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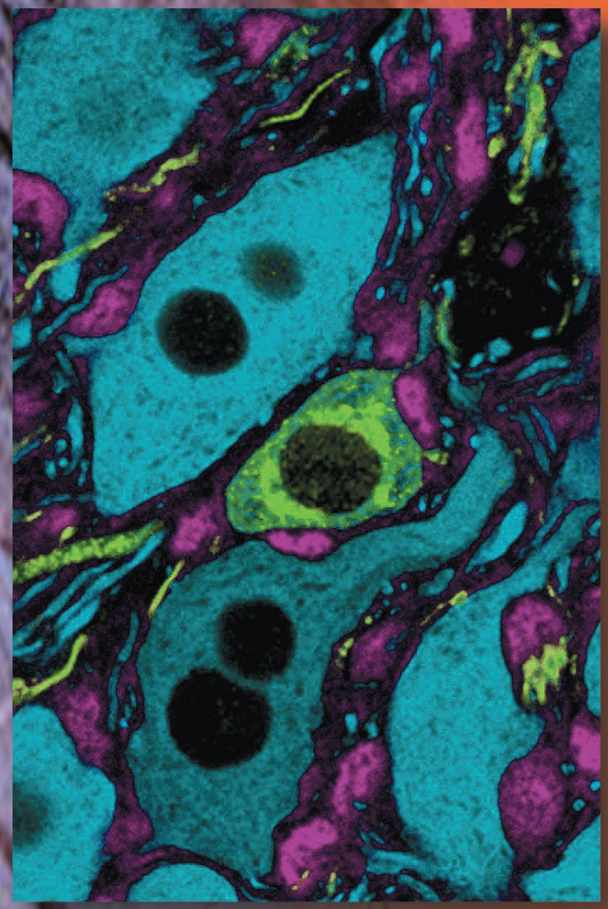

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ORIGINAL ARTICLE

Vaccines against SARS-CoV-2: A discussion of the risk-benefit

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Abstract

The first known infections from SARS-CoV-2 were discovered in Wuhan, China late in 2019. The original source of viral transmission to humans remains unclear, as does when the virus became pathogenic. It was the third mutated coronavirus described with pandemic potential after SARS-CoV-1, first described in February 2003, and the ongoing limited transmission of Middle East Respiratory Syndrome (MERS), first described in Saudi Arabia in 2012 and largely transmitted from dromedary camels.

Both of the first two coronaviruses caused less than 10,000 cases worldwide, both had a much higher mortality than SARS-CoV-2 and had a limited capacity for person-to-person transmission. No vaccine was developed against either coronavirus despite attempts to do so. More concerningly, one candidate vaccine against SARS-CoV-1 demonstrated antibody-dependent enhancement of the coronavirus thus potentially worsening disease and infectivity if used.

Keywords: SARS-CoV-2, coronavirus, pandemic

Initial pandemic control activities

In the absence of vaccines, standard public health infection control measures of hand hygiene, surface cleaning, the use of masks, and physical distancing were used in varying intensity across the world, along with isolation, quarantine and cohorting. Where closure of external borders around an island was possible, these measures largely restricted the spread of the initial wave of disease, allowing temporary elimination in Australia, Taiwan and New Zealand.

As an RNA virus circulating in very large numbers, SARS-CoV-2 often made mistakes in copying itself, and over time some of these mutations proved to be more efficient at transmission and started to become the predominant viral strain circulating first in that area and then more globally over time. Some of the new strains were sufficiently different to avoid the effect of monoclonal therapies in use to treat COVID-19 disease and with the arrival of the Brazilian P1 strain (Gamma), were different enough to avoid antibody produced from previous disease and allow recurrent COVID-19 disease (Singh *et al* 2021).

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Vaccine development

The fastest vaccine developed previously from commencement to use was the mumps vaccine which took four years to develop. These timeframes resulted from a need to ensure interim safety and effectiveness tests in early trials were completed before funds were committed to the next of the three phases of development, and in some cases conducting initial animal trials before any human trials.

With the urgent challenge of finding vaccines against SARS-CoV-2, multiple alliances of vaccine companies, governments, the World Health Organisation, the Gates Foundation, Global Alliance for Vaccines and Immunisation and other groups committed tens of billions of dollars to the cause. Developers were indemnified against loss if their trials failed and nations committed to purchase billions of doses of successful vaccines.

The effect of these fiscal commitments, and the redirection of the greatest virological, infectious diseases and vaccinology specialists to the task, produced a raft of vaccines with high efficacy ready to use in twelve months. Many of these used developing and new technologies not used in vaccines before, with a predominance of technologies targeting the spike glycoprotein of the prototype SARS-CoV-2 strain (Motamedi *et al* 2021; Chung *et al* 2021; Machhi *et al* 2021).

Concerns were expressed about the rapidity of development and the potential for safety steps to have been skipped,

but apart from the deletion of animal studies from some protocols and the decision of the Russian Government to approve the Sputnik V vaccines after promising data on less than 100 patients (Machhi *et al* 2021), no safety steps were skipped and all licensed vaccines met all the criteria for new vaccines.

Vaccine types

Messenger RNA vaccines

Coronaviruses contain crown like spikes on their surface. These spike proteins are 'the keys' that attach to and enable the invasion by the virus into cells and thus are an ideal target for vaccines. The messenger RNA (mRNA) in vaccines is the genetic material that tells the body how to make these foreign spike proteins. After the delivery of the instructions, the mRNA is broken down and the spike proteins without any attached virus are then exposed to the body's defences where antibodies and cellular immune responses are produced. When the virus arrives, the body recognises the spike protein and attacks the virus.

There are two mRNA vaccines available now or will be soon in Australia:

- Comirnaty (Pfizer/BioNTech 162b) 30µg dose;
- Moderna (mRNA-1273) 100µg dose.

One of the major attractions of mRNA vaccines is the speed with which they can be manufactured and scaled up when the genetic code of the viral spike protein is known, and similarly, the capacity to rapidly upgrade the vaccines when the mutations and base substitutions of new strains are identified.

Viral vector vaccines

This is the vaccine, initially developed by Oxford University and AstraZeneca, (AZD 1222, ChAdOx1) which is locally manufactured under licence in Australia and is readily available. With this vaccine, a weakened and modified chimpanzee adenovirus is used as the delivery system to introduce the spike protein into the body. The body then makes antibodies against the spike protein and recognises the spike protein when it enters the body attached to the SARS-CoV-2 virus.

Nanoparticle vaccines

The other vaccine ordered by the Australian Government but not yet available, is the Novovax (NVX-CoV2373) vaccine which is a recombinant nanoparticle vaccine containing the full-length spike glycoprotein with a Matrix-M adjuvant.

Vaccine effectiveness

Initial trials data on all the vaccines available in Australia showed efficacy data after two doses well above 70% against the initial SARS-CoV-2 viral strain. The emergence of new strains with altered spike proteins led to variations in vaccine effectiveness against these strains and restrictions placed on the use of some vaccines. South Africa ceased use of the ChAdOx1 vaccine after preliminary evidence of reduced effectiveness against the prevalent Beta variant circulating in the country. Furthermore, evaluation of trials data attracted criticism due to varying vaccine dosages being used (ChAdOx1), and a lack of data transparency with Chinese and Russian vaccines. All vaccines had varying effectiveness depending on the outcome measure used, the age range of the population studied, living conditions of trial participants, the degree of immune suppression and the prevalent SARS-CoV-2 strain circulating during the study (Tregoning *et al* 2021).

With the current circulating Delta strain, vaccine effectiveness against disease acquisition after a single dose is reduced with vaccines available in Australia and is around 40%. When given at the optimal separation times of 3 weeks for Pfizer and 12 weeks for AstraZeneca, the effectiveness increases to around 75-85% against any disease and over 95% against severe disease requiring hospitalisation or causing death (Lopez Bernal *et al* 2021).

While cases are still occurring in vaccinated individuals and potentially continuing to contribute to community transmission, viral shedding in mild disease is usually less and spread is reduced. Recent data however from the United Kingdom suggests viral loads with the Delta strain may not be as greatly reduced in those who are vaccinated.

Vaccine safety

As always, the decision to vaccinate is a risk-benefit decision. Having cleared the criteria for adoption of a vaccine program generically, the individual decision is a balance of benefit in disease and consequences averted versus vaccination risk. The public debate in the Australian media focused on uncommon side effects has been problematic.

Astra Zeneca (ChAdOx1) serious side effects:

The risk of a hyper-immune response leading to auto-antibodies against platelets (PF4) and clotting in central venous sinuses or splanchnic veins has been well described. The cause of the clotting is now well understood treatment and therefore processes are well developed.

The risk is about 1/80,000 overall but is age dependent and as high as 1/30,000 in those aged 25 years. It also varies with the study population and has shown to be higher in Scandinavia. The risk decreases tenfold for the second dose of vaccine. The mortality after clotting was around 1/800,000 (Pottegård *et al* 2021; Chan *et*

al 2021). In the absence of significant disease rates in the community, recommendations from the Australian Technical Advisory Group on Immunisation (ATAGI) were to not have the AstraZeneca vaccine if under 50 years of age and then after higher rates in those over 50 years, not to have it if under 60 years of age.

With community clusters occurring, younger people were encouraged to have AstraZeneca vaccine but this was more a function of a lack of Pfizer vaccine availability rather than a lessening of the AstraZeneca risk.

There has been a suggestion that the AstraZeneca vaccine is associated with an increase in Guillain-Barre Syndrome, described with another of the viral vector vaccines. At this time data is insufficient to confirm that the rate of this is above background (Rosenblum *et al* 2021).

Pfizer serious side effects

Rates of anaphylaxis are higher with mRNA vaccines with the increased risk being greatest in those known to have a history of atopia and particularly those who carry an epi-pen because of previous allergic reactions. The incidence is about 1/250,000 overall and 95% occur in females. These people should be vaccinated in a major centre (Tregoning *et al* 2021).

In younger persons, particularly younger males, there is an increased risk of pericarditis and myocarditis after Pfizer. The risk is of the order of 1/25,000 (Tregoning *et al* 2021; Bozkurt *et al* 2021). Most of these cases resolve spontaneously over a few days without residual deficits. This has led to recommendations that people with some cardiac conditions should not receive this vaccine (Australian Government 2021).

The future of COVID vaccines

Boosters

Data is becoming increasingly available, particularly with mRNA vaccines, that while the first two vaccine doses produce neutralising antibodies and protect people from serious disease, protection is not durable and antibody levels decrease over time. It is also likely that higher antibody levels are required to protect against the Delta strain (Rosenberg *et al* 2021).

Booster doses will likely be required after 6-8 months and data suggests that the secondary immune response produced by this third dose is faster and stronger increasing antibody titres at least ten-fold. For some groups booster doses are required as part of initial processes. Data is emerging that persons living with immune suppression who are fully vaccinated are much more likely to suffer from breakthrough infections with the Delta strain. Children in this setting are already recommended to have a third dose in the United States. Australia has ordered next generation mRNA vaccines for boosters from both Moderna and Pfizer for delivery early in 2021. While boosters will be necessary for all people, and have commenced in Israel already, the ethics of boosting the people in developed countries when many of the lower and middle income countries have received minimal first doses has been challenged by the World Health Organisation.

Universal Vaccines?

The stem helix of the spike protein has remained conserved during the evolution of a number of beta-coronaviruses, opening up the possibility of vaccines generating antibodies against this machinery. This would enable the coronavirus to fuse with the membrane of host cells and avoid the challenge of those parts of the spike protein which rapidly mutate under pressure from the body's antibody response.

A number of monoclonal antibodies against the stem helix have been studied to look at their expression post infection and post vaccination. A combination of factors including pre-existing priming from common cold coronaviruses appear to be important for their generation (Pinto *et al* 2001).

Other groups are currently studying combinations of monoclonal antibodies from SARS-CoV-1 survivors and animal models in the hope of developing a "cocktail" vaccine against coronaviruses (Tan *et al* 2021). The development of these universal vaccines appears to be some way off still.

Summary

The newer strains of SARS-CoV-2 are proving more infectious, are infecting a younger group of persons, and at times are proving more virulent. In addition, they are resistant to some of the antibody responses provided by previous disease or by vaccination with current vaccines.

While vaccines are "sub-optimal", and public health measures such as physical distancing and masking are less effective against an aerosol transmitted virus than one transmitted by large droplets, we are seeing strong data that severe disease and death is occurring principally in the unvaccinated. Herd immunity is not possible with the effectiveness of currently available vaccines combined with large numbers of unvaccinated children. Despite this, all of us need to be vaccinated to provide a base of protection that can be built on with future boosters.

Just as we line up for annual influenza vaccines hoping to protect us against the likely circulating strains during winter we will need regular SARS-CoV-2 vaccines. Thankfully, the new technologies used in these vaccines will make changes to the vaccine more rapid and accurate than the current slow egg production process of influenza vaccines.

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Is there a role for medical scientists in the detection of unsuspected Covid-19?

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Abstract

While much of the research into laboratory test results seen in COVID-19 patients has centred on prognostic implications, there may also be a role for aiding diagnosis. With the small number of positive cases that have developed in Australia so far and the high rate of RT-PCR testing, it is unlikely that COVID has been an incidental finding. There may be however an emerging role for medical scientists to identify potential unsuspected COVID-19 patients, similar to the current role we play in the detection of unsuspected dengue fever. Ninety percent of experts believe that in the future COVID-19 will become a seasonal endemic viral infection such as Influenza (Phillips 2021). When a patient presents with flu-like symptoms in years to come, RT-PCR testing for SARS-CoV-2 may not be as prevalent. In this setting there may be a role for medical scientists to potentially identify COVID-19 patients through a constellation of characteristic laboratory results. There are several identifiable abnormalities that are seen on presentation in a developing SARS-CoV-2 positive patient. These abnormalities may be present with or without clinical symptoms of COVID-19.

Keywords: *COVID-19, SARS-CoV-2, RT-PCR testing*

COVID-19 Case Study

The following case study is a hypothetical patient based on real patient data and published COVID-19 cases from Australia and internationally (Abdullah *et al* 2021). This case will show the classic presentation that is seen in early COVID-19 and one that may prompt a follow up phone call to the requesting clinician to suggest SARS-CoV-2 RT-PCR testing. It should be noted that it is not typical to have a full range of blood test results available for COVID-19 patients in the current climate, as they are monitored primarily by nasopharyngeal swab.

This case is a 32-year-old female who presented to emergency with a fever, dry cough and flu-like symptoms. The patient was symptomatic for approximately one week. Laboratory results are as follows.

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Biochemistry results

Analyte	Result (units)	R Range	Flag
Sodium	131 (µmol/L)	(135-145)	**(L)**
Potassium	4.3 (µmol/L)	(3.5-5.2)	
Chloride	103 (µmol/L)	(95-110)	
Bicarbonate	25 (µmol/L)	(22-32)	
Anion gap	9 (µmol/L)	(4-13)	
Glucose	6.0 (µmol/L)	(3.0-7.8)	
Urea	4.3 (µmol/L)	(2.9-8.2)	
Creatinine	71 (µmol/L)	(64-108)	
Urate	0.24 (µmol/L)	(0.15-0.5)	
Protein	66 (g/L)	(60-80)	
Albumin	39 (g/L)	(35-50)	
Globulin	27 (g/L)	(25-45)	
Bilirubin	14 (µmol/L)	(<20)	
Bili (Conj.)	<4 (µmol/L)	(<4)	
ALP	59 (U/L)	(30-110)	
GGT	73 (U/L)	(<55)	**(H)**
ALT	84 (U/L)	(<45)	**(H)**
AST	68 (U/L)	(<35)	**(H)**
LD	281 (U/L)	(120-250)	**(H)**

Haematology results

Analyte	Result (units)	R Range	Flag
Haemoglobin	125 g/L	(115-160)	
Platelets	161 (x10⁹/L)	(140-400)	
RBC	4.25 (x10⁹/L)	(3.8-5.2)	
MCV	87 (fL)	(80-100)	
WBC	3.34 (x10⁹/L)	(4.0-11.0)	**(L)**
HCT	0.42	(0.33-0.47)	
MCH	29.9g/L	(310-360)	
Neutrophils	1.84 (x10⁹/L)	(2.0-8.0)	**(L)**
Lymphocytes	0.95 (x10⁹/L)	(1.0-4.0)	**(L)**
Monocytes	0.52 (x10⁹/L)	(0.1-1.0)	
Eosinophils	0.03 (x10⁹/L)	(<0.6)	
Basophils	0.0 (x10⁹/L)	(<0.2)	

Coagulation profile

Test	Result (units)	R Range	Flag
INR	1.2	(0.9-1.2)	
PT	12 (s)	(10-13)	
APTT	38 (s)	(26-41)	
Fibrinogen	5.8 (g/L)	(1.7-4.5)	**(H)**
D-Dimer	0.9 (mg/L)	(<0.28)	**(H)**

Blood film morphology

Moderate number of plasmacytoid reactive lymphocytes noted (Figure 1). Neutrophils show increased granulation. Red cells are unremarkable. Large and giant platelets are present.

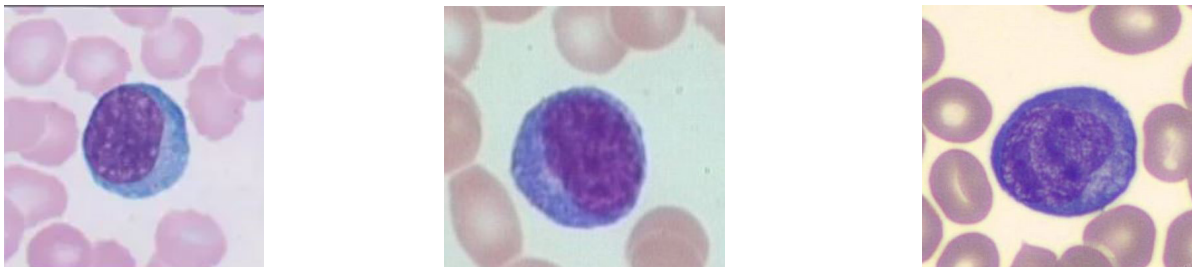

Figure 1. *Reactive plasmacytoid lymphocytes.*

Discussion

Notable laboratory investigations in the case presentation include leucopenia, neutropenia, lymphopenia, elevated ALT and AST and a mildly elevated D-Dimer. These features are commonly seen in COVID-19 patients, along with clinical symptoms of fever, cough and dry mucous membranes (Hashmi *et al* 2020; Thachil *et al* 2020). A Hashmi-Asif COVID-19 chart has been developed that has a reported sensitivity of 95% in detecting symptomatic cases (Figure 2).

Basophilic reactive plasmacytoid lymphocytes are not specific to COVID-19 and may be present in several viral illnesses, including dengue fever. Interestingly, these cells were not typically seen in 2003 SARS infections (Chong *et al* 2020). While these abnormalities when seen together are not diagnostic for COVID-19, they certainly increase suspicion for this causative agent. Clinical symptoms may also be taken into consideration, but many cases are asymptomatic. In the future, when swabbing for RT-PCR testing is not as prevalent, laboratory protocols may benefit from including surveillance for potential COVID-19 patients that have escaped clinical suspicion.

Physical Signs and Symptoms			Scores	
Temperature	≤37 Score-1	<37.5 Score-2	37.5–38 Score-3	>38 Score-3
*Cough	Absent Score-1	Productive Score-2	Dry Cough Score-3	Prudent Score-3
Fatigue	Absent Score-1	From 1 Day Score-2	From 2 days Score-3	>2day Score-3
Nausea and vomiting	Absent Score-1	Nausea with vomiting Score-2	Vomiting with diarrhea Score-3	Vomiting with abdominal discomfort Score-3
Mucus membrane	Normal Score-1	Inflamed Score-2	Dry appearance Score-3	Hyperemic Score-3
				Total
Blood biomarkers				
Leukocytes 3,800–1,100/µl	5,000–11,000 Score-1	3,800–5,000 >11,000 Score-2	3,500–3,800 Score-3	<3,500 Score-4
***Lymphocytes** 1,000–3,900/µl	>2,500 Score-1	1,750–2,500 Score-2	1,000–1,750 Score-3	<1,000 Score-4
Neutrophils 1,900–7,400/µl	1,900–3,500 Score-1	≥3,500 Score-2	1,800–1,900 Score-3	<1,800 Score-4
***Platelets** 150,000–400,000/µl	>250 × 10^3 Score-1	150–250 × 10^3 Score-2	125–150 × 10^3 Score-3	<125×10^3 Score-4
***Alanine aminotransferase** 10–49U/L	<50 Score-1	50–60 Score-2	60–70 Score-3	>70 Score-4
Aspartate Aminotransferase <33 U/L	<35 Score-1	35–40 Score-2	40–50 Score-3	>50 Score-4
	Cumulative Scoring ≥13–22/39 should be considered at high risk to be diagnosed with Covid-19 and considered for RT-PCR for SARV-Cov-2.			**Total Score** (Cumulative) No Disease ≤**12** Mild: **13–22** Moderate: **23–33** Severe: **34–39**

Figure 2. *Hashmi-Asif COVID-19 chart (Hashmi et al 2020). Reproduced with permission.*

Conclusion

A pattern of possible patient symptoms, laboratory results and blood film morphology can help identify a COVID-19 infection. The scientist can aid in this diagnosis by recognising this pattern and alerting the treating team to the possibility of this and therefore play a vital role in the containment and treatment.

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Coagulopathy in COVID-19

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Abstract

The novel coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), was identified as the cause of an outbreak of viral pneumonia in Wuhan, China in 2019. The disease, later named coronavirus disease 2019 (COVID-19), subsequently spread globally and has impacted the lives of the world's population. There have been many millions infected and many deaths.

COVID-19 affects most of the body systems through the ensuing cytokine storm and coagulopathy is a consequence. Effects on multiple components of haemostasis contribute to this coagulopathy: increased fibrinogen due to the acute phase reaction; endothelial injury and activation; platelet activation; loss of coagulation cascade homeostasis and downregulation of the fibrinolytic system.

Keywords: *COVID-19, coronavirus, coagulopathy, disseminated intravascular coagulation, anticoagulant*

Introduction

COVID-19 is the newest member of the betacoronavirus family. This family of viruses has caused previous regional outbreaks of severe acute respiratory syndrome (SARS) in 2002 (SARS) and Middle East respiratory syndrome (MERS) in 2012 as well as the current global pandemic SARS-CoV-2 in 2019 (COVID-19) (NIAID 2020). This infectious disease is thought to have originated in Wuhan, China in late 2019. In the first three months after COVID-19 emerged, nearly 1 million people were infected and 50,000 died. By six months the number of cases exceeded 10 million and there were more than 500,000 deaths. To date there are over 200 million people infected and 4.2 million of these have died (NIAID 2020 COVID-19 dashboard at CCSSE at JHU 2021).

Pathogenesis

The virus enters the respiratory tract and binds to the angiotensin-converting enzyme-2 (ACE-2) receptor which is expressed at high levels on pneumocytes and endothelial cells. It infects vascular endothelial cells and also targets lung epithelial cells and lymphocytes, resulting in the clinical presentation of acute respiratory distress syndrome (ARDS), shock and coagulopathy in severe cases. The clinical syndrome which manifests in individuals is highly variable, ranging from asymptomatic or minimally symptomatic, through to critical cases which lead to death. Definitions of various disease stages and severity are summarised in Table 1.

A cytokine storm is often triggered, leading to a life-threatening systemic inflammatory syndrome involving elevated levels of circulating cytokines, immune-cell hyperactivation and multi-organ dysfunction (Table 2).

Faigenbaum and June (2020) have proposed the following three criteria for defining a cytokine storm: '*elevated circulating cytokine levels, acute systemic inflammatory symptoms, and either secondary organ dysfunction (often renal, hepatic, or pulmonary) due to inflammation beyond that which could be attributed to a normal response to a pathogen (if a pathogen is present), or any cytokine-driven organ dysfunction (if no pathogen is present)*' (Faigenbaum and June 2020).

The cells in the innate immune system that are most often implicated in the pathogenesis of cytokine storm include neutrophils, macrophages and natural killer (NK) cells. Neutrophils can produce neutrophil extracellular traps (NETS), a network of fibres that contribute to thrombus formation and amplify cytokine production during the cytokine storm. Macrophages become activated and secrete excessive amounts of cytokines, ultimately causing severe tissue damage that can lead to organ failure. An increase in haemophagocytic macrophages can be seen in the bone marrow from patients suffering from a

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Table 1. *Definitions of COVID-19 disease stages and severity.*

	Asymptomatic or presymptomatic	Mild illness	Moderate illness	Severe illness	Critical illness
Features	Positive SARS-CoV-2 test, no symptoms	Mild symptoms (fever, cough, change in taste or smell, no dyspnoea)	Clinical or radiographic evidence of lower respiratory tract disease; oxygen saturation ≥94%	Oxygen saturation ≤94%; respiratory rate ≥30 breaths/min; lung infiltrates ≥50%	Respiratory failure; shock; multi-organ dysfunction or failure
Management	Monitor for symtoms	Clinical monitoring and supportive care	Clinical monitoring; if patient high risk then anti-viral Rx (remdesivir)	Hospitalisation; oxygen therapy; specific medications (remdesivir, dexamethasone)	Critical care and specific therapy (dexamethasone and possibly remdesivir)

Table 2. *Organs and body systems affected by the cytokine storm. Modified from Fajgenbaum and June (2020).*

Affected organ/system	Manifestation
Lungs	Pneumonitis, pulmonary oedema, dyspnoea, acute respiratory distress syndrome
Liver	Hepatomegaly, elevated liver enzymes, liver damage, cholestasis, liver failure, increased hepcidin
Kidneys	Acute renal dysfunction or injury, renal failure
Vascular and lymphatic systems	Coagulopathy, cytopenias, anaemia, elevated cytokines, endothelial damage, capillary leak syndrome, increased acute phase proteins
Nervous system	Confusion, delirium, aphasia, seizures
Heart	Hypotension, tachycardia, cardiomyopathy
Constitutional symptoms	Fever, anorexia, fatigue
Rheumatologic system	Vasculitis, arthritis, arthralgia
Gastrointestinal system	Nausea, vomiting, diarrhoea, ascites
Skin	Rash, oedema

cytokine storm and this may contribute to the cytopenias commonly observed in patients (Figure 1). However true haemophagocytic lymphocytosis (HLH) is uncommon in COVID-19 (Hueso *et al* 2020). NK cells have a cytolytic function which can be reduced in some cytokine storms resulting in prolonged antigenic stimulation and difficulty in resolving inflammation (Faigenbaum and June 2020).

This increased production of cytokines, increased levels of damage-associated molecular patterns (DAMPs), the stimulation of haemophagocytic and apoptotic mechanisms and vascular endothelial damage are believed to be the main drivers of coagulopathy in any severe infection (Iba *et al* 2020).

Laboratory testing and results

In COVID-19 patients, there are numerous haematological abnormalities present. Neutrophilia, lymphopenia, some monocytopenia and eosinopenia and sometimes thrombocytopenia can be found in the full blood count (FBC). The white cell count (WCC) has not been found to correlate with disease severity, with variable counts reported in the literature. Neutrophilia was found in severe cases but there were normal numbers in the non-severe cases in the majority of studies reviewed by Khartabil *et al* (2020). Lymphopenia, however, was a consistent finding, with it present on admission and worsening in severe cases. The lymphocyte count was determined to be the most sensitive and reliable parameter in predicting disease severity and outcome. Decreased monocyte numbers were

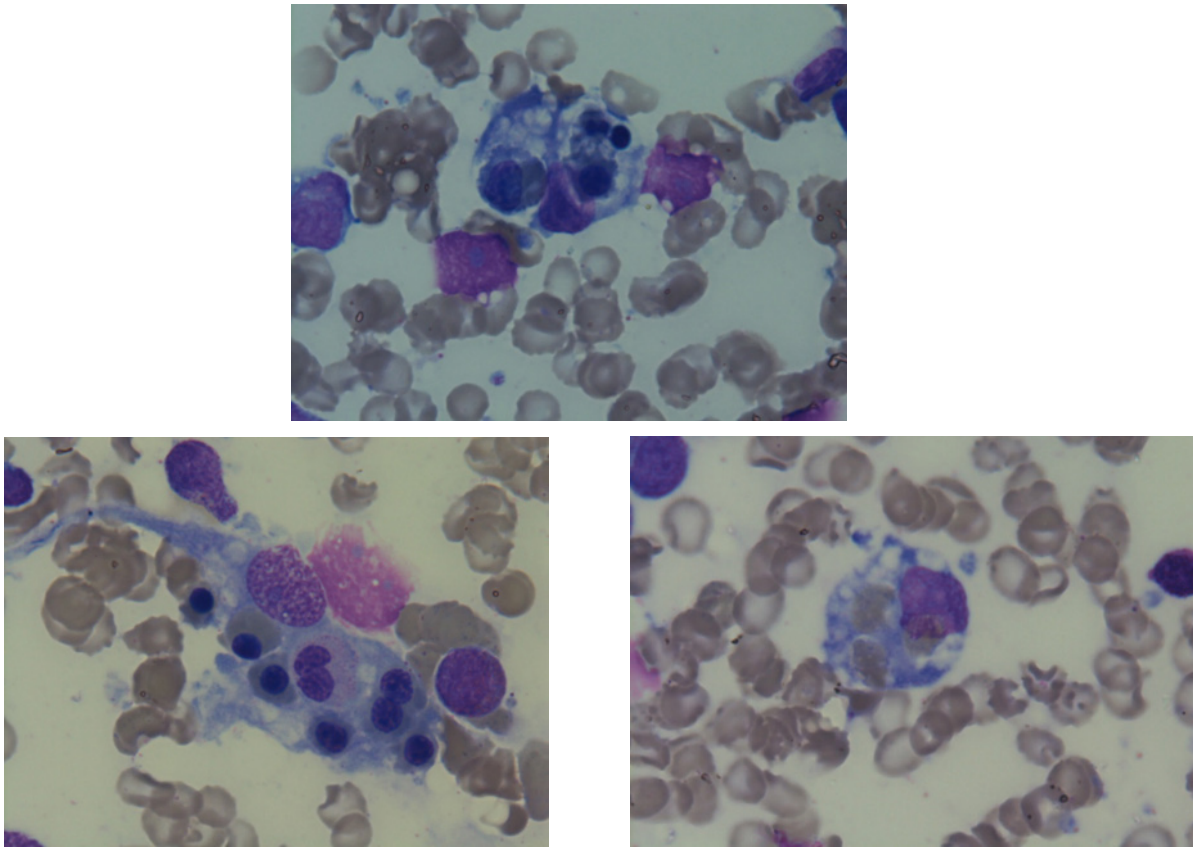

Figure 1. *Increased haemophagocytic macrophages cells in the bone marrow.*

found in the more severe cases but were in the normal range in milder cases. Eosinopenia was also frequently found and was present in almost every patient who died (Khartabil *et al* 2020).

Platelets are normal or decreased in non-severe patients and significantly decreased in severe patients. Approximately half of severe cases have platelets <150 X 10^9/L and in the meta-analysis by Lippi *et al* (2019), a low platelet count is associated with increased risk of severe disease and mortality (Lippi *et al* 2019).

Morphological changes can also be seen in the blood film. Dysplasia manifesting as hyposegmented neutrophils with coarsely clumped chromatin and dark cytoplasmic granulation as well as immature granulocytes, large platelets, apoptotic cells, and hypogranular neutrophils have been seen. Following treatment with anti-viral and anti-inflammatory medications, increased numbers of reactive lymphocytes and large granular lymphocytes can also be observed (Zini *et al* 2020).

Abnormal coagulation results are a consistent finding in COVID-19 with prolonged PT and elevated D-dimer results in the severe cases. The activated partial thromboplastin time (APTT) is usually normal and the fibrinogen is often raised due to it being an acute phase protein (Tang *et al* 2020; Asakura and Ogawa 2021; Xiong *et al* 2020). This is in contrast to the classic disseminated intravascular coagulation (DIC) results of prolonged PT and APTT, increased D-dimer and low fibrinogen and platelet count.

Tang *et al* (2020) looked at the coagulation test results of 183 patients with novel coronavirus pneumonia (NCP) over 14 days (Figure 2). The tests performed were PT, APTT, fibrinogen, D-dimer, fibrin degradation products (FDP) and antithrombin (AT). In this cohort of patients, there were 21 non-survivors and 162 survivors. There was a significant difference ($p < 0.05$) in D-Dimer and FDP levels, and longer PT for non-survivors compared to survivors on admission. After 14 days the fibrinogen and AT levels were also significantly lower in non-survivors. This suggested that conventional coagulation parameters during the course of NCP were significantly associated with prognosis (Tang *et al* 2020). This confirms the findings of many authors that although this is not a classic sepsis or malignancy associated DIC presentation, there is DIC occurring in the severe cases of COVID-19 (Tang *et al* 2020; Asakura and Ogawa 2021; Xiong *et al* 2020).

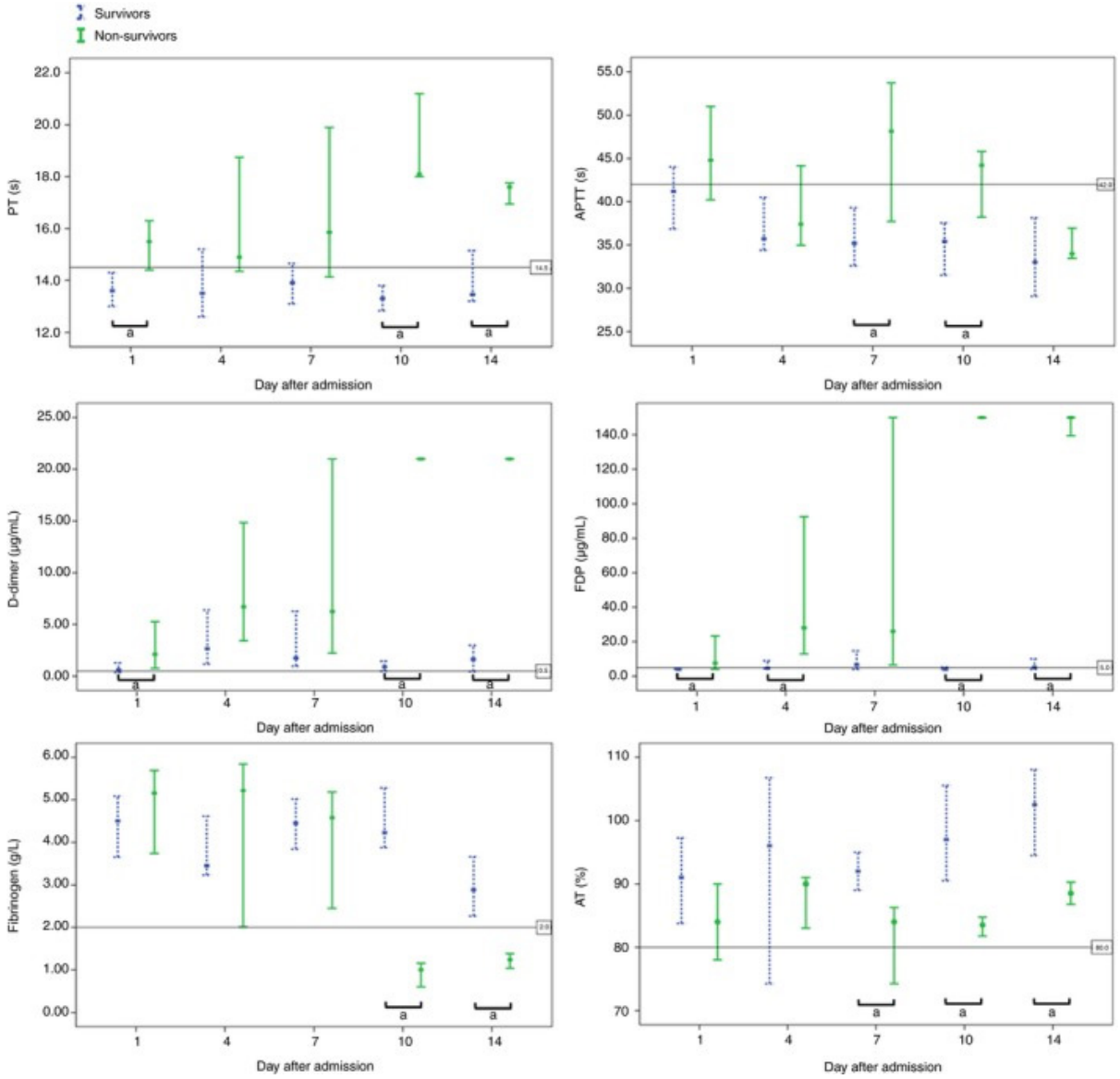

Figure 2. *Coagulation parameters in patients with NCP over 14 days. The error bars show medians and 25% and 75% percentiles. The horizontal lines show the upper normal limits of prothrombin time, activated partial thromboplastin time, D-dimer and fibrin degradation product, and the lower normal limits of fibrinogen and antithrombin activity, respectively. The a = P < 0.05 for survivors versus non-survivors (Tang et al 2020. J Thromb Haemost).*

Additional markers of coagulation and fibrinolysis that have been studied are von Willebrand factor (vWF), disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13 (ADAMTS13), thrombin–antithrombin complex (TAT) and plasmin-α2 plasmin inhibitor complex (αPIC). Platelet function studies have also been conducted.

In a French single centre study conducted by Delrue *et al* (2021), 133 patients were included with moderate to severe COVID-19. Venous thromboembolism (VTE) was present in 38 of those patients and this VTE group had a higher percentage of critically ill patients (72% vs 43%). They also had a higher vWF Ag 5.22 IU/L compared to 4.73 IU/L (RR 0.5-1.5) in the non-VTE cohort and a lower ADAMTS13 level (59% vs 68.5% (RR 50-150%)). They concluded that there was markedly imbalanced vWF/ADAMTS13 axis with extremely high circulating VWF concentrations of up to 14.7 IU/L contrasting with normal or decreased ADAMTS13 activities, partly due to consumption by its substrate vWF and/or inhibition by high interleukin-6 levels. The low ADAMTS13 activity results in ultra-large vWF molecules, which promote pulmonary vascular microthrombi frequently found in the respiratory system in COVID-19 patients. This suggests that the low ADAMTS13 levels along with the increased D-dimer levels may be a poor prognostic marker (Delrue *et al* 2021).

AT levels have been shown to be a prognostic factor and the TAT (coagulation activation marker) and αPIC complexes (fibrinolytic activation marker) have also been studied by Asakura and Ogawa (2021). They reported that the basic coagulation tests as well as these essential markers for coagulation and fibrinolysis activation must be regularly monitored as short-term fluctuations can occur in the non-surviving patients and the changes could be missed. In fatal cases, for example, fibrinogen was as high as about 4.0 g/L on day 7 but dropped sharply to about 1.0 g/L on day 10 (only three days later). FDP and D-dimer levels also increased sharply in just three days. In cases with DIC in which PIC is markedly increased, major bleeding is likely to occur when α2 plasmin inhibitor (α2 PI) is reduced to less than half. In cases where antithrombin activity is significantly reduced, the sensitivity of TAT may be reduced.

Manne *et al* (2020) conducted a US study with the platelets from 41 adult COVID-19 patients (compared to healthy donor platelets) to investigate any alterations in platelet gene expression and function. They found by RNA sequencing that the gene expression profile changes with differential gene-expression changes in pathways associated with protein ubiquitination, antigen presentation, and mitochondrial dysfunction. Resting platelets from COVID-19 patients had increased P-selectin expression basally and upon activation. Platelets from COVID-19 patients became "stickier" and aggregated faster with low-dose agonists (2MeSADP, thrombin, and collagen) and showed increased spreading on both fibrinogen and collagen. This increase in platelet activation and aggregation demonstrate that SARS-CoV-2 infection is associated with platelet hyperreactivity, which may contribute to COVID-19 pathology particularly in the formation of both macro- and microthrombi (Manne *et al* 2020).

Discussion

In a healthy individual, haemostasis occurs with vasoconstriction and endothelial interaction with pro-coagulant and anti-coagulant factors in the vascular system, followed by platelet aggregation forming a platelet plug. The low platelet count in severe infections could be due to platelet directed antibodies, hematopoietic stem cell suppression or increased consumption of platelets. The coagulation cascade is activated to generate a fibrin mesh which combines with the platelet plug to prevent bleeding. The fibrinolytic system keeps this coagulable state in balance.

In the COVID-19 coagulopathy, there is endothelial injury and activation, increased platelet aggregation as they are primed and stickier, the coagulation cascade is activated and thrombosis can occur unchecked as the fibrinolytic system is downregulated. The cytokine storm that results from infection with SARS-CoV-2 causes inflammation that exacerbates this coagulopathy and the increase in vWF and decreased ADAMTS13 activity also contribute. The pathophysiology of the coagulopathy associated with COVID-19 is very different from that of classic sepsis-related DIC, and both thrombotic and haemorrhagic pathologies can be seen. COVID-19 thrombosis includes macro- and microthrombosis, and in particular, VTE. Considerable bleeding has also been observed and this could mean that in fatal cases, there is a possibility that suppressed-fibrinolytic-type DIC has changed to an enhanced-fibrinolytic-type DIC (Asakura and Ogawa 2021). These conditions can only be elucidated with additional testing of markers of both coagulation and fibrinolysis.

It has been proposed that this phenomenon should be named "pulmonary intravascular coagulopathy "(PIC), as opposed to DIC, because the thrombosis occurs overwhelmingly in the lungs rather than the whole body.

The tests to identify PIC in COVID-19 infected patients should include the common coagulation profile tests (PT, APTT, fibrinogen and platelet count) but the extra markers of fibrinolysis such as D-dimer, FDPs and AT should also be performed at regular intervals. There can be marked fluctuations in some of the parameters such as fibrinogen, but in all instances there is a predictable trend in the

levels as the condition worsens, and in all non-survivors evidence of thrombosis is present (Tang *et al* 2020; Asakura and Ogawa 2021; Xiong *et al* 2020). The extent of the PIC can be further demonstrated by the additional laboratory tests such as vWF levels, ADAMTS13 activity, α2 PI, αPIC and TAT, although not all these tests are readily available in routine laboratories.

Conclusions

The pathophysiology of COVID-19 infection is complex but is known to be exacerbated by the cytokine storm resulting in an inflammatory state causing endothelial damage, hypercoagulability and suppression of the fibrinolytic system which results in a systemic thrombotic tendency. The majority of COVID-19 related deaths show evidence of thrombosis and frequent testing of these markers need to be performed to identify the course of this infection.

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COVID-19 vaccine induced (immune) thrombotic thrombocytopenia (VITT)/thrombosis with thrombocytopenia syndrome (TTS): an update

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Abstract

Coronavirus disease 2019 (COVID-19) represents a global pandemic. Several vaccines have been produced to prevent infection and/or severe sequelae associated with infection from severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Rare reports of vaccine associated thrombotic events, in particular for adenovirus-based vaccines have recently emerged. These have been termed vaccine induced (immune) thrombotic thrombocytopenia (VITT) by workers in the field and thrombosis with thrombocytopenia syndrome (TTS) by government agencies. In this review, we provide an update on VITT/TTS, including clinical features, laboratory testing, potential pathogenesis, and treatment. VITT sometimes also needs to be differentiated from heparin induced thrombocytopenia with thrombosis (HITT). In published reports to date, the key initial laboratory clues to VITT are thrombocytopenia, raised D-dimer, and reduced fibrinogen. The main clinical feature is thrombosis, potentially in, but not limited to, unusual sites such as cerebral venous thrombosis and splanchnic vein thrombosis. Confirmation of VITT requires immunological and/or functional assays for platelet factor 4 (PF4) antibodies. Immunologically, PF4 antibodies in VITT can be identified by ELISA based assays, but not by other immunological assays typically positive in HITT. In some assays, standard doses of heparin have been shown to inhibit platelet activation in suspected VITT, but tend to augment activation in HITT. Treatment of VITT involves supportive therapies such as intravenous immune globulin (IVIG) and anticoagulation with a non-heparin anticoagulant.

Keywords: *vaccine induced (immune) thrombotic thrombocytopenia, vaccine associated thrombotic thrombocytopenia, thrombosis with thrombocytopenia syndrome, platelet factor 4 antibodies, laboratory testing, COVID-19*

Introduction

COVID-19 is a recognised global pandemic caused by infection with SARS-CoV-2. This infectious disease is believed to have originated in Wuhan, China, in late 2019, and at time of writing has infected over 189 million people and caused over 4 million deaths (COVID-19 Dashboard, 2021). COVID-19 appears to affect all facets of hemostasis, including primary hemostasis, secondary hemostasis and fibrinolysis (Favaloro *et al* 2021a; Levi and Thachil 2021; Kwaan 2021; Larsen *et al* 2021). In turn, severe COVID-19 primarily reflects a prothrombotic disorder (Lippi *et al* 2021; Thachil and Srivastava 2021; Schulman 2021; Di Minno *et al* 2021; Jenner *et al* 2021; Uaprasert *et al* 2021), potentially arising from disturbances in immune response (Lippi *et al* 2021). Indeed several autoimmune events have also been associated with COVID-19, including presence of antiphospholipid antibodies (Favaloro *et al* 2021b; Favaloro *et al* 2021c) and generation of platelet factor 4/heparin (PF4/H) antibodies (Favaloro *et al* 2021d). Only a portion of identified PF4/H antibodies in COVID-19 are actually associated with the condition called heparin induced thrombocytopenia (HIT), and fewer still are clearly associated with thrombosis (HITT) (Favaloro *et al* 2021e). Of relevance to the current review, a HITT-like syndrome has also been reported in patients who have been vaccinated against COVID-19, and primarily using adenovirus vaccines such as those produced by AstraZeneca (AZD1222, ChAdOx1-S) and Johnson & Johnson/Janssen (Ad26.CoV2.S). This pathological condition has been given many names (Table 1), but two terms are most commonly used: (1) VITT for Vaccine Induced (immune) Thrombotic

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Thrombocytopenia, as used by workers in the field, and (2) TTS, for Thrombosis with Thrombocytopenia Syndrome, which is the term favoured by most official reporting agencies, including the CDC (Centers for Disease Control [and Prevention]) in the USA, the EMA (European Medicines Agency), and TGA (Therapeutic Goods Administration) in Australia. As workers in this field, we will preferentially use the term VITT throughout this review, except where referring to data reported from official reporting agencies, where the term TTS will be used. We do so, in part because VITT is more descriptive of the condition under investigation, and in part because it links to the separate, but presumed pathophysiologically related condition termed HITT. Also, we and other peer clinicians/scientists do not preferentially use the term TTS, as it does not specifically reference any vaccine association, and because the term can encompass any condition where thrombosis can be associated with thrombocytopenia, including HITT, catastrophic antiphospholipid (antibody) syndrome (CAPS), and thrombotic thrombocytopenia purpura (TTP).

Incidence of suspected VITT

It is not possible to be accurate regarding the true incidence of suspected VITT. First, there are limited scientific publications. In a recent review, current as of May 28 2021, a total of only 81-133 cases had been reported in the scientific literature from a total of 16 studies, mostly case studies or small case series (Favaloro 2021). The uncertainty in total published case numbers relates to likely duplication of cases in at least four publications. Most of the original reports have been in women (F:M ~ 3:1), and mostly 'younger' cohorts, although this association is likely biased due to the vaccinations being initially targeted to health care workers, which in Europe were mostly young women.

In Australia, the latest report from the TGA identifies 83 cases of TTS (51 'confirmed' and 32 'probable') from approximately 5.4 million administered doses of the AstraZeneca vaccine, which would therefore estimate the incidence at around 1 in every 65,000 vaccinated people (TGA COVID-19 vaccine weekly safety report 15/07/2021). Similarly, the EMA has estimated the incidence of TTS at around 1 in every 100,000 vaccinated people (European Medicines Agency News 23/04/2021). The incidence from the Johnson & Johnson vaccine may be lower, since a recent safety monitoring report from the CDC reported 17 cases of TTS from 7.98 million administered doses, or around 1 case in every 500,000 doses (Shay *et al* 2021).

It can be noted that the number of cases of TTS in Australia has risen over recent past months, as shown in Figure 1, using data reported by the TGA in its weekly reports. Although the apparent incidence also seems to have increased over time, this is most likely due to increasingly earlier and better case detection. At time of writing, the Australian Technical Advisory Group on Immunisation (ATAGI) recommendation is that the COVID-19 Pfizer vaccine (Comirnaty) is the preferred vaccine for those aged 16 to under 60 years (ATAGI statement on revised recommendations on the use of COVID-19 Vaccine AstraZeneca, 17 June 2021), based on their analysis of relative risks and benefits of vaccination vs COVID-19.

In a further assessment, Figure 2 shows the total number of TTS cases as reported by the TGA, according to Australian State or Territory. Most cases have been identified from Victoria and NSW. Although the numbers somewhat align to population numbers in each state, this is not entirely consistent with expectations based on populations and assuming proportional numbers of Australians are being immunised in each state. We are unable to provide any further analysis according to immunisations in each state, since this data is not available to the public. However, it may also be that Victoria, representing the first state in which a case of VITT was identified (Hocking *et al* 2021) is most attuned to the possibility of TTS post vaccination, and thus is more proactive in data collection.

As a further comparator the TGA also provides data of overall adverse events as reported to the TGA and as a proportion of vaccine doses (both AstraZeneca and Pfizer) according to State/Territory, as shown in Figure 3. Here the leading states are Tasmania and Victoria, with other states perhaps under-reporting events.

Clinical manifestations

The initial reports on VITT appeared to identify unusual sites of thrombosis. For example the first peer-review case series published (Greinacher *et al* 2021b) reported on 11 cases, nine of whom had cerebral venous (sinus) thrombosis (CVT or CVST) and three of whom had splanchnic vein thrombosis. More common thromboses such as pulmonary embolism (PE) were nevertheless also seen in some cases. The next few case series publications (Schultz *et al* 2021; Scully *et al* 2021; Tiede *et al* 2021) also appeared to identify a predominance of unusual and catastrophic thromboses, including cerebral. Such cases were also associated with high mortality rates ranging from 30-60%. It is entirely plausible however that VITT can be found in patients with thrombosis seen more typically in clinical practice, such as PE and deep vein thrombosis (DVT). Why such cases of VITT were not reported in the early literature may be that these common thrombotic presentations, seen frequently in emergency departments, would not have been fully investigated for the syndrome as the association with the vaccine would not have been pursued. For added context, the current Australian fatality for TTS is 2.4% (two fatal cases from 83 confirmed or probable) (TGA COVID-19 vaccine weekly safety report 17/07/2021).

Table 1. *Terms used to describe post COVID-19 vaccine associated thrombosis with thrombocytopenia.*

Abbreviation	Stands for	Comments
VIPIT	Vaccine Induced Prothrombotic Immune Thrombocytopenia	Original term reported by German researchers (Greinacher *et al*, 2021a).
VITT	Vaccine Induced Immune Thrombotic Thrombocytopenia or Vaccine Induced immune Thrombosis with Thrombocytopenia	Term used in subsequent reports by the German group (Greinacher *et al*, 2021b) and separate case series by Norwegian (Schultz *et al*, 2021) and UK (Scully *et al*, 2021) based groups publishing in the NEJM. Reflects a variation of the abbreviation HITT, representing the similar pathological condition of 'heparin induced thrombocytopenia with thrombosis'.
VATT	Vaccine Associated (immune) Thrombotic Thrombocytopenia (or Thrombosis with Thrombocytopenia)	A term supported by some researchers concerned with the terminology 'induced' where a clear pathological link to the vaccine is not clear. A broader term excluding 'immune' potentially captures other thrombotic events associated with thrombocytopenia and vaccine use, but where an immune relationship is unclear.
TTS	Thrombosis with Thrombocytopenia Syndrome	A term favoured by official reporting agencies that does not specifically reference any 'vaccine' association. Term not typically utilised by researchers for the condition associated with COVID-19 vaccine use, since essentially can encompass any condition where thrombosis can be associated with thrombocytopenia.

For suspected VITT, the expected clinical manifestations would align to the presenting clinical entity. For example, for CVT, the most common symptom will be severe headache, particularly one that does not settle with analgesics such as paracetamol. For splanchnic vein thrombosis, the presenting symptoms would include severe gastrointestinal pain, for PE shortness of breath, and for DVT swelling and leg pain (Table 2).

Pathogenesis

Although VITT is described as HITT-like, the clinical and laboratory features of VITT are most similar to those of rare cases of spontaneous HIT-like autoimmune thrombosis with thrombocytopenia in patients not receiving heparin (Warkentin and Greinacher 2021). Such events can occur following a number of presumed triggers including infections and certain types of surgery. VITT appears to be caused by antibodies that target PF4 bound to platelets. These antibodies activate platelets, independent of heparin, through interaction with receptors on the platelet surface that bind the Fc portion of IgG (FcγIIa). Platelet activation, with a possible contribution from other cells such as neutrophils and monocytes, triggers marked activation of the coagulation system and a highly hypercoagulable state resulting in platelet consumption (thrombocytopenia) and thrombosis. While thrombosis in so called typical sites (lower limb DVT and PE) is encountered in VITT, predilection for thrombosis in unusual sites, such as the cerebral venous sinuses and splanchnic veins is a distinguishing feature of VITT, albeit without a clear pathophysiological explanation at present.

Initial investigation of VITT

The initial investigation of VITT requires an assessment of whether thrombosis is present, which would typically

***Table 2**. Typical presenting symptoms for different thromboses.*

Typical presenting symptoms	Presumed site of thrombosis	Diagnostic imaging
Persistent, intense headache Nausea and vomiting Visual changes Change in mental status	Cerebral venous sinuses	Magnetic resonance venography CT brain combined with contrast enhanced CT cerebral venography
Severe abdominal pain Back pain	Splanchnic veins (portal, splenic, mesenteric)	Intravenous contrast enhanced CT of the abdomen and pelvis Doppler ultrasound
Shortness of breath Chest pain	Pulmonary embolism (PE)	CT pulmonary angiography Ventilation/perfusion scan
Leg pain Leg swelling/oedema +/- colour change	Deep vein thrombosis (DVT) of the leg	Doppler ultrasound
Limb pallor and coldness Limb pain	Acute arterial thrombosis/ischaemia	CT angiography Catheter-based angiography

require scans/imaging of the areas of interest (Table 2). These are typically organised by the emergency department or a haematologist (thrombosis clinic), depending on where the patient initially presents. Blood should also be taken for full blood count (FBC), in particular to assess platelet count, and also for D-dimer, a breakdown product of fibrin clots, as a measure of thrombosis. Potentially useful also is estimation of fibrinogen, which is low in most patients with VITT (Favaloro 2021). However, most valuable and potentially suggestive of VITT is thrombocytopenia (i.e. platelet count < $150x10^9$/L) and a highly raised D-dimer (often >5x the upper limit of normal) (Favaloro 2021). Based on these first tier test findings, the patient may then be further assessed by second and third tier tests, specifically for PF4 antibodies by immunological and/or functional assays. In Australia, this investigative process is being coordinated by the Thrombosis and Haemostasis Society of Australia and New Zealand (THANZ) VITT Advisory Group (https://www.thanz.org.au).

Confirmation or exclusion of VITT

According to guidance issued by the THANZ VITT advisory group, VITT is confirmed by positive anti-PF4 ELISA, and positive platelet based functional testing in cases of suspected VITT. VITT is unsupported in the case of negative anti-PF4 ELISA, AND negative platelet based functional testing in cases of suspected VITT. Other gradings in regards to VITT may be possible, depending on th findings of ELISA and functional testing, as summarized in Table 3.

Table 3. *Classification of suspected VITT cases following specific VITT testing. Summarized from the latest THANZ Advisory Statement (https://www.thanz.org.au/news/suspected-vaccine-induced-prothrombotic-immune-thrombocytopenia-vipit-thanz-advisory-statement-check-for-weekly-updates).*

Confirmed	positive anti-PF4 ELISA, AND positive platelet based functional testing in cases of suspected VITT
Strongly supported	strongly positive anti-PF4 ELISA, OR positive by platelet based functional testing in cases of suspected VITT
Possible	VITT remains possible in suspected VITT where anti-PF4 ELISA is positive, but functional platelet based functional testing is negative,
Unsupported	negative anti-PF4 ELISA, AND negative platelet based functional testing in cases of suspected VITT

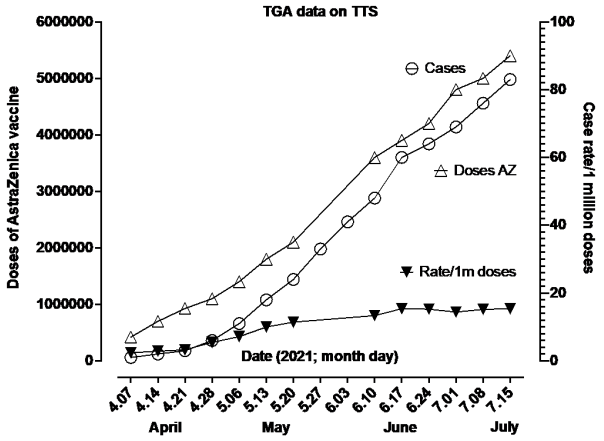

Figure 1. *Data on vaccination and TTS as available from the TGA. Figure shows numbers of AstraZeneca (AZ) vaccine doses delivered to Australians from April to July (as current at time of writing) in millions (left y-axis) as well as the number of TTS cases identified by the TGA over the same time period (right y-axis). This permits calculation of the incidence of TTS as rate of TTS per million doses of AZ vaccine (right y-axis). The rate appears to increase over time, but this is likely due to better and earlier detection of cases as the condition was increasingly recognised.*

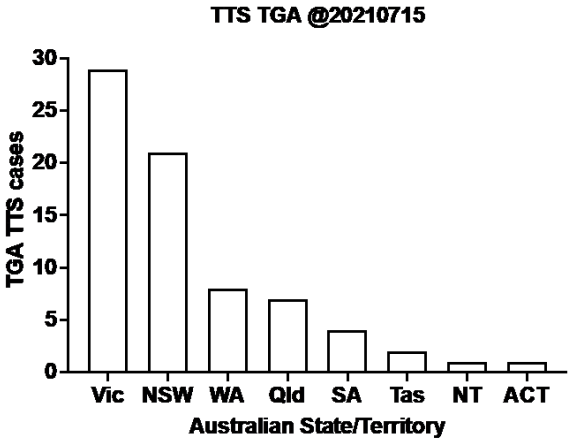

Figure 2. *Data on current total numbers of TTS cases, both 'probable' and 'confirmed', using data reported by the TGA. Figure shows TGA reported TTS cases (left y-axis) according to Australian State or Territory. Current as at 15th July, 2021.*

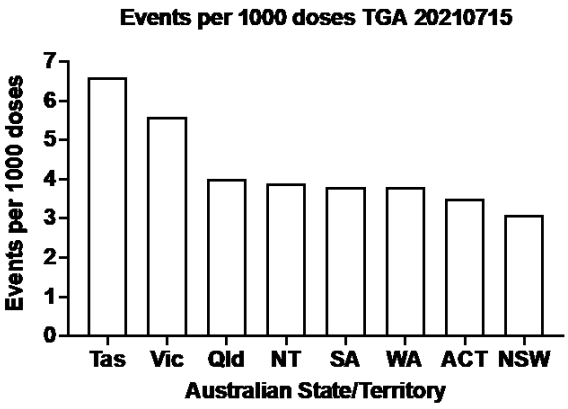

Figure 3. *Data on adverse vaccine related events using data reported by the TGA. Figure shows events per 1000 doses (left y-axis) according to Australian State or Territory. Current as at 15th July, 2021.*

Laboratory based differentials between VITT and HITT

In VITT, PF4 antibodies identified in VITT are consistently only detected by ELISA, with in-house and six commercial methods used in the published studies (Favaloro 2021). Other methods typically used for assessment of PF4/H antibodies, and normally positive in HITT, including latex immunoassay (LIA), chemiluminescence (CLIA), lateral flow (STiC) and gel-based (PAGIA) assays are mostly negative. Thus, VITT is different to HITT, where all these assays are able to detect the PF4 antibodies.

Another interesting observation has been the effect of heparin on ELISA and functional platelet-based test results. In HITT therapeutic levels of heparin generally augment the detection of PF4/H antibodies, whereas in VITT therapeutic levels of heparin often dampens the ability of assays to identify VITT associated PF4 antibodies (Favaloro 2021).

Treatment of VITT

Treatment of VITT evolved rapidly since the first published case series as a result of unprecedented information sharing of various expert groups across the world. The general experience with management of HIT has served as a very useful template for treatment of VITT. Rapid initiation of systemic non-heparin anticoagulation is essential in arresting the often rapidly progressive thrombotic process, which if not treated promptly can involve multiple vascular beds, both venous and arterial, and lead to multiple organ failure and major morbidity and death. The choice of anticoagulant should be guided by a haematologist with expertise in haemostasis and thrombosis. This will be based on the clinical status of the patient, presence/risk of bleeding, anticipated need to stop anticoagulation, and local availability and experience. The main current options are parenteral direct thrombin inhibitors (bivalirudin or argatroban), other parental agents (fondaparinux or danaparoid) and direct oral anticoagulants. Avoidance of heparin was recommended empirically early because of the resemblance of VITT to HITT and reports that some patients treated with heparins suffered clinical deterioration, including death. However with the increasing understanding of the pathophysiology of VITT, heparin may ultimately be found to be a reasonable choice in some patients or some remote settings. Along with anticoagulation high-dose intravenous immune globulin (IVIG) is recommended based on experience with autoimmune HIT, as a way of blocking ongoing platelet activation by VITT antibodies. This is particularly important for cases with severe fulminant thrombosis unresponsive to anticoagulation alone. Platelet transfusions should be avoided, but may be considered in individual patients with serious bleeding and/or need for surgical intervention, after consultation with a haemostasis/thrombosis expert. Similarly, in patients with very low fibrinogen and/or bleeding, replacement of fibrinogen should be considered. Additional therapies, including immunosuppression and plasma exchange have been described in case studies, but there is no wide consensus on their role in management of most patients with VITT, and therefore should be reserved for refractory disease. Anticoagulation duration is unknown but it should probably be limited to 3-6 months, after normalisation of the platelet count. Some expert groups recommend checking for resolution of VITT antibodies prior to cessation of anticoagulation.

Conclusion

VITT/TTS represents serious clinical events that occur in a small proportion, perhaps 1 in 100,000 or so, of people vaccinated with COVID-19 adenovirus-based vaccines (i.e. AstraZeneca, Johnson & Johnson/Janssen). Nevertheless, it is unclear if there is a single immune related event occurring in all cases, given that some cases have shown clinical signs of suspected VITT without positive PF4 antibodies by ELISA, and also given the variety of emerging clinical features, which are much broader than those initially identified. Although a direct vaccine mediated mechanism remains unproven, enhancement of platelet activation by addition of vaccine has been shown in some studies. There are a variety of plausible mechanisms by which adenovirus vaccines could be implicated in VITT/TTS (Makris *et al* 2021; Douxfils *et al* 2021; Dotan and Shoenfeld, 2021), but most cases have shown development of anti-PF4 antibodies

capable of activating platelets and causing thrombosis in a manner similiar to that seen in HITT.

In terms of laboratory tests, the key initial findings in suspected VITT are thrombocytopenia, highly raised D-dimer, potentially reduced fibrinogen, and in almost all patients the presence of PF4 antibodies detected by ELISA assay.

Finally, not all thrombocytopenia post vaccination will be VITT. For example, there have been several cases of apparent secondary immune thrombocytopenia (ITP) after SARS-CoV-2 vaccination with both the Pfizer and Moderna vaccines (Lee *et al* 2021). There has also been a recent case proposed to reflect the first case of VITT caused by a non-adenovirus vaccine (Moderna; Messenger RNA-1273) (Sangli *et al* 2021; Pishko and Cuker, 2021).

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Competing interest

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ORIGINAL ARTICLE

Scaling up COVID-19 testing – a regional experience and perspective

Fleur Francis

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Abstract

The demand for pathology testing for the coronavirus (SARS-CoV-2) has placed a huge demand on pathology laboratories throughout Australia and the world. Not only is the demand unpredictable but there has been uncertainty with reagent and supplies availability, the instrumentation has struggled to analyse the samples within an acceptable timeframe, and staff has been overloaded with the sheer volume of tests. Our regional laboratory in north Queensland has quickly adapted to these demands with the support of all the dedicated staff, the Pathology Queensland organisation, and the Queensland Government.

Keywords: molecular testing, coronavirus, COVID-19, PCR

Introduction

There is an ominous feeling in our laboratory right now, it feels just like we are in the eye of a cyclone. The chaos has passed for the moment, it is calm again and we can see a patch of sunshine through the dark clouds. There is a sense of foreboding however for the storm will inevitably return. But this is not a cyclone, this is the threat of the coronavirus pandemic and another deluge of swabs may be just around the corner.

In the tropical north of Australia, we have considerable experience in disaster management as during summer our communities can become isolated very quickly by extreme weather events such as tropical cyclones. Roads can be cut for days, even weeks, and flights in and out of our region may be delayed or cancelled. We have therefore been dealing with seasonal isolation and logistics issues for decades and we are used to solving problems in an unpredictable environment. The disruptions caused by the pandemic however have been more severe and sustained.

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Just like the early warnings broadcast by the Bureau of Meteorology about developing tropical weather systems, in early 2020 the media kept us regularly informed of the developing threat as the coronavirus (SARS-CoV-2) pandemic spread around the globe. Every year prior to the wet season we plan, prepare and organise ahead of time to be "disaster ready". So we applied our disaster preparedness plans to this new threat to our community. Various disaster management committees were formed in our hospital and health service and many of these included our laboratory management team. We came together to plan and prepare with our hospital, city council, public health, emergency services, and other key stakeholders to tackle this new threat together as a cohesive community. Our organisation, Pathology Queensland, quickly recognised our pivotal role in the response to this pandemic and formed a "COVID Response Team" to direct our state-wide coordinated response and to communicate essential information. We consequently found ourselves in the thick of things locally and in a state-wide forum.

In early 2020, we were the only regional laboratory in Queensland to offer a comprehensive molecular service, the other molecular hub located in Brisbane. Our primary client is our large regional hospital, and our other clients include the many smaller regional hospitals in North Queensland, the sexual health clinics, plus Corrective Services, and some general practice clinics. Specimens are often transported to our laboratory over long distances for we serve a large area of north Queensland, from the Torres

Strait in the north, to Mackay in the south, and west to Mt Isa. Our laboratory has had experience in scaling up testing in the past, such as in 2009 for swine flu, and in 2016 for the Zika virus, but the current coronavirus pandemic has required ramping up on a much larger scale and this has presented new challenges.

Because pathology testing is the only definitive method of diagnosing cases of COVID-19 there has been a massive demand for laboratory testing throughout the world. Never before has pathology had to respond so urgently in a sustained manner. In the beginning, there were no tests available for the newly discovered virus, but in-house (laboratory developed) polymerase chain reaction (PCR) tests to detect the coronavirus were swiftly developed and implemented in pathology laboratories around the world. As of the 1st of August, over 23 million COVID-19 tests have been performed in Australia (KPKH 2021).

Methods

In late January, we were asked by our organisation to order primers and probes to set up our own in-house PCR. This PCR assay was based on a WHO method (Corman *et al* 2020) that targeted the region of the new virus' genome that codes for the envelope (E) gene. The assay had been modified to fit into our standard protocol for our other in-house PCRs and involves the following steps: sample preparation, nucleic acid purification, addition of purified samples to reagents, and then performance of the PCR to amplify and detect a unique sequence of the viral genome (in the region encoding the E gene). Our own PCR reagents (MasterMix) are first prepared using the new primers and probes. Then the nucleic acids in each sample are purified using a robotic instrument (MagNA Pure 96, Roche Diagnostics) that had been purchased for our laboratory by Queensland Health in 2016 to assist with Zika virus testing. This high-throughput instrument had not only given us the capacity to process hundreds of samples for Zika virus detection, but it had also improved the turnaround times for all of our in-house assays since then and it became essential for our coronavirus testing. The next step is loading the sample extracts and our homemade coronavirus MasterMix into test wells using a liquid handling robot (QIAgility, Qiagen) and then the PCR is performed using a Rotor-Gene Q (Qiagen) real-time PCR cycler. At the end of this process, scientists are required to analyse the data from the PCR run to determine if the virus has been detected in the samples. The entire process takes approximately 4 ½ hours to complete. After thorough planning, preparation, and verification testing, we started testing patient samples using the new assay in the first week of March 2020, and we became the first laboratory in regional Queensland to perform molecular testing for SARS-CoV-2.

As test numbers rose swiftly, we identified two bottlenecks in our in-house process. We were fortunate that Queensland Health supported us once again and gave us another robot to load the samples and reagents into test wells and another real-time PCR cycler. Both of these instruments significantly improved our SARS-CoV-2 testing workflow.

While labs around the world were developing and implementing their "in-house" PCR assays for the new virus, commercial suppliers of molecular assays had also been busy developing tests for their systems. Our laboratory had a small, cartridge-based rapid molecular instrument (GeneXpert, Cepheid) which had been placed in our laboratory in 2018 to facilitate rapid Influenza and Respiratory Syncytial Virus (RSV) testing after hours. These types of "desktop" molecular analysers are used in many large and small laboratories throughout Queensland, as well as in countless labs in Australia and around the world, for the rapid molecular detection of many different pathogens. Cepheid soon developed a SARS-CoV-2 specific test cartridge for the GeneXpert and it became available to us in late April 2020. As there was an overwhelming global demand for the cartridges they were in limited supply, so this test was reserved for very select cases only in our laboratory and required Clinical Microbiologist authorisation. While the individual test is rapid (results in less than an hour) our small instrument does not have the capacity for high-throughput testing, so our in-house E gene PCR remained our primary test method due to the ability to test large numbers of samples per day.

In early 2020 transport networks had been disrupted, with few planes flying, and road transport restrictions were affecting our usual specimen transport systems. The isolation and disruption were handled with our usual "can do" attitude. Specimens were transported to our laboratory from afar by buses, couriers, and any other form of transport we could rustle up, including our own vehicles. For example, specimens were driven down from Cairns daily, stopping at Cardwell (about halfway between Cairns and Townsville) where they would be handed over to a courier from Townsville to be brought down to our laboratory for testing. Similar solutions were arranged for specimens to be transported in from the Mackay and Mt Isa regions.

Meanwhile, local drive-through testing clinics were being established in our city and in various other towns in our region, so our test numbers were rising rapidly. It was challenging to maintain reasonable turnaround times for results using our in-house assay and automation of the testing process became essential to keep up with the workload. Fortunately, we had an existing high-throughput, a fully-automated molecular analyser in our laboratory (the Panther Fusion system, Hologic) that we had been using for respiratory virus detection since 2018. Hologic developed

an assay to detect SARS-CoV-2 using the Panther Fusion, which we commenced running in early May 2020. Being a fully-automated and high-throughput analyser, this revolutionised our local test process as it required minimal hands-on time and samples could be loaded continuously. This was more efficient than our in-house assay which required much greater hands-on time and batching of samples.

The Panther Fusion, and other similar "sample to result" molecular analysers, are simple to use and do not need to be located in a dedicated molecular laboratory, so more of these types of analysers (Panther Fusion system, Hologic, and the Cobas 6800 system, Roche Diagnostics) were funded by the Queensland Government and installed in hospital laboratories across our vast state. By the end of the roll-out process these instruments had been installed in 11 metropolitan and regional laboratories in Queensland, reducing the need to transport COVID-19 samples over long distances to testing labs, thus improving turn-around times for results around the state. Two of the regional laboratories that had previously referred their COVID-19 specimens to us had these high-throughput instruments installed, which helped with our workload and reduced their turn-around times. In addition, 34 Pathology Queensland laboratories now had GeneXpert instruments. Late last year four additional high-throughput fully-automated molecular analysers (Alinity m, Abbott Molecular) were installed in hospital laboratories around Queensland, including at our laboratory. Having two high-throughput molecular analysers has further increased testing efficiencies in our laboratory and has given us the much needed capacity to manage the increased workload when testing surges occur.

Another advantage of having numerous assays on site is that we can quickly confirm a positive result, for we have assays that target numerous different regions of the SARS-CoV-2 genome. For example, if a sample gives a positive result in one assay, we can then rapidly test the same sample using a combination of our other assays that have different targets. If the sample gives positive results for additional targets of the SARS-CoV-2 genome we can confidently and promptly report the positive result, avoiding the delay that can be caused by referral of the sample to a reference laboratory for confirmatory testing.

Discussion

The coronavirus pandemic has created an enormous amount of change in our laboratory, yet we have managed to handle all of this whilst maintaining our routine molecular service to our regular clients. I believe this is in part due to good planning and preparation and our experience in solving problems in an unpredictable environment, but also due to the steadfast support that we have received from many sources. For example, our organisation (Pathology Queensland/Queensland Health) has listened to our needs and has provided the resources to enable us to rapidly adapt and scale up our testing. We have been given more staff to work in our molecular department and more state-of-the art technology to perform the tests. They have developed and implemented an SMS result system and have provided call center support. They also frequently communicate with suppliers on our behalf, they help us to solve logistics issues, and much, much more. We are extremely grateful for the assistance that this highly organised team of innovative thinkers continues to provide to us.

Through our frequent state-wide "COVID Response" online meetings we have become even more connected with the other laboratories in our organisation throughout Queensland, enabling us to establish closer collaboration and to share our knowledge and experiences as we tackle the pandemic response together. This collaboration has facilitated a very well-coordinated state-wide response through our network of 36 laboratories. We have shared reagents and consumables between laboratories when the global supply chain has been disrupted and when some labs have experienced a significant surge in testing, and we have been able to refer some of the specimens to other labs around the state who have the capacity to take on the extra work, to ease the burden on each other. The various private laboratories around our great state have likewise stepped up to help during the pandemic, by opening pop-up clinics when needed and also performing massive numbers of tests. As the saying goes "we've got each other's backs".

At a local level, we have been superbly supported by our own laboratory staff during the hectic past 18 months. Our Specimen Reception team members have been working hard at registering all of the swabs, following up on labelling errors, and triaging specimens based on clinical urgency, all the while still providing the same service to the many other departments in our large regional laboratory. Our administration staff has been answering countless calls for results, effectively taking the pressure off those of us in the laboratory performing the testing. Our highly organised stores staff have ensured that the stock we have needed have been ordered and distributed as efficiently as possible which enables us to keep testing the samples. They have also been thinking outside the square to find places to store all of this stock, including converting an office into another storeroom.

Our microbiology department has two staff who are multiskilled in molecular techniques, and they have

generously given of their time when we have needed help. For example, there have been numerous occasions when they have finished their shift in microbiology and then they have worked additional hours helping with our molecular workload. The microbiology department and our anatomical pathology department are our closest neighbours in our laboratory and both departments have graciously relinquished some of their precious bench space so that our molecular department can expand.

Our local management team has provided strong leadership and support. We have appreciated their rational decision-making and common-sense approach during these uncertain times. For example, our clinical microbiologists have acted as a conduit between our laboratory and our clients. They have managed the expectations of all stakeholders by providing clear and consistent communication to us and our hospitals, infection control teams, public health, and various other clients. Effective communication is key to giving our clients the service that they require. In addition, our laboratory manager has actively listened, carefully analysed, and provided solution-based leadership to enable and empower us to consistently achieve our goals.

On a personal note, I am extremely proud of my marvellous molecular team. There have been frequent and major changes to our workload, workflow, team makeup, processes, client expectations, and more, yet they have adapted well and have remained goal focussed. We have kept each other motivated, remaining positive and optimistic even through the tough times. I have encouraged the team to try to include a bit of enjoyment in our workdays to avoid burnout during these unpredictable and challenging times. We had some cheeky fun at our 2020 laboratory Christmas lunch, when we all dressed as reindeer, complete with antlers and face painting. One of the team designed unique t-shirts for our molecular team that we all wear with pride. This same staff member encouraged everyone to bring a picture of their pet to work, and the pictures are now displayed in our department for all to see. So while we may not be able to bring our furry friends into the laboratory, a glance at their picture on the wall gives us a warm and fuzzy feeling and some comfort on tough days. Another team member has enviable cooking skills and we look forward to those days when she gets up early to bake delicious treats for us to share. Kind and caring gestures like these have created cohesion in our department during these challenging times.

Conclusion

Over the past 18 months, the coronavirus pandemic has presented countless challenges to pathology laboratories around the world, requiring us to rapidly develop and implement tests for the newly discovered virus and to scale them up swiftly to cope with the unprecedented and unpredictable workload. In our regional laboratory, the key to our success has been our outstanding staff and their "can-do" attitude, supported by a wide range of people and organisations. I am proud to work with such a hard-working, flexible, adaptive, committed, conscientious, supportive and caring bunch of people. We are extremely grateful for the staunch support from our organisation and from the amazing team of colleagues we have in our laboratory. It is a privilege to work together to serve our communities.

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Know Pathology Know Healthcare 2021. https://knowpathology.com.au/covid-19-testing-thank-you-pathology/ Accessed 10 August 2021

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Journal-based CPD No. 78

Page 1 of 1

Questions relating to the article *'Coagulopathy in COVID-19'* at page 79 of this issue.

1.	COVID-19 affects most of the body systems through the ensuing cytokine storm and coagulopathy is a consequence.	True/False
2.	Effects on multiple components of haemostasis contribute to this coagulopathy – increased fibrinogen due to the acute phase reaction; endothelial injury and activation; platelet activation; loss of coagulation cascade homeostasis and downregulation of the fibrinolytic system.	True/False
3.	COVID-19 is the newest member of the betacoronavirus family.	True/False
4.	This infectious disease is thought to have originated in Wuhan China in late 2019.	True/False
5.	In the first three months after COVID-19 emerged, nearly 1 million people were infected and 50,000 died.	True/False
6.	To over 200 million people infected and 4.2 million of these have died (NIAID 2020 COVID-19 dashboard at CCSSE at JHU 2021).	True/False
7.	It infects vascular endothelial cells and also targets lung epithelial cells and lymphocytes, resulting in the clinical presentation of acute respiratory distress syndrome (ARDS), shock and coagulopathy in severe cases (Guan *et al* 2020).	True/False
8.	A cytokine storm is often triggered, leading to a life-threatening systemic inflammatory syndrome involving elevated levels of circulating cytokines, immune-cell hyperactivation and multi-organ dysfunction.	True/False
9.	Dysplasia manifesting as hyposegmented neutrophils with coarsely clumped chromatin and dark cytoplasmic granulation as well as immature granulocytes.	True/False
10.	The tests performed were PTC, APTCT, fibrinogen, D-dimer, fibrin degradation products (FDPN) and antithrombin (ATH).	True/False

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Journal-based CPD No. 79
Page 1 of 1

Questions relating to the article *'COVID-19 vaccine induced (immune) thrombotic thrombocytopenia (VITT)'* at page 86 of this issue.

1.	Confirmation of VITT does not require immunological and/or functional assays for platelet factor 4 (PF4) antibodies.	True/False
2.	Treatment of VITT involves supportive therapies such as intravenous immune globulin (IVIG) and anticoagulation with a non-heparin anticoagulant.	True/False
3.	Several autoimmune events have been associated with COVID-19, including presence of antiphospholipid antibodies.	True/False
4.	HITT-like syndrome has also been reported in patients who have been vaccinated against COVID-19, and primarily using adenovirus vaccines such as those produced by AstraZeneca (AZD1222, ChAdOx1-S) and Johnson & Johnson/Janssen (Ad26.CoV2.S).	True/False
5.	The incidence from the Johnson & Johnson vaccine may be lower, since a recent safety monitoring report from the CDC reported 17 cases of TTS from 7.98 million administered doses, or around 1 case in every 500,000 doses (Shay *et al* 2021).	True/False
6.	The initial reports on VITT appeared to identify unusual sites of thrombosis.	True/False
7.	The current Australian fatality for TTS is 2.4% (two fatal cases from 83 confirmed or probable) (TGA COVID-19 vaccine weekly safety report 17/07/2021).	True/False
8.	For splanchnic vein thrombosis, the presenting symptoms would include severe gastrointestinal pain, for PE shortness of breath, and for DVT swelling and leg pain.	True/False
9.	VITT is not caused by antibodies that target PTF4 bound to platelets.	True/False
10.	VITT is confirmed by positive anti-PTF4 ELISA, AND positive platelet based functional testing in cases of suspected VITT.	True/False

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Following is a list of books available for review by resource consultants and members of the Institute with particular expertise in the field. The reviewer is invited to retain the complimentary copy of the book once the review is received.

As per our agreement with the book publishing companies, complimentary books are submitted to the Institute provided that all reviews are published in the Australian Journal of Medical Science. These reviews must be of a high quality as buying decisions and the reputation of the book and author are important considerations.

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1. **Bifidobacteria: Genomics & Molecular Aspects** edited by B. Mayo, & D. Van Sinderen. Caister Academic Press. xii + 260 pages.

2. **Medicine and Sport Science Volume 55: Cytokines, Growth Mediators & Physical Activity in Children during Puberty** edited by J. Jurimae, A.P. Hills & T. Jurimae. Karger. viii+178 pages.

3. **Digestive Diseases The Keys to IBD 2010: Treatment, Diagnosis & Pathophysiology.** Edited by G. Rogler & W. Sandborn. Karger. 188 pages.

4. **Else Kröner-Fresenuis Symposia Volume 1: Molecular Mechanisms of Adult Stem Cell Aging** edited by K.L. Rudolph. Karger. xii+108 pages**.**

5. **Endocrine Development Volume 24: Hormone Resistance and Hypersensitivity** edited by M. Maghnie, S. Loche, M. Cappa, L. Ghizzoni & R. Lorini. Karger. viii + 160 pages.

6. **Frontiers of Hormone Research Volume 41: Endocrine Tumor Syndromes and Their Genetics** edited by C.A Stratakis. Karger. xii + 187 pages.

7. **Frontiers of Hormone Research Volume 39: Kallmann Syndrome & Hypogonadotropic Hypogonadism** edited by R. Quinton. Karger. x+174 pages.

8. **Generic: The Unbranding of Modern Medicine** by Jeremy A. Greene. John Hopkins University Press. 368 pages.

9. **Human Pathogenic Fungi: Molecular Biology and Pathogenic Mechanisms** edited by Derek J. Sullivan & Gary P. Moran, Caister Academic Press. x + 342 pages.

10. **Internal Medicine: A Doctor's Stories** by Terrence Holt. Black Inc. 273 pages.

11. **Intolerant Bodies: A Short History of Autoimmunity** by Warwick Anderson and & Ian R. Mackay. John Hopkins University Press. 250 pages

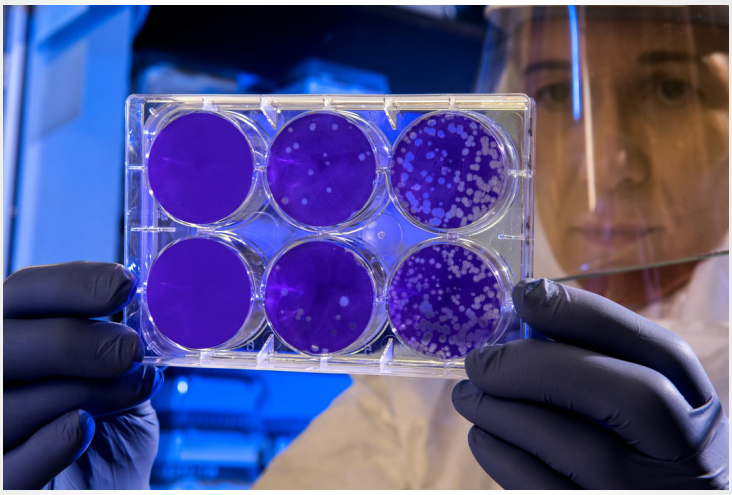
12. **Lyme disease and relapsing fever spirochetes** edited by Justin D. Radolf and D. Scott Samuels. Caiser Academic Press. 760 pages

13. **More Than Hot: A Short History of Fever** by Christopher Hamlin. John Hopkins University Press. 400 pages.

14. **Pediatric and Adolescent Medicine Volume 19: Metabolic Syndrome and Obsesity in Childhood and Adolescence** edited by W. Kiess, M. Wabitsch, C. Maffeis, A.M. Sharma. Karger. x + 202 pages.

15. **Phage Therapy - Current Research and Applications** edited by Jan Borysowski, Ryszard Miedzybrodzki & Andrzej Gorski. Caister Academic Press. 368 pages.

16. **Shigella: Molecular and Cellular Biology** edited by William D. Picking & Wendy L. Picking. Caister Academic Press. 280 pages.



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AUSTRALIAN JOURNAL OF MEDICAL SCIENCE

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Papers published in the *AJMS* are in the form of:

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All individuals listed as authors must have made a substantial contribution to the conception and design of the study, the acquisition of data or the analysis and interpretation of data; the drafting of the article or revising it critically for important intellectual content; and final approval of the version to be published. The corresponding author must take responsibility for obtaining permission from all the authors for the submission of any version of the manuscript and for any changes in authorship.

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Number pages consecutively commencing with the title page.

Arrange the article in the following sequence:

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- Abstract and key words
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- Acknowledgements
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- Tables - each table, complete with title and footnotes, on a separate page
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Authors should ensure that their manuscript communicates their ideas and concepts simply and clearly so that the article is easily read and understood. Authors are strongly recommended to refer to the recommendations on reporting standards as outlined in the statements and checklists of the CONSORT group (see: http://www.consort-statement.org/) and similar groups such as STARD (see: http://www.stard-statement.org/). The principles outlined in these standards may be used as general guidelines and not just as applied to clinical trials and diagnostic studies.

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The title of the article should not exceed three lines (40 characters per line), including punctuation and spacing. All authors must be identified on the title page (e.g., William Smith, Susan Yeo, ..."). Where applicable, the title page should also include the name of the institution with which each author is affiliated and to which the work should be attributed. In the case of multiple authors, the name, postal address, email address, telephone and facsimile number of the author responsible for correspondence relating to the manuscript should be indicated.

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The abstract should be approximately 150 words and should make sense when read alone or in conjunction with the article. The abstract should be a concise overview that describes the important details of the article including the purpose of the study/investigation, basic procedures (study subjects/experimental animals/observational and analytic methods) and the results and principal conclusions. New and important aspects of the work and its implications may also be included. References should not be included.

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- Materials and methods
- Results
- Discussion
- References

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Clearly state the purpose of the article leading the reader from the known to the unknown. Summarise the rationale for the study and state the question to be answered as appropriate. Give only strictly pertinent references, and do not review the subject extensively.

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Present the materials and methods in a logical sequence. Describe the selection of the observational or experimental subjects (patients or experimental animals, including controls) clearly. Notification of ethics approval must be given where relevant. Identify the methods, apparatus and procedures in sufficient detail to allow other workers to reproduce the results. Give references to established methods, including statistical methods. Adequately describe new or substantially modified methods. Identify precisely all drugs and chemicals used, including generic name(s), dosage(s), and route(s) of administration. Do not identify patients or hospitals without consent.

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Present the results in the same sequence as given in the Materials and methods; use tables and illustrations where these will help the reader understand the work being presented. Do not repeat in the text all the data in the tables or illustrations.

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Indicate the new and important aspects of the study and emphasise the conclusions that follow. Do not repeat in detail data given in the Results section and do not add new data. Include in the Discussion the implications of the findings and their limitations and compare the observations to other relevant studies. Recommendations may be included if appropriate. Link the conclusions with the goals of the study and answer the experimental question stated in the Introduction. However, avoid unqualified statements and conclusions not completely supported by your data. Avoid claiming priority and alluding to work that has not been completed. State new hypotheses when warranted, but clearly label them as such.

Acknowledgements

Acknowledge individuals who have made substantial contributions to the study including technical work and financial support. Authors are responsible for obtaining consent from all the individuals acknowledged by name as inclusion may be interpreted as an endorsement of the article's contents.

References

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Throughout the body of the manuscript cite the author/s name and the publication year in parentheses as in the following examples:

(i) Research in this area (Jones 1999) …

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Examples of the correct form for references are given below:

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Osler AG 1976. *Complement: mechanisms and functions*. Englewood Cliffs: Prentice-Hall.

Editor, Compiler, Chairman as Author:

Rhodes AJ, Van Rooyen CE, comps. 1968. *Textbook of virology: for students and practitioners of medicine and the other health sciences*. 5th ed. Baltimore: Williams and Wilkins.

Chapter in Book:

Weinstein L, Swartz MM 1974. Pathogenic properties of invading microorganisms. In: Sodeman WA Jr, Sodeman WA, eds. *Pathologic physiology: mechanisms of disease*. Philadelphia: WB Saunders; 457-472.

Online documents:

National Center for Biotechnology Information. OMIM: online Mendelian inheritance in man. http://www.ncbi.nlm.nih.gov/omim. Accessed February 25, 2007.

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Number tables consecutively with Arabic numerals and supply a brief title for each. Give each column a short or abbreviated heading. Place explanatory matter in footnotes, not in headings. Explain in footnotes all non-standard abbreviations used in each table.

For footnotes, use the following symbols in this sequence:

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In preparing tables, consideration should be given to the page width of the Australian Journal of Medical Science. All tables should be prepared for publication vertically. In the text, cite each table in consecutive order, and mark in the margin of the text its approximate location.

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Photomicrographs must have internal scale markers and the magnification must be stated. Symbols, arrows, or letters used in the photomicrographs should contrast with the background.

Cite each figure in the text in consecutive order, e.g., "Figure 1 illustrates …" or "… as shown (Figure 2)". If a figure has been published, acknowledge the original source and submit with the manuscript written permission from the copyright holder to reproduce the material. Permission is required, regardless of authorship or publisher, except for documents in the public domain.

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Use only standard abbreviations (see list of commonly used abbreviations).

Avoid abbreviations in the title. The full term for which an abbreviation stands must precede its first use in the text unless it is a standard abbreviation for a unit of measurement.

Report measurements in the units in which the measurements were made. In most countries the International System of Units (SI) is standard.

Commonly used abbreviations

Abbreviation or Symbol	Standard Units of Measurement
g	gram
g	gravity
Hz	hertz
h	hour
IU	international unit
K	kelvin
kg	kilogram
L	liter, litre
m	meter, metre
min	min
M	molar
mL	millilitre
mol	mole
N	newton
nm	nanometre
p	probability
rpm	revolutions per min
s	second
wk	week
yr	year

Additional information

The following are useful sources of information. The first two publications are used by the AJMS as standard references.

Style Manual Committee. Council of Biology Editors. *Scientific style and format: the CBE manual for authors, editors, and publishers.* 6th ed. Cambridge University Press, 1994.

Style manual for authors, editors and printers. 6th ed. John Wiley & Sons Australia Ltd, 2002.

O'Connor M, Woodford FP. *Writing scientific papers in English: an ELSE-Ciba Foundation guide for authors.* Amsterdam, Oxford, New York: Elsevier-Excerpta Medica, 1975.

Day RA. *How to write and publish a scientific paper.* Philadelphia, Institute for Scientific Information Press, 1979.

Zeiger M. *Essentials of writing biomedical research papers*. 2nd ed. New York, McGraw-Hill, 2000.

Matthews JR, Matthews RW. *Successful scientific writing: a step-by-step guide for the biological and medical sciences*. 3rd ed. Cambridge, Cambridge University Press, 2007 [Also available in eBook format.]

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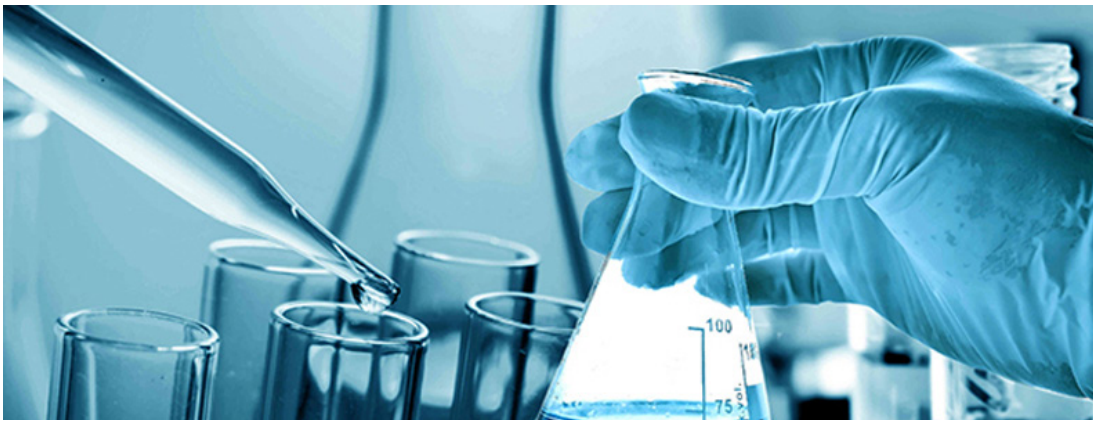

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