.160	0.153	0.085	0.229
Insulin	–0.287	–0.043	–0.219	–0.034	0.126	0.092	–0.035	0.284[a]
HOMA-IR	–0.256	–0.072	–0.267	–0.021	0.096	0.124	0.004	0.324[a]
TC	–0.220	–0.201	0.008	–0.139	0.135	0.337[b]	-0.149	0.050
TG	–0.202	––0.137	–0.159	–0.217	0.234	0.484[b]	-0.351	0.147
LDL	–0.161	-0.220	–0.096	–0.010	0.207	0.327[b]	-0.176	0.023
HDL	0.022	0.130	0.282	–0.105	–0.262	0.038	-0.133	0.009
ICAM-1	–0.101	0.224	0.281	–0.069	–0.095	0.266[a]	0.291	0.416[b]
VCAM-1	–0.029	0.201	0.199	–0.222	0.162	0.257[a]	0.095	0.441[b]

AHI, apnea-hypopnea index; ANGPTL3, angiopoietin-like protein 3; BMI, body mass index; CTRP9, C1q/TNF-related protein; DBP, diastolic blood pressure; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance; ICAM-1, intercellular adhesion molecule 1; LDL, low-density lipoprotein; OSA, obstructive sleep apnea; SBP, systolic blood pressure; SpO_2, saturation of peripheral oxygen; TC, total cholesterol; TG, triglyceride; VCAM-1, vascular cell adhesion protein 1.

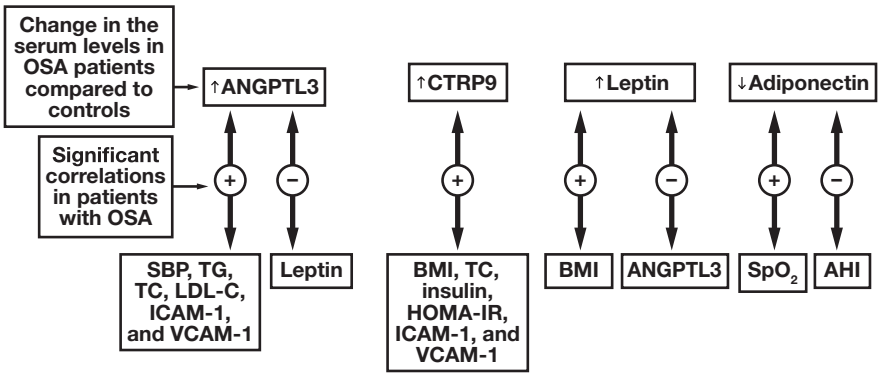
[a] $P < .05$.

[b] $P < .01$.

patient comorbidities. The study by Moradi et al[37] reported a positive correlation between levels of CTRP9 and adhesion molecules in patients with T2DM. Endothelial dysfunction is an important consequence of OSA and increases the risk of cardiovascular disease in these patients. Our study reports higher concentrations of markers of endothelial dysfunction such as ICAM-1 and VCAM-1, which were positively correlated with levels of CTRP9. Studies have shown that CTRP9 has a favorable influence on glucose and lipid metabolism and endothelial function; however, the levels of this adipokine increased in cardiometabolic diseases and OSA. This condition suggests that elevation of CTRP in circulation might be a compensatory response, and there might be a resistance against CTRP9 in these patients. Additional studies are needed to confirm this.

Serum levels of adiponectin and leptin were also measured in patients with OSA. Adiponectin levels were reduced in patients with

FIGURE 3. Schematic representation of the main findings of the study. Changes in the serum levels of ANGPTL3, CTRP9, leptin, and adiponectin in OSA patients compared to controls and their significant correlations in the OSA patient group. AHI, apnea-hypopnea index; ANGPTL3, angiopoietin-like protein 3; BMI, body mass index; CTRP9, C1q/TNF-related protein; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance; ICAM-1, intercellular adhesion molecule 1; LDL, low-density lipoprotein; OSA, obstructive sleep apnea; SBP, systolic blood pressure; SpO_2, saturation of peripheral oxygen; TC, total cholesterol; TG, triglyceride; VCAM-1, vascular cell adhesion protein 1.



OSA. Adiponectin levels were inversely related to AHI and SpO_2 levels, suggesting OSA-induced hypoxia might be a factor related to reduced adipokines. A previous study reported that hypoxia suppressed adiponectin expression in adipocytes.[38] The present study has some limitations: (1) the sample size is relatively small; (2) we used a cross-sectional design, which limited us to conclude causal relations between the variables we measured; (3) the effects of weight loss or treatment with continuous positive airway pressure on CTRP9 and ANGPTL3 were not studied; and (4) markers of subclinical atherosclerosis, such as carotid intima-media thickness, were not measured in the studied population.

Conclusion

A summary of the main findings of this study is illustrated in **FIGURE 3**. This study reports that increases in serum levels of ANGPTL3 and CTRP9 in patients with OSA were related to the risk factors and complications of OSA such as obesity, insulin resistance, dyslipidemia, and endothelial dysfunction. The results support the possible role of these two secretory factors in the pathogenesis of OSA.

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REFERENCES

1. Almendros I, García-Río F. Sleep apnoea, insulin resistance and diabetes: the first step is in the fat. *Eur Respiratory Soc*; 2017;49:1700179.
2. Lévy P, Kohler M, McNicholas WT, et al. Obstructive sleep apnoea syndrome. *Nat Rev Dis Primers.* 2015;1(1):1–21.
3. Al Mutairi S, Mojiminiy OA, Al Alawi A, Al Rammah T, Abdella N. Study of leptin and adiponectin as disease markers in subjects with obstructive sleep apnea. *Dis Markers.* 2014;2014:1–8. doi:10.1155/2014/706314.
4. Robinson G, Stradling J, Davies R. Sleep·6: obstructive sleep apnoea/hypopnoea syndrome and hypertension. *Thorax.* 2004;59(12):1089.
5. Sánchez-de-la-Torre M, Campos-Rodriguez F, Barbé F. Obstructive sleep apnoea and cardiovascular disease. *Lancet Respir Med*. 2013;1(1):61–72.
6. Daniels TF, Killinger KM, Michal JJ, Wright, RW Jr, Jiang Z. Lipoproteins, cholesterol homeostasis and cardiac health. *Int J Biol Sci.* 2009;5(5):474–488. doi:10.7150/ijbs.5.474.
7. Kapsimalis F, Varouchakis G, Manousaki A, et al. Association of sleep apnea severity and obesity with insulin resistance, C-reactive protein, and leptin levels in male patients with obstructive sleep apnea. *Lung.* 2008;186(4):209–217. doi:10.1007/s00408-008-9082-x.
8. Orlando A, Nava E, Giussani M, Genovesi S. Adiponectin and cardiovascular risk. From pathophysiology to clinic: focus on children and adolescents. *Int J Mol Sci.* 2019;20(13):3228. doi:10.3390/ijms20133228.
9. Lin M-T, Lin H-H, Lee P-L, et al. Beneficial effect of continuous positive airway pressure on lipid profiles in obstructive sleep apnea: a meta-analysis. *Sleep Breath.* 2015;19(3):809–817.
10. Eltzschig HK, Eckle T. Ischemia and reperfusion—from mechanism to translation. *Nat Med.* 2011;17(11):1391–1401. doi:10.1038/nm.2507.
11. Tsutsumi K. Lipoprotein lipase and atherosclerosis. *Curr Vasc Pharmacol.* 2003;1(1):11–17. doi:10.2174/1570161033386673.
12. Dijk W, Kersten S. Regulation of lipid metabolism by angiopoietin-like proteins. *Curr Opin Lipidol.* 2016;27(3):249–256. doi:10.1097/MOL.0000000000000290.
13. Mattijssen F, Kersten S. Regulation of triglyceride metabolism by angiopoietin-like proteins. *Biochim Biophys Acta Molec Cell Biol Lipids*. 2012;1821(5):782–789.
14. Li Y, Teng C. Angiopoietin-like proteins 3, 4 and 8: regulating lipid metabolism and providing new hope for metabolic syndrome. *J Drug Target.* 2014;22(8):679–687. doi:10.3109/1061186X.2014.928715.
15. Hassan M. ANGPLT3: a novel modulator of lipid metabolism. *Glob Cardiol Sci Pract.* 2017;2017(1):e201706.
16. Kersten S. Angiopoietin-like 3 in lipoprotein metabolism. *Nat Rev Endocrinol.* 2017;13(12):731–739. doi:10.1038/nrendo.2017.119.
17. Li J, Yang Y, Jiao X, et al. The clinical role of angiopoietin-like protein 3 in evaluating coronary artery disease in patients with obstructive sleep apnea. *Cardiovasc Drugs Ther.* 2020;6:773–780.

18. Al-Terki A, Abu-Farha M, AlKhairi I, et al. Increased level of angiopoietin like proteins 4 and 8 in people with sleep apnea. *Front Endocrinol.* 2018;9:651. doi:10.3389/fendo.2018.00651.
19. Wong G, Wang J, Hug C, Tsao T-S, Lodish H. A family of Acrp30/adiponectin structural and functional paralogs. *Proc Natl Acad Sci USA.* 08/01 2004;101:10302–10307.
20. Si Y, Fan W, Sun L. A Review of the relationship between CTRP family and coronary artery disease. *Curr Atheroscler Rep.* 2020/05/28 2020;22(6):22. doi:10.1007/s11883-020-00840-0.
21. Peterson JM, Wei Z, Seldin MM, Byerly MS, Aja S, Wong GW. CTRP9 transgenic mice are protected from diet-induced obesity and metabolic dysfunction. *Am J Physiol Regul Integr Comp Physiol.* 2013;305(5):R522–R533. doi:10.1152/ajpregu.00110.2013.
22. Uemura Y, Shibata R, Ohashi K, et al. Adipose-derived factor CTRP9 attenuates vascular smooth muscle cell proliferation and neointimal formation. *FASEB J.* 2013;27(1):25–33.
23. Zhao D, Feng P, Sun Y, et al. Cardiac-derived CTRP9 protects against myocardial ischemia/reperfusion injury via calreticulin-dependent inhibition of apoptosis. *Cell Death Dis.* 2018/06/20 2018;9(7):723. doi:10.1038/s41419-018-0726-3.
24. Kambara T, Ohashi K, Shibata R, et al. CTRP9 protein protects against myocardial injury following ischemia-reperfusion through AMP-activated protein kinase (AMPK)-dependent mechanism. *J Biol Chem.* 2012;287(23):18965–18973. doi:10.1074/jbc.M112.357939.
25. Mohammadi H, Rezaei M, Sharafkhaneh A, Khazaie H, Ghadami MR. Serum testosterone/cortisol ratio in people with obstructive sleep apnea. *J Clin Lab Anal.* 2020;34(1):e23011.
26. Khazaie H, Negahban S, Ghadami MR, Sadeghi Bahmani D, Holsboer-Trachsler E, Brand S. Among middle-aged adults, snoring predicted hypertension independently of sleep apnoea. *J Int Med Res*. 2018/03/01 2018;46(3):1187–1196.
27. Santulli G. Angiopoietin-like proteins: a comprehensive look. *Front Endocrinol.* 2014;5:4. doi:10.3389/fendo.2014.00004.
28. Quagliarini F, Wang Y, Kozlitina J, et al. Atypical angiopoietin-like protein that regulates ANGPTL3. *Proc Natl Acad Sci USA.* 2012;109(48):19751–19756. doi:10.1073/pnas.1217552109.
29. Sarwar N, Sandhu M, Ricketts S, et al. Triglyceride Coronary Disease Genetics Consortium and Emerging Risk Factors Collaboration: triglyceride-mediated pathways and coronary disease: collaborative analysis of 101 studies. *Lancet.* 2010;375(9726):1634–1639.
30. Ference BA, Kastelein JJ, Ray KK, et al. Association of triglyceride-lowering LPL variants and LDL-C–lowering LDLR variants with risk of coronary heart disease. *JAMA.* 2019;321(4):364–373.
31. Yu C-G, Yuan S-S, Yang L-Y, et al. Angiopoietin-like 3 is a potential biomarker for retinopathy in type 2 diabetic patients. *Am J Ophthalmol.* 2018/07/01/ 2018;191:34–41. doi:10.1016/j.ajo.2018.03.040.
32. Camenisch G, Pisabarro MT, Sherman D, et al. ANGPTL3 stimulates endothelial cell adhesion and migration via integrin αvβ3 and induces blood vessel formation in vivo. *J Biol Chem.* 2002;277(19):17281–17290. doi:10.1074/jbc.m109768200.
33. Su X, Peng D-q. New insights into ANGPLT3 in controlling lipoprotein metabolism and risk of cardiovascular diseases. *Lipids Health Dis.* 2018/01/15 2018;17(1):12.
34. Li Z, Du Y, Jia L, et al. Association of C1q/TNF-related protein-9 (CTRP9) level with obstructive sleep apnea in patients with coronary artery disease. *Mediators Inflamm.* 2020/08/05 2020;2020:7281391. doi:10.1155/2020/7281391.
35. Wolf RM, Steele KE, Peterson LA, et al. C1q/TNF-related protein-9 (CTRP9) levels are associated with obesity and decrease following weight loss surgery. *J Clin Endocrinol Metab.* 2016;101(5):2211–2217.
36. Katznelson L, Laws ER Jr, Melmed S, et al. Acromegaly: an endocrine society clinical practice guideline. *J Clin Endocrinol Metab.* 2014;99(11):3933–3951.
37. Moradi N, Fadaei R, Emamgholipour S, et al. Association of circulating CTRP9 with soluble adhesion molecules and inflammatory markers in patients with type 2 diabetes mellitus and coronary artery disease. *PLoS One.* 2018;13(1):e0192159. doi:10.1371/journal.pone.0192159.
38. Chen B, Lam KS, Wang Y, et al. Hypoxia dysregulates the production of adiponectin and plasminogen activator inhibitor-1 independent of reactive oxygen species in adipocytes. *Biochem Biophys Res Commun.* 2006;341(2):549–556. doi:10.1016/j.bbrc.2006.01.004.

MLL1:EZH2 Ratio in Uterine Secretions and Endometrial Receptivity in Patients with Endometriosis

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Abbreviations: EM, endometriosis; ET, embryo transfer; MLL1, mixed lineage leukemia; HMT, histone methyltransferase; EZH2, zeste homolog 2; DAPI, 4′,6-diamidino-2-phenylindole; qRT-PCR, quantitative real-time polymerase chain reaction; Ct, comparative threshold cycle; H3K4me3, trimethylation of histone 3 lysine 4

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ABSTRACT

Objective: To establish a novel approach for diagnosing endometriosis (EM) in patients with impaired endometrial receptivity.

Method: Mixed lineage leukemia 1 (MLL1) and enhancer of zeste homolog 2 (EZH2) levels were analyzed. The MLL1:EZH2 ratio in identifying impaired endometrial receptivity has been established and validated.

Results: In normal endometrial tissue, the MLL1:EZH2 ratio increased significantly in the midsecretory phase, compared with that in the proliferative phase. In the midsecretory phase, the MLL1:EZH2 ratio in endometrial tissues and uterine secretions accurately identifies patients with EM who have impaired endometrial receptivity. In the validation group, the sensitivity and specificity of the MLL1:EZH2 ratio in the uterine secretions of the midsecretory phase, in diagnosing patients EM who have impaired endometrial receptivity, were 100% and 96.55%, respectively.

Conclusions: The MLL1:EZH2 ratio in uterine secretions of the midsecretory phase may serve as a marker to diagnose EM in patients with impaired endometrial receptivity.

Endometriosis (EM) is one of the most common gynecological diseases worldwide, affecting approximately 10% of women of reproductive age.[1] Clinically, approximately 30%–50% of female infertility cases are caused by EM.[2] The reasons for infertility in women with EM may range from anatomical distortions such as tubal adhesions, endocrine abnormalities, and immunological disturbances.[3] Patients with EM who have tubal obstruction may undergo IVF and embryo transfer (IVF-ET) to achieve conception. Although IVF-ET is an effective treatment option for patients with EM having less-advanced disease stages, women with more advanced stages of EM have significantly lower success rates,[3] which is partially due to the reduced endometrial receptivity in patients with EM.[4] Impaired endometrial receptivity was defined as ≥3 times failed embryo transfer or early miscarriage (≤12 weeks).[5]

Currently, endometrial thickness and serum levels of estrogen and progesterone are the most commonly used markers of endometrial receptivity.[6] However, based on these markers, the outcome of IVF-ET in patients with EM remains unsatisfactory, with pregnancy rate of 33%, live birth rate of 23%, and miscarriage rate of 36%.[7] These results indicate that for a substantial proportion of patients with EM, IVF-ET treatment would be unsuitable, due to impaired endometrial receptivity in those patients.

Because EM may also cause decreases in ovarian reserve,[8] it is necessary to fully evaluate the endometrial status of patients with EM before embryo transfer, to avoid endangering embryos. Hence, a more effective method should be established to reflect the receptivity of the endometrium and to distinguish patients with EM who have impaired receptivity from those with normal receptivity.

Recent study reports, such as by Liu et al,[9] have revealed that epigenetic modifications play a key role in establishing endometrial receptivity. Mixed lineage leukemia 1 (MLL1), an important histone methyltransferase (HMT), was decreased in the eutopic endometrium of patients with EM experiencing infertility.[10] Also, enhancer of zeste homolog 2 (EZH2), which is another HMT, showed the opposite effect of MLL1 during establishment of endometrial receptivity.[11] Uterine secretions contain exfoliated endometrial cells.[12]

The detection of mRNA levels in uterine-secretion genes has gradually attracted widespread attention.[12,13] In this study, we hypothesized that the MLL1:EZH2 ratio could reflect the receptivity of the eutopic endometrium of patients with EM. We analyzed the MLL1:EZH2 ratio in endometrial tissues and uterine secretions from patients with EM who have or do not have impaired endometrial receptivity, to identify a novel

method to guide the treatment of EM-related infertility and to improve the success rate of IVF-ET.

Material and Methods

Ethics Approval

Our research plans were approved by the Human Ethics Committee of The Maternal and Child Health Hospital of Hunan Province (No. KS-2018.012.2), according to the Declaration of Helsinki. Informed consent was obtained from all individual participants included in the study.

Subjects

Our methods were established and optimized in our hospital from January 2018 to March 2021, and 58 patients with EM were enrolled. Among them, specimens from 25 patients were used for method establishment and specimens from 33 other patients were used for method optimization (**FIGURE 1A**). Method-validation analysis was conducted in our hospital from January 2018 to March 2021, for which 100 patients with EM were enrolled (**FIGURE 1B**). The inclusion criteria were diagnosis of EM complicated with tubal obstruction by laparoscopy, having had sexual intercourse for more than 1 year (without contraception) but having never been pregnant, age younger than 35 years, having regular (21–35 days) menstrual cycles and having had no history of hormonal treatment within the past 3 months, and agreeing to receive IVF-ET treatment. Patients with 1 or more of the following conditions were excluded: male partner having diagnosed infertility; low embryo quality; complications with other diseases, such as cardiovascular and cerebrovascular diseases, immune-system diseases, or other diseases that have a direct impact on pregnancy outcomes; family history of genetic diseases; unwillingness to sign informed-consent paperwork.

Patients enrolled in the analysis of method establishment and optimization (n = 58) provided endometrial tissue and uterine secretions, and patients enrolled in the analysis of method validation (n = 100) provided only uterine secretions. The menstrual cycle phase was confirmed based on menstrual history combined with histological results and/or serum hormone levels.

Collection of Endometrial Tissues and Uterine Secretions

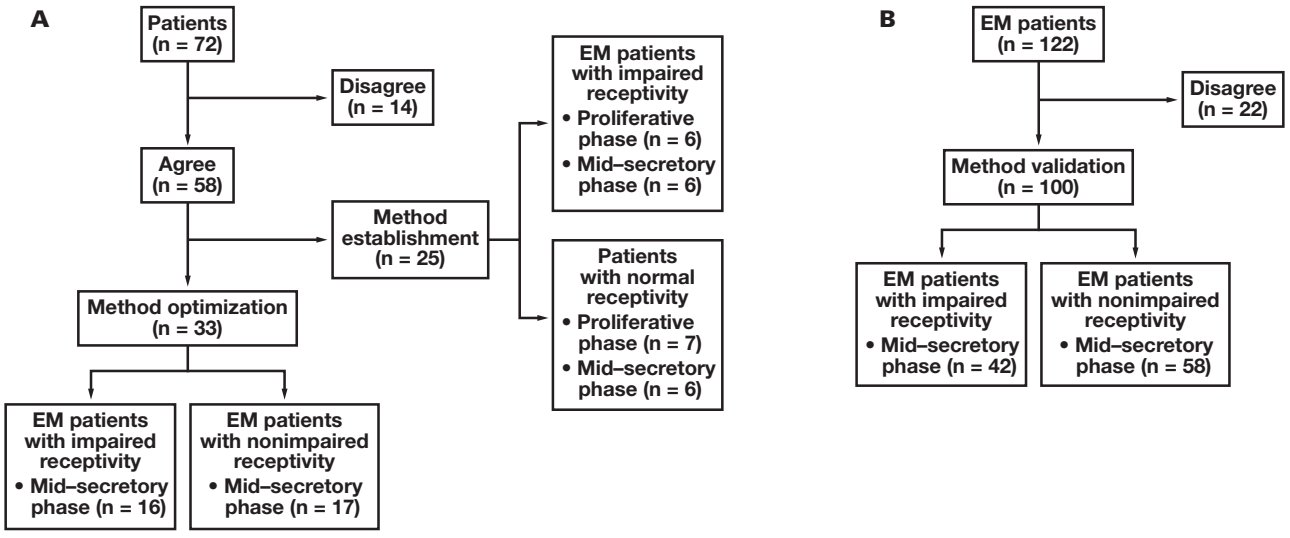
The patient was placed in the lithotomy position. The vagina and cervix were disinfected before the procedure. To collect endometrial biopsies, endometrial tissues were scraped using a sterile curette and immediately immersed in 4% paraformaldehyde for at least 24 hours. The tissues were then embedded in paraffin. To collect uterine secretions, an 11-cm sterile catheter connected to an empty syringe was gently introduced into the uterine cavity. By withdrawing the plunger, approximately 10–30 μL of uterine secretions were sucked into the syringe, and the catheter was removed. The collected secretions were transferred into clean centrifuge tubes containing 100 μL of PBS and stored at 4°C. The research worker analyzing endometrial tissues was masked to the results of the uterine secretions and vice versa.

Immunofluorescence

Immunofluorescence staining was performed on paraffin-embedded sections and cells. For paraffin-embedded sections, after being dewaxed and hydrated, tissues were incubated overnight at 4°C with antibodies anti-MLL1 (14197; Cell Signaling Technology) and anti-EZH2 (612666; BD Biosciences) separately, and were followed by being incubated with goat antirabbit Cy3-Conjugated Igg (Boster Bio) or goat antimouse FITC-conjugated IgG (Boster Bio) for another 0.5 hours in a dark room. Sections were then washed 3 times with PBS, photographed using a Laser Scanning Confocal Microscope (Leica Microsystems), and analyzed using Leica Application Suite X, version 4.0 (Leica Microsystems).

Sloughed-off cells in uterine secretions were smeared onto slides using the StatSpin CytoFuge 2 (Beckman Coulter) at 157*g* for 5 minutes. The cells were fixed using 4% paraformaldehyde for 15 minutes and made permeable with 1% Triton X-100 for another 5 minutes. Cells were incubated with primary antibodies anti-MLL1 (14197, Cell Signaling

FIGURE 1. Procedure for method establishment, optimization, and validation. A, In the analysis of method establishment and optimization, 58 specimen pairs of endometrial tissues and uterine secretions were collected from patients with endometriosis (EM). Among these specimens, 25 pairs were used for method establishment and the other 33 pairs were used for method optimization. B, In the method-validation analysis, 100 uterine-secretion specimens were collected from patients with EM during the mid-secretory phase.



Technology) and anti-EZH2 (612666, BD Biosciences) separately, and then incubated with goat antirabbit Cy3-conjugated IgG (Boster Bio) or goat antimouse FITC-conjugated IgG (Boster Bio) for another 30 minutes in a dark room. We added 4′,6-diamidino-2-phenylindole (DAPI) to stain nuclei. The stained cells were photographed using an inverted fluorescence microscope (Olympus), and the results were analyzed using ImageJ software (developed by the National Institutes of Health).

Quantitative RT-PCR

Total RNAs from endometrial tissues and cells in uterine secretions was isolated using the Invitrogen TRIzol Reagent (Thermo Fisher Scientific). cDNA was synthesized from total RNAs using the PrimeScript RT Master Mix (Takara Holdings). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using SYBR Premix Ex Taq (Takara Holdings). The sequences of primers used were: MLL1 (forward: 5′-CCACCACCAGAATCAGGTCC-3′; reverse: 5′-ATTTGGAATGGACCCAGCGA-3′), EZH2 (forward: 5′-TGCGACTGAGACAGCTCAAG-3′; reverse: 5′-GCTTATAAGTGT TGGGTGTTGC-3′), and GAPDH (forward: 5′- CCATGGGGAA GGTGAAGGTC-3′; reverse: 5′- AGTGATGGCATGGACTGTGG-3′). The quantity of target transcripts was calculated based on the comparative threshold cycle (Ct) using the $2^{-\triangle\triangle^{Ct}}$ method.

Statistics

Statistical analyses were performed using SPSS software, version 22.0 (IBM) and GraphPad Prism, version 6.0 (GraphPad Software). Continuous data are shown as mean (SEM), and the Student *t* test was used to analyze the differences between the 2 groups. Categorical variables were analyzed using the χ^2 test. In this study, $P < .05$ was considered statistically significant.

Results

MLL1: EZH2 Ratio Abnormality in Patients with EM Having Impaired Endometrial Receptivity

We first analyzed the expression patterns of MLL1 and EZH2 in eutopic endometrium from different menstrual-cycle phases. The results showed that in the normal endometrium the expression of MLL1 increased from the proliferative phase to the midsecretory phase, while EZH2 expression decreased (**FIGURE 2A**). Accordingly, in normal endometrial cells, the MLL1:EZH2 ratio increased from 0.45 (0.07) in the proliferative phase to 1.80 (0.16) in the secretory phase, whereas the MLL1:EZH2 ratio in the eutopic endometrium with impaired receptivity showed no significant change from the proliferative phase to the secretory phase (0.40 [0.08] vs 0.51 [0.09]) (**FIGURE 2B**). However, MLL1 expression remained low throughout the menstrual cycle in the eutopic endometrium with impaired receptivity. In contrast, the EZH2 expression pattern was opposite to that of MLL1, which remained high throughout the menstrual cycle (**FIGURE 2C**).

Similar results were observed in sloughed-off cells in uterine secretions—the MLL1:EZH2 ratio in secretions from patients with EM who had normal receptivity increased from 0.36 (0.08) in the proliferative phase to 1.86 (0.17) in the secretory phase, whereas it showed no obvious change in the secretions from patients with EM having impaired receptivity (0.41 [0.07] vs 0.37 [0.07] (**FIGURE 2D**). The clinical characteristics of the patients are provided in Supplemental Tables 1 and 2. Hence, the MLL1:EZH2 ratio in endometrial tissues and uterine secretions can accurately identify patients with EM who have impaired endometrial receptivity.

Next, we explored the value of the MLL1:EZH2 ratio in identifying eutopic endometrial tissue with impaired receptivity, using endometrial tissues and uterine secretions. Results showed that when we set 1.0 as the cutoff value (impaired receptivity, MLL1: EZH2 ≤1.0; normal receptivity, MLL1: EZH2 > 1.0), the groupings of patients with EM according to the MLL1:EZH2 ratio in endometrial tissues and uterine secretions were highly consistent (**FIGURE 3A**). Also, the MLL1:EZH2 ratio in endometrial tissues and uterine secretions could accurately identify patients with EM who had impaired receptivity, with accuracy rates of 97.0% (32/33) and 93.9% (31/33), respectively (**FIGURE 3B** and **3C**). The clinical characteristics of the patients are provided in Supplemental Tables 1 and 2.

qRT-PCR in Detection of the MLL1:EZH2 Ratio in Uterine Secretions

To simplify the detection process, we attempted to detect the MLL1:EZH2 ratio using qRT-PCR instead of using the immunofluorescence. qRT-PCR yields results within 2 hours after specimen acquisition, which is more suitable than other methods for monitoring endometrial status to find the optimal transfer window. Our results revealed that MLL1 transcription levels in endometrial tissues and uterine secretions were highly correlated (**FIGURE 4A**); a similar result was observed for EZH2 transcription levels (**FIGURE 4B**). The results of further studies showed that the MLL1:EZH2 ratio in endometrial tissues and uterine secretions detected by qRT-PCR could accurately identify patients with EM who had impaired receptivity, with accuracy rates of more than 95% (32/33; **FIGURE 4C**). These findings indicate that qRT-PCR is feasible for the detection of the MLL1:EZH2 ratio to reflect endometrial status.

MLL1:EZH2 Ratio in Uterine Secretions Detected by qRT-PCR in Predicting IVF-ET Outcome Accurately in Patients with EM

Finally, we studied 100 patients with EM from another hospital, to validate the value of the MLL1:EZH2 ratio in uterine secretions detected by qRT-PCR in identifying patients with EM who have impaired endometrial receptivity. The clinical characteristics of the analyzed patients are shown in **TABLE 1**. No significant differences in clinical characteristics were observed between the patients with impaired endometrial receptivity and those with normal endometrial receptivity.

Among all studied patients, 44 were reported to have an MLL1:EZH2 ratio ≤1, and 56 were reported to have an MLL1:EZH2 ratio >1. The success rate of IVF-ET within 3 cycles was significantly higher in patients with an MLL1:EZH2 ratio >1 than in patients with an MLL1:EZH2 ratio ≤1 (4.5% vs 100%) (**TABLE 2**). Also, according to the IVF-ET outcomes, 95.5% (42/44) of patients with EM who had an MLL1:EZH2 ratio ≤1 showed impaired endometrial receptivity, and 100% (56/56) of patients with EM who had an MLL1:EZH2 ratio >1 showed normal endometrial receptivity (**TABLE 3**).

Discussion

The results of our pilot study revealed that the MLL1:EZH2 ratio in endometrial tissues can be used as a marker to identify patients with EM

who have with impaired endometrial receptivity. Also, the approach we used to generate this ratio was proven to be rapid and minimally invasive.

Reduced success rates in women with EM receiving intrauterine insemination have been repeatedly demonstrated.[14,15] A recent systematic review explored the associations between impaired endometrial receptivity and endometrial receptivity markers (endometrial thickness, endometrial pattern, endometrial wave-like activity, progesterone, and endometrial biopsy); however, the poor ability of the studies in the review in predicting impaired endometrial receptivity (endometrial thickness: sensitivity = 58%, specificity = 52%; endometrial pattern: sensitivity = 69%, specificity = 35%; endometrial wave-like activity: sensitivity = 27%, specificity = 18%; progesterone: sensitivity = 61%, specificity = 44%; and endometrial biopsy: sensitivity = 83%, specificity = 45%) prevents them from being used as diagnostic tests of endometrial receptivity.[15] Although medical treatment of EM has been shown to improve ART outcomes,[16] there is no reliable method to identify patients who should receive medical treatment before ART, nor any method to reflect the improvement in endometrial receptivity after treatment, which is important for the guidance of EM therapy and ET treatment protocols.

It is not yet clear how EM affects the eutopic endometrial function and leads to reduced endometrial receptivity. Recent studies, such as one by Patel et al,[17] have reported that inflammation is centrally associated with the pathophysiology of EM and contributes to progesterone resistance in the eutopic endometrium, which may further lead to impaired endometrial receptivity.

FIGURE 2. The MLL1:EZH2 ratio is abnormal in patients with endometriosis with impaired endometrial receptivity. A and B, Immunofluorescence results showed that in mid-secretory phase, MLL1:EZH2 ratio in endometrium with impaired receptivity was decreased compared with that in endometrium with normal receptivity (x400).

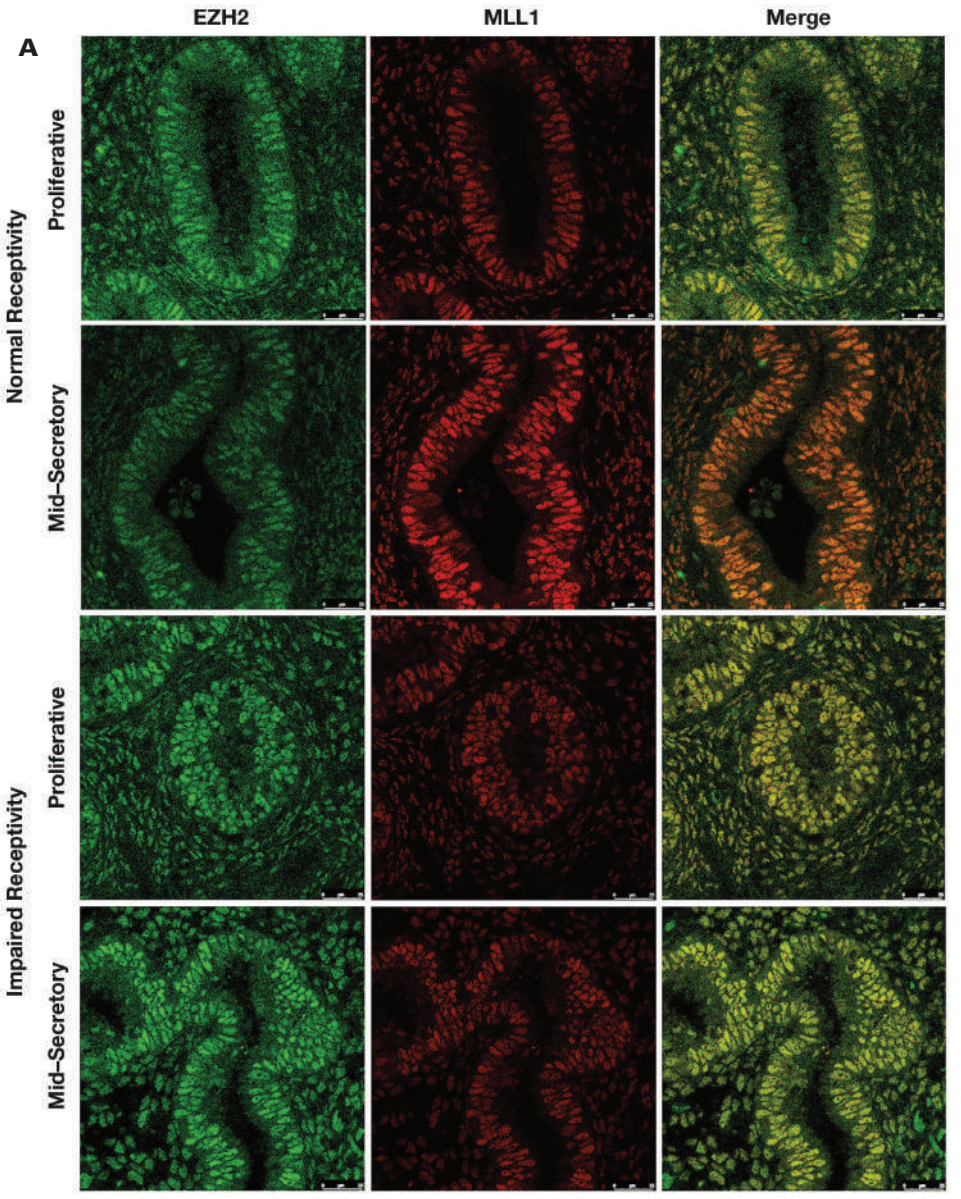
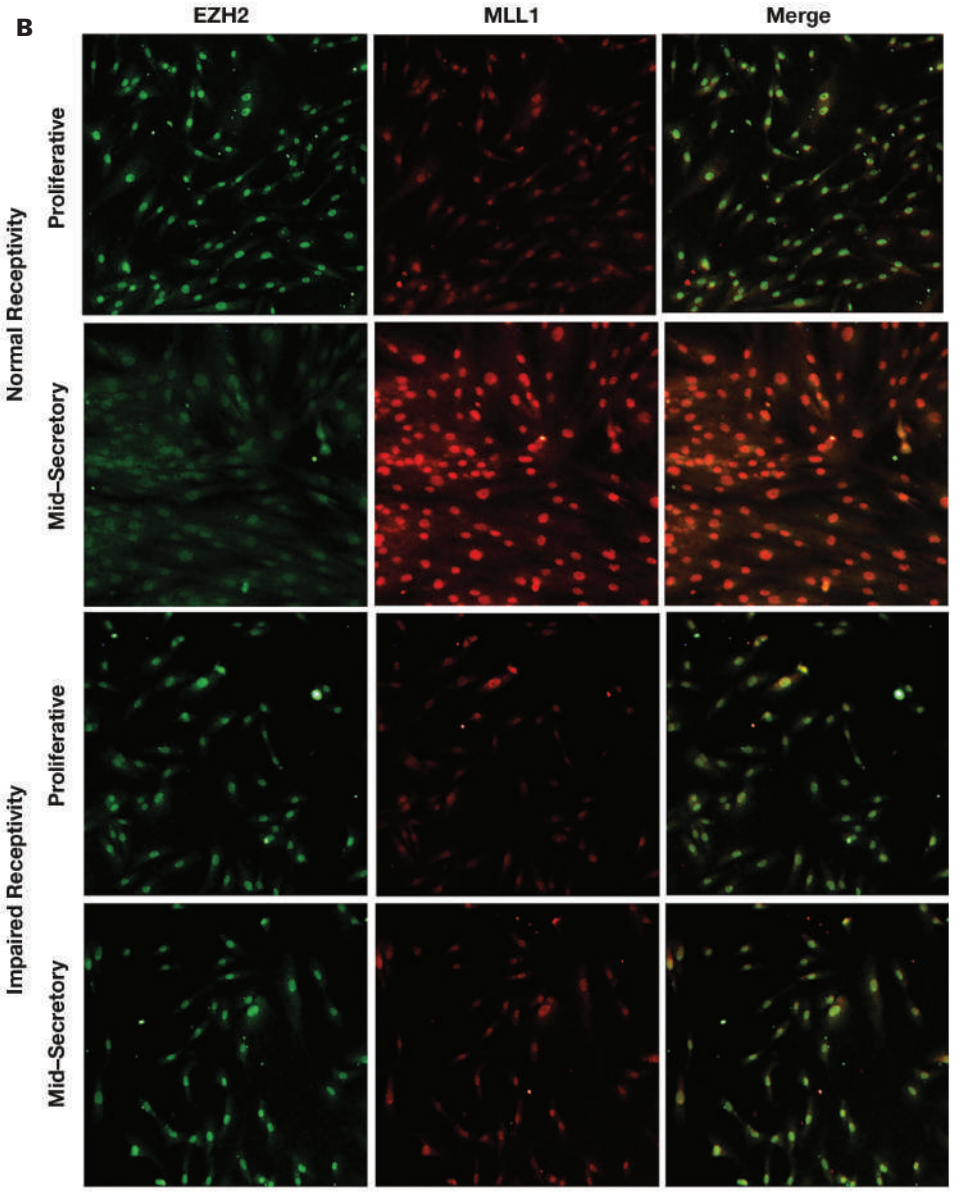
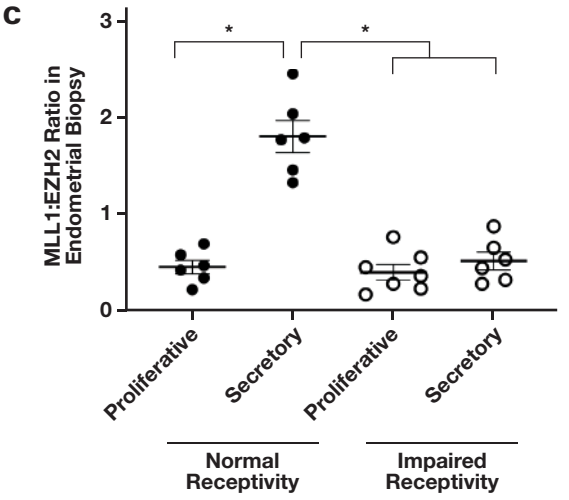


FIGURE 2. (cont) C and D, Similar results were also observed in uterine secretions. Data are expressed as mean ± SE; *$P < .001$.





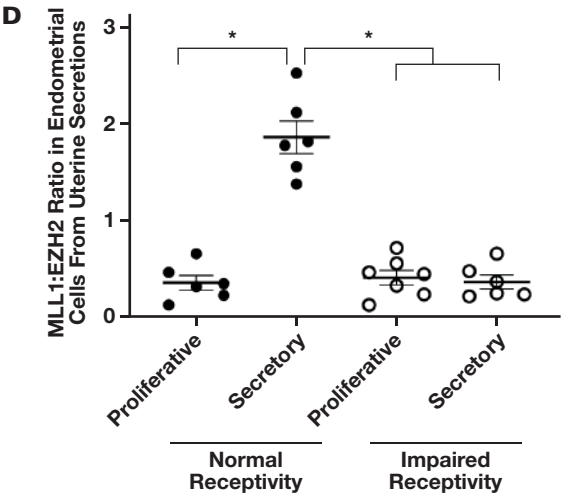
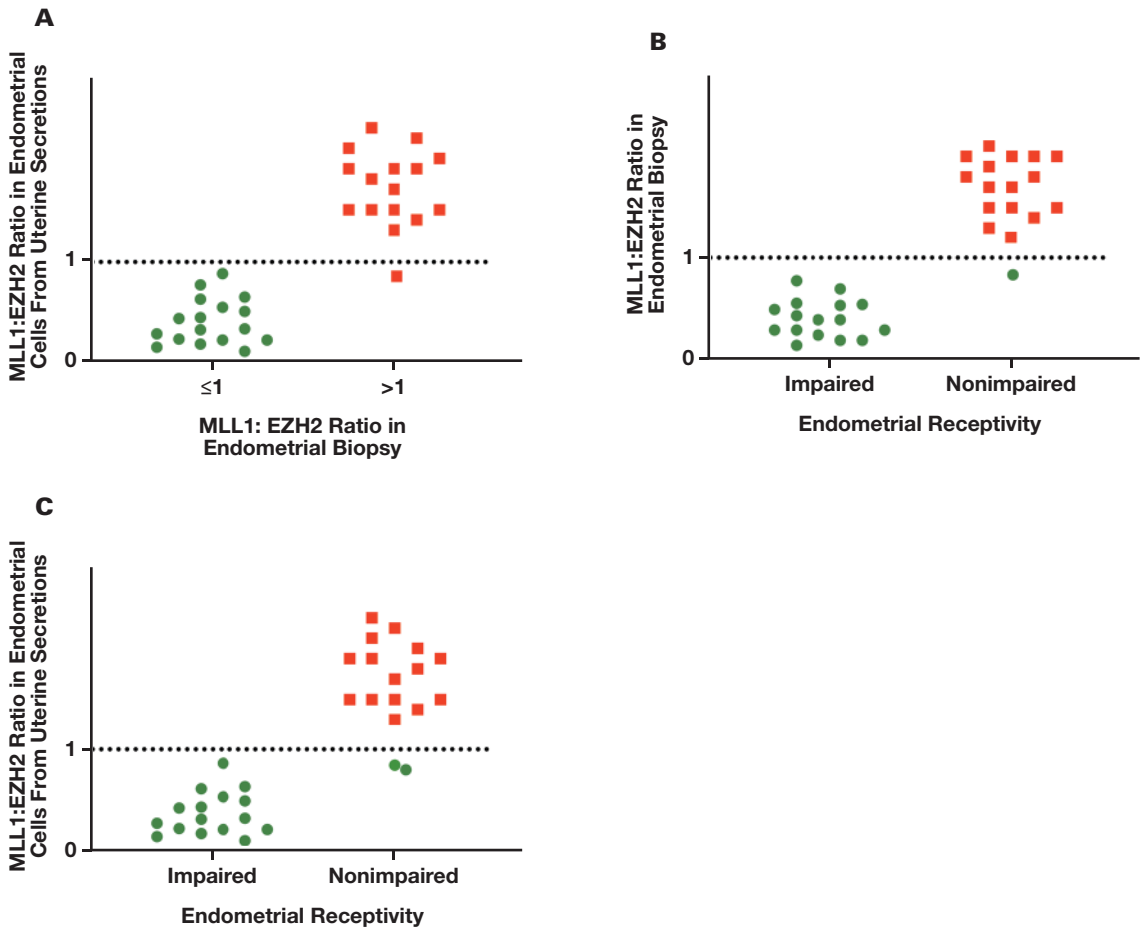


FIGURE 3. **The MLL1:EZH2 ratio in endometrial tissues and uterine secretions can accurately identify patients with endometriosis (EM) who have impaired endometrial receptivity. When setting 1.0 as the cutoff value, the MLL1:EZH2 ratio in uterine secretions was highly consistent with that in endometrial tissues (A), and the MLL1:EZH2 ratio in endometrial tissues (B) and uterine secretions (C) could accurately identify patients with EM who have impaired endometrial receptivity. Data are expressed as mean ± SE.**



Endometrial receptivity is regulated by epigenetic pathways. MLL1 and EZH2 are 2 important HMTs that catalyze histone methylation. The most important downstream effector of MLL1 is the homeobox family (Hox) gene.[18] Hox 10 has been proven to be crucial for embryo implantation and endometrial decidualization, and its ablation results in decidualization defects and embryo-implantation failure.[19] By upregulating the trimethylation of histone 3 lysine 4 (H3K4me3) in the HOXA10 promoter, MLL1 induces HOXA10 expression to regulate embryo implantation during the peri-implantation period. In contrast, EZH2 serves as a transcriptional repressor by upregulating H3K27me3 levels in the gene promoter.[20]

Previous research on EZH2[21] has mainly focused on its role in malignancy. A recent study report[21] indicated that the H3K27me3 pattern and EZH2 levels are abnormal in the eutopic endometrium of patients with EM, compared with those in the endometrium of control individuals. EZH2 has also been reported to target HOXA10 and inhibit its expression, thus affecting endometrial stromal cell decidualization.[11]

We were intrigued to learn that MLL1 and EZH2 are regulated by progesterone.[22,23] Considering that patients with EM usually show progesterone resistance in the eutopic endometrium, we speculate that MLL1 and EZH2 may be used as markers to monitor the eutopic endometrium status in patients with EM.

As we expected, our results indicated that the MLL1:EZH2 ratio can accurately reflect the receptivity of the eutopic endometrium of patients with EM. Also, we simplified the detection method and proposed a rapid and minimally invasive approach by analyzing the MLL1:EZH2 ratio in uterine secretions using qRT-PCR. Although the detection process was simplified, the accuracy was not discounted. In addition, the sensitivity of using the MLL1:EZH2 ratio in uterine secretions to diagnose patients with EM who have impaired endometrial receptivity is 100%. Hence, this method can identify all patients for whom ET is unsuitable, thereby avoiding endangering embryos, which is of particular importance for patients with EM who have decreased ovarian reserve.[8]

This study had some limitations. First, the sample size was small, and the results should be validated in more clinical studies. Second, we did not investigate the value of the MLL1:EZH2 ratio in monitoring the efficacy of different drugs in improving endometrial receptivity; this variable is equally important in guiding the treatment of EM-related infertility. Finally, besides EM, certain other causes can also lead to impaired endometrial receptivity, such as pelvic inflammation and intrauterine surgery. The MLL1:EZH2 ratio perhaps identifies all-cause endometrial-receptivity impairment, or it is perhaps limited to

FIGURE 4. **Feasibility of quantitative real-time polymerase chain reaction (qRT-PCR) for the detection of the MLL1:EZH2 ratio in uterine secretions; data are expressed as mean ± SE. MLL1 (Pearson *r* = 0.982; *P* = .001) (A) and ZEH2 (Pearson *r* = 0.972; *P* = .001) (B) transcript levels in endometrial tissues and uterine secretions are highly correlated. When setting 1.0 as the cutoff value, the MLL1:EZH2 ratio in endometrial tissues and uterine secretions detected by qRT-PCR could accurately identify patients with endometriosis who have impaired endometrial receptivity (C).**

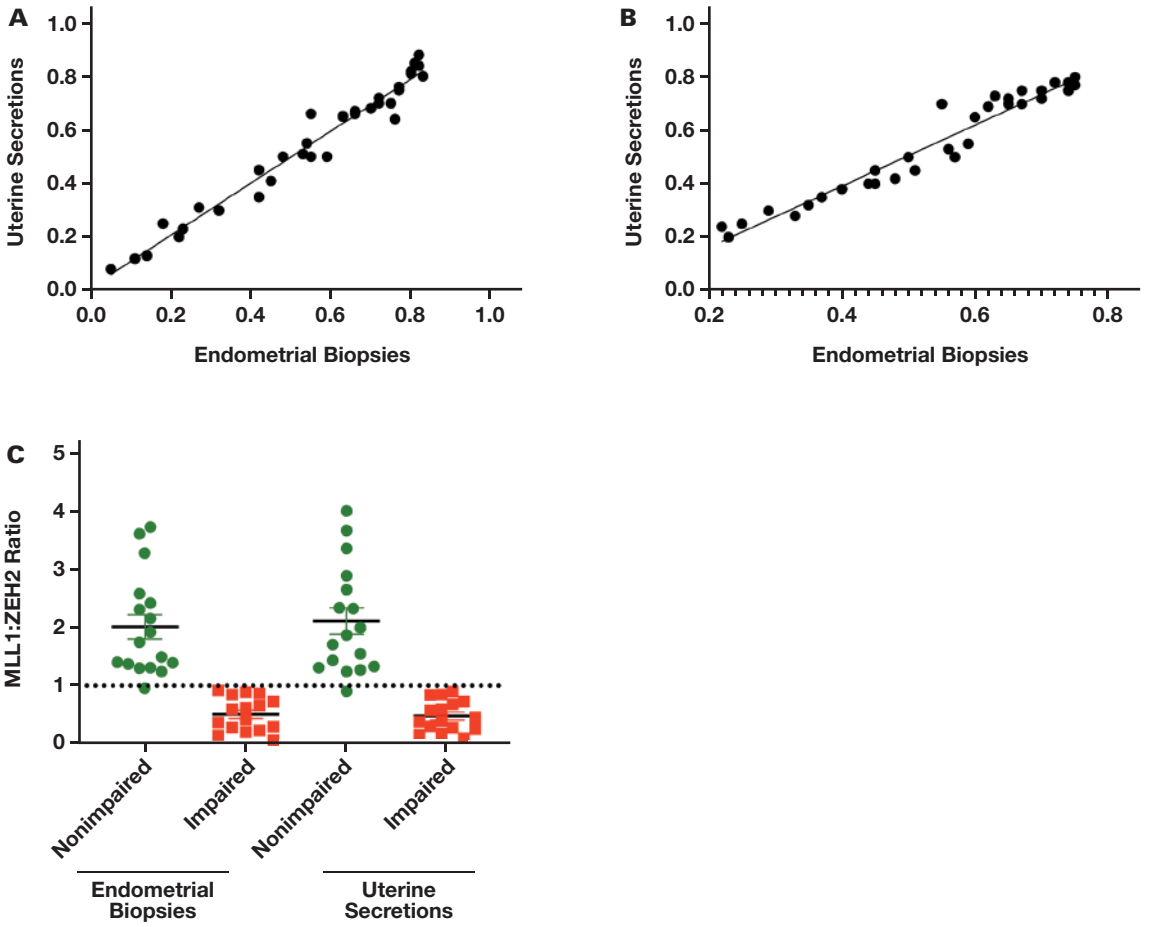


TABLE 1. **Characteristics of Patients Included in the Analysis of Method Validation[a]**

Characteristic	Patients with EM and Impaired Receptivity (n = 42)	Patients with Normal Receptivity (n = 58)	*P* Value
Age, y	30.23 (3.17)	29.41 (3.84)	.88
Age at menarche, y	13.15 (1.82)	13.49 (1.77)	.64
BMI, kg/m^2	20.46 (2.65)	21.32 (2.43)	.56
Nulliparous (%)	78.57 (33)	75.86 (44)	.75
AMH, ng/mL	3.36 (0.67)	3.29 (0.58)	.69
E2 level at baseline, pg/mL	65.72 (12.61)	58.21 (11.43)	.44
P level at baseline, ng/mL	0.96 (0.20)	1.03 (0.24)	.79
E2 level at transfer, pg/mL	255.72 (28.61)	241.33 (35.22)	.56
P level at transfer, ng/mL	12.88 (2.42)	13.07 (3.01)	.60

AMH, anti-Müllerian Hormone; E2, estradiol; EM, endometriosis; P, progesterone.
[a]*Continuous variables were expressed as mean (SE) and were analyzed using Student* t *testing. Categorical variables were shown as percentages and were analyzed using* χ^2 *testing.*

detection of EM-related receptivity impairment—we strongly recommend exploration of this question in future studies.

In conclusion, this study is the first in the literature, to our knowledge, to propose a rapid and minimally invasive approach to identify patients with EM who have impaired endometrial receptivity. The information yielded by this approach can guide the treatment of EM-related infertility and is likely to improve the success rate of IVF-ET in patients with EM.

TABLE 2. The MLL1:EZH2 Ratio in Uterine Secretions Detected by qRT-PCR in Prediction of IVF-ET Outcomes[a]

Cycle, No.	MLL1:EZH2 Ratio, No. (%)		*P* Value
	MLL1: 44 (≤1.0)	EZHR2: 56 (>1.0)	
1	0	45 (80.4%)	.001
2	2 (4.5%)	11 (19.6%)	.03
<3	2 (4.5%)	56 (100%)	.001
≥3	42 (95.5%)	0	.001

EZH2, enhancer of zeste homolog 2; ET, embryo transfer; IVF, in vitro fertilization; MLL1, mixed lineage leukemia 1; qRT-PCR, quantitative real-time polymerase chain reaction.
[a]When setting 1.0 as the cutoff value, patients with EM having a low MLL1:EZH2 ratio in the midsecretory phase had a significantly lower IVF-ET success rate within 3 cycles.

TABLE 3. Sensitivity and Specificity of the MLL1:EZH2 Ratio in Diagnosing Impaired Endometrial Receptivity in Patients with Endometriosis

MLL1:EZH2 Ratio	With Impairment[a]	Without Impairment[b]
≤1.0	42	2
>1.0	0	56
Total	42	58

[a]Sensitivity, 100% (42/42).
[b]Specificity, 96.5% (56/58).

Supplementary Data

Supplemental figures and tables can be found in the online version of this article at www.labmedicine.com.

Acknowledgments

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REFERENCES

1. Chapron C, Marcellin L, Borghese B, Santulli P. Rethinking mechanisms, diagnosis and management of endometriosis. *Nat Rev Endocrinol.* 2019;15(11):666–682.
2. Filip L, Duică F, Prădatu A, et al. Endometriosis associated infertility: a critical review and analysis on etiopathogenesis and therapeutic approaches. *Medicina (Kaunas).* 2020;56(9):460.
3. Tanbo T, Fedorcsak P. Endometriosis-associated infertility: aspects of pathophysiological mechanisms and treatment options. *Acta Obstet Gynecol Scand.* 2017;96(6):659–667.
4. Lessey BA, Kim JJ. Endometrial receptivity in the eutopic endometrium of women with endometriosis: it is affected, and let me show you why. *Fertil Steril.* 2017;108(1):19–27.
5. von Grothusen C, Frisendahl C, Modhukur V, et al. Uterine fluid microRNAs are dysregulated in women with recurrent implantation failure. *Hum Reprod*. 2022;37(4):734–746.
6. Mackens S, Santos-Ribeiro S, van de Vijver A, et al. Frozen embryo transfer: a review on the optimal endometrial preparation and timing. *Hum Reprod.* 2017;32(11):2234–2242.
7. Tamura H, Yoshida H, Kikuchi H, et al. The clinical outcome of Dienogest treatment followed by in vitro fertilization and embryo transfer in infertile women with endometriosis. *J Ovarian Res* 2019;12(1):123.
8. Kitajima M, Khan KN, Harada A, et al. Association between ovarian endometrioma and ovarian reserve. *Front Biosci (Elite Ed)* 2018;10(1):92–102.
9. Liu H, Huang X, Mor G, Liao A. Epigenetic modifications working in the decidualization and endometrial receptivity. *Cell Mol Life Sci.* 2020;77(11):2091–2101.
10. Wen X, Xiong Y, Liu H, et al. Decreased mixed lineage leukemia 1 is involved in endometriosis-related infertility. *J Mol Endocrinol.* 2021;66(1):45–57.
11. Xiong Y, Wen X, Liu H, Zhang M, Zhang Y. Bisphenol a affects endometrial stromal cells decidualization, involvement of epigenetic regulation. *J Steroid Biochem Mol Biol.* 2020;200:105640.
12. Beier HM, Beier-Hellwig K. Molecular and cellular aspects of endometrial receptivity. *Hum Reprod Update*. 1998;4(5):448–458.
13. von Wolff M, Classen-Linke I, Heid D, et al. Tumour necrosis factor–α (TNF-α) in human endometrium and uterine secretion: an evaluation by immunohistochemistry, ELISA and semiquantitative RT-PCR. *Mol Hum Reprod*. 1999;5(2):146–152.
14. Jansen RPS. Minimal endometriosis and reduced fecundability: prospective evidence from an artificial insemination by donor program. *Fertil Steril.* 1986;46(1):141–143.
15. Craciunas L, Gallos I, Chu J, et al. Conventional and modern markers of endometrial receptivity: a systematic review and meta-analysis. *Hum Reprod Update*. 2019;25(2):202–223.
16. Barra F, Laganà AS, Scala C, et al. Pretreatment with dienogest in women with endometriosis undergoing IVF after a previous failed cycle. *Reprod Biomed Online.* 2020;41(5):859–868.
17. Patel BG, Rudnicki M, Yu J, Shu Y, Taylor RN. Progesterone resistance in endometriosis: origins, consequences and interventions. *Acta Obstet Gynecol Scand.* 2017;96(6):623–632.
18. Wang Z-H, Li D-D, Chen W-L, You Q-D, Guo X-K. Targeting protein-protein interaction between MLL1 and reciprocal proteins for leukemia therapy. *Bioorg Med Chem.* 2018;26(2):356–365.
19. Du H, Taylor HS. The role of *Hox* genes in female reproductive tract development, adult function, and fertility. *Cold Spring Harb Perspect Med.* 2015;6(1):a023002.
20. Li Y, Li H, Zhou L. EZH2-mediated H3K27me3 inhibits ACE2 expression. *Biochem Biophys Res Commun.* 2020;526(4):947–952.
21. Duan R, Du W, Guo W. EZH2: a novel target for cancer treatment. *J Hematol Oncol* 2020;13(1):104.
22. Colón-Caraballo M, Monteiro JB, Flores I. H3K27me3 is an epigenetic mark of relevance in endometriosis. *Reprod Sci.* 2015;22(9):1134–1142.
23. Nanjappa MK, Mesa AM, Medrano TI, et al. The histone methyltransferase EZH2 is required for normal uterine development and function in mice. *Biol Reprod.* 2019;101(2):306–317.

Diagnostic Efficiency of Serum-Based Infrared Spectroscopy in Detecting Breast Cancer: A Meta-Analysis

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Keywords: breast cancer, FTIR, serum, diagnostic efficiency, liquid biopsy, meta-analysis

Abbreviations: FTIR, Fourier transform infrared; ATR, attenuated total reflection; IR, infrared; CA, carbohydrate antigen; CEA, carcinoembryonic antigen; PRISMA, Preferred Reporting Items for Systematic Review and Meta-Analyses; NOS, Newcastle-Ottawa Scale; I^2, inconsistency index; T^2, tau-squared; LR+, positive likelihood ratio; LR–, negative likelihood ratio; DOR, diagnostic odds ratio; sROC, summary ROC; IDC, invasive ductal carcinoma; ANNs, artificial neural networks; ctDNA, circulating tumor DNA; CTCs, circulating tumor cells; NGS, next-generation sequencing

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ABSTRACT

Background: The advancement of Fourier transform infrared (FTIR) spectroscopy as a potential diagnostic tool in the clinical setting has been studied over the years, particularly its application in cancer diagnostics.

Objective: To summarize previous research on FTIR spectroscopy in detecting breast cancer using serum specimens.

Methods: Related literature was searched and screened from various databases. Relevant data were then extracted, tabulated, and analyzed using Meta-DiSc 1.4 software.

Results: Sensitivity and specificity rates were 90% to 100% and 80% to 95%, respectively. The area under the receiver operating characteristic curve was at 0.9729, indicating that serum analysis via FTIR spectroscopy can accurately discriminate between healthy individuals and patients with breast cancer.

Conclusion: Overall, FTIR spectroscopy for breast cancer diagnosis using serum specimens shows promising results. However, further studies are still needed to validate these claims.

Breast cancer is one of the most prevalent cancers and the principal cause of cancer-related deaths in women worldwide,[1] with an estimated 2 million cases in 2018.[2] Statistical analyses of the past breast cancer trends in women showed an increasing rate of global burden in terms of incidence and mortality in 102 countries in the past 26 years (1990–2016). We expect that the incidence and mortality rates will escalate further in the succeeding years. Further, these rates are higher in well-developed countries, whereas developing countries have lower incidence with higher mortality rates, which is partly attributed to the lack of breast cancer screening.[3–5]

Mammography is a noninvasive and inexpensive screening method for breast cancer with good sensitivity (63%–98%) with increasing age.[6] However, there have been reports of less-than-optimal values (~30%–48%) in women with dense breasts.[7,8] Further, a triple assessment, which entails multiple tests such as clinical examination, mammography, and ultrasound, has been found to have high sensitivity, specificity, and concordance values. Although ultrasound and MRI are sensitive in detecting invasive breast cancers, the 2 methods also risk overestimating tumor extent.[9] Also, false-positive and false-negative results may occur, especially in young, premenopausal women.[10] Hence, early breast cancer testing in high-risk groups demands new strategies.

Fourier transform infrared (FTIR) spectroscopy is an analytical technique used to characterize solids, liquids, and gases[11] used in organic, inorganic, or polymeric materials.[12,13] An FTIR spectrometer uses an interferometer that merges 2 or more light sources to create an interference pattern that can be measured and analyzed. The infrared light goes from the glowing source to a beam splitter usually made of polished KBr crystal placed at a 45-degree angle. The Fourier standard computer algorithm converts the interferogram from a time domain into a frequency domain spectrum that allows people to see the strength

of absorption as a function of the frequency (or wavelength). The FTIR can also be enhanced with various accessories to fit the specific requirements for specimen analysis, such as ATR (attenuated total reflection), transflection, and transmission mode. An infrared spectrum results from this technique, regardless of the accessories used.[14]

FTIR spectroscopy can detect biochemical compositions such as nucleic acids, proteins, lipids, and carbohydrates by identifying molecular conformations, bonding types, functional groups, and intermolecular interactions. The technique has gained increased interest over the years because it is versatile in detecting biochemical and biological features of specimens. Infrared spectroscopy produces a signature spectral fingerprint based on the absorbance ratios from the specimens. The intensities and positions of peaks depend on the biochemical compositions of a specimen,[15] which may be used to differentiate cell types. It has been used to compare healthy vs cancerous or diseased conditions and even detect degree of tumor aggression.[16] Therefore, FTIR spectroscopy has been recognized as an emerging tool in diagnostics because it can provide pertinent information on patient health status.

Several studies have been published on FTIR spectroscopy that use human blood specimens for disease screening and diagnosis. High-throughput ATR-FTIR spectroscopy has been shown to be capable of delivering a diagnosis within minutes using blood specimens. Blood collection is a relatively simpler method than mammography and CT scans,[17] and the serum is used in routine clinical tests to carry information regarding intra- and extracellular events. Serum is the liquid part of blood that contains no clotting factors or blood cells; it is the most complex biofluid, with more than 20,000 different proteins.[16] Blood perfuses to organs throughout the human body, gaining proteomes from neighboring cells and organs. The peptidome is a component of blood that is thought to have cancer-specific diagnostic information and is abundant in serum.[15] Previously established biomarkers used in aiding breast cancer diagnosis, such as the carbohydrate antigen (CA) 549, CA M26, CA M29, CA 15-3, and the carcinoembryonic antigen (CEA), are also found in serum.[18]

In this study we synthesized the existing literature on the potential of FTIR spectroscopy in detecting breast cancer using serum. The sensitivity, specificity, likelihood ratios (LRs), diagnostic odds ratios (DORs), and area under the curve (AUC) of spectral analysis to diagnose breast cancer were compared to the current criterion standard, which is the microscopic examination of H&E-stained biopsy specimens.

Materials and Methods

Study Selection, Data Extraction, and Methodological Quality Assessment

We used the Preferred Reporting Items for Systematic Review and Meta-Analyses (PRISMA) guidelines to select and identify the included studies. We searched related literature in 4 databases, namely, PubMed, ScienceDirect, Cochrane Library, and Google Scholar, making no restrictions concerning the publication date. The keywords used were a combination of terms divided into 3 components: FTIR spectroscopy (eg, FTIR, ATR-FTIR, spectroscopy, and ATR-FTIR spectroscopy), cancer diagnostics (eg, cancer diagnosis, cancer identification, cancer discrimination, cancer detection, and cancer screening), and blood. The literature search was conducted from August 2021 through November 2021. The abstracts of the published articles were then sorted using Mendeley reference management software.

The titles and abstracts of the resulting studies were initially assessed if they qualified for full-text analysis based on the following criteria: use of FTIR spectroscopy as a diagnostic technique, cancer diagnosis as the purpose of the study, and exclusive use of blood as the specimen. Studies that focused on cancers other than breast cancer, utilized specimens other than blood, utilized spectroscopic techniques other than FTIR, and applied FTIR spectroscopy for purposes other than diagnostics were excluded. Review articles, studies conducted on animal models, and studies that did not contain extractable data for the confusion matrix were also excluded.

In the studies that qualified for the screening, the following information was extracted: first-author surname, country where the study was conducted, number of participants or specimens, age range of participants, study design, FTIR technique used, diagnostic performance, and confusion matrix. The gathered data were then summarized and tabulated in a customized spreadsheet.

The quality of the research articles was assessed and graded using the Newcastle-Ottawa Scale (NOS), an assessment tool for bias. A score of 7–9 indicated high quality; 5–6, moderate quality; and 0–4, low quality.

Data Analysis

The Meta-DiSc 1.4, a Windows-based software for meta-analysis of test accuracy data developed by the Unit of Biostatistics team of the Ramon y Cajal Hospital in Madrid, Spain,[19] was used to analyze the data obtained. Several statistical analyses, such as computation for the sensitivity and specificity, positive and negative LRs, DOR, and threshold analysis were performed. The inconsistency index (I^2) test, Cochran-Q test, and tau-squared (T^2) were used to calculate the heterogeneity between studies. Random-effects models were used to analyze the pooled estimated. Threshold analysis and a summary receiver operating characteristic (sROC) curve plot were generated to evaluate the threshold effect. The AUC index and the index Q* were used to measure test accuracy.

Results

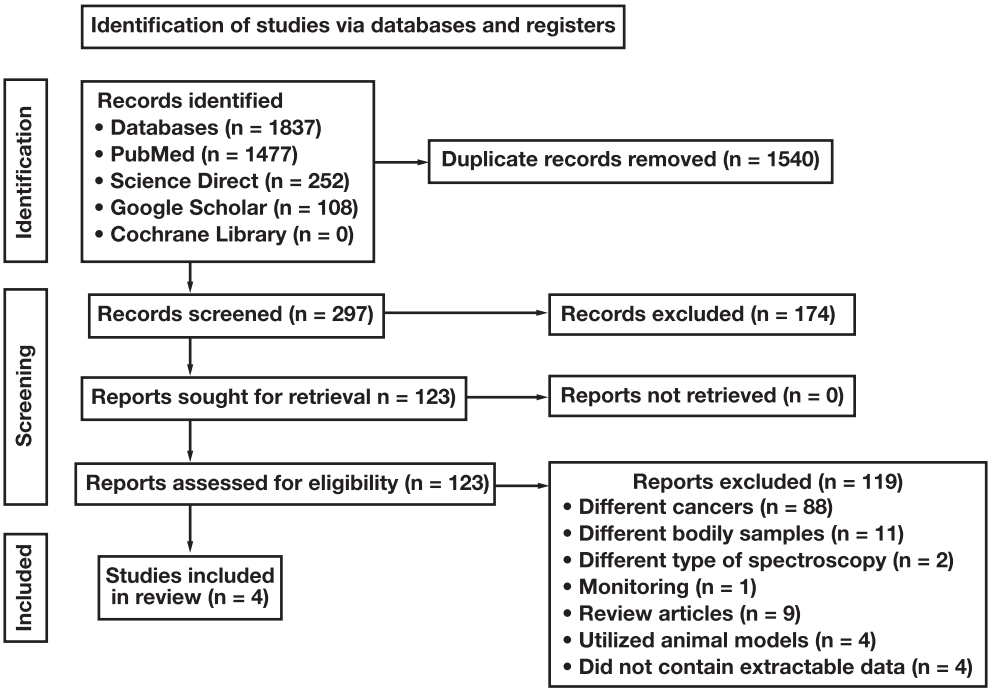
Characteristics of the Included Studies

The database search using the different keywords identified 1837 studies (**FIGURE 1**). After the removal of duplicates (n = 297), exclusion of irrelevant studies (n = 123), and eligibility screening (n = 123), only 4 studies[20–23] were included in the meta-analysis. The selected articles were all case-control studies.

TABLE 1 summarizes the characteristics of the included studies. A total of 591 individuals were included, of whom 289 had breast cancer and 302 were healthy control individuals. The age of the participants with breast cancer ranged from 30–89 years, and the healthy controls were aged 20–75 years. Participants were from Europe, North America, and Asia.

A 2010 study by Backhaus et al,[22] which included 196 serum specimens from patients with breast cancer (n = 98) and healthy controls (n = 98) used transflection and transmission modes. A study from Ghimire et al[20] analyzed 20 serum specimens from healthy controls (n = 10) and patients with breast cancer (n = 10) by ATR-FTIR. Liu et al[21] compared serum specimens from healthy controls (n = 114) and patients with breast cancer (n = 115). They did not mention, however, the specific FTIR sampling technique they used. Lastly, Sitnikova et al[23] analyzed 146 serum specimens from healthy controls (n = 80) and patients with breast cancer (n = 66) using ATR and transmission modes. The NOS assessment of the 4 studies resulted in high-quality ratings, with a mean score of 8.25.

FIGURE 1. Preferred Reporting Items for Systematic Review and Meta-Analyses (PRISMA) flow diagram of included studies.



Diagnostic Efficiency of FTIR in Detecting Breast Cancer Using Serum

The studies by Backhaus et al[22] and Ghimire et al[20] each contained 2 datasets, which were considered 2 data entries. We used Meta-DiSc 1.4 software to measure the accuracy of the individual studies and used forest plots to visually present the sensitivities, specificities, LRs, and DORs. Specifically, transmission and transflection data were included in Backhaus et al.[22] The alpha-beta helices and amide ratio values were taken from Ghimire et al.[20]

Sensitivity and Specificity

The overall sensitivity for detecting breast cancer using serum ranged from 90% through 100% (**FIGURE 2**). The transmission data included in the Ghimere et al[20] and Liu et al[21] studies both had the highest sensitivity of 100%. Pooled sensitivity across all 6 data entries was optimal, at 97% ($P > .05$; $df = 5$).

Specificity varied from 80% through 95%, with the highest being that in the transflection spectroscopy technique reported by Backhaus et al,[22] which rendered approximately 95% specificity. Pooled specificity across the 6 data entries was optimal, at 92% ($P > .05$; $df = 5$). The I^2, which is an indicator of heterogeneity among studies, was determined to assess the consistency of the articles. The measured inconsistency indices were 12.2% for sensitivity and 0% for specificity. Although the computed I^2 values were <40%, these values represent nonsignificant results among the studies, following the general guidelines for interpretation of the I^2.[24]

Likelihood Ratios

LRs are another type of useful statistical data for summarizing diagnostic accuracy. By convention, the acceptable threshold values for positive and negative LRs are 10 and 0.1, respectively. In most circumstances, LRs >10 and <0.1 indicate strong evidence to rule in or rule out a diagnosis, respectively.[25]

The pooled positive LR (LR+) at 95% CI was 10.25 (6.70–15.71), which is viewed as a strong diagnostic value that provides conclusive evidence to rule in breast cancer (**FIGURE 3**). Similarly, the pooled negative LR (LR–) at 95% CI was 0.05 (0.03–0.09), indicating that FTIR spectroscopy could effectively rule out breast cancer. Backhaus et al[22] and Liu et al[21] provided the strongest validity of the positive and negative outcomes.

We used several measures of heterogeneity, namely, I^2 statistic, Cochran-Q test, and T^2, to compute the positive and negative LRs of all included studies. Overall, the LR+ displayed low heterogeneity ($I^2 = 17.1\%$; $P > .05$; $T^2 = 0.0488$). Likewise, the LR– also showed nonsignificant variation between studies ($I^2 = 0\%$; $P > .05$; $T^2 = 0$).

Diagnostic Odds Ratio

The DOR across all 6 datasets was >10 (**FIGURE 4**), which is a good ratio by current standards. Further, pooled DOR across all studies was 252.69 ($P > .05$; $df = 5$). We note that the study by Liu et al[21] and the transflection dataset from Backhaus et al[22] had DOR values >500, which is indicative of high specificity and sensitivity >90% (**FIGURE 2**). This range is considered optimal because it has a low likelihood of false positives and false negatives.

An sROC curve was plotted to visually demonstrate the sensitivity and specificity of all the datasets used in the selected studies (**FIGURE 5**). A qualitative assessment of the sROC did not display a shoulder-arm pattern, suggesting the absence of the threshold effect.

The AUC was 0.9729 (SE = 0.0175), indicating that the analysis of serum by FTIR spectroscopy is a highly accurate method for detecting breast cancer. The Q* index, a global estimate of test accuracy for comparing sROC curves, was 0.9245 (SE = 0.0293), further proving that FTIR spectroscopic analyses of serum specimens can efficiently discriminate between healthy individuals and patients with breast cancer.

TABLE 1. Studies Included in the Systematic Review and Meta-Analysis Using FTIR Spectroscopy as a Diagnostic Tool for Breast Cancer Detection

Author & Year	Country	Sample Size	Study Design	FTIR Technique	Diagnostic Performance	Accuracy	Confusion Matrix	NOS Quality Assessment
Backhaus et al, 2010[22]	Germany	196 serum specimens: patients with breast cancer (n = 98); healthy controls (n = 98)	Case-control	Transflection and transmission	Cluster Analysis: Sensitivity: 98% Specificity: 95% ANN: Sensitivity: 92% Specificity: 100%	Unspecified	Transmission: TP: 93 TN: 89 FP: 7 FN: 4 Transflection: TP: 95 TN: 91 FP: 5 FN: 2	High quality
Ghimire et al, 2020[20]	USA	20 serum specimens: patients with breast cancer (n = 10), healthy controls (n = 10)	Case-control	Attenuated total reflection	Alpha & beta helices: Sensitivity: 90% Specificity: 90% Amide ratio: Sensitivity: 100% Specificity: 80%	Unspecified	Alpha and beta helices: TP: 9 TN: 9 FP: 1 FN: 1 Amide Ratio: TP: 10 TN: 8 FP: 2 FN: 0	High quality
Liu et al, 2020[21]	China	229 serum specimens: patients with breast cancer (n = 115), healthy controls (n = 114)	Case-control	Not indicated	Sensitivity: 91.7% Specificity:100%	95.7%	TP: 33 TN: 34 FP: 3 FN: 0	High quality
Sitnikova et al, 2020[23]	Russia	146 serum specimens: patients with breast cancer (n = 66), healthy controls (n = 80)	Case-control	Attenuated total reflection and transmission	Healthy: Sensitivity: 92.3% Specificity: 87.1%	89.3%	TP: 24 TN: 35 FP: 5 FN: 2	High quality

ANN, artificial neural networks; FN, false negative; FP, false positive; FTIR, Fourier transform infrared; NOS, Newcastle-Ottowa Scale; TN, true negative; TP, true positive.

Discussion

This meta-analysis summarized the results of 4 studies conducted on 289 patients with breast cancer. Overall, sensitivity and specificity rates for the use of FTIR in serum specimens in breast cancer detection were 97% and 92%, respectively. The pooled LR+ was 10.25, indicating that patients with breast cancer who tested positive via FTIR using serum were 10.25-fold more likely to have the disease than those who tested negative. Consequently, the LR– was 0.05, which indicated a low probability of testing negative among individuals with breast cancer. The AUC value was estimated at 0.9729 with a Q* index of 0.9245, proving further that FTIR can detect breast cancer with excellent accuracy using serum specimens.

Although the statistical analysis shows insignificant heterogeneity in the data with good estimates of diagnostic accuracy, the findings in this study should be interpreted with caution, considering several nonthreshold factors that may affect the reliability of the pooled data. First, only a few studies were used in the meta-analysis due to the limited studies on the concept. This limits the sample size of the study, decreasing the power of the analysis. Second, the FTIR sampling mode (ie, attenuated transflection, transflection, and transmission) was not uniform among the reviewed studies. Other possible factors for heterogeneity included preanalytical factors such as specimen collection, preparation, and subsequent storage.

Another possible factor for the heterogeneity of the pooled outcomes was the selection of participants. It should be noted that although FTIR spectroscopy showed promising results in discriminating cancerous vs healthy cells using serum specimens, the included articles lacked coherence and consistency regarding patient selection and categorization. For instance, in the study by Liu et al,[21] 2 benign tumors (fibroadenoma) were reported among the patients with cancer compared with healthy controls. Moreover, some participants were in remission, others were currently undergoing therapy, and still others had been recruited previously to any form of treatment.

Lastly, the criterion for discriminating between healthy and breast cancer sera dramatically varied. Backhaus et al[22] named the spectral region of 685–1250 cm as the best discriminatory factor, whereas Ghimere et al[20] and Liu et al[21] pointed out the significance of the protein (amide I and II) region as a potential biomarker. Further, Sitnikova et al[21] showed negative results, stating that proteins, lipids, and carbohydrates did not provide significant discriminatory potential. Instead, they observed noticeable spectral changes in the functional groups of the nucleic acids (1250–1306 cm^{-1}), supporting the assumption of the potential role of breast cancer–associated alterations in DNA and RNA in cancer development.

Also, we examined studies that reviewed the potential of proteins as breast cancer biomarkers. Ghimire et al[20] used the Student *t*-test (with

FIGURE 2. Forest plot for sensitivity (A) and specificity (B) of using serum to detect breast cancer by Fourier transform infrared spectroscopy.

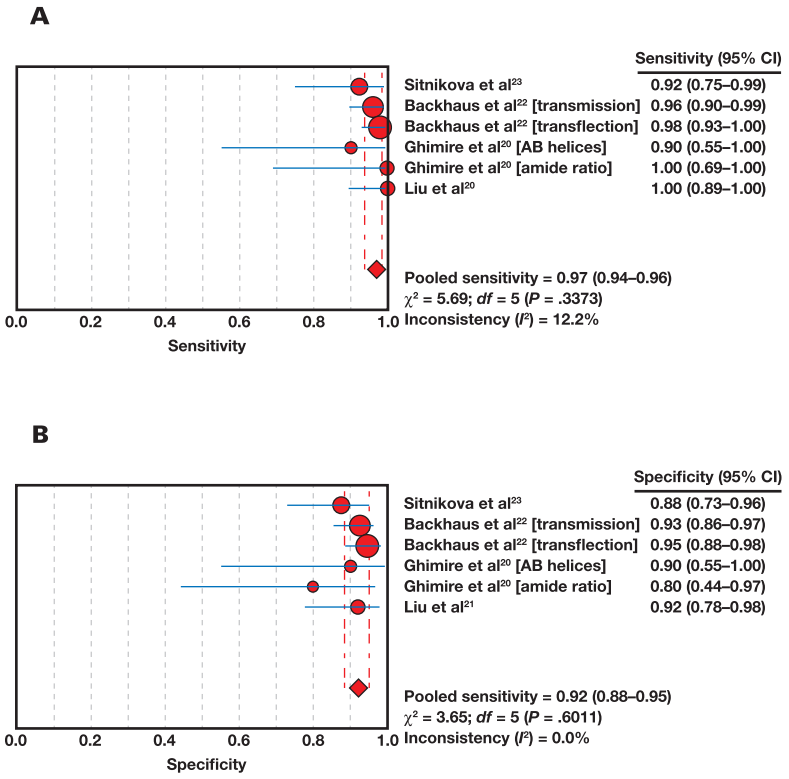


FIGURE 3. Forest plot for likelihood ratios (LRs) for positive (A) and negative (B) test results at 95% CI using serum to detect breast cancer by Fourier transform infrared spectroscopy.

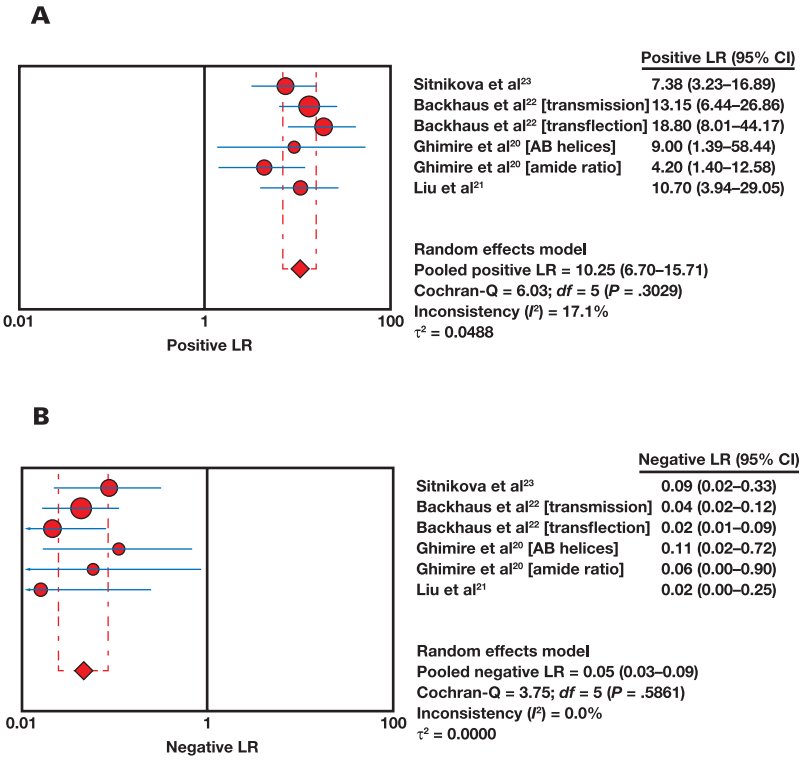


FIGURE 4. **Forest plot for diagnostic odds ratio (OR) to detect breast cancer by Fourier transform infrared spectroscopy using serum specimens.**

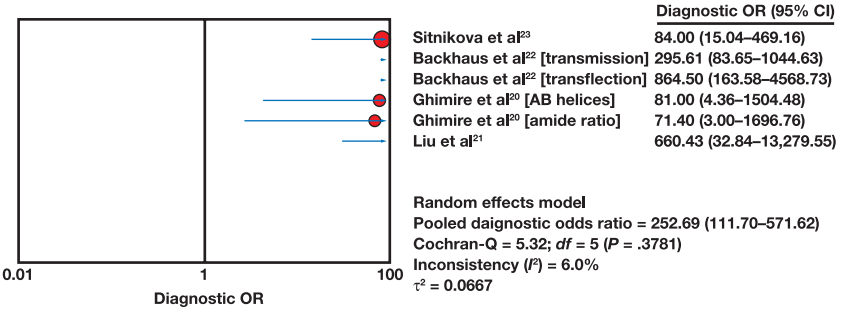
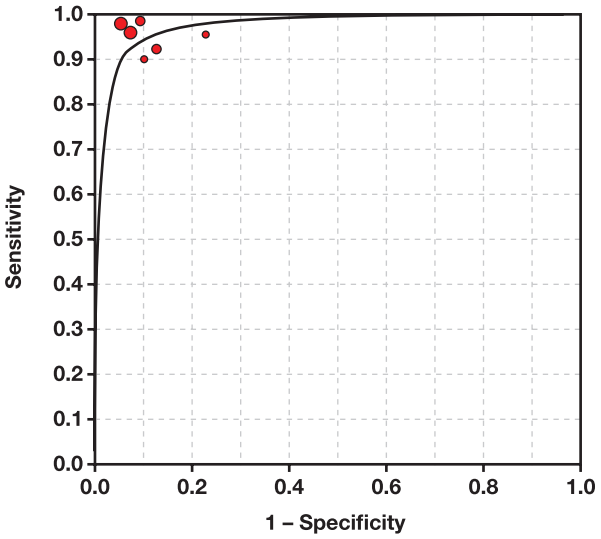


FIGURE 5. **Summary receiver operating characteristic curve depicting the diagnostic accuracy of Fourier transform infrared spectroscopy in detecting breast cancer using serum specimens.**



2-tailed unequal variance) in their spectral data and found discriminatory potential in the amides (1541–1656 cm) with prominent N-H bends. Liu et al[21] also observed significant variation between healthy people, patients with invasive ductal carcinoma (IDC), and patients without IDC in the 1539–1650 cm region, with infrared intensity being highest among healthy patient sera. Backhaus et al[22] performed a cluster analysis of 3 spectral regions, namely, the CH region (2800–3100 cm); the protein, C-C, and C-H deformation region (1300–1770 cm); and the C-O, P-O, and aromatic ring absorption (650–1200 cm) region. They inferred that the area between 684 and 1250 cm yielded the best forecast. They also used artificial neural networks (ANNs) with all measured spectra, which yielded similar results, wherein spectral changes were observed in the CH stretching vibrations (2853–2925 cm), the C-O ribose, the ribose backbone, and the P-O vibrations.

Currently, mammography is the most widely used tool in screening for breast cancer. However, several studies have shown that mammography yields a high rate of false positivity[26,27] and risk exposure through low-dose radiation given to misdiagnosed patients, causing unnecessary trauma and anxiety.[28] The risk of false-positive results from mammogram screening in European women undergoing biennial screening at ages 50–69 years is approximately 20%, and the risk of undergoing a biopsy due to a false-positive result is 3%.[27] Overall, the rate of overdiagnosis by mammography leading to overtreatment is 30%.[26]

Although mammography generally exposes patients to low levels of radiation that are not necessarily harmful, patients who are genetically at risk and with high disposition toward cancer are advised to avoid being exposed to ionizing radiation because the exposure can trigger carcinogenesis.[29,30] Also, the radiation dose is increased in patients with obesity and those with dense breast tissue.[31] Women who received false-positive results were also more likely to experience general anxiety than women with normal results.[28] They had lower scores on general and mental health but higher scores on depression than controls.[32] A report of a study among women in the United States who had received false-positive results noted that these women were more likely to see a mental health professional and scored higher on a standardized test on well-being, indicating increased psychological distress.[33]

Because none of the studies included in this meta-analysis compared benign and malignant breast tumors, and breast cancers with other cancer types, there is not enough evidence to say that FTIR spectroscopy can be used to screen for breast cancer. Our study findings, however, demonstrated that FTIR spectroscopy of serum specimens can be used as an additional test to confirm the presence of cancer.

One study[10] recommended that mammography is best paired with noninvasive liquid biopsies, such as miRNA profiles, as adjunct extensions for diagnosis. Liquid biopsy is a type of cancer detection test that utilizes body fluid specimens, which include but are not limited to blood, urine, and cerebrospinal fluid. These tests detect circulating tumor DNA (ctDNA), cell-free RNA, circulating tumor cells (CTCs), exosomes, and other cancer-derived elements found in bodily fluids.[34]

Current molecular technologies in liquid biopsies include digital polymerase chain reaction (PCR), RT-qPCR, mass spectrometry, and next-generation sequencing (NGS). However, there are several limitations to these methods. PCR is highly prone to bias from specimen extraction to the entire PCR process.[35] Further, it is labor-intensive and requires expensive reagents, and the procedures are only exclusive to detecting changes in tumor-derived, circulating nucleic acids.[36] Meanwhile, mass spectrometry can detect proteins and nucleic acids. However, proteomic analysis via this method is unsuitable for high-plex proteomes in liquid biopsy specimens.[37] Although NGS has a broader approach to genomic readings, much variation exists, and the turnaround time depends on the individual conducting the analysis and the equipment being used.[38]

Studies on FTIR-based liquid biopsy for breast cancer screening have shown optimal sensitivity and specificity values (>90%) and were determined to be cost-effective and associated with lower morbidity and

lower mortality rates due to early detection.[17] Further, it can capture signals from all biomolecules present in the liquid biopsy specimen through the infrared molecular fingerprint region.[39]

However, FTIR-based analysis of liquid biopsy specimens is susceptible to extraneous confounding variables during data acquisition and specimen handling. In particular, in the specimen-deposition method for dried specimens, fractionation of the specimen across the film causes significant variations in thickness and composition, which is termed the "coffee ring effect."[40] Intrinsic biological variations that differ between individual specimens, such as plasma concentrations and their subsequent composition (namely, lactate, carbonate, phosphate, and glucose) may also affect the spectroscopy readings.[41] An onco-IR-phenotype study has shown that breast, bladder, prostate, and lung cancers all had significantly different spectral signatures and were distinguishable.[39] However, sufficient data regarding the capacity of FTIR spectroscopy to distinguish different types of cancer via infrared spectral signatures in the fingerprint region remains to be determined and assessed.

To summarize, liquid biopsy methods show great promise in early cancer detection and can influence treatment options and improve therapeutic efficiency. However, molecular-based liquid biopsies are far too elaborate, compared with FTIR-based protocols, requiring costly equipment and highly trained staff to perform a comprehensive genomic profiling of patients. Hence, FTIR-based liquid biopsy can be a less-expensive and easier-to-perform alternative. Further, the use of FTIR spectroscopy as an adjunct method for breast cancer diagnosis can significantly reduce the subjectivity of histopathologists who rely on cancer-linked morphological changes in tissue specimens.[42]

As shown by this meta-analysis, the percentage of false-positive and false-negative diagnoses using FTIR spectroscopy in the selected studies was relatively smaller than that of the mammogram. This is a good indicator that FTIR spectroscopy has strong potential in breast cancer detection. However, considerable effort may be required before ATR-FTIR spectroscopy can be integrated in the clinical setting. Standardization of methods employed is currently lacking, as shown in the 4 included studies that used different ATR-FTIR techniques. Preanalytical factors may also produce or influence the results, such as blood specimen preparation and storage, which have been found to affect the generated spectra.[43] Studies on ensuring the quality of spectral data acquisition and reproducibility of spectroscopic analyses will be conducive to integrating ATR-FTIR spectroscopy in the clinical setting for breast cancer detection.

Even with the promising results presented, our study has several limitations. There were only a few studies on the topic, making it difficult to generate concrete conclusions from the synthesized data. The selected articles varied greatly in specimen size, and there is limited information on the timing of specimen collection and processing. The studies on this topic have so far only compared the serum specimens of clinically healthy donors and patients with breast cancer. Thus, future studies must address the aforementioned limitations and also compare between benign and malignant breast tumors, early-stage vs late-stage breast cancers, and breast cancers with other cancer types, to warrant the clinical application of FTIR spectroscopy using serum specimens to screen for breast cancer.

The present study could only assess the diagnostic capacity of FTIR spectroscopy in discriminating patients with breast cancer from clinically healthy individuals, using serum as the specimen type. Although the spectral data from the breast cancer cases were significantly different from those of the healthy donors, it is unknown whether these findings would be applicable for identifying patients with early-stage breast cancer.

The articles included in the meta-analysis contained minimal information on the stages of the studied breast cancer cases and the exact time point when the serum specimens were collected. One might infer that many of the cases involved late-stage disease based on the clinical information, such as having undergone partial mastectomy or having tested positive for lymph node metastasis. Therefore, future studies should focus on comparing the spectral data between early-stage and late-stage breast cancer to justify the clinical utility of FTIR spectroscopy as a less-invasive, inexpensive, but highly efficient screening tool for breast cancer.

Conclusion

To our knowledge, ours is the first study that synthesized the efficiency of FTIR spectroscopy as a potential tool in detecting breast cancer using serum specimens. Our results show that the pooled sensitivity of the FTIR spectroscopy was higher than 90%, demonstrating its promising potential in identifying breast cancer cases. Other statistical tests, such as the DOR across the selected studies, were optimal. The pooled negative LR indicated a low probability of testing negative in individuals with breast cancer, and the AUC curve value is high, indicating the good performance of the techniques.

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REFERENCES

1. Ntirenganya F, Twagirumukiza JD, Bucyibaruta G, Rugwizangoga B, Rulisa S. Premenopausal breast cancer risk factors and associations with molecular subtypes: a case-control study. *Int J Breast Cancer.* 2021;2021;5560559.
2. De Guzman BG, Cabaya NF, Ting FIL, Sandoval-Tan J. Factors influencing treatment decisions among breast cancer patients in the Philippine General Hospital Cancer Institute: Medical Oncology Outpatient Clinic. *Ann Oncol.* 2019;30:1–120.
3. Momenimovahed Z, Salehiniya H. Epidemiological characteristics of and risk factors for breast cancer in the world. *Breast Cancer: Targets Ther.* 2019;11:151–164.
4. Tao ZQ, Shi A, Lu C, et al. Breast cancer: epidemiology and etiology. *Cell Biochem Biophys.* 2015;72:333–338.
5. Sharma R. Breast cancer incidence, mortality and mortality-to-incidence ratio (MIR) are associated with human development, 1990–2016: evidence from Global Burden of Disease Study 2016. *Breast Cancer* 2019;26:428–445.
6. Johnson KC, Miller AB, Collishaw NE, et al. Active smoking and secondhand smoke increase breast cancer risk: the report of the Canadian Expert Panel on Tobacco Smoke and Breast Cancer Risk (2009). *Tobacco Control*. 2011;20(1):e2–e2.
7. Kolb TM, Lichy J, Newhouse JH. Comparison of the performance of screening mammography, physical examination, and breast US and evaluation of factors that influence them: an analysis of 27,825 patient evaluations. *Radiology*. 2002;225(1):165–175.
8. Lip G, Zakharova N, Duffy SW, Gillan MGC, Gilbert FJ. Breast density as a predictor of breast cancer risk. *Breast Cancer Res.* 2010;12(suppl 3):P1.
9. Berg WA. Beyond standard mammographic screening: mammography at age extremes, ultrasound, and MR imaging. *Radiol Clin North Am.* 2007;45(5):895–906.

10. Zubor P, Kubatka P, Kajo K, et al. Why the gold standard approach by mammography demands extension by multiomics? Application of liquid biopsy miRNA profiles to breast cancer disease management. *Int J Mol Sci.* 2019;20(22):2878.
11. Sala A, Anderson DJ, Brennan PM, et al. Biofluid diagnostics by FTIR spectroscopy: a platform technology for cancer detection. *Cancer Lett.* 2020;477:122–130.
12. Asmatulu R, Khan WS. Characterization of electrospun nanofibers. In: *Synthesis and Applications of Electrospun Nanofibers*. Elsevier; 2019:257–281.
13. Titus D, Samuel E, Roopan SM. *Nanoparticle Characterization Techniques*. Elsevier; 2019.
14. Lima C, Goulart V, Côrrea L, Pereira TM, Zezell DM. ATR-FTIR spectroscopy for the assessment of biochemical changes in skin due to cutaneous squamous cell carcinoma. *Int J Mol Sci.* 2015;16(4):6621–6630.
15. Su K-Y, Lee W-L. Fourier Transform Infrared spectroscopy as a cancer screening and diagnostic tool: a review and prospects. *Cancers (Basel).* 2020;12(1):115.
16. Shakya BR, Shrestha P, Teppo HR, Rieppo L. The use of Fourier Transform Infrared (FTIR) spectroscopy in skin cancer research: a systematic review. *Appl Spectrosc Rev.* 2020;56(5):1–33.
17. Freitas DLD, Câmara IM, Silva PP, et al. Spectrochemical analysis of liquid biopsy harnessed to multivariate analysis towards breast cancer screening. *Sci Rep.* 2020;10:1–8.
18. Yedema KA, Kenemans P, Wobbes T, et al. Carcinoma-associated mucin serum markers CA M26 and CA M29: efficacy in detecting and monitoring patients with cancer of the breast, colon, ovary, endometrium and cervix. *Int J Cancer.* 1991;47(2):170–179.
19. Zamora J, Abraira V, Muriel A, Khan K, Coomarasamy A. Meta-DiSc: a software for meta-analysis of test accuracy data. *BMC Med Res Methodol.* 2006;6:1–12.
20. Ghimire H, Garlapati C, Janssen EAM, et al. Protein conformational changes in breast cancer sera using infrared spectroscopic analysis. *Cancers* 2020;12(7):1708.
21. Liu J, Cheng H, Lv X, et al. Use of FT-IR spectroscopy combined with SVM as a screening tool to identify invasive ductal carcinoma in breast cancer. *Optik*. 2020;204:164255.
22. Backhaus J, Mueller R, Formanski N, et al. Diagnosis of breast cancer with infrared spectroscopy from serum samples. *Vib Spectrosc.* 2010;52(2):173–177.
23. Sitnikova VE, Kotkova MA, Nosenko TN, et al. Breast cancer detection by ATR-FTIR spectroscopy of blood serum and multivariate data-analysis. *Talanta*. 2020;214:120857.
24. Dees JJ, Higgins J, Altman DG. *Cochrane Handbook for Systematic Reviews of Interventions. Version 6.* Cochrane Library; 2021.
25. Jaeschke R, Guyatt G, Lijmer J. Diagnostic tests. In: *User's Guide to Medical Literature.* 3rd ed. American Medical Association; 2015:121–140.
26. Gøtzsche PC, Jørgensen KJ. Screening for breast cancer with mammography. *Cochrane Database of Systematic Reviews*. 2013;doi:10.1002/14651858.CD001877.pub5
27. Løberg M, Lousdal ML, Bretthauer M, Kalager M. Benefits and harms of mammography screening. *Breast Cancer Res.* 2015;17:1–12.
28. Salz T, Richman AR, Brewer NT. Meta-analyses of the effect of false-positive mammograms on generic and specific psychosocial outcomes. *Psycho-Oncology.* 2010;19(10):1026–1034.
29. Jansen-Van Der Weide MC, Greuter MJW, Jansen L, et al. Exposure to low-dose radiation and the risk of breast cancer among women with a familial or genetic predisposition: a meta-analysis. *Eur Radiol.* 2010;20(11):2547–2556.
30. Pauwels EKJ, Foray N, Bourguignon MH. Breast cancer induced by X-ray mammography screening? A review based on recent understanding of low-dose radiobiology. *Med Princ Pract.* 2016;25(2):101–109.
31. Miglioretti DL, Lange J, Van Den Broek JJ, et al. Radiation-induced breast cancer incidence and mortality from digital mammography screening a modeling study. *Ann Intern Med.* 2016;164(4):205–214.
32. Hafslund B, Espehaug B, Nortvedt MW. Effects of false-positive results in a breast screening program on anxiety, depression and health-related quality of life. *Cancer Nurs.* 2012;35(5):E26–E34.
33. Jatoi I, Zhu K, Shah M, Lawrence W. Psychological distress in U.S. women who have experienced false-positive mammograms. *Breast Cancer Res Treat.* 2006;100:191–200.
34. Lianidou E, Pantel K. Liquid biopsies. *Genes Chromosom Cancer.* 2019;58(4):219–232.
35. Castro-Giner F, Gkountela S, Donato C, et al. Cancer diagnosis using a liquid biopsy: challenges and expectations. *Diagnostics (Basel).* 2018;8(2):31.
36. Eastburn DJ, Sciambi A, Abate AR. Identification and genetic analysis of cancer cells with PCR-activated cell sorting. *Nucleic Acids Res.* 2014;42(16):e128.
37. Ding Z, Wang N, Ji N, Chen Z-S. Proteomics technologies for cancer liquid biopsies. *Mol Cancer.* 2022;21:1–11.
38. Tan O, Shrestha R, Cunich M, Schofield DJ. Application of next-generation sequencing to improve cancer management: a review of the clinical effectiveness and cost-effectiveness. *Clin Genet.* 2018;93(3):533–544.
39. Huber M, Kepesidis KV, Voronina L, et al. Infrared molecular fingerprinting of blood-based liquid biopsies for the detection of cancer. *Elife*. 2021;10:1–20.
40. Hughes C, Brown M, Clemens G, et al. Assessing the challenges of Fourier transform infrared spectroscopic analysis of blood serum. *J Biophotonics.* 2014;7(3-4):180–188.
41. Diem M. Comments on recent reports on infrared spectral detection of disease markers in blood components. *J Biophotonics.* 2018;11(7):1–6.
42. Mamede S, Mamede S, de Carvalho-Filho MA, et al. "Immunising" physicians against availability bias in diagnostic reasoning: a randomised controlled experiment. *BMJ Qual Saf.* 2020;29(7):550–559.
43. Lovergne L, Lovergne J, Bouzy P, et al. Investigating pre-analytical requirements for serum and plasma based infrared spectro-diagnostic. *J Biophotonics.* 2019;12(12):1–12.

Serum Leukocyte Cell-Derived Chemotaxin 2 (LECT2) Level Is Associated with Osteoporosis

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Keywords: LECT2, osteoporosis, bone mineral density, C-terminal telopeptide of type 1 collagen, 25-hydroxy-vitamin D, enzyme-linked immunosorbent assay

Abbreviations: LECT2, leukocyte cell-derived chemotaxin 2; NAFLD, non-alcoholic fatty liver disease; ELISA, enzyme linked immunosorbent assay; BMD, bone mineral density; IL-1, interleukin-1; IL-6, interleukin-6; TNF, tumor necrosis factor; M-CSF, macrophage colony-stimulating factor; hs-CRP, high-sensitivity C-reactive protein; PG, plasma glucose; ALT, alanine aminotransferase; AST, aspartate amino transferase; TC, total cholesterol; TG, triglycerides; P1NP, N-terminal propeptide of type 1 procollagen; OCN, osteocalcin; CTX, C-terminal telopeptide of type 1 collagen; 25-(OH)-D, 25-hydroxy-vitamin D.

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ABSTRACT

Objective: The aim of this study was to examine serum leukocyte cell-derived chemotaxin 2 (LECT2) levels in osteoporosis subjects to confirm its association with osteoporosis.

Methods: A total of 204 adult subjects were recruited. Bone mineral densities (BMD) were assessed and blood samples were collected for measurements of biomedical parameters and the bone turnover markers. Serum LECT2 levels were measured by enzyme-linked immunosorbent assay. The relationships between serum LECT2 levels and other parameters were analyzed using the Spearman correlation coefficient.

Results: Serum LECT2 levels were significantly increased in osteoporosis subjects over controls. We found a significantly negative correlation of serum LECT2 with BMD, 25-hydroxy-vitamin D, and creatinine and a significantly positive correlation with C-terminal telopeptide of type 1 collagen and total cholesterol.

Conclusion: Serum LECT2 levels were significantly upregulated in osteoporosis subjects and correlated with the severity of bone loss. Serum LECT2 could be a potential biomarker to assess the risk of bone loss.

Osteoporosis is a highly prevalent systemic bone metabolic disease that is caused by bone microstructure destruction.[1,2] It is characterized by bone mass reduction leading to increased bone fragility and fracture risk. Osteoporosis negatively affects the quality of life and leads to fracture. It has become a global problem affecting the health of an aging population.[3,4] Osteoporosis occurs when the connection between bone resorption and formation is impaired. Previous studies have showed that the immune system plays a pivotal role in bone homeostasis.[5] For example, inflammatory cytokines interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor (TNF), and macrophage colony-stimulating factor (M-CSF) tightly regulate osteoclastogenesis and bone resorption,[6–9] and their imbalance may lead to osteoporosis.

Leukocyte cell-derived chemotaxin 2 (LECT2), a 16 kDa chemotactic protein with multiple physiological functions, is mainly expressed in human hepatocytes and secreted into the blood.[10] Studies have reported that LECT2, as a regulator of immune and inflammatory response, plays a major role in various pathophysiological processes such as sepsis,[11] hepatitis,[12] arthritis,[13] and hepatocarcinoma.[14] LECT2 is also implicated in metabolic disorder diseases including obesity,[15] diabetes,[16] and non-alcoholic fatty liver disease (NAFLD).[17]

Recently, studies showed that hepatokines were involved in the complex interactions among bone, adipose tissue, and liver.[18–22] Additionally, it has been reported that after LECT2 stimulation, TNF secretion from CD209a$^+$ macrophages and osteolineage cells was downregulated in bone marrow.[23] Obesity and NAFLD are closely related to the occurrence of osteoporosis, and LECT2, as a multifunctional protein mainly secreted by hepatocytes, has been shown to be closely related to obesity and NAFLD, although the clinical significance of serum LECT2 in osteoporosis patients has been unavailable. Thus, we assume that LECT2 may also participate in the osteoporosis process. In this study, we evaluated the serum LECT2 levels, high-sensitivity C-reactive protein (hs-CRP), plasma glucose (PG), creatinine, uric acid, alanine aminotransferase (ALT), aspartate aminotransferase (AST), total cholesterol (TC), triglycerides (TG), N-terminal propeptide of type 1 procollagen (P1NP),

osteocalcin (OCN), C-terminal telopeptide of type 1 collagen (CTX), and 25-hydroxy-vitamin D (25-(OH)-D) in 104 subjects with osteoporosis and 100 control subjects. The relationship between LECT2, osteoporosis, and metabolic parameters was investigated.

Material and Methods

Subjects

From March 2019 to March 2021, a total of 204 adults visiting the Spine Department of the Affiliated Hospital of Medical School of Ningbo University were recruited. Subjects with malignant tumor, renal, liver, or respiratory dysfunction, liver cirrhosis, infection, inflammatory rheumatism, hyperthyroidism, hyperparathyroidism, and those using steroids or other drugs that could cause osteoporosis were excluded. Subjects had never received antiosteoporosis therapies such as salmon calcitonin, bisphosphonates, human parathyroid hormone, and activated vitamin D.

Subjects affected by osteoporosis (lumbar spine and/or femoral neck T score ≤−2.5) and control subjects with osteopenia (−2.5< T score <−1) and normal for these conditions (T score ≥−1) were recruited consecutively. The bone mineral density (BMD) of all subjects was assessed by dual-energy X-ray (DXA, Discovery Wii, Hologic) at the spine (L1-L4) and the hip (femoral neck). A control phantom was scanned every day. After admission, blood samples were collected after overnight fasting for measurements of hs-CRP (serum immunoturbidimetry method, Beckman IMMAGE 800), PG (serum hexokinase method, Beckman AU5800), ALT (serum ultraviolet method, Beckman AU5800), AST (serum ultraviolet method, Beckman AU5800), TC (serum cholesterol oxidase method, Beckman AU5800), TG (serum glycerol phosphate oxidase method, Beckman AU5800), creatinine (serum enzyme method, Beckman AU5800), uric acid (serum enzyme colorimetry method, Beckman AU5800), P1NP (serum electrochemiluminescence method, Roche Cobas E602), OCN (serum chemiluminescence method, Roche Cobas E602), CTX (serum electrochemiluminescence method, Roche Cobas E602), and 25-(OH)-D (serum chemiluminescence method, Roche Cobas E602). All clinical and metabolic data collected from all participants, including the osteopenia and normal groups, are shown in **TABLE 1**.

Blood samples were collected and stored at −80°C immediately from participants with osteoporosis and control subjects, including the osteopenia and normal groups, at admission. Subsequently, serum LECT2 levels were measured by a commercially available human LECT2 ELISA kit (R&D System) according to the manufacturer's protocol.

Written informed consent was obtained from all participants and all experiments were approved by the institutional review board of the Affiliated Hospital of Medical School of Ningbo University.

Statistical Analysis

All statistical analyses were performed using the IBM SPSS Statistics Version 22.0 (IBM). Mean and standard deviations were used for quantitative data analysis. The Student's *t*-test was used to analyze continuous variables. A 2-tailed unpaired Student's *t*-test was used to compare the serum LECT2 levels between osteoporosis and control subjects. The significance of difference was tested by ANOVA for comparisons between more than 2 groups. The relationships between serum LECT2 levels and biomedical parameters were analyzed using the Spearman correlation coefficient. *P* value less than .05 was considered statistically significant.

TABLE 1. Clinical and Metabolic Data of Osteoporosis Patients and Controls[a]

	Osteoporosis (n = 104)	Control (n = 100)	*P*
Age (y)	66.38 ± 7.16	64.72 ± 6.93	.095
Female (n)	81	57	
BMI (kg/m^2)	24.21 ± 1.85	24.30 ± 1.90	.719
Smoking (n)	18	31	.060
Bone facture (n)	65	6	.000
Lumbar BMD (T-score)	−3.13 ± 0.85	−0.47 ± 1.21	.000
Femoral neck BMD (T-score)	−2.14 ± 0.90	−0.62 ± 0.86	.000
Hs-CRP (mg/L)	1.55 ± 0.18	1.64 ± 0.28	.139
ALT (U/L)	19.63 ± 7.45	20.66 ± 8.74	.368
AST (U/L)	20.73 ± 5.27	21.39 ± 6.35	.420
PG (mmol/L)	5.47 ± 0.90	5.61 ± 1.18	.356
TC (mmol/L)	4.61 ± 0.93	4.52 ± 0.98	.495
TG (mmol/L)	1.22 ± 0.54	1.53 ± 0.68	.001
Creatinine (μmol/L)	56.90 ± 13.98	67.99 ± 12.90	.000
Uric acid (μmol/L)	303.33 ± 70.46	319.25 ± 75.33	.120
P1NP (ng/mL)	49.99 ± 10.00	41.14 ± 10.62	.000
OCN (ng/mL)	16.53 ± 8.44	13.50 ± 4.19	.001
CTX (ng/mL)	0.47 ± 0.21	0.28 ± 0.09	.000
25-(OH)-D (ng/mL)	17.14 ± 5.02	22.93 ± 5.24	.000
LECT2 (ng/mL)	30.51 ± 15.57	19.39 ± 9.19	.000

25-(OH)-D, 25-hydroxy-vitamin D; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMD, bone mineral density; BMI, body mass index; CTX, C-terminal telopeptide of type 1 collagen; hs-CRP, high-sensitivity C-reactive protein; LECT2, leukocyte cellderived chemotaxin 2; OCN, osteocalcin; P1NP, N-terminal propeptide of type 1 procollagen; PG, plasma glucose; TC, total cholesterol; TG, triglycerides.
[a]*Data are means ± standard deviations.*

Results

Serum LECT2 levels were significantly higher in osteoporosis subjects than controls (30.51 ± 15.57 ng/mL vs 19.39 ± 9.19 ng/mL, $P < .01$; **TABLE 1**), as well as P1NP, OCN, and CTX, whereas creatinine, uric acid, and 25-(OH)-D serum levels were significantly lower in osteoporosis subjects than in controls (**TABLE 1**).

Considering the severity of bone loss measured according to T-scores, subjects were divided into 3 groups: normal (n = 44), osteopenia (n = 56), and osteoporosis (n = 104). Although higher serum LECT2 levels were found in the osteopenia group than in the normal group (21.63 ± 10.10 ng/mL vs 16.54 ± 6.99 ng/mL, $P = .12$), no statistically significant differences were detected. There were significantly higher LECT2 levels in the osteoporosis group compared with the normal group ($P < .01$) and the osteopenia group ($P < .01$; **FIGURE 1**).

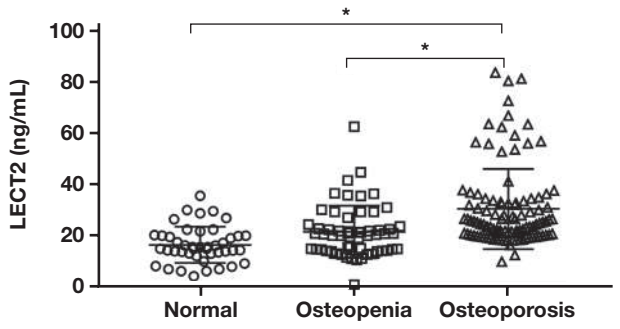
There was a significantly negative correlation in all subjects between serum levels of LECT2 and lumbar BMD ($r = −0.436$, $P < .001$; **FIGURE 2A**), as well as between serum levels of LECT2 and femoral neck BMD ($r = −0.292$, $P < .001$; **FIGURE 2B**). A significantly positive correlation in all subjects was observed between serum levels of LECT2 and CTX ($r = 0.581$, $P < .001$; **FIGURE 3A**), but a significantly negative

correlation was found between serum levels of LECT2 and 25-(OH)-D (r = −0.164, P < .019; **FIGURE 3B**). Serum LECT2 and TC were significantly positively correlated (r = 0.247, P < .001; **FIGURE 3C**), whereas creatinine showed a significantly negative correlation with serum LECT2 levels (r = −0.358, P < .001; **FIGURE 3D**).

Discussion

In this study, we found that the levels of serum LECT2 were higher in participants with osteoporosis than in control subjects. LECT2 is a hepatokine mainly expressed in hepatocytes and endothelial cells.[24] It has been reported to be an immune modulatory factor in inflammatory arthritis,[25] bacterial sepsis, renal amyloidosis,[11,26,27] hepatitis, and hepatic carcinogenesis. Some metabolic syndromes, such as obesity and NAFLD, have been described to be associated with osteoporosis via hepatokines. Yoo et al[17] reported an increase in plasma LECT2 in a NAFLD group compared with control subjects (31.2 ng/mL vs 24.5 ng/mL). Tanisawa et al[28] showed that participants with dyslipidemia had higher levels of plasma LECT2 than those without dyslipidemia, which is consistent with different LECT2 levels between participants with or without metabolic syndrome. Our data suggested that serum LECT2 level is related to osteoporosis, which is consistent with previously reported results of other diseases, including obesity, fatty liver, diabesity,[15] insulin resistance,[29] atherosclerosis,[30] and osteoarthritis.[31]

FIGURE 1. Concentration of serum leukocyte cell-derived chemotaxin 2 (LECT2) in normal (n = 44), osteopenia (n = 56), and osteoporosis groups (n = 104). *P < .01.



We also found that the levels of serum LECT2 were significant negatively associated with lumbar and femoral neck BMD. Additionally, the concentration of serum LECT2 decreased in the order of osteoporosis, osteopenia, and normal groups, although the difference between the osteopenia and normal groups was not statistically significant. The results suggest that serum LECT2 level might be closely related to the severity of osteoporosis.

Our clinical results suggest that LECT2 participates in bone resorption and osteoporosis development. In this study, we found a significant positive correlation between LECT2 and the resorption marker CTX, which seems to confirm that LECT2 enhances osteoclast activity, although the direct effect of LECT2 on osteoclast function is unclear. It was considered that immune cells and osteoclasts share some similar regulatory molecules.[5–7] Hwang et al[30] reported that LECT2 increased the transcript levels of proinflammatory cytokines such as TNF-α and IL-1β in human umbilical vein endothelial cells and human acute monocytic leukemia cells. Similarly, Jung[32] demonstrated that LECT2 enhanced inflammation markers such as IL-6 and TNF-α expression. Zhao et al[33] reported that serum LECT2 levels were related to serum IgE in atopic dermatitis patients, a chronic skin inflammation.

The 25-(OH)-D is generally recognized to promote calcium absorption. In fact, 25-(OH)-D is also closely related to inflammation. For instance, the decreased level of 25-(OH)-D was reported to enhanced inflammatory and stress responses in autoimmune hepatitis patients, and the 25-(OH)-D level was negatively correlated with TNF-α.[34] Wang et al[35] also demonstrated that 25-(OH)-D decreased in diabetic patients with the increase of inflammatory factors such as IL-6 and TNF-α. In this study, we also found a significantly negative correlation between LECT2 and 25-(OH)-D. It is reasonable to assume that LECT2 may play a role in bone resorption and osteoporosis.

TC, creatinine, and other metabolic parameters can reflect the physiological state of human liver and kidney. Okumura et al[15] demonstrated that LECT2 levels correlated with total cholesterol in obesity patients positively. Similarly, in this study, we found that LECT2 levels were significant positively associated with TC. Kan et al[36] reported that higher serum TC levels were associated with greater risk of osteoporosis. Higher lipid levels were associated with higher oxidative stress levels, which could inhibit osteoblast differentiation.[37] Additionally, we found

FIGURE 2. Correlation between serum levels of leukocyte cell-derived chemotaxin 2 (LECT2) and bone mineral density (BMD) in all subjects. A, The correlation between LECT2 and lumbar BMD (r = −0.436, P < .001). B, The correlation between LECT2 and femoral neck BMD (r = −0.292, P < .001).

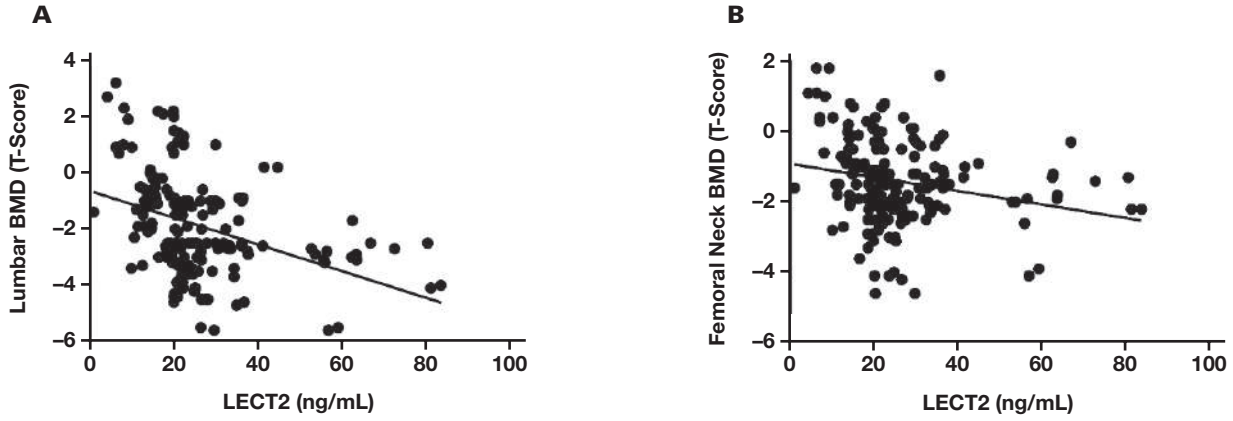
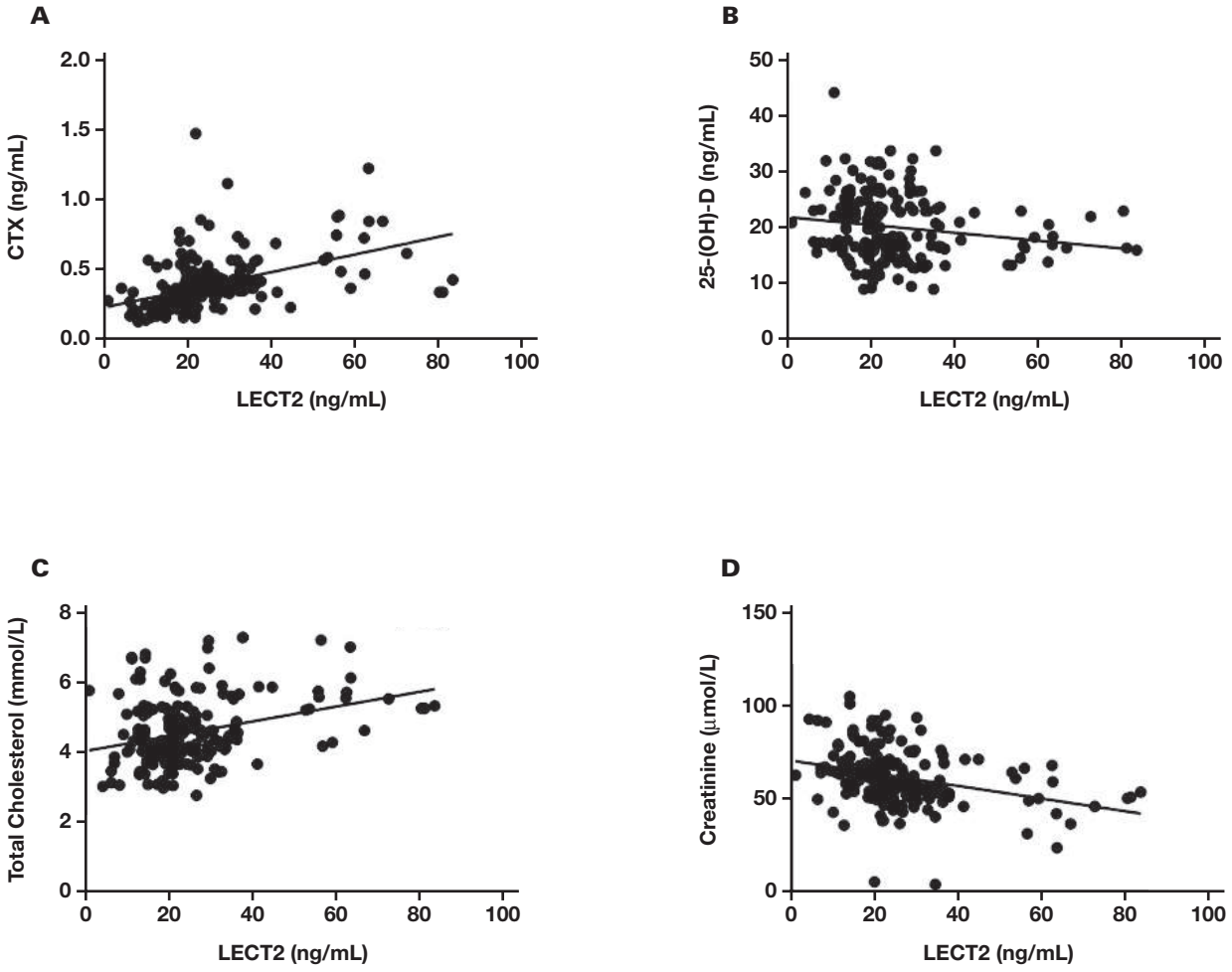


FIGURE 3. **Correlation between serum levels of LECT2 and the biochemical of bone turnover and other parameters in all subjects. A, The correlation between LECT2 and C-terminal telopeptide of type 1 collagen (CTX) (r = 0.581, P < .001). B, The correlation between LECT2 and 25-(OH)-D (r = –0.164, P = .019). C, The correlation between LECT2 and total cholesterol (r = 0.247, P < .001). D, The correlation between LECT2 and creatinine (r = –0.358, P < .001).**



that LECT2 levels were significant negatively associated with creatinine, and lower serum creatinine levels were detected in subjects with osteoporosis. Similarly, Cui et al[38] demonstrated that serum creatinine levels were lower in osteoporosis subjects than in controls. Huh et al[39] also found that serum creatinine was positively associated with BMD in 8648 participants with normal kidney function, and subjects with low serum creatinine have a higher risk for low BMD. The possible explanation is that serum creatinine served as a marker of muscle mass, and low skeletal muscle mass was associated with deterioration of BMD. The cause of the relationship between LECT2 and creatinine needs to be further explored.

Several limitations of the present study should be considered. The first limitation was that the sample size was relatively small in this single-center study. The result of this research should be verified by a multiple-center study with a larger sample size. Second, because of the different morbidity of osteoporosis in males and females, there was a difference in the number of females between the osteoporosis and control groups in our study. Although there was no difference between males and females in the control group, and scores were the same as those for males and females in the osteoporosis group after statistical analysis, the sex effect should be further studied. Third, we did not confirm the cause-effect relationship between serum LECT2 levels and osteoporosis. The mechanism should be further studied by animal experiments and molecular biology experiments.

In conclusion, we describe an increased serum LECT2 level in osteoporosis subjects and found it was related to the severity of bone loss. We also found that LECT2 levels were significant positively associated with CTX and negatively associated with 25-(OH)-D. The serum LECT2 level could be a potential biomarker to assess the risk of bone loss. However, due to the limited number of participants in this study, further large-scaled perspective studies are needed, and the pathological role of LECT2 in the development of osteoporosis should be investigated.

Acknowledgments

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REFERENCES

1. Ensrud KE, Crandall CJ. Osteoporosis. *Ann Intern Med.* 2017;167(3):ITC17–ITC32. doi:10.7326/aitc201708010.
2. Wang Y, Tao Y, Hyman ME, et al. Osteoporosis in China. *Osteoporos Int.* 2009;20(10):1651–1662. doi:10.1007/s00198-009-0925-y.
3. Sanders S, Geraci SA. Osteoporosis in postmenopausal women: considerations in prevention and treatment: (women's health series). *South Med J.* 2013;106(12):698–706. doi:10.1097/SMJ.0b013e3182a0df8b.
4. Hernlund E, Svedbom A, Ivergard M, et al. Osteoporosis in the European Union: medical management, epidemiology and economic burden. A report prepared in collaboration with the International Osteoporosis Foundation (IOF) and the European Federation of Pharmaceutical Industry Associations (EFPIA). *Arch Osteoporos.* 2013;8(1):136. doi:10.1007/s11657-013-0136-1.
5. Ginaldi L, De Martinis M. Osteoimmunology and beyond. *Curr Med Chem.* 2016;23(33):3754–3774. doi:10.2174/0929867323666160907162546.
6. Souza PP, Lerner UH. The role of cytokines in inflammatory bone loss. *Immunol Invest.* 2013;42(7):555–622. doi:10.3109/08820139.2013.822766.
7. Takayanagi H. Osteoimmunology and the effects of the immune system on bone. *Nat Rev Rheumatol.* 2009;5(12):667–676. doi:10.1038/nrrheum.2009.217.
8. Zhang Q, Guo R, Schwarz EM, et al. TNF inhibits production of stromal cell-derived factor 1 by bone stromal cells and increases osteoclast precursor mobilization from bone marrow to peripheral blood. *Arthritis Res Ther.* 2008;10(2):R37. doi:10.1186/ar2391.
9. Brincat SD, Borg M, Camilleri G, et al. The role of cytokines in postmenopausal osteoporosis. *Minerva Ginecol.* 2014;66(4):391–407.
10. Yamagoe S, Yamakawa Y, Matsuo Y, et al. Purification and primary amino acid sequence of a novel neutrophil chemotactic factor LECT2. *Immunol Lett.* 1996;52(1):9–13. doi:10.1016/0165-2478(96)02572-2.
11. Lu XJ, Chen J, Yu CH, et al. LECT2 protects mice against bacterial sepsis by activating macrophages via the CD209a receptor. *J Exp Med.* 2013;210(1):5–13. doi:10.1084/jem.20121466.
12. Saito T, Okumura A, Watanabe H, et al. Increase in hepatic NKT cells in leukocyte cell-derived chemotaxin 2-deficient mice contributes to severe concanavalin A-induced hepatitis. *J Immunol.* 2004;173(1):579–585. doi:10.4049/jimmunol.173.1.579.
13. Graessler J, Verlohren M, Graessler A, et al. Association of chondromodulin-II Val58Ile polymorphism with radiographic joint destruction in rheumatoid arthritis. *J Rheumatol.* 2005;32(9):1654–1661.
14. Okabe H, Delgado E, Lee JM, et al. Role of leukocyte cell-derived chemotaxin 2 as a biomarker in hepatocellular carcinoma. *PLoS One.* 2014;9(6):e98817. doi:10.1371/journal.pone.0098817.
15. Okumura A, Unoki-Kubota H, Matsushita Y, et al. Increased serum leukocyte cell-derived chemotaxin 2 (LECT2) levels in obesity and fatty liver. *Biosci Trends.* 2013;7(6):276–283.
16. Zhang Z, Zeng H, Lin J, et al. Circulating LECT2 levels in newly diagnosed type 2 diabetes mellitus and their association with metabolic parameters: an observational study. *Medicine (Baltimore).* 2018;97(15):e0354. doi:10.1097/MD.0000000000010354.
17. Yoo HJ, Hwang SY, Choi JH, et al. Association of leukocyte cell-derived chemotaxin 2 (LECT2) with NAFLD, metabolic syndrome, and atherosclerosis. *PLoS One.* 2017;12(4):e0174717. doi:10.1371/journal.pone.0174717.
18. Guanabens N, Pares A. Osteoporosis in chronic liver disease. *Liver Int.* 2018;38(5):776–785. doi:10.1111/liv.13730.
19. Handzlik-Orlik G, Holecki M, Wilczynski K, et al. Osteoporosis in liver disease: pathogenesis and management. *Ther Adv Endocrinol Metab.* 2016;7(3):128–135.
20. Filip R, Radzki RP, Bienko M. Novel insights into the relationship between nonalcoholic fatty liver disease and osteoporosis. *Clin Interv Aging.* 2018;13:1879–1891.
21. Pirgon O, Bilgin H, Tolu I, et al. Correlation of insulin sensitivity with bone mineral status in obese adolescents with nonalcoholic fatty liver disease. *Clin Endocrinol (Oxford).* 2011;75(2):189–195. doi:10.1111/j.1365-2265.2011.04038.x.
22. Musso G, Paschetta E, Gambino R, et al. Interactions among bone, liver, and adipose tissue predisposing to diabesity and fatty liver. *Trends Mol Med.* 2013;19(9):522–535. doi:10.1016/j.molmed.2013.05.006.
23. Lu XJ, Chen Q, Rong YJ, et al. LECT2 drives haematopoietic stem cell expansion and mobilization via regulating the macrophages and osteolineage cells. *Nat Commun*. 2016;7:12719. doi:10.1038/ncomms12719
24. Xu M, Xu HH, Lin Y, et al. LECT2, a ligand for Tie1, plays a crucial role in liver fibrogenesis. *Cell.* 2019;178(6):1478–1492.e20. doi:10.1016/j.cell.2019.07.021.
25. Okumura A, Saito T, Otani I, et al. Suppressive role of leukocyte cell-derived chemotaxin 2 in mouse anti-type II collagen antibody-induced arthritis. *Arthritis Rheum.* 2008;58(2):413–421. doi:10.1002/art.23215.
26. Li DY, Liu D, Wang SX, et al. Renal leukocyte chemotactic factor 2 (ALECT2)-associated amyloidosis in Chinese patients. *Amyloid.* 2020;27(2):134–141. doi:10.1080/13506129.2020.1722097.
27. Mejia-Vilet JM, Cardenas-Mastrascusa LR, Palacios-Cebreros EJ, et al. LECT2 Amyloidosis in kidney transplantation: a report of 5 cases. *Am J Kidney Dis.* 2019;74(4):563–566. doi:10.1053/j.ajkd.2018.10.016.
28. Tanisawa K, Taniguchi H, Sun X, et al. Visceral fat area is a strong predictor of leukocyte cell-derived chemotaxin 2, a potential biomarker of dyslipidemia. *PLoS One.* 2017;12(3):e0173310. doi:10.1371/journal.pone.0173310.
29. Lan F, Misu H, Chikamoto K, et al. LECT2 functions as a hepatokine that links obesity to skeletal muscle insulin resistance. *Diabetes.* 2014;63(5):1649–1664. doi:10.2337/db13-0728.
30. Hwang HJ, Jung TW, Hong HC, et al. LECT2 induces atherosclerotic inflammatory reaction via CD209 receptor-mediated JNK phosphorylation in human endothelial cells. *Metabolism.* 2015;64(9):1175–1182. doi:10.1016/j.metabol.2015.06.001.
31. Ikeda D, Ageta H, Tsuchida K, et al. iTRAQ-based proteomics reveals novel biomarkers of osteoarthritis. *Biomarkers.* 2013;18(7):565–572. doi:10.3109/1354750X.2013.810667.
32. Jung TW, Chung YH, Kim HC, et al. LECT2 promotes inflammation and insulin resistance in adipocytes via P38 pathways. *J Mol Endocrinol.* 2018;61(1):37–45. doi:10.1530/JME-17-0267.
33. Zhao K, Xu F, Jiang X, et al. Serum leukocyte cell-derived chemotaxin 2 level is associated with atopic dermatitis patients. *Ann Palliat Med.* 2021;10(10):11006–11012. Doi:10.21037/apm-21-2690.
34. Tao S, Zhang H, Zhao Q, et al. Correlation of vitamin D with inflammatory factors, oxidative stress and T cell subsets in patients with autoimmune hepatitis. *Exp Ther Med.* 2020;19(5):3419–3424. doi:10.3892/etm.2020.8601.
35. Wang JM, Ye SD, Li SM, et al. Correlations of 25(OH)D level with blood lipid, inflammatory factors and vascular endothelial function in

diabetic patients. *Eur Rev Med Pharmacol Sci.* 2018;22(3):731–735. doi:10.26355/eurrev_201802_14303.

36. Kan B, Zhao Q, Wang L, et al. Association between lipid biomarkers and osteoporosis: a cross-sectional study. *BMC Muscloskelet Disord.* 2021;22(1):759.
37. Yamaguchi T, Sugimoto T, Yano S, et al. Plasma lipids and osteoporosis in postmenopausal women. *Endocr J.* 2002;49(2):211–217. doi:10.1507/endocrj.49.211.
38. Cui R, Zhou L, Li Z, et al. Assessment risk of osteoporosis in Chinese people: relationship among body mass index, serum lipid profiles, blood glucose, and bone mineral density. *Clin Interv Aging.* 2016;11:887–895. doi:10.2147/CIA.S103845.
39. Huh JH, Choi SI, Lim JS, et al. Lower serum creatinine is associated with low bone mineral density in subjects without overt nephropathy. *PLoS One.* 2015;10(7):e0133062. doi:10.1371/journal.pone.0133062.

SPECIAL REPORT

Compliance with the Current NCCN Guidelines and Its Critical Role in Pancreatic Adenocarcinoma

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Abbreviations: NCCN, National Comprehensive Cancer Network; MSI/MMR, microsatellite instability/mismatch repair; HRR, homologous recombination repair; PARP, poly(adenosine diphosphate-ribose) polymerase; EUS-FNA, endoscopic ultrasound guided fine needle aspiration.

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ABSTRACT

Objectives: Since 2019, the National Comprehensive Cancer Network (NCCN) has recommended genetic testing for patients diagnosed with pancreatic adenocarcinoma that includes universal germline testing and tumor gene profiling for metastatic, locally advanced, or recurrent disease. However, testing compliance with this guideline has not yet been published in the English literature.

Methods: A quality assurance/quality improvement retrospective review was done to identify patients diagnosed with pancreatic adenocarcinoma from January 2019 to February 2021 to include the patient's clinical status and genetic test results.

Results: There were 20 patient cases identified with pancreatic adenocarcinoma. A total of 11 cases had molecular tumor gene profiling and microsatellite instability/mismatch repair (MSI/MMR) testing performed and 1 case had only MSI/MMR testing by immunohistochemistry performed. Only 3 patients of the 20 in total received germline testing.

Conclusion: There was a significant number of patients for whom tumor gene profiling or germline testing had never been attempted as per recommended NCCN guidelines.

In 2019, the National Comprehensive Cancer Network (NCCN) recommended germline testing for all patients diagnosed with pancreatic adenocarcinoma and also recommended tumor gene profiling along with microsatellite instability/mismatch repair (MSI/MMR) testing in all patients with metastatic, recurrent, or locally advanced pancreatic adenocarcinoma.[1,2] Germline testing involves testing of nontumor tissue (such as peripheral blood) with comprehensive gene panels for hereditary cancer syndromes.[1] Tumor gene profiling (performed preferentially on tumor tissue but also can be performed on tumor cell-free DNA in peripheral blood) involves genetic testing of tumors to identify mutations, particularly actionable somatic mutations.[1] MSI/MMR testing is testing to determine MSI/MMR status, whether performed as part of tumor gene profiling or performed to determine protein expression using an immunohistochemical panel. These recommendations are retained in the latest NCCN guidelines, version 2.2021, for pancreatic adenocarcinoma.[1]

Germline tests as recommended by the NCCN guidelines are helpful, as it is known that up to 20% of patients have cancer predisposition genes that would be important to detect for the guidance of therapy and management, particularly given that pancreatic adenocarcinoma remains one of the most lethal cancers.[3–5] The identification of germline mutations has implications for both potential genetic counseling for relatives and in treatment of the pancreatic adenocarcinoma, particularly if the germline mutation identified suggests the tumor may be susceptible to a particular regimen.[3,4] In addition, it is important to note that germline testing has an additional diagnostic role for tumor genetic findings even if tumor gene profiling had been performed.[6] Lincoln et al[6] suggest that germline testing could potentially detect pathogenic genetic variants for the patient's malignancy that were missed by direct tumor gene profiling in 8.1% of cases. Therefore, the performance of germline testing would be useful not only for genetic predisposition counseling of family members but also in guiding management and tumor gene profiling.

Molecular tests for tumor gene profiling would identify the mutations present within the patient's pancreatic adenocarcinoma that could then be targeted by precision medicine, a paradigm shift from "one size fits all" to targeted therapy.[7–14] Taghizadeh et al[7] found that 28% of pancreatic adenocarcinoma patients in their study were able to receive targeted therapy through a clinical trial. This percentage could increase over time as advances are made in precision medicine for pancreatic adenocarcinoma.[7] Furthermore, it is known that certain mutations (such as those in *BRCA1/2*, *PALB2*, or other homologous recombination repair [HRR]

mutations) suggest a sensitivity to platinum-based agents.[1,15–17] This sensitivity may guide initial chemotherapy decisions by either strongly supporting the treatment decision to start with a platinum-based therapy, if such mutations are present, or making an alternative, less toxic, gemcitabine-based therapy a consideration, if such mutations are absent, in the right clinical context.[16,17] Selection of less toxic therapy has the ability to positively affect the quality of the patient's life in the appropriate clinical setting. Additional germline findings for *BRCA1/2* or *PALB2* appear to play a role in determining who may benefit from maintenance treatment with a poly(adenosine diphosphate-ribose) polymerase (PARP) inhibitor in the case of metastatic disease.[1,16] Therefore, genetic results provide potential guidance for both precision medicine and selection of chemotherapy not otherwise directly targeted to a particular mutation.[15–17]

Previously, it had been observed that noncompliance with NCCN guidelines, such as the treatment recommendations in 2012 for pancreatic cancer, results in worse patient outcomes.[18] There was a study monitoring the relative infrequency of germline testing prior to the NCCN 2019 guidelines recommending universal germline testing of patients with pancreatic adenocarcinoma.[19] However, there has been no study, to the authors' knowledge, about compliance with NCCN guidelines on genetic germline testing or tumor gene profiling. Although there is an important element of individual judgment and decision-making autonomy for each patient case, the heterogeneity in following the NCCN guidelines remains important because NCCN guideline compliance correlates with patient outcomes.[18] This heterogeneity can be motivated by a number of reasons that were not included in the NCCN guidelines, including but not limited to cost, insurance coverage, advanced stage of disease beyond ability to treat, and provider or patient preferences.[1] Here, we present the first quality assurance and improvement study on NCCN testing guideline compliance for patients with pancreatic adenocarcinoma.

Methods

As part of quality assurance and improvement, a retrospective review was performed to identify cases diagnosed as pancreatic adenocarcinoma from all surgical pathology and cytology cases to form a database from January 2019 to February 2021 at a single regional Veteran Affairs Medical Center. For each patient, a review of demographic information, germline test results, molecular test results, MSI/MMR testing, initial chemotherapy choice, and clinical follow-up status was performed. For tumor gene profiling, a record was made of which type of specimen (circulating tumor DNA in blood vs cytology and surgical pathology) was used for the tumor gene profiling. Diagnosis for all cases of pancreatic adenocarcinoma had been reviewed independently by 2 pathologists. The presence of a surgical pathology report would indicate the presence of a biopsy or resection of the tumor in that patient's case. The presence of a cytology report would indicate that there exists a cytology specimen from the tumor (such as from a fine needle aspiration of the tumor). The test results were also recorded for each patient. Paraffin blocks and slides were retained for each patient and were available for additional quality assurance or improvement investigation.

Unstained tumor-containing slides from the cell block or surgical specimen were sent to Foundation Medicine, Inc. (Cambridge, MA) for Tumor gene profiling studies. A pathologist was informed for each tumor gene profiling request and was involved in selecting the best specimen block to send out for this study, ideally a block with a tissue surface area of 25 mm^2 and a tumor content of at least 20% tumor nuclei.[20] Germline testing was sent to Invitae (San Francisco CA) to test for cancer predisposition genes on blood or saliva samples. Immunohistochemical studies for MSI/MMR testing were performed by Integrated Oncology (New York, NY). All tests were performed at the request of the clinician.

For pancreatic endoscopic ultrasound guided fine needle aspiration (EUS-FNA) samples, air-dried smears stained with Diff-Quik (Cancer Diagnostics, Durham, NC), alcohol-fixed slides stained by Papanicolaou staining (Epredia Richard-Allan Scientific, Kalamazoo, MI), and additional samples collected in CytoLyt Solution (Hologic, Marlborough, MA) for both liquid-based preparation (ThinPrep) and cell block preparation were used. Hematoxylin and eosin-stained slides from cell blocks and surgical specimens are stained using the Sakura Tissue Tek Prisma (Torrance, CA).

Data was tabulated on Microsoft Excel (Redmond, WA) for comparison and analysis. Statistical calculations by the χ^2 distribution statistic were performed using the appropriate formula function (CHITEST) in Microsoft Excel.

Results

There were 20 patient cases identified with pancreatic adenocarcinoma from January 2019 to February 2021. These 20 patient cases included 12 African American patients (60%), 7 Caucasian American patients (35%), and 1 patient with mixed ethnicity (5%). The ages of these patients ranged from 51 to 80 years old. The average age was 68 with a median of 69 years. All patients were male. Of the 20 patients, 12 (60%) received at least some of the NCCN-recommended testing, consisting of either pancreatic tumor gene profiling or germline testing. The ethnicity of this tested population of 12 was 10 African Americans (83%) and 2 Caucasian Americans (17%). The age range of the tested population was 51 to 80 years of age (average age 65 years). African Americans were the majority in both the tested population (10 of 12 or 83%) and the total population (12 or 20 or 60%) without a statistically significant difference by the χ^2 distribution statistic.

Of these 12 patients (see **TABLE 1**) only 7 had cytology specimens from EUS-FNA for acquisition of pancreatic tumor tissue for diagnosis, on which the molecular testing was performed. Of the 12 patients, 4 had both cytology and surgical specimens, from which 3 had molecular testing performed on the surgical pathology specimen (1 of these 3 also had an additional liquid biopsy for tumor circulating DNA in the peripheral blood) and the remaining 1 patient of the 4 had molecular testing performed on the cytology specimen. There was 1 patient of the 12 who had only a surgical pathology specimen that was subsequently sent for tumor gene profiling. Of these 12 patients, 11 had tumor gene profiling performed at Foundation Medicine (Cambridge, MA) that also included MSI/MMR testing by PCR (**TABLE 1**) and 1 patient with only a cytology tumor specimen had only the MSI/MMR testing by immunohistochemistry. Examination of the cytology paraffin block from the 1 patient with only MSI/MMR testing by immunohistochemistry revealed only limited tissue, which may have limited the extent of the tumor mutational testing. Of these 12 tested patients, 11 were determined to be MSI/MMR stable and 1 was undetermined without additional orthogonal testing (such as an immunohistochemical panel) to confirm this result. All the patients who underwent tumor gene profiling had identifiable mutations (see **TABLE 1**). There were 2 of

the 12 patients (specifically patient 3, gene mutation in *FANCA*, and patient 11, gene mutation *ATM*, shown in **TABLE 1**) who had HRR gene mutations. Only 3 of the 20 patients (15%) had germline testing. Although no cancer predisposition genes were found on germline testing, the tumor gene profiling of all these patients demonstrated identifiable mutations (see **TABLE 1**). There were no patients who received only germline testing without tumor gene profiling. The remaining 8 of 20 patients (40%) without any attempted molecular testing included 3 patients with only cytology specimens, 4 patients with both cytology and surgical pathology

TABLE 1. Tabulation of the 12 Patients Who Underwent Some of the NCCN Recommended Testing to List Germline and Tumor Molecular Profile Results for Advanced Pancreatic Adenocarcinoma

Patient #	Germline Results	Cytology and/or Surgical Tumor Specimen	Ancillary Tests on Cytology vs Surgical Specimen	Mutations Identified	HRR Mutation Present	Microsatellite Status
1	Negative	Both types of specimen	Surgical specimen	*CCNE1* amplification *KRAS* G12V *SMAD4* L104F *SMAD4* E417fs*11 *TP53* R273H	No	Stable
2	Not done	Both types of specimen	Cytology specimen	*CCNE1* amplification *CDK6* amplification *CDKN2A* CDKN2A(NM_000077)-FLJ35282(NR_038977) fusion (C2:F5) *CDKN2B* CDKN2B(NM_004936) rearrangement exon 2 *ERBB2* amplification *GATA6* amplification *HGF* amplification *KRAS* G12V *SMAD4* loss *TP53* H214R	No	Stable
3	Not done	Both types of specimen	Surgical specimen	*AKT2* amplification *FANCA* S1064fs*15 *FGFR4* amplification *GATA6* amplification *KRAS* G12D TP53I195T	Yes	Stable
4	Not done	Cytology specimen only	Cytology specimen only	*CDKN2A* Y44* *KRAS* G12D *MAP2K4* R287H *TP53* loss	No	Stable
5	Not done	Cytology specimen only	Cytology specimen only	*CDKN2A* loss *CDKN2B* loss *KRAS* G12R *SMAD4* R361H *TP53* R196	No	Stable
6	Negative	Both types of specimen	Surgical specimen	*KRAS* G12V *MAP2K4* R154W *TP53* R273H	No	Undetermined

TABLE 1. Continued

Patient #	Germline Results	Cytology and/or Surgical Tumor Specimen	Ancillary Tests on Cytology vs Surgical Specimen	Mutations Identified	HRR Mutation Present	Microsatellite Status
7	Negative	Cytology specimen only	Cytology specimen only	*CDKN2A* loss *CDKN2B* loss *FGFR1* amplification *KRAS* G12V *MTAP* loss *NSD3 (WHSC1L1)* amplification *SMAD4* S232fs*3 *TP53* R342* *ZNF703* amplification	No	Stable
8	Not done	Cytology specimen only	Cytology specimen only	Not done	Unknown	Stable
9	Not done	Cytology specimen only	Cytology specimen only	*CDKN2A* V126D *KRAS* G12D *SMAD4* *553Wext*41 *TP53* C242fs*5	No	Stable
10	Not done	Cytology specimen only	Cytology specimen only	*CCND3* amplification *CDKN2A* Y44fs*1 *GATA6* amplification *KRAS* G12D *SMAD4* R445* *TP53* G244V	No	Stable
11	Not done	Cytology specimen only	Cytology specimen only	*ATM* E1091* *ATM* F1774fs*B *CDKN2A* loss *CDKN2B* loss *KRAS* G12D *MTAP* loss *SMAD4* T453fs*23	Yes	Stable
12	Not done	Surgical specimen only	Surgical specimen only	*CDKN2A* C72fs*48 *KRAS* G12E *RNF43* A752fs*75 *TP53* I195T	No	Stable

NCCN, National Comprehensive Cancer Network; HRR, homologous recombination repair.

specimens, and 1 patient with only surgical pathology specimens. Interestingly, all specimens that had been submitted for molecular testing were adequate for testing purposes (adequacy in this case defined as tumor gene profiling testing performed successfully with identifiable tumor mutations), although this could be due to specimens being selected prior to send-out. Cytology specimens were sent more often for molecular testing (8 out of 12 cases sent for tumor gene profiling or 67%); therefore, this demonstrates that cytology specimens could provide enough material for testing compared to surgical specimens. As noted in this study (**TABLE 1**), all 8 cytology cases produced adequate results on the ancillary studies, similar to the remaining 4 done on the surgical specimen.

Follow-up of the clinical course of these 20 patients on a chart review 3 months after the study period revealed that 13 of these patients expired from their disease. Of these, 2 patients had germline testing performed, 6 had tumor gene profiling and MSI/MMR performed, and 1 had MSI/MMR by immunohistochemistry performed. Of the 20 patients included in this chart review, 5 had either progression, recurrence, metastases, or an advanced stage of their pancreatic adenocarcinoma. All 5 of these patients had tumor gene profiling and MSI/MMR testing, but only 1 of 5 had germline testing. Only the remaining 2 from the original 20 patients had apparent complete resection followed by no further evidence of recurrence or progression (based on review of clinical follow-up documentation up to 20 and 23 months after the complete resection). Neither of these 2 patients had any germline or molecular test performed. Only 3 of the 20 patients declined chemotherapy (2 expired from pancreatic adenocarcinoma, 1 is alive with apparent complete resection without recurrence or progression). The other 17 of these 20 patients received initial chemotherapy; out of the 17, 5 received gemcitabine and nabpaclitaxel (including 1 patient with an HRR mutation but with significant clinical comorbidities), 11 received FOLFIRINOX, and 1 received FOLFOX. Of the 12 tested patients, 7 had expired from pancreatic adenocarcinoma, and the other 5 were alive with evidence of either recurrence, metastases, or advanced stage of disease.

Discussion

Quality assurance is an important component of medical care in the United States.[18] To improve the quality of cancer care, in the 1990s, the NCCN began to publish clinical practice guidelines to guide the diagnosis, testing, treatment, and palliation of common cancers based on the best available evidence and expert consensus.[18] One of these guidelines involves the diagnosis, testing, treatment, and palliation of pancreatic adenocarcinoma.[1,2] The NCCN guidelines for pancreatic adenocarcinoma continue to undergo revisions with the recent 2019 and 2021 updates including the recommendation for germline testing of all patients with pancreatic adenocarcinoma as well as tumor gene profiling of all patients with at least locally advanced, metastatic, or recurrent pancreatic adenocarcinoma.[1,2] It had previously been demonstrated in a quality assurance study of the general population that failure to follow an NCCN update on treatment protocols for pancreatic cancer in 2012 compromises outcomes in a discernably measurable way by increasing mortality.[18] However, there is no previous literature regarding quality assurance studies on compliance with NCCN guidelines on appropriate genetic and tumor molecular testing for pancreatic adenocarcinoma. Therefore, this is the first study on NCCN guideline compliance with genetic and molecular testing recommendations for patients with pancreatic adenocarcinoma since 2019. Quality assurance studies on compliance with best practice guidelines remain critically important to ensure best patient care and detect issues in compliance. Making improvements in such a scenario would ultimately lead to better outcomes for patients (**FIGURE 1**).

It is notable that a significant number of the Veterans Affairs Medical Center patients in the study did not receive the NCCN recommended testing. Only 15% or 3 patients of the identified 20 patients from 2019 onwards received germline testing. For these 3 patients, the germline testing was negative for relevant germline mutations. However, because 3 is a small number of patients and relevant germline mutations may only be seen in 20% of patients with pancreatic adenocarcinoma, the lack of detected germline mutations in this study does not argue against the usefulness of germline testing.[3,4] Germline testing, which is generally performed on nontumor tissue such as peripheral blood or saliva, remains important to identify germline mutations that may have a role in either treatment or genetic consideration for the patient's relatives. In addition, germline testing represents an important doublecheck of the tumor gene profiling studies performed on tumor genetic material.[6] Lincoln et al[6] demonstrated that in a large diverse population of patients with malignancies, tumor gene profiling can miss pathogenic genetic variants in 8.1% of cases (95% confidence interval

FIGURE 1. A schematic – "The Benefit Hexagon of Appropriate Genetic Testing" – showing six direct benefits of following the NCCN guidelines for genetic testing of patients with pancreatic adenocarcinoma. This schematic diagram shows the importance and benefits of quality assurance to track compliance with the NCCN guidelines. Monitoring to detect heterogeneity or non-compliance represents an important first step to effect the change to follow the guidelines for best clinical practices. NCCN, National Comprehensive Cancer Network.

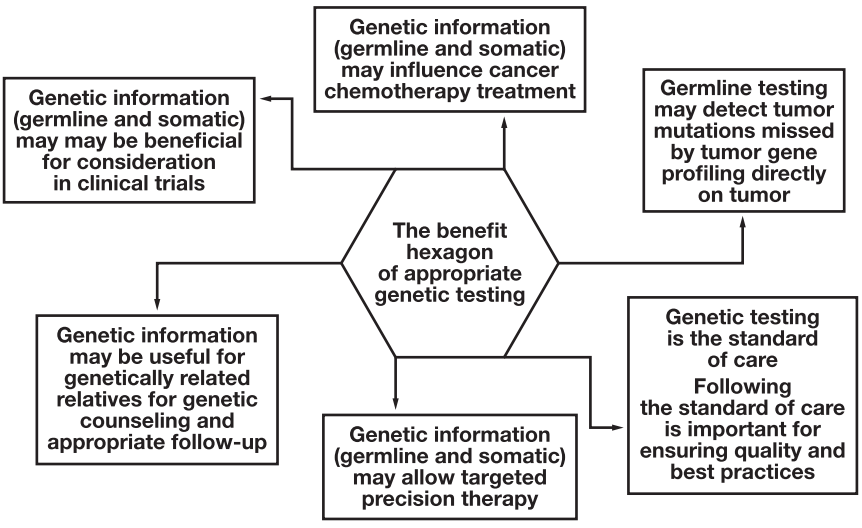
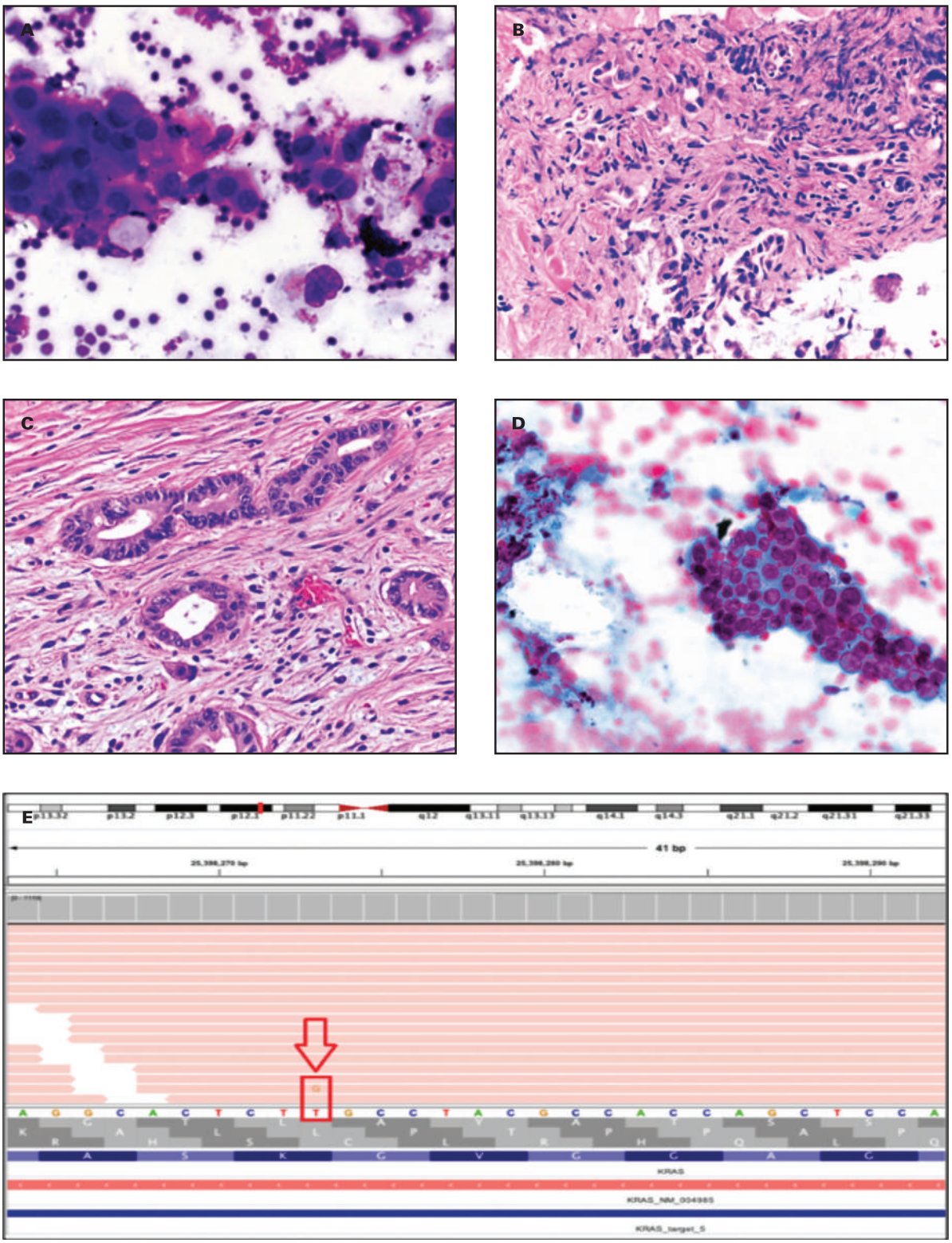


FIGURE 2. **Representative images of pancreatic adenocarcinoma from the patient cases in the case series. All samples sent for molecular testing were adequate for the requested testing. A) Diff Quik stain for rapid on-site evaluation of fine needle aspiration specimen that shows groups of pleomorphic and single cells consistent with adenocarcinoma, B) hematoxylin and eosin stained cell block from fine needle aspiration specimen showing pleomorphic glands consistent with adenocarcinoma, C) hematoxylin and eosin stained surgical resection specimen showing pleomorphic glands consistent with the diagnosis of adenocarcinoma, and D) Papanicolaou stain of fine needle aspiration specimen showing group of cells with pleomorphism consistent with adenocarcinoma. E) a screenshot from a representative Foundation Medicine sequencing data showing the identified KRAS c.35G>T, p.G12V variant that was detected on tumor gene profiling of the pancreatic adenocarcinoma. The red arrow and red rectangle highlight the exact site of the detected mutation.**



6.2%–10.5%) that could be picked up by germline testing. Reasons for tumor gene profiling missing the germline mutation include the well-known technical limitations of tumor gene sequencing; these include the challenges of interpretation (ie, somatic variant interpretation guidelines versus more sensitive germline variant interpretation guidelines), the limitations of the submitted tumor specimen, and the obscuring tumor genetic changes (ie, low copy numbers or insertions/deletions in the tumor's genetics).[6] Hence, every patient should receive germline testing to comply with NCCN guidelines. Without systematic testing of all patients presenting with pancreatic adenocarcinoma, germline mutations may be missed, and appropriate targeted therapy may not be considered for the patient.

Additionally, it is notable that although 18 of the 20 veterans had a locally advanced tumor, tumor progression, metastases, or recurrence that should have been an indication for tumor gene profiling, only 12 of the 18 or 67% had any type of tumor gene profiling, and for 1 patient, this testing was restricted to MSI/MMR testing by immunohistochemistry. All patients with pancreatic adenocarcinoma should have had germline testing; however, only 12 of 20 patients or 60% had any of the NCCN recommended testing. Similar to other studies, common mutations found in our study involved *KRAS*, *TP53*, *CDKN2A*, and *SMAD4*.[21] Two of the patients had a mutation in a HRR gene (specifically, *FANCA* and *ATM*) that suggests a tumor that may be particularly sensitive to a platinum-based chemotherapeutic agent.[16,17] This identification would provide information for the clinician to consider when deciding on the initial chemotherapy.[16,17] Genetic information regarding mutations such as *BRCA1/2*, *PALB2*, or other HRR genes provide information not only on sensitivity to platinum agents but also tumor susceptibility to maintenance therapy with PARP inhibitors.[1,16,17] In our population, the majority or 12 of 20 patients (60%) received a platinum-based chemotherapy regimen (FOLFIRINOX or FOLFOX). Only 2 patients had an HRR mutation detected on tumor gene profiling, of whom 1 received FOLFIRINOX as would be suggested based on the tumor sensitivity to platinum-based regimens, and 1 received the less toxic gemcitabine/nabpaclitaxel as the patient would have otherwise been unable to tolerate FOLFIRINOX due to medical comorbidities. Therefore, although the presence of an HRR mutation may argue for a platinum-based therapy, clinical considerations also play a major role in initial chemotherapy selection even if an HRR mutation is present. For precisely targeted therapy, Taghizadeh et al[7] demonstrated that 28% of a cohort of patients with pancreatic carcinoma qualified for targeted therapy through their institutional trials; this percentage could only be expected to increase with the passage of time as additional therapies are developed.

There clearly exists heterogeneity in clinical practice that represent areas of potential quality improvement.[1] Most veterans (17 out of 20 or 85%) with pancreatic adenocarcinoma in the regional Veteran Affairs Medical Center did not receive any germline testing during the study period. Similarly, there is heterogeneity with tumor gene profiling, as only a 12 out of 18 (67%) received this testing despite these patients having appropriately advanced pancreatic adenocarcinoma. Similar heterogeneity in clinical practice regarding treatment protocols had been previously noted for earlier NCCN recommendations in 2012.[18] We document for the first time in the literature that similar heterogeneity also exists in clinical practice in performing the molecular testing recommended by the current NCCN guidelines for pancreatic adenocarcinoma patients. Further future directions highlighted by this quality assurance study include looking into education about the NCCN guidelines for clinicians or further exploring the clinical reasons for not sending tissue for testing per the NCCN guidelines. Potential limitations that could be further explored in future studies include clinician perception or knowledge of tumor sampling, degree of familiarity with the NCCN guidelines, willingness of the patient to undergo genetic testing or counseling, and whether there are other clinical reasons to not pursue further testing.

The Veteran Affairs Medical Center provides equitable care to all its patients regardless of socioeconomic background and ethnicity, as has been previously published.[22] Similarly, our current study also demonstrated equal access to all, including minority populations. Therefore, the heterogeneity in ordering the NCCN recommended testing

FIGURE 3. Schematic of the pancreatic adenocarcinoma diagnosis/molecular testing and treatment arms. Pathology plays a critical role in diagnosing the adenocarcinoma, advising on tumor tissue sufficiency from the specimen, and providing the available tissue from the specimen for appropriate testing. The clinical team not only collects the tumor specimen and provides appropriate communication and counseling, but also decides on test ordering. As there are standard practice guidelines on what tests should generally be ordered, the monitoring of this test ordering represents a useful avenue for quality assurance.
Black arrows: pathology actions on pathology arm on left.
Green arrows: Clinician actions on clinical arm on right.
Orange arrows: Actions related to genetic and ancillary studies recommended by standard of care NCCN guidelines.

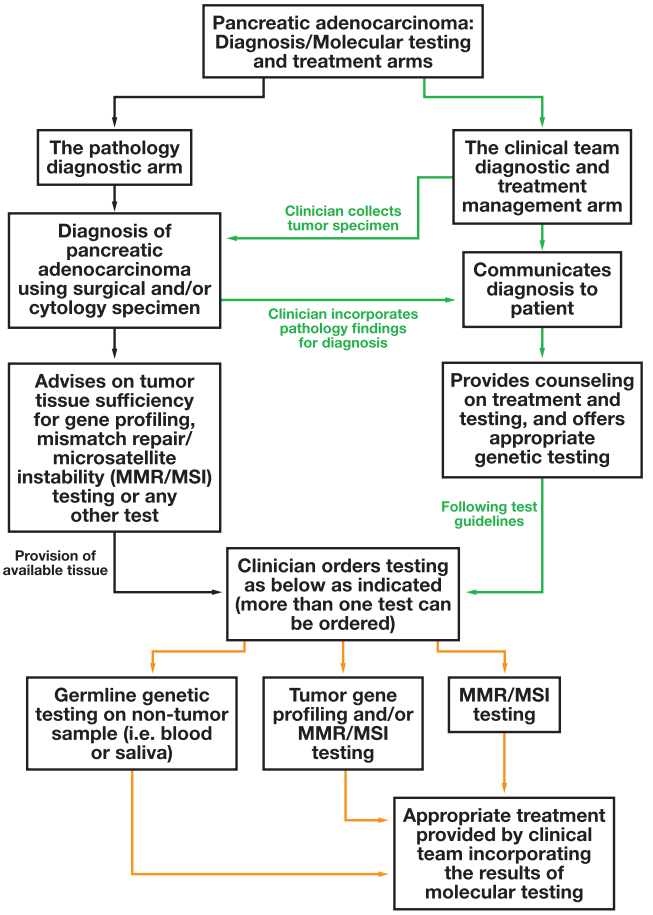
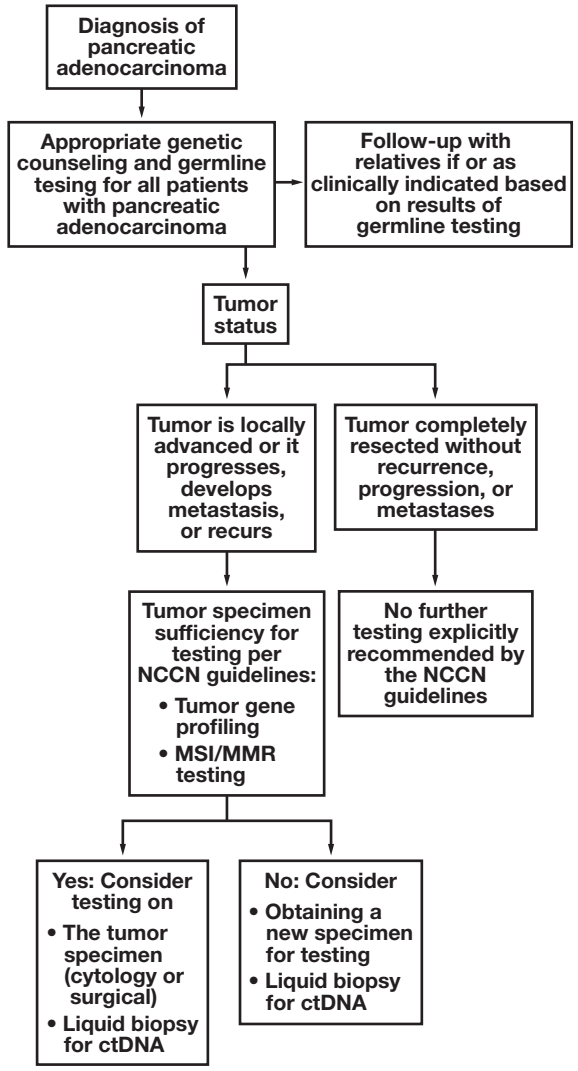


FIGURE 4. A simplified testing algorithm for pancreatic adenocarcinoma to comply with the current NCCN guidelines. All patients diagnosed with pancreatic adenocarcinoma should receive germline testing. Furthermore, for those who have tumor that cannot be resected, that progresses, that develops metastasis, or recurs would be recommended for tumor gene profiling and MSI/MMR testing. The presence of adequate tumor tissue or not would influence choice of specimen for testing, though in the presence of inadequate tumor tissue, options include either obtaining a new tumor specimen or attempting a liquid biopsy of circulating tumor DNA for this testing. If the pancreatic adenocarcinoma is resected and cured without recurrence, progression or metastases, no further testing is explicitly recommended by the NCCN guidelines. NCCN, National Comprehensive Cancer Network; MSI/MMR, microsatellite instability/mismatch repair; ctDNA, circulating tumor deoxyribonucleic acid.



was not affected by the ethnicity of the patient in the small sample for this study.

Sixty-seven percent of molecular testing (8 out of 12 patients) for tumor gene profiling was performed on the cytology specimen. This highlights that EUS-FNA, the gold standard technique for acquiring cytology specimens from pancreatic tumors for diagnosis and studies, is efficacious not only in diagnosis but also in providing sufficient material for a variety of ancillary genetic studies including tumor profiling and next generation sequencing testing (see **FIGURE 2**).[23–28] Sending cytology specimens is in line with NCCN guidance that considers the EUS-FNA to be a preferred modality for exploring pancreatic adenocarcinoma, both for diagnosis and for obtaining the tumor tissue for ancillary studies.[1] Notably, all 12 patients who underwent tumor gene profiling had adequate tissue (adequate defined as enough tumor tissue to allow for completion of the ordered ancillary testing on tumor) to give a result (see **TABLE 1** and **FIGURE 2**). On the other hand, there was no documented evidence that the other 6 patients (out of 18) who did not have tumor gene profiling, despite the NCCN guideline, had any testing for tumor gene profiling attempted. There remain several avenues to perform this testing, including sending the diagnostic tumor tissue (surgical or cytology) or a liquid biopsy (a peripheral blood specimen) to chase the genetics of the pancreatic adenocarcinoma, particularly if the biopsy or aspiration specimen is otherwise inadequate.[29] We have created a simple guiding testing algorithm for pancreatic adenocarcinoma following the NCCN guideline (**FIGURE 3** and **4**) that can aid quality assurance/improvement role for all pancreatic adenocarcinoma cases to ensure that testing proceeds routinely or reflexively. Therefore, there is no true specimen barrier to attempting tumor gene profiling.

Conclusion

In this first quality assurance study of molecular testing compliance with the NCCN guidelines for pancreatic adenocarcinoma patients, there appears to be heterogeneity in practice. This heterogeneity led to a significant percentage of patients who either did not receive tumor gene profiling (40%) or germline testing (85%). Given the advances in precision-based medicine in pancreatic adenocarcinoma, the provision of recommended testing by the NCCN guidelines remains critical and important for the best quality care. This study highlights an important quality assurance avenue both within the Veteran Healthcare System and throughout the country to verify whether the NCCN treatment guidelines are being adhered to.

Future studies with a larger population will be of immense benefit to explore the reasons behind the heterogeneity in ordering molecular testing, the importance of following the NCCN guidelines, and its impact on both outcome analysis and patient management.

REFERENCES

1. National Comprehensive Cancer Network (NCCN). *NCCN Clinical Practice Guidelines in Oncology (NCCN Guidelines®): Pancreatic Adenocarcinoma NCCN Evidence BlocksTM*. Version 2.2021, February 25, 2021.
2. Tempero MA. Highlights of the NCCN 2019 Annual Conference, NCCN guidelines updates: pancreatic cancer. *J Natl Compr Canc Netw.* 2019;17(5.5):603–605.
3. Stoffel EM, McKernin SE, Brand R, et al. Evaluating susceptibility to pancreatic cancer ASCO provisional clinical opinion. *J Clin Oncol.* 2018;37(3):153–164.
4. Salo-Mullen EE, O'Reilly EM, Kelsen DP, et al. Identification of germline genetic mutations in patients with pancreatic cancer. *Cancer.* 2015;121:4382–4388. doi:10.1002/cncr.29664
5. Rawla P, Sunkara T, Gaduputi V. Epidemiology of pancreatic cancer: global trends, etiology and risk factors. *World J Oncol.* 2019;10(1):10–27.

6. Lincoln SE, Nussbaum RL, Kurian AW, et al. Yield and utility of germline testing following tumor sequencing in patients with cancer. *JAMA Network Open.* 2020;3(10):e2019452e2019452.
7. Taghizadeh H, Müllauer L, Mader RM, et al. Applied precision medicine in metastatic pancreatic ductal adenocarcinoma. *Ther Adv Med Oncol.* 2020;12. doi: 10.1177/1758835920938611
8. Aung KL, Fischer SE, Denroche RE, et al. Genomics-driven precision medicine for advanced pancreatic cancer: early results from the COMPASS trial. *Clin Cancer Res.* 2017;24(6):1344–1354.
9. Knudsen ES, O'Reilly EM, Brody JR, et al. Genetic diversity of pancreatic ductal adenocarcinoma and opportunities for precision medicine. *Gastroenterology.* 2016;150:48–63. Doi: 10.1053/j.gastro.2015.08.056
10. Zheng-Lin B, O'Reilly EM. Pancreatic ductal adenocarcinoma in the era of precision medicine. *Semin Oncol.* 2021;48(1):19–33.
11. Zagouri F, Sergentanis TN, Chrysikos D, et al. Molecularly targeted therapies in metastatic pancreatic cancer. *Pancreas.* 2013;42:760–773. doi:10.1097/MPA.0b013e31827aedef
12. Waddell N, Pajic M, Patch A, et al. Whole genomes redefine the mutational landscape of pancreatic cancer. *Nature.* 2015;518:495–501. doi:10.1038/nature14169
13. Regel I, Mayerle J, Mukund MU. Current strategies and future perspectives for precision medicine in pancreatic cancer. *Cancers.* 2020;12(1024):1. doi: 10.3390/cancers12041024
14. Sahin IH, Lowery MA, Stadler ZK, et al. Genomic instability in pancreatic adenocarcinoma: a new step towards precision medicine and novel therapeutic approaches. *Expert Rev Gastroenterol Hepatol.* 2016;10(8):893–905.
15. Pokataev I, Fedyanin M, Polyanskaya E, et al. Efficacy of platinum-based chemotherapy and prognosis of patients with pancreatic cancer with homologous recombination deficiency: comparative analysis of published clinical studies. *ESMO Open* 2020;5:e000578. doi:10.1136/esmoopen-2019-000578
16. Ryan DP. *Initial systemic chemotherapy for metastatic exocrine pancreatic cancer*. In: UpToDate, Post TW (Ed), UpToDate, Waltham, MA. https://www.uptodate.com/contents/initial-systemic-chemotherapy-for-metastatic-exocrine-pancreatic-cancer (Accessed May 28, 2021).
17. Ryan DP, Mamon H. Initial chemotherapy and radiation for nonmetastatic, locally advanced, unresectable and borderline resectable, exocrine pancreatic cancer. In: UpToDate, Post TW (Ed), UpToDate, Waltham, MA. https://www.uptodate.com/contents/initial-chemotherapy-and-radiation-for-nonmetastatic-locally-advanced-unresectable-and-borderline-resectable-exocrine-pancreatic-cancer (Accessed May 28, 2021).
18. Visser BC, Ma Y, Zak Y, et al. Failure to comply with NCCN guidelines for the management of pancreatic cancer compromises outcomes. *HPB.* 2012;14(8):539–547.
19. Walker EJ, Carnevale J, Pedley C, et al. Referral frequency, attrition rate, and outcomes of germline testing in patients with pancreatic adenocarcinoma. *Fam Cancer.* 2019;18:241–251. Doi:10.1007/s10689-018-0106-2
20. *FOUNDATIONONE CDx Specimen Instructions*, 2021.
21. Ryan DP, Hong TS, Bardeesy N. Pancreatic adenocarcinoma. *N Engl J Med.* 2014;371:1039–1049. doi:10.1056/NEJMra1404198
22. Petersen JM, Dalal S, Jhala D. Improved across the board access to SARS-CoV-2 laboratory testing in an integrated medical system; the Veteran Affairs Medical Center (VAMC) experience. *Am J Clin Pathol.* 2020;154(Suppl 1):S144–S145.
23. Jhala NC, Jhala DN, Chhieng DC, et al. Endoscopic ultrasound-guided fine-needle aspiration: a cytopathologist's perspective. *Am J Clin Pathol.* 2003;120:351–367. doi:10.1309/MFRF-J0XY-JLN8-NVDP
24. Wei S, Lieberman D, Morrissette JJD, et al. Using "residual" FNA rinse and body fluid specimens for next-generation sequencing: an institutional experience. *Cancer Cytopathol.* 2016;124(5):324–329.
25. Jhala D, Eloubeidi M, Chhieng D, et al. Fine needle aspiration biopsy of the islet cell tumor of the pancreas: a comparison between computerized axial tomography and endoscopic ultrasound guided fine needle aspiration biopsy. *Ann Diagn Pathol.* 2002;6(2):106–112.
26. Jhala NC, Jhala D, Eltoum I, et al. Endoscopic ultrasound-guided fine-needle aspiration biopsy: a powerful tool to obtain samples from small lesions. *Cancer* 2004;102(4):239–246.
27. Jhala N, Jhala D, Vickers SM, et al. Biomarkers in diagnosis of pancreatic carcinoma in fine needle aspirates. *Am J Clin Pathol.* 2006;126(4):572–579.
28. Jhala N, Srimunta P, Jhala D. Role of ancillary testing on EUS-FNA from cystic pancreatic neoplasms. *Acta Cytol.* 2020;64(1–2):124–135.
29. Aghamir SMK, Heshmat R, Ebrahimi M, et al. Liquid biopsy: the unique test for chasing the genetics of solid tumors. *Epigenetics Insights.* 13:1–7.

SCIENCE

Application of the Fluorescence Method on Sysmex XN9000 Hematology Analyzer for Correcting Platelet Count in Individuals with Microcytosis

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Keywords: platelet count, MCV cutoff, microcytosis, fluorescence method, impedance method, Sysmex XN9000 hematology analyzer

Abbreviations: MCV, mean corpuscular volume; RBC, red blood cells; ROC, receiver operating characteristic; SD, standard deviation.

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ABSTRACT

Objective: Although small red blood cells are a well-known analytical pitfall that could cause artifactual increase of the platelet count, limited information is available on the accuracy of impedance platelet counting in cases with microcytosis. The aim of this study is to assess the accuracy of impedance platelet counting in the presence of small red blood cells, and to establish the optimal mean corpuscular volume (MCV) cutoff to endorse fluorescence platelet counting.

Methods: In this study, platelet counts estimated by the impedance method on the Sysmex XN9000 analyzer (Sysmex, Kobe, Japan) were compared with those provided by the fluorescence method. The accuracy of impedance platelet counting was assessed. Receiver operating characteristic curve was used to evaluate the performance of MCV in predicting falsely increased platelet counts.

Results: There was a tendency for the impedance method to overestimate the platelet count in samples with 70 fL < MCV ≤ 80 fL, 60 fL < MCV ≤ 70 fL, MCV ≤ 60 fL. Receiver operating characteristic curve analysis showed that a 73.5fL cutoff of MCV was highly sensitive in predicting falsely increased platelet counts.

Conclusion: In cases with MCV < 73.5 fL, we strongly suggest that the platelet counts obtained by the impedance method on the Sysmex XN9000 analyzer should be checked and corrected by fluorescence counting.

Automated platelet count is generally used to support the diagnosis of various hematologic disorders and to determine bleeding risk from abnormal platelet counts. The accuracy of automated platelet count is, therefore, very important to ensure appropriate patient management. The impedance method is currently used routinely for platelet counting in most hematology laboratories. This method relies on the measurement of the volume of particles. Platelets are accurately counted in the majority of samples, while the platelet count can be significantly overestimated in cases with interfering particles of a similar size.[1–3] Microcytosis, characterized by the presence of abnormally small red blood cells (RBC) with mean corpuscular volume (MCV) less than 80 fL, is the most common condition that may lead to overestimation of the platelet count. The problem may be more common in countries where microcytic disorders such as thalassemia are prevalent.

To date, the performance of impedance platelet counting has been the subject of ongoing debate, with conflicting reports in the literature as to the accuracy depending on the sample type.[4–6] To overcome this, some analyzers incorporate more than 1 platelet counting technology, such as the Sysmex XN9000 devices, which have impedance and optical platelet counting with the availability of a new fluorescence platelet counting method. This new method is based on a Fluorocell fluorescent dye (oxazine), an extended counting volume, and an extended counting time. Platelet counts by this method are highly accurate (fluorescence platelet counting vs immunological platelet counting, r=0.988).[7–10] If the fluorescence method is enabled on the analyzer, we have an algorithm to give a best "reported count." If interference is detected in the impedance count, for example, when small RBC are present, we will report the fluorescence count. However, there have been very few studies comparing both counting technologies in the presence of small RBC to provide clear and quantitative evidence of the interference on impedance counting. This limitation has therefore made it difficult to ascertain the timing to apply the fluorescence method to overcome the interference. The aim of this study is to assess the accuracy of impedance platelet counting in the presence of small RBC and to establish the optimal MCV cutoff to endorse fluorescence platelet counting.

Materials and Methods

Blood Samples Used in the Study

Blood samples were drawn into Vacutainer tubes, anticoagulated with K_2-EDTA (Becton Dickinson, Plymouth, England), and analyzed within 4 hours after collection. Blood smears were reviewed for the presence of interferences that could impact platelet count (including platelet aggregates, giant platelets, thrombocyte abnormalities, cell fragments). Samples that met 1 of the following criteria were excluded: (i) flags indicating that "platelet clumps" and "fragments" were found by analyzers; (ii) platelet aggregates, cell fragments, and giant platelets were shown in the blood smear. A total of 1740 blood samples used in this study were collected from unique patients admitted to The First Affiliated Hospital of Sun Yat-sen University from March to August 2019. A total of 1374 blood samples collected from March to July were used to assess the accuracy of impedance platelet counting and determine the optimal cutoff of MCV. The 366 blood samples collected in August were used to validate the MCV cutoff value.

Hemocytometry

The calibration status of the Sysmex XN9000 hematology analyzers (Sysmex Corporation, Kobe, Japan) was initially checked by the manufacturer. Quality control samples and maintenance procedures were performed daily according to the manufacturer's instructions. Blood samples were analyzed with the parameter profile "CBC+RET+PLT-F" to ensure that results of all parameters were available.

Definition of Inaccurate Platelet Count

Inaccurate platelet count was defined as platelet count by the impedance method that differed from the fluorescence count by more than 25%. The value of 25% was derived from the allowable total error, as defined by the Clinical Laboratory Improvement Amendments of 1988 (CLIA'88). The frequencies of inaccurate platelet counts were calculated at different levels of MCV.

Statistical Analyses

Statistical analyses were carried out using the software program MedCalc (Version 11.5.1.0, MedCalc Software, Mariakerke, Belgium). Continuous variables with normal distribution were expressed in terms of mean and standard deviation (mean ± SD). Receiver operating characteristic (ROC) curve was used to assess the potential of MCV as a single parameter to discover spurious high platelet counts. The difference in platelet counts between impedance and fluorescence methods was compared using the paired samples *t* test, and the correlation was analyzed by linear regression. In all analyses, statistical tests were 2-sided, and *P* values less than .05 were considered statistically significant.

Results

Frequencies of Inaccurate Platelet Count

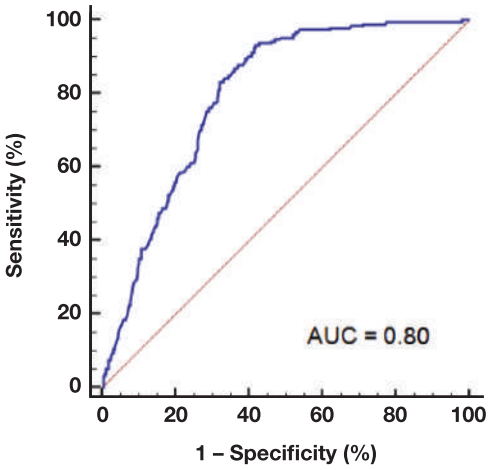
The MCV distribution and the frequencies of inaccurate platelet counts are listed in **TABLE 1**. The 1374 blood samples were divided into 5 groups according to MCV. The data showed clearly that there was a tendency for the impedance method to overestimate the platelet count compared with the fluorescence method at 70.0 fL < MCV ≤ 80.0 fL, 60.0 fL < MCV ≤ 70.0 fL, and MCV ≤ 60.0 fL. Low MCV increased the probability of inaccurate platelet count considerably. In samples with MCV ≤ 80.0 fL, the frequency of inaccurate platelet count was 22.7%, while the frequency was 1.1% in samples with MCV > 80.0 fL. This suggests that small RBC plays a critical role in the overestimation of the platelet count and emphasizes the need for alternative methods to produce the most accurate count.

TABLE 1. The MCV Distribution and the Frequencies of Inaccurate Platelet Counts in Samples.

MCV (fL)	No. of Samples	Frequencies of Inaccurate Platelet Count (%)
≤ 60	235	32.8
60 < MCV ≤ 70	340	24.7
70 < MCV ≤ 80	237	9.7
80 < MCV ≤90	250	1.6
> 90	312	0.6

MCV, mean corpuscular volume.

FIGURE 1. The ROC of MCV for inaccurate platelet count. ROC, receiver operating characteristic; MCV, mean corpuscular volume.



MCV Acted as a Powerful Predictor of Inaccurate Platelet Counts

The ROC of MCV was shown for inaccurate platelet count in **FIGURE 1**. The area under the curve was 0.80 (95% CI: 0.77–0.82, *P* <.0001), suggesting MCV exhibited reasonable predictive performance for a false high platelet count. The optimal cutoff value was determined to be 73.5 fL, and the sensitivity and specificity were 93.16% (95% CI: 88.6%–96.3%) and 58.02% (95% CI: 55.2%–60.9%), respectively. This cutoff presented poor specificity but good sensitivity. This means the impedance method can give an accurate platelet count in samples with MCV higher than the cutoff, while the results should be confirmed in the presence of lower MCV.

Comparison of Platelet Counts by Impedance and Fluorescence Methods

The MCV cutoff was validated in an independent cohort of 183 samples with MCV < 73.5 fL and 183 samples with MCV > 73.5 fL. The mean platelet counts (mean ± SD) were calculated for both methods. These are plotted in **FIGURE 2**. In samples with MCV < 73.5 fL, the impedance method

yielded significantly higher platelet counts and also had poor agreement with the fluorescence method (mean ± SD:339 ± 104 vs 294 ± 103, P <0.0001; r=0.877, P <0.0001). In samples with MCV > 73.5 fL both methods produced similar results (mean ± SD:229 ± 107 vs 230 ± 108, P =0.293; r=0.999, P <0.0001). The impedance method overestimated the count considerably in samples with MCV < 73.5 fL, emphasizing a clear requirement of the fluorescence method to correct the platelet count.

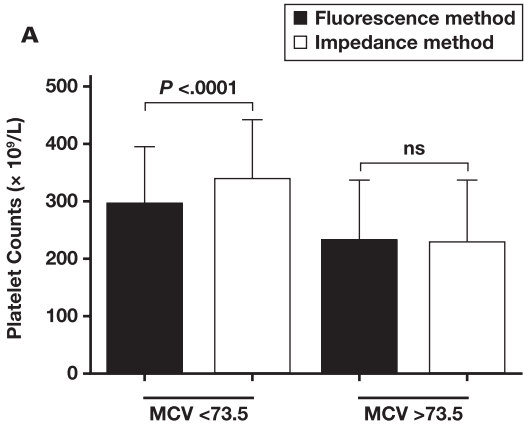
Discussion

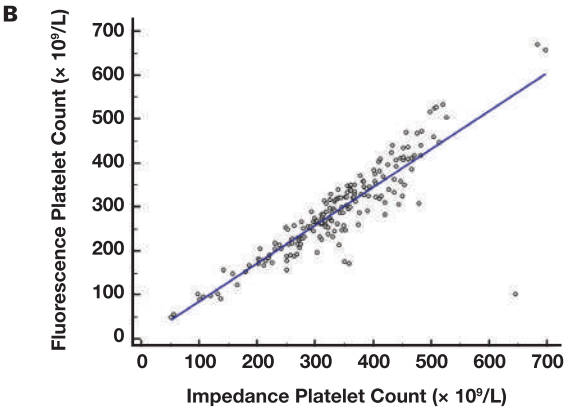
The platelet and RBC counts are performed on the impedance channel of the Sysmex XN9000 hematology analyzers. Under normal conditions, the particle size differs significantly, allowing an easy discrimination between platelets and RBC. In contrast, in pathological situations associated with the presence of numerous small RBC, some of these particles can be wrongly classified as platelets,[11] indicating the device's incapacity to properly distinguish platelets from RBC. Better separation between platelets and small RBC is achieved in most cases using techniques based on optical light scatter, fluorescence, or flow cytometry.[12]

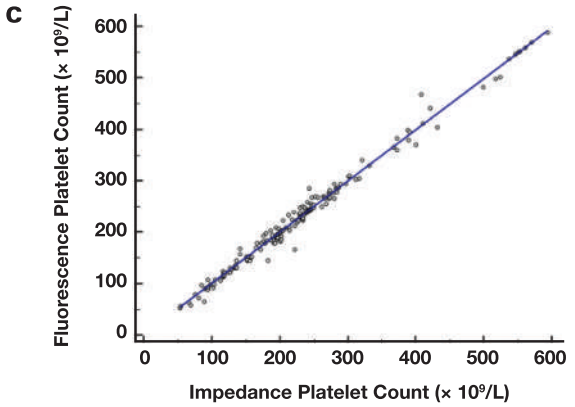
The results of this study highlight the impact of microcytes on the accuracy of impedance platelet counting in routine clinical use and the potential role of MCV in laboratory practice to use the fluorescence method to correct the spurious count in cases with microcytosis. To define the inaccurate platelet count, the allowable total error (25%) provided by CLIA'88 is used. The impedance method overestimates the platelet count considerably, highlighting alternative methods to give an actual count, such as the fluorescence method. The determination of a 73.5 fL MCV cutoff is a highly sensitive parameter for predicting spuriously high platelet counts. In samples with MCV < 73.5 fL, we strongly suggest that the platelet count should be confirmed by the fluorescence method.

The fluorescence method is not widely routinely utilized as it requires extra resources in terms of the Fluorocell reagent with higher cost compared to the impedance method. It is only usually performed to confirm the platelet count in cases of interference. Our hospital, located in Guangzhou, Guangdong, is one of the largest general hospitals in China. Most of the patients come from Guangdong province, in which thalassemia associated with low MCV is prevalent and variable. In Guangdong, 17.70% of pregnant women, 15.94% of their husbands, and 16.03% of neonates are heterozygous carriers of α- or β-thalassemia.[13] Therefore, a significant

FIGURE 2. Comparison of platelet counts between impedance and fluorescence methods according to MCV cutoff. (A) The mean platelet counts in samples with MCV < 73.5fL and in samples with MCV > 73.5fL. Error bars denoted mean ± SD. (B) Correlation analysis of platelet counts in samples with MCV < 73.5fL. (C) Correlation analysis of platelet counts in samples with MCV > 73.5fL. MCV, mean corpuscular volume.







number of patients admitted to our hospital present with microcytosis. In our routine practice, in samples with MCV < 80 fL, automated hematology analyzers flag questionable platelet counts, necessitating review of results with confirmation by an alternative method—usually the fluorescence method. This process thus significantly increases the cost of performing platelet counting and delaying results in thalassemia endemic areas. In this study, we confirm that the impedance method tends to overestimate the platelet count in low MCV samples, and we determine a specific MCV cutoff for reflexing to the fluorescence method for correcting platelet count in samples suspected of having an inaccurate impedance count due to microcyte interference. The use of a 73.5 fL cutoff, instead of 80 fL, contributes significantly to lessening workloads and reducing costs of performing platelet counts in our laboratory. According to our preliminary statistics, 14.5% of samples acquired for a complete blood count show MCV less than 80 fL, and 8.4% show MCV less than 73.5 fL. This means the review rate for platelet counting decreases from 14.5% to 8.4% through the use of a 73.5 fL cutoff. As a result, the workloads and costs of performing fluorescence platelet counting are cut b y 42.1%. As clinical laboratories are continually seeking ways to improve efficiency, the marked reduction in the platelet review rate has favorably affected workflow and laboratory costs. In addition, patient care is improved with more timely reporting of accurate results.

In addition to small RBCs, several types of interferences (cell fragments, cryoglobulin, microorganisms, lipids, etc.) can also induce false thrombocytosis, or inversely incur artificial total increase in platelet counting or partial masking of thrombocytopenia.[1] In practice, their occurrence is suspected by an abnormally high and unexplained platelet count or unexpected increase. Concerning the impedance method of platelet counting, if interference is suspected, the count must be checked using another method that the hematology analyzer is equipped with.[14] Facing any abnormal results, the specialist in laboratory medicine must ensure that all the necessary checks have been done and then interpret the result on its numerical value while integrating other data, such as past results, delta check, and clinical context.

The current study has some limitations. First, the CD61-ImmunoPLT reference method is not used. Second, finding the MCV cutoff at 73.5fL is only valid for the XN9000 analyzer. Further study on evaluation of the 73.5 fL cutoff for other types or brands of hematology analyzers should be conducted.

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REFERENCES

1. Zandecki M, Genevieve F, Gerard J, Godon A. Spurious counts and spurious results on haematology analysers: a review. Part I: platelets. *Int J Lab Hematol.* 2007;29:4–20. doi:10.1111/j.1365-2257.2006.00870.x.
2. Tantanate C. Spuriously high platelet counts by various automated hematology analyzers in a patient with disseminated intravascular coagulation. *Clin Chem Lab Med.* 2015;53:e257–e259. doi:10.1515/cclm-2015-0055.
3. Tantanate C, Khowawisetsut L, Pattanapanyasat K. Performance evaluation of automated impedance and optical fluorescence platelet counts compared with international reference method in patients with thalassemia. *Arch Pathol Lab Med.* 2017;141:830–836. doi:10.5858/arpa.2016-0222-OA.
4. Kunicka JE, Fischer G, Murphy J, Zelmanovic D. Improved platelet counting using two-dimensional laser light scatter. *Am J Clin Pathol.* 2000;114:283–289. doi:10.1309/0GLP-K5NU-N1GD-PR8T.
5. Sandhaus LM, Osei ES, Agrawal NN, et al. Platelet counting by the coulter LH 750, sysmex XE 2100, and advia 120: a comparative analysis using the RBC/platelet ratio reference method. *Am J Clin Pathol.* 2002;118:235–241. doi:10.1309/MK3G-MC3V-P06R-PNV2.
6. Segal HC, Briggs C, Kunka S, et al. Accuracy of platelet counting haematology analysers in severe thrombocytopenia and potential impact on platelet transfusion. *Br J Haematol.* 2005;128:520–525. doi:10.1111/j.1365-2141.2004.05352.x.
7. Briggs C, Longair I, Kumar P, et al. Performance evaluation of the Sysmex haematology XN modular system. *J Clin Pathol.* 2012;65:1024–1030. doi:10.1136/jclinpath-2012-200930.
8. Schoorl M, Oomes J, van Pelt J. New fluorescent method (PLT-F) on Sysmex XN2000 hematology analyzer achieved higher accuracy in low platelet counting. *Am J Clin Pathol.* 2013;140:495–499.
9. Tanaka Y, Gondo K, Maruki Y, et al. Performance evaluation of platelet counting by novel fluorescent dye staining in the XN-series automated hematology analyzers. *J Clin Lab Anal.* 2014;28:341–348.
10. Seo JY, Lee ST, Kim SH. Performance evaluation of the new hematology analyzer Sysmex XN-series. *Int J Lab Hematol.* 2015;37:155–164. doi:10.1111/ijlh.12254.
11. Lawrence C, Atac B. Hematologic changes in massive burn injury. *Crit Care Med.* 1992;20:1284–1288. doi:10.1097/00003246-199209000-00015.
12. Briggs C, Harrison P, Machin SJ. Continuing developments with the automated platelet count. *Int J Lab Hematol.* 2007;29:77–91. doi:10.1111/j.1751-553X.2007.00909.x.
13. Yin A, Li B, Luo M, et al. The prevalence and molecular spectrum of alpha- and beta-globin gene mutations in 14,332 families of Guangdong Province, China. *PLoS One.* 2014;9:e89855. doi:10.1371/journal.pone.0089855.
14. Baccini V, Genevieve F, Jacqmin H, et al. Platelet counting: ugly traps and good advice. proposals from the french-speaking Cellular Hematology Group (GFHC). *J Clin Med.* 2020;9(3):808. doi:10.3390/jcm9030808.

CASE STUDY

Incidental Discovery of a Patient with the Bombay Phenotype

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Abbreviations: RBCs, red blood cells; IAT, indirect antiglobulin test; *FUT1*, fucosyltransferase 1; HDFN, hemolytic disease of the fetus and newborn.

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ABSTRACT

Bombay phenotype, an exceptionally rare blood type in individuals outside of Southeast Asia, occurs in approximately 1 in 1,000,000 individuals in Europe. This blood phenotype is characterized by the absence of the H antigen on red blood cells (RBCs) and in secretions. As the H antigen is the structure on which the ABO system is built, individuals lacking this antigen are unable to produce A or B antigens and appear as type O on routine ABO phenotyping. H deficiency does not cause ill effect; however, these individuals produce an anti-H alloantibody capable of causing severe acute hemolytic transfusion reactions when exposed to RBCs that express the H antigen. In this case study, we highlight the incidental discovery of a patient with Bombay phenotype in a North American hospital system, expected test results, the immunologic and genetic basis underlying the Bombay and para-Bombay phenotypes, and methods to ensure availability of compatible blood.

Case Report

A 48 year old gravida 3 para 3 woman of Pakistani ancestry presented to her obstetrician with complaints of dysmenorrhea and irregular uterine bleeding for greater than 1 year. Otherwise, the patient denied any significant past medical history and had never received a blood transfusion. Magnetic resonance imaging demonstrated evidence of diffuse adenomyosis and a left adnexal dermoid cyst. An endometrial biopsy demonstrated proliferative endometrium with stromal breakdown; there was no evidence of malignancy. Although not significantly anemic (baseline hemoglobin 11.8 g/dL; institutional reference 11.7 – 15.5 g/dL), the patient elected to undergo curative treatment via total laparoscopic hysterectomy given persistent bleeding and associated symptoms.

Routine pre-operative evaluation, including determination of the patient's ABO blood type and assessment for any potential underlying alloantibodies, was ordered. ABO forward and reverse typing was performed via serologic methods using the ID-Micro Typing System gel test (Ortho Clinical Diagnostics, NJ, USA). Anti-A and Anti-B reagents (Ortho Clinical Diagnostics, NJ, USA) did not agglutinate the patient's red blood cells (RBCs) in the forward type, whereas the patient's plasma agglutinated both A_1 and B reagent RBCs (Ortho Clinical Diagnostics, NJ, USA) in the reverse type. These findings were initially interpreted as blood type O.

To assess for potential alloantibodies, the patient's plasma was tested against 3 type O reagent RBCs using an IgG-specific solid phase technology [Capture-R Ready-Screen (3); IMMUCOR, INC, Norcross, GA, USA]. The patient's plasma strongly agglutinated all 3 screening RBCs, showing 4+ reactivity. Alloantibody identification was then attempted by testing the patient's plasma against 14 type O reagent RBCs with unique antigen profiles, again using an IgG-specific solid phase technology (Capture-R Ready-ID; IMMUCOR, INC, GA, USA). Similarly, the patient's plasma agglutinated all 14 RBCs. A direct antiglobulin test, performed using an anti-IgG -C3d polyspecific reagent (IMMUCOR, INC, GA, USA) via standard tube methods, demonstrated no evidence of IgG or C3 coating the patient's RBCs. An autocontrol was negative, illustrating no evidence of an autoantibody in the patient's plasma.

Given the reactivity pattern and the history of multiple pregnancies, the transfusion medicine service was concerned that an alloantibody to a high-incidence antigen was present; thus, further testing was performed by a regional immunohematology reference laboratory (the American Red Cross, Farmington, CT), where the patient's RBCs were determined to be negative for the H-antigen using commercial anti-H lectin (*Ulex europaeus*). An anti-H alloantibody was identified in the patient's plasma, which agglutinated reagent RBCs expressing the H-antigen at multiple phases of testing, including immediate spin in saline, 37°C in low ionic-strength saline, in polyethylene glycol enhancement, and via indirect antiglobulin test (IAT) using monospecific anti-human IgG. Frozen H-negative cells from other Bombay donors were used to rule out other significant red cell alloantibodies. Further testing, including a serologic phenotype of the patient's RBCs, demonstrated expression of the

Lewis A (Le^a) antigen, but not the Lewis B (Le^b) antigen, consistent with nonsecretor status. These findings were compatible with the incidental discovery of the Bombay phenotype (O_h). The surgical team was strongly recommended to postpone surgery while the transfusion medicine service determined the optimal approach for acquisition of compatible blood, including potentially identifying donors with the Bombay phenotype or autologous RBC donation.

A national search for H-negative RBC blood identified 1 liquid RBC unit available for immediate shipping and 3 frozen units located in a neighboring state. The patient returned for surgery after the liquid RBC unit was received and confirmatory testing was completed by our blood bank. The patient's preprocedure hemoglobin was 11.7 g/dL, and the procedure was performed via robotic technique to minimize blood loss, with patient blood management options (e.g., a cell saver) being available as an additional blood conservation method if necessary. The procedure was uncomplicated, and estimated blood loss was 100 mL.

Postoperatively, the patient experienced no significant hemorrhage or complication and was discharged home on postoperative day 2. At the time of discharge, her hemoglobin was 9.5 g/dL (decreased from 10.4 g/dL immediately post-op). She did not require transfusion of the liquid Bombay unit, and it was subsequently frozen for potential future use. The patient and her family members have been encouraged to contact local/regional blood donor centers for consideration of phenotyping (family members) as well as other options (e.g., banking autologous blood). In follow-up, the patient stated her intentions to donate blood following full recovery from her surgical procedure.

Discussion

First described in 1952[1] in 3 individuals from Bombay (now Mumbai), India, the Bombay blood group is exceptionally rare outside of Southeast Asia, occurring in approximately 1 in 1,000,000 individuals in Europe.[2] However, in parts of India, the incidence is estimated to be as high as 1 in 10,000.[1,2] This is of particular significance, as individuals with the Bombay phenotype produce a naturally occurring anti-H alloantibody, which is similar to anti-A and anti-B isohemagglutinins in that it is predominantly IgM, active at 37°C, and is capable of inducing severe intravascular hemolysis and acute hemolytic transfusion reactions if exposed to RBCs with the H antigen.[3,4]

In general, there are 4 possible conventional ABO blood types depending on which antigen is expressed on the RBC surface: A, B, AB, and O. Individuals with type A only express the A antigen on their RBCs, type B individuals express only the B antigen, and type AB individuals express both the A and B antigens. Individuals who do not express either the A or B antigen are considered to be type O; however, O is not an antigen itself, but rather reflects the absence of the A and B antigens. Individuals with type O express only the H antigen on the RBC surface.

The genetic basis of the ABO and H blood group systems depends on 3 genes: fucosyltransferase 1 (*FUT1*), *FUT2*, and *ABO*. The *FUT1* and *FUT2* genes differ in that the enzymes these genes encode act on different tissues. *FUT1* encodes a fucosyltransferase that is responsible for creation of the H antigen on RBCs. *FUT2* encodes a similar fucosyltransferase that catalyzes the formation of the H antigen in secretions, including plasma. Individuals who are capable of forming the H antigen in secretions are therefore known as "secretors".

The H antigen is the precursor substrate on which terminal monosaccharides are attached via the specific transferase(s) encoded by the *ABO* gene, resulting in expression of the A antigen (*N*-acetylgalactosamine) and/or B antigen (galactose). Conversely, type O individuals do not encode an A or B transferase; thus, blood type O represents the absence of A and B antigens, and type O individuals express only the H antigen on their RBCs provided they encode at least 1 functional *FUT1* allele.

The Bombay phenotype results from mutations in both the *FUT1* and *FUT2* genes, precluding individuals with these mutations from producing the H antigen on their RBCs and in their secretions. The most common mutations are an inactivating mutation in Exon 4 of *FUT1* and deletion of Exon 2 in *FUT2*.[2,3] In contrast to "true" Bombay phenotype, the para-Bombay phenotype ($H+^w$) occurs in individuals generally via 1 of 2 mechanisms. In the first, mutations in the *FUT1* gene prevent the formation of the H antigen on RBCs; however, these individuals have at least 1 functional *FUT2* allele, allowing them to produce the H antigen in secretions and plasma.[2,3] As these individuals are capable of producing the H antigen in their plasma, a small amount may become passively adsorbed onto the surface of their RBCs. In the second mechanism, individuals with mutations in *FUT1* that result in a fucosyltransferase with significantly reduced, but intact function, have minute amounts of the H antigen, as well as the A and/or B antigen (depending on the ABO alleles), on their RBCs.

Bombay individuals appear to type as group O on ABO testing regardless of their ABO genotype, as they have no H-antigen structure on which to form the A and/or B antigen on their RBCs; therefore, there is no agglutination in the forward type when anti-A and anti-B reagents are tested against their RBCs. Similar to group O individuals, those with Bombay phenotype produce antibodies against the A and B antigens, and therefore agglutinate both A_1 and B reagent RBCs in the reverse type (an example of expected test results from a Bombay individual is depicted in **TABLE 1**). However, Bombay individuals also produce a clinically significant anti-H antibody, which is capable of causing severe intravascular hemolysis in the setting of incompatible RBC transfusion.

Para-Bombay individuals also tend to type as group O on forward typing using routine serologic testing methods, as they express either no or negligible amounts of H antigen on their RBC surface; however, because these individuals are capable of expressing the H antigen in their secretions, their reverse typing often reflects their ABO genotype (eg, if the patient genotypes as type A, their serum will agglutinate type B reagent cells). In contrast to Bombay individuals, those with the para-Bombay phenotype typically produce an anti-HI antibody, and less frequently, an anti-H antibody. The anti-HI antibody is optimally active at lower temperatures and is essentially universally clinically insignificant.[3] The anti-H antibody that can occur in para-Bombay individuals is often cold-reacting, with optimal reactivity at 4°C; however, these antibodies can have a broad thermal amplitude, and may react at 37°C, particularly in individuals with para-Bombay secondary to reduced *FUT1* activity.[5–8] Therefore, whereas Bombay individuals tend to show equally strong panreactivity on antibody panels, para-Bombay individuals have more variable reactivity patterns (**TABLE 2**). Given that the identity of the antibody present will significantly affect blood product selection and future patient management, testing to differentiate between anti-H and anti-IH is crucial. To differentiate these antibodies, testing the patient's plasma against type O cord RBCs can be performed; anti-H will react strongly with type O cord RBCs due to the presence of the H antigen, whereas anti-IH will react weakly or not at all due to the absence of the I antigen on cord RBCs, as this antibody reacts strongly only in the presence

TABLE 1. Example of Expected Serologic Results from an Individual with the Bombay Phenotype

ABO Type			
Forward Type		**Reverse Type**	
Anti-A	**Anti-B**	**A_1 cells**	**B cells**
0	0	4+	4+
Antibody screen			
Reagent cells		**Reactivity at IAT (AHG phase)**	
Reagent screening cell 1		4+	
Reagent screening cell 2		4+	
Reagent screening cell 3		4+	
Antibody identification			
Reagent cells		**Reactivity at IAT (AHG phase)**	
Reagent panel cell 1		4+	
Reagent panel cell 2		4+	
Reagent panel cell 3		4+	
Reagent panel cell 4		4+	
Reagent panel cell 5		4+	
Reagent panel cell 6		4+	
Reagent panel cell 7		4+	
Reagent panel cell 8		4+	
Reagent panel cell 9		4+	
Reagent panel cell 10		4+	
Reagent panel cell 11		4+	
Reagent panel cell 12		4+	
Reagent panel cell 13		4+	
Reagent panel cell 14		4+	
H antigen phenotyping			
Reagent		**Reactivity of patient RBCs**	
Ulex europaeus		0	

IAT, indirect antiglobulin test; AHG, anti-human globulin; RBC, red blood cells

TABLE 2. Example of Expected Results Obtained from Routine Serologic Testing of an ABO type A Individual with the Para-Bombay Phenotype

ABO Type			
Forward Type		**Reverse Type**	
Anti-A	**Anti-B**	**A_1 cells**	**B cells**
0	0	0	3+
Antibody screen			
Reagent Cells		**Reactivity at IAT (AHG Phase)**	
Reagent screening cell 1		4+	
Reagent screening cell 2		4+	
Reagent screening cell 3		3+	
Antibody Identification			
Reagent Cells		**Reactivity at IAT (AHG Phase)**	
Reagent panel cell 1		4+	
Reagent panel cell 2		3+	
Reagent panel cell 3		4+	
Reagent panel cell 4		4+	
Reagent panel cell 5		2+	
Reagent panel cell 6		3+	
Reagent panel cell 7		4+	
Reagent panel cell 8		4+	
Reagent panel cell 9		3+	
Reagent panel cell 10		3+	
Reagent panel cell 11		2+	
Reagent panel cell 12		4+	
Reagent panel cell 13		4+	
Reagent panel cell 14		2+	
H Antigen Phenotyping			
Reagent		**Reactivity of Patient RBCs**	
Ulex europaeus		1+	

IAT, indirect antiglobulin test; AHG, anti-human globulin; RBC, red blood cells

of both I and H antigens.[9] To assess for the presence of the H-antigen on the surface of RBCs, a substance from the plant *U. europaeus* can be used, which binds to the H-antigen and induces agglutination of RBCs that express the H-antigen. RBCs from Bombay individuals would not agglutinate in the presence of *U. europaeus,* whereas testing may demonstrate weak reactivity in para-Bombay individuals.

In contrast to the severe hemolysis that can occur if Bombay individuals receive RBCs bearing the H antigen, cases of hemolytic disease of the fetus and newborn (HDFN) associated with the anti-H antibody are exceptionally rare. This is likely due to multiple factors, including the rarity of the Bombay phenotype in general, decreased expression of the H antigen on fetal RBCs, interaction between the anti-H antibody and H antigen on other fetal tissues, and the fact that only the IgG isotype of the antibody can cross the placenta.[2] Furthermore, titers of anti-H do not appear to correlate with risk of HDFN in the cases observed, and the greatest risk to both mother and fetus seems to occur at the time of delivery due to potential hemorrhage and need for transfusion of H antigen-negative blood.[3]

As individuals with the Bombay phenotype produce a clinically significant anti-H alloantibody, they must only receive H antigen-negative blood. Therefore, despite being considered the universal red cell donor product, Bombay individuals cannot receive group O RBCs due to this anti-H alloantibody, as group O RBCs express large amounts of the H antigen. This can often prove challenging given the rarity of Bombay blood group donors, particularly in regions of Europe and North America. Thus, options for these patients include (i) autologous donation if the patient is able, (ii) directed donations from related individuals, (iii) querying of rare donor registries to identify potential unrelated Bombay donors, and/or (iv) acquisition of stored frozen Bombay units from blood bank inventories containing rare RBC units. In the setting of pregnancy, the mother may be instructed to undergo autologous donations between pregnancies for potential use during a subsequent delivery.

Given these considerations, optimal care of patients with the Bombay phenotype requires interdisciplinary care and close communication among clinical providers, including transfusion medicine physicians and blood donor services. Furthermore, as the completion of serologic investigation followed by allocation of H-negative RBC unit took more than 4 weeks, this case highlights the importance of early and accurate blood type and antibody screening in patients that may potentially require blood, as well as definitive characterization of any additional alloantibodies present.

REFERENCES

1. Bhende YM, Deshpande CK, Bhatia HM, et al. A “new” blood group character related to the ABO system. *Lancet (Lond).* 1952;1:903.
2. Krigstein M, Cromer N. Navigating the challenges of Bombay phenotype (O_h) and twin pregnancy; a case report and literature review. *Transfusion.* 2022;62(1):247–251.
3. Bullock T, Win N, Jackson B, et al. Bombay phenotype (Oh) and high-titer anti-H in pregnancy: two case reports and a review of the literature. *Transfusion.* 2018;58(12):2766–2772.
4. Reid ME, Lomas-Francis C, Olsson ML. *The Blood Group Antigen Factsbook*. Amsterdam: Elsevier/Academic Press; 2012.
5. Subramaniyan R. AB para-Bombay phenotype: a rare blood group variant and its clinical significance. *Hematol Transfus Cell Ther.* 2018;40(1):96–97.
6. Chacko MP, Mathan A, Daniel D. Para-Bombay: A blind spot in blood grouping? *Asian J Transfus Sci.* 2011;5(2):182–183.
7. Lin-Chu M, Broadberry RE. Blood transfusion in the para-Bombay phenotype. *Br J Haematol*. 1990 Aug;75(4):568–72.
8. Storry JR, Johannesson JS, Poole J, et al. Identification of six new alleles at the FUT1 and FUT2 loci in ethnically diverse individuals with Bombay and Para-Bombay phenotypes [published correction appears in Transfusion. 2007;47(2):360]. *Transfusion.* 2006;46(12):2149–2155.
9. Koh YE, Ryu GY, Kim YH, Jang MA, Yoon SY, Lim CS. Anti-H antibody showing agglutination in the anti-human globulin phase in a Korean patient. *Ann Lab Med.* 2016;36(3):284–286.

Causes of Inappropriate Laboratory Test Ordering from the Perspective of Medical Laboratory Technical Professionals: Implications for Research and Education

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Keywords: clinical laboratory services, medical laboratory personnel, medical laboratory science, allied health occupations, interprofessional skills, laboratory stewardship

Abbreviations: MLPs, medical laboratory professionals; MLT, medical laboratory technologist; MLA, medical laboratory assistant; CSMLS, Canadian Society for Medical Laboratory Science.

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ABSTRACT

Objective: Inappropriate laboratory test ordering is a significant and persistent problem. Many causes have been identified and studied. Medical laboratory professionals (MLPs) are technical staff within clinical laboratories who are uniquely positioned to comment on why inappropriate ordering occurs. We aimed to characterize existing MLP perceptions in this domain to reveal new or underemphasized interventional targets.

Methods: We developed and disseminated a self-administered survey to MLPs in Canada, including open-ended responses to questions about the causes of inappropriate laboratory test ordering.

Results: Four primary themes were identified from qualitative analysis: ordering-provider factors, communication factors, existing test-ordering processes, and patient factors. Although these factors can largely be found in previous literature, some are under-studied.

Conclusion: MLP insights into nonphysician triage ordering and poor result communication provide targets for further investigation. A heavy focus on individual clinician factors suggests that current understandings and interprofessional skills in the MLP population can be improved.

Laboratory testing is the most common diagnostic procedure in Canada. However, an estimated 20% to 50% of testing is inappropriately ordered.[1] Inappropriate testing results in large amounts of wasted resources, misdirected clinical effort, and the potential for patient harm.[2] The causes are numerous, overlapping, and intersecting. Research into contributing factors demonstrates that there is a mix of healthcare professional, patient, and system factors.[3] Healthcare systems globally are responding with a wide variety of interventional approaches, with some having greater success than others. Evidence arising from current reviews encourages approaches that include administrative or computerized strategies that limit access to inappropriate tests.[4–7]For example, ordering forms or processes could be adjusted to prevent ordering a test more inconsistent with guidelines. Interventions could be supported by complementary means, such as providing peer practice variation metrics, educational sessions, or audit and feedback.[4] Despite some gains, inappropriate testing remains a persistent and widespread issue, and the sustainability of interventions is largely unknown.[5,6]

Medical laboratory professionals (MLPs), often called scientists, technologists, technicians, or technical assistants, are underrepresented in literature related to inappropriate laboratory utilization. Unsurprisingly, the predominant intervention target is the test-ordering practitioner, as they steer the trajectory of testing being conducted. Recommended contributions from those in the laboratory are usually focused on high-authority actors, such as pathologists, managers, or PhD-trained clinical scientists. The scope of practice of MLPs in Canada includes the collection and performance of ordered tests, validation and communication of test results, and management of quality assurance processes.[8] For this article, we use the term MLP to encompass multiple designations, primarily medical laboratory technologist (MLT) and medical laboratory assistant (MLA). In general, an MLT has received a baccalaureate degree or diploma from an accredited training program and is required to be certified to practice in Canada. An MLA is typically trained at a vocational or private institution, and certification is not universally required across Canada.[9] There is some overlap in scope of practice, but

MLAs tend to focus on preanalytical activity, whereas MLTs have significantly more oversight and involvement in analytical processes and quality management.[10] MLPs work directly with ordering practitioners and systems as well as with healthcare professionals tangentially involved with test orders. Recent studies have explored the perceptions of other healthcare professionals, such as physicians, nurses, and advanced practice providers, about why they believe inappropriate laboratory testing occurs.[11,12] However, the observations arising from MLP experience in relation to the contributing factors of inappropriate laboratory test-ordering has not been investigated. The purpose of this study is to characterize existing MLP perceptions in this domain, which may reveal new or understudied targets for improvement activities.

Methods

We developed a self-administered online survey of MLPs in Canada, which was to evaluate behaviors, knowledge, and attitudes of MLPs with respect to their participation in laboratory stewardship.[13] The survey also examined MLP perspectives of external factors that contribute to inappropriate laboratory testing, which is analyzed and discussed here. Ethical approval was granted by both the ethics boards of the University of Alberta and the Canadian Society for Medical Laboratory Science (CSMLS), which is the national certifying body and professional society for MLPs in Canada.[14] The survey was sent to the active members of CSMLS in March 2019 and was available in French and English. Reminders were sent via social media, electronic newsletters, and direct emails. An open-ended question asked: "What do you believe are the primary reasons for over-ordering of laboratory tests?" The survey was pretested with a small (n = 20) sample of the target population to assess understandability and question validity. At the time of survey distribution, there were 9440 active CSMLS members with an MLT designation and 1833 who held an MLA designation.[15] According to the Canadian Institute for Health Information, there were 20,048 working MLTs in Canada in 2019.[16] There is no database that tracks practicing MLAs, as in most jurisdictions, they are not required to be registered.

Descriptive analysis of the quantitative demographic questions was performed using open-source statistical software jamovi.[17] We used the framework method as described by Gale et al[18] to identify themes in the open-ended question. This begins with the research team reading all responses, then assigning a brief conceptual label (theme) to excerpts of text. This was completed for 50 responses to develop the working analytical framework that was then applied to the remainder of the responses. Two researchers independently identified codes. Regular discussions occurred with any discrepancies solved by clarifying theme descriptions. The codes arising were then categorized into broad themes with subthemes.

Results

We received 1161 surveys from MLPs with responses to the open-ended question about reasons for inappropriate ordering. 151 respondents provided more than 1 reason. Most respondents hold the professional designation of Medical Laboratory Technologist. Responses were gathered from every province and territory in Canada and were highest from Ontario, the most populous region. The complete demographic profile is displayed in **TABLE 1**.

There were 4 major themes identified in the open-ended responses. Ordered by frequencies, they were ordering provider factors (72%), coordination factors (22%), ordering process factors (15%), and patient factors (4%). These themes, along with associated subthemes and definitions, are found in **TABLE 2**.

Theme 1: Ordering Provider Factors

Most respondents mentioned a cause directly attributed to the ordering provider (physician, nurse practitioner, pharmacist, etc.), including characteristic, confidence, or capability. Many respondents indicated that clinician training or professional development in laboratory medicine is inadequate, particularly in the context of advancements in testing and/or testing parameters. For example, respondents remarked that clinicians may lack "education about the benefits of newer and more accurate testing methods," or may not be aware of the "validity of certain tests when diagnosing their patients."

This knowledge gap was also thought to be related to clinical competency, particularly with less experienced ordering providers. One respondent stated that they see "new [clinicians] order everything to

TABLE 1. Demographic information of survey respondents

Category	n (%)
Professional Designation	
Medical Laboratory Technologist	1052 (91%)
Medical Laboratory Assistant	71 (6.1%)
Other	34 (2.9%)
Years of Clinical Experience	
0-10 years	410 (35.3%)
11-20 years	215 (18.6%)
21 or more years	534 (46.1%)
Location	
Alberta	198 (17.3%)
British Columbia	154 (13.4%)
Manitoba	125 (10.9%)
New Brunswick	77 (6.7%)
Newfoundland & Labrador	37 (3.2%)
Northwest Territories, Nunavut, Yukon	10 (0.9%)
Nova Scotia	135 (11.8%)
Ontario	298 (26.0%)
Prince Edward Island	12 (1.0%)
Quebec	30 (2.6%)
Saskatchewan	69 (6.0%)
Sex	
Male	142 (12.4%)
Female	986 (85.8%)
Other/Prefer not to disclose	21 (1.8%)

TABLE 2. Analysis of Reasons for Overordering

Theme	Subtheme	Description
Ordering provider factors—mentioned by 72% of respondents	Inadequate training in laboratory medicine	Responses here refer to the perception that ordering provider training in laboratory medicine (in university, residency, or continued professional development) may be inadequate.
	Habit	Responses here refer to the perception that there are some ordering providers who order the same tests for their patients most or all the time.
	Shotgun approach	Responses here describe the perception that ordering providers are ordering excessive tests in the hope that one may generate a result that could help with diagnostic decision-making
	Inexpensive tests	Responses here refer to the perception that ordering providers view testing as inexpensive and plentiful, and thus not subject to limits.
	Fear of litigation	Responses here refer to the perception that ordering providers may order too much because they are concerned about litigation arising from not exhaustively investigating each patient case.
	Lack of time	Responses here describe situations where ordering providers order excessive testing because they do not have enough time to obtain complete clinical histories or take a measured, stepwise approach
Coordination factors—mentioned by 22% of respondents	Duplication of efforts	Responses here indicate that problems with disjointed or fragmented care is one of the drivers of inappropriate ordering. Overtesting arises when multiple ordering providers or care settings order duplicate tests.
	Barriers to accessing history	Responses here describe perceptions that inappropriate ordering occurs because there is poor access to previous results. This access may be because of system deficiencies, or the lack of mechanisms to support this action by physicians or other healthcare professionals.
	Ordering before patient is seen by physician	Responses here describe the practices performed by other healthcare professionals that contribute to overordering, particularly the practice of prefilled or nursing/clerk-filled requisitions that are submitted before the patient sees the physician. This leads to testing that may not match symptoms.
Ordering process factors—mentioned by 15% of respondents	Order sets and panels	Responses here describe the belief that many existing testing bundles often contain tests that are not relevant for a given patient.
	Daily or standing orders	Responses here describe how ordering systems may also contribute to extended inappropriate testing through standing orders existing in perpetuity without requiring review.
	No oversight	Responses here indicate that a lack of oversight for ordering allows inappropriate practices to perpetuate
Patient Factors - mentioned by 4% of respondents	None	Responses here refer to the perception that the patient is the primary driver of inappropriate testing.

try to catch something abnormal that might explain the clinical picture." This type of sentiment is echoed in the sense that some ordering providers may lack confidence and are using "tests to make a diagnosis rather than to support a diagnosis." This is closely related to the label of "shotgun medicine" that many participants indicated in their response. At times, this approach to testing was given in the context of a fear of litigation or a lack of time. As 1 respondent described: "Doctors don't have enough time to spend with patients to determine the true cause of the complaint, so they blanket order tests to cover all bases." In some responses, participants linked this defensive ordering approach to cost. If testing is viewed as cheap and plentiful, there is no need to consider restrictions. Frustration was noted frequently, as illustrated by a respondent lamenting that ordering providers "undervalue how much a lab test costs in labour hours and transportation costs."

Some respondents perceived that inappropriate testing is performed because of ingrained ordering practices. As one respondent put it, "it's habit. Clinicians order the same tests that were ordered last time, or the same set of tests for all their patients." This was not necessarily attributed to individual ordering providers but occasionally, to departments. The emergency department was frequently singled out. For instance, a respondent described that "the emergency department orders the same things on everybody. I think it's a convenience for them."

Theme 2: Coordination Factors

Although not mentioned as frequently as clinician-related factors, many respondents indicated that factors related to the way care is communicated and coordinated contribute to inappropriate ordering of laboratory tests. When patients see more than 1 care provider, there can be duplication of efforts. One respondent believed that excess tests are ordered because of a "lack of communication between general physicians and specialists. I see a lot of times a general physician will order tests and then a specialist will order the same tests." This lack of coordination was also noted across locations or departments. One respondent commented that "different locations order the same tests when patient is transferred, not waiting for first result to come back before ordering again." Several survey participants commented that there were challenges when trying to access prior patient test results. One respondent summed this up as "poor information sharing, poor communication between areas, and a lack of a centralized patient care computer system." Respondents also noted that in some clinical areas, tests were ordered by a nurse or clerk before a physician saw the patient in hopes of making triage more effective. A participant remarked that this practice occurs "to have results before the doctor even sees the patient in emergency department."

Theme 3: Ordering Process Factors

Several survey participants brought up ideas related to the ordering system and process itself. One major issue identified is standing orders, particularly those that do not require a stop date. The presence of order sets and testing panels was also identified as potentially problematic, because they are easy to use yet may contain uninformative tests. One respondent described that "unnecessary tests are on an order set so get ordered without a second thought of appropriateness." Some respondents qualified that these order sets are often outdated or lack the proper review needed to update ordering to current best practices.

The relative ease of ordering was frequently listed as a factor contributing to over-testing. A respondent described that it is "very easy for physicians to check off the boxes on requisitions." Some stated that this ease was exacerbated by lack of accountability. This is illustrated by the following quote that ordering providers "are not held responsible for the monetary, and systematic cost incurred by inappropriately ordered lab tests."

Theme 4: Patient Factors

Responses related to patients themselves were infrequent but present. A representative quotation from a respondent is that unnecessary testing occurs because of "pressure exerted by patients and their families on physicians to investigate illness regardless of the opinion of the healthcare professionals."

Discussion

MLPs identified a wide range of factors that contribute to inappropriate ordering of laboratory tests. They mentioned factors related to ordering providers most frequently, which mirrors the emphasis on the ordering provider found in the utilization management literature. Multipronged interventions are the most successful,[4] but they largely focus on the ordering process and those ordering the tests. This focus may miss opportunities to improve the appropriateness of ordering, as illustrated by the variety of factors identified by MLPs. Further research into factors related to health system structure, such as ways care is coordinated, is needed.

MLPs identified several factors related to the coordination or communication of results. They witnessed a lack of access to valid previous results and believe this contributes to overordering. Other research supports that missing results contributes to inappropriate repeat test ordering,[19] as does transferring patients from 1 hospital to another.[20] Being seen by multiple providers also contributes to overordering,[21] but this type of systems-lens research is scant. As clinical information systems continue to evolve and connection between and within sites improves, attention should be paid to how effectively clinicians are able to access test results, particularly when historical results remain valid. As it is within the scope of practice of MLPs to communicate results, they can be part of initiatives to improve result receipt. Research has linked issues of result communication with diagnostic errors.[22] More research is needed to understand relationships between result communication, result receipt, and inappropriate testing, particularly in an era where test results are increasingly being communicated directly to patients.[23]

The emergency department was mentioned specifically as a source of inappropriate test ordering, which aligns with a recent study that also explored MLP perspectives.[24] In our study, MLPs doubted the effectiveness of obtaining laboratory test results before the patient is seen by a physician, or "triage ordering". The emergency department is a place where unnecessary testing is not uncommon.[25] In general, increased numbers of tests and order episodes tends to increase patient length of stay.[26] However, in specific cases, such as patients presenting with chest pain, ordering triage laboratory tests may quicken important clinical decisions.[27] A recent qualitative study revealed that staff believe that this type of advance ordering is important for efficiency and continuity of care.[28] The impact of ordering triage laboratory tests is an emerging, context-dependent area where research is needed. A recent review found that nonphysician-ordered triage laboratory testing did not result in clinically meaningful changes to length of stay in the emergency department, nor did it always line up with what would be ordered once complete clinical information is obtained.[29] This may result in unnecessary phlebotomy events, which are not without risks, such as nerve injury[30] or hospital-acquired anemia.[31] This is complicated by settings where specific tests are required for referral or consultation, although the relevance of these tests is suspect. For example, mandatory screening tests for people with psychiatric complaints in the emergency department rarely affected patient care.[32] Optimizing emergency department ordering sets and test availability can decrease unnecessary laboratory tests,[33] but this approach usually focuses on physician ordering once the patient has been seen.

The factors identified by MLPs with respect to individual ordering providers are present to varying degrees in the literature. Ordering providers agree that habit is an important driver of inappropriate testing.[12,34] Limited laboratory medicine training in many medical schools means that physicians may lack understanding of the basic concepts of laboratory testing.[11,35] Studies indicate a wide variation in test-ordering practices between individual physicians even within the same diagnoses.[36] For every test, performance characteristics and biological variation can affect result interpretation, but these metrics are often poorly understood by ordering providers.[37] MLPs hold expertise on testing requirements and workflow and can have a role in helping provide education on testing parameters, changes in testing, and updates in technology. However, it is not within their scope of practice to advise on clinical decision-making, such as helping understand the diagnostic meaning of a particular result.

The most frequently mentioned cause of inappropriate testing related directly to individual ordering providers, and at times, the phrasing seemed to betray frustration. MLPs do not have an opportunity to interact with ordering providers directly or frequently in a way that allows them to have a full understanding of their role. Other research describes interactions between laboratory staff and physicians about utilization issues as frequently negative.[24] Historically, healthcare has been hierarchical and siloed. This hierarchy can impede stewardship efforts of those who perceive themselves in positions of less power. For example, a recent qualitative study about nursing roles in antimicrobial stewardship identified that lack of clarity of roles has a negative impact on collaboration and integration of nursing expertise, as well as the social dynamics that defer to physician expertise and the desire to avoid the appearance that physician decisions are being questioned.[38] Enhancing teamwork in healthcare has been promoted as a strategy for many contemporary problems.[39] The perceptions held about others can facilitate or impede effective teamwork.[40,41] MLPs may be reluctant to be part of interdisciplinary teams working to solve problems if they perceive that individual ordering providers are primarily responsible for the issue and are intimidated by perceived relative positions of power. Building trust for effective collaboration between healthcare professionals requires understanding and respecting each other's roles, expertise, and efforts to overcome biases.[42] With a goal of enhancing teamwork and collaboration, there is room to improve MLP overall understanding to include more nuanced and system-based factors.

Limitations

We were unable to probe responses to have a full description of existing perceptions. Thus, only the most obvious or top-of-mind responses

were likely given. Our sample was based on nonprobability voluntary response of CSMLS members and was overrepresented by MLTs (91%). Thus, we do not claim that the results are representative of the entire MLP population. In addition, the structure of clinical laboratory service delivery is different across Canada, which likely affects MLP lived experience. We echo other researchers that when addressing inappropriate utilization, a careful study of local contexts is vital.[2,7,43]

Conclusion

Given the nuance added through exploring the observations of MLPs, we encourage their inclusion as stakeholders in utilization management and laboratory stewardship activities. Educational institutions and professional learning programs should focus on supporting enhanced understanding of resource utilization issues and effective participation as members of stewardship teams.

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REFERENCES

1. Naugler C, Wyonch R. What the doctor ordered: improving the use and value of laboratory testing. CD Howe Institute Commentary No. 533. 2019. Available from: https://www.cdhowe.org/public-policy-research/what-doctor-ordered-improving-use-and-value-laboratory-testing. Accessed 14 February 2022.
2. Cadamuro J, Ibarz M, Cornes M, et al. Managing inappropriate utilization of laboratory resources. *Diagnosis.* 2019;6(1):5–13.
3. Whiting P, Toerien M, Salis I, et al. A review identifies and classifies reasons for ordering diagnostic tests. *J Clin Epidemiol.* 2007;60(10):981–989.
4. Baird G. The laboratory test utilization management toolbox. *Biochem Medica.* 2014;24(2):223–234.
5. Kobewka DM, Ronksley PE, McKay JA, et al. Influence of educational, audit and feedback, system based, and incentive and penalty interventions to reduce laboratory test utilization: a systematic review. *Clin Chem Lab Med.* 2015;53(2):157–183.
6. Bindraban RS, Berg MJ, Naaktgeboren CA, et al. Reducing test utilization in hospital settings: a narrative review. *Ann Lab Med.* 2018;38(5):402–412.
7. Rubinstein M, Hirsch R, Bandyopadhyay K, et al. Effectiveness of practices to support appropriate laboratory test utilization. *Am J Clin Pathol.* 2018;149(3):197–221.
8. Medical Laboratory Techonologist Scope of Practice Review [Online]. Health Professions Regulatory Advisory Council; 2008. Available from: https://www.hprac.org/en/projects/resources/HPRAC.Collaboration.MLTScopeLitReviewFINAL.pdf. Accessed 30 March 2020.
9. CSMLS - SCSLM/ Education. (Updated 2022). Available from: https://www.csmls.org/Medical-Laboratory-Professionals/How-to-become-a-Medical-Lab-Professional/Education.aspx. Accessed 2 March 2022.
10. Medical Laboratory Professionals. (Updated 2022). Available from: https://medlabprofessionals.ca/. Accessed 2 March 2022.
11. Vrijsen BEL, Naaktgeboren CA, Vos LM, et al Inappropriate laboratory testing in internal medicine inpatients: prevalence, causes and interventions. *Ann Med Surg* 2020;51:48–53.
12. Korenstein D, Husain S, Gennarelli RL, et al. Impact of clinical specialty on attitudes regarding overuse of inpatient laboratory testing. *J Hosp Med.* 2018;13(12):844–847. doi:10.12788/jhm.2978.
13. VanSpronsen AD, Zychla L, Villatoro V, et al. Engaging laboratory staff in stewardship: barriers experienced by medical laboratory technologists in Canada. *J Appl Lab Med.* 2022;7(2):480–494. doi:10.1093/jalm/jfab103.
14. CSMLS - SCSLM/ Who are Lab Professionals. (Updated 2022). Available from: https://www.csmls.org/Medical-Laboratory-Professionals/About/Who-are-Lab-Professionals.aspx. Accessed June 10, 2020.
15. CSMLS - SCSLM/ Demographics. 2019. Available from: https://www.csmls.org/About-Us/Our-Members/Demographics.aspx. Accessed June 11, 2020.
16. Canadian Institute of Health Information. Health Workforce | CIHI. (Updated 2021). Available from: https://www.cihi.ca/en/health-workforce. Accessed June 11, 2020.
17. jamovi - Stats. Open. Now. 2020. Available from: https://www.jamovi.org/. Accessed February 14, 2022.
18. Gale NK, Heath G, Cameron E, et al. Using the framework method for the analysis of qualitative data in multi-disciplinary health research. *BMC Med Res Methodol.* 2013;13:117.
19. Fieldston ES, Fakeye OA, Friedman DF. Repeat laboratory testing in the pediatric emergency department: how often and how important? *Pediatr Emerg Care.* 2019;35(3):190–193.
20. Bertrand J, Fehlmann C, Grosgurin O, et al. Inappropriateness of repeated laboratory and radiological tests for transferred emergency department patients. *J Clin Med* 2019;8(9):E1342.
21. Tam K, Williamson T, Ma IWY, et al. Association between health system factors and utilization of routine laboratory tests in clinical teaching units: a cohort analysis. *J Gen Intern Med.* 2021. doi:10.1007/s11606-021-07063-2.
22. Barwise A, Leppin A, Dong Y, et al. What contributes to diagnostic error or delay? A qualitative exploration across diverse acute care settings in the United States. *J Patient Saf.* 2021;17(4):239–248.
23. Witteman HO, Zikmund-Fisher BJ. Communicating laboratory results to patients and families. *Clin Chem Lab Med.* 2019;57(3):359–364.
24. Asalmari S. Inappropriate laboratory testing requests: perspectives of clinical laboratory professionals. *Iberoam J Med.* 2021;3(3):249–256.
25. Maughan BC, Rabin E, Cantrill SV. A broader view of quality: choosing wisely recommendations from other specialties with high relevance to emergency care. *Ann Emerg Med.* 2018;72(3):246–253.
26. Li L, Georgiou A, Vecellio E, et al. The effect of laboratory testing on emergency department length of stay: a multihospital longitudinal study applying a cross-classified random-effect modeling approach. *Acad Emerg Med.* 2015;22(1):38–46. doi:10.1111/acem.12565.
27. Hwang CW, Payton T, Weeks E, et al. Implementing triage standing orders in the emergency department leads to reduced physician-to-disposition times. *Adv Emerg Med.* 2016;2016:e7213625.
28. Li J, Dahm MR, Thomas J, et al. Why is there variation in test ordering practices for patients presenting to the emergency department with undifferentiated chest pain? A qualitative study. *J Emerg Med.* 2021;38(11):820–824.
29. Gottlieb M, Farcy DA, Moreno LA, et al. Triage nurse-ordered testing in the emergency department setting: a review of the literature for the clinician. *J Emerg Med.* 2021;60(4):570–575. doi:10.1016/j.jemermed.2020.11.004.
30. Tsukuda Y, Funakoshi T, Nasuhara Y, et al. Venipuncture nerve injuries in the upper extremity from more than 1 million procedures. *J Patient Saf* 2019;15(4):299–301. doi:10.1097/PTS.0000000000000264.
31. Shander A, Corwin HL. A narrative review on hospital-acquired anemia: keeping blood where it belongs. *Transfus Med Rev.* 2020;34(3):195–199.
32. Tobin MR, Hartline JR, Sullivan SB, et al Utility of nonspecific laboratory testing for psychiatric patients undergoing medical screening in a military emergency department. *Mil Med.* 2020;185(11–12):e1941–e1945.
33. Lapić I, Juroš GF, Rako I, et al Changing the electronic request form proves to be an effective tool for optimizing laboratory test utilization in the emergency department. *Int J Med Inf.* 2017;102:29–34.

34. Parker G, Shahid N, Rappon T, et al. Using theories and frameworks to understand how to reduce low-value healthcare: a scoping review. *Implement Sci.* 2022;17:6. doi:10.1186/s13012-021-01177-1.

35. Smith BR, Aguero-Rosenfeld M, Anastasi J, et al. Educating medical students in laboratory medicine: a proposed curriculum. *Am J Clin Pathol.* 2010;133(4):533–542.

36. Sood R, Sood A, Ghosh AK. Non-evidence-based variables affecting physicians' test-ordering tendencies: a systematic review. *Neth J Med.* 2007;65(5):167–177.

37. Emre HO, Karpuzoglu FH, Coskun C, et al. Utilization of biological variation data in the interpretation of laboratory test results – survey about clinicians' opinion and knowledge. *Biochem Medica.* 2021;31(1):010705.

38. Kirby E, Broom A, Overton K, et al. Reconsidering the nursing role in antimicrobial stewardship: a multisite qualitative interview study. *BMJ Open.* 2020;10(10):e042321. doi:10.1136/bmjopen-2020-042321.

39. Rosen MA, Diaz Granados D, Dietz AS, et al. Teamwork in healthcare: key discoveries enabling safer, high-quality care. *Am Psychol.* 2018;73(4):433–450.

40. Hean S, Clark JM, Adams K, et al. Will opposites attract? Similarities and differences in students' perceptions of the stereotype profiles of other health and social care professional groups. *J Interprof Care.* 2006;20(2):162–181.

41. Cook K, Stoecker J. Healthcare student stereotypes: a systematic review with implications for interprofessional collaboration. *J Res Interprofessional Pract Educ*. 2014;4(2). Available from: https://jripe.org/index.php/journal/article/view/151. Accessed October 15, 2021.

42. Supper I, Catala O, Lustman M, et al. Interprofessional collaboration in primary health care: a review of facilitators and barriers perceived by involved actors. *J Public Health.* 2015;37(4):716–727.

43. Lam JH, Pickles K, Stanaway FF, et al. Why clinicians overtest: development of a thematic framework. *BMC Health Serv Res.* 2020;20(1):1–11.

CASE STUDY

Mycoplasma hominis Meningitis Diagnosed by Metagenomic Next-Generation Sequencing in a Preterm Newborn: a Case Report and Literature Review

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Keywords: *mycoplasma hominis*, meningitis, metagenomic next-generation sequencing, preterm, cerebrospinal fluid, case report

Abbreviations: mNGS, metagenomic next-generation sequencing; CSF, cerebrospinal fluid; WBC, white blood cell; N, neutrophil; L, lymphocyte; CRP, C-reactive protein; M, monocyte; LDH, lactate dehydrogenase; PCR, polymerase chain reaction; MP, Mycoplasma pneumoniae; RNA, ribonucleic acid; ADA, adenosine deaminase; IHA, indirect erythrocyte agglutination test.

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ABSTRACT

Mycoplasma hominis is mainly colonized in the genital tract and vertically transmitted to newborns; however, it rarely causes neonatal meningitis. We report a case of *M. hominis* meningitis in a premature infant. She was admitted to our hospital for treatment after 6 days of repeated fever. After admission, repeated cerebrospinal fluid (CSF) analysis showed that leukocytes and protein in CSF increased substantially and glucose decreased, but there was no growth in conventional CSF culture. The patient was diagnosed with *M. hominis* meningitis by metagenomic next-generation sequencing (mNGS). The antibiotic therapy used for the neonate was meropenem, vancomycin, and ampicillin against bacterial infection and azithromycin against mycoplasma infection. The child was subsequently considered cured and discharged from the hospital and followed up regularly in the neurology clinic. The mNGS may be a promising and effective diagnostic technique for identifying uncommon pathogens of meningitis in patients with meningitis symptoms and signs without microbial growth in routine CSF culture.

Mycoplasma hominis is mainly colonized in the human urinary system and the genital tract, causing urinary tract infection, genital inflammation, and adverse pregnancy outcomes.[1,2] Neonates are susceptible to *M. hominis* infection in the uterus or through the colonized birth canal during delivery.[3] However, the organism rarely causes meningitis in newborns, either full-term or preterm infants.[4] Also, it does not grow through routine culture, rendering the diagnosis of *M. hominis* meningitis difficult. Herein, we reported a case of *M. hominis* meningitis in a preterm infant diagnosed by metagenomic next-generation sequencing (mNGS). The infant presented clinical manifestations of the central nervous system infection, but no growth was detected in the conventional cerebrospinal fluid (CSF) cultures.

Case Presentation

After a 14-hour labor, a female was born at 36 weeks of gestation to a gravida 1, parity 1 mother with spontaneous premature rupture of membranes. The neck of the fetus was wrapped by two loops of the umbilical cord, and the Apgar score was 9-10-10 for 1-5-10 min, respectively. The weight of the newborn was 2500 g. The mother had no history of fever and disease during pregnancy and delivery. The baby suffered from neonatal hyperbilirubinemia because of ABO incompatibility and was treated with blue light in the hospital for 3 days. Subsequently, she was admitted to another hospital due to fever and intracranial infection 6 days after birth. Blood routine examination results were as follows: white blood cell (WBC) 15.38×10^9/L, neutrophil (N) 66.1%, hemoglobin 111 g/L, platelet 716×10^9/L, and C-reactive protein (CRP) 40.50 mg/L. The CSF was yellow and slightly turbid, and analysis revealed WBC 480×10^6/L, lymphocyte (L) 83%, protein 1738.90 mg/L, and glucose 0.23 mmol/L. Sputum culture was negative. The treatment plan was ambiguous. Due to the poor treatment effect and recurring fever for 6 days, the patient was transferred to the neonatal department of West China Second University Hospital, Sichuan University.

After admission, the anteroposterior radiograph of the chest and abdomen showed neonatal pneumonia, and the CRP level was 17.8 mg/L. Lumbar puncture was performed, and CSF analysis showed WBC 450×10^6/L (N 31%, L 58%, monocyte [M] 11%), protein 1256.0 mg/L, Glu 0.20mmol/L, chloride 108.0 mmol/L, and lactate dehydrogenase (LDH) 93 U/L. The normal range of protein and glucose in CSF in our hospital is 80 to 430 mg/L and 2.8 to 4.5 mmol/L, respectively, whereas the blood glucose level of the infant was 2.9 mmol/L. Vancomycin,

meropenem, and ampicillin were used for empirical antibiotic therapy. No microorganisms were detected in the CSF or blood samples as assessed by Gram staining. Also, no pathogens were found in the CSF by conventional culture or multiplex polymerase chain reaction (PCR), according to a meningitis pathogens nucleic acid detection kit, which can detect 12 viruses and 7 bacteria, including *Listeria monocytogenes*, *Borrelia burgdorferi*, and *Cryptococcus*. On day 2 after admission, *Mycoplasma pneumoniae* (MP) nucleic acid was detected by quantitative fluorescence PCR and treated with azithromycin. At 4 days after antibiotic treatment, the cytological and biochemical results of CSF were as follows: WBC 290 × 10^6/L (N 41%, L 57%, and M 7%), protein 2907.0 mg/L, Glu 1.10 mmol/L, chloride 121.0 mmol/L, and LDH 69. However, no microorganisms were detected by CSF culture or microscopy. After continued treatment, CSF analysis showed the following: WBC 245 × 10^6/L (N 27%, L 67%, and M 6%), protein 2583.0 mg/L, Glu 1.50 mmol/L, chloride 116.0 mmol/L, and LDH 76U/L. At 11 days after admission, as the symptoms of the child improved and the body temperature returned to normal, vancomycin and azithromycin were discontinued. Nevertheless, 3 days after drug withdrawal, fever was persistent after a combination antibiotic therapy of meropenem and ampicillin. The results of CSF assessment showed WBC 243 × 10^6/L (N 6% and L 94%), protein 2338.0 mg/L, Glu 1.20 mmol/L, chloride 123.0 mmol/L, and LDH 78U/L, which was not a significant improvement compared to results before the treatment. Fluconazole and vancomycin have been used empirically to prevent fungal infection.

The child was transferred from the neonatal department to the pediatric neurology department for further treatment 20 days after admission. Several samples of CSF were collected by lumbar puncture for culture to evaluate the therapeutic effects, and the results are shown in **TABLE 1**. The CSF analysis showed that the protein in the CSF decreased significantly and glucose increased, indicating that antibiotic treatment was effective, but there was no microbial growth, and the pathogens nucleic acid of CSF was always negative. On day 6, *M. hominis* was detected in the CSF by using mNGS in the neurology department (**FIGURE 1**), whereas no growth was observed in the CSF culture; the multiplex PCR was also negative. Since the child's condition was in remission, the therapeutic schedule was not adjusted. The child was in stable condition on day 15 in the neurology department, on which day vancomycin and meropenem were discontinued. Finally, the child was discharged 58 days after admission without neurological complications and sequelae. Except for slight protein elevation (633.6 mg/L), other CSF findings returned to normal, and the child was referred to the neurology clinic for follow-up.

Discussion and Conclusion

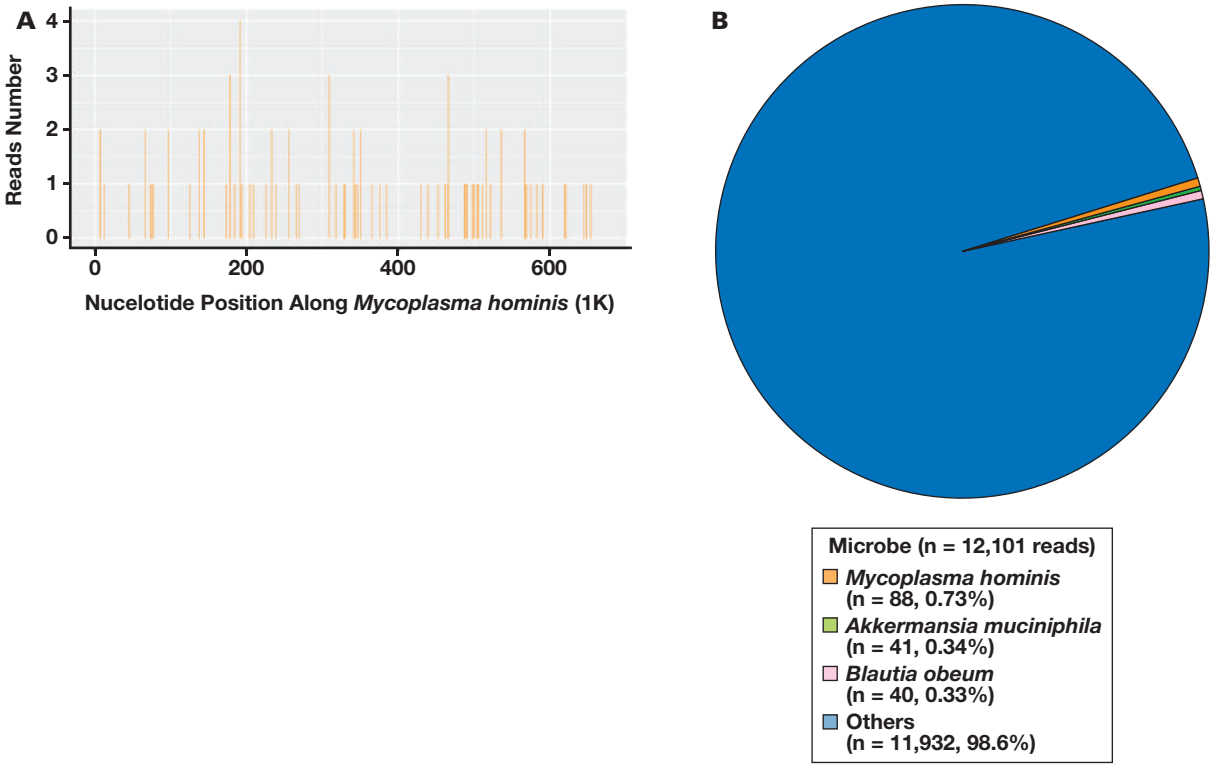
M. *hominis* colonizes in the female genital tract and infects newborns through vertical transmission during delivery, causing neonatal bacteremia, pneumonia, and meningitis.[5] Although *M. hominis* meningitis is rare in newborns, it leads to neurological deficits and can be life-threatening. Previous studies have shown that the risk factors of neonatal *M. hominis* meningitis are preterm birth, low birth weight, and neural tube defects.[5] In this study, we reported a case of *M. hominis* meningitis in a premature newborn diagnosed by mNGS and also retrieved 10 cases of preterm infants from the literature in English[6–15] (**TABLE 2**). There have been 10 detailed reports of *M. hominis* meningitis in preterm neonates, including 2 very preterm infants and

TABLE 1. The Results of CSF Culture and Examination

Enrollment date	WBC	Protein (mg/L)	Glu (mmol/L)	Chloride (mmol/L)	LDH (U/L)	ADA	smear	culture	Nucleic acid detection
Day 1	50 × 10^6/L(N3%, L63%, M34%)	1656.0	1.50	124.0	43	N/A	negative	negative	negative
Day 6	30 × 10^6/L(N16%, L70%, M14%)	1147.4	2.01	125.0	47	0.7	negative	negative	negative
Day 20	10 × 10^6/L	633.6	2.28	124.0	44	0.1	negative	negative	negative
Day 28	25 × 10^6/L(N19%, L71%, M10%)	591.0	2.27	121.0	35	<0.1	negative	negative	negative

WBC, white blood cell; LDH, lactate dehydrogenase; ADA, adenosine deaminase.

FIGURE 1 Metagenomic next-generation sequencing (mNGS) results of this case. A, Coverage of *M. hominis* detected by mNGS was 0.83%. B, A total of 88 specific reads of *M. hominis* were detected by mNGS in this case.



4 extremely preterm infants, and the birth weight of each child was less than 2500g. The analyses of these cases revealed that all patients had a vaginal delivery except 1 who did not know the mode of delivery and 1 who underwent cesarean delivery. This indicated that premature infants delivered vaginally might be infected by *M. hominis*, resulting in *M. hominis* meningitis. One case of death was a 26-week-old newborn who developed *M. hominis* meningitis at 20 days after birth, whereas a 35-week-old premature infant, whose onset age was 15 days after birth, developed severe hemiplegia. The outcome of *M. hominis* meningitis may be related to the onset age but not birth weight and gestational age. This phenomenon could be attributed to the delayed admission time rather than late-onset age, allowing deterioration of the patient's condition and causing poor prognosis. Furthermore, diverse antibiotics, including chloramphenicol, doxycycline, moxifloxacin, and ciprofloxacin, are available for the treatment of *M. hominis* meningitis. Herein, azithromycin was used to resist mycoplasma infection. Some studies have shown that *M. hominis* is sensitive to azithromycin.[16] Moreover, azithromycin combined with other antibiotics was effective in treating adult *M. hominis* meningitis[17] and *M. hominis* orbital abscess.[18] Conversely, some studies proposed that azithromycin is ineffective in treating *M. hominis*[19,20] and hence, combination of azithromycin and vancomycin, meropenem, and ampicillin was used in the present case. Therefore, the therapeutic effect of azithromycin on *M. hominis* is as yet controversial, which could be explained by the loss of drug resistance of the organism due to23S rRNA mutation.[21] Moreover, the combination of antibiotics may also be the key factor for successful treatment. Whether azithromycin can be used in the clinical treatment of *M. hominis* needs to be verified further. Of the 10 cases in the literature review, 8 cases were identified by culture and 2 cases were identified by 16s RNA sequencing. The cases we reported were identified by mNGS. Due to low sensitivity or inactivation of pathogens after antibiotic treatment, traditional culture identification of *M. hominis* is difficult. In addition, compared with 16s RNA sequencing, mNGS has more advantages in species identification, because 16s RNA sequencing can only identify the genus of microorganisms.

Palacios et al[22] identified a new arenavirus from 3 organ transplant patients analyzed by metagenomics in 2008. To the best of our knowledge, this is the first study wherein mNGS was applied to identify pathogenic microbial infectious diseases, especially rare, new, difficult-to-detect, or co-infected pathogens.[23] Subsequently, this technology has been increasingly applied in the clinic. Together, the case symptoms and laboratory results suggested that the child in this study may suffer from bacterial meningitis, but no growth was detected in the CSF culture. The negative culture may be because a BD Bactec Peds Plus pediatric blood culture vial was used for CSF culture in our hospital rather than the specific medium for mycoplasma culture. In addition, the infant was treated with antibiotics in the other hospital before admission, which might cause microbial inactivation. Appropriate clinical medication cannot be determined due to uncertain etiology, thereby delaying diagnosis of the child's condition. Finally, the case was confirmed as *M. hominis* meningitis by mNGS. Since mNGS can detect multiple microorganisms in a panel, it is useful for the rapid diagnosis of unusual pathogens or atypical clinical pathogens in newborns. Therefore, mNGS may be a promising and effective diagnostic technique for identifying uncommon pathogen meningitis when the result of traditional microbial cultivation is negative.

TABLE 2. Characteristics of Published Cases with *Mycoplasma hominis* Meningitis in Premature Infants

Study	Gender	Gestational Age	Birth Weight	Birth Mode	Onset Age	Identification	Treatment	Outcome
Siber, 1977	Male	35 weeks	1530g	Vaginal delivery	15 days after birth	CSF cultures and serologic test	Ampicillin, chloramphenicol	Right hemiparesis and left frontal transillumination
Gewitz, 1979	Female	33 weeks	1740g	Vaginal delivery	8 days after birth	CSF cultures	Chloramphenicol, tetracycline	Normal development
Hjelm, 1980	Male	34-35 weeks	2200g	Vaginal delivery	10 days after birth	CSF cultures and IHA	Doxycycline, lincomycin	Normal development
Kirk, 1987	Male	30 weeks	1080g	Vaginal delivery	12 days after birth	CSF cultures and growth inhibition tests	Chloramphenicol, doxycycline	Normal development
McDonald, 1988	Male	26 weeks	920g	Vaginal delivery	20 days after birth	CSF cultures and growth inhibition tests	Ampicillin, vancomycin, chloramphenicol, doxycycline	Death
Watson, 2008	Male	24 weeks	623g	Vaginal delivery	4 days after birth	CSF cultures	Chloramphenicol	Good clinical progress
Watt, 2012	Male	26 weeks	813g	Not mentioned	6 days after birth	CSF 16s RNA sequencing	Doxycycline, moxifloxacin	Normal development
Nohren, 2020	Female	30 weeks	Not mentioned	Cesarean delivery	2 days after birth	CSF cultures and 16s rRNA sequencing	Ciprofloxacin, gentamicin, moxifloxacin	Normal development
Kersin, 2020	Female	27 weeks	885g	Vaginal delivery	At birth	CSF 16s rRNA gene sequencing	Vancomycin, meropenem, ciprofloxacin	Moderate neurological impairment
Ansari, 2021	Male	25 weeks	Not mentioned	Vaginal delivery	7 days after birth	CSF cultures and 16s rRNA PCR	Moxifloxacin, doxycycline	Normal neurological examination

CSF, cerebrospinal fluid; IHA, indirect erythrocyte agglutination test; RNA, ribonucleic acid; PCR, polymerase chain reaction.

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REFERENCES

1. Wildenbeest JG, Said I, Jaeger B, Hest R.M. van, et al. Neonate with Mycoplasma hominis meningoencephalitis given moxifloxacin. *Lancet Infect Dis.* 2016;16(11):e261–e266.
2. Capoccia R, Greub G, Baud D. Ureaplasma urealyticum, Mycoplasma hominis and adverse pregnancy outcomes. *Curr Opin Infect Dis.* 2013;26(3):231–240. doi:10.1097/QCO.0b013e328360db58.
3. Knausz M, Niederland T, Dosa E, Rozgonyi F. Meningo-encephalitis in a neonate caused by maternal Mycoplasma hominis treated successfully with chloramphenicol. *J Med Microbiol.* 2002;51(2):187–188. doi:10.1099/0022-1317-51-2-187.
4. Hata A, Honda Y, Asada K, Sasaki Y, et al. Mycoplasma hominis meningitis in a neonate: case report and review. *J Infect.* 2008;57(4):338–343. doi:10.1016/j.jinf.2008.08.002.
5. Waites KB, Schelonka RL, Xiao L, Grigsby PL, et al. Congenital and opportunistic infections: Ureaplasma species and Mycoplasma hominis. *Semin Fetal Neonatal Med.* 2009;14(4):190–199. doi:10.1016/j.siny.2008.11.009.
6. Siber GR, Alpert S, Smith AL, Lin JS, et al. Neonatal central nervous system infection due to Mycoplasma hominis. *J Pediatr.* 1977;90(4):625–627. doi:10.1016/s0022-3476(77)80385-5.
7. Gewitz M, Dinwiddie R, Rees L, Volikas O, et al. Mycoplasma hominis. A cause of neonatal meningitis. *Arch Dis Child.* 1979;54(3):231–233. doi:10.1136/adc.54.3.231.
8. Hjelm E, Jonsell G, Linglof T, Mardh PA, et al. Meningitis in a newborn infant caused by Mycoplasma hominis. *Acta Paediatr Scand* 1980;69(3):415–418. doi:10.1111/j.1651-2227.1980.tb07103.x.
9. Kirk N, Kovar I. Mycoplasma hominis meningitis in a preterm infant. *J Infect.* 1987;15(1):109–110. doi:10.1016/s0163-4453(87)91701-4.
10. McDonald JC, Moore DL. Mycoplasma hominis meningitis in a premature infant. *Pediatr Infect Dis J.* 1988;7(11):795–798. doi:10.1097/00006454-198811000-00011.
11. Watson L, Pang YM, Mitchell S, Dodgson A. Mycoplasma hominis meningitis in a 24 week premature neonate: case report and short literature review. *J Pediatr Pharmacol Ther* 2008;13(4):251–254. doi:10.5863/1551-6776-13.4.251.
12. Watt KM, Massaro MM, Smith B, Cohen-Wolkowiez M, et al. Pharmacokinetics of moxifloxacin in an infant with Mycoplasma hominis meningitis. *Pediatr Infect Dis J.* 2012;31(2):197–199. doi:10.1097/INF.0b013e31823980c3.
13. Nohren J, Namtu K, Peloquin C, Messina A, et al. The pharmacokinetics of moxifloxacin in cerebrospinal fluid following intravenous administration: a report of successfully treated infant with Mycoplasma hominis meningitis. *Pediatr Infect Dis J.* 2020;39(8):e183–e184. doi:10.1097/INF.0000000000002655.
14. Kersin SG, Bozan T, Ozdemir H, Bilgen HS, et al. Clinical and laboratory awareness for an under recognized pathogen in newborn meningitis: Mycoplasma hominis: a case report. *Turk J Pediatr.* 2020;62(2):280–283. doi:10.24953/turkjped.2020.02.015.
15. Ansari NS, Asztalos E, Rolnitsky A. Mycoplasma hominis meningitis in an extremely preterm newborn: a case report. *BMC Pediatr.* 2021;21(1):69.
16. Liu Y, Zhang Y, Yang D, Xu C, et al. Chlamydia trachomatis and mycoplasma infections in tubal pregnancy. *Sci Rep.* 2019;9(1):15979.
17. Zhou M, Wang P, Chen S, Du B, et al. Meningitis in a Chinese adult patient caused by Mycoplasma hominis: a rare infection and literature review. *BMC Infect Dis.* 2016;16(1):557.
18. Kersten RC, Haglund L, Kulwin DR, Ma'luf R, et al. Mycoplasma hominis orbital abscess. *Arch Ophthalmol.* 1995;113(9):1096–1097. doi:10.1001/archopht.1995.01100090018009.
19. Jang YS, Min JW, Kim YS. Positive culture rate and antimicrobial susceptibilities of Mycoplasma hominis and Ureaplasma urealyticum. *Obstet Gynecol Sci* 2019;62(2):127–133. doi:10.5468/ogs.2019.62.2.127.
20. Choi JB, Lee SJ, Lee MK, Lee SJ, et al. Prevalence and Antimicrobial Susceptibility of Ureaplasma spp. and Mycoplasma hominis in asymptomatic individuals in Korea. *Microb Drug Resist.* 2018;24(9):1391–1396. doi:10.1089/mdr.2017.0431.
21. Pereyre S, Renaudin H, Charron A, Bebear C, et al. Emergence of a 23S rRNA mutation in Mycoplasma hominis associated with a loss of the intrinsic resistance to erythromycin and azithromycin. *J Antimicrob Chemother.* 2006;57(4):753–756. doi:10.1093/jac/dkl026.
22. Palacios G, Druce J, Du L, Tran T, et al. A new arenavirus in a cluster of fatal transplant-associated diseases. *N Engl J Med.* 2008;358(10):991–998. doi:10.1056/NEJMoa073785.
23. Han D, Li Z, Li R, Tan P, et al. mNGS in clinical microbiology laboratories: on the road to maturity. *Crit Rev Microbiol.* 2019;45(5-6):668–685. doi:10.1080/1040841X.2019.1681933.

SPECIAL REPORT

Coping with the COVID-19 Pandemic: How a Master's in Clinical Laboratory Sciences Program Adapted Through the Modification of Existing Resources

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Keywords: clinical laboratory, masters course teaching, COVID-19, Teaching modification, curriculum adaptation, regulatory compliance

Abbreviations: MCLS, Master of Clinical Laboratory Sciences; LMS, NYMC Learning Management System, Canvas; NYMC, New York Medical College; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2

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ABSTRACT

Objective: Our aim was to describe the rapid adaption of a Master of Clinical Laboratory Sciences (MCLS) program to the abrupt suspension of classroom instruction and laboratory training at affiliated hospitals in compliance with the New York governor's executive order in March 2020.

Methods: Teaching modifications included greater emphasis on Zoom video conferencing, Media Lab assignments, independent self-study, and online testing.

Results: Instruction of academic coursework continued uninterrupted using previously established teaching modalities. Clinical training presented 2-fold concerns, credit hours needed for the master's degree and clinical hours required for New York State licensing. The latter was delayed.

Conclusion: The real-time need to deliver laboratory science education during a time of statewide closure was fulfilled using available teaching modalities. The resulting uninterrupted academic and clinical training ensured the education of the incoming workforce of our clinical laboratories. This teaching strategy may be considered during new curricula development in preparation for times of future crises.

The inaugural class of a newly developed Master of Clinical Laboratory Sciences (MCLS) program at New York Medical College began classes in early July 2019. On March 20, 2020, the governor of New York issued an executive order requiring all nonessential businesses closed and limiting any concentration of individuals outside their home, to prevent the spread of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). For this MCLS program, closure and social distancing requirements in response to the COVID-19 pandemic resulted in the suspension of classroom instruction and clinical laboratory training at affiliated hospitals.[1,2]

Rapid adaptation through modification of teaching modalities already in use by the program allowed uninterrupted learning with short delays in student career progression during this time. This strategy in program development may serve as a model in preparation for future crises.

Materials and Methods

Teaching modalities involved Zoom remote classroom instruction, Canvas, the NYMC Learning Management System (LMS), laboratory simulations available through Medialab, self-direct learning, and on-site training in clinical and academic laboratories with emphasis on maintenance of social distancing.

Following a period of reoccupancy planning,[3] an on-site plan,[5] exposure plan,[4] and notification tree[6] were developed prior to reopening,

Results and Discussion

Prior to March 22, 2020

The MCLS program at NYMC was developed as a 2-year program, with the first year intense with academic and clinical training followed by a year for completion of master's requirements involving a literature review or a capstone research project. This second year has potential for employment under a New York State provisional permit. Resources were acquired to provide students with multiple sources of training and education.

Classroom instruction was provided in person. With clinical affiliates for the program found throughout the New York, New Jersey, and Connecticut tri-state area, the potential existed that students living a distance from Valhalla would be unable to attend lectures in person.

Classroom instruction was, synchronously held on Zoom for these students and any student taken ill. Zoom lectures were recorded, posted on Canvas, and available throughout the semester for student review.

Clinical training for the program included 2 clinical practicum courses earning 22 credit hours of education, including over 1200 hours of hands-on training, well above the 720-hour NYS licensing requirement. Training took place at multiple locations for each student. This provided the student with varying operational experiences, access to the individual strengths of each affiliate, and an opportunity to present themselves as a potential employee upon program completion. The Introduction to Clinical Laboratory Sciences course, which includes a wet laboratory, introduces students to basic clinical laboratory skills in preparation for hospital laboratory entry. Students were further prepared for clinical training with Medialab course assignments and Medialab Simulator training. Assessment included completion of an evaluation by an affiliate designee and online testing through Canvas, allowing access from any affiliate location.

After March 22, 2020

When the governor of New York issued the executive order to prevent the spread of SARS-CoV-2, NYMC had a plan in place for managing the COVID-19 pandemic[7] (see **TABLE 1**). CLS lectures were switched solely to the Zoom platform and made available synchronously and asynchronously. Lecture examinations were administered on the school's LMS. (as a follow-up note, lectures remain remote.)

As of March 26, 2020 and through the spring semester, students were not permitted to return to hospital laboratories to conserve supplies of personal protective equipment. Clinical training presented 2-fold concerns, that of credit hours needed for the master's degree and clinical hours required for New York State licensing. The latter was delayed.

With temporary NYS Department of Education approval, self-directed learning assignments, Medialab course assignments, Medialab Simulator training for hematology differentials and urinalysis/body fluid slide review and YouTube videos substituted for firsthand activities. Students were assessed in a like manner to assessment prior to the executive order and performed similarly. These activities satisfied the credit hours assigned to the clinical practicum course. The 720 hours of clinical hands-on training in specific areas of the laboratory required for NYS licensing awaited hospitals reopening to students.

Clinical internship training resumed on May 28, as hospitals opened to student presence and continued to be supplemented as described previously. The program supplied students with face masks, disposable laboratory coats, and hand sanitizer. Students had needs in varying laboratory areas and hours necessary to reach the NYS 720-hour mark.

TABLE 1. Timeline of Events from March 2020 through June 2021

Date	Event
March 8, 2020	NYMC plan for COVID-19[6] instituted
March 22, 2020	MCLS in-person teaching suspension and home stay ordered[1]
March 26, 2020	Internship for the MCLS course students at WMC clinical laboratory suspension
May 28, 2020	Restoration of internship for the MCLS course students at WMC clinical laboratory to complete any remaining hours of the NYS minimum 720 hands-on clinical hours of training required for licensing
June 16, 2020	MCLS course resumes with the preparedness and plan for COVID-19 exposure[3]
July 22,2020	NYMC phased reopening process started[2]
June 1, 2021	NYMC mandates COVID-19 vaccination for the MCLS course students

For the incoming second class, the Introduction to Clinical Laboratory Sciences course was the only course held in person. The room, intended for use by 25 students and 2 instructors, was limited to 9 students and 2 instructors, all practicing social distancing and using face masks.

It was noted that students acclimated well to the learning modifications described.

Conclusion

The plan outlined here for adapting an MCLS program that relied on in-person teaching and affiliate training to abrupt shutdown did not appear to delay academic progress as available resources were immediately available to compensate. Specific hours required for licensing were delayed.

REFERENCES

1. *AAMC Guidance on medical students' clinical participation*, March 17, 2020.
2. *Guidance on Medical Students' Clinical Participation: Joint recommendation issued by the AAMC and LCME*, March 17, 2020.
3. *NYMC College re-occupancy planning*, July 22, 2020
4. *NYMC Clinical Laboratory Sciences Coronavirus Exposure Plan*, June, 2020
5. *NYMC Clinical Laboratory Sciences On-Site Plan*.
6. *NYMC Clinical Laboratory Sciences Notification Tree*.
7. *Executive Order No 202. Declaring a Disaster Emergency in the State of New York*.

SPECIAL REPORT

A Literature Review on How We Can Address Medical Laboratory Scientist Staffing Shortages

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Keywords: report, address, clinical laboratory, scientist, staffing, shortages

Abbreviations: BLS, Bureau of Laboratory Statistics; MLS, medical laboratory scientist; CLS, clinical laboratory scientist; MT, medical technologist; MLT, medical laboratory technician; CLT, clinical laboratory technician; LT, laboratory technician; NAACLS, National Accrediting Agency for Cliical Laboratory Sciences; CLIA, Clinical Laboratory Improvement Amendments 1988; ASCP, American Society for Clinical Pathology; PT, proficiency testing; CMS, Centers for Medicare & Medicaid Services; MeSH, Medical Subject Headings; ASCLS, American Society for Clinical Laboratory Science; BOR, ASCP Board of Certification; CMP, certification maintenance program; STEM, science, technology, engineering, and mathematics.

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ABSTRACT

Objective: Laboratories are facing a critical shortage of medical laboratory scientists (MLS) and medical laboratory technicians (MLT) to address an increasing demand for laboratory testing. Training program closures, fewer student applicants, and financial decisions have contributed to staffing shortages. Lack of visibility, low wages, and perceived lack of opportunities for upward career mobility contribute to challenges in recruiting and retaining qualified individuals and students who are unaware of laboratory medicine careers. Our goal was to review the literature to determine the current state and consequences of staffing shortages, and potential solutions to address these shortages.

Methods: Medline/PubMed, PubMed Central, MeSH, Google Scholar, and Marshall Digital Scholar were used as resources.

Discussion/Conclusions: A collaboration of stakeholders is needed to identify staffing challenges, barriers, and solutions and to increase visibility of laboratory professionals. Early recruitment is best started in the middle and high school educational process.

Based on the US Bureau of Labor Statistics (BLS) in 2020,[1] there were 335,500 jobs for medical laboratory/clinical laboratory scientists (MLS/CLS) and medical laboratory/clinical laboratory technicians (MLT/CLT), previously known as medical technologists (MT) and laboratory technicians (LT), respectively, with an 11% projected increase in jobs to fill annually between 2020 and 2030.[1] The BLS however, placed medical laboratory professionals into 1 category regardless of education and credentials. The US Department of Health and Human Services, Health Resources and Service Administration placed MLS and MLT in separate categories in their National Center for Health Workforce Analysis, and each category was projected to grow by 22% between 2012 and 2025[2] suggesting we may be faced with an even greater workforce shortage then predicted by BLS.

There is no difference between MLS, CLS, and MT certifications. They all require a 4-year baccalaureate degree in medical laboratory science or a baccalaureate degree in life sciences followed by a 1-to-2-year internship in a clinical laboratory program accredited by the National Accrediting Agency for Clinical Laboratory Sciences (NAACLS) for an individual to perform Clinical Laboratory Improvement Amendments (CLIA) 1988 complex tests and procedures requiring specialized scientific and technical knowledge. In contrast, MLT and CLT certifications typically require either a 2-year associate degree, completion of at least 60 semester hours (including 6 hours of chemistry and 6 hours of biology) of academic credit from an accredited college or university, completion of a US military medical laboratory training course, 3 years of full-time acceptable clinical laboratory experience and successful completion of an accredited MLT program, or a nondegree with a specialized training certificate. Completion of 1 of these tracks allows an individual to perform CLIA-waived and moderately complex tests under the supervision of an MLS, laboratory manager, or director, with a minimal risk of an incorrect result.[3,4] The terms MLS and MLT will be used throughout the article to designate these 2 laboratory professional groups.

Certification, although optional, is required for employment by many laboratories. The ASCP certification, for example, designated MLS(ASCP) in the United States, does not expire if awarded prior to January 1, 2004, whereas individuals certified after that date receive a time-limited certification valid for 3 years.[5] They are required to participate in a credential maintenance program to keep and renew their certification using the designation MLS(ASCP)^{CM}. Besides certification, laboratory professionals may need to be licensed, depending on the state where they will be practicing. Currently there are 11 states plus 1 territory that require licensure.[6]

In small laboratories defined by the number of billable tests and staff employed, individuals often work as generalists, where they perform a variety of different tests. In larger laboratories, employees may

specialize in a specific department or section within the laboratory. Medical laboratory professionals are key members of the clinical care team working in collaboration with physicians, nurses, pharmacists, and other healthcare professionals. They are skilled in testing patient's specimens to provide results that aid clinicians in clinical decision-making for diagnosis, treatment, and management of patients.[7]

Factors Contributing to the Need for Increased Laboratory Staffing

Several factors account for the projected need for increased staffing that require increased testing; for example, population growth, an aging population, chronic diseases, COVID-19, expanded insurance coverage under the Affordable Care Act, newer technology including molecular assays, point-of-care testing that requires laboratory oversight to comply with regulatory and testing compliance, frequent data building for laboratory information system upgrades, and automation.[8,9]

Laboratory Vacancy Rates

According to the ASCP 2020 national vacancy survey of medical laboratories in the United States based on 476 respondents, the Central Northeast region reported the highest overall vacancy rate (10.2%) and the Central Southwest the lowest rate (5.3%) compared to other areas of the United States. Chemistry/toxicology had the highest staff vacancy (13.1%) and 5 year projected retirement (17.9%) rates, whereas cytology (4.3%) and flow cytometry (4.9%) had the lowest vacancy and projected retirement rates, respectively. However, compared to the ASCP 2016–2018 survey data, with few exceptions, laboratory departments saw a decrease in vacancies. This drop was related to the elimination of elective procedures during the Covid-19 pandemic with an accompanying decrease in non-Covid-19 test volumes and subsequent staff furloughs and resignations. The survey also showed an overall decrease in retirement rates, suggesting that those employees projected to retire had already retired.[8]

Qualified scientists and technicians are needed to fill vacancies to address the increased demand for laboratory services. Employment vacancy rates are exceeding the number of MLS and MLT graduates due in part to declining accredited educational programs, related to financial decisions, decreasing hospital rotation sites because of laboratory staffing shortages, and increasing testing demands.[9] Between 2000 and 2022, there was a decrease in NAACLS-accredited programs, from 263 to 243 MLS and 248 to 238 MLT programs, respectively.[10] To address this shortage, some programs have reduced their clinical rotations from 22 weeks to 12 weeks, a reduction that may compromise a student's preparedness to enter the workforce.[11]

Challenges in Recruiting and Retaining Staff

Managers have cited challenges in recruiting, hiring, and retaining personnel. Compared to other professions, low wages, poor health and retirement benefits, no tuition or continuing education reimbursements, and no sign-on bonuses contribute to recruiting challenges. That said, providing incentives like sign-on bonuses and tuition reimbursement may be welcomed, but may not be financially feasible because of reductions in laboratory reimbursements. In addition, budget constraints; administrative justification, hiring freezes, and competition for trained and certified individuals continue to be obstacles in laboratory staffing. To add to the dilemma, there are fewer students pursuing a career in laboratory medicine due to a lack of career awareness, resulting in a decline in staffing with qualified and certified laboratory professionals.[9]

The following reasons have been cited by employees for low retention rates: lack of premium pay for overtime, holidays and weekends, scheduling flexibility, and career ladders; limited upward mobility; high stress levels; failure of administration, public, and other health care providers to recognize medical laboratory professionals; increased workload without additional staff due to assimilation of additional hospitals into their health care system; issues with their manager; and noncompetitive compensation compared to other jobs requiring similar educational backgrounds.[8,9,11] Based on the 2021 US BLS, the annual median wage for a MLS/MLT is $57,800. That said, wages vary based on geographic location, certification, and experience. Currently, the top paying states are New York, Rhode Island, Connecticut, Oregon, and California.[1,12]

An ongoing issue relates to lack of visibility, recognition, and respect for the work of laboratory professionals. The public frequently hears about front-line caregivers but rarely hears about laboratory professionals and their contributions to patient care. One of the main reasons for the lack of recognition and respect for MLSs relates to the fact they do not have 1 standardized designation based on their education and training.[13] They may be referred to as an MLS, CLS, or MT. Likewise, there has not been a clear distinction between an MLS and an MLT. As a result, multiple professional titles have led to public confusion on how to address them, so they end up referring to MLSs as "med techs" or "lab techs."

Consequences of Staffing Shortages

Managers may be faced with several obstacles; for example, staffing shortages to fill night, weekend, and double shift positions, fewer qualified applicants to fill positions, and less time for sufficient training. Also, losing a valued staff member can put additional stress on the remaining staff to pick up the slack, maintain quality, prevent reporting errors, and avoid employee dissatisfaction. The hiring process may take 3 to 6 months to advertise, interview, hire, and train a new employee. During this time, managers usually can expect additional payroll costs to cover overtime until a new employee is hired.[9,14,15]

Staffing shortages have led to less restrictive employment requirements, particularly in those states where there is no requirement for certification through an accredited MLT or MLS program or licensure. To address these staffing shortage challenges, a significant problem in small rural laboratories, managers have resorted to using traveling laboratory professionals who work locum tenens (temporary, part-time, per diem, or pro re nata). Traveling MLSs may receive a renewable contract with benefits including financial incentives, health and flex spending benefits, private housing, and sign-on bonuses. They also may pose a financial hardship to employers related to higher salaries than permanent employees and time-consuming training to fulfill immediate needs.[9]

With increasing workloads and demand for staffing, some managers are compelled to hire personnel with a bachelor's degree in biology or chemistry without certification or laboratory experience. These individuals are given on-the-job training to perform moderate and high complexity testing if they are in a state that does not require certification or a license.[9] They may be eligible for national certification as an MLT or MLS once they have documented proof of qualifying clinical laboratory work experience and successful completion of a certification examination in one or more laboratory specialties. The apparent rise in the

TABLE 1. Strategies for Improving Medical Laboratory Scientist and Medical Laboratory Technician Staffing

	Strategies
Visibility[23]	
1	Consistently use the titles "Medical Laboratory Scientist" and "Medical Laboratory Technician."*[13,23]
2	Offer incentives for those who attend laboratory awareness programs or perform community outreach.[23]
3	Promote the profession to students and educators during career days.[7,8]
4	Encourage professional clinical laboratory educational organizations to invite students and educators to attend their meetings and provide awards to students for their scientific achievements.[23]
5	Engage a national television channel to feature a video on the clinical laboratory profession.[24]
6	Invite the C-suite, Executive Board, and health care professionals to visit their laboratory and educate them on the skills, education, and contributions of laboratory professionals to the health care system.[7,23]
7	Use social media such as Linkedin, Facebook, Instagram, and Twitter to increase public visibility.[23]
*The American Society for Clinical Laboratory Science (ASCLS) and ASCP Board of Certification (BOC) wrote a position paper outlining the importance of adopting unified professional titles "Medical Laboratory Scientist" and "Medical Laboratory Technician."[13,23] In 2009, upon uniting the National Certification Agency and ASCP BOC into a single certifying agency, ASCP BOC declared the credential designations Medical Laboratory Scientist (MLS) and Medical Laboratory Technician (MLT) for these professions. Laboratory professionals who formerly held medical technologist (MT) and clinical laboratory scientist (CLS) certifications are now called Medical Laboratory Scientists (MLS) using the designation MLS(ASCP)^{CM} if they participate in the Certification Maintenance Program (CMP) that requires continuing education and performance of activities to remain current. Individuals who were certified as a MT(ASCP) prior to 2004 and who do not participate in the CMP will retain the designation MT(ASCP) without the superscript CM.[5] All stakeholders are encouraged to endorse the use of the declared designations when engaged in discussions related to laboratory personnel, encourage all educational programs to adopt these terms, and advocate for use of the MLS job title for those with a baccalaureate degree and national ASCP certification.[13,23] Declaring the use of the MLS(ASCP)^{CM} or MLT(ASCP)^{CM} credential designations is a step toward linking standard designations with education, credential, and job title. However, organizations other than ASCP have certified laboratory professionals; for example, American Medical Technologists, American Association of Bioanalysts, and have maintained the name Medical Technologist. Arguably the same designations should be used by all laboratory professionals with consideration to grandfather those individuals holding comparable certifications regardless of the certifying agency. That said, standardizing nomenclature to improve public awareness is a major undertaking that can create challenges for human resources in reevaluating jobs and titles and for certifying agencies to change their program names.[13]	
Recruitment[23,24]	
1	Identify mentors from professional laboratory organizations, the medical community, and volunteers to educate and promote laboratory sciences in the classroom.[23,24]
2	Develop a marketing pipeline plan that includes a website to reach broad audiences of prospective laboratory candidates to fill vacancies.in well-populated areas and attract qualified individuals into laboratories in rural, remote, or hard-to-reach areas of the country.[23,25–27]
3	*Form an outreach program to distribute educational and promotional brochures to local and rural communities. *Use social media such as Facebook, Instagram, and Twitter to recruit.[23,24,27] *Hold virtual information sessions for students and educators. *Produce video tours of your laboratory department and team explaining why they chose a career in laboratory medicine. *Add video links to your institution's webpage and recruiter's website, and *Use a recruitment toolkit that includes videos and student brochures created by ASCLS and the Committee for Educational Programs and Initiatives.[25,26]
4	Plan events for Lab Week and Career Days to educate and recruit high school and college/university students.
5	Develop an electronic clearinghouse to advertise vacancies at MLS and MLT clinical laboratory training sites.[9]
6	Consider marketing rural communities by highlighting information on a blog with their career page; for example, low cost of living, provision of relocation expenses, onsite daycare, and hiring spouses with appropriate backgrounds.[27]
7	Explore government funding to develop and support internships and mentoring programs for students including underserved communities. Advocate to administration and government agencies for higher salaries competitive with other employers and commensurate with their education, skills, and experience, and increases in funding for laboratory training programs, student stipends, and scholarships,[23,24] such as Build Dakota Scholarships, Health Occupations Students of America, Future Health Professionals, and Avera Academy financial assistance programs.
8	Encourage distance-learning to deliver didactic information, particularly beneficial for students in rural areas.[28,29]
9	Change the process of recruiting by taking a more individualistic approach to staff management and not assume that a "one size fits all" approach will be appropriate to suit the values and views of all candidates.[14,23]
10	Provide financial incentives, including attendance in continuing education and training programs, flexible schedules to allow a work-home balance or pursuit of an advanced degree or certification, competitive benefits, paid vacations, job descriptions, career ladders for employee development, and advancements with an increase in pay and title.[8,15,23,30]
11	Educate employers as to the importance and benefits of providing clinical laboratory training rotations for students while supporting laboratory certifying programs.[16,31]
12	Provide nontraditional approaches of student training during off-shifts, virtual or simulation training.[9,24] Offer on-the-job training for candidates seeking to minimize education costs and earn income while in training.[14,23]
13	Provide multiple points of entry for individuals with adult responsibilities to enroll in an accelerated, clinical laboratory-based curricula to enter the workforce again as a laboratory professional.[32]
14	Perform a market salary adjustment at least annually.[30]
15	Hire medical laboratory scientists from other countries.[8]
*The American Association for Clinical Chemistry has recommended that Congress provide Title VII funding to allied health training programs to expand their MLS training programs, allocate funding to support clinical rotation training of MLS students in hospitals and/or reference laboratories, and create a loan forgiveness program to reduce laboratory professional student debt for those who work in underserved areas.	

TABLE 1. Continued

	Strategies
Diversity and Inclusion[23]	
1	Support employer's focus on promoting gender, race, and ethnicity diversity in recruitment.[23,24]
2	Engage in outreach to students, parents, and councilors, and partner with underserved communities for their help in distributing informational brochures in multiple languages.[23]
3	Before interviewing candidates from diverse populations, check out their profile so you can personalize your outreach message rather than sending a general notice.[23]
4	Provide strong student support and participate in mentorship and diversity training programs to help in retaining staff from underrepresented groups.[23,32]
5	Understand the importance of recognizing multigenerational diversities in the workplace and learn strategies to accommodate diverse needs of individuals.[14,24]
6	Search for federal agency programs; for example, Area Health Education Center supports medical laboratory training for diverse, underserved, and rural areas a federal agency for people in rural and underserved areas.[23]
Retention[23,24]	
1	Create a positive environment for new employees using on-boarding to familiarize the new employee with their new work environment.[14,15]
2	Use a mentoring system for new hires during orientation to build relationships, integrate them into the organization, and give them a sense of belonging.[14,15]
3	Motivate staff and provide meaningful, challenging work where employees feel their skills and abilities are used effectively. Staff tend to be engaged if they feel their manager is concerned about their growth.[14]
4	Offer career ladders that outline the responsibilities, skills, and experience required for each job on the organizational ladder, a roadmap of steps required to move up the ladder, and career pathways as a guide to determine progression towards their goal.[15,24,32]
5	Mentor laboratory staff to build leadership skills for upward mobility.[23]
6	Incentivize employees to obtain certifications[5,6,8,23] and encourage noncertified individuals with a postbaccalaureate degree; for example, biology or microbiology and interested in a microbiology career to enroll in an American Society of Microbiology (ASM)-Weber State University program for certification.[33]
7	Encourage online training and bridge programs as part of a career ladder to move from an MLT position to an MLS using their current workplace to obtain advanced laboratory training.[28,29]
8	Show that you trust your staff by giving them autonomy within their position, encourage suggestions, and act on their feedback.[14,15]
9	Promote ways to prevent burnout; for example, develop a laboratory stewardship test utilization committee to decrease unnecessary tests and provide creative staffing.[14]
10	Consider financial incentives; for example, annual increases, sign-on bonus after 1-year anniversary, retention bonus each year thereafter, employee referral bonus, shift differential, employee fund for special needs, tuition support, stipends, or scholarships for employees to pursue educational opportunities.[23,24]
11	Provide flexibility; for example, virtual meetings for staff who cannot regularly attend in-person meetings or educational programs, flexible scheduling, and cross-training.[9,32]
12	Recognize staff for contributions to patient care and team efforts.[14]
High retention rates lead to increased productivity and decreased costs related to recruiting and training new employees. Success in retaining employees depends on strong and creative leadership starting in the C-suite and extending through all levels of management. Using an on-boarding strategy for retention that includes a comprehensive orientation with management and co-workers to familiarize them with laboratory procedures, policies, career development, history of the institution, values, expectations, compliance, and the laboratory's role in the operation, is helpful in engaging and motivating new employees so they will feel they made the right decision in joining the team.[14,15] Unfortunately, some managers fail to understand the importance of motivation or they lack the skills to provide a motivating environment. Clear goals, immediate feedback, and tasks that are neither too difficult nor too simple, help to foster a motivating environment.[18] Understanding the needs of the workforce while addressing the needs of the laboratory will go a long way to improving recruitment and retention of qualified laboratory professionals. A complaint among dissatisfied MLSs is the lack of career advancement opportunities and limited upward mobility. Providing career ladders and encouraging certifications can enhance opportunities for career mobility while improving work quality.[16,31] The odds of career advancement from an entry-level position were found to be close to 70% higher for every additional certification a laboratorian obtains, for example, specialist certifications demonstrating competency (P=.018).[16] As an example, students with a baccalaureate degree in a life science and an interest in microbiology can enroll at Weber State University and then apply to the American Society of Microbiology-MLS program. Students who successfully complete 11 credit hours in clinical microbiology, biostatistics and a 4-week clinical laboratory rotation are eligible to take the Microbiology specialist exam through ASCP. If successful, they will receive a Microbiology certificate.[33] For those motivated to advance their career, they also have the opportunity of enrolling in the Doctor of Clinical Laboratory Science degree program that focuses on patient care management, education, research, and delivery of health care services.	
Student Recruitment[23,24]	
1	Increase career awareness through science fairs, educators, and mentoring[23]
2	Identify opportunities to encourage students' interest in a clinical laboratory career.[34] Emphasize the significant role MLSs play in the management of patients, what a "day in the life of a medical technologist" is like, the importance of certification, what is required to become an MLS, and the opportunities for financial stability and advancement to positions of greater responsibility and renumeration.[7,23]
3	Mentor and provide age-appropriate, hands-on laboratory experiences and shadowing of a laboratory professional.[24,34]
4	Partner with a laboratory or a local middle or high school with science, technology, engineering, and mathematics (STEM) discovery-based, hands-on learning experiences. Mobile laboratories provide access to STEM-based learning for children in several states throughout the United States, including rural areas.[23–25,35]
5	Provide tuition, stipends, or scholarship incentives to potential students to attend laboratory training programs.[24,25]

TABLE 1. Continued

	Strategies
	Students are often unaware of career options in laboratory medicine. Engaging students early in their educational journey can be a significant inducement to focus on a career in laboratory medicine. Strategies promoting student interest in laboratory medicine can best be accomplished using a collaboration of stakeholders; for example, health science educators, K-12, magnet and precollege educational program administrators and academic advisors, hospital administrators and laboratory staff, community and volunteer role model mentors, student and professional organizations, clinical laboratory science NAACLS-accredited program directors, legislators, and county school supervisors responsible for strategic business partnerships that help to provide opportunities for students in laboratory medicine.[23] Many of these individuals can also be valuable resources for providing information on what is required to become an MLS and opportunities for advancement to positions of greater responsibility and renumeration.
	Attention needs to be focused on middle and high school students ages 11–14 years to develop face-to-face educational programs to stimulate their interest for a career in laboratory medicine by emphasizing the opportunities that would be available to those taking the path to becoming an MLS. To that point, the authors performed a national survey of Board-certified physicians, microbiologists, and chemists to confirm an earlier observation that a notable number of doctoral level microbiologists and chemists were ASCP-certified or equivalent and had worked on the bench early in their career prior to or during graduate school as a stepping-stone to becoming a board-certified CLIA laboratory or technical director and/or consultant. Survey results showed a sizable number of individuals were certified medical laboratory scientists early in their career. They gained laboratory experience and then took the opportunity to move up the ladder in their career to a position of more responsibility and remuneration. The results of their survey highlight opportunities students can aspire to once they become an MLS. The authors are currently collaborating with the County Public Schools Supervisor of Business Partnerships for careers, technology, and education, who will be a conduit to health science instructors and staff in the public and magnet school system using interactive learning modules and experiential virtual learning programs. In addition, there are plans to work with a large College of Health Sciences to participate in educational programs they offer; for example, Science Festivals, Career Days, Mayor's Youth program, Explorers "summer science exploratory camp," and pre-freshman college orientation, and increase awareness of college bio/science professors and deans to ensure they are aware of MLS careers and the important work the MLS performs.
	Because of increased accredited training program closures, other educational alternatives need to be explored. One option is distance learning (DL) that can reach more students and provide greater schedule flexibility. Currently, there are 23 ASCLS online MLS programs, 24 online MLT programs, and 30 current MLTs enrolled in a bridging program to pursue an MLS online degree completion program. Reported barriers appear to have limited the number of DL programs, although this modality could increase educational opportunities for career preparation to many, including those in rural and remote areas of the country.[29,30]

number of noncertified individuals that are performing moderate and high-complexity tests with no previous laboratory experience should be a wake-up call to address this concern.

Using noncertified personnel rather than certified and experienced testing personnel raises concerns related to maintaining the quality of laboratory results. A retrospective study[16] was designed to compare the measurable outcome of proficiency testing (PT) performance based on testing personnel's college major, degree, certification, and years of laboratory experience. Participants with an MLS major and those with more than 2 years of experience produced a significantly higher percent of acceptable PT results (P=.035 and P=.042, respectively) compared to staff without a clinical laboratory major (P=.035). Although the study results suggest that managers should hire staff who have completed an accredited certification program to maintain quality and minimize laboratory errors, they may be challenged by a lack of qualified applicants.

In 2016, at least in part due to a shortage of clinical laboratory personnel in rural areas, the Centers for Medicare & Medicaid Services (CMS) issued an unprecedented and controversial policy of concern to the laboratory community.[17,18] As stated in the Survey & Certification Letter 16-18 CLIA, CMS ruled that an associate's and bachelor's degree in nursing are equivalent to an associate's or bachelor's degree in a life science that meet the educational requirements for moderate and high complexity personnel, respectively.[17] Numerous laboratory leaders have opposed this policy, stating a lack of educational equivalency. Whereas a baccalaureate degree in a biological science includes high level science coursework and laboratory experience, nursing degrees have a therapeutic focus with little to no laboratory training. Concerns were raised that testing quality and patient safety may be affected as well as loss of jobs for qualified and experienced MLS and MLT personnel.[19,20] The CMS responded to the question of why this rule was proposed by stating that this was not a new CLIA policy or change but a clarification of an existing policy. Laboratories are not "required" to allow nurses to perform moderate and high complexity testing. They can, and in some cases already do, adopt stricter standards; for example, requiring more experience, specialty certification, or more advanced degrees than CLIA regulations require. Also, state law may impose stricter standards than the CLIA regulations require.[21] In 2014, Congress enacted the Promoting Access to Medicare Act for reimbursement of clinical laboratory services. The significant decreases in reimbursement are a threat to providing services to beneficiaries, particularly true for small community and rural hospitals that need to centralize and consolidate their laboratory services to lower costs. As a result, there could be unacceptable consequences to laboratory staffing levels because of reduced test volume.[22]

Methods

The purpose of this review was to determine the current state of laboratory staffing, factors contributing to the need for increased staffing, challenges in recruiting and retaining staff, consequences of staffing shortages, and strategies and potential solutions to address staffing shortages. Inclusion criteria included information in Medline/PubMed, PubMed Central, Medical Subject Headings (MeSH), and Google Scholar related to the list above. References were deselected if there was insufficient or redundant information related to the purpose of the review. Resources were accessed between 2008 and 2022 to obtain relevant and current information pertaining to staffing shortages, including recent US statistics and survey results, CLIA required personnel qualifications, ASCP certifications, visibility of laboratory medicine, recommended strategies for recruitment of students and rural staffing as well as retention of a diverse and inclusive workforce.

Discussion

Strategies to Improve Visibility, Recruitment, Diversity and Inclusion, and Retention

The ASCP in partnership with the University of Washington conducted a detailed analysis using focus groups and one-on-one interviews of management and staff to gather ideas on how to address workforce concerns.[23] The information gleaned from these interviews was used to create a "Blueprint for Action" for engaging stakeholders to work collectively to improve visibility, recruiting, retention, and diversity and inclusion, and address challenges in recruiting staff in rural communities. An abbreviated list of potential solutions for strengthening the clinical

laboratory workforce can be found in **TABLE 1**. For a more comprehensive list, readers are directed to the Blueprint for Action references.[23,24]

Conclusion

Laboratories are facing a critical shortage of MLSs and MLTs to address an increasing demand for laboratory testing. Challenges in recruiting and retaining personnel have added to the dilemma. Staffing shortages have led to less restrictive employment requirements and the use of traveling MLSs, especially in rural areas. Without a collaboration of stakeholders, laboratory staff shortages will continue to plague our laboratories. Fortunately, organizations such as ASCP in partnership with the University of Washington are leading efforts to improve visibility and recognition of medical laboratory professionals. The Blueprint for Action provides a guide for solutions that will increase the number of qualified MLS and MLT candidates while increasing diversity and inclusion within the clinical laboratory. Early recruitment is best started in the middle and high school educational process in collaboration with STEM programs that highlight the role of MLSs, what it takes to become a laboratory professional, and the potential opportunities afforded students who choose a career in laboratory medicine.

REFERENCES

1. U.S. Bureau of Labor Statistics Occupational Outlook Handbook. *Clinical Laboratory Technologists and Technicians.* https://www.bls.gov/ooh/healthcare/clinical-laboratory-technologists-and technicians.htm. Last modified April 18, 2022.
2. U.S. Department of Health and Human Services, Human Resources and Service Administration (HRSA). *National Center for Health Workforce Analysis*. 2015. https://bhw.hrsa.gov>default>files>data-research.
3. Olea S. CLIA required personnel qualifications. In Annual Education Conference, 2012: 1–23.
4. 42 CFR-Laboratories performing high complexity testing Part 493-Laboratory requirements subpart M-Personnel for non-waived testing subjgrp-Laboratories performing high complexity testing. 2015:634–650. https://www.govinfo.gov>app.CFR-2015-title42-vol5.
5. American Society for Clinical Pathology Board of Certification. *Guide to the Citation of ASCP Board of Certification Credentials.* 2017.
6. American Society for Clinical Pathology. *State Licensure of Laboratory Personnel (Policy Number 05-02). J Lab Med*; Sept 2005:1–9.
7. Wolcott J, Schwartz A, Goodman C. *Laboratory Medicine: A National Status Report*. The Lewin Group; 2008: 1–14.
8. Garcia E, Kundu I, Kelly M, Soles R. The American Society for Clinical Pathology' 2020 vacancy survey of medical laboratories in the United States. *Am J Clin Pathol.* 2022;157:874–889.
9. The American Society for Clinical Laboratory Science (ASCLS). *Position Statement. Addressing the Clinical Laboratory Workforce Shortage*. 2018.
10. Cremeans L. Clinical lab worker shortage: addressing the gap. *Med Lab Sci*. 2022:1–10.
11. Ned-Sykes RM, Pentella MA, Salerno RM, St. George K. Strengthening laboratory partnerships, enhancing recruitment, and improving retention through training and outreach activities: the Minnesota experience. *Public Health Rep.* 2019;134(2 Suppl):11S–15S.
12. US Bureau of Laboratories. *Occupational Employment and Wage Statistics*, May 2021. Last modified March 31, 2022. https://www.bls.gov/oes/current/oes292010.htm Clinical Laboratory Technologists and Technicians (bls.gov).
13. The American Society for Clinical Laboratory Science (ASCLS). Standardizing the professional title of medical laboratory professionals. A Position Paper of ASCLS and ASCP BOC, 2018.
14. Verlander EG, Evans MR. Strategies for improving employee retention. *Clin Leadership Manage Rev.* 2007;21(2):E4.
15. Morrissey M. Steps to retain lab technicians and technologists. *Med Lab Observ.* 2021:1–11.
16. Chiou PZ, Jia Y. Influence of certification on a pathology laboratory professional's career mobility. *Am J Clin Pathol*. 2022;157(6):942–947.
17. Federal Register. *A Proposed Rule by the CMS on January 9, 2018*;83(6):1004–1009.
18. Meadows PD. *Are Higher Education Institutions Addressing the Employment Needs of Clinical Laboratories in Rural Areas*. *Theses, Dissertations and Capsstones* 1160, 2018, 1–55.
19. *Statement of the American Society for Clinical Pathology and the ASCP Board of Certification before the Clinical Laboratory Improvement Advisory Committee*, 2018. https://www.cdc.gov/cliac/docs/fall.2018/PC3 ASCP Personnel Public Comment.pdf.
20. Association of Public Health Laboratories. *APHL Position Statement. Rescind CMS' Nursing Degree Equivalency Determination*. 2016. https://www.aphl.org>Position_Documents>CM...2017:1-2.
21. *S&C-16-18-CLIA Personnel Policies for Individuals Directing or Performing Non-Waived Tests*. 2016:1–63. https://www.cms.gov>Downloads>PSV-FAQs.
22. National Independent Laboratory Association. *The Protecting Access to Medicare Act Jeopardizes the Nation's Community and Regional Independent Clinical Laboratory Infrastructures,* 2014:1–11. https://www.nila-usa.org/images/nila/PAMA%20Key%20Informant%20Summary_FINAL.pdf.
23. Garcia EC, Kundu I, Kelly MA. The clinical laboratory workforce: understanding the challenges to meeting current and future needs. Blueprint for action. *Am Soc Clin Path.* 2021:1–76.
24. Garcia EC, Kundu I, Kelly MA, Guenther GA, Skillman SM, Frogner BK. *The Clinical Laboratory Workforce: Understanding the Challenges to Meeting Current and Future Needs.* Washington, DC: American Society of Clinical Pathology and Center for Health Workforce Studies, University of Washington; 2021: 1–61. https://www.ascp.org>news-detail>2021/05/17>gro.
25. Beazer K, Cummin K. Effective marketing strategies for a medical laboratory science program. *Clin Lab Sci.* 2020;119:002154.
26. The American Society for Clinical Laboratory Science (ASCLS). *Career Recruitment Tool Kit.* 2022. https://ascls.org>career-recruitment-tool-kit.
27. Bland D. *20 Strategies for Rural Hospitals to Attract and Retain Staff.* Clin Lab Manager, 2018:14–17. https://www.clinicallab.com>20 strategies-for-rural-hospi...
28. Montgomery S. Medical laboratory profession needs all hands-on deck approach to build future workforce. *Am J Clin Pathol.* 2022:1–3.
29. Brooks R, Olmsted J. Distance education in the clinical laboratory sciences: part of the solution to the professional labor shortage. *Cur J Appl Sci Tech.* 2021;40(25):1–7.
30. Solutions Training and Assistance for Recruitment & Retention. *Developing Employee Career Paths and Ladders,* 2015:1–16. https://www.shrm.org/resourcesandtools/tools-and-samples/toolkits/pages/developingemployeecareerpathsandladders.asps.
31. Delost MD, Miller WG, Chang GA, Karzun WJ, Nadder TS. Influence of credentials of clinical laboratory professionals on proficiency testing performance. *Am Soc Clin Chem.* 2009;132:550–554.
32. Linder R. Educating medical laboratory technologists: revisiting our assumptions in the current economic and health-care environment. *J Microbiol Biol Educ.* 2012;13(2):150–154.
33. American Society for Microbiology. *ASM-WSU Microbiology Certificate Program*. 2021. https://weber.edu/Admissions/apply.html.
34. Prunuske A, Fisher C, Molden J, Brar A, Ragland R, vanWestrienen J. Middle-school student engagement in a tick testing community science project. *Insets.* 2021;12(2):1136–1140.
35. Jones AL, Stapleton MK. 1.2 million kids and counting-Mobile science laboratories drive student interest in STEM. *PloS Biol.* 2017;15(5):e2001692.

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