UNIVERSITÀ DEGLI STUDI DI TRIESTE

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Nanoscale microanalysis of bile pigments for the diagnosis and prognosis of disabling neurological diseases

Settore scientifico-disciplinare: **05/BIOS-07**

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ABSTRACT

Mild cognitive impairment (MCI) and Alzheimer disease (AD) are linked to the early biochemical changes prior to the evident neurodegeneration. Although cerebrospinal fluid (CSF) biological biomarkers offer strong biological characterization of AD, they cannot be used on large scale and repeatedly due to their invasive collection. Bilirubin metabolism, influenced by genetic variation in transporters and enzymes, has been implicated in oxidative stress, neuroprotection and blood–brain barrier function, but its potential role as a peripheral biomarker in AD remains inadequately explored. The research problem was to examine whether fractionated bilirubinaemia could be used as an ancillary peripheral biomarker of AD and MCI, and to check the effects of genetic polymorphisms in the bilirubin transporters and metabolizing enzymes on bilirubin fractions and cognitive status. The selected polymorphisms in bilirubin transporters were genotyped in patients with AD, MCI with AD and MCI without AD. The HUG assay was used to measure serum bilirubin fractions, unconjugated and conjugated bilirubin. The bilirubin fraction, genetic variation, and ability of cognitive performance were compared. The results indicate that fractionated bilirubinaemia along with genetic profiling can contribute biologically meaningful and minimally intrusive adjunct biomarker of Alzheimer disease and mild cognitive impairment. This strategy could be used together with well-known biomarkers and help to increase the risk stratification and characterization of the disease.

STUDY OBJECTIVE

The goal of this study was to explore if fractionated bilirubinaemia can be exploited as an ancillary disease biomarker in patients with Alzheimer's disease and mild cognitive impairment. Given the evolving links between the metabolism of bilirubin, oxidative stress, transport through blood–brain barrier, and neurodegeneration, this study aimed to determine whether modifications in specific bilirubin fractions are linked with cognitive impairment. To achieve these objectives, this research measured serum bilirubin fractions through the HUG assay and tested their correlation with clinical diagnosis and cognitive status. Along with this, genotyping of major polymorphisms of the bilirubin conjugation and transport genes was done to determine the effect of genetic variation on bilirubin fraction profiles. These objectives together had a goal to develop some mechanistic and translational understanding of the possible role of bilirubin fractionation as a complementary biomarker in cognitive disorders.

Therefore, the study of genetic polymorphisms within the framework of bile pigments metabolism is justified on the logical and scientific grounds by the fact that bilirubin is at the biochemical intersection between antioxidant defense buffering and metabolism clearance pathways. The knowledge of how genetic variation influences the bilirubin metabolic axis can eventually be used in improving the model of predicting AD risk, stratifying patients into categories, and integrating precision medicine approaches in the screening of neurodegenerative diseases.

This study is based on the close collaboration of two research groups, one in Ljubljana, who provided the patients and expertise in genetic polymorphism analysis, and the other in Trieste, who provided advanced analytics on fractionated bilirubinaemia.

BACKGROUND

Bilirubin in health and disease

Bilirubin, long considered mainly as a biomarker of hepatic function, is now getting recognized for its role in the nervous system. Its neuroprotective effects, antioxidant characteristics, and its dysregulation in pathological states make it significant beyond its traditional metabolic roles. Emerging studies are linking its altered levels with many neurological disorders, this is encouraging the researchers to further study the mechanisms involved in its metabolism and their impact on the health of brain. Growing evidences associate altered bilirubin levels with several neurological disorders, prompting further investigation into its mechanisms and impact on brain health. In the subsequent sections, these aspects will be discussed in detail with a focus on neurological implications.

1.1 Bilirubin metabolism

Bilirubin (derived from Latin word for "red bile") is a reddish-orange compound with antioxidant properties, that is an end product of the catabolism of heme in mammals (Vitek et al., 2023a). This catabolic pathway is mainly active in the spleen for the breakdown of abnormally produced and aged red blood cells (Jayanti et al., 2020), though it is found in essentially all cell types. Moreover, the pathway of bilirubin hepatic detoxification is common with some other organic anions, so understanding its metabolism also gives insight into transport, detoxification and removal of these organic anions from body (Erlinger et al., 2014).

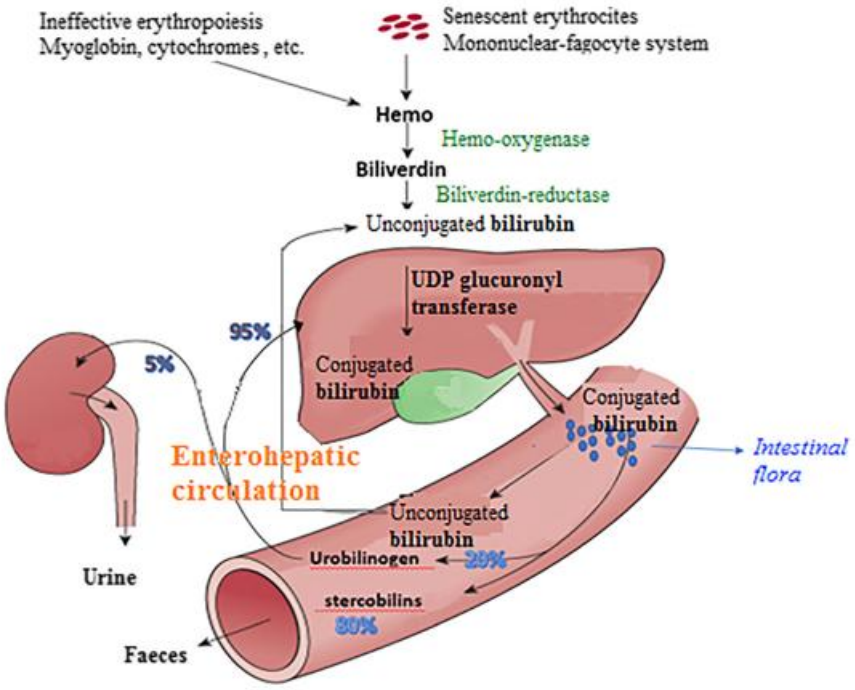


Figure 1.1: Bilirubin metabolism and recirculation (Ruiz et al., 2021).

1.1.1 Biosynthesis

The daily production of bilirubin is approximately 4mg/kg of body weight. The primary erythroid source is haemoglobin, which is released as senescent red blood cells degrade. However, free heme and haemoglobin that is created, but not integrated into

adult red blood cells in bone marrow (ineffective erythropoiesis) also contribute significantly. Normally, additional haemoproteins, mostly in the liver, like cytochromes, catalase, peroxidase, and tryptophan pyrrolase, contribute around 20% of the daily total bilirubin synthesis (X. Wang et al., 2006). Finally, in other particular situations (e.g. cerebral haemorrhages) bilirubin is formed by the heme degradation by macrophages and microglia. In this case, due to the limited drainage capacity, the production of biliverdin and bilirubin occurs directly in the hematoma and, unless the BBB is severely damaged, they are not detectable in serum but in the CSF only.

Heme oxygenase catalyzes the oxidative cleavage of heme, that yields equimolar amounts of biliverdin, carbon monoxide, and iron. In the pathway of bilirubin synthesis, heme oxygenase (HMOX) acts as a rate limiting enzyme. There are two major isoforms of this enzyme identified so far, known as heme oxygenase 1 and 2. The former one is inducible and only expressed during increased concentration of heme or under oxidative stress, and the later one is primarily found in vascular, neuronal, and testicular cells (Nitti et al., 2020).
Biliverdin is subsequently converted into bilirubin, and this NADH/NADPH-dependent reaction is catalysed by biliverdin reductase (Maines, 2005).
Once formed, bilirubin is transported outside the cell and enters into the blood circulation, where it is weakly bound to albumin, a protein carrier, for transport to the liver (Hansen et al., 2020a). The binding affinity of albumin for bilirubin is extremely high, and a very low concentration of free (non-albumin bound) unconjugated bilirubin is measurable in the plasma (Martelanc et al., 2016). To a lesser degree, especially in states of hypoalbuminemia, binding also occurs with high-density lipoprotein. The binding of albumin limits the diffusion of bilirubin from the vascular space, minimizes glomerular filtration and ultimately prevents its precipitation and deposition in tissues. When the albumin-bilirubin complex reaches the liver, the highly permeable intra-hepatic circulation allows the complex to reach the sinusoidal surface of the hepatocyte. This allows the pigment to dissociate from the albumin and enter the liver. This process is not rate-limited (Levitt 2014), but there is a substantial fraction of bilirubin that flows from the liver back to the blood (Berk et al., 1969).

1.1.2 Hepatic conjugation

Once in the liver, bilirubin dissociates from albumin at the hepatocyte sinusoidal membrane where it is taken up through facilitated diffusion into the hepatocyte by the organic anion-carrying polypeptides OATP1B1 and OATP1B3 (Vitek & Ostrow, 2009), though this model has been questioned (Čvorović & Passamonti, 2017). In the cytosol, bilirubin is bound to a group of intracellular binding proteins, one of which glutathione S-transferase isoforms of A1 and A2 specifically bind the hydrophobic pigment and retains it within the cell preventing its outer cellular efflux (Arias et al., 1993; Litwack et al., 1971). This intracellular binding determines a concentration gradient favouring continuous bilirubin uptake from the blood.
Conjugation of bilirubin occurs in the smooth endoplasmic reticulum (ER) of hepatocytes, and is the major process in bilirubin detoxification. The gene of the catalytic enzyme is the UGT1A1 gene, which is a microsomal enzyme called uridine diphosphate-glucuronosyltransferase 1A1 (UGT1A1) (Tukey & Strassburg, 2000). It uses uridine diphosphate glucuronic acid (UDP-glucuronic acid; UDPGA) as the donor of glucuronic acid. The conjugation reaction occurs through nucleophilic attack against the directing carboxyl oxygen of the propionic acid side chain of bilirubin on the anomeric carbon of the glucuronic acid moiety in UDPGA. That leads to the development of an ester bond and the subsequent release of uridine diphosphate

(UDP) (Bosma, Chowdhury, Bakker, Gantla, de Boer, et al., 1995; Fujiwara et al., 2015).
Since bilirubin has two groups of propionic acid, conjugation can proceed two times: first to form bilirubin monoglucuronide (BMG) and then to form bilirubin diglucuronide (BDG) as the main product in adult humans (Blanckaert, 1980). Conjugation at the molecular level significantly changes the physicochemical characteristics of bilirubin. The glucuronic acid residues introduce several hydroxyl and carboxyl groups making the molecule a hydrophilic species instead of a hydrophobic one (Mottino et al., 1983). This mechanism breaks down the intramolecular hydrogen bonds which had so far been keeping bilirubin in its folded hydrophobic state. The resulting conjugates are soluble in water and are excreted in the bile (Fevery et al., 1983).
The glucuronide esters added to the structure of the molecule raise the polarity of the molecule and cause a decrease in its ability to cross lipid membranes, facilitating unidirectional elimination. Bilirubin glucuronides that have been conjugated have to be transported out of the endoplasmic reticulum to the canalicular (bile-facing) surface of the hepatocyte to be released into the bile. This movement occurs via the ATP-binding cassette (ABC) transporter multidrug resistance-associated protein 2 (MRP2, also referred to as ABCC2) that actively transfers the bilirubin mono- and diglucuronides into bile canaliculi across the concentration gradient (JEDLITSCHKY et al., 1997; PAULUSMA et al., 1999).
Conjugated bilirubin can also be released into the sinusoidal circulation via basolateral MRP3 (ABCC3) and this contributes to conjugated hyperbilirubinemia in conditions where the canalicular excretion is impaired (Kamisako et al., 2000a). The defects in any of the elements of this pathway, including a decrease in UGT1A1 activity because of genetic mutations, result in the accumulation of the unconjugated bilirubin and causes the clinical signs of disorders, like Gilbert syndrome and Crigler-Najjar syndromes type I and II (Bosma, Chowdhury, Bakker, Gantla, de Boer, et al., 1995; Kadakol et al., 2000). On the other hand, impairment of canalicular transporters like the MRP2 leads to conjugated hyperbilirubinemia including Dubin-Johnson syndrome. Nuclear receptors have been shown to regulate UGT1A1 expression in response to xenobiotic stimuli, bilirubin level, the constitutive androstane receptor (CAR) and the pregnane X receptor (PXR)(Sugatani et al., 2005). Therefore, bilirubin conjugation is a highly regulated process of bilirubin solubility and excretion which in addition to the bilirubin solubility and excretion is an index of the liver phase II detoxification capability.

1.1.3 Hepatic elimination

As mentioned above, the conjugated bilirubin is then carried into the bile canaliculi which are small ducts in the liver, by the help of multidrug resistance-associated protein 2 (MRP2). It is accumulated into the bile system and is ultimately passed on to the small intestine. Otherwise a fraction of conjugated bilirubin that is transported into the sinusoidal circulation through MRP3 can be taken back into the hepatocytes through sinusoidal memebrane proteins, organic anion transport protein 1B1 and 1B3 (OATP1B1 and OATP1B3). Therefore, a certain amount of conjugated and unconjugated bilirubin can be released from the hepatocyte cytosol into the plasma. However, conjugated bilirubin is the only bilirubin which may be excreted into the bile (Vítek et al., 2006). The hepatic excretory capacity of conjugated bilirubin is the rate-limiting factor in bilirubin throughput. The conjugated bilirubin portion can increase in serum when the biliary excretion of the conjugated bilirubin is abnormal, such as in chronic biliary obstruction or intra-hepatic cholestasis. A small percentage of conjugated bilirubin is covalently conjugated with albumin and is dubbed as delta

bilirubin or delta fraction or biliprotein. Due to binding of the delta bilirubin to albumin, it is cleared slowly in serum (approximately 12-14 days as compared to the half-life of albumin) rather than the normal 2-4 hours (half-life of bilirubin) (Zucker et al., 2004).
Being hydrophilic and having a big molecular weight, conjugation does not allow the bilirubin to be reabsorbed passively in the intestinal mucosa. Moreover, conjugation somewhat reduces the affinity of bilirubin with albumin (Levitt & Levitt, 2014). Conjugated bilirubin is not reabsorbed from the proximal intestine as mentioned above, but can be hydrolyzed to free bilirubin by microbial beta-glucuronidase. Unconjugated bilirubin is partially reabsorbed across the small intestinal epithelium and returns to the liver with the enterohepatic circulation. In the proximal part of small bowel, no further bilirubin metabolism occurs, little deconjugation occurs (Fahmy et al., 1972). Contrastingly, when the conjugated bilirubin is released to the distal ileum and colon it is quickly deconjugated and reduced by the colonic flora to a family of molecules known as urobilinogen. The urobilinogen and stercobilinogen that are the major urobilinoids found in stool will also depend on the presence and composition of the gut bacterial flora. They are colourless yet on oxidation they change to urobilin, which is the cause of the stool colour (VITEK et al., 2006).

1.2 Bilirubin diagnostics

Deviations in bilirubin (total, indirect/unconjugated, direct/conjugated) reveal not just hepatic dysfunction but also extra-hepatic and hematologic anomalies due to the multiple sources of heme. Furthermore, current studies also highlight new roles for bilirubin, including as hormonal, immunomodulatory, anti-inflammatory, and antioxidant properties, which extend its significance in risk assessment and diagnosis (Čepelak et al., 2025; Vítek & Tiribelli, 2021; Yan et al., 2025).
Therefore, the measurement and interpretation of bilirubin fractions is a keystone of liver-function panels, and in result offering a differential diagnostic insight. In the following part the prognostic and diagnostic significance of bilirubin and its fractions is discussed with three different perspectives (Chopra & Griffin, 1985; Limdi & Hyde, 2003).

1.2.1 Liver diseases

The liver plays a crucial role in bilirubin metabolism and elimination, as it takes up unconjugated bilirubin from the blood circulation, converts it into glucurono-conjugated bilirubin in a reaction catalyzed by uridine-diphosphate-glucuronosyltransferase 1A1 (UGT1A1), and ultimately excrets it into the intestine through the biliary pathway (Ramírez-Mejía et al., 2024). Therefore, any significant change in uptake, conjugation, export and excretion can eventually result in abnormal levels of bilirubin. So, this makes bilirubin and its fractions a key component of evaluation of liver function and a hepatic pathology marker in clinical practice. In healthy adults the reference range of total and direct bilirubin level measured by standard laboratory procedures is usually around 0.3-1 mg/dL and 0.1–0.3 mg/dL respectively (Guerra Ruiz et al., 2021; Westwood, 1991a). A further hepatic function evaluation is needed if total or direct bilirubin elevates from the normal levels.
In **acute hepatocellular injury**, for instance caused by toxin exposure, acute viral hepatitis or induced by drugs, a rapid increase in serum bilirubin levels shows hepatic necrosis or a defect in excretory pathways. Such as, recent studies show that bilirubin levels tend to rise quickly in case of acute liver injury eventually correlating to the severity of injury and the chance of hepatic failure (Guerra Ruiz et al., 2021). In this

context the elevated levels of conjugated bilirubin might show intrahepatic cholestasis or impaired canalicular bile flow rather than just increased production of bilirubin. This pattern helps the clinicians to determine the actual etiology and pathogenesis. In the patients with acute on chronic liver failure, the ratio of total and direct bilirubin has been shown in independent correlation with 90-day mortality, thus emphasizing the importance of bilirubin profiling as an important prognostic tool (Ma et al., 2023).

In the case of **chronic liver disease (CLD)**, chronic inflammation, hepatocyte injury and fibrogenesis slowly decrease the ability of liver for conjugation and excretion of bilirubin. It can proceed to liver cirrhosis which involves architectural disruptions like formation of nodules and portosystemic shunt that in the end leads to impaired bilirubin handling (Gan et al., 2025; Sharma & Nagalli, 2025). In this case, an increased bilirubin, especially the conjugated fraction is an indication of advanced anomalies or decline in normal function. In a recent study by (Devarbhavi et al., 2023) it was concluded that global liver disease burden is continuously increasing and one of a key biochemical marker to analyse the severity of disease is serum bilirubin.

In biliary **obstruction or cholestatic conditions**, the hallmark of biochemical change is an increased level of conjugated bilirubin in the blood, whether the obstruction is extrahepatic (like stones in bile duct, malignancy or strictures) or intrahepatic (like cholestasis induced by drug or biliary cholangitis). This happens because the canalicular export and bile drainage are blocked, so conjugated bilirubin leaks back in blood, and it might appear in urine as well (bilirubinuria), eventually resulting in pale and dark stools and urine, respectively. According to clinical guidelines in an adult the direct hyperbilirubinemia is almost always pathologic (Westwood, 1991b).

Prognostic scoring systems are a major clinical application of bilirubin measurement in liver disease. For instance, serum total bilirubin is one of the key components of the commonly used **Model for End Stage Liver Disease** (MELD) score because bilirubin is correlated with hepatic excretory function and, consequently, long-term survival (H. A. Lee et al., 2021). More recently, other models have highlighted the possibility that direct bilirubin may be a better predictor of outcomes than total bilirubin. For example, one study discovered that direct bilirubin was a better predictor of cirrhosis mortality than total bilirubin alone (C. H. Lee & Kim, 2021). Therefore, the elevation and the its pattern for bilirubin fractions (direct and indirect) is of clinical importance.

Bilirubin fractions testing is also crucial in **hereditary biochemical abnormalities** of bilirubin metabolism. For instance, Gilbert's syndrome causes mild intermittent elevation of unconjugated bilirubin due to decreased UGT1A1 activity. Although this pattern is usually benign, it is crucial to identify it so that mild hyperbilirubinemia is not mistakenly linked to hepatic illness (Vitek et al., 2023b). On the other hand, unconjugated and conjugated bilirubin can accumulate in fulminant hepatic failure due to the inability to produce and eliminate conjugated bilirubin; in these severe situations, bilirubin levels may be used as a prognostic and diagnostic indicator (Guerra Ruiz et al., 2021).

Clinicians need to be aware of the ratio and trends of bilirubin fractions from an analytical and diagnostic-interpretive perspective. For instance, predominant direct hyperbilirubinemia often indicates dysfunction of hepatocellular excretion or cholestasis, whereas predominant indirect hyperbilirubinemia with minimal rise of direct bilirubin suggests pre-hepatic processes or conjugation abnormalities (Aragon & Younossi, 2010). Throughout the course of hepatic disease (such as viral hepatitis or drug-induced liver injury), serial bilirubin assessments can provide an insight about the course of the injury and the response to treatment. In fact, a poor prognosis or the

necessity for a transplant referral may be the result of failing to clear bilirubin or its rising levels even after treatment.
Novel aspects of bilirubin metabolism in liver illness have been highlighted by recent studies. A study reported a non-linear correlation between a lower risk of non-alcoholic fatty liver disease (NAFLD) and circulating mild increases of total bilirubin (within physiological range), indicating bilirubin's dual function as a disease marker and modulator (Han et al., 2024) Another recent review highlighted the correlation between the severity of acute-on-chronic liver failure and bilirubin species (such as diglucuronide and monoglucuronide) in chronic liver disease, indicating that more refined bilirubin profiling may have future diagnostic or prognostic value (Y. Zhang et al., 2025).
The implications are complex from a clinical standpoint. First, increased bilirubin should prompt evaluation of the disease stage, functional reserve (particularly excretory capacity), and risk of decompensation in a patient with known liver disease. Second, conjugated hyperbilirubinemia directs towards imaging to focus on the biliary tract in individuals with suspected biliary obstruction. Third, variations in bilirubin levels over time provide information on the response or severity of the disease in therapeutic monitoring (e.g., antiviral therapy for chronic hepatitis or the management of cirrhosis). Lastly, bilirubin continues to be an important biomarker used in prognostic classification and scoring systems for transplant evaluation and referral.
In conclusion, bilirubin measurement and, more specifically, the differentiation of its fractions, retains significant diagnostic, monitoring, and prognostic value in the management and treatment of liver diseases. Clinical decision-making is informed by defects in bilirubin metabolism, which reflect underlying pathophysiological pathways from acute insults to chronic fibrotic development to biliary blockage. Bilirubin's importance in hepatology is still strong and growing as research advances our knowledge of its species, ratios, and trajectories.

1.2.2 Blood diseases

Serum bilirubin levels offer significant insight into hematologic diseases beyond hepatic problems, particularly those due to increased haemolysis. Large volumes of heme are generated when red blood cells are lysed or injured, either intravascularly or extravascularly, and macrophages process them and their heme contents into unconjugated bilirubin (UCB). Elevated indirect (unconjugated) bilirubin in plasma might be a result of this due to a sudden or prolonged haemolytic load overwhelming the hepatic conjugation/excretion capacity, because UCB is linked to albumin and must be taken up and conjugated by hepatocytes before excretion (Jamwal et al., 2020; Kumbhar et al., 2024). Therefore, distinguishing between direct and indirect bilirubin is essential in the diagnostic procedure since increased UCB in the presence of well-preserved hepatic conjugation and excretory processes usually indicates a pre-hepatic process.
Bilirubin production is significantly increased in haemolytic anaemias, such as autoimmune haemolysis, sickle cell disease (SCD), hereditary spherocytosis, thalassemia, and other red-cell enzyme/membrane abnormalities, due to increased breakdown of RBCs. For example, in haemolytic anaemia the count of reticulocyte increases (reflecting upregulated erythropoiesis in bone marrow as a homeostatic compensation), an increase in circulating lactate dehydrogenase (LDH) due to cell lysis is measured, and haptoglobin decreases because of its binding with free

haemoglobin. This all leads to increased unconjugated bilirubin, often clinically manifested as jaundice (Phillips & Henderson, 2018) (Fattizzo & Barcellini, 2022).
In the case, laboratory results of high indirect bilirubin, normal or slightly increased direct (conjugated) bilirubin and preserved synthetic liver functioning hints the destruction of RBCs and not hepatic dysfunction. Therefore, direct/indirect bilirubin differentiation can help the clinicians to differentiate between haemolytic (pre-hepatic) and hepatic or cholestatic (hepatic/post-hepatic) origins of hyperbilirubinemia (Barcellini & Fattizzo, 2015).
As an example, chronic haemolysis is a characteristic feature of sickle cell disease (SCD), leading to a higher rate of bilirubin turnover. Sustained increases of unconjugated bilirubin can be a contributive factor in the formation of pigment gallstones (with a higher bilirubin load in bile) and make long-term management more problematic. Clinicians should also keep in mind the possibility of co-existing hepatic dysfunction (e.g. iron overload or hepatitis viruses) that may change the pattern of bilirubin fractions when interpreting bilirubin levels in SCD patients. Therefore, a high UCB and abnormal hepatic enzymes in the SCD patient should be the reason to conduct additional hepatic investigation (Cappelli et al., 2024) (Bensinger & Gillette, 1974). Also, recent research indicates that even slight co-existence of mild conjugation impairment (as is the case with Gilbert syndrome) in haemolytic disease can enhance hyperbilirubinemia beyond that predicted by the same amount of RBC destruction (Agrawal & Chandra, 2024).
In Thalassemia, especially β-thalassemia either major or intermediate, inefficient erythropoiesis and extravascular haemolysis leads to excessive production of bilirubin. The concentrations of unconjugated bilirubin often rise, and the measurement of the indirect bilirubin may contribute to the evaluation of the haemolytic load and in making decisions regarding the therapy, transfusion planning, or even splenectomy. The rapid red blood cell turnover and the lack of a developed conjugation system in the neonatal group predisposes the group to high unconjugated bilirubin levels, which is the cause of neonatal jaundice. Therefore, unconjugated bilirubin identification is an indispensable requirement in the neonatal field to provide therapeutic interventions, such as phototherapy or exchange transfusion, to avoid undesirable outcomes (Kumbhar et al., 2024).
The unconjugated hyperbilirubinemia as neonatal jaundice has a specific appearance: in this case, high RBC turnover (foetal to neonatal transition), low activity of the UGT1A1 enzyme (capacity to conjugate), and increased enterohepatic circulation play a major role. High levels of UCB in infants may bypass the immature blood-brain barrier and tend to accumulate in the basal ganglia and the brainstem nuclei, leading to a condition termed as kernicterus, resulting in neurotoxicity, cerebral palsy or hearing loss. Therefore, measurement of bilirubin and its fractions is essential in neonatal care to identify the risk population as well as to manage timely to prevent irreversible neurologic damage (Hansen et al., 2020b).
Form a diagnostic point-of-view distinction between fractions of bilirubin direct (conjugated) and indirect (unconjugated) is crucial. A predominant indirect hyperbilirubinemia (with a minimal elevation of direct bilirubin) strongly indicates a haemolytic or defect in conjugation process, whereas a predominant direct hyperbilirubinemia indicates dysfunction of hepatocellular excretion or post-hepatic obstruction. In conditions of haemolysis, the excretion and conjugation pathways of liver are intact, therefore, the pattern is typical isolated indirect bilirubin increase, while in cholestatic or hepatic diseases, the conjugated fraction of bilirubin tends to

dominate. Hence, the bilirubin fractionation turns out to be an effective tool in the differential-diagnostic algorithm of hyperbilirubinemia (Kaplan et al., 2002).
To conclude, bilirubin measurement has a central role in haematologic diagnostics: **(i)** as an indicator of augmented haem-catabolism through RBC rupture; **(ii)** as a method of differentiating between haemolytic, hepatic and obstructive causes of hyperbilirubinemia by fractionation; **(iii)** as a method of monitoring haemolysis complications such as pigment gallstones or hepatic congestion with excess bilirubin; and **(iv)** in neonates to prevent neurotoxicity due to unconjugated bilirubin. The understanding of the bilirubin metabolism (production, conjugation and excretion) dynamics in the context of hematologic disease allows researchers and clinicians to interpret the findings through a proper lens and incorporate bilirubin diagnostics into the larger clinical and laboratory decision-making.

1.2.3 Disease risk

The concept of bilirubin as just a by-product of heme catabolism is outdating rapidly. New findings put bilirubin not only as a biomarker of already existing disease, but also as an emerging prognostic tool of disease progression or risk. This double role is suggested by the fact that overt and borderline hyperbilirubinemia indicate a pathological burden and a health-protecting physiological reserve, respectively.
On its protective side, several observational and mechanistic researches have observed an inverse association between moderately raised levels of serum bilirubin and adverse cardiovascular-metabolic outcomes. For example, a dose–response meta-analysis of over 170,000 participants revealed a U-shaped relationship between risk of Coronary Heart Disease (CHD) and total bilirubin: bilirubin levels raised up to ~13 μmol/L (1.19 mg/dL) were linked with decreasing risk, but beyond that threshold the protective effect decreased and reversed (C. Li et al., 2021; Song & Li, 2025). Mechanistically, bilirubin serves as an effective endogenous antioxidant; for instance, in experimental systems it has been shown to be protective against oxidant loads (like excess hydrogen peroxide) and to hinder oxidative stress in renal and cardiovascular tissues (Adin, 2021; Hinds & Stec, 2018).
In a recent research of maintenance haemodialysis patients, raised indirect (unconjugated) bilirubin levels (≥ 3.0 μmol/L; … mg/dL)) were linked with significantly lesser incidence of cardiovascular events (with ≈0.48 adjusted hazard ratio) as compared to those with lower levels (Y. Chen et al., 2022). These results imply that bilirubin may function as an agent of oxidative/vascular resilience or favourable metabolic equilibrium even in subjects suffering from severe organ failure.
Furthermore, bilirubin's significance in metabolic signalling is gaining more attention now. According to certain findings from humans and animals, bilirubin may influence inflammation, insulin sensitivity, obesity, and lipid metabolism by either directly inhibiting NADPH oxidase in endothelial cells or activating nuclear receptors like PPARα (Hinds & Stec, 2019; Punzo et al., 2024). The observed inverse relationships between bilirubin and type 2 diabetes, metabolic syndrome, atherosclerosis, and even stroke may be explained by such mechanisms. For instance, mild hyperbilirubinemia was demonstrated to enhance the lipid profile and modify adhesion molecule expression and iNOS gene transcription, which may prevent the progression of atherosclerosis this was shown in a 2024 inflammatory-biology study by (Maleki et al., 2024).
On the other hand, increased bilirubin on the pathological side continues to be a sign of a poor prognosis, especially when it is over physiological thresholds or when there

is underlying organ dysfunction. Elevated bilirubin, particularly the conjugated/direct fraction, indicates cholestasis, congestive hepatopathy, or decreased hepatic excretory capacity in individuals with heart failure, chronic liver disease, or another multi-organ injury. Bilirubin elevation in this situation is a warning sign of decompensation and mortality risk rather than a preventative measure. Higher levels of the albumin-bilirubin (ALBI) score, which includes bilirubin, have been found to predict worse outcomes, such as cardiovascular mortality, in populations with non-alcoholic fatty liver disease (MAFLD) (Tang et al., 2025; J. Wang et al., 2024). Furthermore, the protective advantage of elevated bilirubin wanes and risk may increase beyond a specific amount (~13 μmol/L in CHD studies) due to the same U-shaped correlation previously demonstrated (C. Li et al., 2022).

Another aspect to consider is neonatal and developmental risk. Neurotoxicity (kernicterus) can occur in newborns when unconjugated bilirubin surpasses binding capability and passes through the immature blood-brain barrier. Therefore, rather than being protective in this situation, bilirubin is obviously a pathological risk indicator. The standard of care continues to be early measurement, monitoring, and intervention (exchange transfusion, phototherapy).

Bilirubin therefore falls into a "Goldilocks" zone in adult and chronic illness populations: too high may indicate underlying pathophysiology and end-organ damage risk, while too low may indicate weak antioxidant reserve or poor metabolism.

Therefore, the use of bilirubin in preventive medicine and risk assessment is promising. People with favourable metabolic and vascular state may be identified by mildly increased bilirubin, perhaps allowing for tailored preventative strategies. On the other hand, rising bilirubin in a known illness may prompt early intervention or close monitoring. Bilirubin is already included in composite risk scores (like ALBI) to stratify risk in cardiovascular, liver disease, and heart failure cohorts. There is ongoing research into the additional utility of bilirubin diagnostics beyond conventional risk factors.

In conclusion, bilirubin diagnostics plays a dual and complex role in clinical medicine: (i) as an overt pathology marker, where high levels frequently indicate hepatic, biliary, or multi-organ dysfunction; and (ii) as a prognostic biomarker in individuals who appear to be healthy or sub-clinical; moderate levels may indicate protective antioxidant capacity and lower risk, while very high or low levels may indicate increased risk. From a preventive medicine perspective, incorporating bilirubin measurement into standard panels could improve longitudinal prognosis, guided and customized therapies, and early risk detection.

1.3 Bilirubin in brain diseases

The analysis of bilirubin in brain tissues, cerebrospinal fluid (CSF), and serum provides crucial prognostic and diagnostics information in brain diseases. The concentration of bilirubin across these compartments is controlled by a critical pathophysiology that involves blood brain barrier integrity, haemolysis and hepatic function.

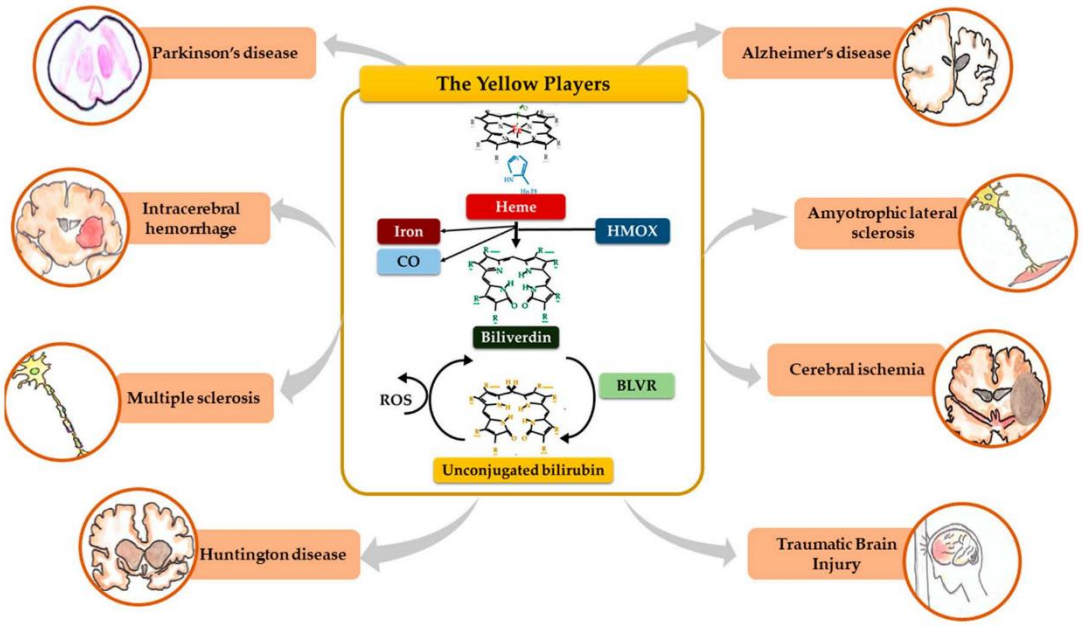


Figure 1.3: Bilirubin in brain disease (Jayanti et al., 2020).

1.3.1 Serum concentrations

Modifications to serum bilirubin levels have been extensively studied in brain diseases especially stroke, neurodegenerative diseases, hepatic encephalopathy, and traumatic brain injury.

In acute ischemic **stroke** and haemorrhagic stroke, various meta-analyses have shown results that elevated bilirubin, especially direct bilirubin, is linked to a greater stroke severity, a higher risk of haemorrhagic transformation, and worse functional outcomes (Petzold et al., 2006). On the other hand, community cohort studies in epidemiology show that slightly increased baseline bilirubin can be associated with reduced stroke occurrence over a long period so that there is a U-shaped relationship between bilirubin level and stroke risk (Zhao et al., 2023).

Evidence is still inconsistent in **neurodegenerative diseases** like the Parkinson disease (PD) and the Alzheimer disease (AD). Huge cohort studies such as the UK Biobank have associated high baseline direct bilirubin to increased risk of PD and AD, but Mendelian-randomisation studies have not established causality, suggesting that bilirubin is more of a systemic oxidative process marker than a direct pathogenic agent (Ramdane & Ramdane, 2021).

Hepatic encephalopathy (HE) and acute liver failure are conditions characterized by severe hyperbilirubinemia that is associated with neuropsychiatric impairment, which is a consequence of hepatic insufficiency and neurotoxicity of the circulating unbound bilirubin (Gelineau-Morel et al., 2024). The mechanisms of interaction between systemic bilirubin and brain dysfunction have several complex mechanisms. To begin with, bilirubin is involved in the biliverdin-bilirubin redox cycle and serves as a potent scavenger of reactive oxygen species (ROS) and prevents the oxidative destruction of lipids and proteins (Ficiarà et al., 2025). Second, bilirubin regulates inflammation via HO-1/BVR axis that controls microglial activation and expression of cytokines (J.

F. Watchko & Tiribelli, 2013). Third, when the albumin-binding capacity is surpassed, such as in extreme hyperbilirubinemia or hypalbuminaemia, the proportion of free UCB rises, and may cross the blood brain barrier, triggering mitochondrial dysfunction, membrane lipid peroxidation, calcium disequilibrium, and neuronal and glial apoptosis (van der Meulen et al., 2024). In this way, bilirubin can serve as a biomarker of systemic oxidative stress and a probable neurotoxin, when its free fraction increases, or barrier integrity collapses. The recent clinical studies show that it is a useful predictor of outcome in acute stroke and traumatic brain injury but its value is not as useful in the normal clinical prediction since it is confounded by hepatic comorbidity and methodological heterogeneity (Song et al., 2022; Zhao et al., 2023). The lack of causal genetic evidence in neurodegeneration suggests that bilirubin could be on the periphery of the pathology (J. Watchko & Tiribelli, 2013).

1.3.2 Tissue concentrations

A significant and dynamic role is played by concentration of bilirubin in brain in neurological pathology than perhaps previously thought. Even though circulating bilirubin is frequently discussed in clinical diagnostics, most relevant to disease state are the pools of bilirubin that are localized, intracellular, and associated to membrane of the neural tissues. Low nanomolar bilirubin levels under physiological conditions play a role in redox homeostasis by being an extremely strong endogenous antioxidant according to scavenging superoxide and other reactive oxygen species. Nevertheless, in a number of neurodegenerative and neuroinflammatory conditions, like, Parkinson's and Alzheimer's disease, multiple sclerosis, and acute ischemic injury, changes in the heme metabolism and HO-1 production result in changes in local bilirubin production. It has been consistently demonstrated in analytical studies that in moderation rises in the concentration of tissue bilirubin can produce defensive effects by neutralizing oxidative stress whereas too much production, typically due to a dysfunctional conjugation or transportation, could impair the function of the mitochondria and make neurons vulnerable to damage. Such changes highlight the importance of accurate quantification of bilirubin pools in individual brain regions as serum analysis do not reflect local neuroprotective or neurotoxic gradients (Vasavda et al., 2019).

Bilirubin distribution in brain tissue is dependent on pathology and developmental conditions. Immature BBB activity in neonates permits free unconjugated bilirubin to selectively enter high-metabolic areas of the brain, including the basal ganglia, hippocampus, cerebellar nuclei, and brainstem, to result in the neuropathology of kernicterus (Ghersi-Egea et al., 2009a). The histological observation shows that in the affected areas, neuronal necrosis, gliosis, and yellow pigment deposition are observed, which are in line with the lipophilic nature of the bilirubin and oxidative properties. In adults, bilirubin accumulation is associated with local haemoglobin breakdown, and local bilirubin retention is controlled by transporters like organic anion-transporting polypeptides (OATPs) and multidrug resistance-associated proteins (MRPs) (Brito et al., 2013; Gazzin et al., 2012).

Bilirubin neurotoxicity at the cellular level occurs in several mechanisms. Overload of unconjugated bilirubin interferes with neuronal membrane integrity, Na^+/K^+-ATPase block, intracellular Ca^{2+} increase and mitochondrial depolarisation and cytochrome-c release resulting in caspase-dependent apoptosis (Pranty et al., 2023). Excitotoxic damage is also enhanced by astrocytic dysfunction and glutamate transporter

inhibition, and microglial activation and oligodendrocyte progenitor loss are another contributor to neuroinflammation and white-matter damage (Pranty et al., 2023; van der Meulen et al., 2024). Nevertheless, at low levels bilirubin has neuroprotective effects because of redox cycling and inhibition of pro-inflammatory signalling (Ficiarà et al., 2025; Paul & Pieper, 2024). This dualism, which is concentration-dependent, is the basis of the paradoxical epidemiological results of stroke and neurodegeneration.

These mechanisms have been elucidated in an experimental context by the use of animal models (Ugt1-deficient mice and neonatal hyperbilirubinaemic rats) to recapitulate selective neuronal vulnerability and demonstrate mitochondrial and glial responses to bilirubin exposure (Pranty et al., 2023). The use of modern methods of analysis such as high-performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS/MS), and other advanced spectrophotometric assays have made it possible to quantify bilirubin isomers and oxidation products in neural tissue (van der Meulen et al., 2024). The new methodological improvements are in line with the new perception that the process of localized bilirubin production and build-up in the damaged brain areas can be used as a marker and mediator of oxidative stress.

1.3.3 Cerebrospinal fluid concentrations

Bilirubin in cerebrospinal fluid is a complex interface between overall bilirubin metabolism and, central nervous system pathology. Physiologically, bilirubin is closely bound to albumin in the blood, and beyond a minute proportion of free bilirubin (unconjugated) exists in the state of equilibrium. This is a highly lipophilic but poorly permeable form with an intact blood brain barrier and blood-CSF barrier. In pathology, however, e.g. inflammation, hypoxia, bleeding or immaturity in the neonatal stage, these barriers are breached and UCB and other bilirubin derivatives enter the cerebrospinal fluid and parenchymatous regions of the brain. Bilirubin may have protective and deleterious effects of concentration and context when it enters the central nervous system. On the physiological level, bilirubin is involved in redox cycling through the biliverdin-bilirubin pathway as an antioxidant and regulator of cellular stress responses. On the contrary, excessive amounts of free bilirubin cause the oxidative stress, dysfunction of mitochondria, and excitotoxic neuronal and glial cell damage (Ficiarà et al., 2025; Pranty et al., 2023).

The most successful clinical use of cerebrospinal fluid bilirubin measurement is in the diagnosis of subarachnoid haemorrhage. Red blood cells lysis in the subarachnoid space following vascular rupture or aneurysmal bleeding, leads to release of haemoglobin which is progressively converted to bilirubin by the heme oxygenase (HMOX-1) and biliverdin reductase. This biochemical transformation gives the yellow coloration of the cerebrospinal fluid called xanthochromia. Bilirubin in the cerebrospinal fluid may be spectrophotometrically identified as an absorbance peak at a wavelength of about 455 nm (Mendelow, 2024). The clinical significance of cerebrospinal fluid bilirubin as a direct indicator of intracranial heme metabolism and oxidative mechanisms can be explained by its robustness as a diagnostic biomarker of subarachnoid haemorrhage.

High cerebrospinal fluid bilirubin in neonatal neurology has been central in the pathogenesis and diagnosis of bilirubin-induced neurological dysfunction and kernicterus. Neonates have immature structures of blood brain barrier and blood-CSF

barrier in addition to having low serum albumin-binding due to which the proportion of free bilirubin is higher and is free to diffuse into the central nervous system. The observations of studies have shown that cerebrospinal fluid bilirubin levels are strongly correlated with serum bilirubin concentrations in hyperbilirubinaemic new-borns, with cerebrospinal fluid bilirubin being the better predictor of neurotoxic exposure (Gelineau-Morel et al., 2024). Bilirubin deposition in the selective parts of the brain like basal ganglia, hippocampus, and cerebellar nuclei is confirmed by post-mortem and neuroimaging studies and is parallel to the high levels of bilirubin in cerebrospinal fluid (van der Meulen et al., 2024). The results underline the significance of cerebrospinal fluid bilirubin testing in high-risk neonates and especially in preterm babies, where neurotoxicity levels of bilirubin are lower, and timely therapeutic interventions can be implemented by detecting the condition at an earlier stage.

In addition to subarachnoid haemorrhage and kernicterus, elevation of cerebrospinal fluid bilirubin has been noted in various other neuropathological situations, but their ability to have a diagnostic specificity is less. In meningitis caused by bacteria and viral meningitis, as well as in encephalitis due to viruses, bilirubin in the cerebrospinal fluid may be mildly increased locally due to local haemolysis or oxidative stress in inflammation and not due to the general entry of bilirubin into the system. Likewise, the bilirubin found in cerebrospinal fluid in traumatic brain injury and intracerebral haemorrhage can be due to the parenchymal blood breakdown and not diffusion across blood brain barrier systemically (Zhao et al., 2023). More refined evaluation of these bilirubin fractions has been possible in recent years by the use of more developed assessment technology to perform the tests. Liquid chromatography-mass spectrometry (LC-MS) and high-performance liquid chromatography (HPLC) can be used to determine the bilirubin isomers and oxidation products with high sensitivity and specificity, as opposed to traditional spectrophotometry (Y. Zhang et al., 2025). These techniques have shown that bilirubin species in the cerebrospinal fluid are unique after haemorrhagic injury, which confirms the bilirubin as a secondary biomarker of oxidative stress and catabolism of heme in the central nervous system.

By applying a novel fluorometric method, bilirubin and biliverdin concentrations in the CSF fluid of 50 subjects presenting early symptoms of neurological disease were found to range 14−340 and 0−66 nM, respectively (Sist, Tramer, et al., 2025) .

Mechanistically, the bilirubin being in cerebrospinal fluid indicates the equilibrium between the systemic manufacture, barrier permeability, and local metabolism. P-glycoprotein, multidrug resistance-associated proteins (MRPs) and organic anion-transporting polypeptides (OATPs) are the transporters that control the efflux of bilirubin into the adult blood. A dysfunction or saturation of these systems as seen in oxidative or ischemic states could enhance bilirubin levels in the central nervous system (Ficiarà et al., 2025). High cerebrospinal fluid bilirubin is therefore an indicator of both secondary neural damage via mitochondrial injury and pro-inflammatory signalling and a byproduct of pathological heme turnover.

Overall, bilirubin levels in the cerebrospinal fluid offer an important biochemical association between the bilirubin metabolism in the system and central nervous system pathology. Cerebrospinal fluid bilirubin in clinical practice has long been considered to diagnose subarachnoid haemorrhage, as well as to give significant diagnostic and prognostic information in neonatal hyperbilirubinemia. In some studies,

it is used as an indicator of the integrity of blood brain barrier, oxidative stress, and neuroinflammatory events. Though its specificity in the non-haemorrhagic and non-neonatal setting is not well understood, the combination of increased analytical tools with transporter biology research has a potential of making cerebrospinal fluid bilirubin a more generalized biomarker of central nervous system injury. Future studies are needed to measure the kinetics of free bilirubin in cerebrospinal fluid compared to serum levels, to determine threshold levels that are predictive of neurotoxicity as well as to implement bilirubin metabolism as a possible therapeutic intervention in neurologic disease.

Bilirubin analytics

2.1 Standard methods

Conventional bilirubin detection systems have contributed to the history of clinical biochemistry and diagnostic medicine over the past 100 years and were used by default as the principal systems in the identification of total and direct bilirubin levels. These classical methods have been extensively applied in hospital labs all over the world and are still the foundation of standardized clinical reference processes and calibration systems (Ngashangva et al., 2019).

The **diazo method**, first described by Ehrlich at the end of the 19th century and then optimized into the Malloy Evelyn method, is one of the first and most widely used procedures. The principle of this technique is the diazotization reaction chemistry, in which bilirubin reacts with diazotized sulfanilic acid to form azobilirubin, which is a colored product that can be measured colorimetrically (Ehrlich, 1884; Ngashangva et al., 2019). This technique supports the normal determination of total and direct (conjugated) bilirubin and has been incorporated into automated chemistry analyzers, which are the foundation of international reference workflow and calibration protocols. Although the diazo method is common, it has a number of established limitations, such as the interfering ability of hemolysis, lipemia, and drug compounds, less sensitivity at low bilirubin levels, and lack of scalability to the microscale and real-time levels. Despite this the diazo method still continues to be the gold-standard in clinical chemistry.

Later, diazo chemistry was also improved, leading to the development of the **Jendrassik Grof method**, that is internationally recognized standard reference method for the quantification of bilirubin in the guidelines of many clinical laboratories. In this technique caffeine-benzoate was used as an accelerator to enhance the efficiency of the reaction and facilitate quantification of unconjugated and conjugated bilirubin under controlled alkaline conditions (Doumas et al., 1985; JENDRASSIK, 1938). This method is widely referred to in literature on laboratory standardization, because of its better performance and less susceptibility to interference.

Another important type of standardized methods of estimating bilirubin is **spectrophotometric** assay, **vanadate oxidase method**. The mechanisms of these are based on the fact that bilirubin has a characteristic absorbance spectrum, particularly at a wavelength of 450 nm, which can allow to quantify bilirubin directly with no extensive chemical modification. Bilirubin is oxidized by vanadate (V) ions in an acidic environment, which causes a detectable drop in absorbance at the characteristic wavelength of bilirubin, which is usually between 440 and 460 nm. Total bilirubin can be determined without the generation of chromogenic coupling products since the pace or amount of this bleaching reaction is proportional to bilirubin concentration. However, due to the photo-lability of bilirubin, optical interference of lipids and hemoglobin, spectrophotometric assays need careful handling, dark

storage, and controlled sample preparation to prevent spectral contamination (Ameri et al., 2011; Penhaker et al., 2013).
Historically, in the instrumentation, basic UV-vis spectrophotometers were used to measure bilirubin and eventually **automated clinical chemistry instruments** were developed, which incorporated multi-wavelength optical systems. Sulfanilic acid diazo components, coupling accelerators, stabilizers, and matrix-specific buffers are normally used as reagents. The process typically included the dilution of samples, mixing of reagents, controlled timing of reaction, and endpoint or kinetic optical detection. Automatic analyzers contributed to an increase in reproducibility, standardization and reduction in manual error (Golub, 1964).
The conventional techniques are appreciated due to their accuracy and precision specifically the Jendrassik Grof technique which has been shown to have high clinical reproducibility and reliability in clinical laboratories. The measurement of total bilirubin by these classical techniques is very precise in the routine diagnostic test in the screening of hepatobiliary diseases, neonatal jaundice and liver functional analysis (Doumas et al., 1985; Ehrlich, 1884; JENDRASSIK, 1938; Ngashangva et al., 2019). Most clinical diagnostic guidelines continue to use them as benchmark comparators to new technologies of bilirubin sensing.
However, traditional methods have a number of limitations. The diazo reaction is not selective enough on bilirubin and can react with other biological chromogen interfering with the diazo reaction. The spectrophotometric methods have a high overlapping interference in absorbance with hemoglobin, carotenoids and lipoproteins, and can interfere with the measurement reliability in lipemic and hemolyzed samples (van der Woerd-de Lange et al., 1983; Zucchini et al., 2024). These interference problems compromise sensitivity, and make quantification in low-concentration matrices e.g. cerebrospinal fluid more challenging.
Moreover, the fact that bilirubin has been well-documented to degrade under light (Cremer et al., 1958) is a significant problem to all of the conventional procedures. Since bilirubin changes quickly in the presence of light, there is need to observe strict protocols for sample handling, dark reaction conditions, and reagent stability issues. An inability to follow these guidelines can decrease the accuracy of the assays, produce false results, and introduce poor repeatability, especially in decentralized clinical practice.
Traditional assays play a critical role in the bilirubin's clinical quantification for diagnostics as they are reliable reference systems because of their long-standing reliability. Their contributions to establish the accuracy standard still influence the benchmarking, standardization, and validation of next-generation chemical and biosensing methodologies, which is why they are not only historically crucial but also technically at the heart of the current bilirubin quantification research.

2.2 Advanced methods

Recent developments in analytical sciences resulted in the innovation of methods to quantify bilirubin that are modern and capable of addressing the short-comings related to conventional chemical assays. These new platforms bring with them increased sensitivity, reduced detection limits, increased selectivity to bilirubin fractions and increased integration with automated and portable diagnostic systems. Compared to historically determined diazo and spectrophotometric assays, the new models of analysis use multi-modal transduction, improved technologies of sample handling and amplified signal processing that facilitate more accurate determination of concentration in complex biological samples in which levels of interference are often very high.

The **HPLC** method has emerged as one of the most popular advanced analytic platforms owing to its higher capability of separation of bilirubin isomers. Through reversed-phase stationary column and optimized mobile phase gradients, HPLC is capable of isolating unconjugated bilirubin from conjugated fractions and bilirubin photo isomers with high efficiency. This ability facilitates precise quantification with less interference as compared to the traditional colorimetric systems, especially where the composition of samples is extremely variable (Lauff et al., 1983; Maeder et al., 2000; Osawa et al., 2006) .

The bilirubin quantification can be further improved by liquid chromatography with tandem mass spectrometry (**LC-MS**/MS), which uses high-resolution measurement and structural analysis. LC–MS/MS methods possess very accurate molecular quantification limits at very low concentration levels, which make them applicable in cases of assessing neonatal bilirubin, CSF analysis, and pharmacokinetic bilirubin quantification in the researches. LCMS/MS systems also facilitate the cross validation of bilirubin markers of biotransformation, which are useful in the deep phenotyping of biomarker system research in hepatology (Jašprová et al., 2020).

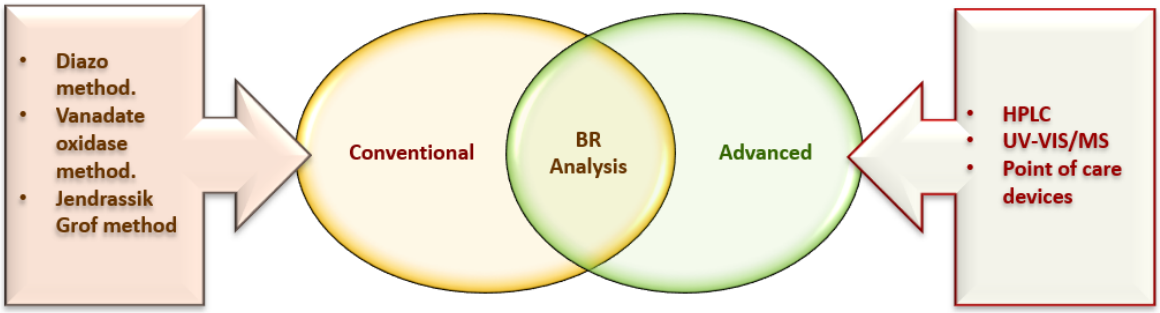
The new trends in bio-sensor-based bilirubin detection have also greatly broadened the analysis competences in biomedical diagnostics. Electrochemical bilirubin biosensors, graphene-enhanced transducers, fluorescent-based systems and enzymatic biorecognition platforms have shown promising analytical improvements in the form of amplified signal response, improved binding kinetics and fast turnaround time. These bio-molecular sensor systems allow point-of-care (POC) bilirubin testing, which is beneficial in emergency clinical units, neonatal units, and decentralized health care. Some of them are discussed below.

Other transformative diagnostic technologies have also come in the form of point-of-care bilirubin devices that use microfluidics. **Microfluidic systems** minimize the amount of sample volume needed, enhance the speed of reaction, support the automation of reagents, and provide multiplex detection of analytes on a single portable chip. The integration of optical readers, micro-pumps, smartphone connectivity and nanomaterial recognition layers is fast-tracking decentralized bilirubin diagnostics and enabling the testing models that are more patient-centric (Ahnood, 2025; Ndabakuranye et al., 2025; Tan et al., 2020).

Nanomaterial-based biosensors have demonstrated great potential for the quantification of bilirubin with their quick analysis, high sensitivity, and compatibility with portable diagnostic formats. A lot of studies are being carried out that shows that these technologies have great potential for point-of-care bilirubin monitoring, especially in the treatment of infant jaundice, but more extensive clinical validation is still required (Anzar et al., 2022; Pan et al., 2019; sabah Ahmed et al., 2023; Xiao et al., 2023).

Wireless sensor-based bilirubin quantification is also becoming more widely acknowledged as a crucial part of creating real-time jaundice monitoring systems, existing research shows important limitations that need to be solved for major clinical translation. Current research mostly uses non-invasive optical sensing modalities, such as transcutaneous spectrophotometry or multi-wavelength reflectance, integrated with wearable or node-level sensor platforms to deduce bilirubin content from its distinctive absorption spectra. However, these methods are extremely vulnerable to physiological and extrinsic factors that affect signal stability and measurement accuracy, such as skin pigmentation, ambient light variability, regional blood perfusion, and irregular sensor to skin contact. In order to accomplish precise,

real-time bilirubin monitoring, integrated, clinically verified WSN-based sensing frameworks that merge efficient optical hardware, reliable communication designs and adaptive algorithms are still desperately needed (Hakimi et al., 2016).
Future digital health integration also provides the transformation of bilirubin sensing to wearable monitoring ecosystems. **Continuous bilirubin monitoring** in settings outside the hospital can be assisted with the help of biosensors linked to smartphone, cloud-linked POC analyzers, and IoT-enabled laboratory test networks. It is especially applicable in the practice of neonatal jaundice and its management, where real-time bilirubin risk prediction could be clinically significant as compared to single point sampling. This type of system integration allows rapid clinical feedback systems and enhances patient risk stratification models (Inamori et al., 2021).
Even though there has been great advancement, there are still a couple of challenges that the administrative usage of contemporary bilirubin sensors has not attained. The



biosensors need to be better long-term stable in non-homogeneous biological matrices, nanomaterial reproducibility should be standardized, and false positive mitigation strategies are needed to make biosensors optimize limit of detection (LOD). Inter-laboratory validation studies should be conducted in order to set the reliability standards similar to classical clinical assays. Before the contemporary bilirubin sensing platforms can be approved to be used on a routine basis, efforts in validating tests and conducting massive cohort clinical trials and development of global calibration standards are necessary. Unless regulation is adapted in a structured manner, advanced sensing platforms can continue to be mostly an academic prototype but not an accepted clinical diagnostic tool.

Figure 2: Methods to quantify bilirubin

2.3 The HUG – based fluorometric method

This is a method based on a fusion protein named as HELP-UnaG. Below I will describe how this protein was developed, its characteristics and properties and how it is efficient in quantification of bilirubin and its fractions.

2.3.1 History of HUG protein

The creation of the HUG protein which is also called the HELP-UnaG fusion protein is an interplay of two significant streams in biomolecular innovation. The former one was first discovered with the identification of UnaG, a novel and rare fluorescent protein found in the Japanese eel initially reported by (Kumagai et al., 2013). The unique thing about UnaG is that the fluorescence of UnaG is exclusively triggered by the non-covalent and high affinity binding of unconjugated bilirubin, an attribute that provides this bioindicator with unmatched specificity and sensitivity to detect bilirubin (Kumagai

et al., 2013). The later research direction was focused on the engineering of human elastin-like polypeptides (HELPs), an evolution of the elastin-based biopolymers aimed at replicating the modularity, biocompatibility, and thermoresponsive properties of human tropoelastin. Having been developed through the efforts of scientists, HELP constructs were useful as scaffolds to biomaterials and novel fusion partners that could be transformed by reversible inverse temperature transitions, permitting simplified and non-chromatographic methods of purification. Its thermal behaviour (the reverse phase transition) allows it to work in a peculiar way; at the temperatures below its transition temperature, it acts in hydrophilic way and its free chains exist in a disordered and completely hydrated state, at temperatures above the transition temperature, it is hydrophobic with the chains properly organized in orderly structure (Bandiera et al., 2005, 2023; B. Li et al., 2001).

These developments from both biomolecular streams formed the principle framework of a synthetic amalgamation of exploiting HELP's material properties and UnaG exquisitely sensitive ligand-dependent fluorescence. Integration of this idea was achieved in 2019 with the creation of a fusion protein the HELP-UnaG (HUG) as it was reported by (Bandiera et al., 2020) that UnaG could retain its bilirubin activated fluorescence in the HELP scaffold and that the fusion protein was capable of working in solution and in engineered matrices. This study was the genesis of HUG as a bifunctional biosensor which could exploit the thermoresponsive response of HELP and retain the analytical precision with which UnaG confers. The next significant innovation was by (Sist et al., 2022) who in 2022 reported a comprehensive biophysical description of HUG which studied its phase behaviour, domain interactions, and bilirubin-binding properties. This study confirmed that in spite of the fusion which minimally modulated the affinity and fluorescence intensity of UnaG, HUG retained the critical functional properties of a sensitive bilirubin detector and retained the inverse temperature transition behaviour of HELP.

This biophysical basis was extended through additional efforts by (Sist et al., 2023; Sist, Tramer, et al., 2025) between 2023 and 2024 to define HUG's use as just a characterized biomolecule, but an operational analytical reagent. They have shown that HUG has the capability to measure bilirubin at nanoscale concentrations in all but the most complex biological samples including serum, cerebrospinal fluid and blood supporting its translational application to biochemical and clinical studies. It was also during this time that a standardized, chromatography-free production protocol, following the thermal cycling properties of HELP, was published that considerably lowered the technical and economic impediments to produce HUG to be used in everyday laboratory use (Sist et al., 2024). The result of these collective efforts was made known in 2025 when (Sist, Urbani, et al., 2025) released a review to place HUG into a more wide technological and biomedical framework. This study highlighted the developmental path of the protein, made structure functionality connections clear, sensing applications within the matrices, and future research directions such as engineered spectral variants and enhanced sensing functions. Through the series of these collaborative studies over the past years, HUG has transformed out of the original idea of a conceptual merger of two ground-breaking proteins to a fully developed, well characterized and versatile biosensing platform with far reaching implications to biomaterials engineering, analytical biochemistry and translational diagnostics.

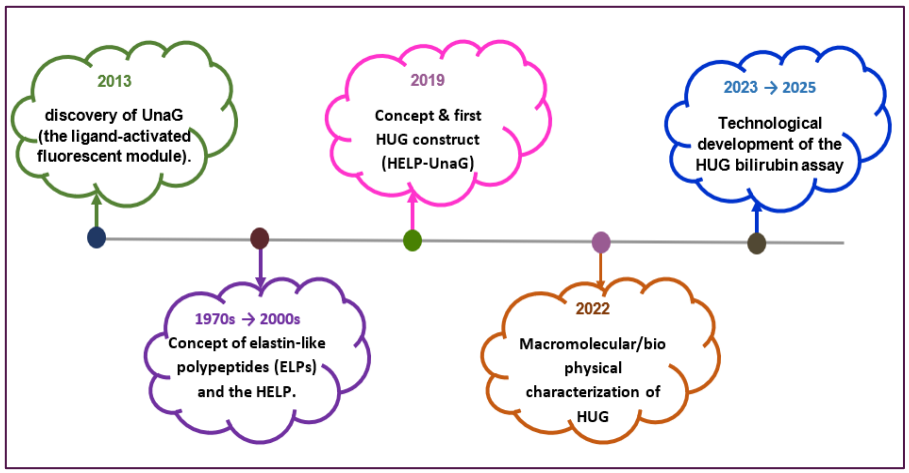


Figure 2.3.1: Timeline for the development of HUG bilirubin assay.

2.3.2 Characteristics of HUG

The recombinant fusion protein HUG, which is composed of human elastin-like polypeptide (HELP) domain conjugated with the fluorescent protein UnaG, shows a clear set of macromolecular and solution properties that come about as a result of the interplay between the two functional components. On the molecular scale, HUG retains the key structural characteristics of both domains; the HELP segment acts like a typical elastin like biopolymer with the ability to undergo an inverse temperature transition, whereas UnaG retains its β-barrel fold and its ligand-activated high-affinity fluorescence upon binding with unconjugated bilirubin. So, in this way the structural independence is preserved for both domains, even though their physical interaction introduces some minor but quantifiable effects on their solution and thermodynamic properties (Sist et al., 2022).

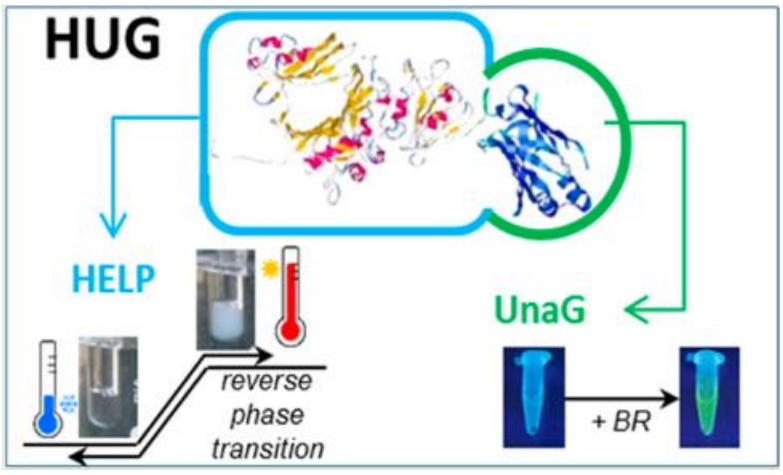


Figure 2.3.2: The recombinant fusion protein HUG (HELP-UnaG) (Sist et al., 2022)

HUG in aqueous solution shows a concentration and temperature dependent organization which is mainly governed by the HELP domain. HUG, like other elastin-like polypeptides, has a typical characteristic of coacervation (inverse temperature transition), which allows the protein to transform itself to a fully soluble, monomeric or

oligomeric form at low temperatures to a coacervated, aggregated form at high temperatures. (Sist et al., 2022) demonstrated that this transition temperature is slightly shifted in HUG protein as compared to the actual HELP polymer, this indicates that the globular fluorescent domain (UnaG) has a contribution to the overall hydrodynamic behaviour of this fusion molecule. Nevertheless, the assembly of HUG due to heating can still be completely reversed, and the protein can revert to its soluble form upon cooling, which supports the fact that the solid phase behaviour is governed by the HELP segment.

The functional properties of UnaG are also fully maintained in this fusion construct. HUG has a very high binding affinity for unconjugated bilirubin and the process of fluorescence activation occurs by the same molecular mechanism that is present in isolated UnaG molecule. However, the fusion structure presents mild changes in the interaction between the ligand and protein: the quantum yield and affinity are a bit modulated due to the proximity of the HELP polymer probably because of the alteration in local environment, steric circumstances or dynamic flexibility. Notably, the effects do not reduce the practical performance of HUG, which still maintains good fluorescence activation and great selectivity to bilirubin in the relevant physiological conditions. Thus, this fusion offers a structurally stabilizing framework as well as a functionally sensitive fluorescent module. Notably, unlike UnaG, the bilirubin-dependent HUG fluorescence is maintained in buffers containing up to 30 % (vol: vol) DMSO (Sist et al., 2023). This property is very useful when the experimental buffers contain natural compound or drugs that need DMSO for their solubility.

Altogether, the macromolecular and biophysical characterization of HUG makes it an ideal well-behaved, modular protein whose two domains are mutually interactive yet do not impair each other. The HELP component provides predictable and tuneable thermal responsiveness, allowing aggregation to be successfully controlled and purification to be easily performed, and UnaG provides a specific and sensitive optical output directly coupled to ligand binding. These solution properties reported by (Sist et al., 2022) confirm the HUG as a stable, reproducible and analytically reliable construct that can be used both in biomaterial applications and in quantitative assays of bilirubin and other related tetrapyrrole ligands.

The study conducted by (Sist et al., 2022) also reported the differences between the BR binding constants with respect to albumin and HUG proteins, which point to a competitive displacement process of BR from albumin protein in presence of HUG because of the presence of UnaG domain. So, all of these characteristics exhibited be HUG protein show its versatility that can be further exploited to quantify BR and its fractions in different biological settings. In conclusion this proves HUG as powerful tool for the detection of BR.

2.3.3 Performance of the HUG assay

The HUG assay, as described earlier is a fluorescence detection based quantitative analysis designed to measure unconjugated bilirubin in a highly sensitive manner and with little sample preparation. The HUG assay is performed in a multiwell plate, which enables many samples and standards to be processed all in once in a uniform and robust manner. A working concentration of HUG protein is made by dissolving the concentrated solution of polymer and milliQ water in phosphate-buffered saline (PBS)

at 1 g/L. In every assay well, a specific amount of the HUG solution (usually 10 µL) is placed in black, flat-bottom fluorescence-compatible microplates to reduce optical cross-talk. To plot a calibration curve, bilirubin standards, made in the nanomolar range (e.g., 0-50 nM to make high-sensitivity), are added to known wells. The samples to be analysed are then added to the wells. A gentle mixing is carried out either manually or using plate shaking in order to allow uniform interaction of bilirubin and the HUG protein. The mixing is then followed by incubation at room temperature until the binding equilibrium is reached. (Sist et al., 2023) states that the level of fluorescence rapidly stabilizes because of UnaG's high binding affinity for bilirubin. Since, bilirubin is light sensitive, all of this is done with minimal exposure to high ambient or UV light to avoid photodegradation.

A standard microplate reader is used to measure the fluorescence with specific excitation and emission wavelengths set for the UnaG-bilirubin complex, usually 498-500 nm excitation, 527-530 nm emission depending on the optical settings of the instrument. The gain or sensitivity parameters of the reader are supposed to be adjusted according to the intensity of wells with standards for calibration curve to avoid the saturation of detectors. Interpolation of the fluorescence intensity of each sample is done against the calibration curve which is plotted by the use of linear regression within the validated concentration range. (Sist et al., 2023) verified strong linearity within their established calibration limits and recommended using separate calibration curves when switching between the 10 nM and 50 nM to preserve accuracy.

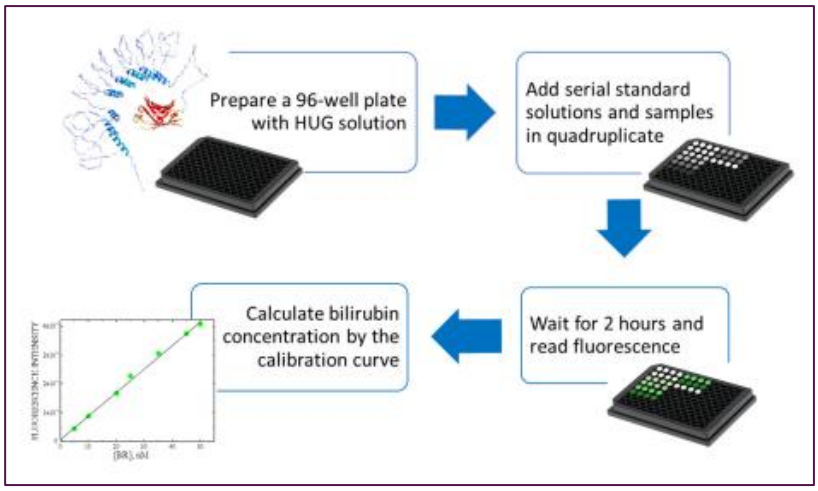


Figure 2.3.3: Workflow of the HUG assay. (Sist, Urbani, et al., 2025)

The HUG assay needs to be validated to ensure the reliability of the analysis in terms of precision, sensitivity, accuracy and matrix effects. The technique has a limit of quantification of about 1.1 nM and relative standard error of approximately 4.5 percent in controlled laboratory conditions. The comparison of method to other methods, such as application to human plasma, has demonstrated a good concordance with the diazo reference method, and relative standard error was about 6.7%, highlighting its appropriateness in the study of plasma bilirubin. The assay has been demonstrated to be robust in physiologically relevant conditions such as pH between 7.4 to 9.5, temperatures between 25-37 ºC and in the presence of serum albumin at common concentrations in diluted biological samples. In addition, the assay stays functional in buffer systems having up to ~30% DMSO, allowing integration with certain

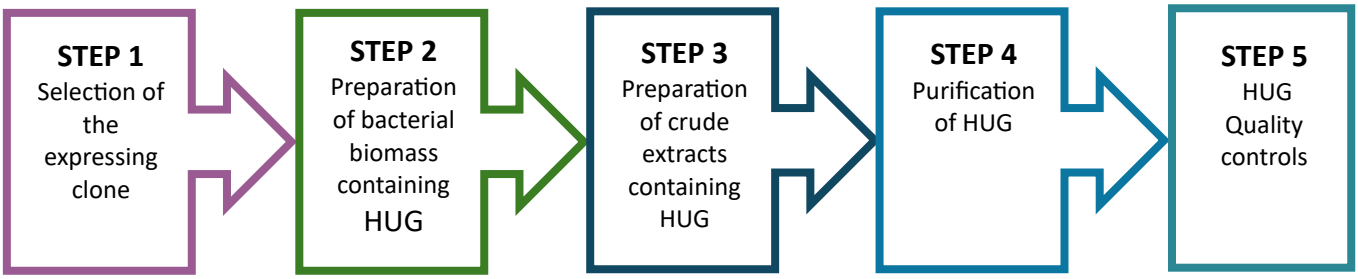
biochemical reaction mixtures. Though HELP domain weakens the bilirubin-binding affinity of UnaG slightly, from pm to low nm K_d the fusion protein still possesses a sufficiently high affinity to effectively compete with albumin for binding with unconjugated bilirubin, guaranteeing the quantitative extraction of bilirubin in standard biological matrices (Sist et al., 2023).

Additional methodological considerations are suggested to be applied in the case of biological applications of this assay. While working with protein rich matrix like plasma or serum, a suitable dilution (usually 10-20X in PBS) is done to reduce matrix effects and bring samples in the linear nanomolar range of assay. To perform experimental workflows that involve time-course measurements, HUG can be directly added to small volume aliquots collected at predefined time intervals, this allows quantification with minimum processing.

In general, the HUG assay's methodological framework can be used to detect unconjugated bilirubin quickly, even in small volumes and with high sensitivity in a variety of biological systems. It is easy to operate, has high throughput compatibility, and robust to changing physicochemical circumstances, all of this contributes to its high compatibility with research in translational studies, hepatobiliary physiology, and precision medicine.

2.3.4 Production of HUG

The HUG protein as is a fusion product of HELP and UnaG (Sist, Urbani, et al., 2025). Here I will briefly describe the standardized lab scale production of HUG protein from *E. coli* extracts. The principle of this method is based on the thermoreactive behaviour of HELP domain. The method is easily reproducible and does not generate hazardous waste products (Sist et al., 2024). The production comprises of 5 steps.



STEP 1: Selection of the expressing clone

During this phase, the focus is on choosing and confirming an *E. coli* clone that can effectively express the HUG protein guaranteeing that only effective clones advance to protein production. The method includes introducing the HUG plasmid into the C3037 strain of *E. coli* and growing the colonies in the presence of antibiotics to preserve plasmid stability. Selected clones are then cultured and observed for growth with samples taken both prior, to and following IPTG induction to evaluate protein synthesis. By comparing pre and post induction samples through electrophoretic analysis, we can confirm whether the HUG protein is being produced. Ultimately, only clones showing clear and sufficient expression are selected for subsequent preparative scale work, ensuring efficiency and reliability in purification steps.

STEP 2: Preparation of bacterial biomass containing HUG

During this stage the primary focus is on the cultivation and regulated induction of the chosen HUG-expressing clone to produce ample bacterial biomass for subsequent protein isolation. A confirmed clone is initially utilized to create a starter culture guaranteeing genetic stability and robust metabolic function prior to expansion. This starter culture is subsequently expanded into 1L flasks filled with nutrient-dense culture media with growth continuously observed until the cells attain the ideal optical density, for induction. At this point, IPTG is added to trigger expression of the HUG polypeptide, allowing the culture to synthesize the target protein over a defined period. Finally, the induced biomass is harvested by centrifugation and stored in extraction buffer at low temperature, preserving the integrity of the expressed protein for subsequent purification and analysis. This process (Fig. 2.3.4.a) requires 2 days.

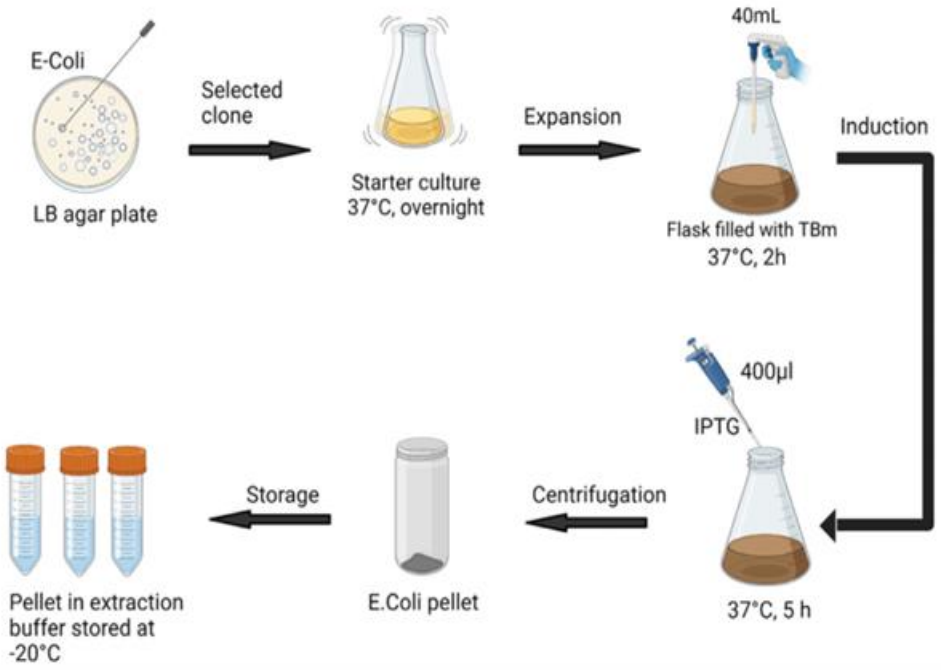


Figure 2.3.4 (a): Preparation of bacterial biomass containing HUG (Sist et al., 2024)

STEP 3: Preparation of crude extracts containing HUG

At this stage the fundamental concept involves disrupting bacterial cells at low temperatures to produce a clarified crude extract rich in the HUG protein while avoiding proteolysis and clumping. Initially the frozen biomass is resuspended in an extraction buffer, with protease inhibitors to maintain protein stability after which mechanical disruption is performed to achieve the uniformity of solution. High-pressure cell lysis is subsequently conducted in a cold-room environment to effectively break the cells while reducing thermal denaturation. The lysis is then followed by the addition of reducing agents for proper solubilization of the HUG protein, this is an important factor that influence the yield and purification of protein. In the end, the clarification of lysate is done by centrifugation to remove cellular debris, and the supernatant is collected containing the soluble protein fraction and is and stored at low temperature for the subsequent purification step. This all process (Fig. 2.3.4.b) requires 4-5 hours.

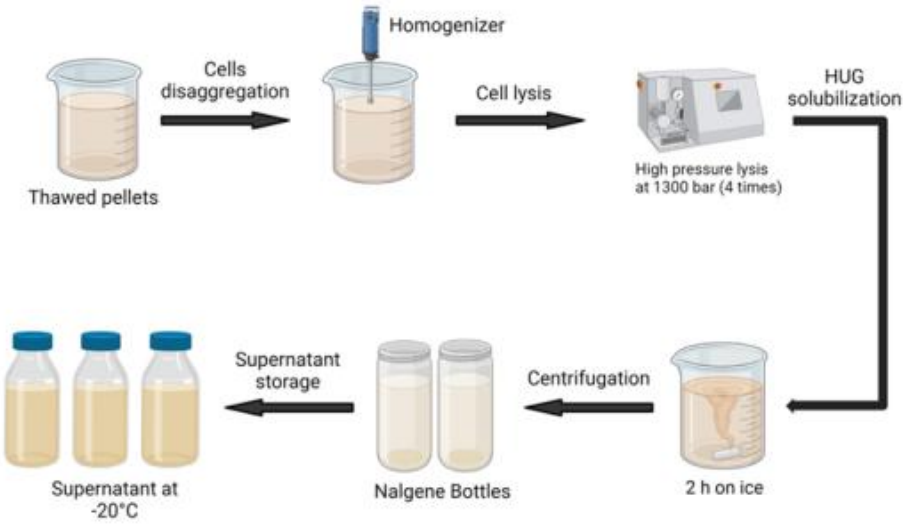


Figure 2.3.4 (b): Preparation of crude extracts containing HUG (Sist et al., 2024)

STEP 4: Purification of HUG

The basic principle of this purification step is the exploitation of unique thermal inversion and salt-dependent aggregation properties of HUG to selectively precipitate, solubilize, and re-precipitate the desired protein under controlled temperature conditions. The crude extract obtained at previous stage is first exposed to high NaCl concentrations and gentle heating to trigger selective clustering of HUG, enabling its separation from soluble impurities. The protein-concentrated pellet formed is then gently resuspended in a solubilizing buffer aimed at counteracting HUG's lipid-binding characteristics and entirely recovering the protein in solution. Subsequently a second cycle of thermal-and-salt coacervation is done to reaggregate HUG with increased purity, utilizing its reversible temperature sensitive properties. Subsequently a second cycle of thermal-and-salt coacervation is done to reaggregate HUG with increased purity, utilizing its reversible temperature sensitive properties. The purified protein is collected, rehydrated gently, and cautiously handled to prevent loss. Finally, a stable and dry form of polymer is achieved by lyophilization for storage and use of polymer in different applications. The process of purification (Fig. 2.3.4.c) takes around 6 hours and the lyophilization usually requires at least 3 days. Below is the visual representation of this step.

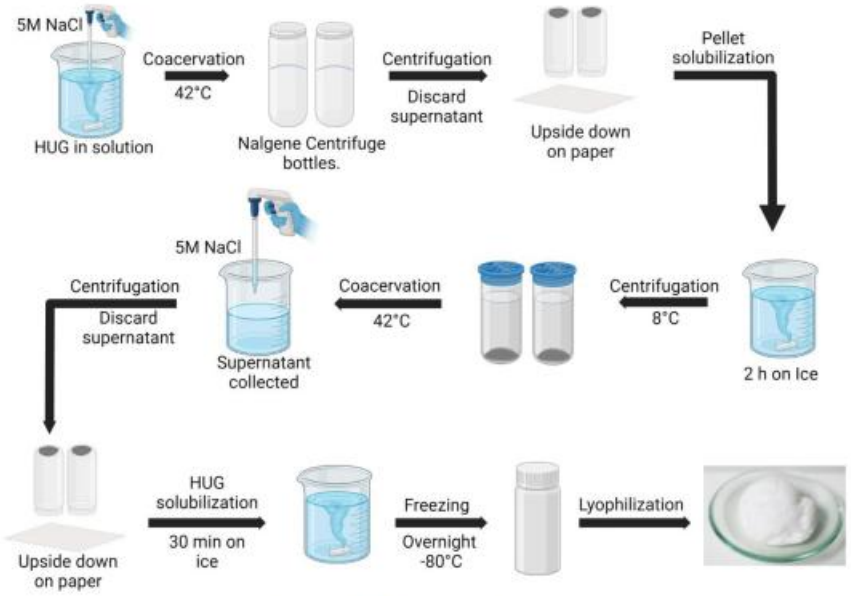


Figure 2.3.4 (c): Preparation of crude extracts containing HUG (Sist et al., 2024)

STEP 5: HUG quality controls

This step (Figure 2.3.4.d) comprises of checking the quality of the polymer obtained after lyophilization. At first the amount of HUG is determined in 2mg/ml working solution by the help of UV-VIS spectroscopy at the wavelength of λ=280nm. After this the quality of polymer is determined with the help of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). At last, the binding affinity of the obtained polymer with bilirubin is checked by the help of a standard calibration curve.

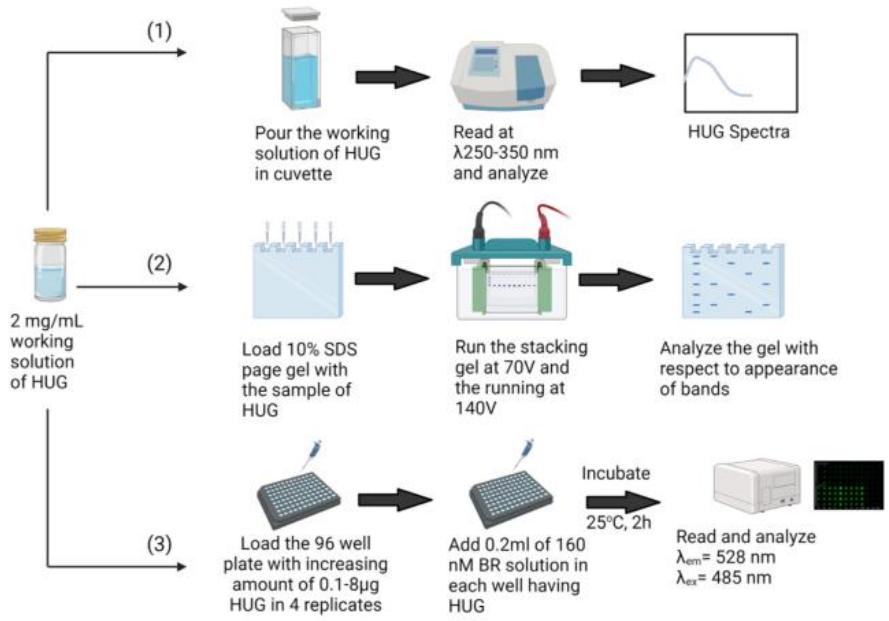


Figure 2.3.4 (d): Preparation of crude extracts containing HUG (Sist et al., 2024)

2.3.5 The HUG assay and its calibration

The assay is based on the high affinity binding of bilirubin to the recombinant protein HUG this in turn leads to a strong and highly detectable fluorescent signal. Mechanistically, HUG has a very high affinity in binding unconjugated bilirubin and so stabilizing it in physiological buffers or fluids. The essentially irreversible HUG-bilirubin complex is a strongly emitting fluorophore. The direct relationship between signal

output and bilirubin concentration allows its quantification in biological samples in the nM range. As compared to traditional diazo chemistry or transcutaneous optical analyses, the HUG fluorometric assay is specific for the bilirubin-protein complex, which significantly lowers the biochemical interference and significantly increases the analytical specificity.
The assay is calibrated using serial dilutions of high-purity bilirubin, prepared in light and pH-controlled conditions to avoid aggregation and photodegradation. The standards are added into a constant amount of HUG (0.05 g/L) under specified assays conditions and fluorescence intensity is recorded at the ideal excitation/ emission wavelengths of the complex. Standard curves are then drawn over physiologically relevant ranges, exhibiting strong linear behaviour at low to moderate concentrations and nonlinear behaviour at saturation. More importantly, the assay needs matrix-matched calibration, which means that the calibration curves should be prepared separately against plasma, serum or other biological fluids.

The assay has a very low limit of detection (LOD= 0.36nM) and a limit of quantification (LOQ= 1.1nM), and a precision of 4.5% (Sist et al., 2023), thus able to detect even minor changes in free bilirubin levels. It has a dynamic range applicable throughout the clinically significant bilirubin rates, and quantitative performance is confirmed and supported through high linear correlation coefficients ($R^2 > 0.99$ in optimized settings).

Introduction to cognitive diseases

Cognitive diseases are a wide category of neurological disorders, which are progressive in nature, associated with the inability of the brain to perform cognitive functions including memory, learning, language, attention and executive abilities. These disorders are an increasing health problem in the world given the ageing population and one of the contributors of disability and dependence among the elderly. Cognitive impairment is a continuum between normal aging and mild cognitive impairment (MCI) and the end result of which is dementia, with Alzheimer disease (AD) being the most common cause of dementia on a global basis (Portet et al., 2006).
There are a few major categories of the cognitive disorders that are classified clinically. The mild cognitive impairment is a transitional phase where people experience a significant cognitive impairment but to a large extent, they are still independent in their daily lives. Even though not every case of MCI leads to dementia, the risk of developing Alzheimer is much higher in persons with MCI, especially in amnestic MCI. Dementia is diagnosed when cognitive impairment has reached the level that it affects the daily functioning and independence. There are also several major types of dementia such as Alzheimer disease, vascular dementia, dementia with Lewy bodies, and frontotemporal dementia, all with different pathological and clinical features (McKhann et al., 2011).
The pathophysiology of cognitive diseases is complicated and multidimensional and includes abnormal aggregation of proteins, neuroinflammation, synaptic dysfunction, vascular damage and loss of neurons. The typical neuropathological changes in Alzheimer's disease are extracellular plaques of amyloid-β (Aβ) and intracellular neurofibrillary tangles of hyperphosphorylated tau protein. Notably, these pathological alterations start appearing years or even decades before the development of clinical symptoms, giving rise a long preclinical phase that which is a challenge as well as an opportunity for the early diagnosis and intervention (Jack Jr. et al., 2018).

Cognitive diseases are traditionally diagnosed based on clinical examination (medical history, neurological examination, and standard neuropsychological testing). The Mini-Mental State Examination (MMSE) and the Montreal Cognitive Assessment (MoCA) are commonly used as tools to evaluate cognitive domains such areas as memory, language, attention, and executive function. Although these tests are effective in identifying cognitive impairment, they are not specific to underlining disease pathology and there is a chance that they are influenced by several factors like education, language and culture (Ng et al., 2018; S. Zhang et al., 2021).

The use of neuroimaging methods has grown to be an important part of the clinical work up. Structural magnetic resonance imaging (MRI) is frequently used to determine patterns of atrophy in the brain, especially in the hippocampal atrophy in Alzheimer disease, and in order to find vascular lesions or other abnormalities related to structure. The techniques for functional imaging, including positron emission tomography (PET) can be used to visualize cerebral glucose metabolism and in vivo visualization of amyloid and tau pathology. Even though imaging enhances the accuracy of the diagnostic process, its high price and inaccessibility make its use limited, particularly in large-scale screening contexts (Frisoni et al., 2010; Meldolesi, 2021).

The pathophysiology of the Alzheimer disease is based on Amyloid-β peptides, specifically $A\beta_{40}$ and $A\beta_{42}$. The $A\beta_{42}$/ $A\beta_{40}$ ratio in blood has demonstrated good correlations with brain amyloid burden measured with the PET imaging. A decrease in plasma $A\beta_{42}$/ $A\beta_{40}$ ratio indicates a higher amyloid deposition in the brain and can be used to spot individuals at the preclinical phases of the Alzheimer disease. Despite the fact that plasma amyloid levels are affected by peripheral generation and clearance, they are still useful screening tests, when combined with other biomarkers (Luebke et al., 2023; Nakamura et al., 2018).

The Tau protein biomarkers add complimentary information to that of neuronal injury and neurodegeneration. The total tau in plasma shows the general damage to the neurons, not specific to the Alzheimer disease, since high levels can be also seen in other neurological disorders. Conversely, phosphorylated tau species are highly specific and sensitive towards Alzheimer disease. Plasma p-tau is associated with tau PET scan, cognitive impairment, stage of the disease, and increases early during the progression of disease, and in many cases even before the onset of evident cognitive impairment (Janelidze et al., 2020; Palmqvist et al., 2020).

The cerebrospinal fluid (CSF) biomarkers have greatly contributed to the biological diagnosis of the Alzheimer disease. The typical changes in the CSF are low levels of amyloid and high levels of total tau (t-tau) and phosphorylated tau (p-tau). These biomarkers have close correlations with neuropathological outcomes and progression of disease. However, lumbar puncture is not always acceptable by patients to collect CSF because of its invasiveness, and this limits the repeated or population-wide testing. Recent developments in ultrasensitive analytical technologies have enabled precise measurement of Aβ and tau biomarkers in peripheral blood, ensuring a less invasive alternative. Circulating AD-related biomarkers not only reflect key pathological processes like inflammation and neurodegeneration but they also have a significant potential for the detection of disease at earlier stages, progression monitoring, and making the decisions for guiding the treatment (Blennow & Zetterberg, 2018; Gaetani et al., 2020).

Circulating or blood-based biomarkers are now emerging as potential alternatives in the diagnosis and monitoring of cognitive diseases in the past few years. Blood-based biomarkers have a number of benefits, such as a low level of invasiveness, reduced

costs, and the possibility to be applied to large-scale screening and longitudinal follow-up. Recent developments in the most sensitive assays, including single-molecule array (Simoa) have made it possible to detect brain-derived proteins in peripheral blood in a very low concentration range reliably (Gaetani et al., 2020; Luebke et al., 2023; Zetterberg & Burnham, 2019).
The application of the circulating biomarkers in clinical and research settings has revolutionized the diagnostic landscape of the cognitive diseases. Early detection, differentiation diagnosis, monitoring disease progression, and choosing the participants to include in the clinical trials are some of the areas where these biomarkers are finding increased applications. Their use is associated with the shift toward a biologically defined paradigm for Alzheimer's disease when the diagnosis is made based on the underlying pathology rather than the clinical symptoms alone (Jack et al., 2024; Jack Jr. et al., 2018).

Analysis of genetic polymorphisms

Genetic polymorphisms are naturally occurring variations in the DNA sequence of individuals belonging to a population which are at a frequency of more than 1%. These variations cause phenotypic diversity and variable degrees of susceptibility to disease development, response to drugs, metabolism, and biomarker expression patterns in biomedical studies (Karki et al., 2015).
The single nucleotide polymorphism (SNP) is the most common form of polymorphism, that is a genetic variant affecting one base pair at a specific locus. Other forms of polymorphisms are insertion and deletions, copy number variation (CNV), microsatellites and structural rearrangements (Storey et al., 2007). Polymorphisms may be either in the coding, non-coding (e.g promoter regions) or in intronic regions. These changes have the potential to modify gene expression (mRNA translation), protein folding or stability, and biological activity (e.g. catalytic power of enzyme).
Genetic polymorphisms may have a direct impact on metabolic pathways, through changes in expression of gene, potential of regulatory feedback, transport kinetics, substrate affinity and protein turnover. Mutations in enzyme's active or cofactor-binding sites have the potential to alter biochemical equilibrium states and promote a particular pattern of metabolite accumulation. In toxicological and neurodegenerative disease studies, metabolic variability mediated by polymorphisms is considered to play a major role in inter-individual differences in disease risk, disease onset timing, and severity of disease (Karki et al., 2015).
Several genetic variants can influence the metabolism of bilirubin. Genetic variations that modify the activity of enzymes including the *UGT1A1* have a direct impact on the conjugation efficiency of bilirubin, level of bilirubin in plasma bilirubin, and the liver excretion capacity in the bilirubin metabolism. *UTG1A1* polymorphism rs8175347 is associated with a significant decrease in transcriptional activity causing decrease in bilirubin clearance and an increase in bilirubin levels in the blood (Bosma, Chowdhury, Bakker, Gantla, Boer, et al., 1995; Kaplan, 2001). This finding is clinically applicable in people with Gilbert syndrome in whom conjugation deficiency is genetically mediated, resulting in chronic mild hyperbilirubinemia.
In the same way, variations in antioxidant regulatory genes, such as KEAP1-NRF2 signaling axis regulation genes, metabolism of glutathione, expression of heme oxygenase and oxidative stress response pathways, can influence the biotransformation of bilirubin indirectly. These polymorphisms determine the relationship between bilirubin as an antioxidant and bilirubin as a toxic metabolite in

the susceptibility of oxidative stress to neurological diseases (Y. Wang et al., 2024). There is experimental evidence that bilirubin has two biochemical functions; bilirubin at concentrations in the physiological setting shows endogenous antioxidant activity, by neutralizing reactive oxygen species (ROS) and consequential lipid peroxidation cascades (Adin, 2021). However, at elevated levels that go beyond physiological range, bilirubin can be cytotoxic and add to the process of protein oxidation, mitochondrial dysfunction, and dysfunction of cells (Berthelot et al., 1982). Polymorphisms in genes that regulate the bilirubin level are thus significant in defining the predominant bilirubin functional role.

In Alzheimer's disease (AD), oxidative stress is a characteristic pathophysiological element associated with progressive neurodegeneration and neuronal death. Genetic polymorphisms in genes that are involved in the oxidative stress response have recently been recognized as a crucial determinant of AD susceptibility, rate of amyloid aggregation, mechanism of tau hyper-phosphorylation, and trajectory of cognitive decline (Vogrinc et al., 2023b).

The recent conceptual frameworks have proposed the concept of mechanistic overlap between metabolic regulation of bilirubin, genetic polymorphism, and pathology of AD. The change in bilirubin clearance has the potential to affect systemic oxidative stress burden, buffering capacity of reactive oxygen species, the activation of microglial cells, and neuroinflammatory signaling cascades. This provides a possible biochemical signaling pathway between the dysregulation of bile pigment metabolism and neurodegeneration-associated redox instability (Lanzillotta et al., 2020; Mao et al., 2025; Nitti et al., 2020).

GWAS has been particularly successful in mapping AD genetic susceptibility loci that influences synaptic maintenance, neuroinflammatory homeostasis, lipid metabolism, oxidative stress response and innate immune modulation (Lambert et al., 2013; Sims et al., 2017). The same rationale may be adopted in the pathophysiology of bilirubin metabolism by finding the genotype to phenotype associations between variants of *UGT1A1* and risk modulation in neurological decline, mediated by oxidative stress.

Finally, the interaction between the bilirubin biochemical physiology and AD pathology can be modeled as a tri-axis mechanistic model between (i) genetic polymorphisms that control the metabolic activity of enzymes, (ii) stability of the bilirubin concentration, and (iii) burden of oxidative stress in the neuronal systems. For instance, if genetic polymorphisms increase the bilirubin or disrupt its biochemical processing, the resulting amplification of oxidative stress may increase neuronal death and trigger AD progression pathways.

4.1 Association of polymorphisms with bile pigments

Many genes play a critical role in the metabolism of bilirubin, conjugation, transport in the cell, and systemic pathways of disposition, and polymorphisms of these genes are major contributors to inter-individual fluctuation of serum bilirubin level. The *UGT1A1* gene, which codes the bilirubin glucuronidation hepatic enzyme is the most researched gene. Along with this, there are membrane efflux and influx transporters like *ABCB1, ABCB10, ABCC2*, *ABCG2, SLCO1A2, SLCO1B1, SLCO1B3,* and *SLCO2B1*, that collectively play a role in regulation of intracellular bilirubin traffic, hepatocellular uptake, sinusoidal reuptake, canalicular secretion and enterohepatic recirculation (Čvorović & Passamonti, 2017; Kamisako et al., 2000b). Thus, the phenotypic differences in bilirubin load, elimination kinetics, hepatic elimination, and downstream oxidative stress buffering functions can be a result of variation in any of these gene loci.

Transcriptional efficiency and conjugation activity of *UGT1A1* are decreased by polymorphisms in *UGT1A1* promoter regions, especially TA repeat variants of *UGT1A1* *28 rs8175347. Carriers of this type of variation have high levels of unconjugated bilirubin, high serum bilirubin retention, and low conjugation capacity in the liver, a typical biochemical phenotype of Gilbert Syndrome. There is clinical evidence that antioxidant buffering can be altered by low *UGT1A1* activity and can indirectly alter the effects of oxidant mediated pathology, which is relevant when taking into account systemic effects of oxidative stress on neurodegenerative diseases such as AD (de Vries et al., 2012; Lin et al., 2006; Schwertner & Vítek, 2008).

Transporters that are part of the ATP-binding cassette also play a role in bilirubin dynamics. For instance, *ABCB1* regulates the transport of xenobiotics and affects biliary excretion of bilirubin related substances by the liver (W. Li et al., 2024a). Likewise, *ABCB10* plays a role in the mitochondrial metabolism of heme and regulation of bilirubin precursor via the heme turnover processes (Liesa et al., 2012). *ABCC2* (MRP2) is specifically of interest since it is the major export efflux transporter of conjugated bilirubin into bile, and polymorphisms in *ABCC2* have been linked to mild hyperbilirubinemia and efficiency of canalicular transport (Wu et al., 2020).

Other transporters like multidrug resistance transporter (*ABCB1*), *ABCG2* and solute carrier (SLCO) family transporters like *SLCO1A2, SLCO1B1, SLCO1B3,* and *SLCO2B1* control the hepatic uptake and redistribution of bilirubin in the liver. *SLCO1B1* and *SLCO1B3* variants can have a drastic effect on the bilirubin influx into hepatocytes and ultimately affecting the rate of conjugation and the concentration of the systemic pool. The significant associations of *SLCO1B1* polymorphisms with serum bilirubin variability have been reported in a series of ethnic cohorts, with a high degree of association confirmed in population studies, making transporter genotypes important predictive factors of bilirubin phenotype (Kang et al., 2010; Liu et al., 2013; Sanna et al., 2009).

Many population studies have suggested significant genotype to phenotype correlation that link *ABCC2, SLCO1B1* and *UGT1A1* transporter with different patterns of bilirubin distribution. As an example, cohort studies of *UGT1A1* function based on genome of Asian and European populations showed that carriers of *UGT1A1*28* had steadily higher bilirubin levels than wild-type homozygotes, and the magnitude of the effect varied in relation to the prevalence of alleles linked to ancestry (Beutler et al., 1998; G. Chen et al., 2012a; Lin et al., 2006; Memon et al., 2016). These results show that metabolic regulation varies globally in bilirubin biochemical systems.

The neurobiological implications of these polymorphism induced effects are that, the bilirubin interacts with oxidative stress pathways, which are suggested to lead to the development of AD pathology. Associated variants with high unconjugated bilirubin levels can also alter systemic redox homeostasis, which can successively alter the threshold of neuroinflammatory activation, ROS clearance capacity, and cumulative oxidative burden of the brain, which is known to trigger neurodegenerative cascades associated by AD (Cioffi et al., n.d.). Thus, the combination of bilirubin genetic variation data and AD risk mapping constructs has a mechanistic interpretive value in precision neurobiology.

Nevertheless, regardless of many observational genotypes to phenotype findings, methodological limitations constrain the reproducibility of genetic association outcomes. Bilirubin is affected by multifactorial regulatory interactions, environmental cofactors, polygenic contributions, comorbid hepatic conditions and dietary antioxidant exposure, making reproducibility sensitive to population sampling bias (Dai et al., 2013; Miranda et al., 2024). Additionally, other parameters may affect bilirubin

metabolism. There are also reported sex-based differences in bilirubin expression where male subjects are statistically higher in the trends of total bilirubin compared to female subjects in large epidemiological studies. This might be connected with sex hormone dependent variations in the transcription of bilirubin regulating enzymes and hepatic transporter adjustment (Coltell et al., 2019; Rosenthal et al., 1984). The changes that occur by age are also noteworthy; the effectiveness of bilirubin metabolism decreases with age as a result of cumulative mitochondrial damage, decrease in hepatic elasticity, and changes in conjugation substrates which can interact with polymorphic variation to enhance systemic oxidative stress over lifespan (Adin, 2021).

Moreover, small sample sizes, lack of demographic heterogeneity, inadequate mechanistic modeling, and inadequate multivariate control of confounding factors are common causes of variability in published associations. Future research should combine multi-omic modeling, stratified subgroup design, well-powered ethnic comparative analysis, and standard protocols of bilirubin fractionation to enhance the reproducibility of the results.
To recap it all, genetic polymorphisms in the bilirubin metabolic enzymes and transporters play a crucial role in determining the systemic bilirubin levels, their clearance potential as well as modulation of oxidative stress and such variations are an indirect contributor in disease risk profiling. Their relevance is not limited only to hepatology but rather to neurodegeneration biomarker studies such as AD that makes a mechanistic rationale for the integration of bilirubin variability driven by polymorphism into molecular risk stratification models.

4.2 Association of polymorphisms with AD biomarkers

Genetic polymorphisms influencing the regulation of oxidative stress, metabolism of heme and biotransformation of bilirubin are becoming actively studied in the context of determining the biomarker signatures of AD. AD biomarkers, like Amyloid-β (Aβ) levels in cerebrospinal fluid (CSF), phosphorylation status of tau, and amyloid positron emissions tomography (PET) uptake, indicate the underlying pathophysiological processes which are synaptic dysfunction, protein aggregation, mitochondrial damage and progressive neuronal loss. Since oxidative stress is a key factor in the acceleration of AD pathogenesis, genetic variations that modify the antioxidant capacity or redox balance can modify the course of the biomarkers and the risk of clinical conversion (Butterfield, 2011).

Apolipoprotein E *(APOE)* genotype variation is one of the most common polymorphic risk factors of AD that have been investigated widely. *APOE-ε4* allele has been established to enhance amyloid deposition, facilitate low efficiency of amyloid clearance, enhance the susceptibility to oxidative stress and accelerate biomarker transformation during preclinical and prodromal stages of AD (Belloy et al., 2019; DiBattista et al., 2016). Many imaging studies have found an increased uptake of amyloid PET tracer and a reduced CSF $A\beta_{42}$ in *APOE-ε4* carriers compared to non-carriers (Reiman et al., 2009). APOE has been linked with **altered levels of bilirubin and liver function markers**, showing that APOE genotype may influence systemic metabolism of bilirubin and pathways of oxidative stress, linked to cognitive impairment and changes in cerebral blood flow (H. Wang et al., 2024). Since *APOE* also modulates lipid metabolism and redox-regulating processes, its relationships with

bilirubin metabolic equilibrium can be an understudied factor in the development of AD as a biomarker.

The genetic variation influencing bilirubin metabolism may indirectly affect AD biomarkers patterns by changing endogenous antioxidant buffering capacity (Nitti et al., 2020). High systemic oxidative load has been observed to be associated with both lowering of CSF $A\beta_{42}$ levels and raising of phosphorylated tau levels in agreement with redox driven amyloidogenic acceleration and tau-associated neurodegeneration (Ahmad et al., 2024; Di Domenico et al., 2016; Vogrinc et al., 2023a). Genetic variants that lower bilirubin antioxidant stability can therefore possibly serve to enhance positive oxidative conditions that enhance abnormalities of biomarkers.

Since oxidative stress is one of the factors of metabolic failure in the mitochondria, the polymorphisms affecting antioxidant molecule turnover may also change the threshold of ROS production and mitochondrial respiration resilience (W. Wang et al., 2020). Redox dysregulation also amplifies tau phosphorylation processes, synaptic vulnerability and lipid membrane peroxidation, which are major mechanisms of divergence in AD biomarkers (Barone et al., 2012). This gives strength to the argument of studying bilirubin pathway polymorphisms as an indirect modulator of the expression of AD molecular biomarkers.

Recent reports have looked at *UGT1A1* polymorphisms in the AD biomarker stratification models. Variants linked to low bilirubin conjugation ability might elevate the availability of unconjugated bilirubin, resulting in changing the blood-brain redox buffering interactions (Gil & Sąsiadek, 2012a) and may potentially cause subtle changes in the CSF biomarker signatures. Though studies are in very early stages, initial studies have indicated that the *UGT1A1* polymorphisms may have an impact on the rate of amyloid accumulation (Langhans et al., 2025).

Genetic variations in heme oxygenase-1 (*HMOX1*) have a direct effect on the heme catabolic flux and rate of bilirubin synthesis. The polymorphisms in *HMOX1* promoter regions causing transcription repressions were linked to less formation of bilirubin, as well as a decreased endogenous capacity to produce antioxidants (Jirásková et al., 2023). These variants have been associated with greater susceptibility to AD and have been linked with increased lipid oxidation patterns and altered tau burden in experimental work and human biomarker cohorts (Schipper, 2007).

At the blood brain barrier (BBB), the ATP-binding cassette (ABC) and organic anion-transporting polypeptide (SLCO/OATP) transporters are crucial in managing molecular exchange including the ones relevant to in Alzheimer disease (AD). This includes several transporters implicated in bilirubin metabolism. One of the most characterized transporters is ABCB1 (P-glycoprotein), which is very abundant in luminal endothelial cells of the BBB. It serves as a significant efflux transporter of amyloid-β (Aβ) into the systemic circulation; decrease in its activity is linked to the accumulation of Aβ in the brain due to its impaired clearance (Cirrito et al., 2005; Van Assema et al., 2012). ABCG2, a co-localizing efflux transporter with ABCB1 on the endothelial cells of brain, works in cooperation with ABCB1 to improve the efflux of Aβ, and inhibition or knockout of ABCG2 speeds up the deposition of Aβ in vivo (Abuznait & Kaddoumi, 2012; Xiong et al., 2009). Comparatively, ABCC2 (MRP2) is mostly hepatic and intestinal with little

expression at the BBB which implies that it has a minimal direct effect in the transport of Aβ as compared to other transporters discussed earlier (W. Zhang et al., 2023).

In the family of OATP (SLCO) transporters the isoform SLCO1A2 is one of the rarest expressed in the BBB and has been suggested to facilitate the transport of organic anion and xenobiotics, although its direct link with Aβ is still to be studied (Franke et al., 2009a). Other than this, localized primarily in the liver, SLCO1B1 and SLCO1B3 are not expressed in the CNS but aid in endogenous metabolite systemic clearance, which may be useful as the peripheral sink effect once Aβ is released into the circulation (DeMattos et al., 2001; Smith et al., 2005). SLCO2B1 is expressed in hepatic and intestinal tissues, mediating the uptake of several endogenous bioactive lipids and drugs, there are some studies that suggest its role as a transporter of peptides and neuropeptides but its link to the trafficking of Aβ in AD remains uncertain (Drozdzik et al., 2019; Kinzi et al., 2021). The differential expression of these transporters makes them a potential target for therapeutic interventions to increase the clearance of Aβ and delay the progression of AD.

Biomarker studies of CSF have also established the genetic polymorphisms involving oxidative stress response to increase changes in biomarkers associated with tau pathology. The high level of CSF phosphorylated tau (p-tau$_{181}$) and total tau is often associated with genetic factors that give rise to more sensitivity to oxidative stress and weakened antioxidant buffering (Butterfield, 2011). These results support the biological feasibility that genetic variants related to bilirubin may cast synergistic or additive effects on downstream AD biomarkers.

A number of imaging genomics cohort studies have combined genome-wide polymorphism data with amyloid PET levels and MRI volumetric neurodegeneration measures. It has been shown by these studies that genetic variation that can influence antioxidant defense is linked to regional patterns of cortical amyloid deposition and neurodegeneration in the hippocampus, which can be found especially in individuals carrying high risk gene sequences like APOE ε4 (Chico et al., 2013; Vogrinc et al., 2023a). Integrating polymorphisms that are related to bilirubin in such frameworks may increase the accuracy of interpretation of oxidative stress sensitive imaging signatures.

Although there is growing research on bilirubin metabolism polymorphisms and their relationship with the AD biomarkers, it is still at very early stage and most studies are confined due to small sample sizes, lack of variation in their ancestry, and limited integration of biomarkers over time. Genetic variants that influence bilirubin levels especially common *UGT1A1* polymorphisms are well studied over the time across multiple ethnic groups (G. Chen et al., 2012b; González-Iglesias et al., 2024; Hanafusa et al., 2022). However, major PET imaging studies, including recent large multicenter cohorts (Ossenkoppele et al., 2022), have not introduced these genes related to bilirubin systemically into their biomarker models. Therefore, the role of *UGT1A1* variation in PET-based Alzheimer's disease markers remains largely unexplored. So, this represents a significant methodological gap to incorporate bilirubin genotype profiling in AD precision research carried out by biomarkers.

With the increasing use of advanced multi-omics frameworks in AD studies, a potential solution to this is including the polymorphisms related to bilirubin in combined

transcriptomic, metabolomic, and imaging biomarker models, which can significantly enhance the stratification of risk prediction. This approach might also be used to differentiate AD endophenotypes driven by oxidative stress and subtypes entirely based on proteinopathy.

Lastly, narrowing these associations to a translational application may result in new disease staging approaches that are informed by biomarkers that might enable redox-targeted therapeutic interventions to be individually tailored based on bilirubin metabolic genetic background. Multi-cohort replication, better phenotype harmonization and ethnicity-adjusted genetic baseline models need to be incorporated into future work to describe the full contribution of bilirubin polymorphism to the heterogeneity of the AD biomarker.

MATERIALS AND METHODS

5.1 Patients' description

61 individuals with cognitive impairment were included in study. All were treated at Department of Neurology, The University Medical Centre Ljubljana, Slovenia between June 2019 and December 2022. Age over 55 and a diagnosis of Alzheimer's disease or mild cognitive impairment were the criteria for inclusion. Participants with dementia and comorbidities from conditions other than AD that substantially impair cognitive function were not included in the study. Clinical and demographic information was gathered through a structured interview with patients and their caretakers. Medical records were consulted for more details.
The National Medical Ethics Committee of the Republic of Slovenia accepted the study protocol (0120-523/2017-4), and all participants gave written informed permission in compliance with the Declaration of Helsinki.
Structural brain imaging, blood laboratory testing, neuropsychological evaluation, and cerebrospinal fluid (CSF) dementia biomarker testing were all part of the extensive diagnostic work-up. The Mini-Mental State Examination (MMSE) was used for cognitive screening. Following a consensus meeting with neuropsychologists and doctors, patients were diagnosed with dementia based on DSM V criteria, considering all relevant data.
The CSF biomarkers analysis was performed at the Laboratory for CSF diagnostics, Department of Neurology, University Medical Centre Ljubljana, Slovenia. $A\beta_{1–40}$, $A\beta_{1–42}$, total tau and p-tau_{181} were measured with the help of the INNOTEST® (Fujirebio, Europe) immunoassays, as per the instructions by manufacturer as described earlier (Vogrinc et al., 2023a).
Patients were divided into three groups: AD, MCI (NOT AD), and MCI (AD) based on CSF biomarker levels, dementia criteria, and Winblad & Peterson MCI diagnostic criteria (Petersen et al., 1999). For $A\beta_{42}$ (>570 pg/mL), $A\beta_{42/40}$ (>0.07), p-tau_{181} (<60 pg/mL), and total tau (<400 pg/mL), locally confirmed biomarker cut-off levels were employed. The AD group consisted of patients with decreased $A\beta_{42}$ and $A\beta_{42/40}$ levels, higher total and p-tau_{181}, and impaired daily activities. The MCI (AD) group consisted of patients with normal daily functioning and CSF biomarker profiles for both AD and MCI. The MCI (NOT AD) group consisted of patients with MCI who maintained their everyday functioning and had normal biomarker levels (Vogrinc et al., 2023b).

5.2 Analysis of fractionated bilirubin in plasma

The bile pigments (bilirubin, biliverdin and bilirubin glucuronide) were measured in plasma with the help of BR-HUG assay. The characteristics and production of HUG is described above in detail (Section 2).

5.2.1 Sample analysis

Plasma samples were analysed to determine bilirubin levels using the HUG assay according to the paradigm of methodology presented by (Sist et al., 2023). Blood samples were stored in anticoagulant tubes which were subsequently centrifuged to yield plasma. The isolated plasma was aliquoted in low-binding tubes, and kept at -80 ºC till the analysis.

For calibration, already prepared bilirubin 5mM stock solution in DMSO was used, it is prepared in the lab and aliquoted to reduce the number of freeze-thaw, and kept at -20 ºC in darkness to avoid photodegradation. For making the working calibration solutions the stock was diluted in phosphate-buffered saline (PBS) supplemented with 0.4 g/L bovine serum albumin (BSA), and a standard curve was prepared within the analytical linear range (usually 0-50 nM). To ensure the saturation independent response to fluorescence, the HUG solution was prepared in such a way that its final concentration in assay was 0.05g/L. Plasma samples were diluted in PBS containing 0.4 g/L BSA to reduce the influence of the matrix and to adjust the bilirubin concentration of the sample with the linear range of the assay. For the quantification of total bilirubin, NaOH and glucuronidase, were added to the samples to hydrolyse the conjugates of bilirubin.

The assay reactions were loaded on a 96-well microplate for fluorescence by adding diluted samples or calibrating solutions to the working solution of HUG, ensuring a uniform final volume in each well. Incubation of the plates was then done at room temperature for about 1-2 h without any light exposure until the stability of the fluorescence signal was reached. Fluorescence intensity was then read with the help of a microplate reader that was configured to an excitation wavelength of around 498 nm and emission detection of 527-530 nm. Before the processing of data, the background fluorescence from reagent blanks was subtracted.

A standard curve was plotted with the fluorescence units versus the concentration of bilirubin fractions and to obtain calibration parameters linear regression was used. Regression results were used to derive actual sample concentrations in µM, utilising dilution factor corrections. To determine the reliability of the methods every batch of the experiment had blank controls and low, medium and high quality-controlling levels. The accuracy was ensured by replicating the sample measurements and accepting the results when the coefficient of variation was within acceptable limits of analysis. The assay had a detection limit in the nanomolar range and was linear within the calibration range, similar to that described by (Sist et al., 2023).

5.3 Analysis of genetic variability of bilirubin enzymes and transporters

5.3.1 DNA isolation and determination of concentration

Genomic DNA was isolated from peripheral blood samples using the E.Z.N.A.® SQ Blood DNA Kit II (Omega Biotek, Inc., Norcross, GA, USA) according to the manufacturer's protocol.

The purity check and concentration measurement of DNA samples was done with UV/VIS spectrophotometry by checking the absorbance at 260nm and 280nm wavelengths. The Beer-Lambert law was applied to the absorbance obtained at 260nm to calculate the DNA concentration. The purity of sample was determined by checking the ratio of absorbances at both wavelengths i.e. 260 and 280nm. The ratio should be around 1.8-2, while the lower value indicating the presence of protein residues and higher value indicating the presence of RNA in the sample (Vogrinc et al., 2023a).

5.3.2 Bioinformatic analysis

For genotyping, potentially functional single nucleotide polymorphisms (SNPs) were selected in genes encoding transporters previously associated with bilirubin: *ABCB1*, *ABCB10*, *ABCC2*, *ABCG2*, *SLCO1B1*, *SLCO1A2*, *SLCO1B3*, and *SLCO2B1*. The selection of polymorphisms in bilirubin transporter genes was done by bioinformatic analysis. SNPs were obtained from different databases like PharmGKB, dbSNP, SNPinfo, LDlink and ForgeDB. Only common polymorphisms with minor allele frequency (MAF) above 5 % in the European population were considered for genotyping. At first all SNPs within each gene and +/- 5000 bp away with frequency above 5% were selected. Additionally, the list of SNPs within each gene previously associated with drug response was retrieved from PharmGKB. After this, the *in silico* predicted function of each SNP was checked using SNP Function Prediction. Then with the help of dbSNP we shortlisted the SNPs that were already reported in literature. In the end linkage disequilibrium analysis (LD) was done to avoid redundant SNPs and to ensure the representative tagging of haplotype blocks. The Variants with high Forge DB scores were prioritized as candidates having potential regulatory impact. LDtrait was then used to identify the SNPs associated previously with relevant phenotypes or in LD with known trait-associated variants. This strategy was used to maintain effective genotyping coverage while optimizing biological relevance and with this, 15 potentially functional polymorphisms in 8 genes were selected for genotyping with the help of already published literature, with at least 0.05 minor allele frequency (MAF): *ABCB1* (rs1128503, rs1045642, rs2032582), *ABCC2* (rs717620, rs2273697, rs2804402), *ABCG2* (rs2231142, rs2231137), *SLCO1B1* (rs11045879, rs4149056, rs2306283), *SLCO1A2* (rs10841795), *SLCO1B3* (rs4149117), *SLCO2B1* (rs12422149), and *ABCB10* (rs1053513).

5.3.3 Genotyping

Polymerase chain reaction (PCR) is a laboratory technique for amplifying particular DNA sequences. Deoxyribonucleotide triphosphates (dNTPs), adenine, thymine, cytosine, and guanine (A, T, C, and G), as well as DNA polymerase, are necessary for PCR. DNA polymerase catalyzes the synthesis of a DNA chain, which is built up of dNTPs. Primers are brief single-stranded DNA fragments that enable the start of amplification by binding complementary to the target DNA sequence. A complementary pair of primers, one on the left (5') and one on the right (3') side of the

DNA sequence to be amplified, are used in PCR. These primers hybridize to opposite DNA strands. PCR usually involves three fundamental stages.
In the first stage the double-stranded DNA is denatured at a high temperature and split it into two complementary strands, then in the second stage the primers are annealed on the complementary regions on the two distinct DNA strands. In the third step a new complementary strand is synthesized by help of DNA polymerase, eventually the DNA is amplified. The number of copies of the target DNA segment increases exponentially when the entire procedure is done multiple times.
Genotyping of *ABCB1* (rs1128503, rs1045642), *ABCC2* (rs717620, rs2273697, rs2804402), *ABCG2* (rs2231142, rs2231137), *SLCO1B1* (rs11045879, rs4149056, rs2306283), *SLCO1A2* (rs10841795), *SLCO1B3* (rs4149117), and *SLCO2B1* (rs12422149) was done by competitive allele specific PCR using the KASP assays by LGC Biosearch Technologies, Hoddesdon, UK as per manufacturer's instructions.
Genotyping of *ABCB1* (rs2032582) was done by classic PCR and electrophoresis.
TaqMan SNP Genotyping assay by Applied Biosystems, Thermo Fisher Scientific, Waltham, Massachusetts, USA was used to genotype *ABCB10* (rs1053513) according to the instructions by manufacturer. The genotyping results of *UGT1A1* rs8175347 were already provided by a previous study from same lab and it was also genotyped by allele specific PCR using the KASP assays by LGC Biosearch Technologies, Hoddesdon, UK as per manufacturer's instructions (Vogrinc et al., 2023b, 2023a).
Additionally, APOE rs7412 and rs429358 were previously genotyped for the assessment of APOE4 status using real-time PCR-based Taqman assay (Applied Biosystems, Thermo Fisher Scientific, Waltham, Massachusetts, USA) in all patients. Combination of APOE rs429358 (p.Cys112Arg) and rs7412 (p.Arg158Cys) defines three polymorphic alleles, APOE2, APOE3 and APOE4 (Vogrinc et al., 2023b).

5.3.3 (a) Genotyping – KASP

Competitive allele-specific PCR (KASP) is a genotyping technique that uses fluorescence signals to identify single nucleotide polymorphisms, insertions, and deletions at certain genomic regions. Three oligonucleotides: two allele-specific primers and one common reverse primer with a universal reaction mix comprising fluorescence resonance (FRET) probes, a ROX reference dye, DNA polymerase, free dNTPs, and magnesium chloride in buffer solution are added to a DNA sample in the KASP method. A fluorescent dye is affixed to the 5' end of the double-stranded oligonucleotide, and a quencher is affixed to the 3' end of the FRET probe. Each allele is identified with a unique fluorophore, such as HEX or FAM, and the fluorescence signal is detected to read the results.

The allele-specific primers attach to complementary regions of distinct DNA strands during PCR cycles. Depending on whether a certain polymorphism is present, the primer's 3' end sequence dictates which primer will bind effectively. In order for the FRET probe to bind and cause the emission of a fluorescent signal, a new strand with an allele-specific tail sequence that is not complementary to the target DNA is created during DNA synthesis. When the probe binds, FAM or HEX dye is released, enabling genotype differentiation. Heterozygotes produce a mixture of signals from both dyes, enabling accurate genotype detection, whereas homozygotes only detect a signal from one dye (FAM or HEX).

The method used for genotyping in this study was touchdown PCR protocol. The method is based on a PCR with the decreasing annealing temperature, where higher starting temperatures make sure that specific base pairs are formed so that the desired

sequence is amplified efficiently. The temperature is lowered in subsequent cycles allowing the selective amplification of the target sequence and improving the reaction's specificity.

Procedure for performing genotyping with the KASP PCR:

The isolated DNA samples were pipetted first into 96-well plates and then diluted with water to obtain equal concentrations of DNA (15 ng/µL). Then, the sample (3 µL) was transferred to KASP PCR suitable 386-well plates. To reduce the quantity of reagents required for reaction, the DNA was dried by placing the plates in oven at 50°C. The reaction mixture was then prepared in 1.5 mL microcentrifuge tubes by mixing KASP Master mix, specific primers and sterile water. The reaction mixture contained KASP Genotyping Assay (0.042 µL), KASP Master Mix (1.5 µL), and sterile water (1.458 µL) per sample. 3 µL of reaction mixture was added to each well of plate with dried DNA samples. The plates were then sealed with optically transparent foil and centrifuged, after this the genotyping was performed using the Proflex PCR System under specific conditions for each polymorphism as shown in table 5.3.3 a (i). KASP thermal cycling conditions for all protocols are presented in table 5.3.3 a (ii). The fluorescence signals were then measured with the FLUOstar Omega, and then the results were analysed using the KlusterCaller™ software, which enabled the genotype determination for all polymorphisms based on the measured fluorescence of the HEX and FAM dyes.

Table 5.3.3 a (i): PCR protocols for each SNP

Gene	rs	Change	FAM/VIC	PCR conditions	PCR protocol	Reference allele	Polymorphic allele
ABCB1	rs1128503	p.Gly412Gly	C/T	1.8mM MgCl2	KASP TD68-62	T	C
	rs1045642	p.Ile1145=	C/T	1.8mM MgCl2	KASP TD61-55	T	C
	rs2032582	p.Ala893Ser/Thr	electrophoresis			G	T/A
ABCC2	rs717620	c.-24C>T	A/G	1.8mM MgCl2	KASP TD61-55	G	A
	rs2273697	p.Val417Ile	A/G	1.8mM MgCl2	KASP TD61-55	G	A
	rs2804402	c.-1019A>G	C/T	1.8mM MgCl2	KASP TD61-55	C	T
ABCG2	rs2231142	p.Gln141Lys	A/C	1.8mM MgCl2	KASP TD61-55	C	A
	rs2231137	p.Val12Met	A/G	1.8mM MgCl2	KASP TD61-55	G	A
SLCO1B1	rs11045879	c.1865+4846T>C	C/T	1.8mM MgCl2	KASP TD61-55	T	C
	rs4149056	p.Ala174Val	T/C	1.8mM MgCl2	KASP TD65-57	T	C
	rs2306283	p.Asn130Asp	C/T	1.8mM MgCl2	2Step57	T	C
SLCO1A2	rs10841795	p.Ile13Thr	A/G	1.8mM MgCl2	KASP TD61-55	A	G
SLCO1B3	rs4149117	p.Ser112Ala	T/G	1.8mM MgCl2	KASP TD61-55	G	T
SLCO2B1	rs12422149	p.Arg312Leu	A/G	1.8mM MgCl2	KASP TD61-55	G	A
ABCB10	rs1053513	c.*1339A>G	T/C	40x assay	TaqMan	T	C

Table 5.3.3 a (ii): KASP thermal cycling conditions for all protocols used.

KASP TD61-55			
Step in the protocol	**Temperature**	**Duration**	**Repeat**
Step 1 Denaturation	**94 °C**	15 min	x 1
Step 2 Annealing	**94 °C**	20 s	x 10
	61 °C With each cycle, the temperature decreased by 0.6 °C to reach a final temperature of 55 °C	60 s	
Step 3 Amplification	**94 °C**	20 s	x 26
	55 °C	60 s	
KASP TD68-62			
Step 1 Denaturation	**94 °C**	15 min	x 1
Step 2 Annealing	**94 °C**	20 s	x 10
	68 °C With each cycle, the temperature decreased by 0.6 °C to reach a final temperature of 62 °C	60 s	
Step 3 Amplification	**94 °C**	20 s	x 26
	62 °C	60 s	
KASP TD65-57			
Step 1 Denaturation	**94 °C**	15 min	x 1
Step 2 Annealing	**94 °C**	20 s	x 10
	65 °C With each cycle, the temperature decreased by 0.6 °C to reach a final temperature of 57 °C	60 s	
Step 3 Amplification	**94 °C**	20 s	x 26
	57 °C	60 s	
KASP 2 step 57°C			
Step 1 Denaturation	**94 °C**	15 min	x 1
Step 2 Amplification	**94 °C**	20 s	x 36
	57 °C	60 s	

5.3.3 (b) Genotyping – classic PCR and electrophoresis

The standard PCR is a popular molecular approach for achieving exponential amplification of a particular DNA segment in vitro. The enzymatic replication of DNA using synthetic oligonucleotide primers flanking the region of interest is the basis for the reaction. Denaturation, annealing, and extension are temperature-dependent procedures that are repeated 25–40 times in a cyclic sequence to perform PCR. In order to separate the double-stranded DNA into single strands, the reaction mixture is heated to roughly 94 to 95 °C during the denaturation step. The next step is the annealing phase, in which the primers are allowed to hybridize to their complementary sequences on the template strands by lowering the temperature (usually between 50 and 65 °C). Taq polymerase then catalyzes the addition of dNTPs to the 3′ ends of the annealed primers during the extension phase, which is at around 72 °C. This creates new DNA strands that are complementary to the template. The target sequence experiences exponential amplification through iterative repetition of these procedures, potentially doubling with each subsequent cycle to provide enough DNA for further analysis.

After amplification, the agarose gel electrophoresis is done to check the presence and specifications of products obtained from PCR. It separates the fragments of nucleic acids based on molecular size. The DNA molecules being a negatively charged

particle move towards anode. The gel enables separation of molecules based on size by acting as a molecular sieve. When the electrophoresis is finished, the gel is stained with a fluorescent dye, usually ethidium bromide to make DNA visible under ultraviolet light. A molecular weight standard (DNA ladder) is used to compare the migration pattern of the PCR products in order to confirm the anticipated amplicon size and assess the amplification reaction's specificity. The PCR conditions for the protocol used are described in Table 5.3.3 (b).

Procedure for performing genotyping with classic PCR and gel electrophoresis:

The samples were diluted to final working concentration of 15 ng/µL. The reaction mixture was prepared for all three alleles (G, T, A) by adding 10X buffer (1 µL), $MgCl_2$ (0.48 µL), dNTP (0.8 µL), G, T or A (0.4 µL), reverse primer (0.4 µL), forward primer (0.15 µL), dH_2O (2.7 µL) and Taq polymerase (0.067 µL) per reaction. The reaction mixture (6 µL) and the DNA (4 µL) were then loaded on 96 well plate. After mixing both well the wells were closed with cap strips. The plate was then centrifuged for 1-2 minutes at 2500 rpm. The PCR was then performed under conditions described in Table 4.3.3 b on PCR Veriti Thermal Cycler. At the end of PCR, the caps were removed gently and 6X Loading dye (2.5 µL) was added to each product.

The agarose gel (2.5%) was prepared and then the end product of PCR mixed with dye was loaded on the gel. 10 µL was loaded in each well in triplicates, for standard the molecular weight ladder of 100 bp was used. The electrophoresis was then ran at 90-100 V for 20-25 min. At the end the results were checked by exposing the gel to UV light.

Table 5.3.3 b: Classic PCR conditions for the protocol used.

Classic PCR			
Step in protocol	**Temperature**	**Duration**	**Repeat**
Initial denaturation	95	10 min	1x
Denaturation	94	40	8x
Annealing	57	40	
Extension	72	60	
Denaturation	94	30	25x
Annealing	53	30	
Extension	72	30	
Final extension	72	10 min	1x

5.3.3 (c) Genotyping – TaqMan

For the genotyping of *ABCB10* rs1053513 quantitative PCR -the 5'-exonuclease assay (TaqMan) was used.

This method of amplifying target DNA segments is based on Taq polymerase's 5'-3' exonuclease activity. Two fluorogenic probes that are specific to each allele allow the detection of the buildup of a particular PCR product during amplification.

Every Taqman Genotyping Assay comprises of a pair of unlabelled primers and two allele-specific TaqMan probes containing a quencher at the 3'-end and a different reporter dye at the 5'-end (fluorophore).

The molecule that fluoresces when exposed to light is called a reporter. A quencher is a chemical that, when present near the reporter, like in a Taqman probe, absorbs fluorescence. Because of this fluorescence resonance energy transfer (FRET) takes place, that is the process by which energy is transferred from the reporter to the quencher when it is illuminated with light. A Taqman probe attaches itself to a particular sequence on the DNA being examined during PCR, and unlabelled primers also attach to the DNA. Until it comes across a Taqman probe next to the SNP region, Taq polymerase starts synthesizing DNA from the 5' end to the 3' end. DNA polymerase concurrently synthesizes a complementary DNA strand and cleaves the Taqman probe due to its 5'-3' exonuclease activity. The reporter and quencher are physically separated upon probe cleavage, which enables the reporter to fluoresce when exposed to light. The fluorescence intensity during PCR rises in proportion to the quantity of DNA products produced because additional dye molecules are released in this manner with each PCR cycle. The TaqMan conditions for the protocol used are described in Table 5.3.3 (c)

Procedure for performing genotyping with the Taqman test:

A reaction mixture for each SNP was prepared, containing Taqman SNP Genotyping Assay (Applied Biosystems, Thermo Fisher Scientific), Taqman Master mix (Applied Biosystems, Thermo Fisher Scientific), sample DNA and distilled water.

The Taqman Master mix contains Taq polymerase, universal ROX marker, dNTPs and buffer with an optimal concentration of magnesium ions.

The Taqman master mix (5 µL), Taqman Genotyping Assay (0.25 µL), distilled water (2.25 µL) were added to each 2.5 µL of DNA sample making the total volume of reaction mixture 10 µL. Then amplification and detection were performed in a 7500 Real Time PCR System.

Table 5.3.3 c: TaqMan conditions for the protocol used.

TaqMan PCR			
Step in the protocol	**Temperature**	**Duration**	**Repeat**
Activation	50 ºC	2 min	
Starting denaturation	95 ºC	10 min	
Denaturation	92 ºC	15 s	x 50
Annealing and amplification	60 ºC	1 min	

5.4 Statistical analysis

The median and interquartile range (25%–75%) were used to characterize continuous variables and frequencies were used for describing categorical variables. If there were more than three samples in the group, the interquartile range was calculated using weighted averages; if there were only three samples, Tukey's hinges were used. The categorical characteristics of patients were compared between groups using Fisher's exact test. The continuous characteristics of patients were compared between groups using the Kruskal-Wallis test with *post hoc* Bonferroni corrections for pairwise comparisons.

The agreement of genotype frequencies with Hardy–Weinberg equilibrium (HWE) was tested using chi-squared test. The subsequent analysis was done using both additive and dominant genetic models. Only the dominant model was employed if fewer than five patients were homozygous for the polymorphic allele.

For comparing genotype frequencies between groups, the Fisher’s exact test was used.

The association between polymorphisms and CSF biomarker levels, MMSE scores, and bilirubin/bilirubin glucuronide/biliverdin concentration or bilirubin glucuronide to bilirubin/biliverdin to bilirubin ratio was assessed using the Mann-Whitney test (dominant model) or the Kruskal-Wallis test with *post hoc* Bonferroni corrections for pairwise comparisons (additive model). ANCOVA was used for the adjustment with *UGT1A1* genotype.
IBM SPSS Statistics version 29.0 (IBM Corporation, Armonk, NY, USA) was used for all statistical analyses. Every test was two-sided, and 0.05 was the set level of significance. The figures were created using GraphPad Prism version 10 (GraphPad Software, LLC., San Diego, CA, USA).

5.5 Lab equipment

The lab equipment used are enlisted below.

Laboratory equipment	Distributer
Centrifuge 5810R	LLG Labware
Microcentrifuge MiniSpin	Eppendorf
Multichannel pipette 96 tip Viaflo384	Integra
Meridian assay dispensor	LGC Genomics/Bioresearch tehnologies
Kube heat sealer	LGC Genomics/Bioresearch tehnologies
UV/VIS spektrometer Lamba Bio	PerkinElmer
PCR Veriti	Applied Biosystems
PCR Proflex	Applied Biosystems
Fluorescence reader FLUOstar Omega	BMG Labtech
qPCR RT 7500	Applied Biosystems
Gel electrophoresis system	
Centrifuge Avanti J-26 XP	Beckman coulter
APV model 1000 homogenizer	SPX Brand
Homogenizer OV5	VELP Scientifica
Thermostat Cabinet	
Shaker 709	Asal
Magnetic stirrer	(Icamag® Rec-G)
Multi-purpose water purification system	(Crystal EX, Androna®)
Centrifuge Avanti J-26 XP	Beckman coulter
APV model 1000 homogenizer	SPX Brand
Centrifuge 5804 R	Eppendorf
WTB-2000 Balance	RadWAG
Analytical Balance ABT 120–4NM (KERN 770)	
Centrifuge Rotor JA-14	Beckman coulter
Centrifuge Rotor 25.5	Eppendorf
Vortex Shaker	IKA
Cary 4E, Varian	
SynergyH1, Biotek platereader	

RESULTS

6.1 Patients' characteristics

The study included 61 patients with cognitive impairment: 29 with Alzheimer's disease (AD) or 32 mild cognitive impairment (MCI). Patients' characteristics are presented in Table 6.1. Based on the criteria we used to stratify the patients into groups, MMSE score, tau, $A\beta_{42}$, $A\beta_{42/40}$ ratio differed significantly between the groups with (all P<0.001) for all. Additionally, sex distribution (P=<0.001) and BMI (P=0.015) also differed between the groups.

Table 6.1: Clinical characteristics of all patients with cognitive impairment (N=61) and of patients with AD (N=29), MCI (AD) (N=17) and MCI (not AD) diagnosis (N=15).

Characteristic	Category/Unit	All patients	Patients with AD	Patients with MCI (AD)	Patients with MCI (not AD)	P
Sex	Male, N (%)	23 (37.7)	8 (27.6)	3 (17.6)	12 (80.0)	**<0.001***
	Female, N (%)	38 (62.3)	21 (72.4)	14 (82.4)	3 (20.0)	
Age at onset	Years, Median (25-75%)	77 (73-80)	79 (74.5-81.5)	76 (73.5-79.5)	74 (69-79)	0.054†
Education	Years, Median (25-75%)	12 (8-14.5)	12 (8-12)	12 (11.5-16)	11 (8-15)	0.058†
BMI	kg/m^2, Median (25-75%)	23.9 (20.7-27.1) [12]	23.4 (19.8-26.5) [3]	22.8 (20.5-24.2) [7]	26.4 (24.7-29.3) [2]	**0.015**† **Pairwise: 2 vs 0: 0.021
MMSE	Score, Median (25-75%)	25 (20-27) [16]	20 (15-23.5) [8]	27 (25-28.5) [4]	26 (24-27) [4]	**<0.001**† **Pairwise: 2 vs 0: 0.004 1 vs 0: <0.001
$A\beta_{42}$	pg/ml, Median (25-75%)	738 (551.5-1013.5)	638 (540.5-765)	647 (497-794)	1463 (1220-1663)	**<0.001**† **Pairwise: 2 vs 0: <0.001 2 vs 1: <0.001

$A\beta_{42/40}$ ratio	Median (25-75%)	0.06 (0.04-0.08) [2]	0.05 (0.04-0.06)	0.06 (0.04-0.07) [1]	0.11 (0.09-0.13) [1]	**<0.001**† **Pairwise: 2 vs 0: <0.001 2 vs 1: <0.001
Total tau	pg/ml, Median (25-75%)	571 (410.5-1007)	894 (573.5-1109)	567 (444-976)	324 (253-404)	**<0.001**† **Pairwise: 2 vs 0: <0.001 2 vs 1: 0.002
p-tau_{181}	pg/ml, Median (25-75%)	86 (64.5-128)	117 (82.5-143)	87 (74.5-135)	56 (44-65)	**<0.001**† **Pairwise: 2 vs 0: <0.001 2 vs 1: 0.001
APOE	E3/E3	26 (42.6)	12 (41.4)	6 (35.3)	8 (53.3)	0.075*
	E2/E3 or E2/E4	7 (11.5)	1 (3.4)	2 (11.8)	4 (26.7)	
	E3/E4	28 (45.9)	16 (55.2)	9 (52.9)	3 (20.0)	

AD: Alzheimer's disease; Aβ: amyloid-β; BMI: body mass index; MCI: mild cognitive impairment; MMSE: The Mini-Mental State Examination; p-tau: phosphorylated tau; *Fisher's exact test; †Kruskal–Wallis test. Number of missing data is presented in [] brackets. For **pairwise comparisons: 0= Patients with AD, 1= Patients with MCI (AD), 2= Patients with MCI (not AD).

6.2 Analysis of fractionated bilirubinaemia in patients with Alzheimer's disease and mild cognitive impairment

Concentration of bilirubin (BR), bilirubin glucuronide (BRG) and biliverdin (BV) in patients with Alzheimer's disease (AD), mild cognitive impairment with AD cerebrospinal fluid biomarker levels (MCI (AD)) and mild cognitive impairment with normal cerebrospinal fluid biomarker levels (MCI (not AD)) is presented in Table 6.2.

Patients with AD or MCI (AD) had slightly higher BR levels compared to patients with MCI (not AD), but the difference was not statistically significant (P=0.079). No differences among groups were observed for BRG and BV concentration.

Table 6.2: Analysis of BR, BV and BRG concentrations in all groups.

Characteristic	Category/Unit	All patients	Patients with AD	Patients with MCI (AD)	Patients with MCI	P^a	P^b	P^c

					(not AD)			
BR concentration	(µM), median (25%-75%)	5.15 (3.92-6.94)	5.98 (4.11-7.35)	5.28 (4.38-7.54)	4.40 (3.25-5.78)	0.213	0.319	0.079
BRG concentration	(µM), median (25%-75%)	0.05 (0.01-0.08)	0.05 (0.00-0.07)	0.05 (0.01-0.11)	0.03 (0.01-0.07)	0.650	0.647	0.667
BV concentration	(µM), median (25%-75%)	0.07 (0.00-0.11)	0.07 (0.00-0.11)	0.03 (0.00-0.14)	0.08 (0.00-0.11)	0.942	0.873	0.866

[a] comparison of all three groups, [b] comparison of AD vs all MCI, [c] comparison of AD and MCI (AD) vs MCI (not AD)

6.3 Analysis of genetic variability of *UGT1A1*

As *UGT1A1* genetic variability is a known predictor of bilirubin levels, we first analysed the genotype frequencies of *UGT1A1* rs8175347 and their association with bilirubin levels in our study group. Genotype frequencies for *UGT1A1* rs8175347 are presented in Table 5.3 (I). Variant allele frequency was 45.1% and the genotype distributions was in agreement with HWE. There were no significant differences in genotype distribution among different patient groups (Table 6.3 (II)), therefore all further analyses were performed on the whole cohort of patients combined.
Association *UGT1A1* rs8175347 with bilirubin, bilirubin glucuronide and biliverdin concentration is presented in Table 6.3 (III). Carriers of at all (-:-, TA:-, TA:TA) polymorphic allele of *UGT1A1* rs8175347 had higher bilirubin concentration (P=0.001). Therefore, for association of other polymorphisms with bilirubin concentration, adjustment for *UGT1A1* rs8175347 genotype was also performed.

Table 6.3 (I): Genotype frequencies.

Gene	SNP	Genotype	All patients N (%)	VAF	pHWE
UGT1A1	rs8175347	-:-	17 (27.9)	45.1	0.470
		TA:-	33 (54.1)		
		TA:TA	11 (18.0)		

HWE: Hardy-Weinberg equilibrium, VAF: variant allele frequency, pHWE: p-value for agreement of genotype frequencies with Hardy–Weinberg equilibrium.

Table 6.3 (II): Comparison of genotype frequencies between groups.

Gene	SNP	Genotype	Patients with AD	Patients with MCI (AD)	Patients with MCI (not AD)	P
UGT1A1	rs8175347	-:-	8 (27.6)	4 (23.5)	5 (33.3)	Padd=0.438
		TA:-	13 (44.8)	11 (64.7)	9 (60.0)	
		TA:TA	8 (27.6)	2 (11.8)	1 (6.7)	
		TA:- + TA:TA	21 (72.4)	13 (76.5)	10 (66.7)	Pdom=0.872

Table 6.3 (III): Association of selected polymorphisms with bilirubin, bilirubin glucuronide and biliverdin concentration.

Gene	SNP	Genotype	BR concentration Median (25%-75%)	P
UGT1A1	rs8175347	-:-	3.8 (3.2-4.7)	**Padd=0.001**
		TA:-	5.3 (4.5-6.6)	Pairwise comparisons: TA:- vs -:- P=0.034 TA:TA vs -:- P=0.001
		TA:TA	7.0 (5.4-10.8)	
		TA:- + TA:TA	5.9 (4.6-7.2)	**Pdom=0.001**
			BRG concentration Median (25%-75%)	
UGT1A1	rs8175347	-:-	0.05 (0.00-0.07)	Padd=0.465
		TA:-	0.04 (0.00-0.08)	
		TA:TA	0.06 (0.00-0.13)	
		TA:- + TA:TA	0.05 (0.01-0.09)	Pdom=0.344
			BV concentration Median (25%-75%)	
UGT1A1	rs8175347	-:-	0.03 (0.00-0.09)	Padd=0.214
		TA:-	0.09 (0.02-0.13)	
		TA:TA	0.07 (0.00-0.10)	
		TA:- + TA:TA	0.08 (0.00-0.13)	Pdom=0.119

Padd: p-value for additive genetic model, Pdom: p-value for dominant genetic model

6.4 Analysis of genetic variability of bilirubin transporters

6.4.1 Genotype frequencies

Genotype frequencies for all investigated polymorphisms are presented in Table 6.4.1 (I). Variant allele frequency ranged from 9 to 59%. All genotype distributions were in agreement with HWE. There were no significant differences in genotype distribution among different patient groups Table 6.4.1 (II), therefore all further analyses were performed on the whole cohort of patients.

Table 6.4.1 (I): Genotype frequencies of selected polymorphisms.

Gene	SNP	Genotype	All patients N (%)	VAF	pHWE
ABCB1	rs1128503	TT	11 (18.03)	59.0	0.690
		TC	28 (45.90)		
		CC	22 (36.06)		
	rs2032582	GG	20 (32.77)	40.5 (T)	/
		GT/GA	25 (40.98)	3.4 (A)	
		TT/TA	13 (21.31)		
	rs1045642	TT	19 (31.15)	47.5	0.259
		TC	26 (42.62)		
		CC	16 (26.23)		
ABCC2	rs2804402	CC	12 (19.67)	53.3	0.499

		CT	33 (54.10)		
		TT	16 (26.23)		
	rs717620	GG	40 (65.57)	19.7	0.604
		GA	18 (29.50)		
		AA	3 (4.92)		
	rs2273697	GG	43 (70.50)	17.2	0.284
		GA	15 (24.59)		
		AA	3 (4.92)		
ABCG2	rs2231137	GG	56 (91.80)	4.1	0.739
		GA	5 (8.20)		
		AA	0 (0)		
	rs2231142	CC	52 (85.25)	8.2	0.315
		CA	8 (13.11)		
		AA	1 (1.64)		
SLCO1B1	rs2306283	TT	20 (32.79)	40.2	0.327
		TC	33 (54.10)		
		CC	8 (13.11)		
	rs4149056	TT	38 (62.30)	18.9	0.070
		TC	23 (37.70)		
		CC	0 (0)		
	rs11045879	TT	37 (60.65)	19.7	0.056
		TC	24 (39.34)		
		CC	0 (0)		
SLCO1A2	rs10841795	AA	42 (68.85)	15.6	0.150
		AG	19 (31.15)		
		GG	0 (0.0)		
SLCO1B3	rs4149117	GG	45 (73.77)	13.9	0.844
		GT	15 (24.59)		
		TT	1 (1.64)		
SLCO2B1	rs12422149	GG	51 (83.61)	9.0	0.431
		GA	9 (14.75)		
		AA	1 (1.64)		
ABCB10	rs1053513	TT	33 (62.3) [8]	22.6	0.314
		TC	16 (30.2)		
		CC	4 (7.5)		

HWE: Hardy-Weinberg equilibrium, VAF: variant allele frequency

Table 6.4.1 (II): Comparison of genotype frequencies between patient groups.

Gene	SNP	Genotype	Patients with AD	Patients with MCI (AD)	Patients with MCI (not AD)	P
ABCB1	rs1128503	TT	7 (24.1)	2 (11.8)	2 (13.3)	Padd=0.493
		TC	14 (48.3)	6 (35.3)	8 (53.3)	
		CC	8 (27.6)	9 (52.9)	5 (33.3)	
		TC+CC	22 (75.9)	15 (88.2)	13 (86.7)	Pdom=0.628
	rs2032582	GG	7 (25.9)	8 (47.1)	5 (35.7)	Padd=0.563
		GT+GA	14 (48.1)	7 (41.2)	5 (35.7)	
		TT+TA	7 (25.9)	2 (11.8)	4 (28.6)	
		GT+GA+TT+TA	20 (74.1)	9 (52.9)	9 (64.3)	Pdom=0.379
	rs1045642	TT	9 (31.0)	4 (23.5)	6 (40.0)	Padd=0.840
		TC	13 (44.8)	7 (41.2)	6 (40.0)	
		CC	7 (24.1)	6 (35.3)	3 (20.0)	
		TC+CC	20 (69.0)	13 (76.5)	9 (60.0)	Pdom=0.677
ABCC2	rs2804402	CC	5 (17.2)	5 (29.4)	2 (13.3)	Padd=0.430
		CT	16 (55.2)	10 (58.8)	7 (46.7)	

		TT	8 (27.6)	2 (11.8)	6 (40.0)	
		CT+TT	24 (82.8)	12 (70.6)	13 (86.7)	Pdom=0.540
	rs717620	GG	21 (72.4)	9 (52.9)	10 (66.7)	Pdom=0.422
		GA+AA	8 (52.9)	8 (47.1)	5 (33.3)	
	rs2273697	GG	18 (62.1)	14 (82.4)	11 (73.3)	Pdom=0.363
		GA+AA	11 (82.4)	3 (17.6)	4 (26.7)	
ABCG2	rs2231137	GG	26 (89.7)	15 (88.2)	15 (100.0)	Padd=0.582
		GA	3 (10.3)	2 (11.8)	0 (0.0)	
	rs2231142	CC	24 (82.8)	14 (82.4)	14 (93.3)	Pdom=0.720
		CA+AA	5 (17.2)	3 (17.6)	1 (6.7)	
SLCO1B1	rs2306283	TT	10 (34.5)	4 (23.5)	6 (40.0)	Padd=0.251
		TC	16(55.2)	12 (70.6)	5 (33.3)	
		CC	3 (10.3)	1 (5.9)	4 (26.7)	
		TC+CC	19 (65.5)	13 (76.5)	9 (60.0)	Pdom=0.565
	rs4149056	TT	18 (62.1)	8 (47.1)	12 (80.0)	Padd=0.170
		TC	11 (37.9)	9 (52.9)	3 (20.0)	
	rs11045879	TT	18 (62.1)	7 (41.2)	12 (80.0)	Padd=0.089
		TC	11 (37.9)	10 (58.8)	3 (20.0)	
SLCO1A2	rs10841795	AA	19 (65.5)	12 (70.6)	11 (73.3)	Padd=0.937
		AG	10 (34.5)	5 (29.4)	4 (26.7)	
SLCO1B3	rs4149117	GG	22 (75.9)	11 (64.7)	12 (80.0)	Pdom=0.609
		GT+TT	7 (24.1)	6 (35.3)	3 (20.0)	
SLCO2B1	rs12422149	GG	24 (82.8)	14 (82.4)	13 (86.7)	Pdom=1.000
		GA+AA	5 (17.2)	3 (17.6)	2 (13.3)	
ABCB10	rs1053513	TT	19 (76.0)	6 (40.0)	8 (61.5)	Pdom=0.075
		TC+CC	8 (24.0)	9 (60.0)	5 (38.5)	

6.4.2 Association of selected polymorphisms with bile pigments

First, we evaluated the association of bilirubin transporter polymorphisms with plasma bilirubin concentration and the concentration of its metabolites.

The association of investigated polymorphisms with plasma bilirubin concentration is presented in Table 6.4.2 (I). Carriers of at least one polymorphic *ABCB1* rs1128503 C allele had lower bilirubin concentration compared to carriers of two normal alleles (Pdom=0.031) (Figure 6.4.2 (I)). None of the other transporter polymorphisms was associated with bilirubin concentration.

Table 6.4.2 (I): Association of selected polymorphisms with bilirubin concentration and adjustment for *UGT1A1* rs8175347 genotype.

Gene	SNP	Genotype	BR concentration Median (25%-75%)	P	Padd
ABCB1	rs1128503	TT	6.2 (5.3-7.4)	Padd=0.097	Padd=0.310
		TC	4.8 (3.9-6.8)		
		CC	4.9 (3.6-6.9)		
		TC+CC	4.8 (3.8-6.8)	**Pdom=0.031**	Pdom=0.124
	rs2032582	GG	4.6 (3.6-6.7)	Padd=0.082	Padd=0.438
		GT+GA	4.7 (3.5-7)		
		TT+TA	6 (5.2-7.4)		
		GT+GA+TT+TA	5.2 (4.3-7.3)	Pdom=0.303	Pdom=0.437
	rs1045642	TT	5.8 (4.8-7.4)	Padd=0.165	Padd=0.362
		TC	4.9 (3.3-6.9)		
		CC	4.3 (3.7-6.7)		
		TC+CC	4.8 (3.6-6.8)	Pdom=0.073	Pdom=0.192

ABCC2	rs2804402	CC	4.8 (4-6.3)	Padd=0.726	Padd=0.730
		CT	5.3 (4-6.9)		
		TT	5.7 (3.6-7.4)		
		CT+TT	5.3 (3.8-7.1)	Pdom=0.586	Pdom=0.803
	rs717620	GG	5.3 (3.8-7.1)	Pdom=0.485	Pdom=0.330
		GA+AA	4.8 (4-6.4)		
	rs2273697	GG	4.8 (3.8-7.2)	Pdom=0.548	Pdom=0.546
		GA+AA	5.9 (4.3-6.9)		
ABCG2	rs2231137	GG	5 (3.8-7.1)	Padd=0.750	Padd=0.710
		GA	5.3 (4.6-6.7)		
	rs2231142	CC	5 (3.8-6.9)	Pdom=0.555	Pdom=0.760
		CA+AA	5.4 (4.5-8)		
SLCO1B1	rs2306283	TT	4.8 (3.8-6.7)	Padd=0.917	Padd=0.993
		TC	5.1 (4-7.1)		
		CC	5.5 (3.3-7.3)		
		TC+CC	5.3 (4-7.1)	Pdom=0.678	Pdom=0.990
	rs4149056	TT	4.9 (3.7-6.9)	Padd=0.413	Padd=0.315
		TC	5.4 (4.2-7.3)		
	rs11045879	TT	4.9 (3.7-6.8)	Padd=0.210	Padd=0.077
		TC	5.6 (4.3-7.4)		
SLCO1A2	rs10841795	AA	4.8 (3.8-6.9)	Padd=0.166	Padd=0.458
		AG	5.7 (4.7-7.4)		
SLCO1B3	rs4149117	GG	4.9 (4.0-6.9)	Pdom=1.000	Pdom=0.485
		GT+TT	5.5 (3.6-7.4)		
SLCO2B1	rs12422149	GG	5.3 (3.9-7.0)	Pdom=0.682	Pdom=0.846
		GA+AA	4.7 (3.8-7.5)		
ABCB10	rs1053513	TT	5.4 (3.8-7.2)	Pdom=0.727	Pdom=0.961
		TC+CC	5.6 (4.2-6.9)		

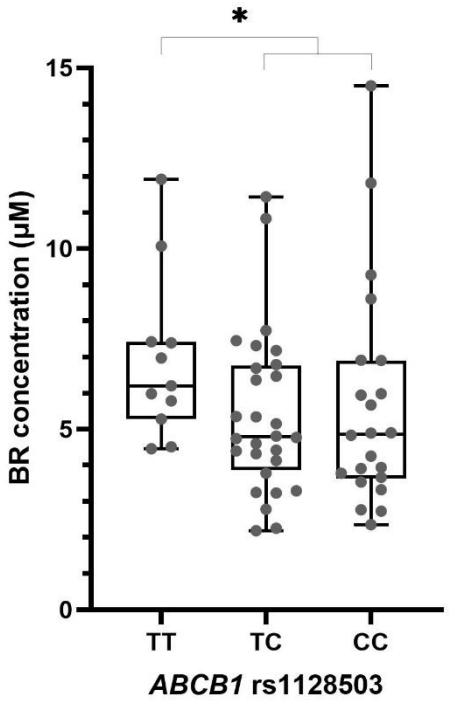


Figure 6.4.2 (I): Bilirubin (BR) concentrations (µM) across three genotypes of the *ABCB1* rs1128503 polymorphism (TT(N=11), TC(N=28), and CC(N=22)).

After adjustment for *UGT1A1* rs8175347 genotype, there were no significant associations between investigated polymorphisms and bilirubin concentration.

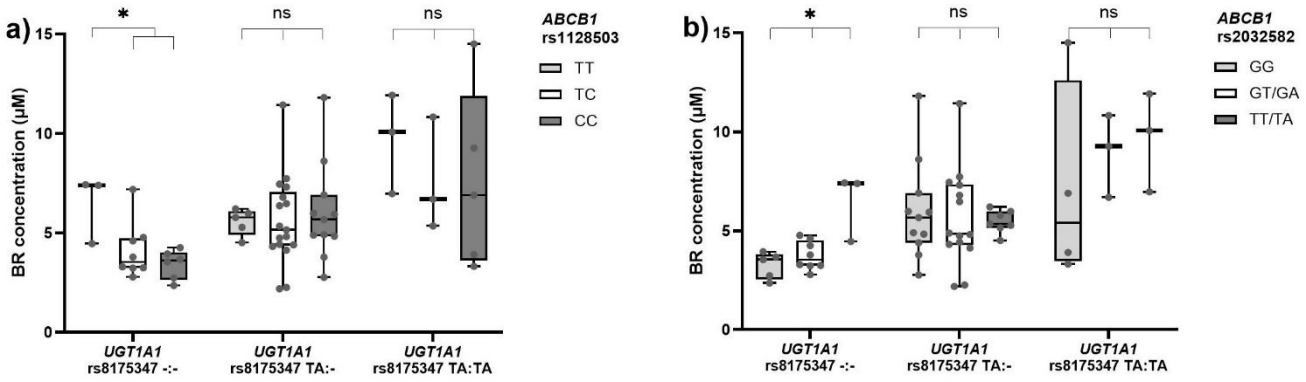
Then, we stratified the patients according to *UGT1A1* rs8175347 genotype (Table 6.4.2 (II)) and it showed that the *ABCB1* polymorphisms were associated with bilirubin concentration only in carriers of two *UGT1A1* rs8175347 reference alleles. Carriers of at least one polymorphic *ABCB1* rs1128503 C allele had lower bilirubin concentration compared to carriers of two normal alleles (Pdom=0.021). Carriers of two polymorphic *ABCB1* rs2032582 alleles (genotype TT or TA) had higher bilirubin concentration compared to carriers of two normal alleles or one polymorphic allele (Padd=0.041) (Figure 6.4.2 II).

Table 6.4.2 (II): Association of selected polymorphisms with bilirubin concentration – separate analysis based on *UGT1A1* rs8175347 genotype.

***ABCB1* SNP**	**Genotype**	***UGT1A1* rs8175347 -:-**		***UGT1A1* rs8175347 TA:-**		***UGT1A1* rs8175347 TA:TA**	
		BR concentration Median (25%-75%)	**P**	**BR concentration Median (25%-75%)**	**P**	**BR concentration Median (25%-75%)**	**P**
rs1128503	TT	7.4 (5.9-7.4) *	Padd=0.056	5.8 (4.9-6.1)	Padd=0.896	10.1 (8.5-11) *	
	TC	3.5 (3.2-4.7)		5.1 (4.4-7)			

	CC	3.6 (2.6-4)		5.7 (4.8-6.9)			
	TC+CC	3.6 (3.1-4.3)	**Pdom=0.021**	5.2 (4.4-6.9)	Pdom=0.865	6.8 (4.3-10.4)	Pdom=0.279
rs2032582	GG	3.5 (2.5-3.8)	**Padd=0.041**	5.7 (4.4-6.9)	Padd=0.915	5.4 (3.5-12.6)	
	GT+GA	3.5 (3.2-4.5)		4.9 (4.3-7.3)			
	TT+TA	7.4 (5.9-7.4) *		5.3 (5.1-6)			
	GT+GA+TT+TA	4.2 (3.3-4.8)	Pdom=0.115	5.3 (4.5-6.6)	Pdom=0.845	9.7 (6.9-11.1)	Pdom=0.352
rs1045642	TT	4.8 (3.6-7.4)	Padd=0.200	5.3 (4.7-6)	Padd=0.861	8.5 (6.9-11.5)	
	TC	3.3 (3.2-4.6)		4.9 (4.3-6.6)			
	CC	3.7 (2.9-4.1)		5.8 (4.2-7.3)			
	TC+CC	3.6 (3.2-4.2)	Pdom=0.082	6.7 (3.9-10.8)	Pdom=0.923	6.7 (3.9-10.8)	Pdom=0.0412

*calculated using Tukey's hinges



(a) (b)

Figure 6.4.2 (II): Bilirubin (BR) concentrations (µM) stratified by *UGT1A1* rs8175347 genotypes (-/-, TA:-, TA:TA) and *ABCB1* rs1128503 (a) and rs2032582 (b) .

Secondly, the association of bilirubin transporter polymorphisms with plasma biliverdin and bilirubin glucuronide was evaluated.

The association of investigated polymorphisms with plasma biliverdin and bilirubin glucuronide concentration is presented in Table 6.4.2 (III) & (IV) respectively.

SLCO1A2 rs10841795 was associated with biliverdin concentration: carriers of one polymorphic allele had higher biliverdin concentration compared to carriers of two normal alleles (P=0.038) (Figure 6.4.2 (III)).

Carriers of at least one polymorphic *ABCB1* rs2032582 T or A allele had lower bilirubin glucuronide concentration compared to carriers of two normal alleles (Pdom=0.009) (Figure 6.4.2 (IV)).

Table 6.4.2 (III): Association of selected polymorphisms with biliverdin concentration.

Gene	SNP	Genotype	BV concentration Median (25%-75%)	P
ABCB1	rs1128503	TT	0.09 (0.04-0.1)	Padd=0.754

		TC	0.04 (0-0.13)	
		CC	0.07 (0-0.12)	
		TC+CC	0.06 (0-0.12)	Pdom=0.656
	rs2032582	GG	0.08 (0-0.13)	Padd=0.883
		GT/GA	0.05 (0-0.13)	
		TT/TA	0.07 (0.02-0.1)	
		GT+GA+TT+TA	0.07 (0-0.11)	Pdom=0.621
	rs1045642	TT	0.09 (0-0.14)	Padd=0.654
		TC	0.03 (0-0.11)	
		CC	0.07 (0-0.11)	
		TC+CC	0.06 (0-0.11)	Pdom=0.358
ABCC2	rs2804402	CC	0.1 (0.02-0.14)	Padd=0.460
		CT	0.07 (0-0.12)	
		TT	0.06 (0-0.11)	
		CT+TT	0.06 (0-0.11)	Pdom=0.217
	rs717620	GG	0.04 (0-0.11)	Pdom=0.226
		GA+AA	0.09 (0.01-0.13)	
	rs2273697	GG	0.07 (0-0.12)	Pdom=0.714
		GA+AA	0.07 (0-0.11)	
ABCG2	rs2231137	GG	0.07 (0-0.11)	Padd=0.514
		GA	0.14 (0-0.16)	
	rs2231142	CC	0.07 (0-0.11)	Pdom=0.608
		CA+AA	0.03 (0-0.11)	
SLCO1B1	rs2306283	TT	0.03 (0-0.1)	Padd=0.253
		TC	0.07 (0.01-0.13)	
		CC	0.1 (0.01-0.11)	
		TC+CC	0.08 (0.01-0.13)	Pdom=0.110
	rs4149056	TT	0.1 (0-0.12)	Padd=0.135
		TC	0.03 (0-0.09)	
	rs11045879	TT	0.09 (0-0.12)	Padd=0.300
		TC	0.03 (0-0.09)	
SLCO1A2	rs10841795	AA	0.03 (0-0.11)	**Padd=0.038**
		AG	0.1 (0.06-0.12)	
SLCO1B3	rs4149117	GG	0.07 (0-0.11)	Pdom=0.817
		GT+TT	0.08 (0-0.13)	
SLCO2B1	rs12422149	GG	0.07 (0-0.11)	Pdom=0.275
		GA+AA	0.1 (0-0.25)	
ABCB10	rs1053513	TT	0.07 (0.01-0.11)	Pdom=0.803
		TC+CC	0.09 (0-0.14)	

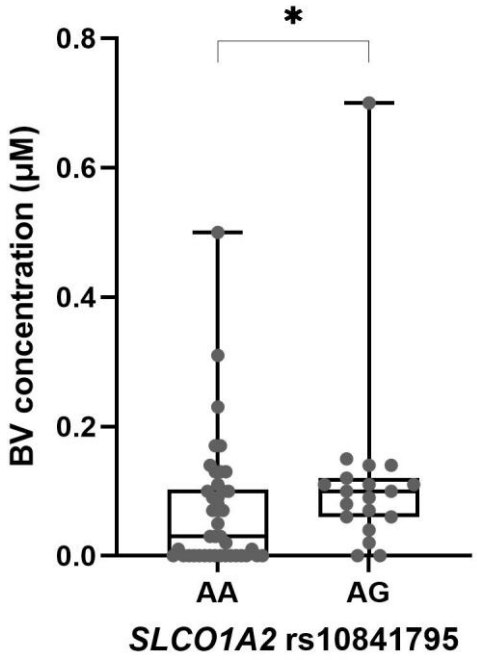


Figure 6.4.2 (III): BV concentrations (µM) in subjects with *SLCO1A2* rs10841795 genotypes AA (N=42) and AG (N=19).

Table 6.4.2 (IV): Association of selected polymorphisms with bilirubin glucuronide concentration.

Gene	SNP	Genotype	BRG concentration Median (25%-75%)	P
ABCB1	rs1128503	TT	0.05 (0-0.1)	Padd=0.174
		TC	0.02 (0-0.07)	
		CC	0.06 (0.02-0.1)	
		TC+CC	0.04 (0.01-0.07)	Pdom=0.970
	rs2032582	GG	0.06 (0.04-0.1)	**Padd=0.032**
		GT+GA	0.02 (0.01-0.05)	Pairwise comparisons GT+GA vs GG Padj=0.032
		TT+TA	0.04 (0-0.09)	
		GT+GA+TT+TA	0.02 (0-0.06)	**Pdom=0.009**
	rs1045642	TT	0.04 (0-0.1)	Padd=0.844
		TC	0.05 (0.02-0.07)	
		CC	0.04 (0-0.07)	
		TC+CC	0.05 (0.01-0.07)	Pdom=0.701
ABCC2	rs2804402	CC	0.07 (0.04-0.09)	Padd=0.207
		CT	0.04 (0-0.08)	
		TT	0.05 (0-0.07)	
		CT+TT	0.04 (0-0.08)	Pdom=0.077
	rs717620	GG	0.05 (0-0.07)	Pdom=0.658
		GA+AA	0.05 (0.01-0.09)	
	rs2273697	GG	0.04 (0.01-0.08)	Pdom=0.830
		GA+AA	0.05 (0-0.08)	

ABCG2	rs2231137	GG	0.05 (0.01-0.07)	Padd=0.618
		GA	0.01 (0-0.09)	
	rs2231142	CC	0.05 (0.01-0.07)	Pdom=0.846
		CA+AA	0.04 (0-0.09)	
SLCO1B1	rs2306283	TT	0.04 (0-0.07)	Padd=0.360
		TC	0.05 (0.01-0.08)	
		CC	0.08 (0.01-0.19)	
		TC+CC	0.05 (0.01-0.09)	Pdom=0.304
	rs4149056	TT	0.05 (0.01-0.08)	Padd=0.070
		TC	0.02 (0-0.06)	
	rs11045879	TT	0.05 (0.01-0.08)	Padd=0.176
		TC	0.03 (0-0.07)	
SLCO1A2	rs10841795	AA	0.04 (0-0.07)	Padd=0.131
		AG	0.05 (0.01-0.1)	
SLCO1B3	rs4149117	GG	0.05 (0-0.08)	Pdom=0.748
		GT+TT	0.04 (0.01-0.07)	
SLCO2B1	rs12422149	GG	0.04 (0.01-0.07)	Pdom=0.322
		GA+AA	0.06 (0-0.22)	
ABCB10	rs1053513	TT	0.05 (0.01-0.08)	Pdom=0.824
		TC+CC	0.05 (0-0.10)	

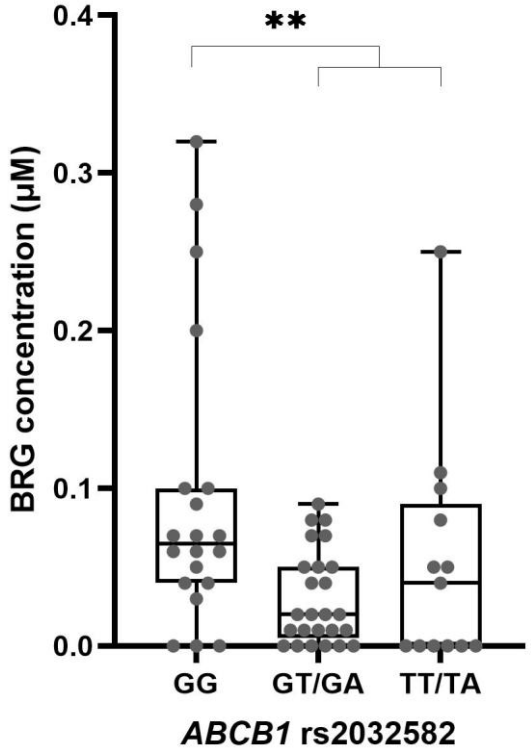


Figure 6.4.2 (IV): BRG concentrations (µM) in subjects with *ABCB1* rs2032582 genotypes GG (N=20), GT/GA (N=25) and TT/TA (N=13).

The association of bilirubin transporter polymorphisms with bilirubin to bilirubin glucuronide ratio and bilirubin to biliverdin ratio were also evaluated This was evaluated as the indicator of genetically determined enzyme activity in the metabolism of bilirubin, enabling to interpret the effects of polymorphisms. (Table 5.4.2 (V)).

ABCB1 rs1128503 polymorphism was associated with BRG to BR ratio: carriers of two polymorphic alleles had higher BRG to BR ratio (Padd=0.046). *ABCB1* rs2032582 polymorphism was associated with BRG to BR ratio: carriers of at least one polymorphic allele had lower BRG to BR ratio (Pdom=0.001). *SLCO1B1* rs4149056 polymorphism was associated with BRG to BR ratio: carriers of one polymorphic C allele had higher BRG to BR ratio (Padd=0.048) Figure 6.4.2 (V).
No association was found between selected polymorphisms and biliverdin to bilirubin ratio.

Table 6.4.2 (V): Association of selected polymorphisms with bilirubin glucuronide to bilirubin ratio (BRG/BR*100%) and biliverdin to bilirubin ratio (BV/BR*100%).

Gene	SNP	Genotype	BRG/BR Median (25%-75%)	P	BV/BR Median (25%-75%)	P
ABCB1	rs1128503	TT	0.64 (0-1.68)	**Padd=0.046**	1.03 (0.48-1.64)	Padd=0.631
		TC	0.44 (0.06-1.24)		1.06 (0-1.89)	
		CC	1.7 (0.39-2.16)		1.51 (0-3.23)	
		TC+CC	0.95 (0.19-1.77)	Pdom=0.462	1.08 (0-2.24)	Pdom=0.880
	rs2032582	GG	1.77 (0.9-2.21)	**Padd=0.006**	1.51 (0.04-3.81)	Padd=0.588
		GT/GA	0.38 (0.09-1.03)		1.08 (0-2.34)	
		TT/TA	0.44 (0-1.3)		1.03 (0.24-1.6)	
		GT+GA+TT+TA	0.41 (0-1)	**Pdom=0.001**	1.06 (0-1.82)	Pdom=0.322
	rs1045642	TT	0.44 (0-1.68)	Padd=0.523	1.09 (0.15-1.99)	Padd=0.917
		TC	0.93 (0.32-1.64)		0.95 (0-2.24)	
		CC	0.99 (0.03-1.84)		1.1 (0-1.89)	
		TC+CC	0.97 (0.27-1.77)	Pdom=0.256	1.06 (0-1.97)	Pdom=0.730
ABCC2	rs2804402	CC	1.3 (0.56-1.81)	Padd=0.166	1.7 (0.56-2.92)	Padd=0.269
		CT	0.64 (0.02-1.63)		0.93 (0-1.87)	
		TT	0.77 (0-1.91)		0.96 (0-1.87)	
		CT+TT	0.66 (0-1.72)	Pdom=0.063	0.93 (0-1.87)	Pdom=0.107
	rs717620	GG	0.77 (0.05-1.65)	Pdom=0.636	0.88 (0-1.9)	Pdom=0.150
		GA+AA	0.93 (0.09-1.82)		1.64 (0.27-2.21)	
	rs2273697	GG	0.88 (0.21-1.77)	Pdom=0.905	1.09 (0-2.15)	Pdom=0.744
		GA+AA	0.88 (0-1.73)		0.96 (0-1.93)	
ABCG2	rs2231137	GG	0.9 (0.17-1.75)	Padd=0.465	1.06 (0.02-1.89)	Padd=0.548
		GA	0.21 (0-1.87)		3.09 (0-3.16)	
	rs2231142	CC	0.88 (0.16-1.77)	Pdom=0.767	1.12 (0-2.11)	Pdom=0.552
		CA+AA	0.88 (0-1.65)		0.6 (0.03-1.87)	
SLCO1B1	rs2306283	TT	0.77 (0-1.51)	Padd=0.246	0.88 (0-1.62)	Padd=0.255

		TC	0.88 (0.17-1.63)		1.15 (0.28-2.54)	
		CC	1.87 (0.31-2.84)		1.55 (0.23-2.11)	
		TC+CC	0.97 (0.23-1.79)	Pdom=0.366	1.45 (0.28-2.34)	Pdom=0.105
	rs4149056	TT	0.99 (0.35-1.81)	**Padd=0.048**	1.35 (0.13-2.62)	Padd=0.127
		TC	0.38 (0-1.5)		0.6 (0-1.75)	
	rs11045879	TT	0.97 (0.33-1.79)	Padd=0.105	1.17 (0.09-2.34)	Padd=0.267
		TC	0.41 (0-1.6)		0.72 (0-1.81)	
SLCO1A2	rs10841795	AA	0.79 (0-1.54)	Padd=0.186	0.88 (0-1.82)	Padd=0.072
		AG	1.62 (0.28-2.23)		1.53 (0.85-2.52)	
SLCO1B3	rs4149117	GG	0.88 (0.02-1.77)	Pdom=0.674	1.08 (0-2.07)	Pdom=0.974
		GT+TT	0.81 (0.17-1.09)		1.1 (0.04-1.83)	
SLCO2B1	rs12422149	GG	0.69 (0.14-1.63)	Pdom=0.144	1.03 (0-1.84)	Pdom=0.219
		GA+AA	1.74 (0-2.52)		1.77 (0-5.04)	
ABCB10	rs1053513	TT	0.97 (0.33-1.65)	Pdom=0.644	1.15 (0.16-1.79)	Pdom=0.796
		TC+CC	0.53 (0-2.03)		0.89 (0.00-2.85)	

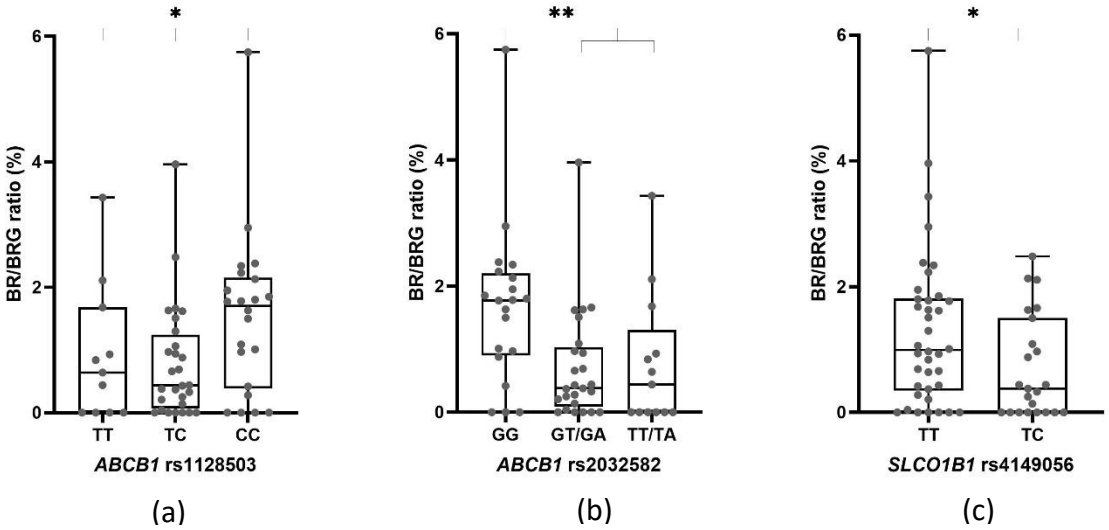


Figure 6.4.2 (V): BR/BRG ratio in subjects with *ABCB1* rs1128503 genotypes TT (N=11), TC (N=28) and CC (N=22) (a), rs2032582 genotypes GG (N=20), GT/GA (N=25) and TT/TA (N=13) (b), *SLCO1B1* rs4149056 genotypes TT (N=38) and TC (N=23).

6.4.3 Association of selected polymorphisms with AD biomarkers

In the second part of the study, we evaluated the association of bilirubin transporter polymorphisms with AD biomarkers ($A\beta_{42}$, total tau and p-tau_{181}) concentration in CSF. The association of transporter polymorphisms with MMSE score was also evaluated.

The association of investigated polymorphisms with $A\beta_{42}$ concentration and $A\beta_{42/40}$ ratio is presented in Table 6.4.3 (I). *SLCO1B1* rs2306283 polymorphism was associated with $A\beta_{42}$ concentration. Carriers of two polymorphic *SLCO1B1* rs2306283 C alleles had higher $A\beta_{42}$ concentration (Padd=0.006) (Figure 6.4.3 (I)). On the other hand, no association was found between selected polymorphisms and $A\beta_{42/40}$ ratio.

Table 6.4.3 (I): Association of selected polymorphisms with $A\beta_{42}$ concentration and $A\beta_{42/40}$ ratio.

Gene	SNP	Genotype	$A\beta_{42}$ concentration Median (25%-75%)	P	$A\beta_{42/40}$ ratio Median (25%-75%)	P
ABCB1	rs1128503	TT	770 (575-1072)	Padd=0.338	0.06 (0.05-0.07)	Padd=0.495
		TC	759.5 (587-1071.5)		0.06 (0.04-0.09)	
		CC	613.5 (514-851.8)		0.05 (0.04-0.08)	
		TC+CC	731.5 (544-944.8)	Pdom=0.586	0.06 (0.04-0.08)	Pdom=0.992
	rs2032582	GG	613.5 (514-903.8)	Padd=0.244	0.05 (0.04-0.08)	Padd=0.418
		GT/GA	759 (554-901)		0.06 (0.04-0.08)	
		TT/TA	774 (611-1248.5)		0.06 (0.05-0.1)	
		GT+GA+TT+TA	765 (573.8-1071.3)	Pdom=0.164	0.06 (0.05-0.08)	Pdom=0.228
	rs1045642	TT	774 (556-1277)	Padd=0.219	0.06 (0.04-0.1)	Padd=0.520
		TC	751 (594-926)		0.06 (0.05-0.08)	
		CC	613.5 (518-808)		0.05 (0.04-0.07)	
		TC+CC	710 (546.8-874)	Pdom=0.297	0.06 (0.04-0.08)	Pdom=0.670
ABCC2	rs2804402	CC	759.5 (580.8-911.3)	Padd=0.475	0.06 (0.05-0.06)	Padd=0.686
		CT	689 (526.5-936)		0.06 (0.03-0.08)	
		TT	752.5 (584.8-1421.5)		0.06 (0.05-0.08)	
		CT+TT	725 (545-1091)	Pdom=0.730	0.06 (0.04-0.08)	Pdom=0.844
	rs717620	GG	710 (543.8-1043)	Pdom=0.574	0.06 (0.04-0.08)	Pdom=0.457
		GA+AA	766 (582-1006)		0.06 (0.04-0.08)	
	rs2273697	GG	743 (547-1071)	Pdom=0.492	0.06 (0.04-0.08)	Pdom=0.894
		GA+AA	689 (551.5-904.3)		0.06 (0.05-0.07)	
ABCG2	rs2231137	GG	759.5 (559.5-1071.8)	Padd=0.114	0.06 (0.04-0.08)	Padd=0.482
		GA	647 (469.5-713.5)		0.05 (0.04-0.07)	
	rs2231142	CC	759.5 (579.8-1042.3)	Pdom=0.102	0.06 (0.04-0.08)	Pdom=0.147
		CA+AA	547 (478-870.5)		0.05 (0.03-0.06)	
SLCO1B1	rs2306283	TT	843 (613.3-1183)	**Padd=0.006**	0.06 (0.04-0.08)	Padd=0.259

		TC	638 (518.5-786.5)	Pairwise comparisons CC vs TC Padj=0.017	0.06 (0.04-0.07)	
		CC	1034.5 (739.8-1620.5)		0.08 (0.05-0.1)	
		TC+CC	725 (536.5-841)	Pdom=0.108	0.06 (0.04-0.08)	Pdom=0.718
	rs4149056	TT	763 (566.5-1234.3)	Padd=0.151	0.06 (0.04-0.08)	Padd=0.319
		TC	695 (514-822)		0.05 (0.04-0.07)	
	rs11045879	TT	766 (582-1248.5)	Padd=0.071	0.06 (0.04-0.08)	Padd=0.151
		TC	682 (488.5-810)		0.05 (0.04-0.07)	
SLCO1A2	rs10841795	AA	759.5 (544-1071.3)	Padd=0.674	0.06 (0.04-0.08)	Padd=0.387
		AG	731 (556-799)		0.06 (0.03-0.07)	
SLCO1B3	rs4149117	GG	760 (572.5-1071.5)	Pdom=0.265	0.06 (0.04-0.08)	Pdom=0.480
		GT+TT	667.5 (534-816.5)		0.06 (0.04-0.08)	
SLCO2B1	rs12422149	GG	738 (570-1071)	Pdom=0.386	0.06 (0.05-0.08)	Pdom=0.198
		GA+AA	686.5 (496.5-973.8)		0.05 (0.03-0.07)	
ABCB10	rs1053513	TT	766 (584.5-1013.5)	Pdom=0.474	0.06 (0.04-0.08)	Pdom=0.273
		TC+CC	671 (544-1100.5)		0.06 (0.04-0.08)	

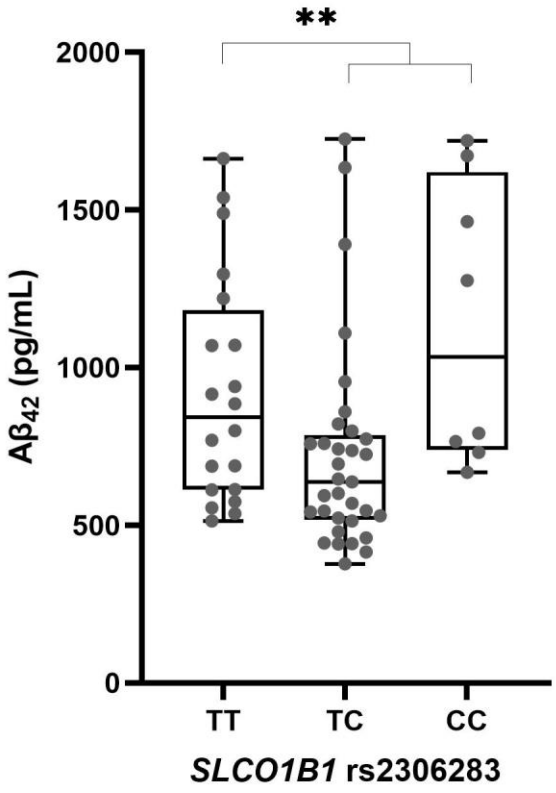


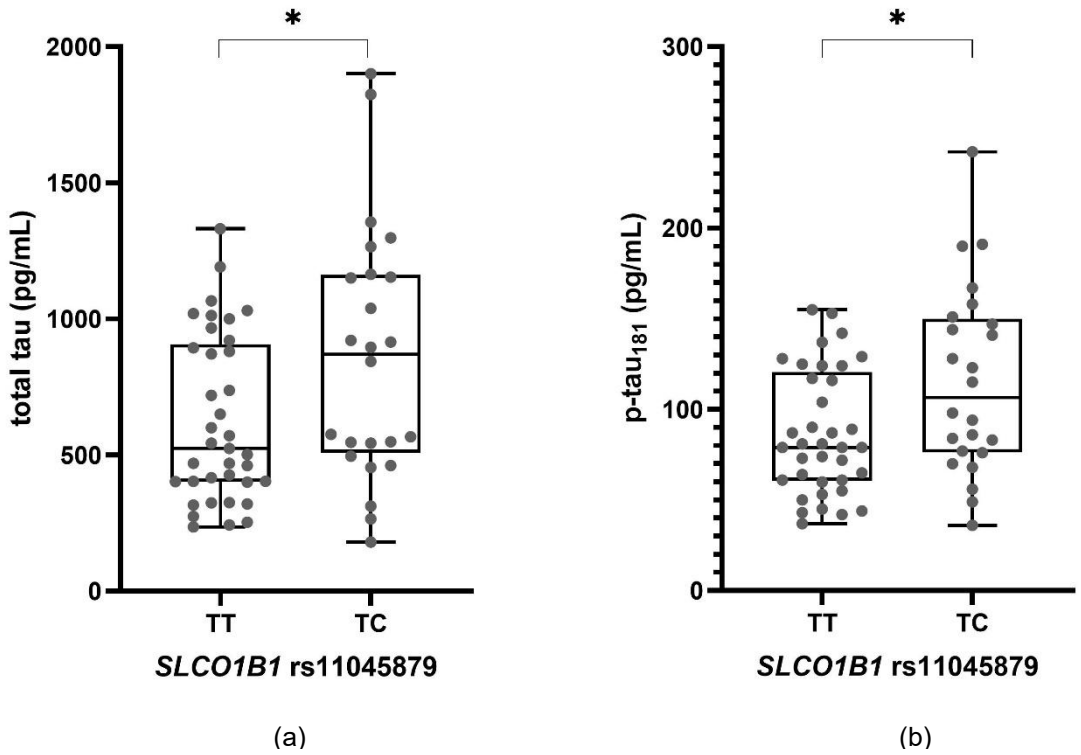
Figure 6.4.3 (I): $A\beta_{42}$ concentrations (pg/mL) in subjects with *SLCO1B1* rs2306283 genotypes TT (N=20), TC (N=33) and CC (N=8).

The association of investigated polymorphisms with total tau concentration and p-tau$_{181}$ is presented in Table 6.4.3 (II). *SLCO1B1* rs11045879 polymorphism was associated with total tau concentration: carriers of one polymorphic C allele had higher total tau concentration (P=0.038) (Figure 6.4.3 (II)). *SLCO1B1* rs11045879 polymorphism was associated with p-tau$_{181}$ concentration: carriers of one polymorphic C allele had higher total tau concentration (P=0.027) (Figure 6.4.3 (II)).

Table 6.4.3 (II): Association of selected polymorphisms with total tau and p-tau$_{181}$ concentration.

Gene	SNP	Genotype	Total tau concentration Median (25%-75%)	P	p-tau$_{181}$ concentration Median (25%-75%)	P
ABCB1	rs1128503	TT	549 (403-1265)	Padd=0.915	84 (61-144)	Padd=0.713
		TC	586 (407.3-919.5)		85 (61-123.8)	
		CC	560 (421.3-1040)		90 (68.8-131)	
		TC+CC	573.5 (413.8-978.5)	Pdom=0.918	86.5 (64.8-125.8)	Pdom=0.778
	rs2032582	GG	728.5 (409.8-1058)	Padd=0.750	104.5 (66.3-135)	Padd=0.725
		GT/GA	601 (470-918)		87 (71-123.5)	
		TT/TA	496 (332.5-1133)		81 (54.5-143)	
		GT+GA+TT+TA	569 (413.5-941)	Pdom=0.629	85 (60.8-124.3)	Pdom=0.447
	rs1045642	TT	549 (400-1013)	Padd=0.788	84 (60-141)	Padd=0.504
		TC	569 (459.3-899.3)		82 (69.5-117.8)	
		CC	741 (344.8-1160.8)		105.5 (63.8-151.5)	
		TC+CC	573.5 (424.5-983)	Pdom=0.821	86.5 (69.5-128)	Pdom=0.803
ABCC2	rs2804402	CC	534 (463.3-630.3)	Padd=0.426	81 (74.5-86.8)	Padd=0.250
		CT	872 (364.5-1109)		117 (62.5-144)	
		TT	523 (403.3-914.8)		78 (62-115.8)	
		CT+TT	719 (403.5-1029.5)	Pdom=0.364	94 (62.5-139)	Pdom=0.235
	rs717620	GG	588.5 (463.3-1010)	Pdom=0.208	90.5 (70.5-127.3)	Pdom=0.327
		GA+AA	470 (314-976)		81 (52-135)	
	rs2273697	GG	576 (417-1013)	Pdom=0.675	87 (68-128)	Pdom=0.462
		GA+AA	546.5 (324.8-1005.8)		80.5 (58.3-123.3)	
ABCG2	rs2231137	GG	558 (407.3-955.5)	Padd=0.335	83.5 (64.3-128.8)	Padd=0.548
		GA	1013 (590-1043.5)		123 (83-124.5)	
	rs2231142	CC	558 (403.3-955.5)	Pdom=0.140	83.5 (61-127.3)	Pdom=0.149

		CA+AA	844 (481.5-1172.5)		104 (75-155.5)	
SLCO1B1	rs2306283	TT	525.5 (344-878.8)	Padd=0.236	82.5 (55.5-124)	Padd=0.469
		TC	719 (457.5-1095)		90 (69-142)	
		CC	507 (334.8-1095.8)		72 (57.3-137)	
		TC+CC	576 (435.5-1095)	Pdom=0.138	87 (64.5-140.5)	Pdom=0.345
	rs4149056	TT	534 (402.3-932.5)	Padd=0.097	80 (60.8-124)	Padd=0.073
		TC	844 (496-1154)		98 (76-147)	
	rs11045879	TT	524 (401.5-907.5)	**Padd=0.038**	79 (60.5-120.5)	**Padd=0.027**
		TC	870 (508-1161.5)		106.5 (76.3-150)	
SLCO1A2	rs10841795	AA	548 (404-932.5)	Padd=0.123	82 (67-125.8)	Padd=0.338
		AG	894 (470-1154)		98 (61-144)	
SLCO1B3	rs4149117	GG	571 (429-984)	Pdom=0.941	84 (64.5-126.5)	Pdom=0.806
		GT+TT	572.5 (347.3-1123)		86.5 (60.3-142.3)	
SLCO2B1	rs12422149	GG	567 (404-1001)	Pdom=0.430	86 (61-128)	Pdom=0.654
		GA+AA	654.5 (533.5-1022.8)		94 (71.8-128.3)	
ABCB10	rs1053513	TT	549 (403.5-1043.5)	Pdom=0.666	86 (62.5-139.5)	Pdom=0.399
		TC+CC	631.5 (407.3-1002)		79 (53-126.8)	



(a) (b)

Figure 6.4.3 (II): total tau and p-tau$_{181}$ concentrations (pg/mL) in subjects with *SLCO1B1* rs11045879 genotypes TT (N=37) and TC (N=24).

The association of investigated polymorphisms with MMSE score is presented in Table 6.4.3 (III).

ABCB1 rs20322582 polymorphism was associated with MMSE score: carriers of at least one polymorphic allele had lower MMSE score (Pdom=0.040). *ABCC2* rs2804402 and rs2273697 polymorphisms were associated with MMSE score: carriers of at least one polymorphic allele had lower MMSE score (Pdom=0.019 and Pdom=0.009, respectively) Figure 6.4.3 (III). No other associations were observed.

Table 6.4.3 (III): Association of selected polymorphisms with MMSE score.

Gene	SNP	Genotype	MMSE score Median (25%-75%)	P
ABCB1	rs1128503	TT	21 (16-26)	Padd=0.141
		TC	24 (19.3-26)	
		CC	26.5 (23-27.8)	
		TC+CC	25 (20.3-27)	Pdom=0.409
	rs2032582	GG	26 (23-27.3)	Padd=0.121
		GT/GA	23.5 (16.3-26.3)	
		TT/TA	22 (17-25)	
		GT+GA+TT+TA	23 (17-26)	**Pdom=0.040**
	rs1045642	TT	22 (17-25)	Padd=0.094
		TC	25 (18-26)	
		CC	27 (21.5-28)	
		TC+CC	25.5 (20.8-27)	Pdom=0.105
ABCC2	rs2804402	CC	27 (26-27.5)	**Padd=0.032**
		CT	24.5 (20-26.3)	Pairwise comparisons TT vs CC Padj=0.026
		TT	21.5 (15-25.3)	
		CT+TT	23.5 (19.3-26)	**Pdom=0.019**
	rs717620	GG	23.5 (19.3-26)	Pdom=0.226
		GA+AA	26 (22.5-28.5)	
	rs2273697	GG	26 (22.5-27)	**Pdom=0.009**
		GA+AA	20.5 (15-25)	
ABCG2	rs2231137	GG	25 (20-27)	Padd=0.220
		GA	23 (16-25)	
	rs2231142	CC	25 (20.8-27)	Pdom=0.071
		CA+AA	20 (13-26)	
SLCO1B1	rs2306283	TT	23.5 (20-27)	Padd=0.944
		TC	25 (20-26)	
		CC	25.5 (15-27.5)	
		TC+CC	25 (19.5-26)	Pdom=0.981
	rs4149056	TT	25 (20.8-27)	Padd=0.328
		TC	24 (17-26)	
	rs11045879	TT	25 (20.8-27)	Padd=0.328

		TC	24 (17-26)	
SLCO1A2	rs10841795	AA	25 (20.8-27)	Padd=0.176
		AG	22 (15-26)	
SLCO1B3	rs4149117	GG	24 (18-26.5)	Pdom=0.198
		GT+TT	25.5 (21.8-27.8)	
SLCO2B1	rs12422149	GG	25 (20.5-27)	Pdom=0.405
		GA+AA	23 (13.5-26.8)	
ABCB10	rs105351 3	TT	23 (17-27)	Pdom=0.305
		TC+CC	25 (23.5-27)	

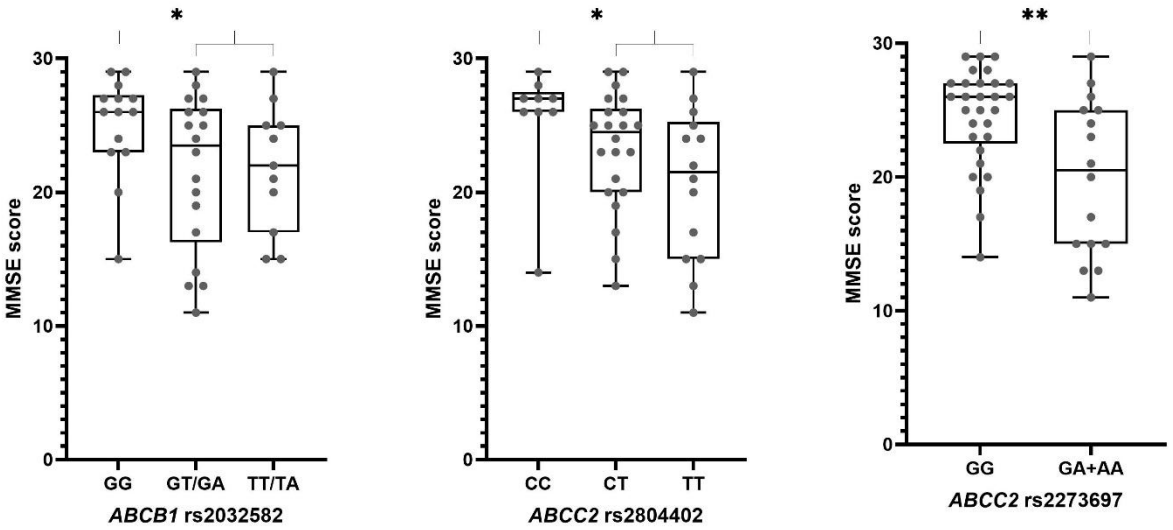


Figure 6.4.3 (III): MMSE score in subjects with *ABCB1* rs2032582 genotypes GG (N=20), GT/GA (N=25) and TT/TA (N=13), *ABCC2 rs2804402* genotypes CC (N=12), CT (N=33) and TT (N=16), *ABCC2 rs717620* genotypes GG (N=40) and GA+AA (N=21) *and rs2273697* genotypes GG (N=43) and GA+AA (N=18).

DISCUSSION

This study examined the hypothesis of genetic polymorphisms of major bilirubin transporters and metabolic enzymes allowing the variation in plasma bilirubin and changes in Alzheimer disease (AD) related biomarkers and cognitive functioning among individuals with AD and mild cognitive impairment (MCI) in this study. The rationale of the research is the increasing evidence that bilirubin, traditionally viewed as a heme degradation product, has strong antioxidant, anti-inflammatory, and cytoprotective effects that can mediate neurodegenerative processes (Sedlak & Snyder, 2004; Stocker et al., 1987; Vitek et al., 2023a; Vitek & Ostrow, 2009, p. 2). In addition to this, changed expression or activity of transporters in the ABC and SLCO/OATP families that take up, conjugate and efflux bilirubin in peripheral tissues and at the blood-brain barrier (BBB) have been associated with diverse neurological and metabolic disorders ((Čvorović & Passamonti, 2017; Ghersi-Egea et al., 2009b). However, their possible contribution to the susceptibility of AD and in the alteration of biochemical indices of AD pathology is still poorly known.

The study showed no statistically significant differences between the concentration of plasma bilirubin, bilirubin glucuronide and biliverdin among the patient groups. Although the levels of BR were moderately higher in the AD/MCI(AD) groups, the difference was not significant. These results were consistent with other studies that reported inconsistent or weak correlations between peripheral bilirubin concentration and AD. An example is the lower or altered levels of bilirubin in AD in some reports (Kim et al., 2025), while other reported no or slight increase (H. Wang et al., 2022). This variability probably represents the complex interactions among the systemic redox status, BBB transport efficiency, peripheral metabolism and local CNS bilirubin production via heme oxygenase pathways (Schipper, 2007; Schipper et al., 2009). Thereby, genetic variation in the transport and metabolism of peripheral bilirubin can be a more informative mechanistically relevant target than just peripheral bilirubin.

The genotype frequency for all polymorphisms included in study were in Hardy-Weinberg equilibrium between all patient groups implying that there was no enrichment of the transporter variants at the population level in AD. The *UGT1A1* polymorphism, rs8175347 behaved as anticipated i.e. showing high levels of BR with carriers of polymorphic (TA) repeats. These results were aligned with extensive studies on the genetics of Gilbert syndrome, where low UGT1A1 promoter activity results in impaired glucuronidation eventually causing elevated levels of unconjugated bilirubin (Bosma, Chowdhury, Bakker, Gantla, de Boer, et al., 1995; Gil & Sąsiadek, 2012b; Gu et al., 2022; Raijmakers et al., 2000). The strong association in our cohort supports the validity of the methods used to perform the analysis and offers the biological reference to explain the effects of the transporter.

Primary analysis revealed that there was a relationship between C allele of *ABCB1* rs1128503 and reduced BR concentration. Although, this effect was found to disappear after the adjustment for the reference *UGT1A1* genotype. However, when stratified analysis was done, transporter effects were only found in individuals with the *UGT1A1* reference genotype. This interaction between the genes indicates that when the conjugation capacity of bilirubin is intact the modulation of systemic bilirubin levels

may be regulated by variability in the transporters, especially at the efflux sites. Pharmacogenomic literature supports such interactions well with evidence that *UGT1A1* genotype is a strong modulator of phenotype effects of variants of ABC transporter (Guillemette, 2003; M. Li et al., 2018). This study extends this concept to endogenous bilirubin metabolism.

Additional associations were observed for bilirubin metabolites. The *SLCO1A2* polymorphism rs10841795 which was associated with increased levels of BV was likely to indicate the alteration in cellular uptake or peripheral distribution. One of the key BBB expressed organic anion transporters is SLCO1A2 (Schäfer et al., 2021) that is not characterized as much as SLCO1B1 and 1B3 in hepatocytes, but its functional polymorphisms can influence the transport of endogenous ligands and drugs (Franke et al., 2009b). Meanwhile, lower levels of BRG were related to *ABCB1* rs2032582, which according to the results, coincides with the changes in the efficiency of the conjugated bilirubin efflux. ABCB1 (P-glycoprotein) is an important transporter of amphipathic xenobiotics and metabolites at the BBB, intestine and liver; thus, its genetic variation may theoretically affect the systemic handling of bilirubin and CNS exposure (W. Li et al., 2024b; J. F. Watchko et al., 1998; Wolking et al., 2015).

Correlations among transporter variants and ratios of BRG/BR were also observed which may reflect changes in metabolic or transport equilibrium. The well-characterized loss of function polymorphism *SLCO1B1* rs4149056, which has been determined to influence hepatic statin and organic anion uptake (Niemi et al., 2011), was linked to an increased BRG/BR ratio, indicating that SLCO1B1 plays a role in the handling of conjugated bilirubin. All these findings indicate that the genetic variation in bilirubin transporters meaningfully contributes to interindividual differences in BR and its metabolite profiles, even in the absence of apparent metabolic disease.

One of the objectives was to identify the relationship between transporter genotypes and AD related CSF biomarkers or cognitive status. We have found significant relations between *SLCO1B1* polymorphism and $A\beta_{42}$, total tau, and p-tau181 levels. Although SLCO1B1 has been described as a hepatic uptake transporter, there is growing evidence of its expression in brain endothelia and it may have a role in the BBB transportation (Bao et al., 2020; Bronger et al., 2005; Geier et al., 2013; *SLCO1B1 Protein Expression Summary - The Human Protein Atlas*, n.d.). Transporter variability may theoretically alter the exposure of the CNS to bilirubin or other endogenous ligands with neuroprotective effects, in result altering the biochemical environment that determines the development of amyloid or tau pathology. The correlation between the transporter polymorphism and neuronal integrity is further supported by the fact that *ABCB1* rs2032582 are associated with lower scores in MMSE. ABCB1 and ABCC2 are crucial BBB efflux transporters that are associated with the clearance of amyloid-β (Cascorbi et al., 2013a; Pahnke et al., 2014; Šerý et al., 2025). A decrease in activity or expression, as is observed with aging and AD, was identified to be linked to a higher amyloid burden and a decline in cognition. Variations in genetics can thus promote or eliminate these processes.

Even though these associations cannot be used to prove causality, they support the hypothesis that genetic variability in transport pathways affects processes associated with AD that are beyond the metabolism of bilirubin. These results correspond with the

reports on the association of the *ABCB1* polymorphisms with the changed risk or progression of AD (Cascorbi et al., 2013b; Fehér et al., 2014; Zhong et al., 2016) and the study showing that transporter deficiencies affect the ability of amyloid to be cleared (Van Assema et al., 2012). This literature is supplemented by our findings that put emphasis on bilirubin as a potential mechanistic intermediate or biomarker-modulating molecule.

Collectively, the findings of this study suggest that though peripheral bilirubin levels fail to significantly discriminate between AD and MCI, genetic diversity of bilirubin transporters has an effect on bilirubin management and correlates with CSF biomarkers of AD and cognition. This supports a model where bilirubin metabolism and transport does not directly reflect a neurodegenerative status but can interplay with molecular mechanisms of interest in AD pathophysiology such as oxidative stress, inflammation and altered BBB transport dynamics.

The strengths of the study are that it included metabolic profiling of bilirubin in detail with a sensitive bilirubin assay, metabolic profile analysis of the different bilirubin fractions and combined the analysis of transporter genotypes with clinical and CSF biomarker data. The limitations are small sample size and cross-sectional design, which may restrict the ability to detect small effects and preclude the ability to make a temporal inference. Based on the dispersion of data on bilirubin concentration, we evaluated the differences in bilirubin concentration between different genotype groups in the dominant model that we could detect with 80% power in our study. For common polymorphisms with MAF above 10%, we could detect differences in bilirubin concentration of approximately 2-2.5 μM (Dupont & Plummer, 1990).

Despite these limitations, the study suggests biologically plausible and clinically relevant associations between transporter and biomarker which should be replicated.

Future studies ought to determine longitudinal changes in cognitive decline relative to bilirubin transporter genotypes, to examine transporter expression and activity at the BBB in AD models as well as to determine whether bilirubin-related pathways are therapeutically adjustable. The understanding of the interaction between bilirubin metabolism and neurodegeneration can be used to lead to the creation of new biomarkers or a personalized risk stratification strategy.

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