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SARS-CoV-2 (COVID-19) Continues to Challenge Health Care, Workforce and Families Across the Globe

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Editorial

SARS-CoV-2 (COVID-19) Continues to Challenge Health care, Workforce and Families Across the Globe

Patricia Tille Ph.D MLS(ASCP) AHI (AMT) FASC
IJBLS Editor in Chief

In 2019 in Wuhan China, a new coronavirus, SARS-CoV-2 or Covid-19, was identified as the cause of multiple cases of acute respiratory distress syndrome. What we did not know at the time was that this would cause a world-wide pandemic lasting more than three years and resulting in more than 23,686,690 deaths globally. But the loss of millions of individuals is only a small fraction of the devastation we have seen in the United States and other countries. Health care workers have left their careers to avoid mandatory vaccination and for the mental health and safety for themselves and their families. This has caused an increase in health care workforce shortage in an already strained system. In addition to the drain on the health care system, families who continue to battle for love one's care and survival continues despite the knowledge and scientific advancements in the care for patients infected with Covid-19. The lingering effects and continued battle with the virus can be seen in survivors, patients, families, loved ones, health care workers and in the health care industry.

As a patient, I witnessed what I would consider, less than optimal health care early on during the pandemic when I was admitted to the hospital in August of 2020 for Covid-19. Recall that this was before any vaccinations were available and all treatment options were new and experimental. As a health care professional, myself, I am haunted by the memories of my experience being locked away for five days in what I would consider a suboptimal care situation. In addition, I continue to experience "long haul" symptoms that includes significant shortness of breath and at times unexplainable fatigue.

But what about those who become critically ill with Covid-19? How do they move forward and how does their family manage their care for recovery? Beginning in December of 2021, a family friend reached out to me for advice. Her husband, Roy, was suffering from Covid-19 symptoms and tested positive on December 23rd. Roy had chosen to not receive the Covid-19 vaccine. She took him to the emergency room when he began to experience severe shortness of breath. During that visit he was diagnosed with low blood oxygenation, an abnormal EKG (electrocardiogram), and a severely elevated glucose. Despite his condition, he was sent home and told to return if his symptoms worsened and to consider getting the Covid-19 vaccine when he recovered. He was not given monoclonal antibodies because the clinic that provided the infusion, would not be open until three days later. Approximately, 3 days later, December 26th, they returned to the emergency room, and Roy was immediately admitted for severe Covid-19 in what one would consider a critical state.

Roy and his family experienced what I would consider a situation I would not want for any patient in any country or within any health care system. Roy is a 63 year old male, who was unvaccinated. Would he have been observed or even admitted upon his first presentation to the emergency room to determine why he had an abnormal EKG and elevated glucose, independent of Covid-19 had there not been a pandemic? Was the health care system simply overwhelmed? Within 4 days, Roy rapidly progressed to requiring intubation and being placed in a coma and on a ventilator. Roy was sedated and given a paralytic to put a feeding tube through his nasal passage. This first attempt resulted in a punctured lung and his lung collapsing. The medical staff was able to reinflate his lung, but it again collapsed about two hours later. Two days later, Roy again suffered a third collapsed lung from the damage that occurred when the feeding tube was put in place. The damage to the lung and the combined effects of the Covid-19 virus continued to make it difficult to keep Roy's blood oxygenation at an acceptable level. On January 11th, following just over two weeks "standard" Covid-19 treatment which consisted of steroids, anticoagulants, remdesivir, enteral nutrition and respiratory ventilation, the medical team was ready to discuss "comfort care" with the family. The medical team was concerned that the inflammatory response induced by Covid-19 known as a cytokine storm, where the body's own defense mechanisms attack and cause damage to the lungs and other organs had destroyed approximately 95% of Roy's lung tissue making it difficult to nearly impossible for him to recover. The family reached out for a consultation, and as a laboratory professional I visited with the medical team and the family on Roy's behalf.

During the lengthy stay in the Covid-19 intensive care unit

March 2022, after battling severe Covid-19, Roy is now recovering in a specialized rehabilitation

During that initial visit to the intensive care unit, I saw numerous patients, isolated and attached to life-saving devices and health care workers experiencing Covid-19 fatigue. The medical team was receptive to discussing Roy's care and all his diagnostic tests with the family's approval. After that discussion, one of the primary care providers on the case, looked at Roy's wife and commented, "I think Roy needs more time." Roy was given more time; he was not placed on comfort care, and he continued to improve. On January 24th, nearly a month after being placed in an induced coma and on a ventilator Roy had an MRI (magnetic resonance imaging test), to determine if his brain activity was normal in hopes of him regaining consciousness. When

the hospital staff brought him back to his room, “his eyes were open, and he looked at me,” reported his wife.

It is now March 25, 2022. 90 days from the day Roy was admitted to the hospital with acute respiratory distress and critically ill from Covid-19. Roy is still recovering in a specialized rehabilitation center 3 ½ hours from his home. His family takes shifts staying in a nearby apartment to help with his recovery and care. Roy is still receiving enteral nutrition and is unable to care for himself. How much longer will his recovery take and how much financial and personal challenges remain for the family are still unknown.

However, in this story, there is an amazing group of individuals that we often forget about. That is the family, friends and health care workers affected in Roy’s story and everyone’s story that has been touched by Covid-19. Throughout the experience, the Hoefert family and friends began to bring food to the nurses, valets and other health care workers caring for Covid-19 patients during Roy’s nearly 72 days at the primary hospital. It was a small gesture, but one that not only gave the health care providers strength and hope but inspired the family to keep fighting for Roy and other Covid-19 patients and families alongside all the health care workers!

Pam Hoefert, Roy’s wife, with a Valentines day celebration for the nurses and other health care workers in the pulmonary wing of the hospital.

So, what is my point of this editorial? The pandemic is not over. The devastation to families and the health care system is not over! I encourage you to share your stories, your laboratory experiences and your challenges as we work to determine how to continue to move forward and look at what we have learned not only in relation to Covid-19 care, prevention and treatment, but how globally we can better prepare for the next pandemic and improve all health care systems and workforce development.

This article is dedicated to the memory of all those lost, and an eternal thank you to all the researchers, health care workers, family, friends, and patients who have battled and continue to do battle with Covid-19.

Sincerely, IJBLS Editor in Chief,

Patricia Tille Ph.D. MLS(ASCP) AHI(AMT) FACSc

Permission to publish the information regarding the patient’s case and experience was received from the Hoefert family.

Editorial

Can You Teach an Old Dog New Tricks?

Hassan A. Aziz PhD, FACSs, MLS(ASCP)^{cm}
Associate Editor of Education and Administration

I admit that every once in a while, I find myself dealing with a student that is struggling to learn the basic and essential principles of the material we are teaching while the rest of the class has moved on and ready for new material. By no means, these are weak students. They have met the rigorous admission criteria and their academic records prior to entering the program prove that they possessed the necessary intellectual abilities (and the grades) to succeed in the program.

So, I decided to dig deeper into this issue and talked to colleagues in different disciplines. I became more convinced that we, the teachers, need to better understand our own neurological strengths and weaknesses to reach all of our students.

Students come to us from diverse learning styles that require different teaching approaches. So how can we adapt our teaching to reach and engage as many of them as possible?

Interestingly, the answer lies in first knowing ourselves as teachers. To do this, one must understand your own "neurological style" and the way it could influence the way we teach. We all have a left-, a right-, or a middle-brain preference, that influences our teaching patterns.

The neurological profile guides the way we teach our classes; left-brain teachers tend to teach in a "left-brain style," right-brain teachers teach in a "right-brain style," and middle-brain teachers tend to vary between the two approaches.

Teachers are more inclined to reach students who share the same neurological strengths. A left-brain teacher needs to make a conscious effort in order to better reach a right-brain student in the classroom.

Left-brain teachers prefer to teach using lecture and discussion. They follow outlines, and they like to adhere to prepared time schedules. They challenge their students to work on problems and assignments independently and they like to assign more research and writing than their right-brain peers. They maintain a reasonably quiet, structured classroom.

Left-brain students prefer to work alone. They like to read independently and incorporate research into their papers. They favor a quiet classroom without a lot of distraction.

Right-brain teachers prefer to use hands-on activities over a lecture format. They incorporate more visual aids into their lessons. Right-brain teachers assign more group projects and activities, and prefer a busy, active, noisy classroom environment.

Unlike left-brain students, right-brain students prefer to work in groups. They absolutely do not like to write another tedious term paper.

Students with left- or right-brain tendencies prefer to be taught to their neurological strengths. Although they can learn by different methods, they get most excited and involved when they can learn and do assignments in their area of strength.

To be more successful in your classroom, step outside your comfort zone and try to incorporate new neurological teaching methods. If you are a left-brain teacher, add at least one right-brain methodology (such as role playing or group project) into your lessons. If you are a right-brain teacher, consider lecturing more often, or assigning more individual and/or research-oriented projects. If you are a middle-brain teacher, select and incorporate something new from either area.

Better yet, give your students a variety of assignments to choose from. You may be pleasantly surprised to see students gravitating towards their own neurological strengths when given a choice of assignments.

The good news is that we, even the seasoned ones, can strengthen the weaker parts of our brains because they are always searching for new meanings and connections.

So, yes, you can teach an old dog new tricks!

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Editorial: Laboratory Spotlight

Medi3 Healthcare - Managing SARS-CoV-2 in Norway

Ann-Kristen Tveten Ph.D.
Associate Editor of Molecular Biology

When the world went into global lockdown due to Covid-19, and the regional industry in a corner of Norway could not get workers to cover shifts, Medi3 expanded their laboratory facilities to include SARS-CoV-2 analysis. This gave the industry a way to clear the staff for duty and help limit the spread of the coronavirus. In charge of the project, and quality of the analysis, was the dynamic duo that devoted their time in this laboratory spotlight, Ole Andreas Erstad and Karoline Valkvae.

About Medi3, and their role in SARS-CoV2 testing during the pandemic

Medi3 is a private healthcare service, and Medi3 Aalesund provides medical services for the marine and maritime industry in the region, in addition to traditional medical services. The region is a global supplier of maritime technology and ships as well as seafood. A new situation occurred during the covid pandemic, when otherwise healthy industrial workers needed to confirm that they were SARS-CoV-2 negative. The Norwegian healthcare system was pushed to the limits analyzing samples from patients with symptoms or close contacts of confirmed infected patients. The public healthcare system could not prioritize analysis of healthy individuals. This was a huge challenge for the regional industry that had to prevent the SARS-CoV-2 virus from spreading on site, to keep the wheels turning. Medi3 became a key factor in solving the problem and they were able to decrease the burden on the public healthcare system by taking over routine screening for SARS-CoV-2 virus as preventive measures among industrial workers and occupational travelers.

After some time, they were also able to provide testing for shipping crew, in nasopharynx sampling and point of care covid testing, so that crew could verify SARS-CoV-2 status at sea. When ships are far away from the harbor they needed to be able to determine whether they would have to abort the current mission and return to harbor or if they could continue towards their destination.

A new branch of the lab in the middle of a pandemic

In the start of the pandemic, limited clinical information was available concerning SARS-CoV-2 symptoms, analytical methodology and how to collect samples from a patient. Joint forces from multiple medical professions collaborated to overcome certain obstacles such as the identification of areas for patient sampling and developing a pipeline for distributing samples. The interprofessional approach to building a completely new molecular biology laboratory has been crucial to establish quality control (QC) and quality assurance (QA) systems and procedures for SARS-CoV-2 analysis during the pandemic. The analytical

pipeline, from the sampling of individuals, the laboratory pipeline and distribution of results, had to be determined based on basic knowledge and the broad experience from multiple contributors. The broad interprofessional approach enabled an analytical pipeline of the highest standards and has resulted in a laboratory capacity that includes analysis, quality assurance and the release of results within 24 hours. The dedication from the team at the laboratory has made this possible, and the routine screening has ensured a safer working environment on industrial sites.

Managing SARS-Cov2 variants

Initially, the requirements for laboratory results only included positive and negative results. When the laboratory chose analytical technology and considered different approaches, the traditional Real-time quantitative reverse transcriptase polymerase chain reaction (RT-qPCR) with three marker genes and an internal standard was utilized. The analysis was robust enough to detect the mutated variants, and with some experience, the lab was able to identify differences such as the Delta variant. The Delta variant demonstrated strong amplification of all three marker genes, while omicron had laps (gene dropout or gene target failure) in the S gene. The laboratory spent a lot of time working on QC/QA protocols for interpretation of results, and as any other RT-qPCR analysis, amplification curves are as individual as the patients the samples come from. The laboratory built a library of amplification curves for interpretation, and continuously improved the analytical pipeline to optimize the results. There were strict protocols for validation of results, and at some point, the quality of the positive control became an issue when the control sample temperature was too high.

Biomedical laboratory scientist Karoline Valkvae, who oversees the training and routines at the laboratory, carefully monitors all the details. Full scale RT-qPCR analysis can have a lot of critical analytical errors and preventing those requires the attention to details that biomedical laboratory scientists get from their education and professional training. Undoubtedly, continued monitoring of quality and the development of standards will continue as SARS-CoV2 remains a challenge to the laboratories across the globe.

Ann-Kristin Tveten, Ph.D.

Research article

Evaluation of the Effects of Freeze-Thaw Cycles on the Stability of Diabetes-Related Metabolic Biomarkers in Plasma Samples

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Background: **Repeatedly freezing and thawing of samples can affect the stability of biomarkers in plasma samples. There is a lack of studies reporting how these preanalytical factors affect the stability of diabetes-related metabolic biomarkers. This study investigated the effects of repeated freeze/thaw cycles (FTC) on the analysis of insulin, c-peptide, glucagon, total glucagon like peptide-1 (GLP-1), total glucose-dependent insulinotropic polypeptide (GIP), leptin and polypeptide YY (PYY).**

Material and Methods: **Plasma was prepared from blood samples collected from 10 healthy individuals. Each plasma sample was divided into 3 aliquots. An aliquot from each sample was analyzed immediately after preparation. The remaining aliquots were exposed to 3 and 5 repeated FTC. Samples were measured using a MESO Quickplex SQ 120 from Meso Scale Diagnostics LLC. (MSD) and the U-Plex Diabetes Combi 1 (hu) panel kit from MSD.**

Results: **The concentrations of GIP, GLP-1, insulin and PYY were statistical significantly affected by repeated FTC. After 5 FTC, the concentration of GIP was increased by 44 %, GLP-1 by 35 % and PYY by 22 %. There were no significant changes in the concentrations of glucagon, c-peptide and leptin after repeated FTC.**

Conclusions: **GIP, GLP-1 and PYY were significantly affected by repeated FTC. The concentration of these markers increased by 22-44 % with repeated FTC. Hence, repeated FTC can cause significant changes in the concentrations of the biomarkers. Our results suggest that caution should be exercised when comparing results of biomarkers between plasma samples that have been subject to FTC.**

Key words: Preanalytical, laboratory test, plasma, freezing and thawing.

Introduction

In relation to diabetes research, large-scale epidemiological studies and research projects are being carried out, collecting large amounts of human biological material. For logical reasons, all this material cannot be analyzed at the time of collection. The Clinical and Laboratory Standards Institute (CLSI) has developed guidelines for the proper handling of biological specimens and recommends that plasma specimens are frozen and thawed only once.[1] Despite this recommendation, the same

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samples are often used repeatedly up to several times to recreate measurements and/or to determine additional results of multiple analytes.

Over the past decades, the expansion of biobanks has been excessive, which has led to decentralization of the biobanks due to limitations in storage capacity. A disadvantage of this is the fact that there may not be similar guidelines for how to collect and store the biobank samples across the different sectors of the biobanks. This heterogeneity between biobanks makes it difficult to properly compare various biospecimens among samples stored in different biobanks.

Forty-five percent of biobanks in Denmark do not have a Standard operating procedure (SOP) for sample handling. Based on a questionnaire sent to biobanks in Denmark, it has been found that only half of the participants thought that quality control of blood samples in biobanks would alter data quality and test results.[2] This indicates that the knowledge about how preanalytical factors such as FTC affect different biomarkers in the blood samples, is limited.

One of the initiatives to solve this issue, has been establishing the International Organization for Standardization (ISO) standard for biobanks, ISO/DIS 20387. The Danish National Biobank is following the guidelines in this document but the biobank is not yet accredited.[3] The ISO 20387 standard neither mentions the need for temperature monitoring nor contains guidelines for keeping track of how many times samples are thawed and frozen.[4] Studies have shown that repeatedly freezing and thawing of samples affect the stability of various markers in plasma and serum samples.[5,6] To date, however, the stability of the diabetes-related metabolic factors insulin, c-peptide, glucagon, GIP, GLP-1, leptin and PYY has only been investigated in a few studies and with other methods than the MSD.[7–10] These biomarkers play a major role in the prognosis, diagnosis, treatment and research of diabetes.[7,10–14] Insulin for instance, is a hormone produced by beta cells in the pancreas and promotes the absorption of glucose from the blood into the liver, fat and skeletal muscle cells.[8,9] C-peptide is a polypeptide that can be measured to distinguish between T1 and T2 diabetes.[8,15] Glucagon has the opposite effect of insulin and activates the secretion of glucose from the liver cells into the blood.[13,16]

The seven biomarkers included in this study are easily degraded in the body and are therefore very unstable. Hence, this study hypothesized that the stability of the biomarkers will be further affected by FTC.[7,17–20] The aim of this study was to investigate how repeatedly freezing and thawing of plasma samples affect the stability of insulin, c-peptide, glucagon, GLP-1, GIP, leptin and PYY. This information is very relevant for diabetes research both at Steno Diabetes Center Copenhagen (SDCC) and worldwide.

Methods and materials

Setup/specimen collection

In this study venous blood samples were collected from ten healthy employees at SDCC, nine females and one male. All test subjects volunteered and gave spoken informed consent. The ten test subjects ate a meal consisting of bread, yogurt, cheese, jam and butter 30 min before sample collection. The calorie content of the meal was 729 kcal. The program used to design the meal and its composition was LifeSum from LifeSum AB. Inspiration for the composition and calorie content of the meal was taken from previous studies.[21,22] All test subjects were fasting for a minimum 12 hours before participating in the project, due to the fact, that the biomarkers investigated are secreted in response to food intake. The participants were permitted to drink water. Non-diabetic test subjects were recruited to ensure that the response to the food intake was normal, as well as the concentrations of the seven biomarkers were not in the low area of the reference values.

Blood samples were collected in BD™ P800 Blood Collection system containing DPP4 inhibitor, from Becton Dickinson A/S by

venipuncture. The samples were placed on ice immediately after collection, and stored for a maximum of 30 minutes, before centrifugation. All samples were centrifuged at 1000 x g for 10 minutes at 5° C as recommended by MSD. Plasma was extracted and aliquoted into three Eppendorf tubes per sample. One aliquot was analyzed immediately. The second aliquot was frozen at -80° C for at least 12 hours and thawed 3 times and the third aliquot was frozen at -80° C for at least 12 hours and thawed 5 times. All samples were thawed at room temperature for 15 minutes followed by 3 hours on ice (0°C).

MESO Quickplex SQ 120

The samples were analyzed in duplicates from the same aliquot on the Meso Quickplex SQ 120 from MSD. This method uses the sandwich enzyme-linked immunosorbent assay and electro chemiluminescence principle for detection of analytes. In this project, the U-Plex Diabetes Combo (hu) panel (MSD) measuring the total levels of insulin, c-peptide, glucagon, GLP-1, GIP, leptin and PYY was used to analyze the samples. Prior to analyzing, the samples were centrifuged at 2000 x g for 3 minutes by recommendations from MSD.

For each plate analyzed, a standard curve using two different calibrators was included. The calibrators containing known concentrations of analytes were included in the MSD kit.

Data handling and statistics

Bland-Altman plots were used to compare the baseline measurements with the 3 and 5 freeze/thaw measurements for each variable. This was used to evaluate systematic errors and to see if the differences between two measurements increased or decreased with changes in marker concentrations.

Differences between the baseline test result and the test result at each FTC were examined by a 2-sided paired Wilcoxon test. Statistical significance was considered as a p-value <0,05. Data was converted into relative values (%). This was completed using the mean value for all measurements at 0 FTC as the baseline value. Then, the mean value from the relevant FTC (analysis value) was divided with the baseline value times 100 %.

$$\text{Relative value } \% = \frac{\text{Analysis result}}{\text{Baseline value}} \times 100\%$$

To assess whether the stability of the biomarkers was affected beyond the analytical variation, the results were illustrated using the mean relative value from every freeze/thaw cycle with a 95 % confidence interval (95% CI) where an analytical uncertainty of 20 % was included. The calculation was completed using the following formula:

$$95\%\ \text{CI} = \frac{Mean\ of\ relative\ analysis\ value\ \pm 1{,}96 \times \sqrt{2} \times CV\%ana}{\sqrt{\text{n}}}$$

When illustrating the results, a 5 % and 10 % bias line were included to assess the clinical significance of the results.

Results

The Bland-Altman plots (figure 1) illustrate that for most of the measured factors, several measurements were higher at 3 and/or 5 FTC as compared to the baseline (0 FTC). This was particularly evident for insulin, GIP, GLP-1, PYY and glucagon. For GIP, the higher measured concentrations were especially pronounced after 5 FTC, where nine out of ten measurements showed increased concentrations. For c-peptide and leptin there were no consistent differences between the measured values between baseline and 3/5 FTC.

The higher measured concentrations of insulin, GIP, GLP-1 and PYY after 3 and/or 5 FTC as compared to baseline were confirmed statistically significant by the Wilcoxon test. P-values showed significant differences between 0 and 3 FTC for GLP-1 (*p=0,005*), insulin (*p=0,048*) and PYY (*p=0,002*).

The test further showed significant differences between 0 and 5 FTC for GIP (*p= 0,013*), GLP-1 (p=0,048) and PYY(*p=0,019*). For GIP and GLP-1, none of the data points at 5 FTC or the total 95 % CI were within the 10 % bias

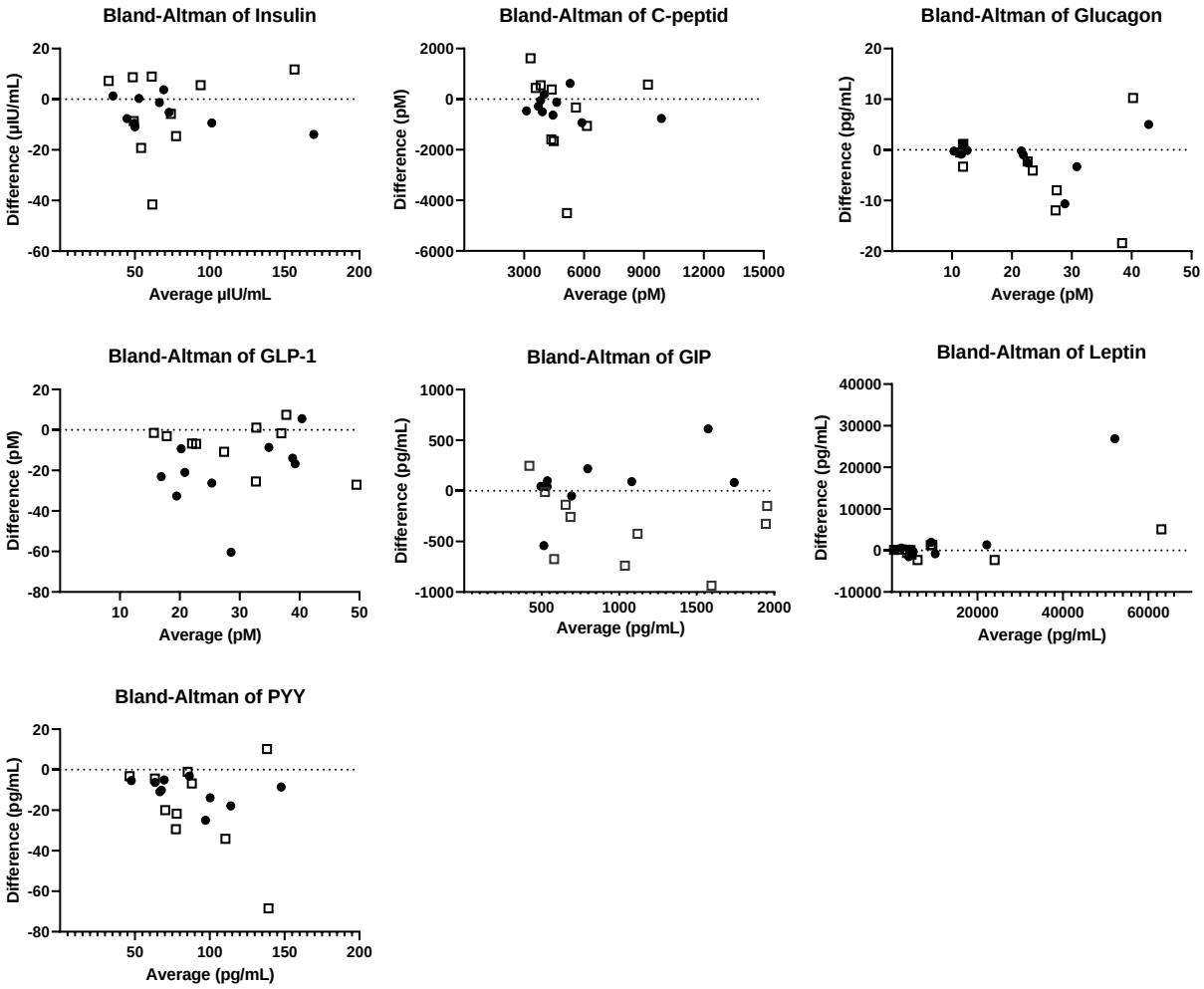

Figure 1. Bland-Altman plot for each biomarker, comparing 0 and 3, and 0 and 5 FTC's. Black circles marks `baseline – 3 FTC's and clear squares marks `baseline – 5 FTC's. The Y-axis shows the difference between the two paired measurements (0-3 or 0-5) and the x-axis represents the average of these measurements.

line, confirming that FTC affect the measured values beyond simple analytical variation. For GLP-1, the 95 % CI was
not within the 5 % bias line after 3 FTC, and for PYY the 95 % CI was not within the 5 % bias line after 5 FTC (figure 2).

Discussion

In this study, the effect of repeated FTC on the measurement of 7 metabolic plasma markers was evaluated using the MSD multiplexing technology. It was found that the measured concentrations of insulin, GIP, GLP-1 and PYY significantly increased as a result of FTC. For glucagon (p=0,23 + 0,16), c-peptide (p=0,08 + 0,62) and leptin (p=0,32 + 0,92) the results showed a tendency in increase in concentration due to multiple FTC, but this did not reach statistical significance.

When evaluating the performance of analysis in clinical practice, the observed bias should be compared to the desirable allowed bias.[23]

Data from Westgard shows that the desirable specification for inaccuracy (B%) for serum c-peptide is 7,1% and for serum insulin it is 15,5%. For the remaining analytes included in this study, B% was not available from

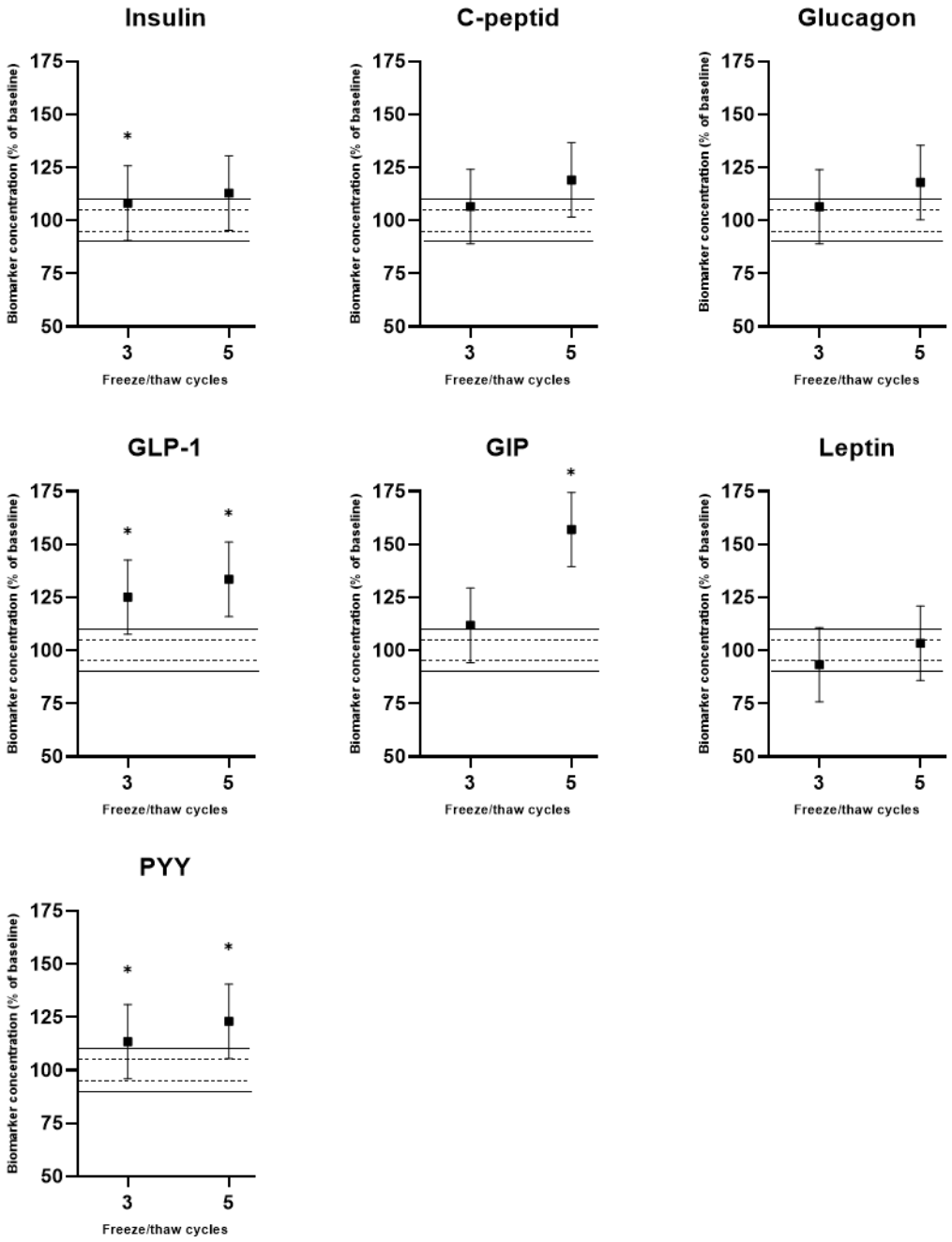

Figure 2. The change in concentration illustrated as means with 95%CI of the 7 biomarkers after 3 and 5 FTC's. The y-axis represents the relative values with 100 % being the baseline value. The dotted line shows 5 % bias limit. The black line shows 10 % bias limit.

95% CI was calculated as $\frac{Mean\ of\ relative\ analysis\ value \pm 1{,}96 \times \sqrt{2} \times CV\%ana}{\sqrt{n}}$

* = p<0,05.

Westgard. Further, the biological variation of the analytes were not available and we were not able to calculate the B% using the formula from Westgard.[23] At SDCC biochemical validated analyses has a maximum inaccuracy/bias between 5-10%. With all this in mind, 5 % and 10 % bias was used when illustrating the results.

For insulin, glucagon, c-peptide, and leptin it is unclear whether the changes in concentrations of the measured biomarkers are due to repeated freezing and thawing of the samples or analytical errors, since the 95 % CI are only partially within the bias lines. If the maximum allowed analytical error (CV% ana) was lower, the range of the 95 % CI would

decrease and might alter the reliability of the results. However, the 95 % CI of GLP-1 and GIP were not within the 10 or 5 % bias line after 5 FTC and the 95 % CI was not within the 5 % bias line after 3 FTC for GLP-1 and after 5 FTC for PYY. This indicates that FTC affects the concentration of these 3 biomarkers beyond analytical variation. A limitation in this study is the sample size of only ten. If the sample size were bigger, the 95 % CI might also have been smaller and thereby the statistical power of the results more robust.

The results show that GLP-1 concentrations in plasma are significantly affected by 3 (p=0,005) and 5 (p=0,048) FTC and GIP is significantly affected after 5 FTC (p=0,013). For both of these biomarkers the concentration increased by approximately 35% following FTC. Insulin was significantly affected after 3 FTC (p=0,048) and PYY after 3 (p=0,0002) and 5 (p=0,019) FTC. A study measured twenty-seven cytokines in plasma after 6 FTC and found that IL-1B concentration decreased and CCL5 concentrations increased, both changes being statistically significant.[24] Another study by Chen et. al used LC-MS method and $^{12}C/^{13}C$ – dansyl-chloride labeling of several metabolites when evaluating the effects of FTC. The results from this study showed a great difference in changing patterns as the FTC increased for each metabolite. Some concentrations decreased and some increased.[25] A possible mechanism being responsible for the increase in concentration of the metabolites could be the degradation of plasma proteins due to the number of FTC. Hoshiyama et. al found that GIP binds to albumin, IgG and transferrin, all plasma proteins found in blood.[26] When the blood samples are subjected to repeated FTC these proteins could degrade, and thereby release more GIP, which will result in an increase in concentration of the biomarker in the sample. Today many studies are investigating the importance of GIP and GLP-1 in diabetes and how these biomarkers can be used in the diagnostic phase and treatment of diabetes. If the samples used in a study are frozen 5 or more times, the results could be misinterpreted and would not reflect the true concentration of GLP-1 in the sample. This would cause poor reproducibility and development of a treatment based on wrong results. Overall, the quality of the samples can be altered by repeated FTC and this can greatly affect the validity and reliability of research studies.

As shown both in this and in many other studies, different biomarkers are affected differently by preanalytical variables.[24,27] This is especially problematic when samples are collected and stored in population-based biobanks, where the samples are used for multiple different purposes and studies, investigating a wide range of (patho) physiological conditions and diseases. The evolution of multiplex analyses like the one from MSD used in this study, also increases the need for knowledge about how the different preanalytical variables affect different biomarkers.

Ensuring that samples are not affected at all by any preanalytical variables is very difficult. It should be standard procedure to track and document every variable the sample is subject to. This can accomplished by using the Standard Pre-analytical Code (SPREC) system. This system makes it easy to document how samples have been handled from collection. It includes preanalytical values such as type of sample, primary container, delay of centrifugation and long term storage conditions.[28] Implementing this system in every biobank, will make it easier for scientists to select the right samples for their studies. However, the SPREC system fails to document the of number of freeze/thaw cycles. Scientists should also improve the documentation of the condition and quality of the samples used for their research and be very transparent in this area.

Conclusion

The results in this study underline that repeated FTC of plasma samples can affect the measured concentrations of protein markers including the total concentrations of PYY and

the incretin hormones GIP and GLP-1. The results show that repeatedly freezing and thawing of samples can cause a statistically significant difference in the results, but not necessarily have a clinical significance. The study emphasizes the need for precaution when repeatedly thawing and freezing samples meant for measuring PYY, GIP and GLP-1. Future studies should include a larger sample size, and from both non-diabetic and diabetic patients in order to cover a larger range in measurements.

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Mini-review

Hemostasis Pathophysiology Associated With Increased Risk of Thrombosis in Acute COVID-19 Infection

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The aim of this mini review is to understand how COVID-19 contributes to thrombotic events in patients. The recent and ongoing coronavirus 19 pandemic (COVID-19) has presented tremendous challenges to healthcare, with approximately 219 million cases worldwide and 4.5 million deaths associated with infection to date. Patients experience a significant immunological response to the virus, and this is often followed by a state of acute respiratory distress syndrome (ARDS). It has become increasingly evident that hemostatic dysregulation and thrombotic events are prevalent complications of acute COVID-19 infection and may persist chronically in the manifestation of "long-COVID." Current anticoagulant therapies are insufficient in mitigating the risk of thrombosis in COVID-19 patients and further understanding regarding the pathophysiological mechanisms of hemostatic dysregulation following COVID-19 infection is critical to improve clinical management. This manuscript endeavors to summarize the current understanding based on the recent clinical literature and to identify potential future research directions to best inform clinicians on how to optimize patient outcomes.

Key words: COVID-19, hemostasis, thrombosis

Introduction

One of the largest public health crises in the modern era is associated with the pathophysiological mechanisms that follow infection with the globally disseminated severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), commonly referred to as COVID-19. It is well understood that COVID-19 can induce ARDS in patients following a marked immunological response to the virus. A markedly increased inflammatory response increases the probability of thrombosis as there is a direct link that can be established when studying the molecular mechanisms of hemostasis and subsequent thrombosis. This can be reflected in the observation of increased predisposing thrombotic markers such as D-Dimer and other procoagulant changes in patients suffering from severe COVID-19 infection, along with reports of increased arterial and venous thrombotic events. This minireview endeavors to investigate and summarize the current clinical literature describing the possible mechanisms and to comment on further impacts on clinical outcomes that occur because of thrombosis in COVID-19 infections. To adequately investigate the thrombotic mechanisms that occur through COVID-19 infection, a baseline understanding of hemostasis must be present.

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Background

Hemostasis

Hemostasisis the process of arresting the loss of blood and maintaining it as a fluid in its compartments. It is highly regulated and physiologically involved, as hemostatic dysregulation can often lead to thrombotic events when disproportionally upregulated, or bleeding disorders when disproportionally downregulated. An understanding of the processes that occur in the body to induce hemostasis is highly clinically relevant. Clinical intervenetions (such as prophylactic heparin) can be conducive to hemostatic dysregulation or may not be feasible due to an underlying presence of pre-existing hemostatic dysfunction.

Hemostasis occurs in three primary stages within the body, the first stage being agonistic blood vessel activity combined with platelet adhesion to form a mechanical primary plug. The second stage of hemostasis is secondary activation of a cascade of coagulation proteins commonly referred to as coagulation factor interactions that contribute to clot formation and significant strengthening of the formed primary plug. The final stage is activation of the fibrinolytic system to regulate hemostatic activity by breaking down fibrin to dissolve the clot after healing has taken place, as demonstrated in Figure 1.

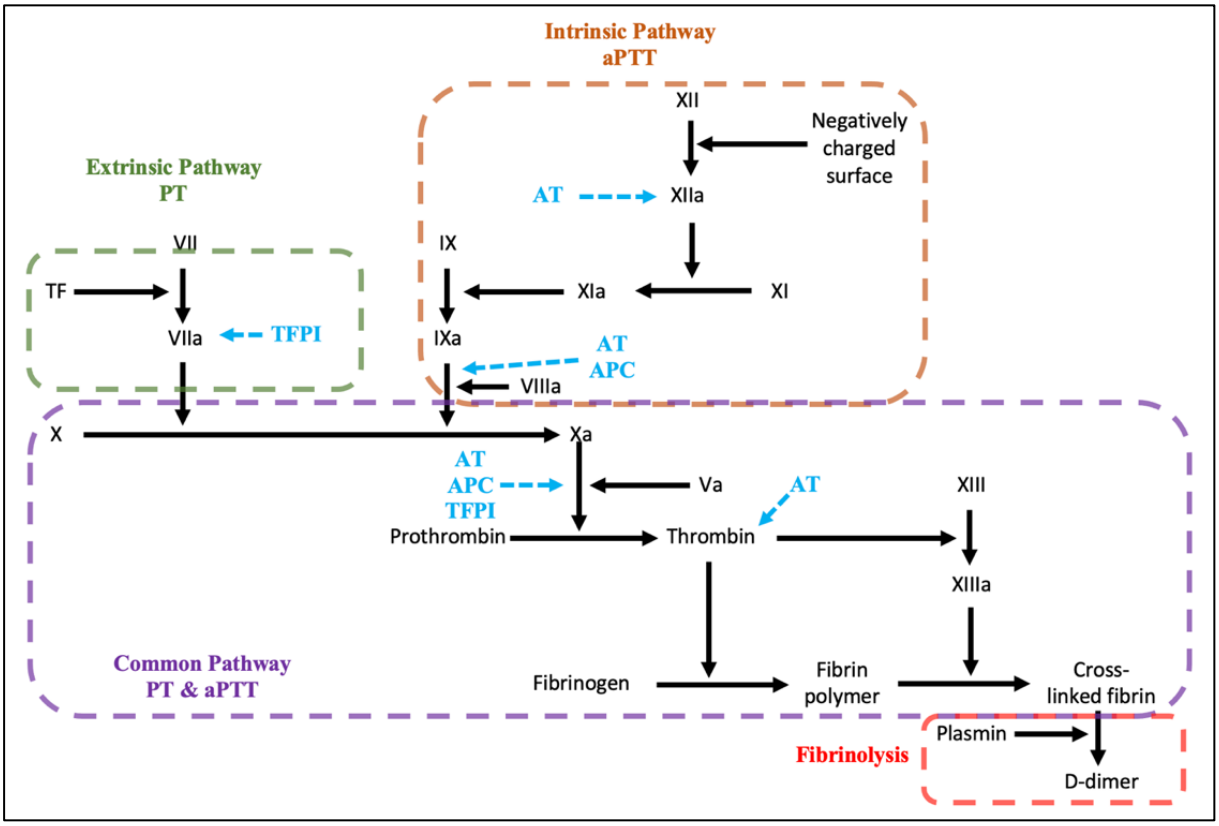

Figure 1: Coagulation Cascade Depiction

Blood coagulation cascade involving an amplification system in which activation of various coagulation factors occurs in sequence leading to a cascade or domino effect. Each reaction is promoted by the preceding reaction. If there is a deficiency of any one of the factors, the following may result: The rate of blood coagulation is slowed, initiation of the next reaction in the sequence is delayed, the time taken to form a clot is prolonged and bleeding from an injured blood vessel is not effectively arrested. Hyperactivation of these procoagulant factors would conversely result in a hypercoagulable state and present risk of uncontrolled clot formation and eventual thrombosis, as is being demonstrated in COVID.

Figure based on (adapted) information in Rodak's hematology textbook: Keohane EM, Otto CN, Walenga JM. Rodak's Hematology: Clinical Principles and Applications. 6th ed. St. Louis, Missouri: Elsevier; 2020.

Discussion

Hemostasis in COVID-19

A high prevalence of increased thrombotic events and prothrombotic pathophysiology has been observed through the infection of COVID-19, due to dysregulation of the coagulation cascade caused by an imbalance between procoagulation and anticoagulation.[1, 2] This imbalance in the coagulation system is evidenced by the finding of both microvascular and macrovascular thrombosis and embolism in COVID-19 patients in multiple organ systems.[3] There are many hypotheses regarding the reason for the dysfunctional coagulation seen in COVID-19, including a cytokine storm, which consequently leads to a hyperinflammatory state.[1] The study by Pretorius, Vlok showed hypercoagulation induced by inflammation along with hypofibrinolysis in COVID-19 affected patients.[2] A particularly high proportion of thromboembolic complications have been reported to arise in hemodialysis circuits and in young, otherwise healthy patients with no underlying predispositions to hypercoagulation despite the administration of prophylactic anticoagulant medications. This would suggest that COVID-19 induces mechanisms which increase the risk of thrombosis in not only patients with underlying medical conditions, but also young and healthy patients. Furthermore, thromboembolism has been reported in acute care and in weeks following illness, suggesting that the mechanisms of hypercoagulation in COVID-19 infection may not only lead to a transient increase of risk for thrombosis, but may last several weeks or longer after hospitalization.[4] This would warrant further study into the pathophysiology of COVID-19 and long COVID.

It has been previously mentioned that a direct link can be observed in the physiological mechanisms between inflammation and hemostasis; however, there is still a significant gap in the literature and a further understanding of this potentially causal link that would suggest anti-inflammatory prophylaxis and therapeutic treatment could mitigate the risk of thrombosis in COVID-19. If there is no causal link, the use of targeted anti-coagulation medication would likely be more favorable in reducing the risk of thrombosis, as anti-inflammatory medication would not have clinical utility. This could also be described as being a significant gap in the current literature, as an understanding of the exact mechanisms of action of COVID-19 infection that give rise to a pathophysiological increased level of hypercoagulation which has been demonstrated in healthy and ill patients would provide a basis for the development of preventative therapies that could appropriately target the cause of thrombosis post infection and potentially reduce rates of mortality and morbidity as a result of COVID-19 infection.

Several studies have found increases in factor VIII and the presence of ultra-large von Willebrand factor (UL-VWF) multimers in COVID-19 patients.[5-8] Under normal circumstances, UL-VWF multimers are cleaved into smaller, functional units, which allows platelet adhesion in the required conditions, such as an active bleed. The increased factor VIII and UL-VWF may produce or augment the hypercoagulable state seen in COVID-19. Currently, the implication of conventional preventative therapies is largely unreliable in reducing thrombotic events due to COVID-19 infection, and treatments generally target the effects on organ systems that occur because of thrombosis and / or embolism. This may be due to the unique pathophysiological processes of COVID-19. The most reported cases of thrombotic events in COVID-19 are deep vein thrombosis and subsequent pulmonary embolism.[9]

Thrombotic Events in COVID-19

Pulmonary embolism has been shown to be extremely prevalent in patients experiencing COVID-19 infection, with about a 20-30% incidence in critically ill patients.[10] Pulmonary embolism (PE) is a pathology where a thrombus has formed generally in a deep vein of the leg, known as a deep vein thrombosis (DVT), dislodged into the vascular circulation

(embolized) and occluded a pulmonary artery. The occlusion of pulmonary vessels causes pulmonary infarction, meaning the death of tissue (tissue necrosis) as a result of a lack of blood supply. The dissemination of micro-thrombi formation throughout the pulmonary vasculature has been hypothesized to contribute to the extremely high incidence of thrombosis overall, PE and the unique presentation and physiology associated with ARDS following COVID-19 infection. Ackermann *et al.,* compared autopsies of lung specimens from 7 patients who died because of COVID-19 with 7 patients who died because of H1N1 influenza ARDS complications.[11] Alveolar capillary microthrombi were found to be approximately 9 times as prevalent in COVID-19 patients when compared to influenza patients, supporting the hypothesis that the COVID-19 virus contributes to thrombotic mechanisms through its unique pathophysiology. Marked increase in endothelial injury in addition to intracellular virus could also be observed in areas of increased microthrombus formation, also supporting the hypothesis that there is a causal link between inflammatory response and thrombus formation. It has also emerged recently that a condition of reduced platelet count in the peripheral blood can be induced by vaccine administration, called vaccine-induced immune thrombotic thrombocytopenia (VITT) in rare cases.

Anticoagulation in COVID-19

Currently, there is insufficient literature to identify and describe the role, significance, and appropriate use of anticoagulant therapies on a large clinical scale in the mitigation of hypercoagulation in COVID-19 afflicted patients. The enormous prevalence of this virus and the high rate of mortality resulting from thrombosis following infection should sufficiently motivate clinical studies in this area to ascertain the true mechanisms of action and therefore develop appropriate therapies. There is currently no targeted strategy for treating the hypercoagulative state observed in COVID-19 infection and the current guidelines suggest thromboembolism prophylaxis for all patients in the absence of significant contraindications. Recent clinical trials have shown conventional thromboprophylaxis was ineffective in reducing inappropriate coagulation due to COVID-19.[12] Low molecular weight heparin has also been demonstrated to be ineffective in recent studies.[13] This would suggest that the hypercoagulation demonstrated in COVID-19 cases are most probably linked to inflammatory pathophysiology.

Conclusion

The emergence and persistence of the COVID-19 pandemic has propelled widespread investigations into the complex pathophysiological mechanisms interacting between multiple organ systems to lead to an increase in patient outcomes; particularly in the management of ARDS frequently developed in hospitalized patients infected with the virus. The aim of this manuscript was to review the current state of literature to ascertain the current level of understanding of these mechanisms, specifically regarding the induction of hypercoagulation in patients infected with COVID-19, to infer possible gaps in the current literature and to form an educated proposal for future directions.

Recent studies demonstrate that a direct link can be observed in the physiological mechanisms between inflammation and hemostasis, but it has not yet been determined whether this is a causal relationship, or if the illness simply induces simultaneous pathophysiological pathways in parallel.[14] This is a significant gap in the literature and a further understanding of this potentially causal link would suggest that anti-inflammatory prophylaxis and therapeutic treatment could mitigate the risk of thrombosis in viral infection from COVID-19. If there is no causal link, the use of targeted anticoagulation medication would likely be more favorable in reducing the risk of thrombosis, as anti-inflammatory medication would not have clinical utility. The presence or

lack thereof a link between these mechanisms should be addressed in future studies.

Currently, there is insufficient literature to identify and describe the role, significance, and appropriate use of anticoagulant therapies on a large clinical scale in the mitigation of hypercoagulation in COVID-19 afflicted patients. The enormous prevalence of this virus and the high rate of mortality resulting from thrombosis following infection should sufficiently motivate clinical studies in this area to ascertain the true mechanisms of action and therefore develop appropriate therapies.

Overall, there is a rising comprehension of the mechanisms of COVID-19 and how these contribute to thrombotic events in patients, however, the current literature is insufficient in establishing standardization of care and appropriately informing clinicians on how to manage cases of COVID19 infection to optimize patient outcomes.

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Review article

Hematologic Abnormalities Associated with Post-Acute COVID-19 Sequelae or "long-COVID"- a Systematic Review

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Objective: SARS-CoV-2 emerged late 2019 and quickly spread globally. Acute COVID-19 effects were quickly elucidated; however, some patients were found to suffer from persistent symptoms in the absence of an acute infection. This places unnecessary pressure on healthcare systems and affects patient quality of life. Literature indicated lymphopenia, hyperferritinemia and coagulopathies were common among those with persistent symptoms. This systematic review aims to summarize the association between these hematologic abnormalities and long-COVID.

Methods: A systematic search of five electronic databases, PubMed, Google Scholar, Science Direct, Griffith University library and Cochrane, was conducted using specified search terms described in the methods section. Studies were refined using the Preferred Reporting Items for Systematic Reviews and Meta Analyses (PRISMA) tool. Data was retrieved from studies that passed the risk of bias (ROB) and met the inclusion and exclusion criteria, as follows; number of participants (≥10), hematologic testing, timing of testing, and studies with full text available in English.

Results: The search strategy identified 14 studies that passed the ROB, met the inclusion and exclusion criteria, and were selected for the systematic review. Though some patients experiencing long-COVID had lymphopenia, hyperferritinemia and coagulopathies, there was inconsistencies found. Some patients with long-COVID had limited evidence of hematologic abnormalities.

Discussion: Lymphopenia was a frequent anomaly identified in post-acute COVID, however, not exclusive to long-COVID patients. New research has shown the absence of specific T and B lymphocyte subsets may be exclusive to long-COVID patients, along with the sustained activation of other immune cells. Evidence has also emerged showing sustained inflammation beyond the acute infection in long-COVID patients. Coagulopathies have been shown to persist due to an elevated D-dimer in post-acute COVID-19 analyses.

Conclusion: There is evidence of hematologic features that are exclusive to long-COVID, however, research is still limited. The cause and effect of these abnormalities are yet to be determined. With future directions, further supporting evidence may emerge elucidating the potential hematological causes and mediators of long-COVID.

Key words: Long-COVID, Persistent symptoms, Lymphopenia, Iron dysregulation, Coagulopathy.

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Introduction

The coronavirus disease 2019 (COVID-19) pandemic has resulted in global consequences, including deaths, lockdowns, economic breakdowns, and more. One consequence of COVID-19 referred to as "long-COVID," occurs when symptoms of the viral infection persist even after the acute infection, or virus, has been cleared. A clear definition of the timeline associated with long-COVID has not yet been established and varies between articles (discussed later). Long-COVID symptoms include fatigue, shortness of breath, general malaise and more.[1-3] These lingering symptoms affect the patient's quality of life and, thus, adds unnecessary additional pressure on the healthcare system due to the extra care required for patients following the acute phase of the disease. The purpose of this study is to assess the changes seen in the blood components, such as red cells, white cells, and platelets, of COVID-19 patients who have recovered from an active infection and are suffering from long-COVID symptoms. Elucidating how long-COVID occurs, how it affects the blood, how to predict it, and how it could possibly be treated could relieve the burden on both healthcare and patients. Samples are often used repeatedly up to several times to recreate measurements and/or to determine additional results of multiple analytes.

Study Aims and Objectives

This systematic review aims to analyze and compare current literature regarding long-COVID with a focus on hematologic parameters to determine the commonly seen changes and the possible associated pathophysiology to assist in future care and rehabilitation. This will be achieved via the following objectives:

1. A planned systematic review of the literature to assess and compare the most frequent abnormal hematologic findings in long-COVID.
2. Comparisons between literature methods and findings to determine the reliability of abnormal hematologic markers for predicting long-COVID and severity.
3. Discussions of the theorized pathophysiology to elucidate possible directed therapy or rehabilitation for affected patients to assist in recovery and return to baseline quality of life.

Multiple studies investigating hematologic parameters in patients with long-COVID were critically evaluated and compared. Establishing disease or diagnostic patterns could provide critical information to determine prognosis and guide patient therapy or rehabilitation.

SARS-CoV-2 and Hematologic System

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a single-stranded, positive sense, enveloped RNA virus belonging to a large family of coronaviruses.[4] With an unestablished, but highly debated origin, SARS-CoV-2 emerged in late 2019 and in March 2020, the World Health Organization (WHO) declared SARS-CoV-2 a global pandemic. Numerous studies of SARS-CoV-2 have provided key information regarding COVID-19 transmission, pathogenesis, and possible treatment options.[5,6] An unexpected aspect of the pandemic, however, was the persistence of symptoms in the absence of an acute COVID-19 infection. This phenomenon has been coined "long-COVID," "chronic COVID," or post-acute COVID syndrome (PACS). Although many patients experience mild respiratory symptoms during the acute phase, select studies have found that many patients are affected by long-COVID. This places significant pressures on healthcare systems and the patient's quality of life.[3,7,8] The hematologic system is central to the basic functions of the human body and based on recent literature, SARS-CoV-2 affects the hematologic system in various ways. This analysis provides insight into the hematologic pathophysiology of long-COVID, providing some key prognostic indicators that may help predict the severity of disease, which can be used for directed therapy and rehabilitation for future patients.

Background/Literature Review

Long-COVID, the persistence of symptoms in the absence of an acute COVID-19 infection,

affects the quality of life of many patients. Establishing patterns in hematologic abnormalities could be used to determine suitable treatment and possible rehabilitation strategies for patients.

Definition of Long-COVID

An official definition for long-COVID has not yet been established. Some studies have followed the National Institute for Health and Care Excellence (NICE) definition of long-COVID, which, in collaboration with other institutes, have defined "ongoing symptomatic COVID-19" as persistent signs and symptoms lasting 4 to 12 weeks, while signs and symptoms lasting more than 12 weeks which cannot be explained by differential diagnoses is defined as "post-acute COVID-19 syndrome (PASC)."[1,3,9,10] Other studies have defined long-COVID as symptoms persisting in the absence of the virus or post-acute COVID-19 if symptoms persist for 3-12 weeks and chronic COVID-19 if symptoms persist for more than 12 weeks.[11-13] For the purposes of this review, long-COVID was defined as persistent COVID-19 symptoms with a negative COVID-19 polymerase chain reaction amplification (PCR) test or at least 1 month following the onset of symptoms (where PCR results were not available). This definition was used to establish a timeline to identify the significant diagnostic changes associated with the persistence of COVID-19 symptoms.

Risk Factors for Long-COVID

Comorbidities are known to increase the severity of COVID-19 and may also be the cause of some patients' suffering from long-COVID. Some of these comorbidities are listed include age (more than 60 years of age), obesity, diabetes mellitus (DM), hypertension, ischemic heart disease, chronic obstructive pulmonary disease (COPD), asthma, and chronic kidney disease (CKD).[1,14,15] The presence of these comorbidities may have pre-existing effects on hematologic parameters or contribute to the abnormalities seen in long-COVID.

Hematologic Abnormalities in Long-COVID

After extensive evaluation of the literature, the most common hematologic abnormalities found in long-COVD included lymphopenia, hyperferritinemia, and coagulopathies. Figure 1 depicts a basic schematic of the hematologic abnormalities identified in this systematic review.

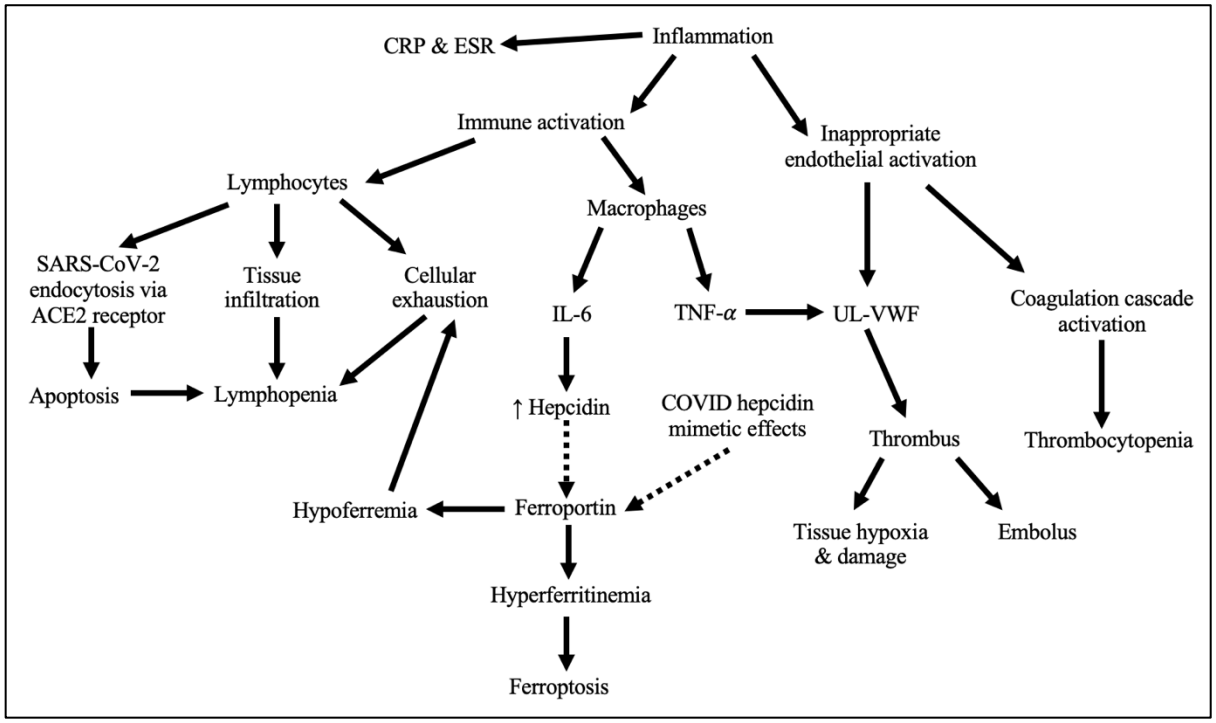

Figure 1: Summary depicting the effects of COVID-19 on various aspects of the hematologic system. C-reactive protein (CRP); erythrocyte sedimentation rate (ESR) tumor necrosis factor-alpha (TNF-α); ultra-large von Willebrand factor (UL-VWF); interleukin-6. (IL-6). Made by authors.

Cytokine Storm

Various studies have shown SARS-CoV-2 induces a cytokine storm, which is an excessive cytokine release due to uncontrolled immune regulation.[4,16] Some of the cytokines noted in COVID-19 includes proinflammatory and procoagulant molecules, such as interleukin (IL)-2, IL-6, interferon-gamma (IFN-γ), interferon-beta (IFN-ß), and tumor necrosis factor alpha (TNF-α) to list a few.[16-18] These cytokines have a multitude of effects on the hematological system. Another notable inflammatory marker that is elevated in SARS-CoV-2 infections is C-reactive protein (CRP). CRP is an acute phase protein and is increased during inflammation due to the release of IL-6.[19] Elevated CRP typically decreases when an active infection is cleared by the immune system, however in long-COVID patients CRP remains elevated indicating a potential significance to the persistent of a patient's symptoms.

Lymphopenia

Lymphopenia, a significant decrease in lymphocyte counts, was noted as a common anomaly found in long-COVID patients.[1,15,20] It was also found in acute infections and was shown to be a good predictor of COVID-19 severity, where patients with lymphopenia were found to have more severe symptoms.[14,20] Lymphopenia is abnormal in viral infections as lymphocytes are known to be one of the primary immune cells elevated in viral infections and involved in the clearance of the virus.[21] It should also be noted that neutrophilia with abnormalities in granulocytes and monocytes were seen in addition to lymphopenia in post-acute infections, which was postulated to contribute to morbidity via facilitation of infections caused by other microorganisms due to the patient's immunocompromised state.[20,22] These leukocyte abnormalities may exacerbate the negative effects of post-COVID contributing to long-COVID symptoms.

Numerous theories have been postulated as to the cause of lymphopenia seen in COVID-19 infections. A narrative review by Korompoki *et al*. indicated that lymphopenia may result from cell lysis due to SARS-CoV-2 infecting the cells via endocytosis mediated by binding of the viral spike protein to angiotensin converting enzyme 2 (ACE2) receptors.[17] Another theory suggested that the cytokine storm seen in COVID-19, as noted earlier, resulting in hyperinflammation was associated with cytopenia and may induce lymphocyte apoptosis.[16,17] Ramakrishnan *et. al*. postulated the possibility of "COVID-associated immune exhaustion," which occurs in chronic viral infections due to prolonged antigen stimulation.[1] Lymphocytic infiltration may also contribute to lymphopenia in COVID-19 patients. Lymphocytic infiltration has been reported in multiple organs, including the lungs, hepatic portal tract, kidneys, and myocardium.[1]

In addition to lymphopenia, iron is central to erythropoiesis and lymphocyte activity. Lymphocytes require iron to produce an effective immune response to infections when the cell initially interacts with the viral protein (antigen) or is primed.[23] Therefore, iron dysregulation may also contribute to lymphopenia and long-COVID symptoms.

Iron Dysregulation

Iron dysregulation has been associated with severe acute COVID-19 infections, producing hyperferritinemia (elevated ferritin concentrations), and has been shown to persist for up to 2 months post-acute infection.[22,24] During inflammation, the cytokine IL-6 is released, which stimulates hepcidin synthesis. Therefore, the hepcidin stimulation functions as a host defense mechanism by limiting iron availability to invading organisms.[11,23,24]

Hepcidin, a peptide hormone produced by the liver, is central to iron homeostasis; it functions to inhibit iron absorption by inactivation of ferroportin, the transmembrane protein responsible for iron exportation out of cells.[21,25] Increases in hepcidin inhibits the release of iron from cells, thus, increasing ferritin concentrations, causing hyperferritinemia, in both serum and macrophages.[11,23] This hyperferritinemia alters iron metabolism, affecting red blood cell (RBC) indices and leading to apoptosis, termed ferroptosis, which is a

type of necrosis induced by excessive iron. Ferroptosis may also cause neighboring tissue damage, which further exacerbates inflammation creating a vicious cycle and continued inflammatory response.[11,24,26] Interestingly, a study by Ehsani found sequence similarities between the SARS-CoV-2 spike protein and hepcidin, suggesting SARS-CoV-2 could possibly have a hepcidin-mimetic effect, further exacerbating the effects of hepcidin.[27] This brings into question whether hepcidin upregulation is due to host defense or a pathological process due to COVID-19.

Coagulopathy

It is well established that inflammation plays a crucial role in infections and often activates clotting and impairs fibrinolysis promoting thrombosis. In long-COVID, it has not been clearly defined whether inflammation is the cause or effect of coagulopathy. Coagulopathy is the dysregulation of the coagulation system resulting in inappropriate coagulation or bleeding. A simplified diagram of the coagulation cascade with select anticoagulant factors and part of the fibrinolytic system is presented in Figure 2. Coagulopathies are the most frequently identified hematologic abnormality in COVID-19 infections, including low platelet counts (thrombocytopenia), low fibrinogen (fibrinogenemia), and an elevated D-dimer.[4,16] This has been coined "COVID-induced coagulopathy" (CIC) or "COVID-19-associated coagulopathy" (CAC).[4,17]

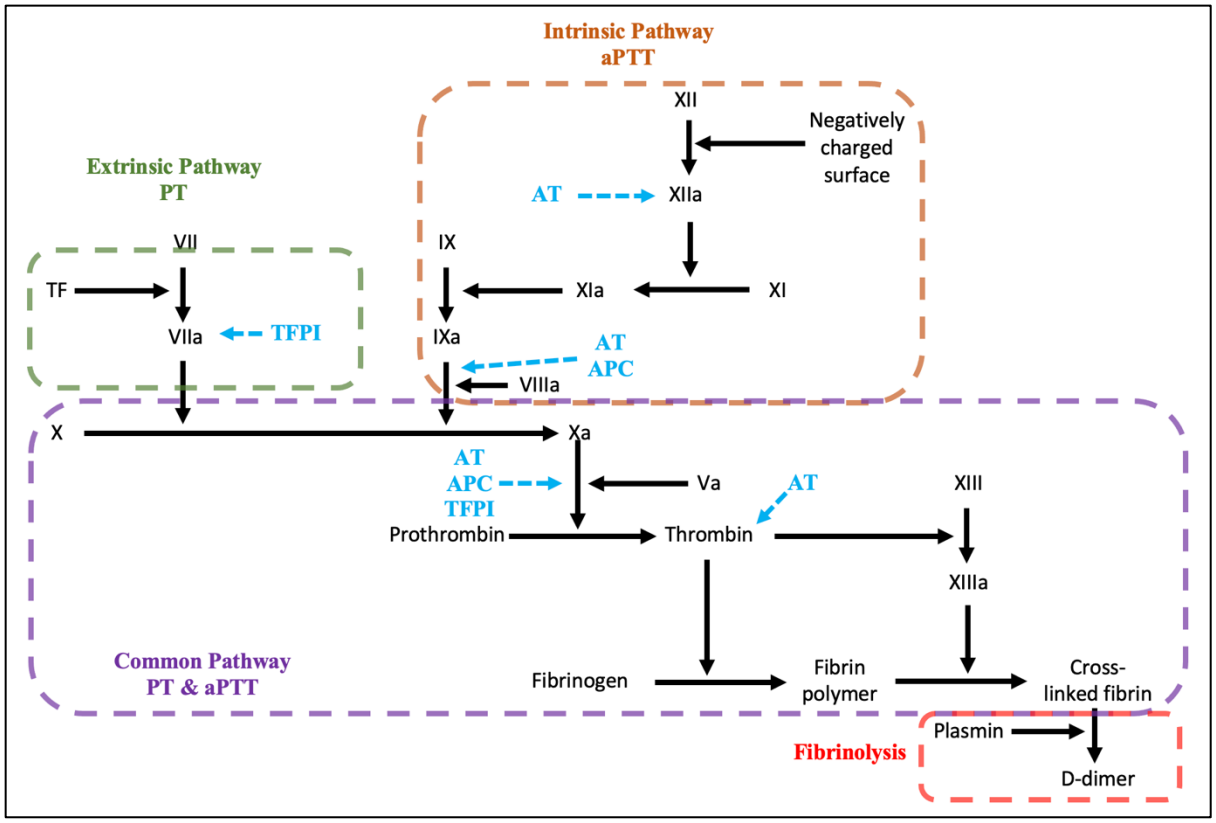

Figure 2: Simplified diagram depicting the coagulation cascade with the intrinsic pathway (measured using activated partial thromboplastin time [aPTT] in orange, the extrinsic pathway (measured using prothrombin time [PT]) in green, and the common pathway (measured using both PT and aPTT) in purple. Select anticoagulant factors and where they act on the cascade are presented in blue. A small segment of the fibrinolytic system is also depicted (red) for convenience.
Abbreviations: Aantithrombin (AT); activated protein C (APC); tissue factor pathway inhibitor (TFPI).
Modified from Keohane EM, Otto CN, Walenga JM. Rodak's Hematology: Clinical Principles and Applications. Sixth edition. ed. St. Louis, Missouri: Elsevier; 2020.

Prothrombin time (PT) and activated partial thromboplastin time (aPTT), which are laboratory tests used to analyze the coagulation system, may be prolonged in acute COVID-19.[4] These features were found to be due to a prothrombotic state, in conjunction with hypo fibrinolysis, caused by COVID-19.[28] A study by Pretorius *et al*. demonstrated that inflammation induced hypercoagulation, hyperactive platelets (figure 3), and ineffective

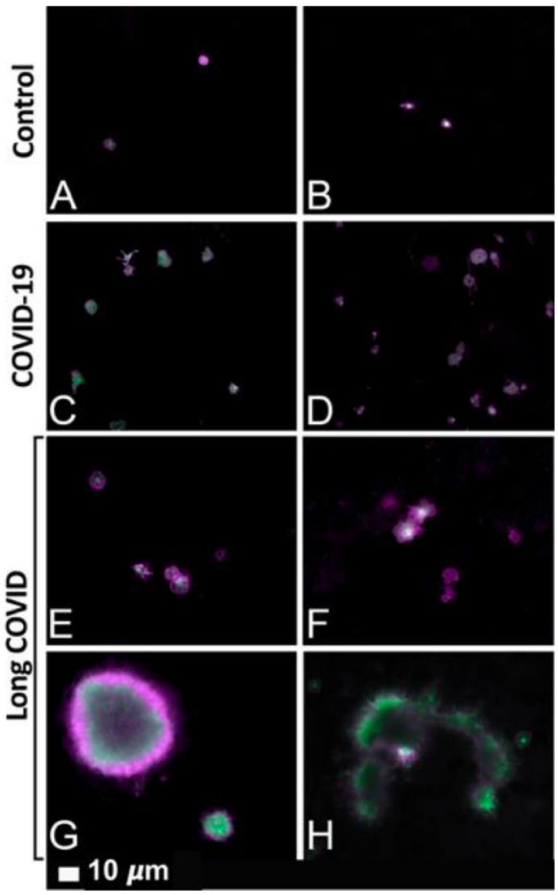

Figure 3: Fluorescence microscopy depicting the hyperactivity of platelets in acute COVID (C & D) and long-COVID (E to H) in comparison to minimally activated control platelets (A & B). From Pretorius E, Vlok M, Venter C, Bezuidenhout JA, Laubscher GJ, Steenkamp J, et al. (2021). Persistent clotting protein pathology in Long COVID/Post-Acute Sequelae of COVID-19 (PASC) is accompanied by increased levels of antiplasmin. Cardiovasc Diabetol, 20(1), 172. PMC8381139 (Open access journal).

fibrinolysis occurs in both acute and long-COVID patients.[28] Hyperactive platelets, which results in inappropriate coagulation, may be the cause of thrombocytopenia in some long-COVID patients; however, it may also be due to platelet aggregation with leukocytes and/or engulfment by leukocytes.[28,29] Furthermore, with persistent symptoms such as shortness of breath noted up to 6 months post-acute infection, the symptoms may be due to fibrinolytic-resistant clotting, which blocks blood flow causing ineffective oxygen exchange.[28] Again, the cytokine storm induced by SARS-CoV-2 may be central, due to disease pathology and the resulting hyperinflammatory state, leading to inappropriate endothelial cell activation and coagulation cascade activation.[4,28] Furthermore, release of TNF-α has been shown to induce the release of ultra large von Willebrand factor (UL-VWF) multimers from vascular endothelial cells.[29] UL-VWF is normally fragmented into small multimers, producing functional VWF, which is required for platelet adhesion. The UL-VWF augments thrombus formation due to the larger size. The cytokine storm also causes dysregulation of the anticoagulant systems, where antithrombin III, tissue factor pathway inhibitor (TFPI) and protein C have been affected (Figures 1 and 2).[4]

Elevated markers of fibrinolysis were also identified, suggesting fibrinolysis was taking place, however, ineffective compared to coagulation.[28] Serum amyloid A (SAA) type 4 was found to be significantly increased in fibrinolytic-resistant clots of long-COVID samples. SAA is an acute phase protein, which increases during inflammation and has been shown to bind fibrin, promoting coagulation and thrombus formation.[30]

Thrombosis can lead to serious complications, including vascular occlusion resulting in tissue hypoxia due to the lack of blood flow and oxygen, which was seen in select patients who suffered from ischemic strokes, limb ischemia and myocardial infarctions.[4,17] This indicates abnormal coagulation parameters are significant in post-acute COVID-19 infections and should be investigated during the recovery phase to prevent serious consequences.

Methods

A systematic search for literature, which assessed the hematologic parameters and

discussed possible connections to post-acute COVID-19 symptoms, was completed following the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta Analyses) guidelines.[31]

Search Strategy

The search strategy used key terms associated with the scope of this study. The key terms were combined in searches to further refine literature. Key terms included "post-acute COVID" OR associated synonyms, which was then combined with "hematology" OR "bio-markers" OR "coagulation" OR "lymphocyte" OR "inflammation" OR other terms and associated synonyms as defined in table 1.

Table 1: Primary key terms with synonyms used in data collection

Key term	Synonyms
Post-acute COVID	Post-acute COVID syndrome, long-COVID, COVID long-hauler(s), post-acute COVID sequelae, chronic COVID. ± hyphens. Interchange COVID with "Covid" OR "SARS-CoV-2" OR "coronavirus" ± "19" OR "nCoV2"
Hematology	Hematologic, clinical hematology. Interchange European (haematology) and American (hematology) spelling.
Biomarkers	Laboratory biomarker(s), parameter(s), laboratory parameter(s), clinical laboratory parameter(s), markers. Interchange laboratory with "lab".
Coagulation	D-dimer, fibrin degradation products (FDP), plasmin, plasminogen.
Lymphocyte	Leukocyte, leucocyte, lymphopenia, leukopenia.
Ferritin	Hyperferritinemia, hyperferritin, iron, iron dysregulation.
Hemolysis	Hemolytic anemia Interchange European (haemolytic) and American (hemolytic) spelling.
Anemia	Anemic. Interchange European (anaemia) and American (anemia) spelling.

Five electronic databases were used to search the relevant terms and synonyms. The databases used included:

- PubMed
- Google Scholar*
- Science Direct**
- Griffith University Library***
- Cochrane

*Time range was adjusted to articles from 2020 to present to reduce non-specific results.

**Advanced search was used for Science Direct. Key terms were searched in the "Title, abstract or author-specified keywords" to reduce non-specific results, due to high numbers of out-of-scope results.

***Search was refined to "Journal Articles."

Inclusion Criteria

The PRISMA guidelines were used to formulate and refine the study methods (Figure 4).[32] Initially, duplicates were removed using EndNote 20. Studies were then screened, and selected studies were sought for retrieval and assessed for eligibility. Eligibility was determined as follows:

1. Number of patients (at least 10 participants),
2. Tests completed (hematologic parameters, e.g., D-dimer, hemoglobin, leukocyte counts, etc.), and
3. Timing of testing (negative COVID-19 PCR or minimum 1 month after onset of symptoms).

Literature was limited to English and original research articles. Though other types of articles were excluded, their reference lists were analyzed for relevant articles.

Exclusion Criteria

Reports without hematologic parameters were excluded. Single case studies and articles without full text were excluded. Studies reporting on acute-phase parameters were excluded (parameters during an active infection). No restrictions were placed on sex, age, or ethnicity.

Results

Literature Search Results

Figure 4 depicts the results of the PRISMA guided database searches, which identified a total of 3221 publications using the search terms presented in table 1. After removal of

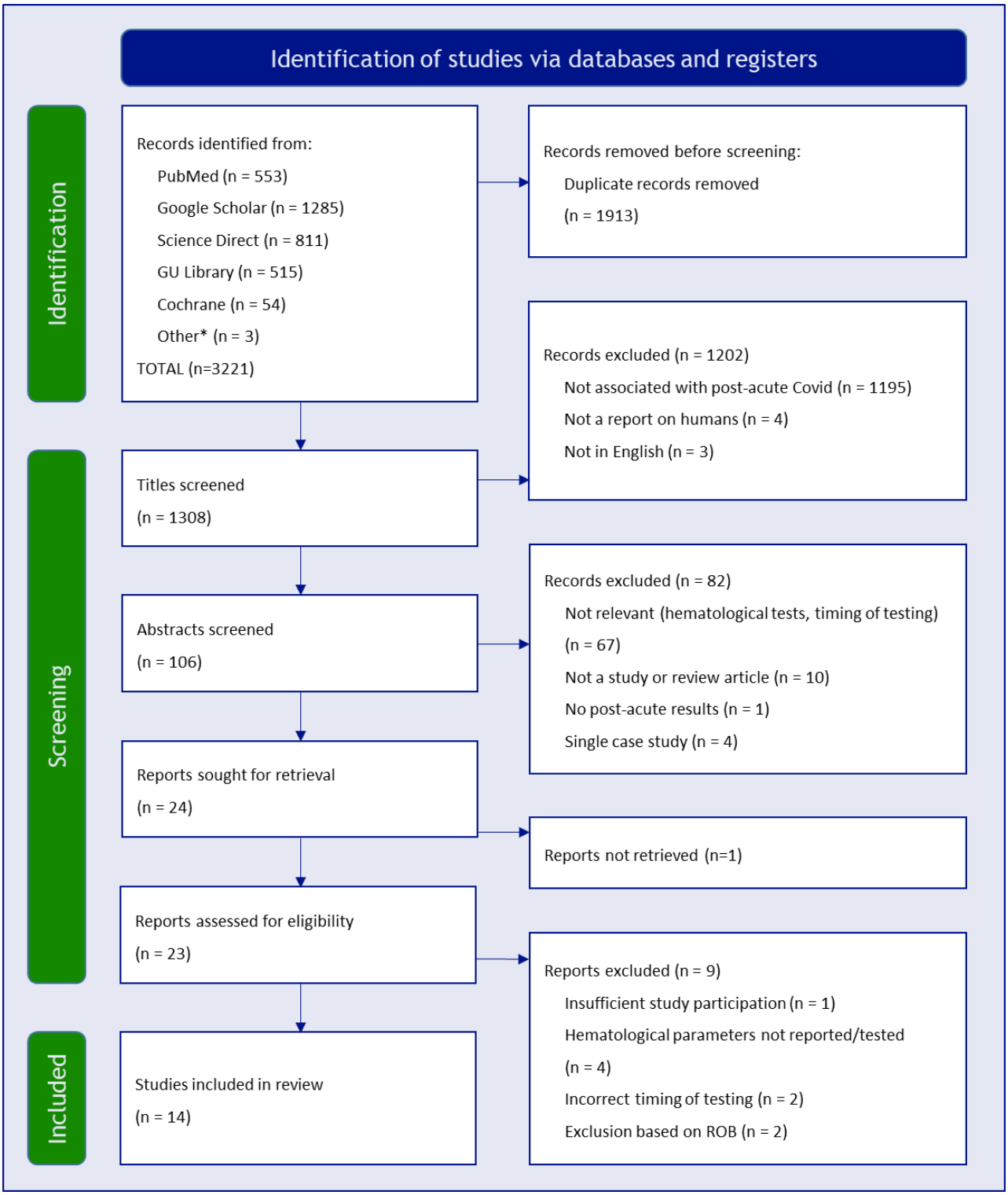

Figure 4: PRISMA flow chart using key terms as outlined in Table 1 (12/11/2021).31 Adapted from Page MJ, McKenzie JE, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, et al. (2021). The PRISMA 2020 statement: An updated guideline for reporting systematic reviews. Int J Surg, 88, 105906.
Key:* Studies sourced and retrieved from other articles.
Abbreviations: Griffith University (GU); risk of bias (ROB).

duplicates, there was 1308 potentially eligible articles. Initial screening of titles and abstracts resulted in 24 possible articles, which were sought for retrieval and assessed for eligibility. After eligibility and risk of bias (ROB) assessment, a total of 14 articles were included in this review.

Quality Assessment and Risk of Bias

Studies meeting the inclusion criteria were assessed using the Specialist Unit for Review Evidence (SURE) Critical Appraisal Tool (CAT) to limit bias.[33] The SURE CAT method was selected as it presents a straightforward appraisal of the selected studies. Bias was assessed by awarding a score of "1" to any checklist criteria met and a "0" for unmet criteria. A percentage for each study evaluated was allocated based on met criteria and only studies with a score equal to or above 70% were included (Tables 2a and 2b).

Sixteen studies were evaluated against the 10 checklist criteria, each was scored as 1 (criteria met) or 0 (criteria not met). Only studies meeting ≥70% of criteria were included. Fourteen studies passed appraisal and were included in the systematic review. Two articles had a score of <70%. These studies were excluded as they did not have a score of ≥70% and were therefore deemed unreliable. Although all the studies included in the review reported statistical significance, only 6 out of the 14 studies included reference ranges (RR) in the reported results (Table 3). Without the respective RR, it was difficult to determine the clinical significance of the reported results. Due to the variable scopes of the included studies, Table 3 provides a summary of the articles and indicates if the respective articles included RR in the reported results for comparison in this analysis.

Table 2a: Table of Critical Appraisal with Checklist Criteria Adapted from SURE CAT (Specialist Unit for Review Evidence (SURE), 2018).[33]

Critical Appraisal Checklist Criteria	Articles							
	2	9	10	11	12	13	15	24
Is the study design clearly stated?	1	1	1	1	1	1	1	1
Does the study address a clearly focused question?	1	0	1	1	1	1	0	1
Are participant characteristics provided?	1	1	1	1	1	1	1	1
Number of participants (≥10)	1	1	1	1	1	1	1	1
Test timing (negative COVID PCR or min. 1 month after symptom onset)	1	0	1	1	1	1	1	1
Are the statistical methods well described?	1	1	1	1	1	1	0	1
Were coagulation or D-dimer tests performed?	1	1	1	1	1	1	1	0
Were ferritin or inflammation markers (e.g., CRP) analysed?	1	0	1	1	1	1	1	1
Were leukocyte or lymphocyte analysis performed?	0	1	1	1	1	1	1	1
Platelet-related disorders or confounding addressed?	1	0	0	0	1	1	0	0
Results out of 10	9	6	9	9	10	10	7	8
Percentage Score (%)	90	60	90	90	100	100	70	80
Included or Excluded	In.	Ex.	In.	In.	In.	In.	In.	In.

Abbreviations: Polymerase chain reaction (PCR); C-reactive protein (CRP); included (In); excluded (Ex).

Table 2b: Table of Critical Appraisal with Checklist Criteria Adapted from SURE CAT (Specialist Unit for Review Evidence (SURE), 2018).[33]

Critical Appraisal Checklist Criteria	Articles							
	28	37	38	39	40	41	42	67
Is the study design clearly stated?	1	1	1	1	1	1	1	1
Does the study address a clearly focused question?	1	1	1	0	1	1	1	1
Are participant characteristics provided?	0	1	1	1	1	1	1	0
Number of participants (≥10)	1	1	1	1	1	1	1	1
Test timing (negative COVID PCR or min. 1 month after symptom onset)	1	1	1	1	1	1	1	1
Are the statistical methods well described?	1	1	1	0	1	1	1	0
Were coagulation or D-dimer tests performed?	1	1	1	1	1	1	1	0
Were ferritin or inflammation markers (e.g., CRP) analysed?	1	1	1	1	1	1	1	0
Were leukocyte or lymphocyte analysis performed?	0	1	1	1	1	1	1	1
Platelet-related disorders or confounding addressed?	1	0	1	1	1	1	1	0
Results out of 10	8	9	10	8	10	10	10	5
Percentage Score (%)	80	90	100	80	100	100	100	50
Included or Excluded	In.	In.	In.	In.	In.	In.	In.	Ex.

Abbreviations: Polymerase chain reaction (PCR); C-reactive protein (CRP); included (In); excluded (Ex).

Table 3: Scope of included studies in analysis and indication if reference ranges (RR) were included in respective studies.

Article	Scope of study	RR
2	Grouped COVID recovered patients based on DD levels (normal vs high).	Y
10	Compared post-severe COVID-19 in patients at discharge, 1 and 3 months.	Y
11	Post-COVID-19 assessment of patients with PS after hospital discharge.	Y
12	Compared symptomatic and asymptomatic long-COVID-19 patients.	N
13	Compared patients whose musculoskeletal symptoms were aggravated post-COVID-19 infections vs. no change post-COVID-19.	N
15	Assessed post-COVID-19 patients with PS post-hospital discharge.	N
24	Post-hospital COVID patients; compared mild, moderate and severe patients.	N
28	Investigated coagulopathies in long-COVID using proteomics.	N
37	Investigated persistent endothelial activation in long-COVID-19. Compared convalescent patients with controls.	Y
38	COVID-19 recovered patients with persistent cardiac symptoms; compared positive CMR and negative CMR imaging patients.	N
39	Assessed post-hospital discharge COVID-19 patients.	N*
40	Compared post-COVID patients with normal vs. abnormal CT scans.	Y
41	Post-COVID-19 recovery assessment in patients with PS vs non-PS.	Y
42	Post-COVID-19 recovery assessment in patients with PS vs non-PS	N*

* No RR provided, however, indicated if results were within, above or below RR.
Abbreviations: No (N); yes (Y); cardiac magnetic resonance (CMR); persistent symptoms (PS); D-dimer (DD); computed tomography (CT).

Data

The data extraction information was adapted from the Centre for Reviews and Dissemination guidelines (2009).[34] Tables 4a, 4b, and 4c present the extracted data of the included studies. The information extracted includes:

- General information: date & identification features of study
- Study characteristics: study design
- Participants: number, age, gender, confounding factors
- Analysis: parameters tested/reported

Variable units were used throughout all studies, for the purposes of accuracy, the units reported by the studies were kept as is.

Table 4a: Extracted Data included in Review

Articles	N	Age (years, mean)	Gender	Study	N in laboratory tests
2	150	47.3 ±15.4	F = 85, M = 65	Retrospective	150
10	199	60.5 ±13.9	F = 73, M = 126	Prospective	Variable◆
11	75	72 ±7	F = 33, M = 42	Retrospective	75
12	315	47.9 ±14.8	F = 158, M = 157	Retrospective	351
13	280	47.45 ±13.92	F = 183, M = 97	Retrospective	182
15	384	59.9 ±16.1	F = 38, M = 62	Retrospective	Variable◆
24	109	58 ±14	F = 44, M = 65	Retrospective	109
28	11	55.7 ±5.8	F = 8, M = 3	Observational	11
37	50	50 ±17	F = 20, M = 30	Retrospective	50
38	109	58 ±14	F = 44, M = 65	Retrospective	109
39	767	63 ±13.6✧	F = 252, M = 515	Retrospective	Variable◆
40	94	48.11 ±11.9	F = 40, M = 54	Retrospective	94
41	1021	Variable◆	F = 256, M = 764	Retrospective	Undefined◆
42	116	41✧	F = 17, M = 99	Retrospective	Undefined

◆ See respective article for further details
✧ Median
Abbreviations: Female (F); male (M).

Table 4b: Extracted Data included in Review (Continued)

Articles	Test timing	Confounding factors
2	6 weeks post symptoms or HD	HTN (18%), DM (9.3%) ◆
10	1- and 3-months post HD	44.4% HTN✣, 25.6% CVD✣, & more◆.
11	60 days post HD	N/A
12	1 month post-acute	20.6% smokers, 42.2% had comorbidities◆
13	Neg. COVID-19 PCR.	Anticoagulant therapies indicated
15	Median 54 days	41.9% HTN, 9.7% IHD & more◆
24	Mean 60 (±12) days post symptom onset	N/A
28	2 months post symptom onset	N/A
37	Median 68 days post-symptom	62% had comorbidities◆
38	Mean 60 (±12) days post symptom onset	8% HTN. CVD patients excluded.
39	Median 81 days post HD	HTN (21.7%), CAD (9.5%) ◆
40	1 year post HD	HTN (17.02%), DM (9.57%) ◆
41	Neg. COVID-19 PCR.	5.7% HTN
42	Median 66 days post symptom onset	Patients with comorbidities excluded

◆ See respective article for further details

✣ Not all participants included

Abbreviations: Hospital discharge (HD); hypertension (HTN); diabetes mellitus (DM); cardiovascular disease (CVD); polymerase chain reaction (PCR); ischemic heart disease (IHD); coronary artery disease (CAD).

Table 4c: Extracted Data included in Review (Continued)

Articles	D-dimer	Ferritin	CRP	Lymphocyte
2	327 ng/mL	N/A	1.23 mg/mL	N/A
10	1 mo. 446 µg/L 3 mo. 322 µg/L	1 mo. 179 µg/L 3 mo. 95 µg/L	1 mo. 5.6 mg/L 3 mo. 2.7 mg/L	1 mo. 29.6% 3 mo. 31.4%
11	900.71 ng/mL	496.24 ng/mL	9.12 mg/L	N/A★
12	0.44*	65.3*	62.55*	26.7*
13	S: 0.38* AS: 0.26*	S: 117* AS: 75*	S: 5.4* AS: 4.45*	S: 1.82* AS: 2.16*
15	384 ng/mL	169 µg/L	1 mg/L	1.94 x 109/L
24	N/A	Mild = 139 µg/L Mod = 260 µg/L Severe = 317 µg/L	Mild = 0.2 mg/dL Mod = 0.2 mg/dL Severe = 0.4 mg/dL	Mild = $5.7x10^9$/L Mod = $6.1x10^9$/L Severe = $6.4x10^9$/L
28	★	N/A	✦	N/A
37	377 ng/mL	N/A	1.1 mg/mL	★
38	0.28 µg/mL	N/A	1.4 mg/L	1.6x109/L
39	700 ng/mL ±1021	N/A	0.36 mg/dL ±0.85	⍰
40	NCT = 290 µg/L ACT = 290 µg/L	N/A	NCT = 5 mg/L ACT = 15 mg/L	NCT = $1.69x10^9$/L ACT = $1.18x10^9$/L
41	Measured, but not defined◆	Measured, but not defined◆	Measured, but not defined◆	Measured, but not defined◆
42	⋆	PS: 191.48 µg/L NPS: 177.03 µg/L	PS: 0.41 mg/dL NPS: 0.41 mg/dL	PS: 29.62% NPS: 31.77%

★ Other associated parameters (e.g., fibrinolytic markers, inflammation markers, or leukocyte counts).

* No units provided.

✦ Reports fold change between healthy controls and long-COVID.

⍰ Reports number of patients within specified ranges.

◆ See respective article for further details.

Abbreviations: C-reactive protein (CRP); month (mo); symptomatic (S); asymptomatic (AS); moderate (Mod); normal computed tomography (NCT); abnormal computed tomography (ACT); persistent symptoms (PS); non-persistent symptoms (NPS).

Discussion

Two years after the emergence of the novel SARS-CoV-2 virus, some patients continue to experience symptoms in the absence of an active infection. After a systematic literature search and ROB assessment, 14 studies that investigated hematological parameters in patients with persistent symptoms (PS) were included in the analysis. Though the included articles addressed statistical significance, clinical significance was often omitted or overlooked. The statistical significance of the included articles may indicate the reliability of the results; however, clinical significance indicates the impact the results have in clinical practice.[35] The statistical significance of results addresses the second aim of this review, while the clinical significance addresses the third aim; therefore, both

statistical and clinical significance are taken into consideration.

Lymphopenia

Lymphopenia is frequently reported in acute COVID-19 infections and has been hypothesized to contribute to symptoms seen in the post-acute phase.[16,36] Twelve of the 14 included studies reported lymphocyte or leukocyte counts (Table 4c). Out of these 12 studies, 5 reported no statistically significant difference in lymphocyte/leukocyte counts between their respective comparison groups.[11,12,24,37,38] RR was included in the studies by Fogarty, *et al.* and Pasini, *et al.*, and showed all participants to be within the reported RR.[11,37] The remaining 7 studies found either statistically or clinically significant differences. Bakilan, *et al.* and Venturelli, *et al.* found statistically significant differences in lymphocyte/ leukocyte count between their comparison groups, however, did not include RR with their results; therefore, it could not be established if the results were clinically significant.[13,39] Darcis, *et al.* and Zhao, *et al.* found statistically significant differences between the comparison groups and included RR in the results.[10,40] When comparing the results to the included RR, the lymphocyte/leukocyte counts were still within the accepted RR, indicating although there is a statistical difference between the groups, it was not clinically significant. The study by Mandal, *et al.* reported 7.3% of the 247 participants showed persistent lymphopenia and Mannan, *et al.* found lymphopenia in 3% of patients experiencing PS, however, ~50% of asymptomatic patients also had lymphopenia.[15,41] In the study by Varghese, *et al.*, 12% of participants were found to have lymphopenia, where 31% of these participants had PS and 9% had none.[42] Furthermore, the results reported 91.07% of the cohort was within RR, however, it was undefined what percentage had PS and what percentage did not.[42] These results indicate some patients will experience lymphopenia with post-acute COVID-19; however, it is not a common abnormality nor a reliable indicator of PS. Further follow-up results were not available; it is undetermined if the lymphocyte population returned to within RR for patients with lymphopenia.

Although Varghese, *et al.* indicated not all patients with lymphopenia had PS post-COVID, a significant difference was noted in immunoglobulin A (IgA) concentration between patients with and without PS.[42] The study indicated IgA concentrations at certain time points in disease progression may be central to PS. High concentrations of IgA during the acute phase can indicate or predict severe disease, while high concentrations post-acute indicates less PS. IgA antibodies are produced by B lymphocytes, or plasma cells, in the lamina propria, and transported to the mucosal surface via receptors to aid in the defense against invading pathogens.[19] Although lymphopenia was not found to be a common factor in patients with PS, the association of PS and reduced IgA may indicate either a pathogenic mechanism of SARS-CoV-2 affecting B lymphocytes or an ineffective immune response. Only one study evaluated the immunoglobulins post-COVID-19 infections; future analyses can elucidate if this is in fact a common factor among other cohorts.

A study by Gao, *et al.* analyzed the frequencies of T lymphocyte subsets in both acute and convalescent patients.[43] The results showed decreased lymphocytes, total T cells, CD4+ T cells and CD8+ T cells during the acute phase of infection and a further reduction noted post-acute infection. B lymphocytes were not assessed. Similarly, a review by Ramakrishnan, *et al.* indicated the ability of SARS-CoV-2 to impair T lymphocyte functionality, leading to immune exhaustion, thus facilitating long-COVID symptoms.[1] This is supported in the study by Peluso, *et al.*, which found patients with PS had decreased CD8+ T lymphocyte responses over time.[44] In contrast to these findings, an additional study found patients with PS had increased and sustained T lymphocyte activity in the late convalescent phase and patients without PS had a gradual decrease in T lymphocyte activity over time.[45]

This suggests immune overactivity may be a cause of PS. No difference was found in B lymphocyte activity between patients with or without PS in the study by Files, *et al*.[45] Interestingly, the study by Phetsouphann, *et al*. analyzed 24 cell clusters 3 months post-acute COVID-19 infection, which showed 5 lymphocyte subsets were absent in long-COVID patients.[18] A further 3 remained absent when analyzed at the 8-month interval, which included CD8+ and CD4+ T lymphocytes and B lymphocyte subsets. Furthermore, CD8+ T lymphocyte activation and exhaustion markers were also found to be higher in long-COVID patients. It was also identified that sustained monocyte and plasmacytoid dendritic cell activity occurred in the long-COVID cohort compared to the matched controls.[18] These results indicate there is a decrease in certain lymphocytes in long-COVID patients with a chronic and sustained activation of a CD8+ T cell subset, monocytes and plasmacytoid dendritic cells, which may contribute to long-COVID symptoms. Other theories described in literature surrounding the cause of lymphopenia include viral bone marrow suppression or immunosuppression, resulting in not only lymphopenia, but also at times neutropenia and thrombocytopenia.[17,46] There are multiple studies reporting neutropenia, however, it was not addressed in the articles included for this review.[47-49]

Blood Morphological Changes

Studies with morphological analysis of peripheral blood (PB) smears on long-COVID patients are limited. There are, however, studies analyzing morphology during the acute phase. Of particular interest are the dysplastic myelocyte features, such as neutrophils with pseudo-Pelger-Huët anomalies, which are atypical for viral infections and have only been evident in human immunodeficiency virus (HIV) infections.[50-52] Analysis of the blood morphological features in PB of long-COVID patients could be useful to determine the persistence of abnormal cells and potential contributors to PS.

Although studies on the PB of post-acute COVID-19 patients are significantly limited, flow cytometry cell analysis has been completed. The study by Kubankova, *et. al* investigated 14 post-acute COVID-19 patients who were, on average, 7 months post-infection, using real-time deformability cytometry (RT-DC).[53] RT-DC is a fast and high-throughput method of analysis the phenotypical features of cells. The study found marked changes in cell phenotypes during the acute phase, including smaller erythrocytes with decreased deformability, monocytes with increased cell size, and lymphocytes with decreased stiffness. Some of these abnormalities were also noted in the post-acute COVID-19 group indicating the effects of COVID-19 persists in the hematologic system for some time. There was a significant difference in the deformation of erythrocytes between the post-acute COVID-19 cohort and the acute and healthy cohorts; the erythrocytes had not returned to "healthy state" in the post-acute COVID-19 group.[53] A study by Thomas, *et al*. found oxidative stress induced by COVID-19 infections resulted in damage of essential erythrocyte proteins.[54] Mature erythrocytes cannot repair or resynthesise these proteins; the persistence or survival of these damaged cells, possibly due to lack of splenic clearance or inefficient damage to induce hemolysis, may contribute to ineffective oxygen transport, resulting in the symptoms seen in long-COVID sufferers.[54] Furthermore, the study by Kubankova, *et al*. found lymphocyte size and deformation was not significantly different from the heathy control group, however, the analysis of neutrophil parameters indicated significant changes between the post-COVID-19 and healthy groups, including cell cross-sectional area, volume, and deformation.[53]

Interestingly, the study by Kannan and Soni, which analyzed the PB smears of acute-phase COVID-19 patients, found one patient, approximately 100 days post COVID-infection, had presented with neutrophilic nuclear abnormalities, coined by the authors as

acquired neutrophilic nuclear projections (ANNP).[55]

There are no clear diagnostic criteria regarding lymphopenia, PB abnormalities and long-COVID yet, however, there is evidence the absence of certain lymphocyte subsets or sustained activation of immune cells may have a connection to long-COVID symptoms. In addition, lymphocyte abnormalities, dysregulated inflammation was also reported in long-COVID patients, which results in high ferritin and CRP.

Iron Dysregulation and C-Reactive Protein (CRP)

Ferritin and CRP are seen in the acute-phase response during inflammation.[19] Of the 14 studies included, 8 reported results for ferritin analysis (Table 4c). Only one study found no significant difference in ferritin between their comparison groups.[13] The remaining 7 studies reported a significant difference in ferritin results between the respective comparison groups. Four studies reported a statistically significant increase in ferritin; however, RR was not stated and, therefore, it could not be determined if there was any clinical significance.[12,15,24,42] The remaining three studies showed a clinically significant increase in ferritin. Darcis, *et al*. reported ferritin concentrations above the RR at hospital discharge, which normalized at the 1 and 3-month assessment. ~37% of symptomatic and ~40% of asymptomatic participants in the study by Manna, *et al*. had ferritin concentrations above RR, indicating possible persistent inflammation in the absence of an active COVID-19 infection.[10,41] Similarly, ferritin concentrations were above RR in both male and female participants in the study by Pasini, *et al*.[11] As stated earlier, the SARS-CoV-2 spike protein was found to have sequence similarities to hepcidin, which may play a role in the hyperferritinemia seen in post-acute COVID-19.

Furthermore, Sonnweber, *et al*. identified iron deficiency anemia in 30% of their participants two months post-acute COVID-19. Of these participants, 90% had severe acute COVID-19 infections.[24] Eighty percent of participants in another study had clinically significant low hemoglobin concentrations.[11] This suggests it may be beneficial to include iron studies as part of a panel for laboratory investigations into long-COVID.

All studies reported CRP results. Six studies reported no significant increase in CRP, or the CRP results were within the RR.[2,13,24,37,38,42] Three of the studies reported a statistically significant increase in CRP in patients with PS, however, it was unclear if the increase was clinically significant (no RR for comparison).[12,15,28] The remaining 5 studies reported CRP above the RR. Darcis, *et al*. found CRP decreased at 1 month and within RR at the 3 month follow up, indicating a gradual return to normal concentration.[10]

The results indicate hyperferritinemia and elevated CRP may be a consequence of COVID-19 infections, however, these markers are non-specific and, hence, may not be specific to long-COVID and PS. Nevertheless, the presence of hyperferritinemia and elevated CRP in recovered patients, with or without PS, indicates there is iron dysregulation and/or persistent post-acute inflammation. Evidence of persistent and sustained inflammation was evident in a study by Phetsouphanh, *et al*., which found persistently elevated IFN-β and IFN-λ1 in the long-COVID cohort compared to matched controls.[18] Inflammation is known to affect the coagulation system, resulting in coagulopathies. This has been noted in both acute and post-acute COVID-19 infections.

Coagulopathies

Coagulopathies are a well-known consequence of COVID-19. There is evidence SARS-CoV-2 invades vascular endothelial cells, resulting in endothelial dysfunction, which triggers a procoagulant environment and, along with the hyperinflammatory response, results in endothelitis.[17,28] This systematic analysis revealed that three of the 14 studies found D-dimer results to be within RR or found no

statistical significance (Table 4c).[37,38,40] Eight studies found either statistically or clinically significant increases in D-dimer in affected participants. Two studies had further follow-up results and reported a decrease in the D-dimer over time, indicating the resolution of COVID-19 induced coagulopathy. The study by Mannan, *et al*. Mannan, *et al*. [41], which compared symptomatic and asymptomatic participants, found elevated D-dimer in both cohorts at similar frequencies; approximately 40%. Similarly, found high D-dimer in ~38% of their participants.[39] In addition, Pretorius, *et al*. reported significant failure in the fibrinolytic processes in convalescent patients, which was evidenced by the presence of clots that were resistant to fibrinolysis.[28] These results indicate there is a combination of hypercoagulation and hypo fibrinolysis occurring in some post-COVID patients.

Although an increased D-dimer is not exclusive to patients with PS, it is still a significant marker. This is highlighted in a retrospective case study concerning an 82-year-old Japanese male whose autopsy findings indicated the patient died due to portal and mesenteric vein thrombosis.[56] This thrombosis caused portal hypertension, which consequently resulted in extensive gastrointestinal necrosis. The patients' D-dimer was reported to be consistently elevated, which emphasizes the importance of investigating persistent coagulopathies in post-acute COVID-19 cases, particularly persistently elevated D-dimer in post-acute COVID-19, which may be valuable in determining patient care and treatment to prevent fatal thrombotic events.

Other markers of the coagulation system may also provide insights into the hypercoagulable state of some patients. Interestingly, although the D-dimer has been found to be within the RR of some patients, a significant increase in factor VIII has been found in convalescent patients.[37] No RR was reported however; thus, clinical significance is undetermined.[37] Similarly, another study also found significantly increased factor VIII in convalescence patients.[17] A comparison of fibrinogen between participants with and without PS indicated higher fibrinogen (hyperfibrinogenemia) in individuals with PS (311.65±78.52 mg/dL) compared to those without (294.34 ±48.33 mg/dL).[42] Though these results are not statistically significant, it may indicate there is more deranged coagulation occurring in individuals experiencing PS. Interestingly, the finding of hyperfibrinogenemia was noted to be contrary to other literature, which reported fibrinogenemia, indicating there may be variations in coagulopathy patterns among long-COVID patients.[4,16] Furthermore, an article by Fan, *et al*. reported significant thrombotic events in 4 young patients (median 38.5 years of age). Laboratory analysis of these patients showed increased factor VIII, VWF, D-dimer and hyperfibrinogenemia.[57] Although these results are not exclusive to patients with PS, analysis of patients' coagulation profile, including D-dimer, fibrinogen, factor VIII, and VWF, may be beneficial in determining post-acute COVID-19 care and to prevent significant thrombotic events. Although lymphopenia, iron dysregulation/inflammation and coagulopathies are the predominant reported abnormalities in long-COVID, some studies have also found other abnormalities secondary to COVID-19 infections.

Abnormalities Secondary to COVID-19 Infections

Clinically significant abnormalities secondary to COVID-19 infections in post-acute patients has briefly been noted in the literature. Abnormalities include alterations in glucose metabolism, development of hemophagocytic lymphohistiocytosis (HLH) and autoimmune diseases.

Abnormal Glucose Metabolism

An increase in hemoglobin A1c (HbA1c) has been noted in post-acute COVID-19 patients who had no prior diabetes mellitus (DM) diagnoses.[36,58] HbA1c is a glycated from of hemoglobin, which becomes elevated when plasma glucose levels are increased for long periods of time, as seen in DM.[21] Multiple

studies have found patients with long-COVID have indications of altered glucose metabolism as evidenced by increases in HbA1c.[58,59] HbA1c has been shown to increase blood viscosity, endothelial inflammation and vascular dysfunction, thus, elevated HbA1c may be the cause of or contribute to the coagulopathies and sustained inflammation seen in post-acute COVID-19.[60]

Hemophagocytic Lymphohistiocytosis (HLH)

Although rare, another consequence of COVID-19 infection is secondary hemophagocytic lymphohistiocytosis (HLH), a life-threatening and rapidly progressive inflammatory syndrome leading to multiorgan failure.[61,62] Characteristics commonly seen in HLH includes excessive cytokines, cytopenia, and hyperferritinemia.[62] These features have been seen in long-COVID, as discussed previously. Due to the high mortality rate seen with HLH, this is certainly a significant consequence associated with COVID-19 that should be given due consideration when assessing patients.[63]

Autoimmune Diseases

There have been multiple reports of immune-related diseases developing after resolution of COVID-19 infections. One study reported seven cases of warm and cold autoimmune hemolytic anemia (AIHA), which developed after confirmed COVID-19 infection and without differential diagnosis.[64] Furthermore, a case report presented a patient with immune thrombocytopenia (ITP) secondary to COVID-19.[65] Viral-induced ITP is caused when antibodies produced by B lymphocytes in response to the viral infection are cross-reactive with thrombocytes, resulting in the antibodies binding to and causing the destruction of thrombocytes, leading to thrombocytopenia.[66]

Limitations of the Review

There are limited studies investigating the long-term effects of SARS-CoV-2 infection. Many of the studies did not include the results of all participants or had variable numbers of participants at different time points, thus, the study may not present an accurate representation of the study groups. Furthermore, the participants were not grouped based on age, ethnicity, sex, or comorbidities, therefore, it could not be determined if one group or characteristic was more prone to PS compared to others. None of the studies defined COVID-19 variants, therefore, it is unestablished whether one strain is more likely to cause long-COVID compared to others.

Future Directions

New emerging studies have found absent lymphocyte subsets or activity in long-COVID patients with sustained activation of other immune cells; studies correlating these results may allow for predictions of long-COVID and potential directed therapeutics. Further research into the cause and prevalence of elevated HbA1c and HLH in post-acute COVID patients may assist in determining the significance and requirements for further observation in potentially affected patients. Many of the studies included in this review were not solely focused on hematologic parameters, therefore, future analyses which focus on the hematologic system would be beneficial, which include the PB smears of long-COVID patients. Furthermore, there are many COVID-19 variants; none of the studies addressed which strains were detected or which were predominant in the respective cohorts. Future studies may reveal one COVID-19 strain implicated in long-COVID more often than another. Finally, although comorbidities were addressed in the included studies, patients were not grouped according to comorbidities; elucidating which comorbidities are more often associated with long-COVID and whether there is a causal relationship may help with prognosis, recovery, and rehabilitation.

Conclusion

Although lymphopenia was not found to be exclusive to long-COVID patients, new studies are emerging with evidence of certain features exclusive to long-COVID. These studies have

shown there is an absence of T and B cell subsets, along with sustained activation of monocytes and plasmacytoid dendritic cells, in long-COVID, however these are not found in non-long-COVID cohorts. Collated evidence also suggests there is sustained inflammation occurring in long-COVID, which may drive the persistent signs and symptoms, including coagulopathies for which these is strong evidence, due to the elevated D-dimer seen in the majority of COVID-19 recovered patients. There is still limited research addressing long-COVID and the effects seen in the hematological system, however, the evidence presented to date indicates the promise of elucidating the potential hematological causes and mediators of long-COVID.

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Research article

Laboratory Automation and Sensitive Analytes - National Study from Clinical Biochemistry Departments in Denmark

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Increased laboratory automation (LA) is becoming a necessity for high throughput centralized laboratories, however, LA provides new pre-analytical challenges. Prolonged air exposure may cause spurious analytical results for sensitive analytes when the de-capped open blood tubes are transported on assembly lines for prolonged periods and at different temperatures. This study maps LA systems in Denmark and investigates if sensitive analytes and LA is an issue of concern in Danish laboratories.

To nationally map LA and LA procedures for two sensitive analytes, blood alcohol and total carbon dioxide, a questionnaire was sent to all clinical biochemistry departments in Denmark (n=36 with inhouse analysis). Three departments were selected for further short interviews in 2020. In total, 86% (31/36) responded. Of respondents, 84% (26/31) had implemented LA: 65% with total laboratory automation and 35% with partial. When LA operated smoothly in the 26 laboratories, the median transport time was 5 minutes (range 2-90) from de-capping of blood tubes to blood analysis. Local laboratory guidelines on open tube stability of the analytes varied considerably: Blood alcohol 60 (0-300) minutes, and total carbon dioxide 60 (0-360) minutes. Consequently, some laboratories still handled sensitive analytes manually off the LA assembly line. This study demonstrated a diversity in how laboratories manage sensitive analytes and LA. This may jeopardize analytical results and patient safety, and evidence-based stability studies, international guidelines and LA technical adaptions are warranted for sensitive analytes to adopt to the contemporary LA setting.

Key words: Preanalytical; laboratory automation; blood alcohol; carbon dioxide; un-stoppered; de-capped; sensitive analytes.

Introduction

Implementation of laboratory automation (LA) has become a prerequisite in the contemporary clinical biochemistry laboratory to increase analytical capacity and efficiency.[1-6] The LA systems are often built with a separate tube decapper function; blood samples are transported in open tubes on the assembly line before reaching the analytical instruments.[5-7] However, prolonged air exposure to blood

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samples may cause spurious analytical measurements for sensitive analytes.[8,9] Transporting the open tubes at increased temperatures, during technical downtime or within larger LA systems may jeopardize patient safety. Some blood analytes may be more vulnerable than others, for instance, blood alcohol and total carbon dioxide are suspected to be sensitive due to the volatile nature of the substances.[8,9]

Guidelines and thorough studies concerning the stability of sensitive analytes in open tubes have not previously been described. This may result in lack of standardization for the LA pre-analytical handling of sensitive analytes. To investigate if sensitive analytes and LA is an issue of concern in the laboratories, this study mapped local laboratory stability guidelines and how laboratories handled blood alcohol and total carbon dioxide in LA. In addition, the differences in LA systems and the open tube transportation time was also reviewed. A survey was created and distributed to all clinical biochemistry laboratory departments in Denmark supported by short qualitative interviews.

Materials and methods

In April 2020, a questionnaire was distributed to all clinical biochemistry departments with inhouse analysis in Denmark. The questionnaire focused on whether the department had LA or not; type; time from de-capping to start of analysis; local guidelines regarding the stability of the blood alcohol and total carbon dioxide analytes in open tubes. Three departments representing dissimilar answers in the questionnaire were interviewed in May 2020. Informants signed a written consent before the audio recorded short semi-structured telephone interview. The interview included: 1) LA system and de-capping procedure and 2) blood alcohol and total carbon dioxide stability in open tubes. Interviews were completed and transcribed in Danish. GraphPad Prism 8.4.3 (GraphPad Software, USA) illustrated data. Fisher's exact test was applied to two-group comparisons, $\alpha = 0.05$. Ethical approval was not required according to the Danish ethical committees.[10]

Results

In total, 86% (31/36) of the departments responded to the questionnaire.

Automation in Denmark

Laboratories with automated assembly lines and choice of LA system in Denmark are shown in Figure 1. The questionnaire included the open-ended question "*How long does it take to transport a blood sample on the automated assembly line from de-capping to analysis on a day when everything runs smoothly?*" and Table 1 shows the LA median open tube time and the difference within the same manufacturer of the LA system. Table 1 also shows if tubes for blood alcohol and total carbon dioxide measurements were chosen to be transported on or off the assembly line. There were no differences between sensitive analytes for this choice (Table 1, $p>0.9$). There were no differences in reported stability time between blood alcohol and total carbon dioxide (Table 2, $p>0.9$).

Bispebjerg and Frederiksberg Hospital, Copenhagen (BF Copenhagen) started to operate a total LA system in January 2020, but a specialized biomedical laboratory scientist (BLS) expressed concerns about flow related difficulties and having manual steps for *e.g.* sensitive analytes: "... this was not expected with implementing total LA."

Blood alcohol and automation

When measuring blood alcohol, tubes were not always transported on the automated assembly lines in Danish laboratories, Table 1. BF Copenhagen initially transported the open tubes on the assembly line. However, samples continuously exceeded the 30 minutes stability warned by the alarm system. Even by drawing blood into a separate tube at phlebotomy for alcohol measurement only, the time issue was still not resolved. This resulted in blood alcohol testing in separate tubes and handled manually off the assembly line. The BLS from Zealand

University Hospital Roskilde (ZUH Roskilde) reported samples were centrifuged in the TLA system and sorted to the output station still capped from where they were manually handled for analysis to avoid evaporation.

There was an interlaboratory variation in local guidelines for the stability of blood alcohol in open tubes, Table 2.

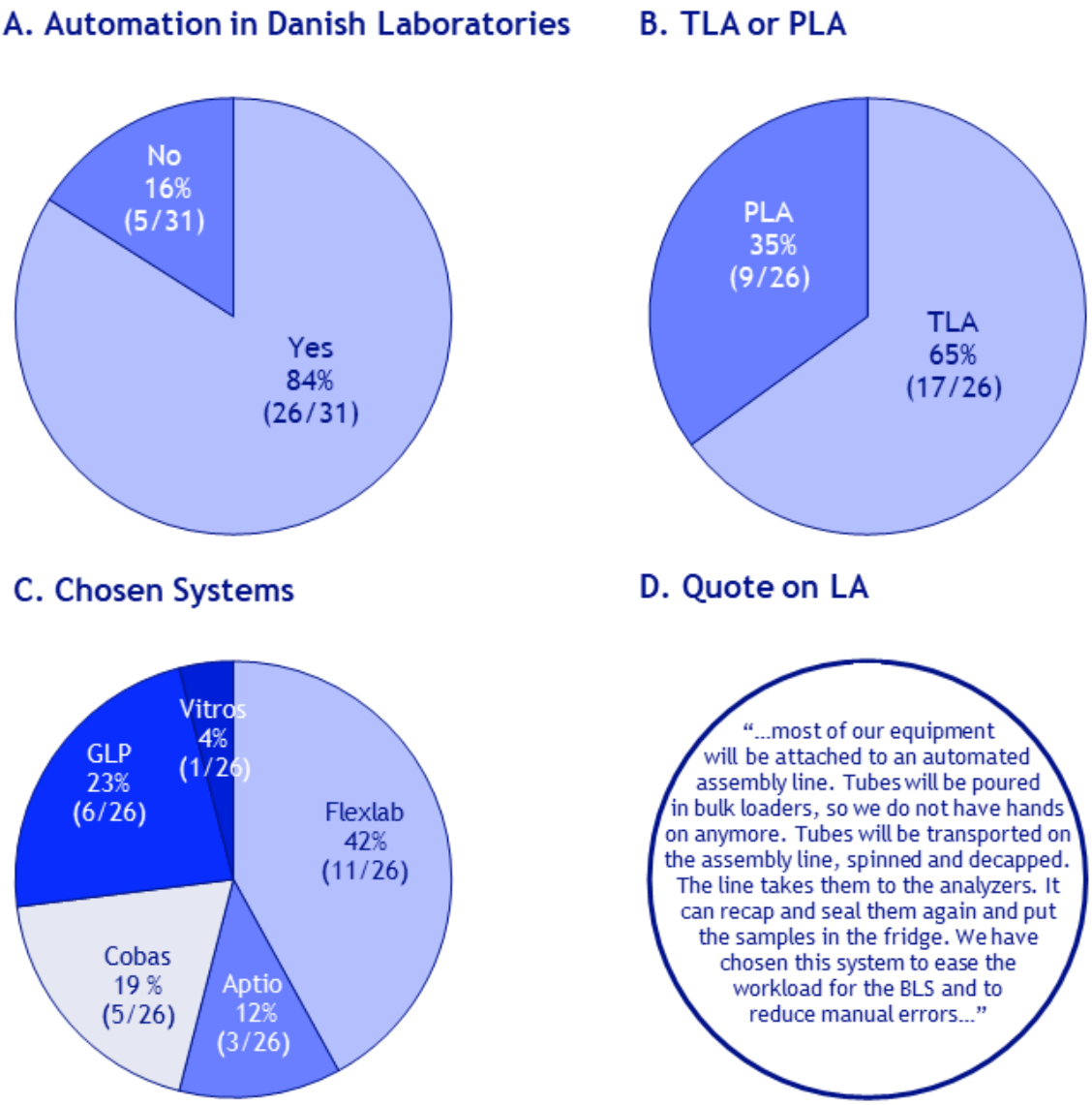

Figure 1: Laboratory Automation (LA) in Denmark in year 2020.

A: Laboratories with and without LA among departments with in-house analysis.

B: Distribution of total laboratory automation (TLA) and partial laboratory automation (PLA) among laboratories with LA.

C: Distribution of laboratories choice of type of LA systems. Note Aptio is based on the Flexlab system from Inpeco, but with a Siemens instrumental collaboration.

D: Quote from a biomedical laboratory scientist (BLS) from Regional Herning Hospital who elaborated benefits of their awaited new LA system and the decapping function.

Abbreviations: Aptio = Aptio Automation (Siemens Healthineers, Germany & Inpeco SA, Switzerland). Cobas = Cobas Connection Modules (Roche Diagnostics, Switzerland). Flexlab = Flexlab Automation (Inpeco SA, Switzerland). GLP = GLP Systems (Abbott Laboratories, USA - IL). Vitros = VITROS Automation Solutions (Ortho Clinical Diagnostics, USA - NJ).

Table 1: The reported laboratory automation (LA) system at clinical biochemistry departments in the Danish health care system (n = 26). The table shows open tube transportation time on assembly lines (i.e. time from de-capping tubes to analysis). The table also shows whether the laboratories measure blood alcohol/total carbon dioxide or not; and if they use the assembly line or not.

LA system	N	Minutes from de-capping to analysis,median [range]	Transport type	Blood Alcohol			Total Carbon Dioxide		
				On assembly line (%)	Off assembly line (%)	Do not analyze (n)	On assembly line (%)	Off assembly line (%)	Do not analyze (n)
Aptio	4	10 [4-60]	Individual	75 (3/4)	25 (1/4)	0	100 (4/4)	0 (0/4)	0
Cobas	5	60 [5-90]	Racks of 5 samples	60 (3/5)	40 (2/5)	0	60 (3/5)	0 (0/5)	2
Flexlab	10	5 [2-30]	Individual	90 (9/10)	0 (0/10)	1	60 (6/10)	0 (0/10)	4
GLP	6*	3.5 [2-15]	Individual	67 (4/6)	33 (2/6)	0	0 (0/6)	50 (3/6)	3
VITROS	1	20 [-]	Individual	0 (0/1)	100 (1/1)	0	0 (0/1)	0 (0/1)	1
Total	26	5 [2-90]	-	76 (19/25)	24 (6/25)	1	81 (13/16)	19 (3/16)	10

No difference between sensitive analytes in use of assembly line or not, p>0.9.
*) Medan [range] based on four answers, as two respondents did not specify their time range from decapping to analysis.
Aptio = Aptio Automation (Siemens Healthineers, Germany & Inpeco SA, Switzerland); Cobas = Cobas Connection Modules (Roche Diagnostics, Switzerland); Flexlab = Flexlab Automation (Inpeco SA, Switzerland); GLP = GLP Systems (Abbott Laboratories, USA - IL); VITROS = VITROS Automation Solutions (Ortho Clinical Diagnostics, USA - NJ).

Table 2: Danish clinical biochemistry department's reported open tube stability on blood alcohol and total carbon dioxide according to their laboratory local guideline. The stability according to the LA system of the laboratory is also shown. Of the 26 laboratories with LA, 96% (25/26) measured blood alcohol, but only 62% (16/26) measured total carbon dioxide.

After decapping: Reported stability in minutes*	Blood Alcohol (n=25 laboratories)		Total Carbon Dioxide (n=16 laboratories)	
	%	Laboratory LA System	%	Laboratory LA System
0-30	32	Aptio, Flexlab, GLP	31	Aptio, GLP
31-60	20	Flexlab, Vitros	31	Cobas, Flexlab,GLP
61-90	0	-	0	-
> 90	28	Cobas, Flexlab, GLP	19	Flexlab
Not established	20	Flexlab, Cobas, GLP	19	Flexlab, Cobas

No difference between blood alcohol and total carbon dioxide reported stability time guidelines (p>0.9).
*) Median (range) for reported stability of blood alcohol was 60 min (0-300 min), and for total carbon dioxide it was 60 min (0-360 min).
LA = laboratory automation; Aptio = Aptio Automation (Siemens Healthineers, Germany & Inpeco SA, Switzerland); Cobas = Cobas Connection Modules (Roche Diagnostics, Switzerland); Flexlab = Flexlab Automation (Inpeco SA, Switzerland); GLP = GLP Systems (Abbott Laboratories, USA - IL); Vitros = VITROS Automation Solutions (Ortho Clinical Diagnostics, USA - NJ).

Total carbon dioxide and automation

When measuring total carbon dioxide, not all laboratories transported the blood tubes on the LA assembly line, Table 1. The majority of the laboratories, 62 % (10/16), had an open tube stability guideline of one hour or less, Table 2, which also shows an interlaboratory variation in local guidelines.

According to the BLS from Regional Hospital, Herning (RH Herning), open tubes for total carbon dioxide measurements were transported on a partial LA system, which would warn if a test result and stability was about to be exceeded. The laboratory stress-tested the system regularly for turnaround time during peak periods. ZUH Roskilde claimed that staff, once every hour, ensured measurement did not expire by checking if test results were available. If no results were available, the staff would manually take the

open tubes off the assembly line to ensure the sample was properly analyzed to avoid evaporation.

Discussion

Even though LA systems significantly improve capacity and efficiency, and reduce human errors, the systems possess pre-analytical challenges that laboratories must address.[11] This includes sensitive analytes transported on assembly lines in open tubes, which may evaporate or otherwise react to prolonged air exposure at various temperatures.[1-4] It was observed that local open tube stability guidelines varied greatly from 0 to 300 minutes for blood alcohol and 0 to 360 minutes for total carbon dioxide among different laboratories. Two previous studies addressed the open tube concern for blood alcohol and total carbon dioxide analytes and suggested that analytical measurements are acceptable when analyzed within 120 minutes after de-capping.[8,9] Nielsen *et al*. suggested that the majority of common analytes (20 of 23 analytes) were not sensitive to de-capping and plasma evaporation with a stability of 6 hours or more at room temperature. The study did not include blood alcohol and carbon dioxide.[12] In practice and without downtime, this study demonstrated that open tubes in general were transported on assembly lines for a median five minutes (after automated de-capping and until analysis), however, some Danish laboratories reported up to 90 minutes transportation time. Many laboratories avoided the problem by handling the tubes for sensitive analytes manually and off the assembly lines. This again confirms preanalytical issues are handled differently among laboratories, sometimes even despite international guidelines exists, like procedures of blood tube order of draw.[13]

De-capped open blood tubes transported on automated assembly lines may be a new preanalytical LA based challenge and could jeopardize the quality of analytical results and patient safety. For quality assurance and standardization of stability guidelines, this study suggests that there is a requirement for evidence-based temperature and time stability studies on sensitive analytes in open tubes. LA manufactures may also assist in solving this preanalytical issue with certain LA adaptions.

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