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Status of unconjugated bilirubin as a biomarker in a South African Long COVID cohort

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List of Abbreviations

ATP ..... *Adenosine Triphosphate*
AUC ..... *Area under the curve*
BLVRA ..... *Biliverdin reductase A*
BMI ..... Body mass index
BP ..... *Blood Pressure*
CAR ..... *Constitutive androstane receptor*
COVID ..... *Coronavirus disease*
COVID-19 ..... *Coronavirus disease 2019*, *Coronavirus disease 2019*
CRP ..... *C-reactive protein*
CV ..... *Coefficient of variation*
DMSO ..... *Dimethyl sulfoxide*
FDG-PET ..... *Fluorodeoxyglucose positron emission tomography*
FRAP ..... *Ferric reducing ability of plasma*
HIV ..... *Human immunodeficiency virus*
HMOX ..... *Heme oxygenase*
HPLC ..... *High-performance liquid chromatography*
ICU ..... *Intensive care unit*
igG ..... *Immunoglobulin G*
IgM ..... *Immunoglobulin G*
IL-6 ..... *Interleukin-6*
MRP2 ..... *Multidrug resistance-associated protein 2*
OATP1B1 ..... *Organic anion transporting polypeptide 1B1*
OATP1B3 ..... *Organic anion transporting polypeptide 1B3*
PPARα ..... *Peroxisome proliferator-activated receptor alpha*
PPARα ..... *Peroxisome proliferator-activated receptor alpha*
PXR ..... *Pregnane X receptor*
QC ..... *Quality control*
ROC ..... Receiver operating characteristic
ROS ..... *Reactive oxygen species*
RP-HPLC ..... *Reversed-phase high-performance liquid chromatography*
SARS-CoV-2 ..... *Severe acute respiratory syndrome coronavirus 2*, *Severe acute respiratory syndrome coronavirus 2*
TB ..... Total bilirubin
TBARS ..... *Thiobarbituric acid reactive substances*
UCB ..... *Unconjugated bilirubin*
UGT1A1 ..... *Uridine diphosphate glucuronosyltransferase 1A1*
UV ..... *Ultraviolet*
WHO ..... *World Health Organization*

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1. Introduction

Most people recover from coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, within weeks. Yet many continue to feel unwell for much longer, often with fatigue, “brain fog”, or shortness of breath. The World Health Organization (WHO) refers to this as Long COVID and sets the duration at least two months after the first three post-infection months (World Health Organization, 2023). Clinics still face problems in monitoring the condition. Standard blood markers, such as C-reactive protein (CRP) or interleukin-6 (IL-6), rise and fall quickly and do not always show how patients feel (Chen et al., 2022).

Unconjugated bilirubin (UCB) is the main breakdown product of haem. For decades it was mainly used to indicate liver damage or newborn jaundice. In recent years, studies have shown that slightly higher UCB can be linked to stronger antioxidant capacity, lower background inflammation and fewer cardiometabolic events (Vítek & Tiribelli, 2021a; Wagner et al., 2015). These features resemble what is seen in Long COVID, where redox stress, immune activation and mitochondrial dysfunction play a role (Kedor et al., 2022; Said et al., 2023).

Findings from acute COVID-19 also suggest that bilirubin may carry clinical information. Higher total bilirubin was associated with a longer hospital stay and a greater need for intensive care (ICU) (Liu et al., 2020). Very low bilirubin, by contrast, has been associated with poor vascular health in non-infectious groups (Hinds & Stec, 2018). Whether the unconjugated fraction shows similar signals after the acute stage is still unclear. Routine blood tests rarely report UCB, and measurements can be affected by light exposure during handling.

This thesis aims to address these gaps. UCB was measured in serum from South African adults with Long COVID using reversed-phase high-performance liquid chromatography (RP-HPLC). Samples were taken at baseline and again after twelve months. The analysis considered stability over time and possible links with age, comorbidities, lifestyle factors and clinical parameters.

2. Literature Review

2.1. Bilirubin structure

Bilirubin is a yellow-orange bile pigment that is formed as the final product of haem degradation. Two enzymes are involved in this metabolism: first, haem oxygenase (HMOX) opens the haem ring. This produces biliverdin, carbon monoxide and iron. Then, biliverdin reductase A (BLVRA) reduces biliverdin to bilirubin IXα, the main form found in humans. These steps turn the ring-shaped haem into a linear tetrapyrrole. Because of this shape, bilirubin is grouped as a porphyrinoid (Vitek & Ostrow, 2009; Wagner et al., 2015).

UCB is the main form of bilirubin in human blood. Chemically, it has a linear structure made up of four pyrrole rings (A to D), connected by three carbon (methene) bridges. These form two nearly planar units, joined in the middle by a methylene group that gives the molecule some flexibility. UCB also carries two propionic acid side chains and other functional groups like methyl, vinyl and lactam groups. Together, these features make the molecule asymmetric and optically active (Bulmer et al., 2008; Wagner et al., 2015).

In the body, UCB mostly occurs as the IXα form with a 4Z, 15Z configuration. This conformation allows the molecule to fold into a compact, ridge-like structure. Within the fold, six hydrogen bonds form between the acid groups and nearby atoms. These internal bonds hide the polar regions and help to explain why the molecule does not mix well with water, even though it has polar parts (Bulmer et al., 2008; Vitek & Ostrow, 2009). At physiological pH, the acid groups stay mostly uncharged because of their high pKa values (around 8.1 and 8.4). That keeps bilirubin in a neutral form and makes it even less water-soluble (Fevery, 2008). **Figure 1** shows the linear and folded structure of bilirubin IXα and how internal hydrogen bonds contribute to its compact shape and poor solubility.

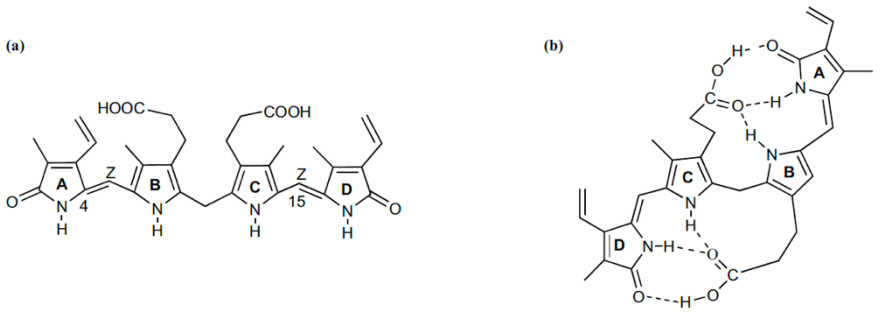

Figure 1: *Structure and folding of unconjugated bilirubin Ixα (Vítek & Ostrow, 2009)*

(a) Linear tetrapyrrole with conjugated double bonds and carboxylic acid side chains. (b) Folded 'ridge-tile' conformation stabilised by six intramolecular hydrogen bonds.

Because it does not dissolve well in water, UCB depends on transport proteins to stay in circulation. In the bloodstream, it binds tightly to serum albumin, which helps to carry it to the liver and prevents it from diffusing into other tissues. This binding is important because free bilirubin can be toxic. If bilirubin levels rise too high, for example due to liver dysfunction or increased haem breakdown, the binding capacity of albumin may be exceeded. In that case, unbound bilirubin can cross tissue barriers and enter the brain, where it may cause neurological damage (Vitek & Ostrow, 2009).

UCB is highly light-sensitive. Because of its structure with alternating double bonds, it absorbs visible light and has a yellow colour. When the molecule is exposed to blue or UV light, its shape can change – this is also known as photoisomerization. In this process, the natural 4Z,15Z form is converted into more water-soluble isomers like 4Z,15E or lumirubin. These forms do not need conjugation in the liver and can be excreted more easily (Bulmer et al., 2008; Mreihil et al., 2010).

This transformation forms the basis of phototherapy in newborns with jaundice. Light allows the infant's body to remove bilirubin even if liver function is not yet fully developed. However, the same property also matters during clinical and laboratory work. If samples are exposed to light for too long, bilirubin may isomerise unintentionally, which can reduce the accuracy of measurements (Bulmer et al., 2008). Other isomers such as IIIα or XIIIα can appear under artificial conditions, especially when samples are exposed to heat or light during storage. These forms do not occur naturally in the human body but may interfere with analytical results if not handled properly (Bulmer et al., 2008).

The structural properties of UCB, including its folding, internal hydrogen bonding and light sensitivity, influence how it behaves in the body. They determine how the molecule is transported, processed and ultimately excreted. These points, discussed by Bulmer et al. (2008), Vítek & Ostrow (2009), and others, are explored further in the next chapter.

2.2. Bilirubin metabolism and excretion

2.2.1 Transport to the liver

After its formation from haem, UCB is released into the bloodstream. Because of its low water solubility, it binds tightly to serum albumin. This complex allows safe transport to the liver and prevents free bilirubin from diffusing into tissues, where it could be toxic (Vitek & Ostrow, 2009; Wang et al., 2021).

2.2.2 Hepatic uptake and conjugation

In the liver, UCB is taken up by the basolateral membrane of hepatocytes. This process mainly involves the organic anion transporting polypeptides OATP1B1 and OATP1B3, which supports the transport of bilirubin and other hydrophobic molecules into the cell (Kamisako et al., 2000). Inside the cytoplasm, UCB binds to proteins such as ligandin, a variant of glutathione S-transferase, which shields it from oxidative damage and helps to direct it to the smooth endoplasmic reticulum for further processing (Bosma, 2003; Kamisako et al., 2000).

A central step of hepatic metabolism is the glucuronidation of bilirubin. This reaction is catalysed by the enzyme UGT1A1, which attaches one or two glucuronic acid molecules to the propionic acid side chains. This results in the formation of mono- and diglucuronides, which can then be excreted through the bile (Iyanagi et al., 1998). In the first days after birth, the activity of UGT1A1 is still low. As a result, levels of UCB often rise temporarily in newborns, which is one of the main reasons for neonatal jaundice (Wang et al., 2006). **Figure 2** summarises the hepatic uptake and conjugation steps of bilirubin metabolism (Wagner et al., 2015).

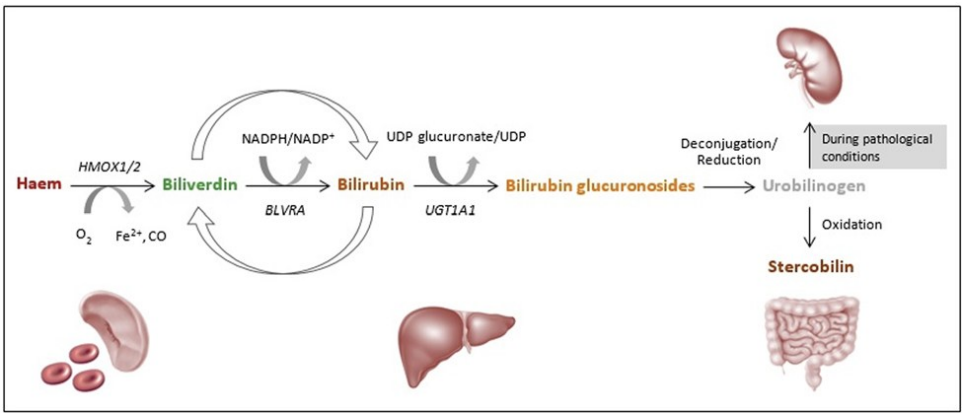

Figure 2: *Bile pigment metabolism (Wagner et al., 2015)*

From haem degradation to bilirubin conjugation

2.2.3 Biliary excretion and intestinal metabolism

Conjugated bilirubin is actively transported into the bile canaliculi by MRP2, an adenosine triphosphate (ATP)-dependent export protein located in the canalicular membrane of hepatocytes (Kamisako et al., 2000). From there, it passes through the bile ducts into the intestine. In the gut, bacterial enzymes convert it into urobilinogen and stercobilin. Most stercobilin is excreted in faeces, which gives stool its brown colour. A part of the urobilinogen is reabsorbed and travels back to the liver through the enterohepatic circulation. A smaller fraction enters the bloodstream and is filtered out by the kidneys, where it ends up in the urine and contributes to its yellow colour (Roy-Chowdhury & Roy-Chowdhury, 2012).

2.2.4 Clinical relevance and regulatory mechanisms

If the liver is damaged or bile cannot drain properly, bilirubin handling in the body changes. In cases like cholestasis or hepatocellular injury, conjugated bilirubin accumulates in plasma and is excreted in the urine because it is water-soluble. In contrast, UCB remains bound to albumin and usually does not pass into the urine, unless severe proteinuria is present (Vitek & Ostrow, 2009). Genetic disorders also play a role. In Gilbert syndrome, for example, reduced UGT1A1 activity causes mild, fluctuating increased UCB. Crigler-Najjar syndrome type I involves a complete loss of UGT1A1 function and requires intensive treatment (Bosma, 2003).

More recently, researchers have discovered that bilirubin metabolism is also regulated at the transcriptional level. Nuclear receptors such as PPARα, CAR and PXR respond to various stimuli including metabolic stress, inflammation or drug exposure. By modulating transporter and enzyme expression, these pathways allow the liver to adapt bilirubin clearance to the organism's physiological state (Creeden et al., 2021; Wagner et al., 2015). This regulatory flexibility may become particularly relevant in post-infectious conditions such as Long COVID, where persistent immune activation and hepatic stress could alter bilirubin dynamics. **Figure 3** shows the pathway of bilirubin from haem degradation to excretion (Zhang et al., 2025).

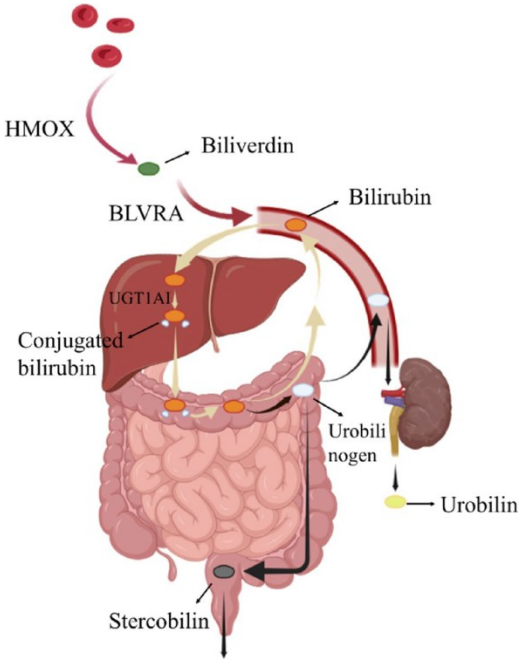

Figure 3: *Overview of bilirubin metabolism (Zhang et al., 2025)*

It shows the conversion of haem to bilirubin, its conjugation in the liver (UGT1A), and the breakdown to urobilinogen, urobilin and stercobilin with excretion via urine and stool.

2.3. Bilirubin as a biomarker: from oxidative stress to chronic disease

Bilirubin, especially in its unconjugated form, was long seen as a waste product from haem breakdown. It was mainly relevant in liver diagnostics. In the last years, though, more research has pointed to a broader role. UCB might support the body's own defense against stress and inflammation, and it seems to interact with metabolic processes too (Vítek & Tiribelli, 2021a).

Its antioxidant effect is especially well studied. UCB can bind and neutralize reactive oxygen species (ROS) like free radicals. These can damage cells, especially in organs like the liver, brain or blood vessels. What makes bilirubin especially effective is its ability to regenerate through

redox cycling with biliverdin. This cycle allows it to persist longer as an antioxidant than most dietary molecules (Vítek & Tiribelli, 2021a; Wagner et al., 2015).

In most healthy adults, total bilirubin stays in a low range, often around 3 to 20 μmol/L. The unconjugated form makes up the largest part of this. Exact numbers can shift a bit, for example with age, gender or genetic differences (Čepelak et al., 2025). In people with Gilbert's syndrome, which is a harmless genetic trait, levels are often higher, sometimes reaching 50 μmol/L or more. These people usually have no symptoms. Some studies even suggest they might have a slightly lower risk for cardiovascular or metabolic diseases (Vítek, 2017a).

People with bilirubin levels near the upper end of the normal range may have some health advantages. Slight increases, just above 10 μmol/L, have been linked to less inflammation, healthier blood lipids and fewer heart problems (Poynard et al., 2023; Wagner et al., 2015). One study from Korea found that men with levels over 10.8 μmol/L had a lower mortality risk from heart disease or stroke than those with lower levels (Kimm et al., 2009).

A meta-analysis that pooled ten cohort studies found that people with slightly higher bilirubin levels had a 22% lower risk of type 2 diabetes (T2D) (RR 0.78) and a 30% lower risk of metabolic syndrome (RR 0.70) (Nikouei et al., 2024). Values between 10 and 20 μmol/L, though, still within the normal range, may therefore already offer some protective effect.

Apart from its antioxidant role, bilirubin might also play a part in how the body handles fat and energy. It can activate the receptor PPARα, which controls several genes related to metabolism. In laboratory experiments, this was linked to better functioning of mitochondria and reduced inflammation (Creeden et al., 2021). Such effects might help explain why people with slightly higher bilirubin levels often show healthier metabolic markers.

UCB could also help to protect the brain. Experimental studies suggest that it can counter oxidative stress and inflammation in neural tissues, which may support neuronal resilience in disease-related conditions (Creeden et al., 2021; Wagner et al., 2015).

These effects might matter for chronic diseases where brain inflammation plays a role. Human data are still rare, but basic research points to a broader role for UCB in the nervous system.

Lower bilirubin levels, especially below 10 μmol/L, have also drawn attention. Several studies linked such values to higher risk for vascular damage, stroke and even overall mortality (Fulks et al., 2009; Higuchi et al., 2018). One explanation is that the body may have lower internal defense capacity against oxidative stress. In this context, the term “hypobilirubinemia” is being used more often, even though it is not yet an official diagnosis (Hinds & Stec, 2018).

In post-viral conditions like Long COVID, persistent oxidative stress and immune dysregulation may interfere with recovery processes. Because of its involvement in redox homeostasis and cellular defense, bilirubin has been discussed as a possible indicator of how well the body can cope with ongoing physiological strain. Even small shifts in unconjugated bilirubin levels could reflect individual differences in this adaptive capacity. Although direct clinical data remain scarce, earlier studies have already highlighted the potential regulatory role of UCB in stress-related pathways (Hinds & Stec, 2018; Wagner et al., 2015).

These associations are summarized in **Table 1** which gives an overview of cohort studies linking bilirubin concentrations with cardiometabolic outcomes. Most population studies report total bilirubin, which mainly consists of the unconjugated fraction. Mildly elevated UCB levels were consistently associated with lower risk for cardiovascular disease, T2D and metabolic syndrome. In contrast, lower bilirubin levels were linked to increased risk of stroke, vascular damage or mortality.

Table 1: *Studies on bilirubin and chronic disease risk (Fulks et al., 2009; Higuchi et al., 2018; Hinds & Stec et al., 2018; Kimm et al., 2009; Nikouei et al., 2024; Poynard et al., 2023)*

Study	Population / Design	Bilirubin type	Main finding	Direction
Kimm et al. (2009)	Korean cohort (~78,700)	Total (mainly UCB)	Higher bilirubin linked to lower stroke risk in men	Protective (↑)
Nikouei et al. (2024)	Meta-analysis (10 cohorts)	Total	Higher bilirubin linked to lower risk of diabetes and metabolic syndrome	Protective (↑)
Poynard et al. (2023)	Gilbert's carriers	UCB	Higher bilirubin associated with lower cardiometabolic risk	Protective (↑)
Higuchi et al. (2018)	Japanese cross-sectional	Total	Low bilirubin associated with higher stroke risk	Risk (↓)
Fulks et al. (2009)	US insurance applicants (~2 Mio.)	Total	Low bilirubin linked to higher mortality	Risk (↓)
Hinds & Stec (2018)	Review	UCB	Low UCB may reduce resilience	Risk (↓)

These protective and risk associations underline why UCB is now being discussed as a potential marker also in post-infectious conditions such as Long COVID.

2.4. Relevance in infectious and post-infectious syndromes: focus on COVID-19

Bilirubin has long been studied in infections like hepatitis, HIV and sepsis. In these settings, changes in its level often reflect liver damage or inflammation. More recently, researchers have begun to explore whether bilirubin also support the body's response to infection, for example through effects on the immune system or metabolism (Barañano & Snyder, 2001; Wagner et al., 2015).

Since the start of the COVID-19 pandemic, altered bilirubin profiles have been consistently reported. While hyperbilirubinemia is often linked to drug-induced liver injury or hepatic congestion, several studies have found that elevated total or direct bilirubin levels also correlate

with disease severity, ICU admission and mortality (Brito et al., 2020; Cai et al., 2020; Liu et al., 2020). These changes likely reflect hypoxic liver injury, systemic inflammation, cholangiocyte damage and oxidative stress (Lin et al., 2021; Wang et al., 2021). Case reports further document post-COVID cholangiopathy with features similar to secondary sclerosing cholangitis, suggesting sustained biliary injury in critically ill patients (Roth et al., 2021).

An umbrella review shows that COVID-19 induces measurable injury in several organs, most often liver, kidney and heart, even months after discharge, underlining that altered bilirubin handling may be only one sign of a broader metabolic stress response (Ewing et al., 2024). Dedicated hepatology reviews now characterize "post-COVID cholangiopathy" and describe rising numbers of chronic hepatic and biliary disorders in convalescents (Caballero-Alvarado et al., 2023; Lebbe et al., 2024; Nasir, 2024).

Severe forms of COVID-19 are frequently associated with cytokine overactivation and endothelial dysfunctions. Under such conditions, bilirubin might serve as a compensatory molecule, because of its antioxidant and immunomodulatory effects (see 2.3).

Prognostic data support this assumption. In a cohort of hospitalized COVID-19 patients, (Liu et al., 2020) found that total bilirubin had moderate predictive value for disease severity, with an area under the curve (AUC) of 0.71 **(Figure 4).**

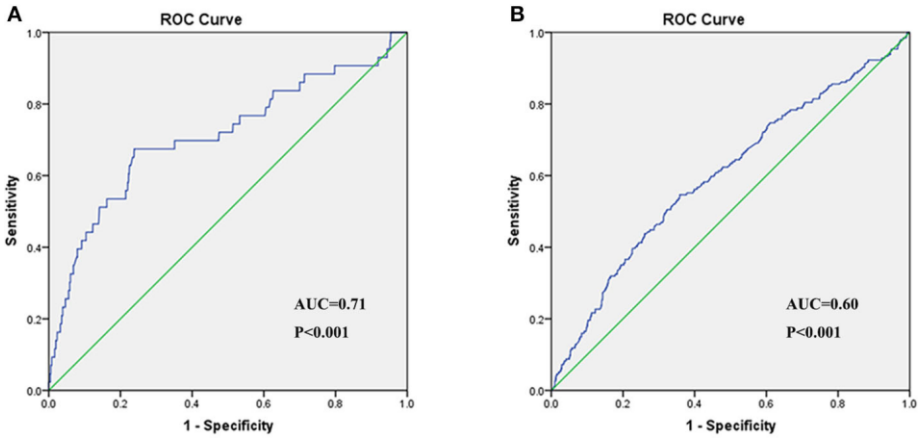

***Figure 4**: ROC curves of total bilirubin in COVID-19 patients (Liu et al., 2020)*

(A): severe cases (AUC = 0.71), (B): moderate cases (AUC = 0.60). $p < 0.001$

Furthermore, a Kaplan-Meier analysis revealed that patients with elevated bilirubin levels had significantly reduced 60-day survival compared to those with normal levels ($p < 0.001$, **Figure 5**).

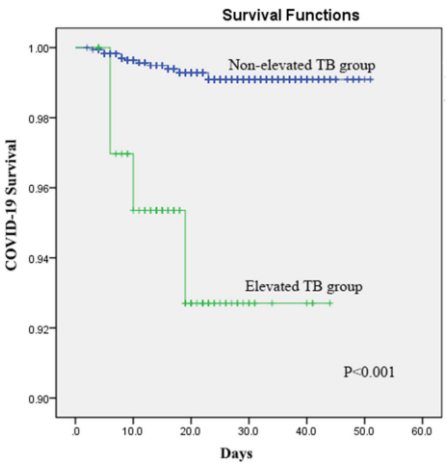

Figure 5: *Kaplan-Meier survival curves according to bilirubin levels (Liu et al., 2020)*

The analysis compared patients with elevated vs. non-elevated total bilirubin levels. Elevated TB was associated with reduced 60-day survival.

Some studies suggest that different bilirubin fractions may have distinct clinical implications. In mild COVID-19 cases, total bilirubin often stays within the normal range or rises only slightly. In more severe cases, however, a disproportionate increase in the conjugated fraction has been observed (Paliogiannis & Zinellu, 2020). Whether changes in UCB specifically – rather than total levels – are of diagnostic value remains uncertain. This is partly because standard laboratory tests rarely distinguish clearly between the two forms.

The role of UCB may gain special importance in post-viral conditions such as Long COVID. Even after the acute phase, many patients continue to suffer from symptoms like ongoing fatigue, cognitive slowing, autonomic dysregulation and exercise intolerance (Kedor et al., 2022; Said et al., 2023). These symptoms have been linked to persistent inflammation, redox imbalance and mitochondrial dysfunction (Creeden et al., 2021). As outlined in section 2.3, UCB interacts with these processes and may therefore both reflect and modulate the biological stress response.

Although bilirubin is easy to measure, it is rarely part of post-infectious follow-up. This is partly because it is still seen mainly as a liver marker. Yet UCB levels are influenced by light exposure, fasting, time of day and genetic traits such as Gilbert's syndrome (Bosma, 2003). To interpret bilirubin effectively as a biomarker, future studies should account for these variables and monitor levels longitudinally (Wagner et al., 2015).

Overall, current studies suggest that bilirubin, especially its unconjugated form, could serve as a useful marker in both acute and post-viral illness.

In COVID-19 and related conditions, it may reflect the systemic stress response while also supporting recovery (Creeden et al., 2021; Kedor et al., 2022; Liu et al., 2020).

2.5. Long COVID: current research and biomarker gaps

Most people recover from a SARS-CoV-2 infection within a few weeks. A smaller proportion, however, develop new or recurrent symptoms after an initial recovery. To describe this pattern, the WHO released a formal clinical case definition in 2021, now widely known as Long COVID or post-COVID-19 condition. Under this definition, symptoms usually appear about three months after the acute illness, persist for at least two months and are not better explained by an alternative diagnosis (World Health Organization, 2023).

According to current WHO reports, persistent fatigue is the symptom most often described. Other common complaints include shortness of breath, non-restorative sleep, diffuse musculoskeletal pain, and cognitive impairment often summarized as “brain fog”. Collectively, these problems disrupt everyday activities and substantially reduce quality of live (Horowitz et al., 2024; World Health Organization, 2023). **Figure 6** shows the most frequently reported symptoms of Long COVID (Kedor et al., 2022; World Health Organization, 2023).

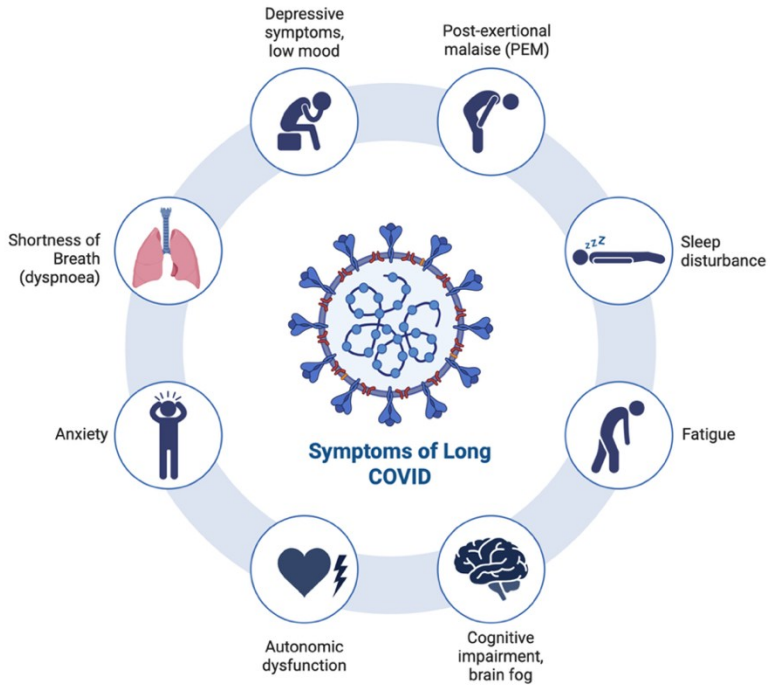

Figure 6: *Common symptoms of Long COVID (Kedor et al., 2022; WHO, 2023). Created with BioRender.com*

Findings from laboratory and imaging studies in Long COVID patients are as mixed as the symptoms. In the Berlin cohort, for example, people who started out with more interleukin-8 often ended up with worse fatigue months later. Many also had high ferritin. At the same time, capillary density was lower, which fits with the idea of persistent inflammation and stress on small vessels (Kedor et al., 2022). Other studies using metabolomic profiling in convalescent patients found that changes in how mitochondria produce energy, along with shifts in the redox balance – a pattern that hints at oxidative stress long after the virus has gone (Creeden et al., 2021). In patients with mainly cognitive symptoms, such as "brain fog", elevated glial markers pointed to mild but ongoing neuroinflammation (Said et al., 2023). All of this supports a view that immune activity, oxidative imbalance, and vascular changes feed into one another over time.

Recent reviews provide further support. They describe persistent immune activation with higher cytokines and complement fragments, combined with oxidative and nitrosative stress and the presence of microclots that may impair capillary flow (Almulla et al., 2024). Imaging studies with repeated FDG-PET scans revealed sustained hypometabolism in limbic and brainstem regions, a pattern consistent with the constant fatigue and cognitive slowing reported by patients (Horowitz et al., 2024). These results strengthen the view that Long COVID involves a loop of immune dysregulation, oxidative stress and small-vessel changes.

Bilirubin is closely linked to disease severity. As shown in acute COVID (see 2.4), higher bilirubin levels were associated with ICU admission and mortality. In a Turkish cohort, a bilirubin-to-albumin ratio predicted cardiac involvement even in moderate cases (Cosgun, 2021). A meta-analysis confirmed that hyperbilirubinemia is more frequent than in non-severe infections (Kumar-M et al., 2020). These associations have often been interpreted as markers of hepatic stress, but bilirubin's biological properties – antioxidant capacity, mitochondrial protection and immune modulation – also suggest a protective role.

Some observations suggest that bilirubin may still play a role after the initial infection has passed. In the Berlin follow-up study, participants with higher UCB also tended to have elevated ferritin and more severe symptom scores. This pattern could reflect a connection between changes in haem breakdown and the fatigue oft seen after viral illness (Kedor et al., 2022). A separate case description told of a COVID-19 patient with Gilbert syndrome, a condition where UCB is naturally higher. That patient experienced only mild symptoms and recovered quickly, which has been taken as a possible sign of protection (Al-Kuraishy et al., 2021). Other authors have noted that

persistently low UCB might do the opposite, leaving the body less able to cope with ongoing oxidative stress (Creeden et al., 2021).

Despite these signals, UCB is rarely included in Long COVID follow-up panels. One reason is practical: levels are affected by fasting status, time of day, certain medications and even light exposure during sample handling (Bosma, 2003). In standard laboratory workflows, TB is measured, and the unconjugated fraction is only estimated. High-performance liquid chromatography (HPLC) allows direct quantification with high precision and has been used in metabolic research, but it has not yet been applied systematically to Long COVID cohorts.

Overall, current findings indicate a persistent symptom load, ongoing inflammatory and oxidative stress responses, and a possible role of UCB in these processes.

3. Study Design and Materials

3.1 Study design

This thesis is part of the Africa-UniNet Research Cooperation Project on Long COVID, carried out in South Africa. The study was designed as a longitudinal observational cohort and included individuals with a confirmed SARS-CoV-2 infection who continued to experience symptoms for weeks to months after the initial infection. The aim of the overall project was to better understand post-infectious changes in this group, using a broad panel of clinical, metabolic and inflammatory markers.

Within this framework, UCB was one of several parameters selected for detailed analysis. It was included because changes in UCB might reflect oxidative stress or be associated with post-infectious recovery.

3.2 Study population

Adults with confirmed SARS-CoV-2 infection were included if they still had symptoms several weeks after the acute phase. Most had mild to moderate illness. At the time of sampling, common complaints included fatigue, brain fog, or other lasting issues. A total of 132 UCB serum samples were analysed at baseline, and 103 participants provided follow-up samples after twelve months.

Besides UCB levels, the dataset also contained anthropometric, demographic and clinical details. More details on inclusion criteria, symptom profiles and comorbidities are provided in Chapter 5 (Results).

3.3 Sample collection and handling

After shipment to Austria, the samples were stored at -80 °C until analysis. To protect bilirubin from light, sample handling was done under dimmed conditions. Aliquots were thawed on ice and processed right away. All serum samples were analysed at the Department of Nutritional Sciences, University of Vienna, from March to June 2025, using HPLC. Further details are given in Chapter 4 (Methods).

3.4 Chemicals and reagents

Analytical or HPLC-grade substances were used for all steps of sample preparation and chromatographic analysis.

Table 2 lists the chemicals, solvents and lab materials used during the process.

Table 2: *Chemicals and materials used in this master thesis*

Material	Specification/Purity	Supplier	Product Code
Bilirubin	Powder, purity > 98 %	Sigma-Aldrich	/
Dimethyl sulfoxide	≥ 99.5 %	Sigma-Aldrich	102736951
Methanol	LiChrosolv® Reag. Ph Eur, gradient grade	Supelco	/
Water	HPLC, HiPerSolv CHROMANORM	VWR Chemicals	/
Dioctylamine	Purity 95 %	Thermo Scientific	/
Acetic Acid	99–100 %, glacial, ReagentPlus®	Sigma-Aldrich	1003420491
Isopropanol	2-Propanol für HPLC, 99.9 %	Sigma-Aldrich	/
Mobile phase	Composition detailed in **Table 6** (see Chapter 4.1.2)	/	/
Microtubes	1.6 mL, amber	Biozym	710169
HPLC vials	1.5 mL, brown glass, hydrolytic class 1, wide opening	Bruckner Analysetechnik	11 09 0520
Inserts	0.2 ml micro insert with flat bottom vial, clear 1st hydrol, glass glass, flat bottom, 31 × 6 mm	Shimadzu	961-10020-12
Vial caps	/	/	09150852

Nylon membrane filters	0.2 µm pore size, 47 mm diameter	Supelco	16E06203
Parafilm	Parafilm® "M"	Bemis	/
Glass Pasteur Pipettes	Disposable, 230 mm length	VWR	/
Helium	5.0 grade, purity >99.999%	Messer	56143882

3.5 Laboratory equipment

The following instruments were used to measure UCB by HPLC. Their specifications are listed in **Table 3**.

Table 3: *Laboratory instruments used in this master thesis*

Equipment	Model/Description
Analytical Balance I	Mettler Toledo
Analytical Balance II	Sartorius BP1200
Centrifuge	Eppendorf Centrifuge 5427 R
HPLC-System	Shimadzu Nexera X2
Micropipettes	Eppendorf Research plus (20-200 µL, 100-1000 µL)
Ultrasonic Bath	Allpax Palssonic
Magnetic Stirrer	Heidolph MR 3001 K
Vortex Mixer	Heidolph REAX 2000
Water Bath	GFL 1002 (Gesellschaft für Labortechnik)

4. Methods

UCB in serum was measured using RP-HPLC. All measurements were performed at the Department of Nutritional Sciences, University of Vienna.

4.1. HPLC method

HPLC is a method used to separate different parts of a liquid sample. The sample moves through a column filled with solid material. In reversed-phase HPLC, this material is non-polar. The more non-polar a substance is, the longer it stays in the column. More polar substances pass through faster. Bilirubin, being non-polar, stays longer and leaves the column after several minutes (Moldoveanu & David, 2013).

A constant mobile phase composed of 96.5 % methanol and 3.5 % ultrapure water was used in all runs. Serum samples were always injected under the same conditions to ensure consistency.

Separation was performed using a Fortis C18 column with a flow rate of 1.0 mL/min and an operating pressure between 120 and 140 bar.

Detection was set at 450 nm with a UV/Vis detector. This wavelength was chosen because bilirubin absorbs light strongly in this region (Moldoveanu & David, 2013). The peak for UCB was usually seen between 8 and 12 minutes.

Concentrations were calculated using standard curves from reference solutions. Peak areas and times were analysed with LabSolutions software. **Figure 7** gives an overview of the HPLC system used.

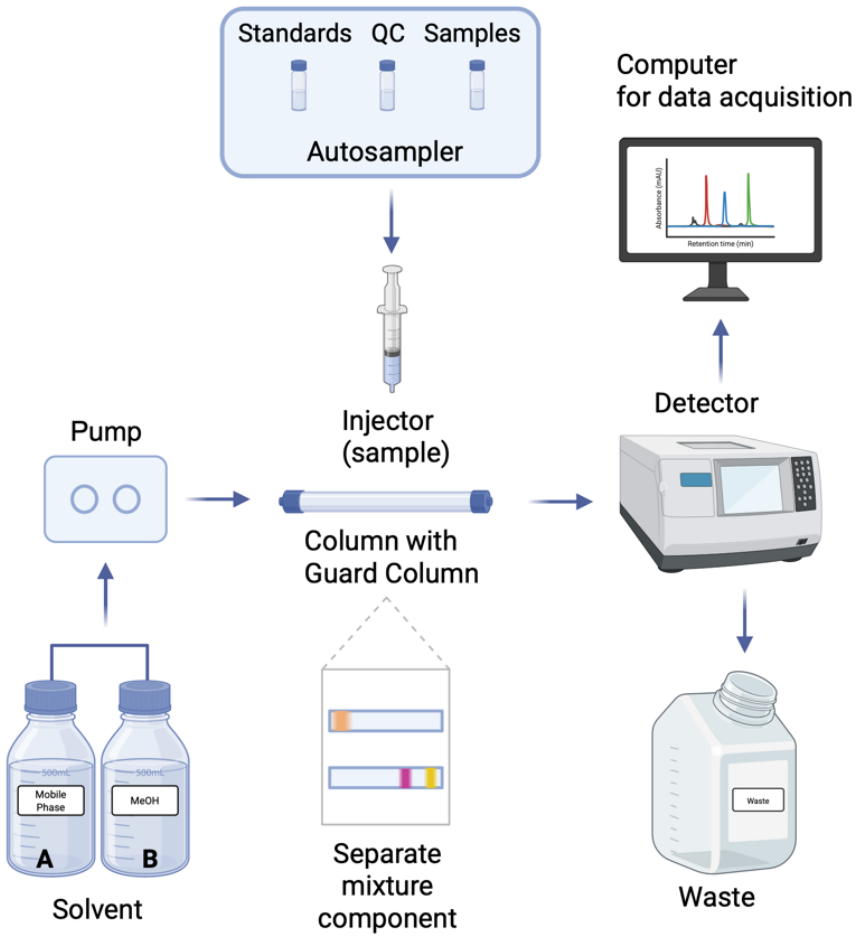

***Figure 7**: Overview of HPLC setup in this master thesis. Created with BioRender.com*

4.1.1 HPLC system and specifications

The analysis was performed on a Shimadzu Nexera X2 HPLC system. It consisted of a binary pump, a degassing unit, a cooled autosampler, a column oven, and a UV-Vis detector. The autosampler was set to 5 °C, and the column oven to 35 °C. Separation was carried out on a Fortis C18 column (dimensions 4.6 x 150 mm, 3 µm).

A reversed-phase method was used. UCB usually eluted within the expected retention time range (see **Table 5**). Before each run, the system was flushed with mobile phase for at least 30 minutes. A methanol blank was injected to check the baseline.

Bilirubin is sensitive to light. For this reason, all samples, standards and solvents were handled in amber vials and covered with aluminium foil whenever possible.

Table 4 lists the components of the system. Operating conditions used throughout the analysis are summarised in **Table 5**. An overview of the general system setup is shown in **Figure 7**.

Table 4: *HPLC system components*

Component	Description
System Controller	Nexera HPLC/UHPLC System Controller – SCL-40 by Shimadzu
Control Software	LabSolutions
Pump	Nexera HPLC/UHPLC Pump – LC-40D by Shimadzu
Degassing Unit	Nexera HPLC/UHPLC Degasser – DGU-405 by Shimadzu
Autosampler	Nexera HPLC/UHPLC Autosampler – SIL-40C by Shimadzu
Column Oven	Nexera HPLC/UHPLC Column Oven – CTO-40C by Shimadzu
Column	Fortis™ HPLC-Column, 150 x 4.6 mm, C18 3 µm
Detector	Nexera HPLC/UHPLC UV-VIS Detector – SPD-40V by Shimadzu

Table 5: *HPLC operating conditions*

Parameter	Setting
Column Oven Temperature	35 °C
Autosampler Temperature	5 °C
Injection Volume	20 µL
Flow Rate	1.0 mL/min
Detection Wavelength	450 nm
Run Time	12 minutes
Retention Time (UCB)	8 – 12 minutes
Column/Pre-column	No leakage
Pressure Range	120 – 140 bars

All runs were checked for pressure, temperature and flow rate. These parameters stayed within their normal ranges. No leakage was observed from the column or pre-column.

4.1.2 Mobile phase preparation

The mobile phase was prepared fresh once or twice a week, depending on the schedule and sample load. All solvents were HPLC-grade.

Its exact composition is listed in **Table 6**. The preparation steps are shown in **Figure 8** and described in more detail below.

***Table 6**: Mobile phase composition*

Chemical	Volume
Methanol (CH_4O)	2.0 L
Dioctylamine ($CH_3(CH_2)_7NH(CH_2)_7CH_3$)	48.4 mL
Acetic acid (CH_3COOH)	12.02 mL
Water (H_2O)	72 mL

Before starting, all glassware, including flasks, cylinders and pipettes, were rinsed with methanol to avoid contamination.

First, 200 mL of methanol were poured into a clean flask. Then, 48.4 mL of dioctylamine were added and stirred briefly. The flask was sealed with Parafilm and cooled on ice for five minutes. After that, 12.02 mL of acetic acid were added slowly while stirring, followed by another 400 mL of methanol.

The full mixture was poured into a 1000 mL measuring cylinder. The flask was rinsed with some methanol, which was also added. Methanol was then added until the 930 mL mark was reached.

Next, 72 mL of ultrapure water was added. The cylinder was sealed and gently inverted three times to mix.

The freshly prepared solution and 1 L of pure methanol were filtered through a vacuum filtration unit. The setup included a metal filter, filter paper, and a nylon supporting ring, all of which had been pre-rinsed with methanol. The two filtered solutions were combined in an amber glass bottle.

To remove any gas bubbles, the solution was placed in an ultrasonic bath for five minutes and then degassed with helium for 20 minutes while stirring. The final mobile phase contained 96.5 %

methanol and 3.5 % water and was used directly in the HPLC system. A visual summary of the preparation steps is shown in **Figure 8**.

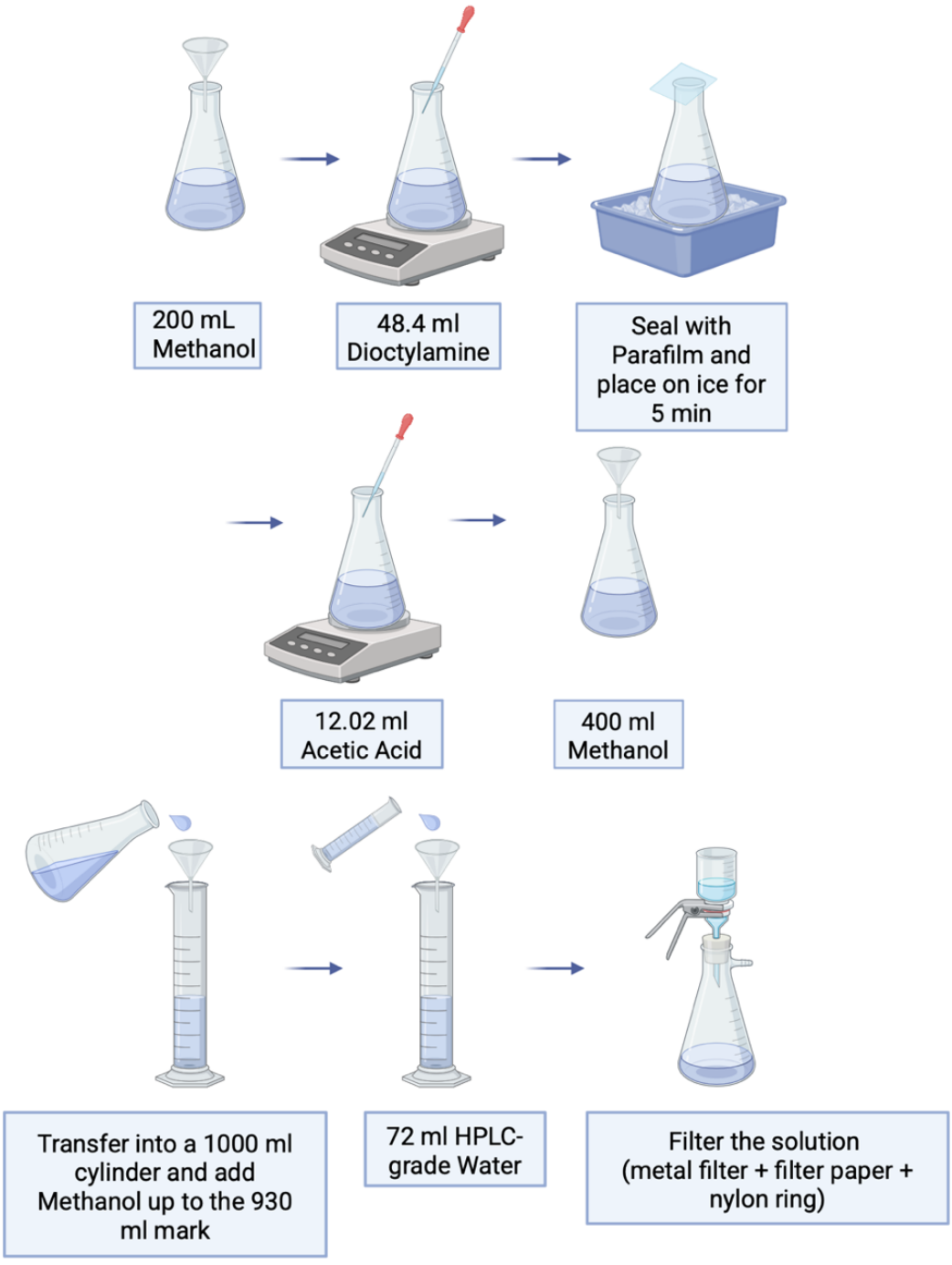

Figure 8: *Mobile phase preparation. Created with BioRender.com*

4.1.3 Preparation of the bilirubin stock solution

For the bilirubin stock solution, about 3 to 5 mg of unconjugated bilirubin powder (Sigma Aldrich, purity ≥ 98%) were weighed into an amber Eppendorf tube. The exact amount was noted. To make a 10 mM solution, the required dimethyl sulfoxide (DMSO) volume was calculated based on the molecular weight (584.66 g/mol). This was done using the molarity calculator from GraphPad (see **Figure 9**).

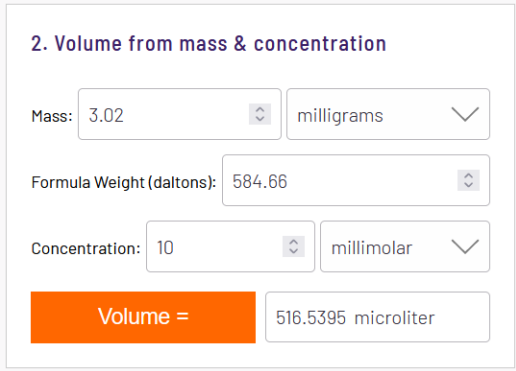

Figure 9: *Calculation of the bilirubin stock solution*

The weighed bilirubin was dissolved in the calculated amount of DMSO. The tube was vortexed and then placed in a 70 °C water bath for five minutes. After that, the sample was sonicated for around one minute, vortexed again and centrifuged at 14,000 rpm for 30 seconds at 4 °C. If the solution remained cloudy or a pellet was still visible, the heating and mixing steps were repeated.

Once the solution was fully dissolved, 50 µL aliquots were transferred into labelled amber microtubes and stored at –80 °C. These aliquots were used to prepare fresh standards before each measurement. The preparation steps are summarised in **Figure 10**.

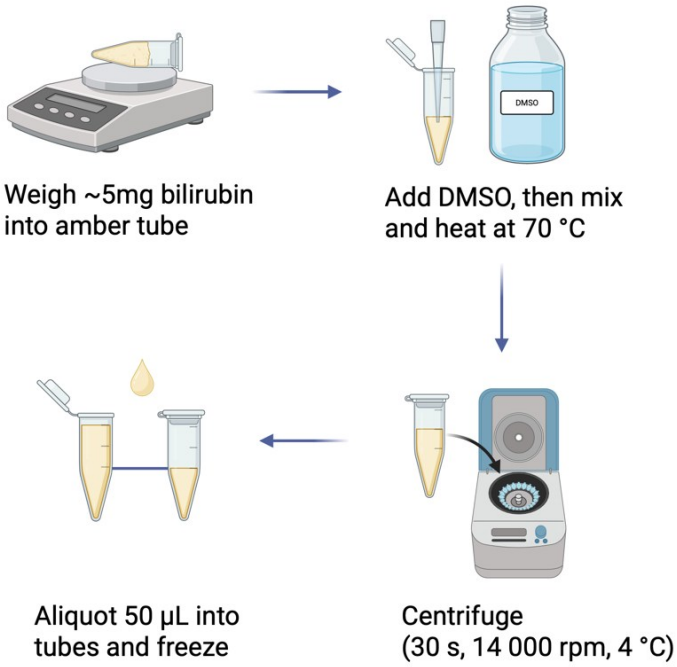

Figure 10: *Preparation of 10 mM bilirubin stock. Created with BioRender.com*

4.1.4 Preparation of the standard dilution

On every analysis day, a fresh set of bilirubin standards was prepared from the frozen stock solution. One tube with 50 µL of the frozen 10 mM stock was taken from the freezer and thawed on ice. After thawing, it was mixed with 450 µL of DMSO in an amber microtube. This gave the first standard (S1) with a concentration of 1000 µM.

To prepare S2, 50 µL of S1 were mixed with 450 µL of DMSO and vortexed. The resulting solution had a concentration of 100 µM.

From there, further dilutions were done. For S3, 250 µL of S2 were pipetted into a new tube with 250 µL of DMSO. The mixture was vortexed. Then, 250 µL of S3 were mixed with 250 µL of DMSO to get S4. The same step was repeated for S5, S6, S7, S8 and S9. All standards were always pipetted into amber tubes to protect them from light. After each step, the solution was vortexed again. The concentrations of all standards are shown in **Table 7**.

Before injection into the HPLC, 40 µL of each of the lower standards (S4 to S9) were pipetted into new tubes and mixed with 160 µL of mobile phase. The mixtures were briefly vortexed. Then, 120 µL were filled into amber HPLC vials with glass inserts. The vials were placed in the autosampler.

All concentrations and dilution steps are listed in **Table 7**. **Figure 11** shows how the standards were prepared and diluted before injection.

Table 7: *Calibration standards*

Standard dilution	Concentration (µmol/L)	Volume from previous standard (µL)	DMSO (µL)	Final volume (µL)
S1	1000	50 µL (from 10 mM stock)	450	500
S2	100	50 (from S1)	450	500
S3	50	250 (from S2)	250	500
S4	25	250 (from S3)	250	500
S5	12.5	250 (from S4)	250	500
S6	6.25	250 (from S5)	250	500
S7	3.13	250 (from S6)	250	500
S8	1.5	250 (from S7)	250	500
S9	0.75	250 (from S8)	250	500

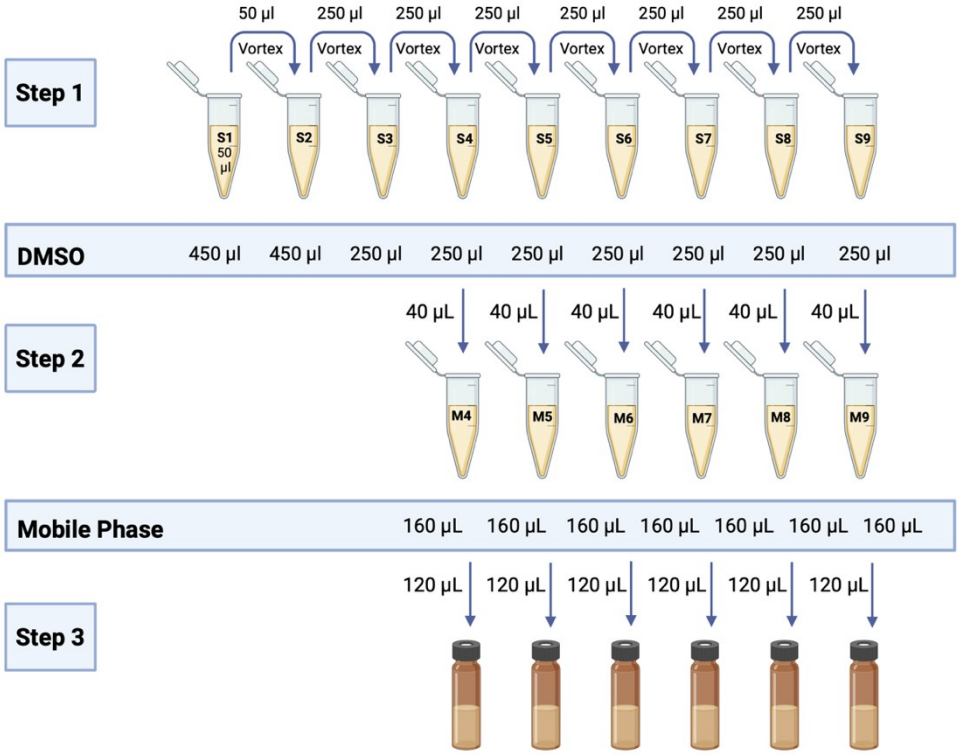

Figure 11: *Preparation of the standards (S1-S9). Created with BioRender.com*

4.1.5 Sample preparation

All serum samples were stored at -80 °C. On each measurement day, the required tubes were taken from the freezer and placed in a rack on ice. The rack remained cold during the entire preparation process. The centrifuge was set to 4 °C and 14,000 rpm for 10 minutes.

For each sample, 200 µL of mobile phase were pipetted into amber Eppendorf tubes. The tubes were labelled with the correct sample numbers. While thawing, the samples stayed in the ice box. Once thawed, the serum tubes were vortexed. Then, 50 µL of serum were added to the tubes with the mobile phase. These tubes were vortexed again for about 5 seconds.

The tubes were centrifuged at 14,000 rpm at 4 °C for 10 minutes. After centrifugation, a small pellet was visible. The clear supernatant was pipetted off. From each tube, 120 µL were transferred into HPLC vials with glass inserts. The vials were labelled with the same sample ID as the corresponding Eppendorf tubes.

All steps were performed under low light conditions. After filling, the vials were sealed and placed into the autosampler. The sample list and vial positions were written into the sequence file. This helped to match each measurement with the correct study code. An overview of all preparation steps is presented in **Figure 12**.

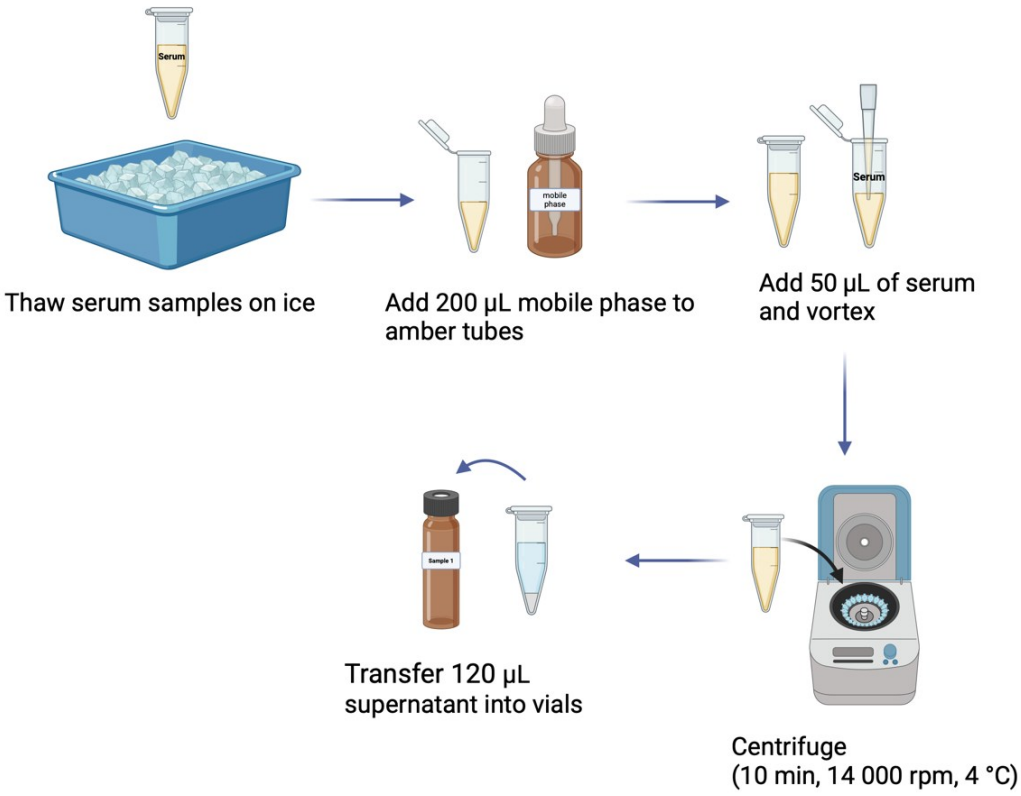

Figure 12: *Sample preparation steps. Created with BioRender.com*

4.1.6 Data analysis

All chromatograms were recorded using LabSolutions with detection at 450 nm. The software showed each run, including standards, serum samples and quality controls.

In the bilirubin standard, three peaks could be observed: IIIα, Ixα, and XIIIα. Of these, the Ixα peak was the most stable and clearly defined, so it was used for calculating the concentration. In serum samples, only the Ixα peak was visible. This matches what is expected, since Ixα is the only isomer normally found in human plasma and serum.

Figure 13 shows the chromatograms. In the serum, only the Ixα peak is visible at ~ 10 min. In the standard, the same peak appears as the reference signal, while additional bilirubin isomers are not clearly resolved in this representation.

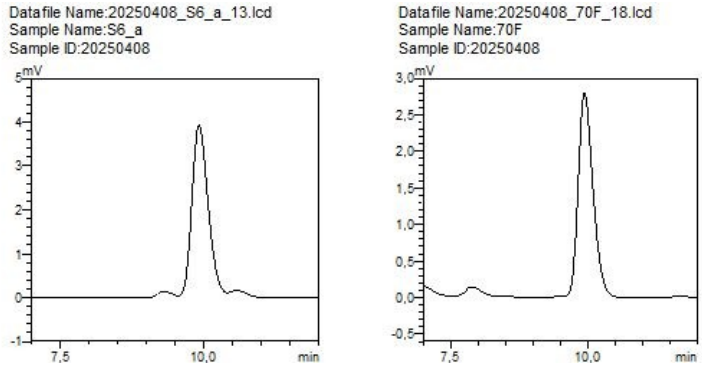

Figure 13: *Chromatograms of (a) sample and (b) standard*

Each chromatogram was checked manually before export. The Ixα peak was compared with the standard. If the peak looked unusual or appeared at the wrong place, the sample was excluded. Only clean and matching peaks were accepted. When everything looked correct, the AUC was taken and noted in Excel.

Bilirubin concentrations were calculated using the standard curve from the same run. For this, standards S4 to S9 were measured first. Their AUC values were plotted against the known concentrations. A linear regression was generated.

The following formula was used:

$\boldsymbol{y = k * x + d}$ **where:** y = AUC (area under the curve)
x = bilirubin concentration (µmol/L)
k = slope of the calibration curve
d = intercept

To formula was used to get the concentration from the AUC:

$$\boldsymbol{Concentration\ [\mu mol/l]\ = \frac{AUC\ (Ix\alpha) - Intercept}{Slope}}$$

Only calibration curves with an R^2 of 0.995 or above were accepted. If this was not reached, the run was repeated. Across all runs, R^2 values ranged between 0.9998 and 1.0000, with a coefficient

of variation (CV) of 0.0066%. The IXα peak was also checked for shape and clarity. Peaks that were broad, noisy or had tailing were excluded and the sample was remeasured.

Figure 14 shows a standard curve based on AUC values from standards S4 to S9. The curve was linear across all concentrations and reached an R^2 of 1.000. **Table 8** shows the corresponding values.

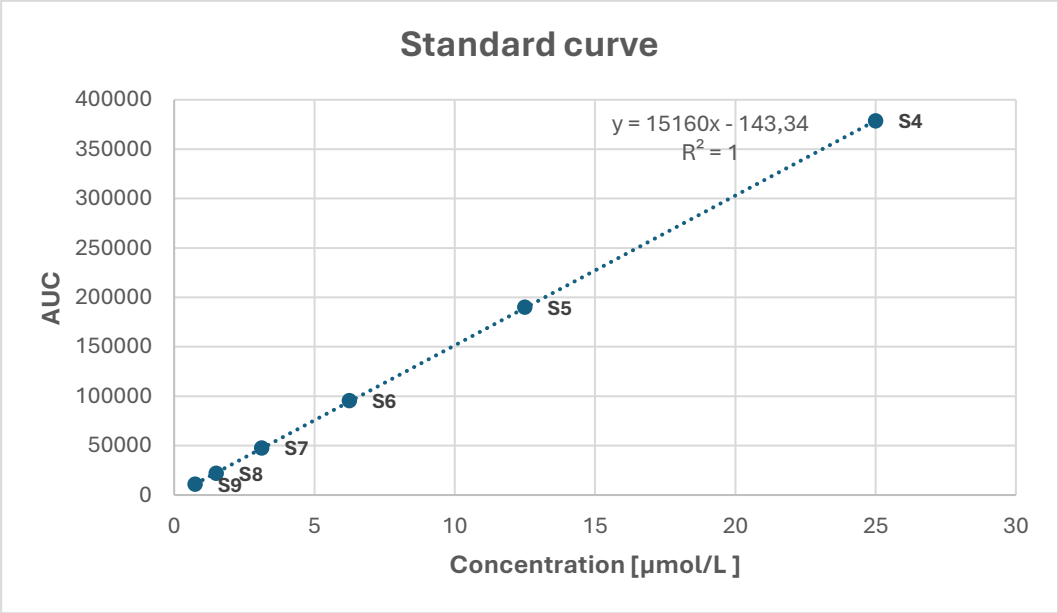

Figure 14: *Standard curve of a bilirubin standard (08.04.2025)*

Table 8: *Bilirubin standard concentrations and AUC values*

Standard	Concentration [µmol/L]	AUC IIIα	AUC Ixα	AUC XIIIα	Total AUC	Actual Concentration [µmol/L]
S9	0.75		10893		10893	0.73
S8	1.5		21802		21802	1.45
S7	3.125	1563	44098	1850	47511	3.14
S6	6.25	2984	90935	1359	95278	6.29
S5	12.5	5952	181279	2764	189995	12.54
S4	25	11763	361130	5492	378385	24.97

4.1.7 Quality control and validation

Each measurement run included a quality control (QC) sample to monitor system performance. The QC consisted of pooled human serum with a stable bilirubin concentration. It was aliquoted into 80 µL portions and stored at -80 °C.

On measurement days, one aliquot was thawed on ice. Without transferring, 320 µL of mobile phase were added directly into the same tube. The mixture was vortexed and centrifuged using the same procedure as for the regular samples. All preparation steps were kept identical.

To allow for duplicate measurement, two vials were filled with 120 µL of the resulting supernatant. The QC sample helped to verify system stability, especially the shape and position of the Ixα peak, as well as signal consistency.

Across all measurement days, 27 QC values were recorded. The average measured concentration was 8.46 µmol/L, with a variation of about 6.6 %. Retention time remained stable, with a CV of 1.9 % (**Table 9**).

Table 9: *QC results from 27 measurements*

Parameter	Mean	SD	CV (%)
QC Concentration [µmol/L]	8.46	0.56	6.6
Retention Time [min]	9.89	0.19	1.9

4.1.8 Statistical analysis

All analyses were performed using IBM SPSS Statistics 30. Data distribution was checked with the Kolmogorov-Smirnov and Shapiro-Wilk tests. Depending on distribution and group size, independent-samples t-tests (with Welch correction when variances were unequal) or the Mann-Whitney U test were applied for two-group comparisons, and one-way ANOVA or Kruskal-Wallis tests for comparisons across three or more. Paired data (baseline vs. follow-up) were analysed using paired t-tests or Wilcoxon signed-rank tests. Correlations were examined with Pearson or Spearman coefficients as appropriate.

5. Results

Descriptive statistics (see 5.1) were based on all available data at each time point, while statistical analyses included only participants with valid UCB measurements. As a result, sample sizes (n) may vary slightly between tables and figures. At baseline, UCB was analysed in 132 serum samples. For the follow-up, 103 samples were available because some participants did not return for the second visit. The measurements followed the HPLC procedure presented in Chapter 4. After completion, the dataset was imported into IBM SPSS Statistics 30, where it was inspected for missing entries and extreme values. Statistical tests were run with a threshold of $p < 0.05$.

5.1. Overview and demographics

To provide a clear picture of the study cohort, descriptive analyses of baseline and follow-up characteristics were performed (**Table 10, Table 11).**

A total of 132 participants took part at baseline. The average age was 45 years (range 33-65), and about 70% were women. At follow-up, serum samples for UCB analysis were available from 103 participants. Descriptive statistics were based on all available data at each time point, while paired comparisons between baseline and follow-up included only participants with UCB data at both time points (n = 103).

The mean body mass index (BMI) at baseline was 33.0 kg/m^2 (SD 7.5). Two individuals were underweight. Eleven percent of the participants had a normal weight, 25% were overweight, 23% obese, and 39% extremely obese. At follow-up, the distribution was almost the same. The largest group remained in the extremely obese category (41%).

At baseline, blood pressure (BP) averaged 137/84 mmHg. More than half of the cohort showed elevated values. Lifestyle factors showed a mixed pattern. Fourteen percent were current smokers and nearly half reported alcohol intake.

About one quarter of the participants had been hospitalised with COVID-19 (32 of 125 with available data), and some required medical support. At follow-up, 61% reported that they had recovered. Vaccination rates were high, with 86% having received at least one dose. Previous infection was confirmed in nearly all cases, as 98% were IgG positive.

At baseline, the mean UCB level was 4.7 µmol/L (SD 3.2). At follow-up, the mean level was slightly lower at 4.1 µmol/L (SD 2.7).

In summary, most participants were middle-aged women, many of them were obese or had high BP. UCB levels were low at both time points and remained stable. These values were taken as the basis for the later analysis on Long COVID.

Table 10: *Baseline characteristics of the study population*

Parameter	**n (%)**	**Mean ± SD**	**Range**
Demographics			
Age [years]	132	45.1 ± 7.9	33.0 - 65.0
Gender			
– *Female*	93 (70.5)		
– *Male*	39 (29.5)		
Anthropometry			
BMI [kg/m²]		33.0 ± 7.5	15.8 - 52.3
BMI categories			
– *Underweight (< 18.50 kg/m²)*	2 (1.5)	16.1 ± 0.4	15.8 - 16.3
– *Normal (18.50 - 24.99 kg/m²)*	15 (11.4)	22.3 ± 2.0	18.6 - 24.9
– *Overweight (25.00 - 29.99 kg/m²)*	33 (25.0)	27.5 ± 1.7	25.0 - 29.7
– *Obese (30.00 - 34.99 kg/m²)*	30 (22.7)	32.7 ± 1.5	30.0 - 34.9
– *Extremely obese (≥ 35.00 kg/m²)*	52 (39.4)	40.4 ± 4.4	35.0 - 52.3
Systolic BP [mmHg]		136.9 ± 20.7	76.0 - 204.0
Diastolic BP [mmHg]		83.5 ± 13.1	54.0 - 128.0
COVID-19 baseline			
COVID illness severity score	131	- 0.43 ± 9.04	- 20 to 20
Lifestyle			
Smoking status			
– *Smoker*	18 (13.6)		
– *Non-smoker*	114 (86.4)		
Alcohol consumption			
– *Alcohol use*	62 (47.0)		
– *No alcohol use*	70 (53.0)		

Biomarker		
UCB baseline [µmol/L]	4.7 ± 3.2	0.5 - 19.8

Note: The number of participants was 132 for all parameters at baseline, except for the COVID illness severity score (n = 131).

Table 11: *Follow-up characteristics of the study population*

Parameter	**n (%)**	**Mean ± SD**	**Range**
Anthropometry			
BMI [kg/m²]	103	33.4 ± 8.0	18.7 - 53.1
BMI categories			
– Normal (18.50 - 24.99 kg/m²)	15 (14.6)	22.1 ± 2.0	18.7 - 24.6
– Overweight (25.00 - 29.99 kg/m²)	24 (23.3)	27.3 ± 1.2	25.7 - 29.6
– Obese (30.00 - 34.99 kg/m²)	22 (21.4)	32.9 ± 1.3	30.1 - 34.8
– Extremely obese (≥ 35.00 kg/m²)	42 (40.8)	41.1 ± 5.0	35.2 - 53.1
Systolic BP [mmHg]	104	135.6 ± 18.6	99.0 - 189.0
Diastolic BP [mmHg]	104	82.3 ± 12.1	54.0 - 123.0
Lifestyle			
Smoking status	102		
– Smoker	17 (16.7)		
– Non-smoker	85 (83.3)		
Alcohol consumption	102		
– Alcohol use	42 (31.8)		
– No alcohol use	60 (58.8)		
COVID-19 follow-up			
Hospitalisation	125		
– Hospitalised	32 (25.6)		
– Not hospitalised	93 (74.4)		
Hospital support	12		
– Hospital support	11 (91.7)		
– No support	1 (8.3)		
Recovery status	81		

– *Recovered*	49 (60.5)		
– *Not recovered*	32 (39.5)		
Vaccination	101		
– *Vaccinated*	87 (86.1)		
– *Not vaccinated*	14 (13.9)		
Antibody IgG	100		
– *neg.*	2 (2.0)		
– *pos.*	98 (98.0)		
Antibody IgM	100		
– *neg.*	97 (97.0)		
– *pos.*	3 (3.0)		
Biomarker			
UCB follow-up [µmol/L]	103	4.1 ± 2.7	1.4 - 21.0

Note: The number of participants differs between parameters because not all participants provided complete follow-up data. Descriptive statistics were calculated using all available values for each variable.

5.2 Group differences in UCB

To examine the role of UCB, several ways of dividing the cohort were tested. The lower reference limit of serum bilirubin is commonly set at around 5 µmol/L. Slightly higher concentrations have been linked to antioxidant and protective properties (Vítek & Tiribelli, 2021b). Based on this, three strategies were used in the analysis: a fixed cut-off at 5 µmol/L, a split at the cohort median, and a division into quartiles.

Cut-offs at 10 µmol/L and 17 µmol/L were also considered, since these values are frequently reported in epidemiological and clinical studies. However, in this cohort only very few participants reached levels above 10 µmol/L (n = 8 at baseline, n = 3 at follow-up), and concentrations in the Gilbert syndrome range (> 17 µmol/L) were almost absent (n = 2 at baseline, n = 1 at follow-up). For this reason, these thresholds were not applied, as they would not have resulted in meaningful subgroup comparisons.

5.2.1 Group comparisons by cut-off (5 µmol/L)

Using the cut-off 5 µmol/L, participants were separated into a low and a high UCB group. At baseline, 89 individuals were in the low group (< 5 µmol/L), while 43 participants were classified as high (≥ 5 µmol/L). At follow-up, 81 participants were in the low group, and 22 in the high group. Mean concentrations differed clearly between the groups at both time points ($p < 0.001$) (**Table 12).**

Table 12: *UCB by subgroup (cut-off 5 µmol/L)*

Timepoint	Group (UCB)	n	Mean (µmol/L) ± SD	Range	p-value
Baseline	Low (< 5)	89	3.1 ± 1.0	0.5 - 4.9	< 0.001
	High (≥ 5)	43	7.9 ± 3.6	5.0 - 19.8	
Follow-up	Low (< 5)	81	3.1 ± 0.9	1.4 - 5.0	< 0.001
	High (≥ 5)	22	7.9 ± 3.7	5.1 - 20.9	

5.2.2 Group comparisons by median

The cohort was further split by the median to get two groups. At baseline, the lower group had UCB values around 2-3 µmol/L, while the higher group was clearly above, with mean values around 6-7 µmol/L. At follow-up it looked quite similar. The lower half stayed near 2-3 µmol/L, the higher half was roughly double. The ranges overlapped, so it was not a sharp cut, but the difference between the group was still obvious (**Table 13**).

Table 13: *UCB at baseline by median split*

Timepoint	Group (UCB)	n	Mean (µmol/L) ± SD	Range	p-value
Baseline	≤ Median	66	2.7 ± 0.8	0.5 - 3.9	< 0.001
	> Median	66	6.7 ± 3.4	4.0 - 19.8	
Follow-up	≤ Median	51	2.6 ± 0.5	1.4 - 3.4	< 0.001
	> Median	52	5.7 ± 3.0	3.5 - 20.9	

5.2.3 Group comparisons by quartiles

For a finer resolution, UCB concentrations were also divided into quartiles. At baseline, the mean ranged from 2.0 µmol/L in Q1 up to 8.7 µmol/L in Q4, with Q2 and Q3 lying in between. The pattern at follow-up was similar, starting at 2.1 µmol/L in the lowest quartile and rising to 7.4 µmol/L in the highest. The overall group difference was clear and statistically significant ($p < 0.001$) (**Table 14**).

***Table 14**: UCB at baseline by quartiles*

Timepoint	Group (UCB Quartiles)	n	Mean (µmol/L) ± SD	Range	p-value
Baseline	Q1 (lowest)	33	2.0 ± 0.6	0.5 - 2.9	< 0.001
	Q2	33	3.4 ± 0.3	2.9 - 3.9	
	Q3	33	4.7 ± 0.5	4.0 - 5.5	
	Q4 (highest)	33	8.7 ± 3.8	5.5 - 19.8	
Follow-up	Q1 (lowest)	25	2.1 ± 0.4	1.4 - 2.6	< 0.001
	Q2	26	3.0 ± 0.2	2.6 - 3.5	
	Q3	26	4.0 ± 0.4	3.5 - 4.6	
	Q4 (highest)	26	7.4 ± 3.6	4.7 - 20.9	

The subgrouping showed consistent differences in UCB concentrations. Whether split by 5 µmol/L, by the median, or by quartiles, the same picture was seen at baseline and follow-up.

5.3 Baseline vs. follow-up comparison

After the subgroup analyses, the next step was to check whether UCB levels changed over time within the same individuals. For this purpose, baseline values were compared with follow-up data in participants who had measurements at both time points (n = 103).

At baseline, the mean UCB concentration was 4.5 µmol/L ± 3.1. At follow-up, the mean was slightly lower at 4.1 µmol/L ± 2.7.

The paired t-test showed no significant difference between baseline and follow-up values ($t(102) = 1.34$, $p = 0.173$). The calculated effect size was small (Cohen's $d = 0.14$) (**Table 15**).

Table 15: *Baseline vs. follow-up for UCB values*

Timepoint	Mean (µmol/L) ± SD	n	t(df)	p-value	Cohen's d
Baseline	4.5 ± 3.1	103			
Follow-up	4.1 ± 2.7	103	1.34 (102)	0.173	0.14

Baseline and follow-up values were, however strongly related ($r = 0.69$, $p < 0.001$). This means that individuals with higher or lower levels at baseline tended to remain in the same range at follow-up, even though the overall group average did not change (**Figure 15**). These findings suggest that UCB levels in this cohort remained largely stable over the 12-month period.

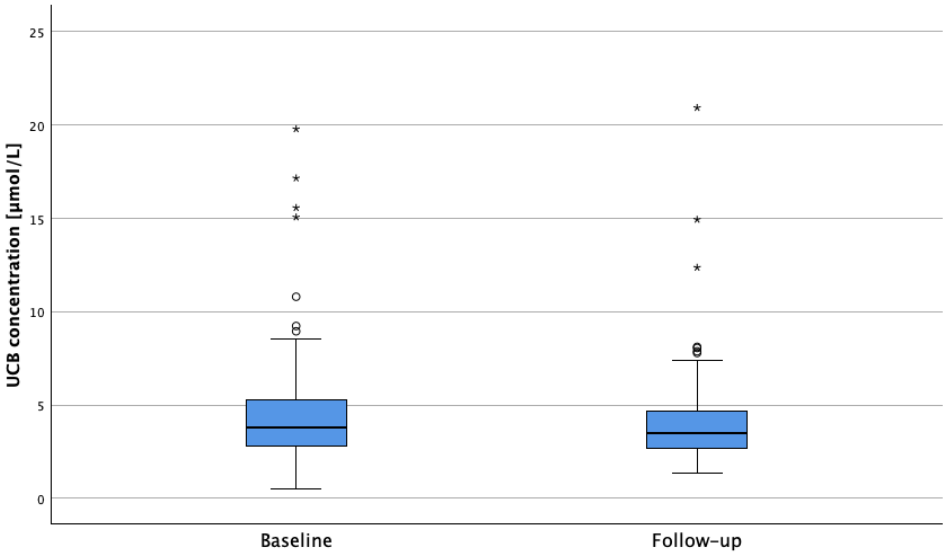

Figure 15: *Boxplot of baseline and follow-up UCB serum values (n = 103)*

No significant difference was found between timepoints (p = 0.173).

5.4. Associations with covariates

To better understand what might influence UCB, its associations with gender, age, BMI and selected clinical factors were examined. Both baseline and follow-up values were included, using continuous data and subgroup comparisons where appropriate. Analyses were based on participants with valid UCB and corresponding covariate data (n = 132 at baseline, n = 103 at follow-up).

5.4.1 Gender and UCB

At baseline, UCB was measured in 39 men and 93 women. Men had higher average values than women (5.3 ± 3.4 µmol/L vs. 4.4 ± 3.1 µmol/L). At follow-up, 31 men and 72 women were included. The same picture was seen again, with men showing higher levels (4.6 ± 2.4 µmol/L vs. 3.9 ± 2.8 µmol/L) (**Table 16**).

Independent t-test did not show a significant difference between men and women (baseline p = 0.152, follow-up p = 0.242). Still, the overall direction fits with earlier studies, where men usually had higher bilirubin than women (Vítek & Tiribelli, 2021b). In this dataset, the smaller number of men and the wide spread of values probably made it harder to see a clear separation (**Figure 16, Figure 17**).

These results pointed in the same direction as earlier studies, although the difference between men and women did not reach statistical significance here.

Table 16: *UCB by gender at baseline (n = 132) and follow-up (n = 103)*

Gender	Baseline UCB (µmol/L, Mean ± SD)	Follow-up UCB (µmol/L, Mean ± SD)
Male	5.3 ± 3.4	4.6 ± 2.4
Female	4.4 ± 3.1	3.9 ± 2.8
p-value	0.152	0.242

Note: p-values are based on independent t-tests.

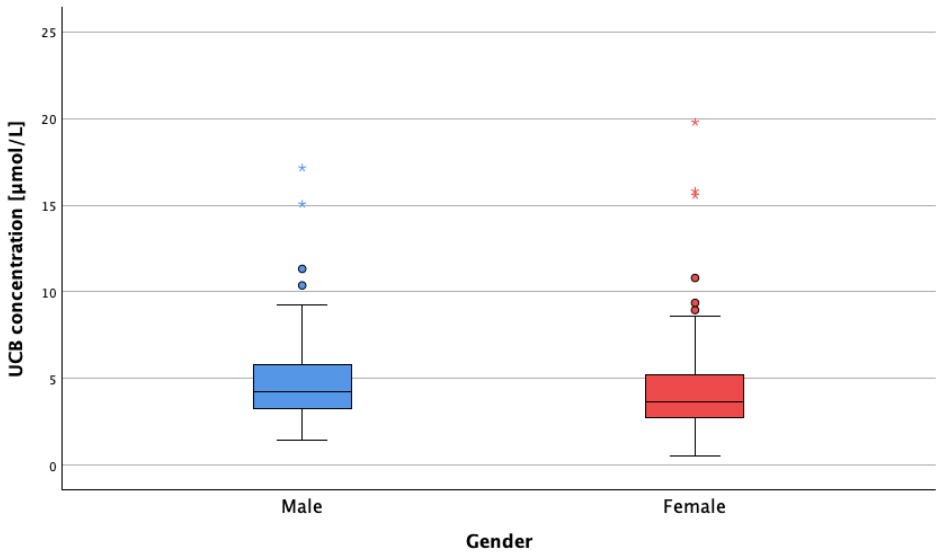

Figure 16: *Baseline UCB values by gender*

Men tended to show higher values, but the difference was not statistically significant (p = 0.152).

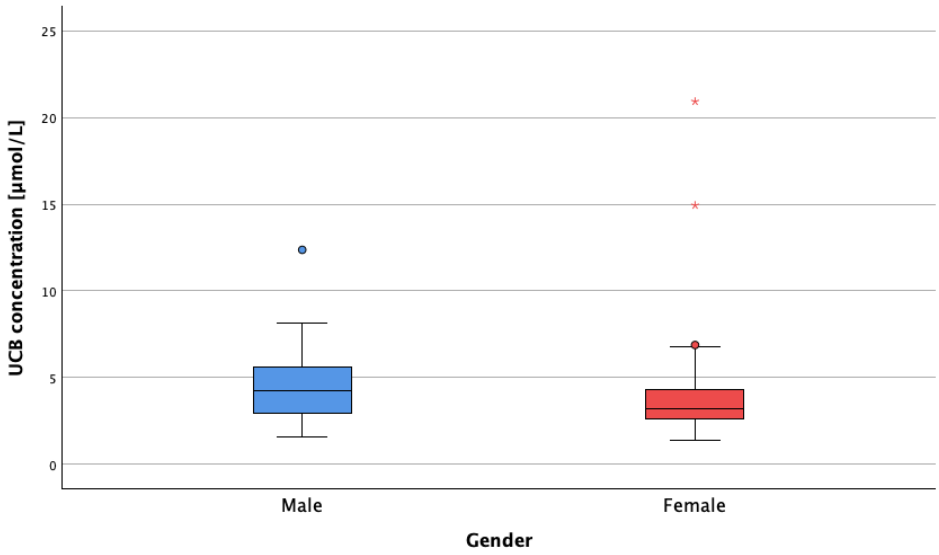

Figure 17: *Follow-up UCB values by gender*

Again, men had higher mean values, but no significant difference was observed (p = 0.242).

5.4.2 Age and UCB

Participants were on average 45 years old, with ages ranging from 33 to 65 years. Age was first analyzed as a continuous variable using Pearson's correlation. At baseline, there was a weak positive correlation with UCB (r = 0.196, p = 0.024, **Figure 20**). At follow-up, no correlation was found (r = 0.152, p = 0.125, **Figure 21**).

For subgroup analysis, a cut-off at 50 years was used. This cut-off was used, as it matched the age distribution of the cohort and provided a clear separation of UCB levels. Most participants were 50 or younger. This group showed lower UCB values. Participants older than 50 had higher levels at both time points. At baseline, means were 4.2 ± 2.6 µmol/L for the younger group and 5.8 ± 4.0 µmol/L for the older (p = 0.029). At follow-up, the difference remained (3.7 ± 2.0 vs. 5.2 ± 3.8 µmol/L, p = 0.062, **Table 17**, **Figure 18**, **Figure 19**).

***Table 17**: UCB by age group at baseline (n = 132) and follow-up (n = 103)*

Age group	**Baseline UCB (µmol/L, Mean ± SD)**	**Follow-up UCB (µmol/L, Mean ± SD)**
≤ 50 years	4.2 ± 2.6	3.7 ± 2.0
> 50 years	5.8 ± 4.0	5.2 ± 3.8
p-value	0.029	0.062

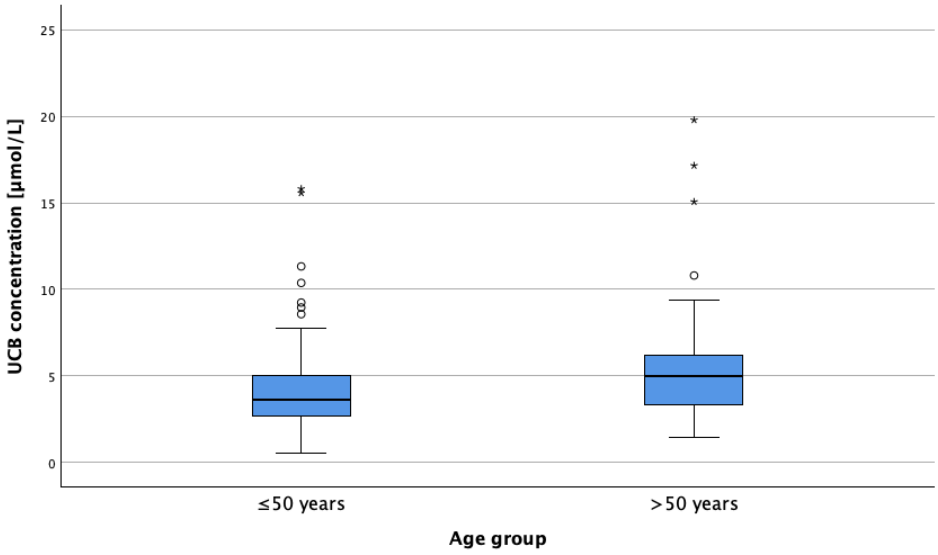

***Figure 18:** Baseline UCB concentrations by age group (≤ 50 vs. > 50 years, n = 132)*

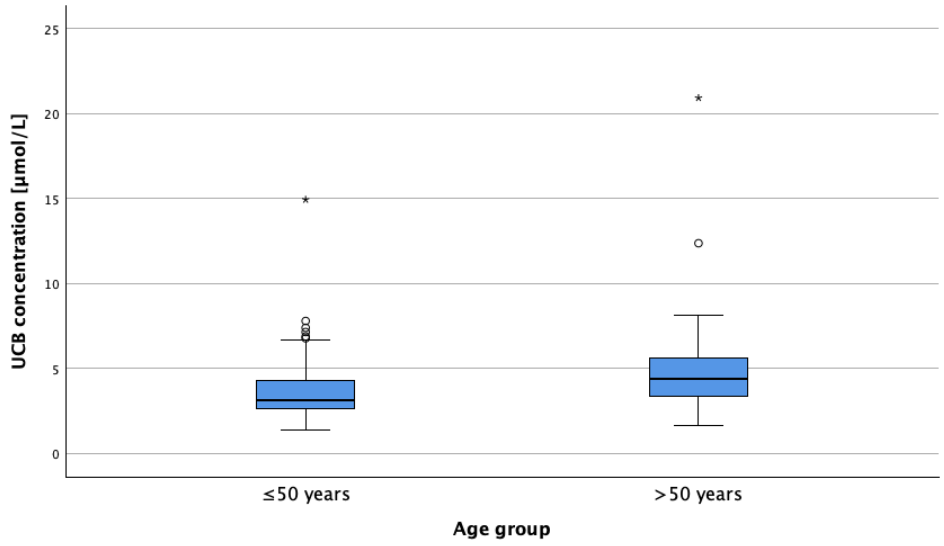

Figure 19: *Follow-up UCB concentrations by age group (≤ 50 vs. > 50 years, n = 103)*

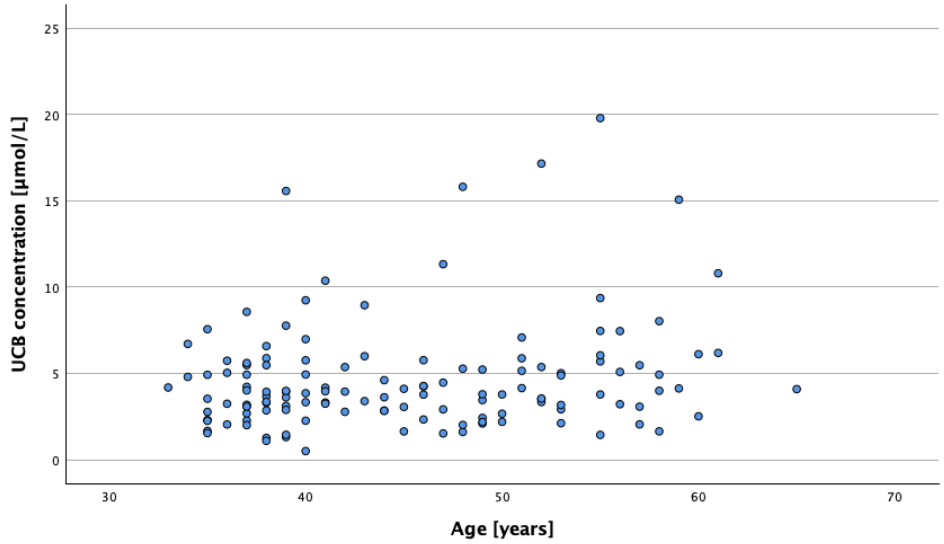

Figure 20: *Correlation between UCB values and age at baseline*

n = 132, a weak positive correlation was observed (r = 0.196, p = 0.024)

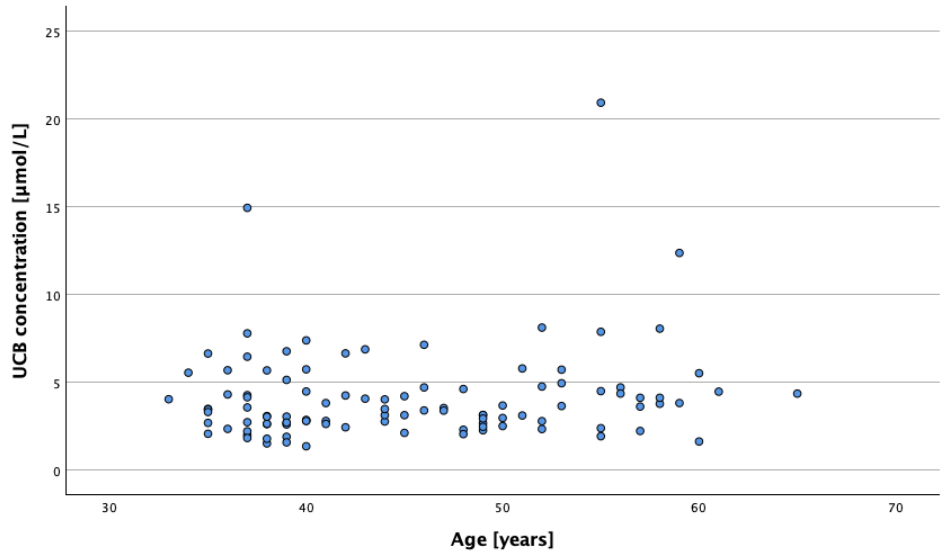

Figure 21: *Correlation of UCB values and age at follow-up*

n = 103, no significant correlation was found (r = 0.152, p = 0.125)

Taken together, age showed only a weak link to UCB, but participants above 50 years consistently had higher concentrations at both time points.

5.4.3 Body mass index and UCB

BMI was compared with UCB at baseline and follow-up. The correlation was weak in both cases. At baseline, the association was r = - 0.144 (p = 0.100), and at follow-up r = - 0.166 (p = 0.136). Spearman's rho showed a similar negative trend, but also without significance.

Looking at BMI groups, normal weight and overweight participants had higher mean UCB values. Obese and extremely obese groups had lower values. At follow-up the same picture was seen. Group differences were not statistically significant (baseline ANOVA p = 0.320 (**Table 18**), follow-up ANOVA p = 0.244 (**Table 19**)). This trend is also shown in **Figure 22** which shows the distribution of baseline UCB across BMI categories.

For follow-up analyses, only participants with valid data for both BMI and UCB were included (n = 82). The smaller sample compared to the demographic overview (n = 103 for BMI) reflects missing values in either variable.

In summary, higher BMI went along with lower bilirubin values in this cohort. The reduced number of follow-up cases (n = 82) may have influenced the results.

Table 18: *Baseline UCB concentrations by BMI categories*

BMI category	n	Mean UCB (µmol/L) ± SD	Range	p-value
Underweight (<18.5)	2	2.6 ± 0.4	2.3 - 2.9	
Normal (18.5–24.9)	15	5.3 ± 4.8	1.5 - 19.8	
Overweight (25.0–29.9)	33	5.5 ± 4.1	1.1 - 17.2	
Obese (30.0–34.9)	30	4.4 ± 1.8	1.7 - 9.4	
Extremely obese (≥ 35.0)	52	4.2 ± 2.5	0.5 - 15.8	
Total	132	4.7 ± 3.2	0.5 - 19.8	0.320

Table 19: *Follow-up UCB concentrations by BMI categories*

BMI category	n	Mean UCB (µmol/L) ± SD	Range	p-value
Normal (18.5 – 24.9)	9	3.8 ± 1.7	1.8 - 6.6	
Overweight (25.0 – 29.9)	20	4.9 ± 3.1	2.0 - 14.9	
Obese (30.0 – 34.9)	18	3.7 ± 1.5	1.4 - 6.8	
Extremely obese (≥ 35.0)	35	3.7 ± 2.0	1.5 - 12.4	
Total	82	4.0 ± 2.2	1.4 - 14.9	0.244

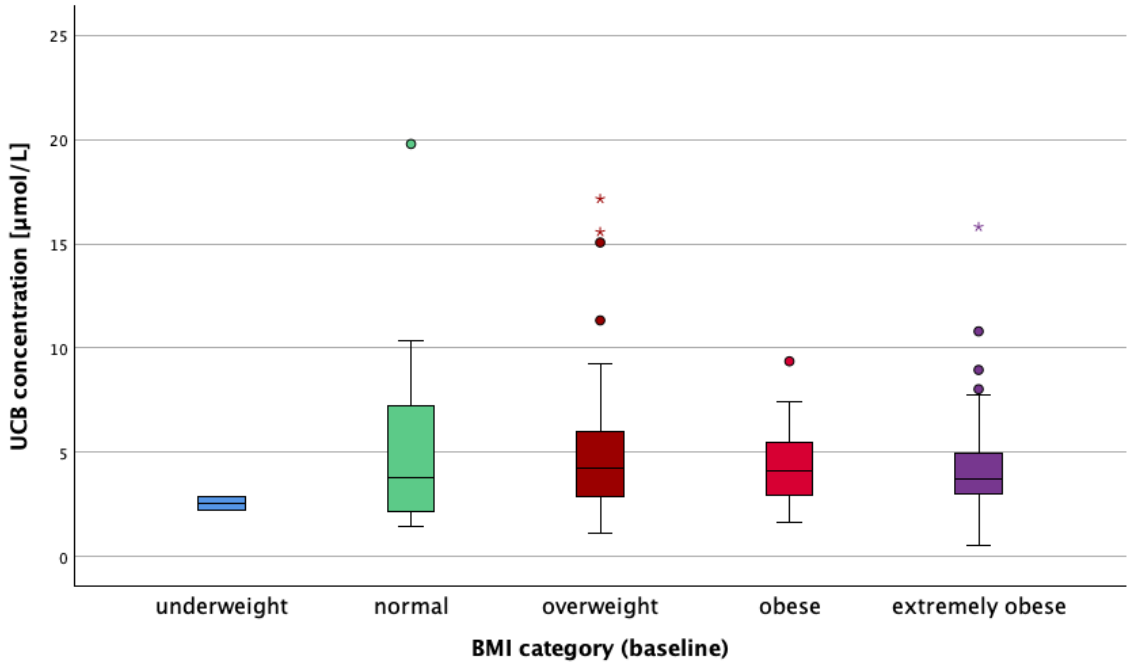

Figure 22: *Boxplot of baseline UCB concentrations by BMI categories*

5.4.4 Blood pressure and UCB

When compared with UCB, no clear relation was seen. Correlation tests gave very weak values, both for systolic and diastolic pressure, and none of them were significant. The results were similar at baseline and follow-up and confirmed by non-parametric testing (**Table 20**).

Taken together, bilirubin concentrations in this cohort did not vary with BP.

Table 20: *UCB concentrations and blood pressure*

Timepoint	Parameter	n	Mean UCB (µmol/L) ± SD	r	p-value
Baseline	Systolic BP (mmHg)	132	4.7 ± 3.2	0.023	0.793
	Diastolic BP (mmHg)	132	4.7 ± 3.2	- 0.067	0.448
Follow-up	Systolic BP (mmHg)	101	4.0 ± 2.2	- 0.074	0.460
	Diastolic BP (mmHg)	101	4.0 ± 2.2	- 0.004	0.968

Note: Mean ± SD represents average UCB concentrations of included participants. Correlations are Pearson's r; only cases with complete UCB and BP data were analyzed (n = 101 at follow-up).

5.4.5 Type 2 Diabetes and UCB

Type 2 diabetes status was also checked in relation to UCB. At baseline, participants with T2D (n = 15) showed lower mean levels (3.5 ± 2.2 µmol/L) than those without T2D (4.8 ± 3.3 µmol/L, n = 117). The difference, however, was not statistically significant (p = 0.127).

At follow-up, the pattern remained the same. Participants with T2D (n = 13) again had lower UCB levels (3.6 ± 1.5 µmol/L) compared to non-diabetic ones (4.2 ± 2.8 µmol/L, n = 90), but this difference was also not significant (p = 0.405, **Table 21**, **Figure 23**, **Figure 24**).

Taken together, bilirubin concentrations were lower in participants with T2D at both timepoints. The small number of cases limits the strength of this finding, but it may point to a link between glucose metabolism and reduced antioxidant capacity.

Table 21: *UCB concentrations and T2D*

Timepoint	Type 2 Diabetes	n	Mean UCB (µmol/L) ± SD	p-value
Baseline	T2D	15	3.5 ± 2.2	0.127
	No T2D	117	4.8 ± 3.3	
Follow-up	T2D	13	3.6 ± 1.5	0.405
	No T2D	90	4.2 ± 2.8	

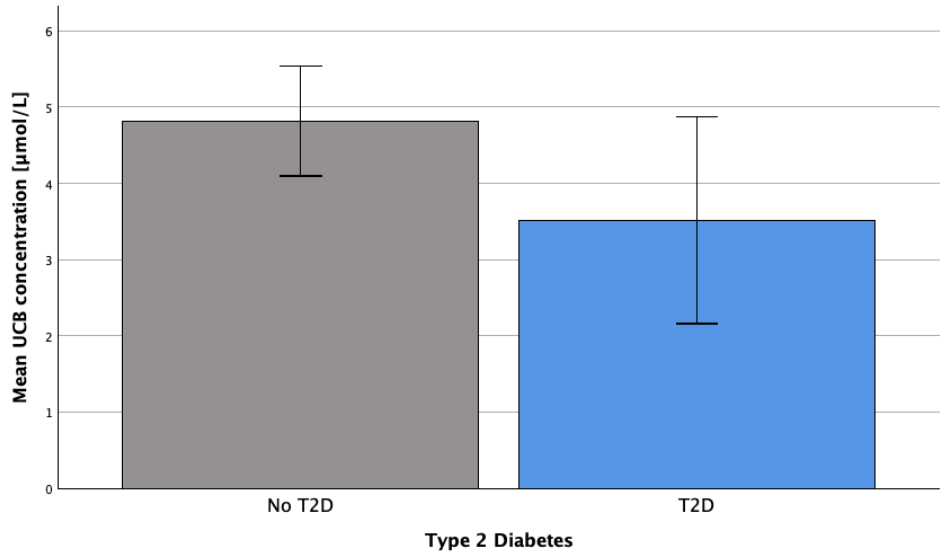

Figure 23: *Mean UCB concentrations and T2D status at baseline (n = 132, p = 0.127)*

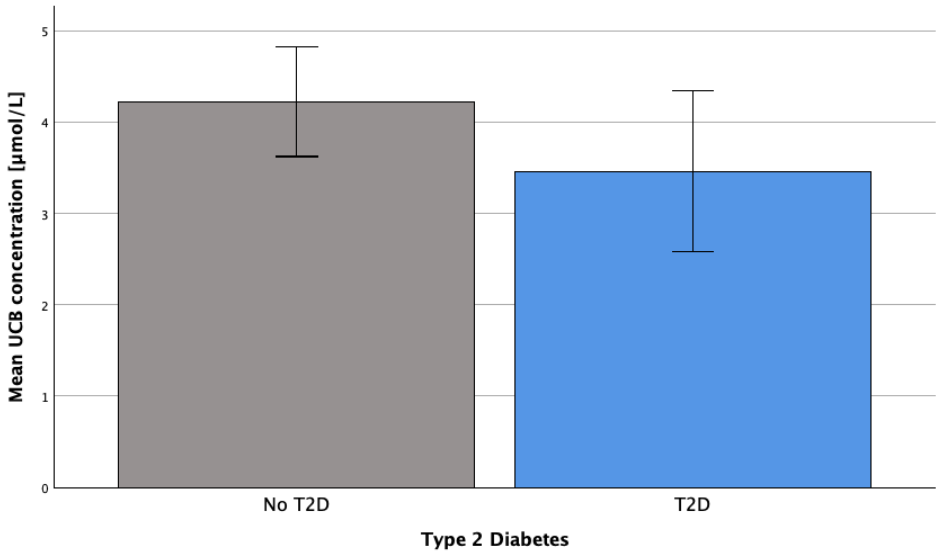

Figure 24: *Mean UCB concentrations and T2D status at follow-up (n = 103, p = 0.405)*

5.4.6 Lifestyle parameters and UCB

UCB was also checked in relation to lifestyle. The focus was on smoking and alcohol use at baseline and follow-up.

Participants were grouped by their self-reported smoking status in the questionnaire, categorized as yes (current smokers) or no (non-smokers). At baseline, smokers had clearly lower UCB values than non-smokers. The mean concentration was 3.1 ± 1.8 µmol/L in smokers (n = 18) compared to 4.9 ± 3.3 µmol/L in non-smokers (n = 114), a difference that reached statistical significance (p = 0.024).

At follow-up, the same pattern was observed. Smokers (n = 16) showed mean values of 3.4 ± 1.7 µmol/L, while non-smokers (n = 84) reached 4.3 ± 2.9 µmol/L. The difference was smaller and no longer significant in the standard t-test (p = 0.214), although the test correcting for unequal variance pointed to a borderline effect (p = 0.083, **Table 22**, **Figure 25**, **Figure 26)**.

Taken together, bilirubin concentrations tended to be lower in smokers at both time points.

Table 22: *UCB serum concentrations by smoking status*

Timepoint	Smoking status	n	Mean UCB (µmol/L) ± SD	p-value
Baseline	Non-smoker	114	4.9 ± 3.3	0.024
	Smoker	18	3.1 ± 1.8	
Follow-up	Non-Smoker	84	4.3 ± 2.9	0.214 (equal var) 0.083 (unequal var)
	Smoker	16	3.4 ± 1.7	

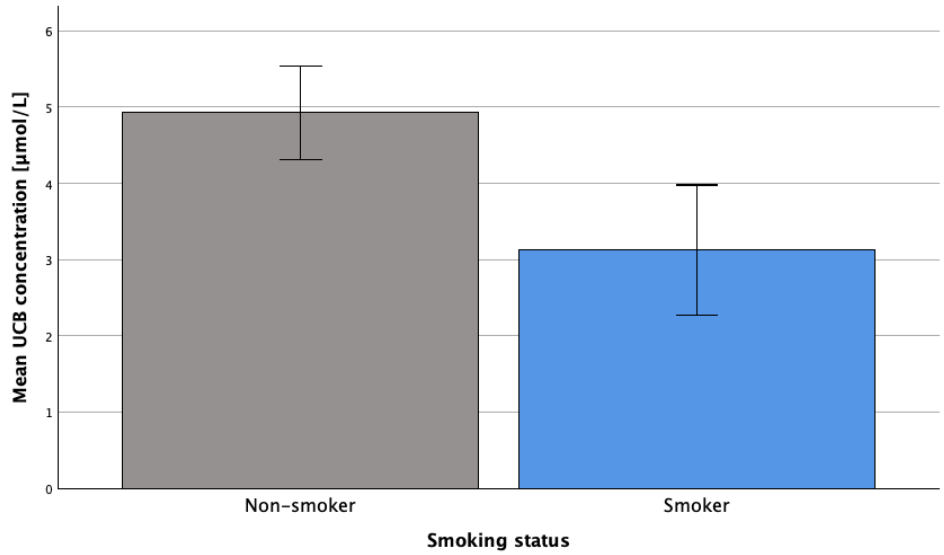

Figure 25: *Mean UCB concentrations by smoking status at baseline (n = 132, p = 0.024)*

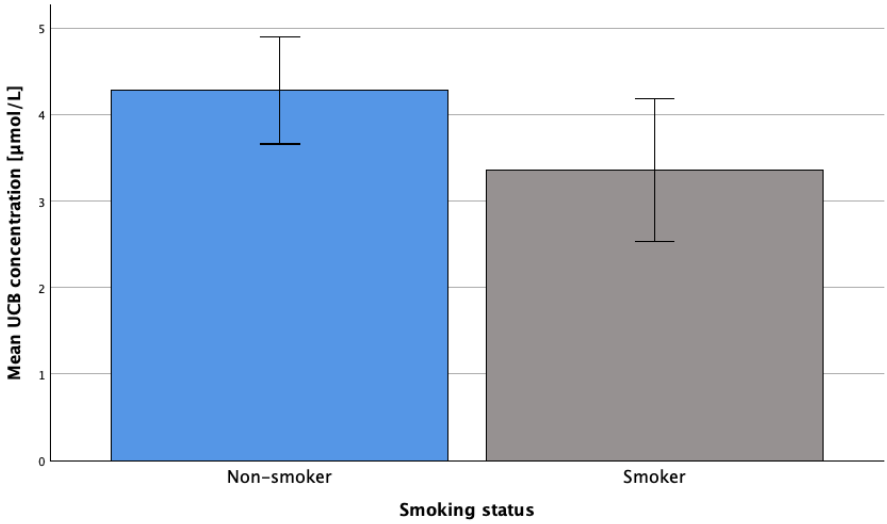

Figure 26: *Mean UCB concentrations by smoking status at follow-up (n = 100, p = 0.214)*

The following analysis focused on alcohol consumption. Based on the questionnaire data, participants were divided into two groups according to their reported alcohol use ("yes" for any current alcohol intake, "no" for none). At baseline, UCB levels were very similar between both groups. Mean concentrations were 4.7 ± 3.0 µmol/L in participants reporting no alcohol use (n = 70) and 4.6 ± 3.4 µmol/L in those reporting alcohol consumption (n = 62) (**Table 23**). The difference was not significant (p = 0.863). At follow-up, the same pattern was observed (p = 0.429).

Taken together, alcohol use, defined as any self-reported current intake, had no detectable effect on UCB concentrations in this cohort.

Table 23: *UCB serum concentrations by alcohol consumption*

Time point	Alcohol consumption	n	Mean UCB (µmol/L) ± SD	p-value
Baseline	No alcohol use	70	4.7 ± 3.0	0.863
	Alcohol use	62	4.6 ± 3.4	
Follow-up	No alcohol use	59	4.0 ± 2.4	0.429
	Alcohol use	41	4.4 ± 3.2	

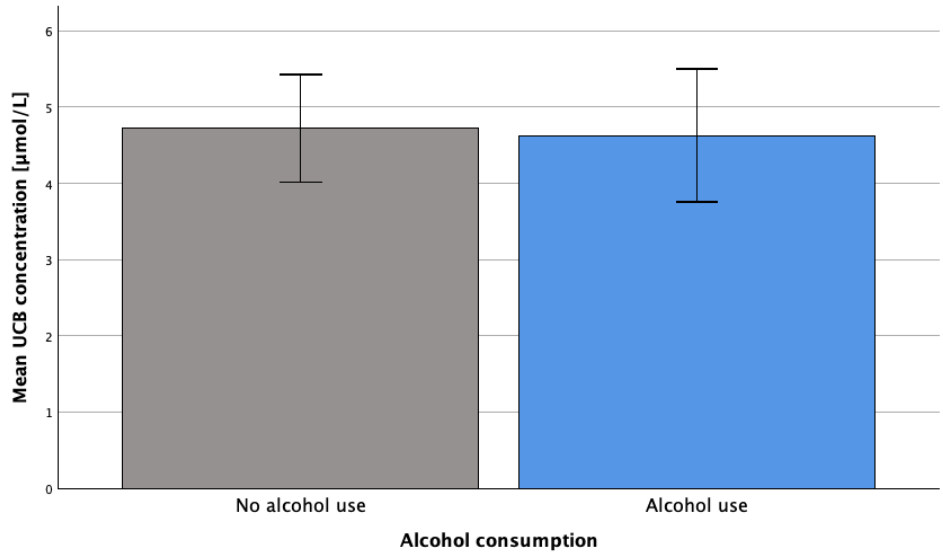

Figure 27: *Mean UCB concentrations by alcohol consumption at baseline (n = 132)*

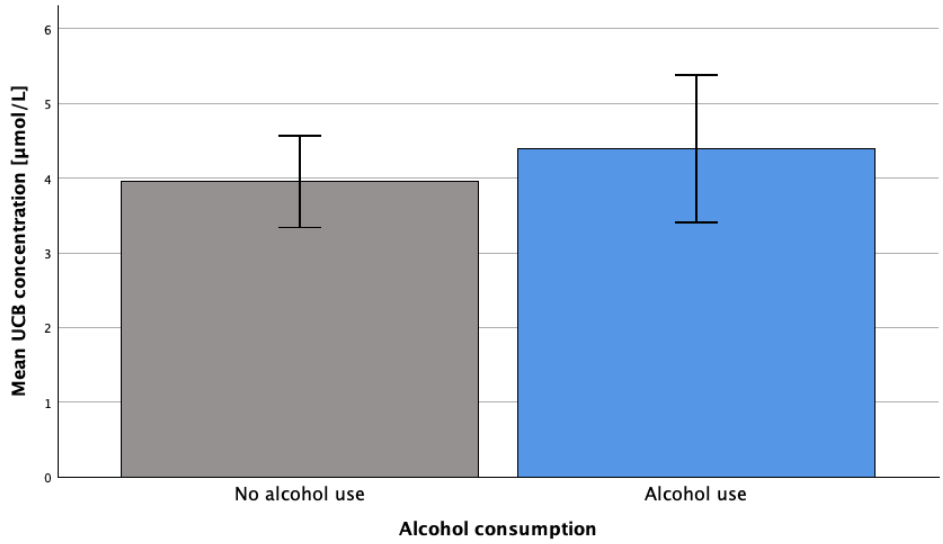

Figure 28: *Mean UCB concentrations by alcohol consumption at follow-up (n = 100)*

5.5 Long COVID and UCB

5.5.1 Long COVID status

To test a possible link between bilirubin and recovery, UCB levels were compared between participants with and without persistent symptoms.

At follow-up, 63 participants gave information on their status. Mean UCB concentrations were 3.9 ± 1.8 µmol/L in the recovered group (n = 38) and 4.0 ± 1.8 µmol/L in the non-recovered group (n = 25). The difference was not significant (t-test p = 0.876). A Mann-Whitney test gave similar results (p = 0.715). In this cohort, UCB at follow-up was not related to recovery status (see **Table 24**, **Figure 29**, **Figure 30**).

Table 24: *Recovery status and follow-up UCB concentrations*

Timepoint	Recovery status	n	Mean UCB (µmol/L) ± SD	p-value
Follow-up	Not recovered	25	4.0 ± 1.8	0.876 (t-test, two-tailed); 0.715 (Mann-Whitney)
	Recovered	38	3.9 ± 1.8	

Note: Analyses were performed on participants with complete data for recovery status and follow-up UCB (n = 63).

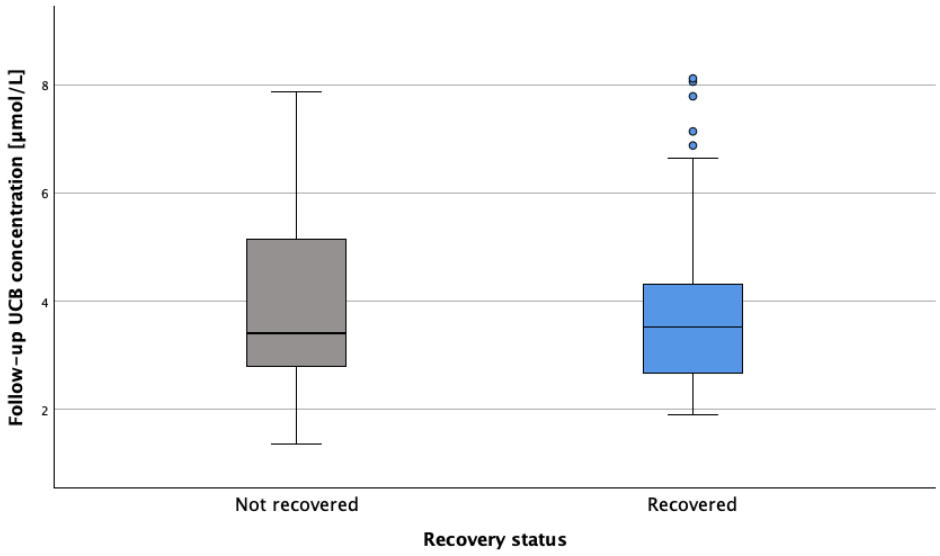

Figure 29: *Follow-up UCB serum concentrations by recovery status (n = 63)*

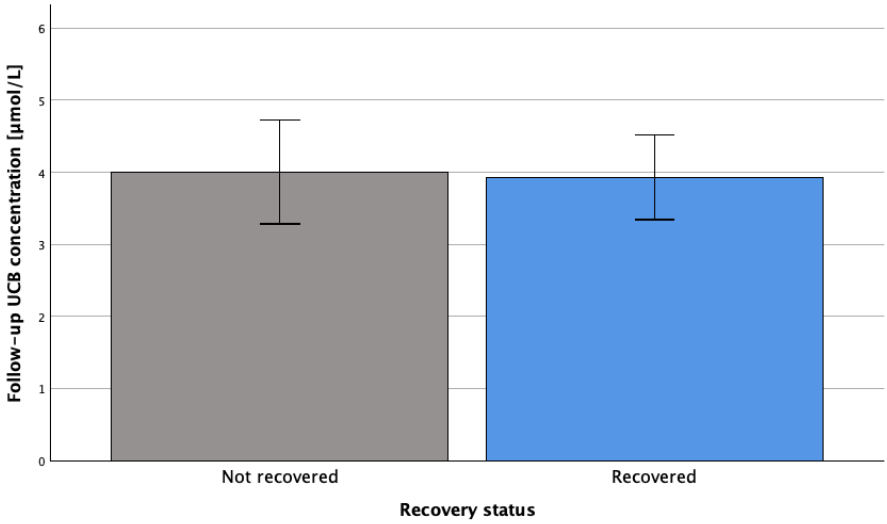

Figure 30: *Mean follow-up UCB serum concentrations by recovery status (n = 63)*

To test whether recovery status differed by bilirubin group, UCB was also split at the 5 µmol/L cut-off. Among participants without recovery, 72% fell into the lower-UCB group and 28% into the high group. In the recovered group, the proportions were similar (79% vs. 21%). Chi-square and Fisher's test showed no significant association (p = 0.526, p = 0.558) (**Table 25**). This confirms that UCB, when categorized by the 5 µmol/L threshold, was not related to recovery status.

Table 25: *Recovery status by UCB subgroup at follow-up (cut-off 5 µmol/L)*

Recovery status	Low UCB (< 5 µmol/L)	High UCB (≥ 5 µmol/L)	Total	p-value
Not recovered	18 (72%)	7 (28%)	25	
Recovered	30 (79%)	8 (21%)	38	
Total	48 (76%)	15 (24%)	63	0.526 (Chi^2), 0.558 (Fisher)

In addition, groups were tested using median split, and quartiles. None of these showed significant difference between recovered and non-recovered participants.

5.5.2 COVID illness severity score

The COVID illness severity score was used to rate the acute SARS-CoV-2 infection. It combined several self-reported symptom domains, such as fatigue and pain, into a single value ranging from -20 to +20. Higher score meant more severe illness. Data were available for 131 participants at baseline.

When baseline UCB was tested against this score, no link appeared. The correlations were very small and not significant (Pearson r = 0.04, p = 0.664; Spearman rho = 0.08, p = 0.364). To see if groups might show clearer differences, this cohort was divided into three severity levels (**Table 26**, **Figure 32, Figure 33**). Mean UCB varied only slightly, and the ANOVA showed no significance (p = 0.464).

The same test was repeated from the other side. First with a cut-off at 5 µmol/L, then by median split, and with quartiles. In every case, the distribution of severity scores stayed balanced. None of these groupings pointed to higher illness severity at low bilirubin (all p > 0.6).

Overall, UCB did not track with severity in this cohort. This is in line with the recovery analysis after twelve months (section 5.5.1), where no excess of low UCB was seen in non-recovered participants.

Table 26: *Baseline UCB concentrations by COVID-19 illness severity groups*

Illness severity group	n	Mean UCB (µmol/L) ± SD	95% CI (Lower–Upper)	Range
Low severity	49	4.4 ± 2.8	3.6 - 5.2	1.4 - 14.9
Moderate severity	39	4.5 ± 3.1	3.4 - 5.5	1.8 - 12.4
High severity	43	5.3 ± 3.7	4.0 - 6.3	1.5 - 20.9
Total	131	4.7 ± 3.1	4.1 - 5.2	1.4 - 20.9

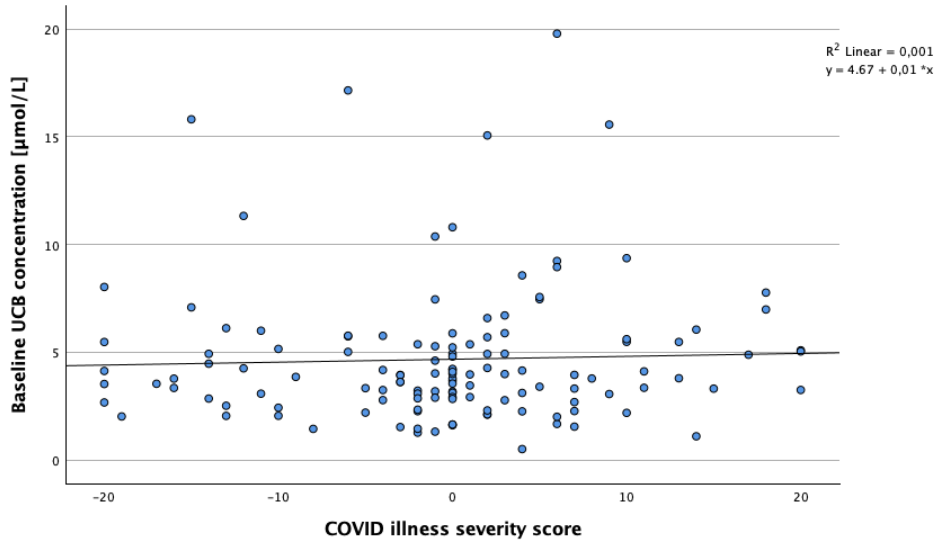

Figure 31: *Scatterplot of COVID-19 illness severity score and baseline UCB concentrations (n = 131)*

Group differences were not statistically significant (ANOVA, p = 0.464). The regression line shows the absence of a significant correlation (Pearson r = 0.04, p = 0.664).

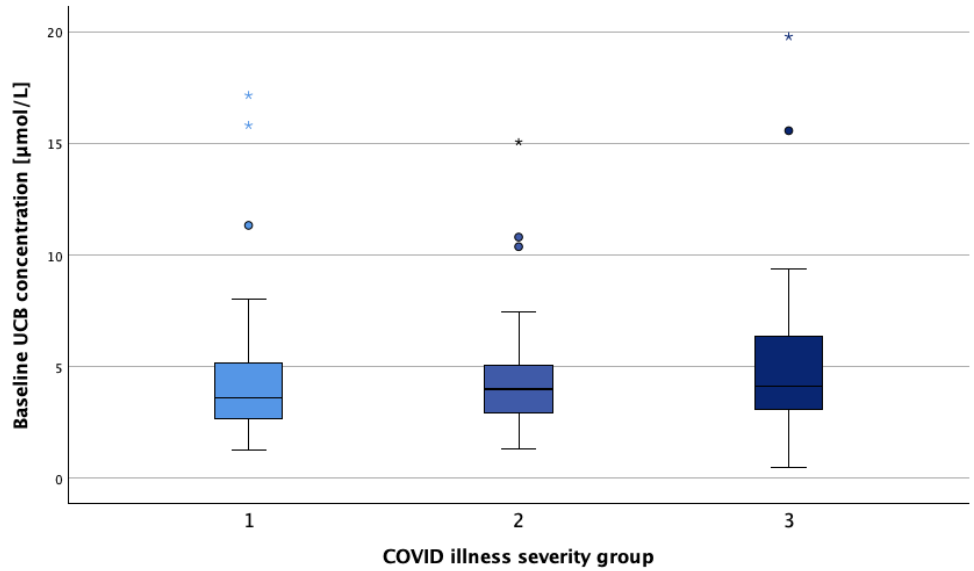

Figure 32: *Baseline UCB concentrations by COVID-19 illness severity groups*

1 = low, 2 = moderate, 3 = high. No significant differences were observed between groups.

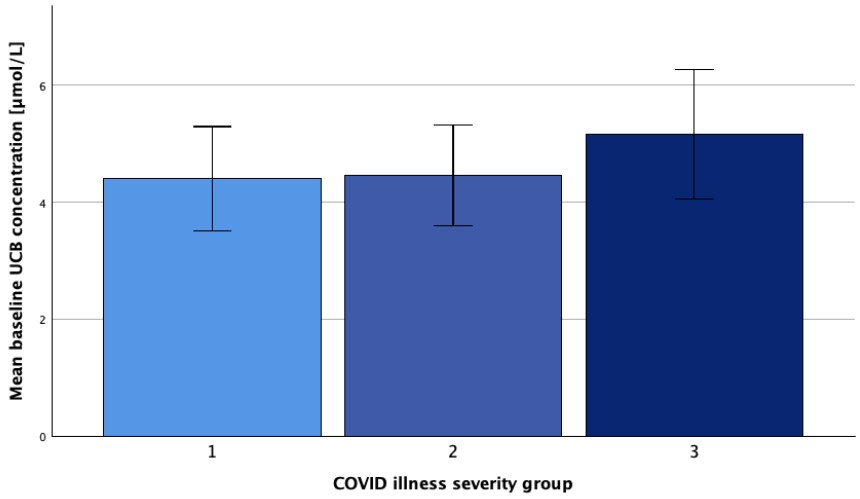

Figure 33: *Mean baseline UCB concentrations by COVID-19 illness severity groups (n = 131)*

Hospitalization and hospital days

To check whether hospitalization in the acute phase was linked to later UCB, participants were divided into two groups (hospitalized vs. not). At follow-up, mean UCB was 4.4 ± 3.9 µmol/L in hospitalised (n = 23) and 4.1 ± 2.3 µmol/L in non-hospitalised participants (n = 74). The difference was not significant (p = 0.637, t-test, **Table 27**, **Figure 34**).

Table 27: *Follow-up UCB concentrations by hospitalisation status*

Hospitalisation status	n	Mean UCB (µmol/L) ± SD	p-value
Not hospitalised	74	4.1 ± 2.3	
Hospitalised	23	4.4 ± 3.9	
Total	97	4.2 ± 2.7	0.637

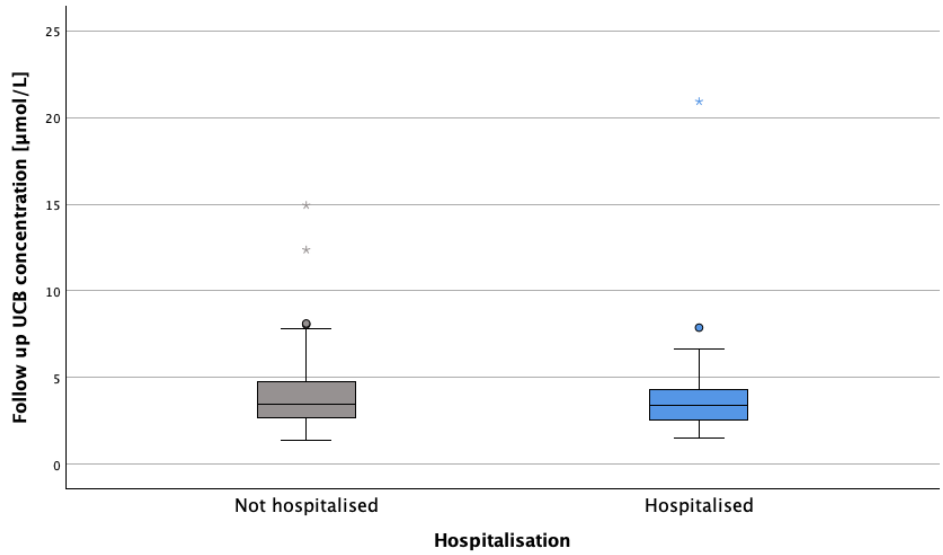

Figure 34: *Follow-up UCB concentrations in participants with and without hospitalisation for COVID-19*

Next, participants were grouped by length of hospital stay (0 days, 1-14 days, 15-28 days, > 42 days). Mean UCB ranged from 2.1 to 4.8 µmol/L across these groups. ANOVA showed no significant difference (p = 0.457). Post-hoc tests also gave no pairwise differences (all p > 0.05; **Table 28**, **Figure 35**).

Table 28: *Follow-up UCB concentrations by hospital stay duration*

Hospital days	n	Mean UCB (µmol/L) ± SD	Range	p-value
0 days	74	4.1 ± 2.3	1.36 - 14.94	
1–14 days	19	4.8 ± 4.2	1.52 - 20.93	
15–28 days	2	2.1 ± 0.5	1.83 - 2.51	
≥ 42 days	2	2.8 ± 0.2	2.64 - 2.97	
Total	97	4.2 ± 2.7	1.36 - 20.93	0.457

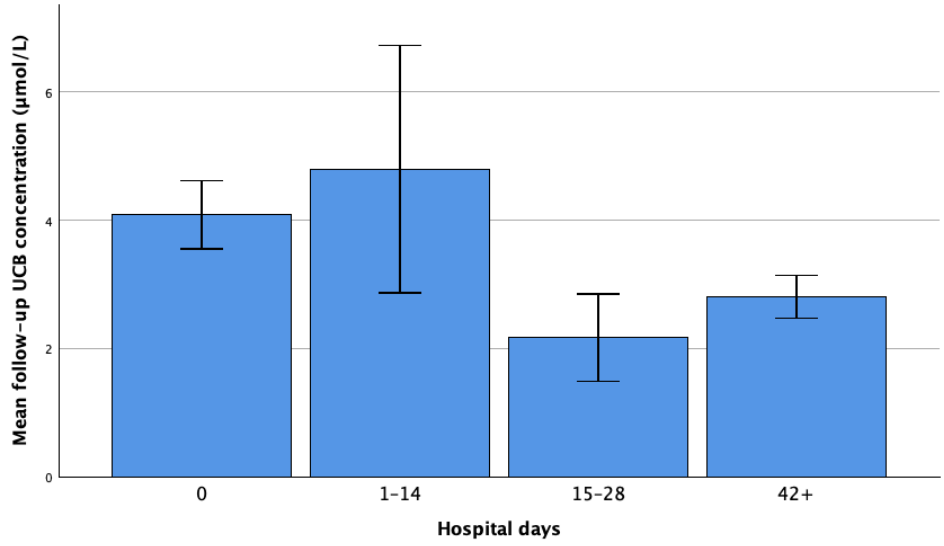

Figure 35: *Mean follow-up UCB concentrations by length of hospital stay*

In short, neither being hospitalised nor the number of days in hospital showed a clear link with UCB at follow-up.

ICU/Oxygen

To explore more severe cases, participants who had required ICU or oxygen support (n = 7) were compared with the single case without such support (n =1). Follow-up UCB levels were slightly higher in the ICU/oxygen group (4.1 ± 2.3 µmol/L) compared to the one without ICU/oxygen (2.6 µmol/L). The difference was not significant (p = 0.567; **Table 29**). Because of the extremely small reference group, these findings cannot be interpreted reliably and should be viewed as descriptive only **(Figure 36).**

Table 29: *Follow-up UCB concentrations by ICU/oxygen support*

ICU/oxygen support	n	Mean UCB (µmol/L) ± SD	p-value
No support	1	2.6 ± -	-
ICU/oxygen support	7	4.1 ± 2.3	0.567

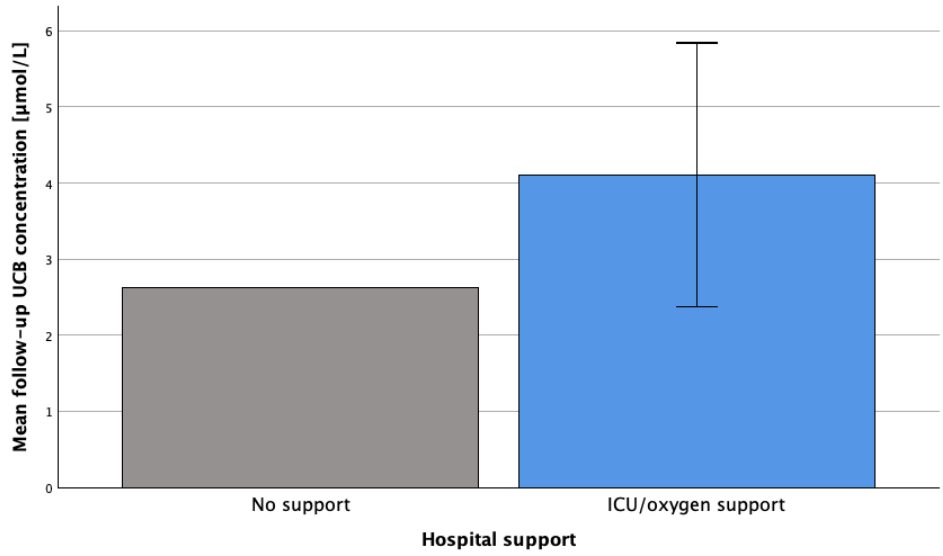

Figure 36: *Mean follow-up UCB concentrations by hospital support*

5.5.3 Vaccination status

Vaccination status was tested in relation to UCB at follow-up. Data were available for 99 participants. Eighty-five had received at least one vaccine dose, and 14 reported no vaccination.

Mean UCB was higher in the vaccinated group (4.3 ± 2.9 µmol/L) compared to the non-vaccinated group (2.7 ± 0.7 µmol/L; **Table 30**). The difference was significant in the t-test (p = 0.046).

In this cohort, vaccinated participants showed higher UCB levels than non-vaccinated participants. Because the non-vaccinated group was small (n = 14), these findings should be interpreted with caution (**Figure 37**).

Table 30: *Follow-up UCB concentrations by vaccination status*

Vaccination status	n	Mean UCB (µmol/L) ± SD	p-value
Not vaccinated	14	2.7 ± 0.7	
Vaccinated	85	4.3 ± 2.9	0.046

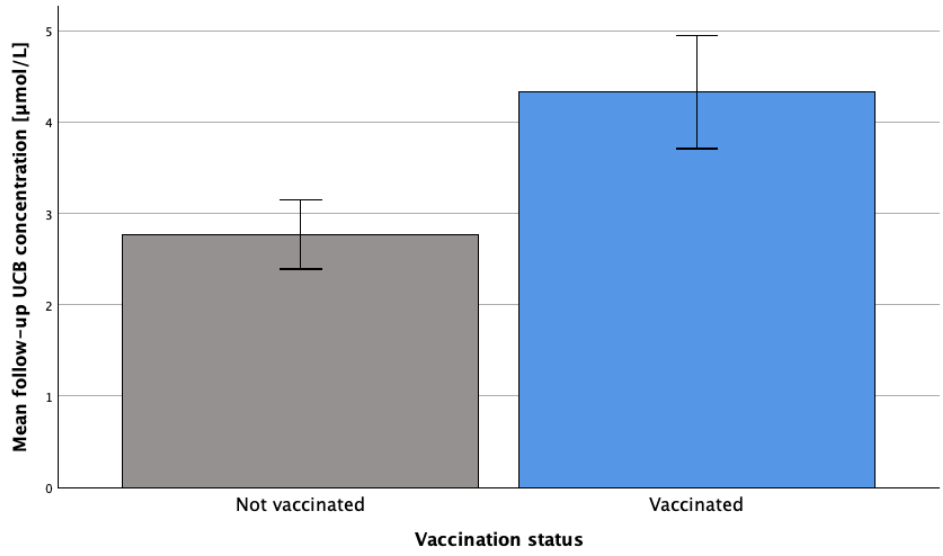

Figure 37: *Mean follow-up UCB concentrations by vaccination status*

5.5.4 Comparison with Rooibos Cohort

Baseline UCB values were compared between the Long COVID cohort and the Rooibos cohort, both recruited in South Africa. In total, data from 132 Long COVID participants and 40 Rooibos participants were available. The Rooibos group included healthy men who had originally taken part in a nutrition and exercise study, and for the present analysis only their baseline values before any intervention were used.

Mean UCB levels were very similar in both groups: 4.7 ± 3.2 µmol/L in the Long COVID cohort and 5.0 ± 2.8 µmol/L in the Rooibos cohort (**Table 31**). Median values were 3.9 and 4.6 µmol/L, respectively. The Long COVID group showed a wider range with some higher outliers (maximum 19.8 µmol/L), while the Rooibos participants reached up to 14.2 µmol/L. Statistical analysis confirmed that the difference between the two cohorts was not significant ($t(170) = -0.53$, $p = 0.596$; Welch's correction $p = 0.568$).

Overall, baseline UCB concentrations were comparable between the Long COVID and Rooibos cohorts (**Figure 38**, **Figure 39**).

Table 31: *Baseline UCB concentrations in the Long COVID and Rooibos cohorts*

Cohort	n	Mean (µmol/L) ± SD	Median	Range
Long COVID	132	4.7 ± 3.2	3.9	0.5 - 19.8
Rooibos	40	5.0 ± 2.8	4.6	0.4 - 14.2

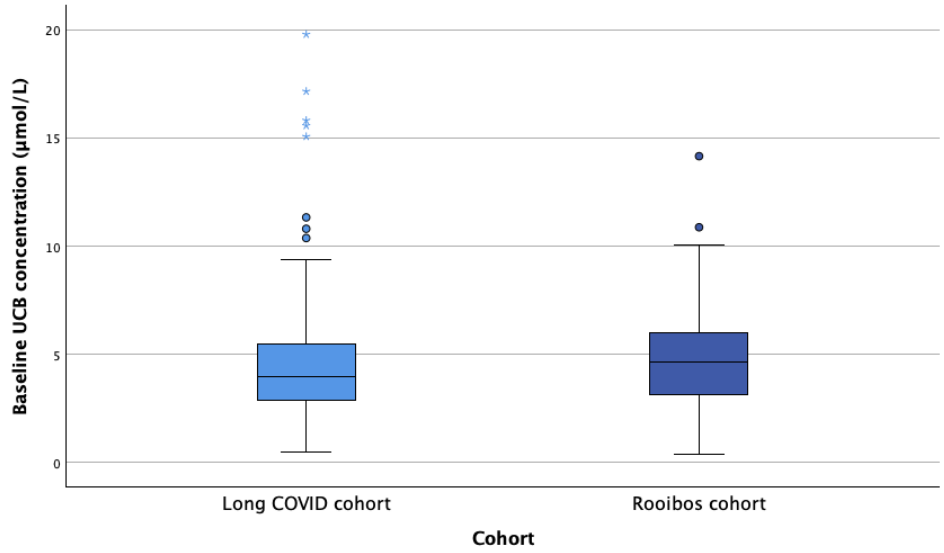

Figure 38: *Baseline UCB concentrations in the Long COVID and Rooibos cohorts*

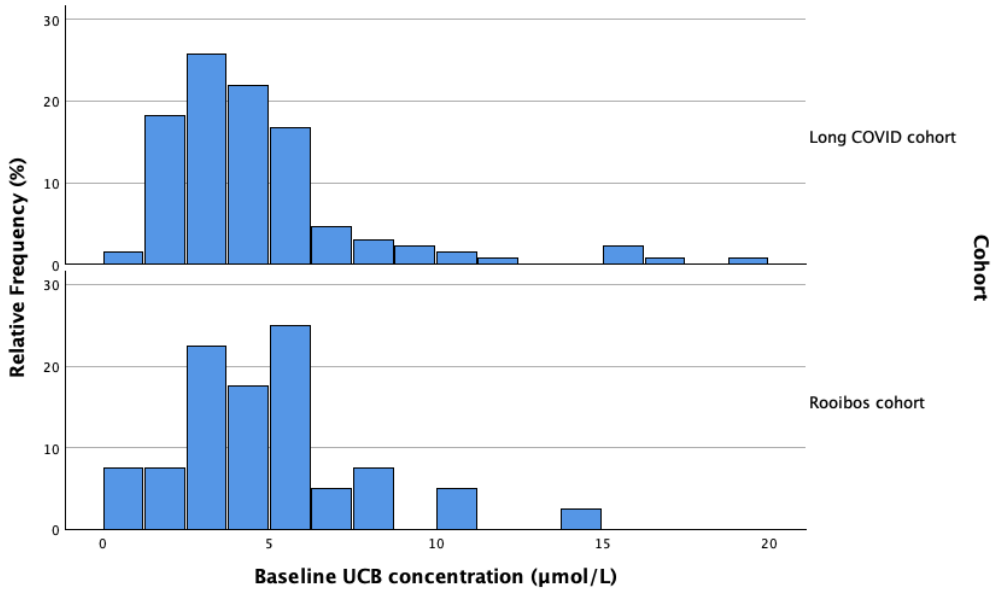

Figure 39: *Histograms of baseline UCB concentrations for both cohorts*

The Rooibos study included only men, so baseline UCB levels were compared with male participants from the Long COVID cohort. In the Long COVID group, the mean value was 5.3 ±

3.4 µmol/L. The Rooibos group showed a mean of 5.0 ± 2.8 µmol/L. Median values were similar, at 4.2 µmol/L for Long COVID and 4.6 µmol/L for Rooibos (**Table 32**).

Tests showed no relevant group difference. Levene's test indicated equal variances (p = 0.583). The t-test was not significant (t(77) = 0.46, p = 0.651). A Mann-Whitney U test gave the same outcome (U = 781, p = 0.992). Overall, baseline UCB values in men were comparable in both cohorts (**Figure 40**).

Table 32: *Baseline UCB concentrations in male Long COVID participants and the Rooibos cohort*

Cohort	n	Mean (µmol/L) ± SD	Median	Range
Long COVID	39	5.3 ± 3.4	4.2	1.5 - 17.2
Rooibos	40	5.0 ± 2.8	4.6	0.4 - 14.2

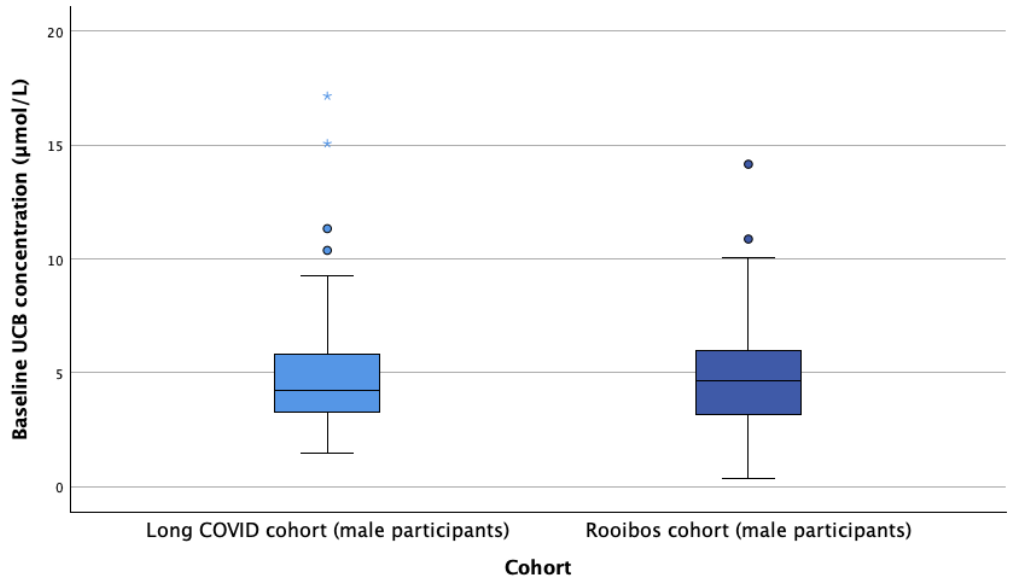

Figure 40: *Baseline UCB values in male Long COVID participants and the Rooibos cohort*

6. Discussion

The aim of this study was to look at UCB in a Long COVID cohort from South Africa. UCB was measured at baseline and again after twelve months at follow-up. Results were also compared with a Rooibos cohort from the same region. Overall, values were in the normal range, stayed stable over time, and subgroup differences were small.

Stability of UCB over time

The UCB concentrations stayed within the normal physiological range and no major changes were observed between the two measurements. This finding points to UCB being a stable parameter in people with Long COVID. Even though symptoms may persist for many months, bilirubin itself did not show marked shifts during that period. This is different from what has been reported in acute COVID-19. Several studies found that bilirubin levels tended to fall in severe disease and that low values were linked to complications and poor outcome (Cosgun, 2021; Liu et al., 2020). Such an association was not visible in this cohort. Once patients had moved beyond the acute stage, bilirubin appeared to remain steady. This aligns with the finding that baseline and follow-up values did not differ significantly ($p = 0.173$), supporting the interpretation of UCB as a stable marker over twelve months.

This suggests that UCB reflects processes of long-term metabolic regulation rather than the rapid immune and inflammatory activity that dominates the acute infection. The timing of measurement therefore plays an important role. Bilirubin values taken during hospitalisation for acute disease cannot easily be compared with values measured months after recovery from the initial infection. The role of bilirubin may differ depending on whether one looks at the acute or the chronic phase. In the chronic stages, as shown here, UCB seems less affected by fluctuations in symptoms and more tied to underlying physiological balance. This contrasts with hypotheses from earlier literature that considered UCB as a compensatory antioxidant during viral stress (Creeden et al., 2021).

Subgroup differences

In the subgroup analysis, some differences became apparent. Participants with baseline concentrations above 5 µmol/L also had higher levels at follow-up, while those with lower baseline values remained low. This suggests that a 5 µmol/L threshold may serve as a useful reference point for research purposes. Age was another factor. Participants over 50 years showed higher UCB

compared with younger ones. This matches what has been described before, since ageing affects the liver and also the body's ability to deal with oxidative stress (Hinds & Stec, 2018; Wagner et al., 2015). The finding in this cohort goes in the same direction. For gender, the difference was smaller. Men tended to have higher levels, which agrees with data from larger population studies (Fulks et al., 2009). Still, in this group the difference was not significant, and there was a lot of overlap between men and women. BMI showed no clear relation to UCB. One reason could be that BMI values in this study were all within a limited range. Which such a narrow spread, it is hard so see clear trends.

Clinical parameters

In this cohort, BP showed no connection with UCB. Participants with T2D had lower bilirubin levels at both time points, although the differences were not statistically significant. Similar results have been described before, where diabetes and metabolic disorders were linked to reduced bilirubin (Creeden et al., 2021).

Findings from this study differ from reports in acute COVID-19, where higher bilirubin often went along with severe disease and poor outcome (Liu et al., 2020). In people with Long COVID, this link was not seen. This is interesting, since earlier work was pointed to oxidative stress and persistent immune activity as central features of the condition (Kedor et al., 2022; Said et al., 2023). The present result suggests that UCB does not clearly reflect symptom persistence, even though it is tied to redox processes.

Lifestyle factors

Smokers had lower UCB values than non-smokers. At baseline, the difference was statistically significant, while at follow-up it was smaller and no longer significant. This matches reports that smoking can reduce bilirubin, probably because of higher oxidative stress (Jo et al., 2012; Vítek, 2017b). Alcohol use, by contrast, showed no links with UCB here. The results suggests that lifestyle may shape bilirubin, but the effects were small and not consistent over time.

UCB and Long COVID

In this study, bilirubin was also checked against clinical features of Long COVID. No relation was seen with the persistence of symptoms. The same was true for the severity of the first infection. People who had been in hospital, needed oxygen, or spent time in ICU showed very similar values

to those who stayed at home. Hospitalisation status also showed no significant differences at follow-up ($p = 0.637$), with nearly identical mean values between groups. The duration of hospital stay also showed no group differences (ANOVA $p = 0.457$), and post-hoc tests did not reveal significant pairwise contrasts. Participants who required ICU or oxygen support also showed no significant difference ($p = 0.567$), although case numbers were very small.

Vaccination was looked at separately. At follow-up, vaccinated participants had higher UCB levels, but the non-vaccinated group was small ($n = 14$), limiting interpretability. Taken together, bilirubin did not mirror illness severity, hospital stay, or symptom persistence. Other blood markers, such as CRP, IL-6, ferritin or D-dimer, are known to show stronger links with inflammation and outcome (Kedor et al., 2022; Said et al., 2023).

Comparison with the Rooibos cohort

The comparison with the Rooibos cohort was an important part of the study. Both groups were recruited in South Africa, but the Rooibos participants were healthy men who had originally joined a nutrition and exercise intervention trial. For the present analysis, only their baseline values prior to intervention were used. This makes the cohort a useful local reference group, though the restriction to male participants limits comparability with the mixed-gender Long COVID sample.

When baseline values were compared, mean and median UCB concentrations were very similar in both cohorts, and statistical testing confirmed that the differences were not significant. The Long COVID group showed a wider distribution with some high outliers, while the Rooibos group appeared more homogeneous. Despite this variation, no systematic group effect was seen.

Taken together, the results indicate that bilirubin concentrations in Long COVID patients are not markedly different from those in healthy individuals from the same region.

Strengths and Limitations

This work comes with clear strengths. On is that it looks at bilirubin in a South African Long COVID cohort, which has not been done often. A further strength is the use of HPLC, a method known for its accuracy and reliability. The study design with measurements at baseline and again after twelve months made it possible to follow the course over time. Including the Rooibos cohort added value as well, since it provided a second group from the same region for comparison. The

analysis also covered a wide range of variables, not only age and gender but also comorbidities, lifestyle habits and clinical factors.

There are, however, also important limitations. The number of participants was limited, with 132 at baseline and 103 at follow-up, and some subgroups were very small. For example, only a few participants were not vaccinated and there were few smokers, which makes results from these groups difficult to generalise. Another point is that only UCB was measured. Other oxidative stress markers mentioned in the report, such as TBARS, FRAP or protein carbonyls, were missing, which prevented placing bilirubin in a wider redox context. The clinical definition of Long COVID is broad, and patients presented with diverse symptoms, making it challenging to connect a single biomarker to such a heterogeneous condition. Lastly, the Rooibos cohort consisted only of men, while the Long COVID group included both gender, which reduces comparability.

7. Conclusion

The aim of this study was to examine UCB in a South African Long COVID cohort. Values were measured at baseline and again after twelve months. They stayed stable and within the normal range. No major shifts were seen over time.

Participants with higher levels at baseline also showed higher levels at follow-up. Age was the main factor, with older participants having higher UCB. Other factors such as gender, BMI, smoking or diabetes showed smaller and less consistent effects.

Clinical features of Long COVID, including hospitalisation, severity of the first infection, ICU stay, oxygen therapy and symptom persistence, were not linked to bilirubin. Hospitalisation and the duration of hospital stay showed no significant differences. Vaccination showed slightly higher levels, but the non-vaccinated group was too small to draw conclusions.

The comparison with the Rooibos cohort showed similar mean and median values. The Long COVID group had a wider spread but no systematic difference. This was supported by non-significant results in both t-tests and Mann-Whitney tests.

These results suggest that UCB is a stable marker. It reflects age and individual background but does not clearly separate Long COVID patients from controls. Still, it may contribute as part of a broader panel of biomarkers for oxidative stress and metabolic health. Its stability over time could make it as a useful background measure in studies that combine it with more dynamic inflammatory markers such as CRP, IL-6, ferritin or D-dimer.

8. Summary

Background:

Long COVID affects a considerable number of people after SARS-CoV-2 infection and is marked by persistent symptoms such as fatigue, cognitive problems, and autonomic dysfunction. Clear diagnostic tools are still lacking, and reliable biomarkers are urgently needed. Unconjugated bilirubin (UCB), known for its antioxidant and metabolic roles, has been proposed as a candidate but has rarely been studied in post-COVID cohorts.

Methods:

This master thesis analysed UCB in a South African Long COVID cohort. Blood samples were taken at baseline (n = 132) and again after twelve months (n = 103). UCB was measured by RP-HPLC, which allows precise quantification of bilirubin isomers. Clinical and lifestyle variables were included in the analysis, and results were compared with a healthy Rooibos cohort from the same region (n = 40 men).

Results:

UCB values remained stable over twelve months and were within the normal physiological range. Participants with higher baseline levels also had higher values at follow-up. Age was the most consistent factor, with older participants showing higher UCB. Gender differences were small, and BMI showed no clear relation. Smokers and participants with diabetes had lower levels at baseline, but these effects were not consistent at follow-up. Alcohol use showed no association. Vaccinated participants had slightly higher UCB, though the non-vaccinated group was too small to firm conclusions. Clinical features of Long COVID, including hospitalisation, disease severity, ICU stay, oxygen therapy, and persistence of symptoms, were not linked to bilirubin. The Rooibos comparison showed very similar mean and median values, with the Long COVID cohort displaying a wider spread but no significant group differences.

Conclusion:

The study shows that UCB is a stable parameter over time and mainly reflects age and background physiology rather than the clinical course of Long COVID. Bilirubin did not separate Long COVID patients from healthy controls but may still contribute as part of a broader biomarker panel for oxidative stress and metabolic health. Further studies with larger and more diverse cohorts, and in combination with other markers, are needed to clarify the role of UCB in post-infectious conditions.

9. Zusammenfassung

Hintergrund:

Long COVID betrifft einen erheblichen Teil der Bevölkerung nach einer Infektion mit SARS-CoV-2 und äußert sich durch anhaltende Beschwerden wie Fatigue, kognitive Störungen oder autonome Dysfunktionen. Verlässliche diagnostische Marker fehlen bislang, weshalb die Suche nach geeigneten Biomarkern von großer Bedeutung ist. Unkonjugiertes Bilirubin (UCB), das für seine antioxidativen und stoffwechselregulierenden Eigenschaften bekannt ist, wird als möglicher Kandidat diskutiert. Bisher liegen jedoch nur wenige Untersuchungen in Post-COVID-Kohorten vor.

Methoden:

In dieser Arbeit wurde UCB in einer südafrikanischen Long-COVID-Kohorte untersucht. Blutproben wurden sowohl zu Studienbeginn (n = 132) als auch nach zwölf Monaten im Rahmen eines Follow-ups (n = 103) analysiert. Die Bestimmung erfolgte mittels RP-HPLC, die eine präzise Quantifizierung der Bilirubin-Isomere ermöglicht. Zusätzlich wurden klinische und lebensstilbezogene Faktoren berücksichtigt. Als Kontrollgruppe diente eine Rooibos-Kohorte gesunder Männer aus derselben Region (n = 40), deren Basiswerte in die Analyse einbezogen wurden.

Ergebnisse:

Die UCB-Konzentrationen bleiben über den Beobachtungszeitraum hinweg stabil und bewegten sich im physiologischen Bereich. Personen mit höheren Ausgangswerten wiesen auch nach zwölf Monaten erhöhte Werte auf. Alter war der stärkste Einflussfaktor: ältere Teilnehmer*innen hatten höhere UCB-Spiegel. Geschlechtsunterschiede waren gering ausgeprägt, und ein Zusammenhang mit dem BMI konnte nicht festgestellt werden. Raucher und Personen mit Diabetes zeigten zum Ausgangszeitpunkt niedrigere Werte, diese Unterschiede bestätigten sich jedoch im Follow-up nicht. Alkoholkonsum stand in keinem Zusammenhang mit UCB. Geimpfte Teilnehmende wiesen tendenziell höhere Werte auf, wobei die Gruppe der Ungeimpften sehr klein war und daher keine belastbaren Schlussfolgerungen zulässt. Klinische Merkmale von Long COVID, darunter Hospitalisierung, Schweregrad der Erstinfektion, Intensivbehandlung, Sauerstoffgabe und das Fortbestehen von Symptomen, zeigten keinen Zusammenhang mit UCB. Der Vergleich mit der Rooibos-Kohorte ergab sehr ähnliche Mittel- und Medianwerte. Die Long COVID Gruppe wies zwar eine größere Streuung auf, systematische Unterschiede zwischen den Gruppen bestanden jedoch nicht.

Schlussfolgerung:

Die Ergebnisse deuten darauf hin, dass UCB ein stabiler Marker ist, der vor allem durch Alter und individuelle Ausgangsfaktoren geprägt wird, jedoch nicht direkt den Verlauf von Long COVID widerspiegelt. Zur Abgrenzung von Long COVID Patienten gegenüber gesunden Kontrollen scheint Bilirubin allein nicht geeignet. Es könnte jedoch im Rahmen eines umfassenderen Biomarker-Panels, das Marker des oxidativen Stresses und der Stoffwechselregulation einschließt, eine Rolle spielen. Um den Stellenwert von UCB in postinfektiösen Syndromen abschließend beurteilen zu können, sind größere und diversere Kohorten sowie die Kombination mit weiteren biologischen Parametern notwendig.

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