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List of abbreviations

APC .....Adenomatous polyposis coli

ATP..... Adenosine triphosphate

CB..... Conjugated bilirubin

CO ..... Carbon monoxide

CRC .....Colorectal cancer

DMSO .....Dimethyl sulfoxide

DM .....Diabetes mellitus

EPIC ..... European Prospective Investigation into Cancer and Nutrition

ER.....Endoplasmic reticulum

GP..... General practitioner

GS.....Gilbert's syndrome

HO-1 ..... Heme Oxygenase 1

HRT .....Hormone replacement therapy

HPLC ..... High-performance liquid chromatography

IARC ..... International Agency for Research on Cancer

LDL ..... Low-density lipoprotein

MRP2.....Multi-drug resistance protein 2

NADH.....Nicotinamide adenine dinucleotide plus hydrogen

NADP.....Nicotinamide adenine dinucleotide phosphate

NADPH .....Nicotinamide adenine dinucleotide phosphate hydrogen

NHANES..... National Health and Nutrition Examination Survey

NSAID..... Nonsteroidal anti-inflammatory drugs

OR ..... Odds ratio

RPM.....Rounds per minute

UCB ..... Unconjugated bilirubin

UDP .....Uridine diphosphate

UGT1A1..... Uridine diphosphate glucuronosyltransferase 1A1

WBCs.....White blood cells

WHO.....World Health Organization

Wnt ..... Wingless-related integration site

WS.....Wistar rats

µL..... Microliter

µmol..... Micromole

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1. INTRODUCTION

Bilirubin, an orange-yellow bile pigment bound to albumin in the blood, is the end product of heme catabolism. Bilirubin is neurotoxic at higher concentrations, which occurs when it exceeds the albumin can no longer binding capacity. Until recently, it was thought to be a toxic waste product of heme breakdown. This changed with the discovery of the biological importance of bile pigments and the potential they hold in health promotion (Wagner et al., 2015).

Experimental studies further confirmed the positive effect of bilirubin by discovering its antioxidant, anti-mutagenic and antiviral properties (Bulmer et al., 2007; Santangelo et al., 2012). As a result, it has led researches to investigate the association between mild hyperbilirubinemia and chronic diseases including cardiovascular diseases and diabetes mellitus type 2. Research on bilirubin and cancer is relatively new and has not been thoroughly explored (Wagner et al., 2015).

The incidence of CRC is on the rise globally. Worldwide, it is the third most common form of cancer and the second leading cause of death occurring as a result of cancer. The numbers are expected to increase by an alarming 60% by the year 2030 (World Health Organization, 2019). Several studies have reported a potential correlation between bilirubin and CRC (Ioannou et al. 2006; Jirásková et al. 2012).

We hypothesized that circulating bilirubin levels are inversely associated with CRC risk. In order to investigate that hypothesis, plasma samples from the European Prospective Investigation into Cancer and Nutrition and the Estonian biobanks were analyzed using high-performance liquid chromatography (HPLC) for their levels of UCB and linked to their CRC status.

2. LITERATURE REVIEW

2.1. Bilirubin Overview

Bilirubin is a yellow-orange colored waste product of the degradation process of heme that has been shown to possess antioxidative properties and can occur in conjugated (CB) and unconjugated (free) forms. UCB is lipophilic, highly soluble in organic solvents and lipids and can, therefore, diffuse readily throughout cell membranes. CB, on the other hand, is hydrophilic and cannot easily cross cell membranes (Bhagavan, 2002).

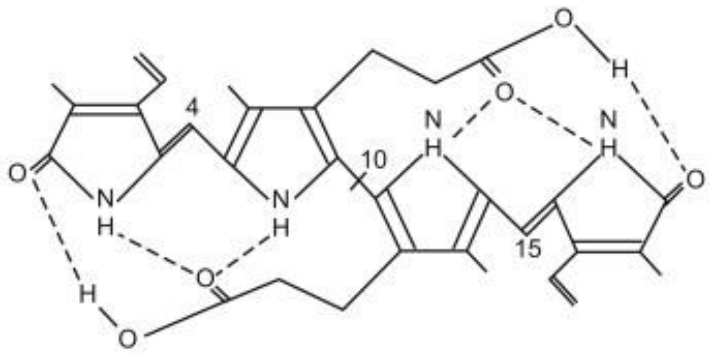

Figure 1: Bilirubin structure (Hydrogen-bonded) (Chowdhury et al, 2013).

The reason UCB is insoluble in water, despite having four amino groups and two propionic acid side chains, is due to the internal stabilization caused by the hydrogen bonding (figure 1) between both of the external pyrrolenone rings (specifically the lactam oxygen and amino groups) and the propionic acid carboxyls. The molecule is constrained into a ridge tile configuration by the aforementioned hydrogen bonds causing the molecule's polar groups to engage, thus causing it to become insoluble (Chowdhury et al, 2013).

UCB consists of four pyrrole rings that are joined together by carbon bridges. Two planar dipyrrole groups are connected to one another at the center by a saturated methylene group giving the UCB molecule a roughly symmetrical configuration. The pair of monopyrroles present in each dipyrrolic part are joined by an unsaturated methene group and are situated in the same plane. Each of the pyrrole rings in the center has a carboxyethyl sidechain, while each

of the external pyrrole rings carries a polar lactam group. Vinyl and ethyl substituents occupy the rest of the sites on the pyrrole rings.

The form of bilirubin species found in humans is the UCB IXα, which is split by a nonenzymatic process called molecular scrambling forming other isoforms such as III and XIII isomers. The reconstructed bilirubin particles are different in their structure than one another as well as the primary compound due to the asymmetry of the UCB IXα molecule (figure 2) (Vitek and Ostrow, 2009).

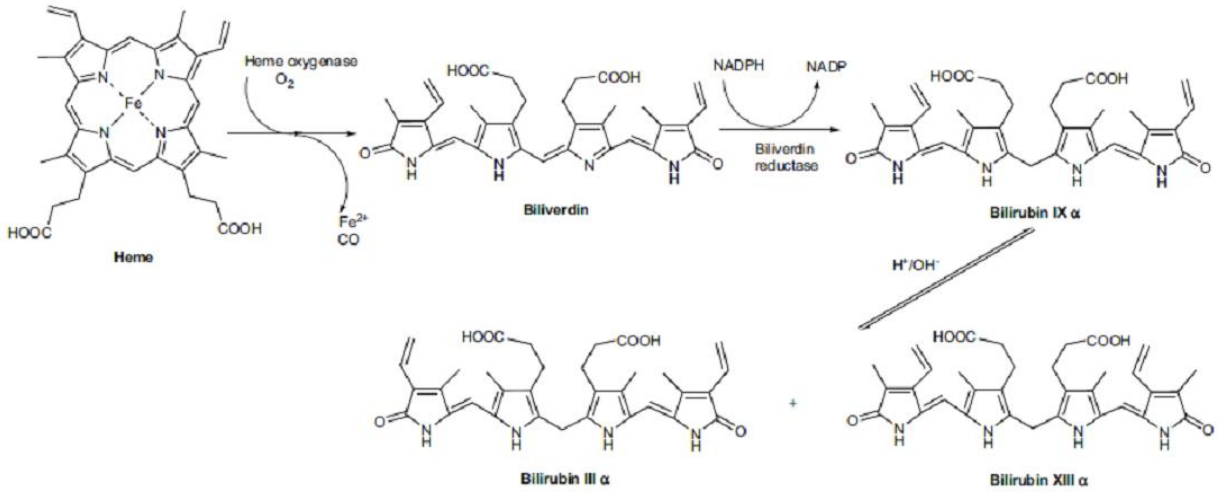

Figure 2: Conversion of Heme to Bilirubin and its Isomers (Vitek and Ostrow, 2009).

2.2. Bilirubin Metabolism

Hemoglobin found in senescent red blood cells is responsible for approximately 80% of the endogenous amount of bilirubin produced in the time frame of 24-hours. The remaining 20% comes from several heme-containing resources such as proteins and enzymes, heme that has not been bound into a protein in the liver (free heme), and red blood cells that remain in the bone-marrow caused by an error in hemopoiesis (Bhagavan, 2002; Chowdhury et al, 2013).

2.2.1 Bilirubin Formation

Heme catabolism is the first step in the formation of bilirubin. Heme, which is also known as ferroprotoporphyrin IX, consists of an iron molecule in its center surrounded by four pyrrole rings which are linked together by methane bridges. A group of enzymes known as heme oxygenase located in the endoplasmic reticulum catalyzes the oxidation of the α-methene bridge carbon causing the ring to open. Oxygen and NADPH must be present for this reaction.

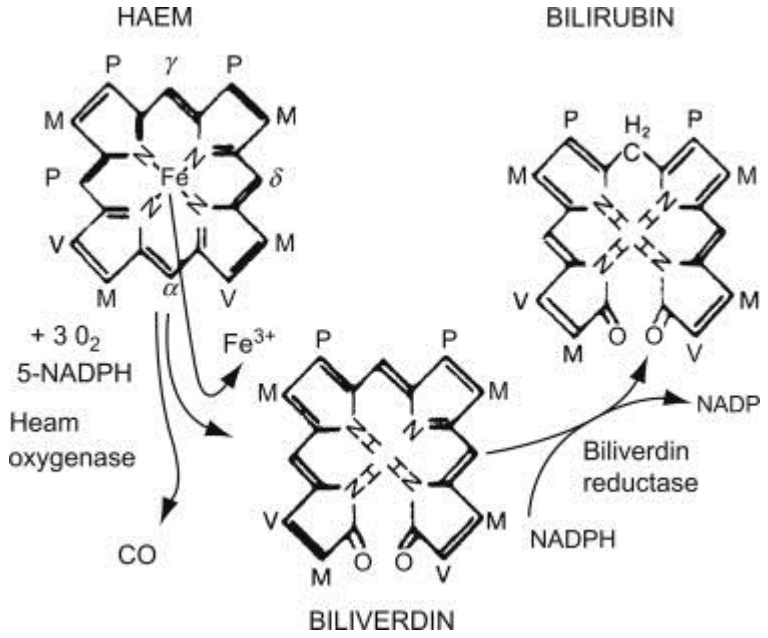

Figure 3: Heme ring opening mechanism (Chowdhury et al, 2013).

The reaction causes the α-methene bridge carbon to be eliminated in the form of carbon monoxide as well as the release and reutilization of iron and the formation of a green pigment known as biliverdin.

HO has high activity levels in organs where the breakdown of hemoproteins takes place, such as the spleen. Kupffer cells and hepatocytes in the liver both have heme oxygenase activity, with that of Kupffer cells being as high as the activity in the spleen. There are three forms of HO that have been discovered. The stress response protein, HO-1, is activated by an array of factors which are stress related, such as heavy metals, multiple cytokines, hypoxia, reactive oxygen species, endotoxins, protoheme IX, and the stress caused by cirrhosis on endothelial cells in the liver. HO-2 is found mainly in the testis and brain and

is considered a constitutive protein. The last isoform, HO-3, has a very low breakdown ability; its function maybe that of a protein that binds heme (Chowdhury et al, 2013).

Biliverdin reductase, a cytosolic enzyme that is present in all normal functioning tissues, converts biliverdin to bilirubin by reducing the methane bridge located in the center of the biliverdin molecule at C-10. The activity of BVR is pH-dependent and has a dual co-factor utilization. At pH 6.7, it uses NADH but resorts to NADPH at pH levels that are more basic (8.7). Reductase activity of BVR can be increased by autophosphorylation, causing an increase in the production of bilirubin which in terms improves protection against oxidative stress (Vitek and Ostrow, 2009; Iyanagi et al. 1998).

2.2.2 Bilirubin Transport and Uptake

Bilirubin cannot be transported unbound in the blood and must be bound noncovalently to plasma proteins, mainly albumin. This mechanism prevents bilirubin diffusion into various extrahepatic tissues, thus inhibiting bilirubin toxicity (Bhagavan, 2002). Once the albumin-bilirubin molecule reaches the liver sinusoids, it diffuses across the fenestrated sinusoidal membrane to the hepatic basolateral plasma membrane domain, where the albumin-bilirubin particle is broken down. Bilirubin is then absorbed by the hepatocyte through facilitated diffusion with the help of inorganic anions (Chowdhury et al, 2013). Two groups of proteins that bind organic anions attach to UCB in the cytosol making it impermanently solubilized and enabling UCB to diffuse freely across the hepatocyte. This also prevents UCB from passively flowing back into the plasma as well as UCB binding to intracellular membranes. One of those hepatic proteins is ligandin (protein Y), a glutathione S-transferase alpha isoform, that aids the UCB uptake in the hepatocytes by keeping it in the cytosol. The second protein is the fatty acid binding protein 1 (FABP1), or protein Z, which helps long chain fatty acids move within the cell (Vitek and Ostrow, 2009).

2.2.3 Bilirubin Conjugation

Bilirubin becomes permanently water soluble through the disruption of the internal hydrogen bonds. This, in turn, happens through propionic acid carboxyls conjugation with the sugar portion of the molecule, more specifically glucuronic acid. As a result, either bilirubin monoglucuronide or diglucuronide is produced, which is determined by whether one or both of the propionic acid carboxyls being glucuronidated. Around 85% of the bilirubin found in bile is in the diglucuronide form; the rest is in the form of monoglucuronides.

Uridine diphosphoglucuronate glucuronsyltransferases, a group of enzymes found in the ER as well as in the nuclear envelope in various cells, are responsible for the transport of the UDPglucuronate's glucuronosyl fraction to aglycone substrates, resulting in the production of thiol, ether, ester, and N-glucuronides. A wide variety of substances, such as toxins, hormones, drugs, and endogenous metabolites, can act as substrates for this family of enzymes (Bhagavan, 2002; Chowdhury et al, 2013).

2.2.4 Bilirubin Excretion

In order to be transported into the bile, bilirubin conjugates use an active process that requires of ATP and is controlled by MRP2. Bilirubin is then carried with the bile across what is known as the biliary tree. The biliary tree is a complex structure of ducts, which start narrow and then widen in diameter, that serve multiple functions such as secretion of bile and immune regulation. Bilirubin moves through the structure starting from the canals of Hering and advancing thorough bile ductules, followed by a series of interlobular and major ducts before being transported into the extrahepatic bile ducts, where bile is finally transferred to the gallbladder and then to the duodenum by relaxing the sphincter of Oddi. The gallbladder stores bilirubin between meals, where less than 2% of the conjugated form is hydrolyzed to UCB. CB cannot be efficiently absorbed in the small intestines and is therefore hydrolyzed to UCB by a group of enzymes called ß-glucuronidases which are produced by coliform bacteria as

well as enterocytes. Finally, some parts of the unmetabolized UCB, in addition to bacterial metabolism by-products, are disposed of in the fecal matter. The methylene bridge located at the center of a urobilinogen molecule can undergo dehydrogenation, producing a yellow-orange urobilin, giving stool it's distinct color (Zhang et al., 2018; Vitek and Ostrow, 2009).

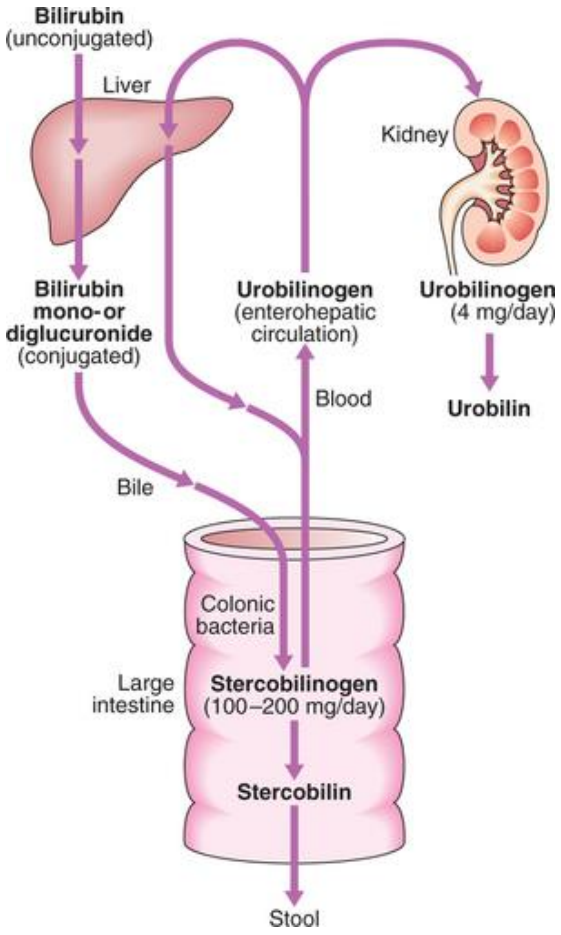

Figure 4: Bilirubin excretion (UFO Themes, 2017)

2.3 Bilirubin Metabolism Disorders: Gilbert's syndrome

GS is a harmless autosomal recessive metabolic disorder, first described in 1901 by Augustine Gilbert and his team, which causes a mild, nonhemolytic, increase in the levels of UCB in the blood (Radlović, 2014). The syndrome is rather prevalent in the general population, affecting between 3-17% with the percentage varying according to ethnicity (Bulmer et al., 2008).

The diagnostic tool for GS is the measurement of circulating bilirubin concentration in fasting status, and for this concentration to be higher than 17.1 μmol/l or 1 mg/dl. Levels of serum transaminases as well as markers of biliary blockage or damage must be within normal range, and the elevated bilirubin levels have been detected at least two times within six consecutive months (Wagner et al., 2018).

Reduced activity in bilirubin UGT1A1, the enzyme responsible for bilirubin conjugation, is the cause of GS. The gene responsible for the production of the enzyme is located on the 2q37.1 chromosome and its defect causes a 70%-80% decrease in the synthesis of UGT1A1 and as a result, a reduction in bilirubin conjugation. In addition to hyperbilirubinemia, the enzyme shortage induces an increase bilirubin monoglucuronide in the bile (Radlović, 2014).

2.4 Health Promoting Aspects of Bilirubin

Extremely high plasma concentrations of bilirubin (>300μM) are neurotoxic and could be damaging to brain tissue. On the other hand, mildly elevated bilirubin concentrations, such as those observed in subjects with GS, could have health promoting effects such as: anti-oxidative properties *in vitro*, vasoprotective and anti-inflammatory properties *in vivo*, and anti-genotoxic effects (Stocker et al., 1987; (Mölzer et al., 2013).

Bilirubin acts as an antioxidant by scavenging peroxyl radicals, under the condition that oxygen concentrations are considerably low and when incorporated into liposomes, more efficiently than α-tocopherol, which is considered the best single antioxidant to protect against lipid peroxidation (Stocker et al., 1987). However, α-tocopherol and bilirubin demonstrate a synergistic effect when present in a liposome membrane. In the presence of bilirubin, α-tocopherol must not act as the only antioxidant agent thus saving it from being depleted (Stocker, 2004).

Vitamin E is not the only fat-soluble micronutrient affected by the presence or absence of bilirubin. A couple of studies have observed that rats that suffer from

thoracic duct fistula were unable to adequately absorb vitamin A as well as ß-carotene due to the absence of bile; Small quantities of bilirubin had a stabilizing effect on both forms, causing them not to be destroyed in the intestines prior to uptake. They also observed that linoleic oxidation has been significantly reduced following the addition of low amounts of bilirubin (Bernhard et al., 1954). This was further investigated by Dr. Wu and his team in vitro by intentionally oxidizing LDLs through exposure to peroxyl radicals that were produced using AAPH (2,2'-Azobis(2-amidinopropane) dihydrochloride), an azo-initiator, and reported a high lipid antioxidant level of both conjugated and UCB (Wu et al., 1996).

The antioxidant properties of bilirubin could have a role in protecting against cardiovascular diseases. Two groups of navy men were examined; the first group consisted of 619 men with a completely available health profile and a second group of 258 men whose complete risk factor information was not available. Analysis of the data showed an inverse correlation between levels of serum bilirubin and severity of the coronary artery disease category: a 50% drop in bilirubin serum values was linked to a 47% disease severity increase (Schwertner, et al., 1994). In addition, individuals with GS have greater flow-mediated vasodilation (figure 5) compared to the control group (7.2%±2.2% and 5.9%±1.7% respectively) and the flow-mediated vasodilation demonstrated a significant correlation (figure 6) with serum bilirubin concentration ($r=0.44$, $P<0.001$) (Maruhashi et al., 2012).

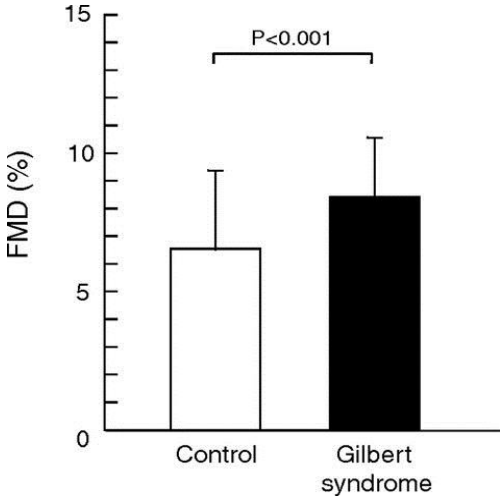

Figure 5: Flow mediated dilation % in control and Gilbert 's syndrome individuals (Maruhashi

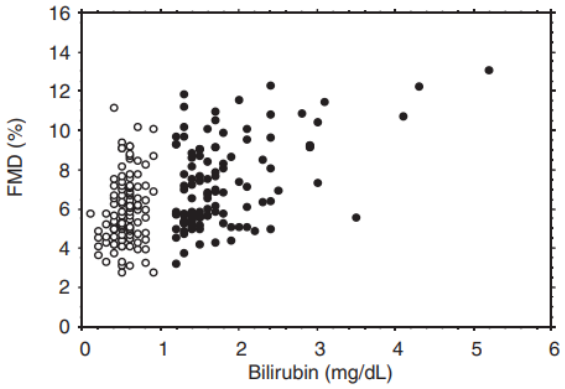

Figure 6: correlation between bilirubin and Flow mediated dilation (Maruhashi et al., 2012)

It has been shown that mild hyperbilirubinemia plays a role in reducing the prevalence of diabetes type 2. A group of Korean scientists injected a group of Wistar rats and a group of Gunn rats with streptozotocin, a β-cell toxin, to induce diabetes by impairing insulin production. After only 3 days, 100% of the Wistar rats became diabetic, while only 40% of the Gunn rats developed DM2 after 7 days of being injected. A week later without the use of insulin supplements, the Wistar rats group had greater levels of fasting blood glucose (510±50.3 mg/dL vs. 236.4 ±42.5 mg/dL, P=0.003) as well as HbA1c (5.0±0.1: 3.9±0.1, P=0.001) than the Gunn rats group. Additionally, the Wistar rats experienced pancreatic islet cell apoptosis along with NADPH oxidase activation, while the Gunn rats did not (Fu et al., 2010). Similar results were obtained by another study using the same methodology resulting in the diabetic Gunn rats exhibiting less albuminuria as well as inhibition of an important characteristic of diabetic nephropathy known as mesangial expansion (Fujii et al., 2010). The same effects were also observed in human subjects. In Korea, a large cross sectional population-based analysis of approximately 94,000 subjects, with an age range of 18-96 years and 53% male, was conducted to examine the relationship between elevated serum bilirubin and diabetes or chronic kidney diseases associated with diabetes. Increased levels of serum bilirubin were shown to be significantly related to decreased prevalence of DM2 in both women (P=0.014) and men (P=0.001) (Han et al., 2010). In another study, data collected by NHANES from roughly 16,000 patients with a 10-year

follow-up (1996-2006) has shown a 20% decrease in risk of DM2 in subjects with bilirubin concentrations higher than 10 µmol/l (Cheriyath, 2010).

2.5 Colorectal Cancer

CRC is the medical term used to describe malignant tumors that are formed from the colorectal mucosa of the large intestines and/or the rectum. Over 95% of CRC cases are Adenocarcinoma: Tumors that are formed in the epithelial tissue accompanied by production of mucin or glandular differentiation (Thrumurthy et al., 2016; Gazdar and Maitra, 2001)

2.5.1 Epidemiology

Statistics have shown that in the year 2018 1.8 million new cases of CRC have been reported as well as approximately 700,000 deaths, making it the third most prevalent cancer and the second leading cause of death due to cancer worldwide. By the year 2030, the CRC global burden is predicted to rise by 60% causing the number of new cases to increase to 2.2 million and the deaths to 1.1 million (Arnold et al., 2016). A more recent statistical analysis done by the Global Cancer Observatory for the WHO in 2018 has shown a worldwide increase in both incidence and mortality to 1.8 million and 880,000, respectively (WHO, 2019).

In Europe, CRC is the second most prevalent cause of cancer death, with lung cancer being the first. In 2018, an estimated number of 500,000 Europeans were determined to have CRC and for approximately half of those (243,000) it was fatal. The incidence number for European women that same year was an estimated 228,000 and at 272,000 for men (Ferlay et al., 2018). In Austria in the year 2016, the incidence rate of CRC for both sexes was 4,516 with 2,213 deaths related to the disease, making it the third leading cause of death by cancer in the country (Statistik Austria, 2018).

2.5.2 Pathogenesis and Risk Factors

CRC develops mainly through the disruption of the wnt signaling pathway either by mutations of the β-catenin gene or the APC gene or by deletion of the APC gene (Arnold et al., 2005). In the majority of the cases, cancer manifests itself as a polyp located on the epithelial tissue lining the colon or rectum. These polyps are not necessarily malignant, they could also be benign or pre-malignant (Bardhan and Liu, 2013).

While genetic predisposition increases the risk of developing CRC, most of the cases are linked to environmental factors such as lifestyle, diet, and food-borne carcinogens. Other important risk factors include the gastrointestinal microbiota as well as chronic inflammation of the intestines that usually occur before tumor development. In approximately 20% of the cases, chronic inflammation, caused by microbiota dysregulation in the gut, caused tumor development (Bardhan and Liu, 2013). Other risk factors include: being male, increasing age (≥50), tobacco smoking, being obese, and excessive alcohol consumption (Thrumurthy et al., 2016).

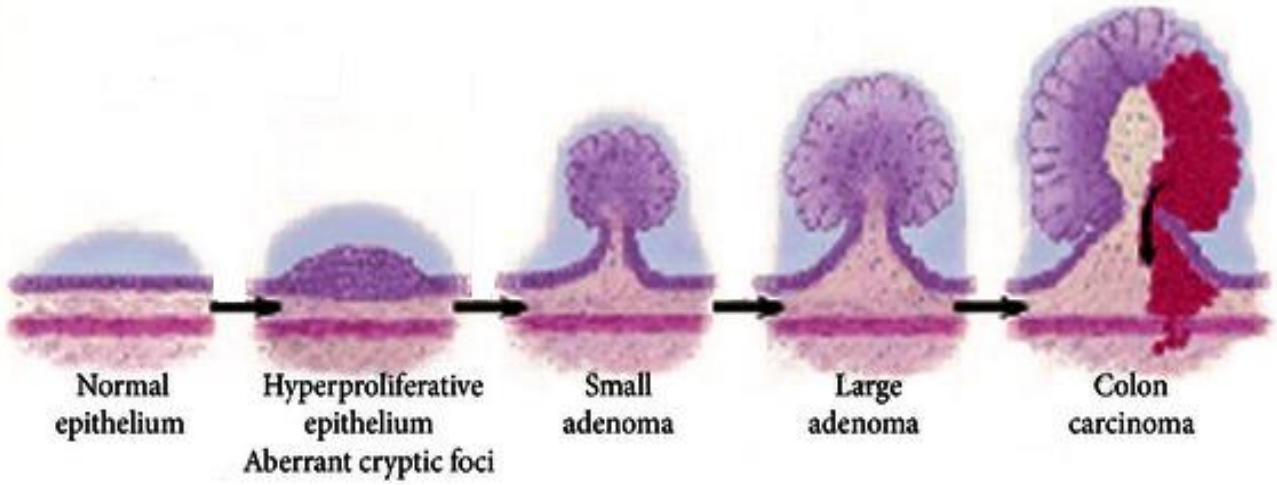

Figure 7: Development of colorectal cancer (modified from Sandouk, Al Jerf and Al-Halabi, 2013)

2.5.3 UCB and CRC

The antioxidant capacity of bilirubin has led scientists to investigate the effect it could have on several forms of cancer, including CRC. An analysis of a thorough survey in the United States, NHANES III, was conducted to determine the correlation between CRC and bilirubin levels in the general population. Results have shown a decreased risk of developing CRC with each 1mg/dL increase in serum bilirubin. This effect was more noticeable in women (OR=0.186) than in men (OR=0.295) (Zucker et al., 2004).

Promising results were reported in a study that investigated the association between bilirubin and CRC both *in vivo* and *in vitro*. BALB/c mice carrying HRT-18 colon cancer cells were treated with bilirubin to study its effect on tumor growth. Simultaneously, western blots were used to examine the association between bilirubin and tumor development in HRT-18 cells *in vitro*. Results have shown inhibition in the spread of tumor cells *in vivo* and apoptotic and cytostatic effects *in vitro* (Ollinger et al., 2007).

UCB has been shown to trigger apoptosis in colon cancer cells after they have been exposed to concentrations that range from 0-50 µM. Furthermore, the study demonstrated that the apoptosis induced by UCB was activated via the mitochondrial pathway (Keshavan et al., 2004).

In 2012, a group of scientists studied the impact of promoter variations of the *UGT1A1* and the *HMOX1* genes, both are enzymes that play a significant role in maintaining bilirubin balance, as well as the association between serum bilirubin levels and the risk of CRC. After adjusting for age, results have shown a 20% decreased CRC risk in UGT1A1*28 allele carriers. The observed protective association was more pronounced in men than in women. It was also observed that patients with CRC had relatively lower levels of serum bilirubin, with the association again being more significant in men than in women. They also observed a 7% increase in CRC risk with each 1µmol/l decrease in bilirubin concentration (Jirásková et al., 2012).

A Macedonian study investigated the association between CRC risk and the *UGT1A1* promoter length polymorphism. They observed an increased frequency of genotypes which contain the UGT1A1*28 in both the heterozygous and homozygous state as of those of the wild type UGT1A1*1/*1 when compared with controls (OR=2, P=0.007). The allele was found to be a risk factor for men (P=0.005) but not for women (P=0.15) (Bajro et al., 2012).

The Belgium-Inter-university Research on Nutrition and Health provided mortality data from a 10-year follow up which was in turn used to investigate the relationship between serum bilirubin cardiovascular, all-cause, and cancer mortality. An inverse association between cancer mortality and serum bilirubin was observed (P=0.004) with this being more prominent in men than in women. Men with the highest bilirubin concentrations had a 58% decreased risk of cancer than those with the lowest bilirubin concentrations, while a 24% risk in women was not found to be significant (Temme et al., 2001)

Contrasting results were reported after an analysis of the first National Health Examination Survey that examined serum bilirubin levels of the participants and the incidence of CRC. No connection was found even after adjusting for gender, age, smoking, and other factors. However, the authors point out the fact that only total bilirubin, and not UCB, was measured (Ioannou et al., 2006).

3. MATERIALS AND METHODS

A total number of 1368 plasma samples were obtained from Estonia, Denmark, and Sweden for the purpose of this study. The Biobank of the Estonian Genome Center at the University of Tartu (EGCUT) provided 680 of the samples, while 688 were collected as part of the European Prospective Investigation into Cancer and Nutrition (EPIC for short) study. Both data sets included personal as well as follow up information about each volunteer and the UCB concentration was measured using High-performance liquid chromatography.

3.1 Estonian Cohort

The Estonian Biobank Project was established by the Estonian Genome Project Foundation in 1999. The creation of a biobank from a large fraction of the Estonian population was a key factor for the examination of the behavioral, environmental, and genetic background of various diseases.

A longitudinal, population-based, cohort study of the collected data combined with participants' clinical information were used to examine the volunteers' health status (Leitsalu et al., 2014).

3.1.1 Study participants

Participant selection, as well as sample collection, was carried out randomly by a group of general practitioners with the help of other various medical professionals in private clinics and hospitals or in the offices of the EGCUT. A total of 454 GPs (corresponding to 56% of registered GPs in Estonia) together with 186 nurses took part in the project.

An estimate of 52,000 volunteers (age ≤18 years) had their DNA, health records, white blood cells (WBCs) along with plasma samples collected for the Biobank. This number represents approximately 5% of the Estonian population while also reflecting a near accurate representation of the adult population of Estonia based on sex, age, and geographical distribution.

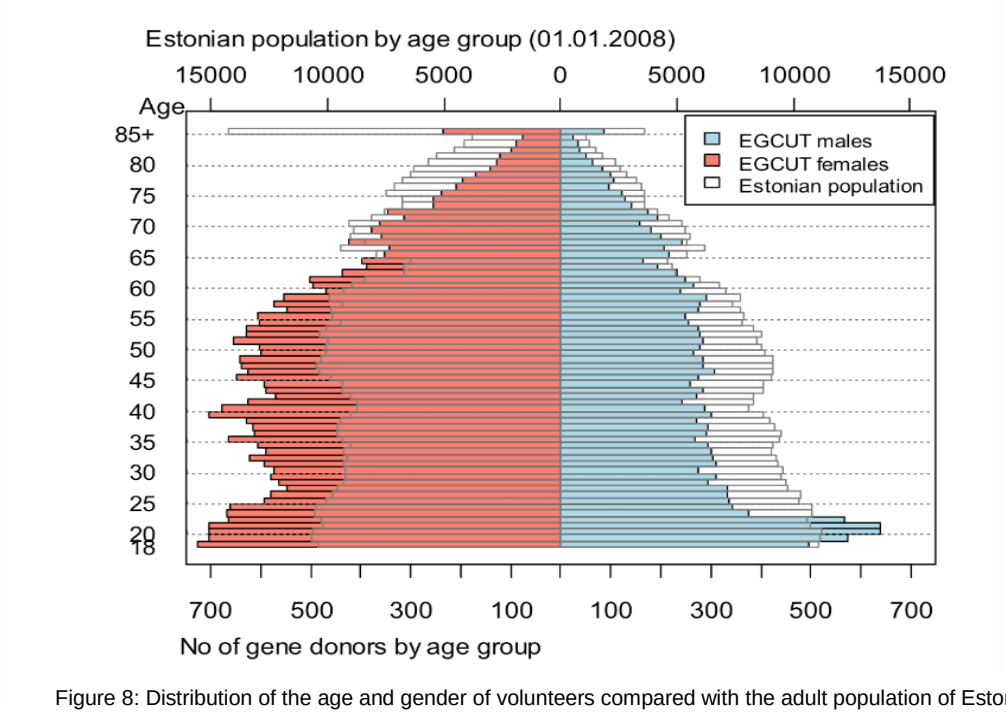

Figure 8: Distribution of the age and gender of volunteers compared with the adult population of Estonia at time of recruitment. (Geenivaramu.ee, 2018)

A close inspection of the database reveals a higher female participation ratio than that of males which is generally the case in other volunteer-based population studies. The biobank's samples represent 3.4% of adult males and 5.5% of adult females in Estonia. The percentage of samples from males was 34% and 66% from females. The ratio in the general population is 45:55, indicating that males are under-represented in the data (Leitsalu et al., 2014).

3.1.2 Data collection

A questionnaire, which was created with the help of the WHO's IARC, containing roughly 330 personal, lifestyle, educational, and medical history questions together with 1000 data fields was filled using a computer-assisted

personal interview. Upon completion, the finished questionnaires were delivered to the EGCUT in the form of an encrypted document electronically.

The collected plasma, DNA, and WBCs samples were moved to the EGCUT's main laboratory within a timeframe between 24-48 hours after extraction and stored at -196°C in CryoBioSystem high-security 0.5mL straws in liquid Nitrogen. In order to ensure anonymity, the coding center labeled each sample with a code consisting of 16 digits along with a barcode. The coding system was divided into 4 groups: a participant ID unique for each sample, a laboratory code, a release code that enables the data to be used for other projects, and a data transport code.

3.1.3 Follow-up

In order to ensure the data is up-to-date, the volunteers have been re-contacted for follow-up on 2 occasions: in 2008 and in 2011. A group of 822 study participants (age range: 20-74 years) was chosen to be re-contacted. The process took place between 2008 and 2010, with its response rate being 57.2%. in 2011, a total of 1072 participants, were asked to visit the recruitment once again in order to confirm the data from the first questionnaire. The response rate of this group was 41.1%. The different re-contacting approaches could be the reason behind the variation in the response rate. The participants of the group with the higher response rate were approached by their GP, whereas those in the group with the lower response rate were contacted via mail (Leitsalu et al., 2014; Geenivaramu.ee, 2018).

3.2 EPIC samples

The initiation of EPIC was governed and planned by IARC-WHO with the help of supporting centers in 1999. The goal of this cohort is to help examine the correlation between cancer and nutrition. Initially, participants from only four countries (Italy, Spain, the United Kingdom, and France) took part in the collection of data and biological samples in 1993 with six additional countries

(Norway, Greece, the Netherlands, Germany, Denmark, and Sweden) joining between 1994 and 1998. Enrollment of participants along with data gathering was finished in 1999 (Epic.iarc.fr, 2018).

One of the advantages of this study taking place in Europe is the diverse nature of dietary habits throughout the continent, with consumption patterns ranging from the Nordic diet in Denmark and Sweden to central European eating habits of the Netherlands and Germany and to the Mediterranean diet of Spain, southern Italy, and Greece (Riboli, 1997).

3.2.1 Study participants

The number of participants taking part in the study is estimated to be more than 521,000 (367,903 females and 153,427 males) with plasma, erythrocytes, leukocytes, and serum samples that were collected at baseline from 387,889 volunteers. The IARC-WHO houses over 9 million aliquots, making it one of the biggest biobanks for the genetic and biochemical cancer research in the world (Epic.iarc.fr, 2018).

In every participating country, high subject cooperation as well as guaranteeing follow-up over an extended period of time were deciding factors on the selection of study volunteers. Differences in the selection process were also noticed amongst countries; in Oxford 50% of the selected volunteers were non-meat eaters, which includes vegans, Lacto-ovo vegetarians, and pescetarians. In France, members of the health insurance for teachers were recruited. In Norway, France, Utrecht, and Naples the recruitment included female participants only.

For the purposes of investigating premenopausal breast cancer, including a sufficient number of premenopausal women, was important. For that reason, the lower age limit for women was set at 35 while that of men at 40. The upper age limit, however, was dependent on the recruitment methods used in each particular center and was set between 60 and 74 for both men and women (Riboli, 1997; Riboli, E, et al., 2002).

3.2.2 Data collection

a. Assessment of dietary intake: gathering information regarding the eating habits of the participants was done using three methods:

• Quantitative dietary questionnaires, consisting of as many as 260 food items, were the method of choice in Greece, Germany, and the Netherlands.

• Semi-quantitative food frequency questionnaires, containing standards food portions given to all members, were the preferred option in Norway, Denmark, Umeå, and in Naples.

• Combined dietary methods, such as 7-day records, 14-day records, and semi-quantitative food –frequency questionnaires, were used in Sweden and the UK.

Comparability problems may arise due to the fact that various methods of dietary assessment were used across the 23 centers. In order to overcome this particular issue, supplementary dietary information was gathered using a computer-assisted 24-hour dietary recall program in representative sub-samples of 8% of volunteers in each and every subcohort. A 24-hour dietary recall was applied as the reference method for the correction of both systematic and random errors in classification in baseline dietary collection.

b. Anthropometric measurements: standardized techniques were used in all participating centers to measure waist and hip circumference, weight, and height. There are two reasons why this step is important. First, physical activity and energy intake and metabolism are associated with anthropometric patterns. Second, the incidence of several forms of cancer may be associated with the aforementioned measurements.

c. Lifestyle-questionnaires: categories include, but are not limited to: physical activity level, reproductive and menstrual history, education, socio-economic status, medical history, alcohol consumption, and tobacco smoking during lifetime, use of contraception, and HRT.

d. Biological samples: collection of blood samples (30 ml per participant) took place at recruitment centers. The samples were then separated to plasma,

serum erythrocytes, and WBCs using centrifugation, followed by aliquoting into 28 plastic straws with each straw containing 0.5 ml. The samples were then divided into two identical halves consisting of 14 aliquots (6 plasma, 4 serum, 2 buffy coat, and 2 erythrocytes) for each half. One of the straw sets was stored at a local location, while the other set was shipped to IARC and stored in liquid nitrogen (-196°C) in the main biorepository.

3.2.3 Follow-up

Two types of follow-up took place during the course of this study: a follow-up for lifestyle and health status and a follow-up for mortality and incidence of cancer.

• Lifestyle and health status: in order to document any changes that might have occurred in the different lifestyle aspects (weight, tobacco smoking, alcohol consumption, etc.) of the cohort members, participants were approached regularly every few years (3-5).

• Cancer and mortality: the population cancer registries of 7 countries (Norway, Italy, Denmark, the Netherlands, the UK, Sweden, and Spain) were used to determine the incidence of cancer amongst the participants. In the remaining 3 countries (Germany, France, and Greece) a combination of approaches including registries of pathology and cancer, records of health insurance, and regular follow-up were used to keep the data up-to-date (Epic.iarc.fr, 2018).

3.3 HPLC: High-performance liquid chromatography

The amount of bilirubin present in each sample was measured using an HPLC machine. A high-pressure pumping device pushes the liquid solvent (mobile phase), along with the sample, through a column containing a highly adsorbent material (stationary phase), where the components of the sample are separated according to their molecular composition and structure (Lindsay, 1987).

The device is connected to a computer with a special program that is used to control the settings and to show the concentration of bilirubin detected in every sample.

3.3.1 HPLC specifications

An HPLC consists of multiple parts, each with a particular function that is crucial for the analysis of the samples. A more detailed list of the parts used for the purposes of this study, along with model name or specification, can be found in table 1.

Table 1: Parts of HPLC

Part	Model/Specification
Pump	LaChrom Elite Hitachi L-2130
Auto-Sampler	LaChrom Elite, Hitachi L-2200, Auto-Sampler 5 °C, Injection Volume: 2x 20 µL
Column Oven	LaChrom Elite, Hitachi L-2300 Column Oven, Temperature 35 °C
Detector	SPD-M20A, Shimadzu, Photodiode Array Detector
Wavelength	450 nM
Column	Fortis™ HPLC-Column F18-050703, 150x4.6 mm, 3 µM C18
Flow	1 ml/Minute
Pressure	140-170 bar
Software	EZ Chrome, Hitachi

3.3.2 Mobile phase preparation

Listed below are the detailed steps for the mobile phase preparation along with table 2 which contains all the chemicals needed as well as their volumes. Figure 9 contains details of the preparation steps.

Table 2: Mobile phase chemicals

Chemical	Amount
Methanol	Approximately 2 liters
Dioctylamine	48.4 ml
Acetic acid	12.02 ml
Water	72 ml

a. An Erlenmeyer flask is placed on a scale and 200 ml methanol (gradient grade for liquid chromatography) is poured into the flask.

b. The scale is switched on and 48.4 ml Dioctylamine are added to the methanol. The flask is then closed with paraffin film and placed on ice for 5 minutes while occasionally shaking it.

c. Thereafter, the flask is removed from the icebox and put back on the scale. 12.02 ml acetic acid are added to the mixture followed by 400 ml methanol.

d. After shaking the solution, it is transferred to a 1000 ml measuring cylinder. The previously used Erlenmeyer flask is flushed with methanol that is then poured into the measuring cylinder until it is filled to the 930 ml mark.

e. 72 ml gradient grade water is added and the cylinder is then closed tightly with a cap and turned gently 3 times in order to properly mix the solution.

f. Using a mobile phase filtration apparatus, 1 L of methanol is filtered, followed by the prepared solution. The two are then mixed together resulting in a 2 L mobile phase that is 3.5 % water and 96.5 % methanol.

g. The last step is degasification (5 minutes) in an ultrasonic bath and addition of helium (20 minutes).

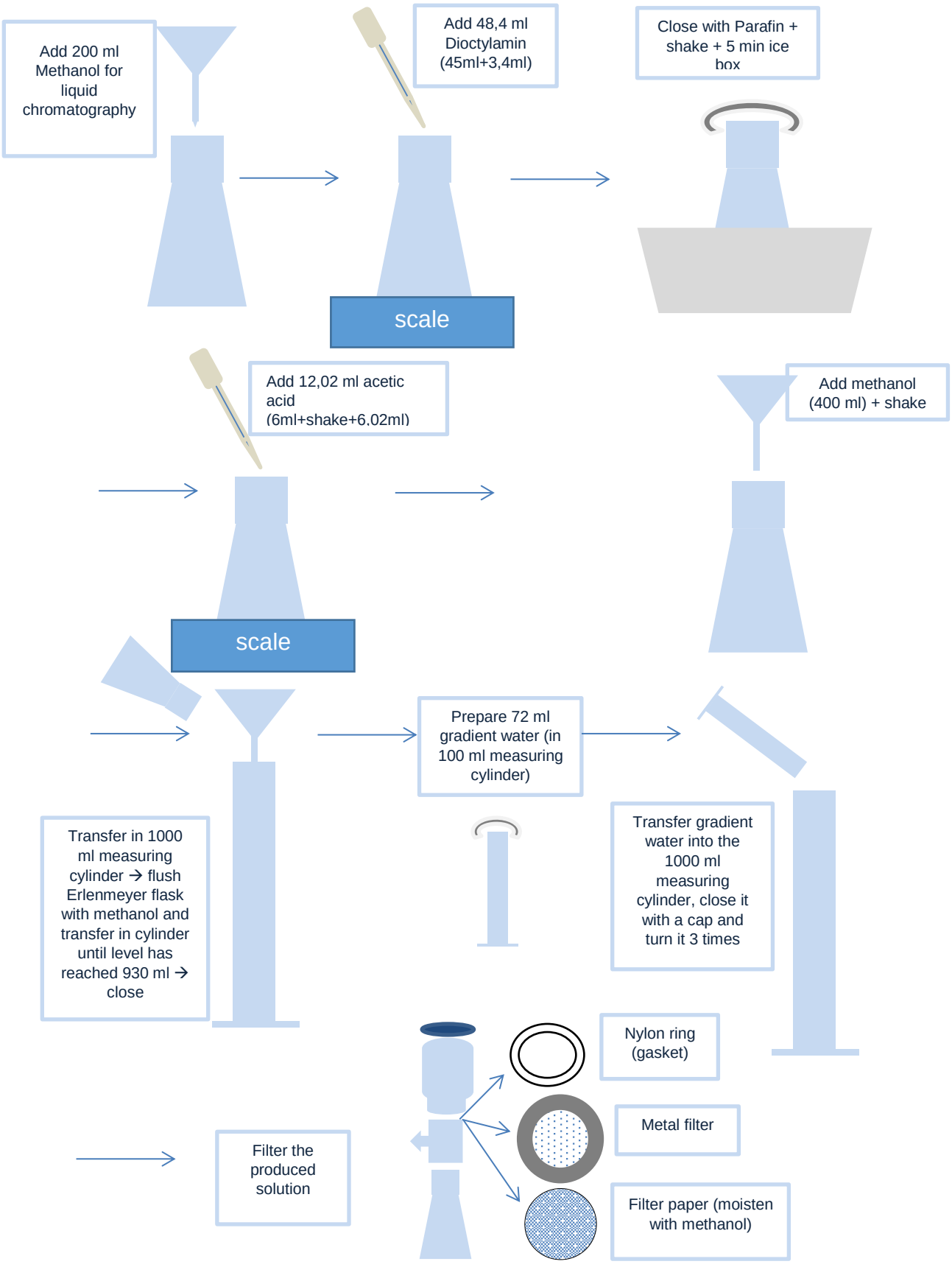

Figure 9: Schematic preparation of mobile phase

3.3.3 standard preparation

The concentration of bilirubin was calculated daily using a standard curve, which in turn was determined by preparing a serial dilution. A stock solution of bilirubin with a concentration of 10mM(S0) is prepared by weighing 3-5 mg bilirubin powder into a 1.5 ml microtube. The exact amount of DMSO required to dissolve bilirubin is determined using the following link: https://www.graphpad.com/quickcalcs/molarityform.cfm.The weighed amount is then entered in the “Mass” field and the amount of DMSO needed is shown in the “Volume” filed as shown in figure 10. Next, DMSO is pipetted and the solution is homogenized using a Vortex mixer and placed in a water bath for 5 minutes at 70°C then mixed with the Vortex

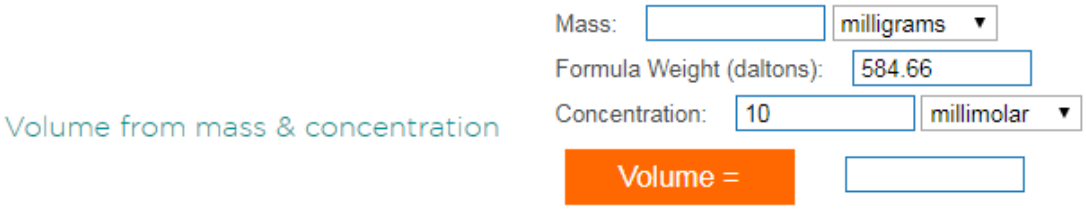

Figure 10: Determination of DMSO volume

The microtube is then placed in an ultrasonic bath for one minute followed by mixing with the Vortex once again. Lastly, the solution is centrifuged for 30 seconds at a temperature of 4°C and a speed of 14000 RPM and vortexed. In case a pellet is present after the last step of the preparation process, the entire procedure must be repeated. 50 µl of the stock solution is aliquoted in brown microtubes labeled “*UCB 10 mM 50 µl* “each and stored in a box in the freezer.

A standard dilution is prepared in accordance with table 3 by adding 450 µl DMSO into the microtube containing the 50µl stock solution, thus making the concentration 1000µl (S1). The rest of the dilution process is shown in detail in part one of figure 11.

Table 3: Standard Dilution

Standard dilution	Concentration(µmol/l)	DMSO(µl)
S0	10 000	0
S1	1000	450
S2	100	450
S3	50	250
S4	25	250
S5	12.5	250
S6	6.25	250
S7	3.125	250
S8	1.5	250
S9	0.75	250

40µl are then transferred from the microtubes S4 through S9 into microtubes containing 160µl mobile phase as depicted in part 2 of figure 11 followed by mixing with a Vortex. Finally, 120µl are pipetted into vials which in turn are placed in the HPLC for analysis.

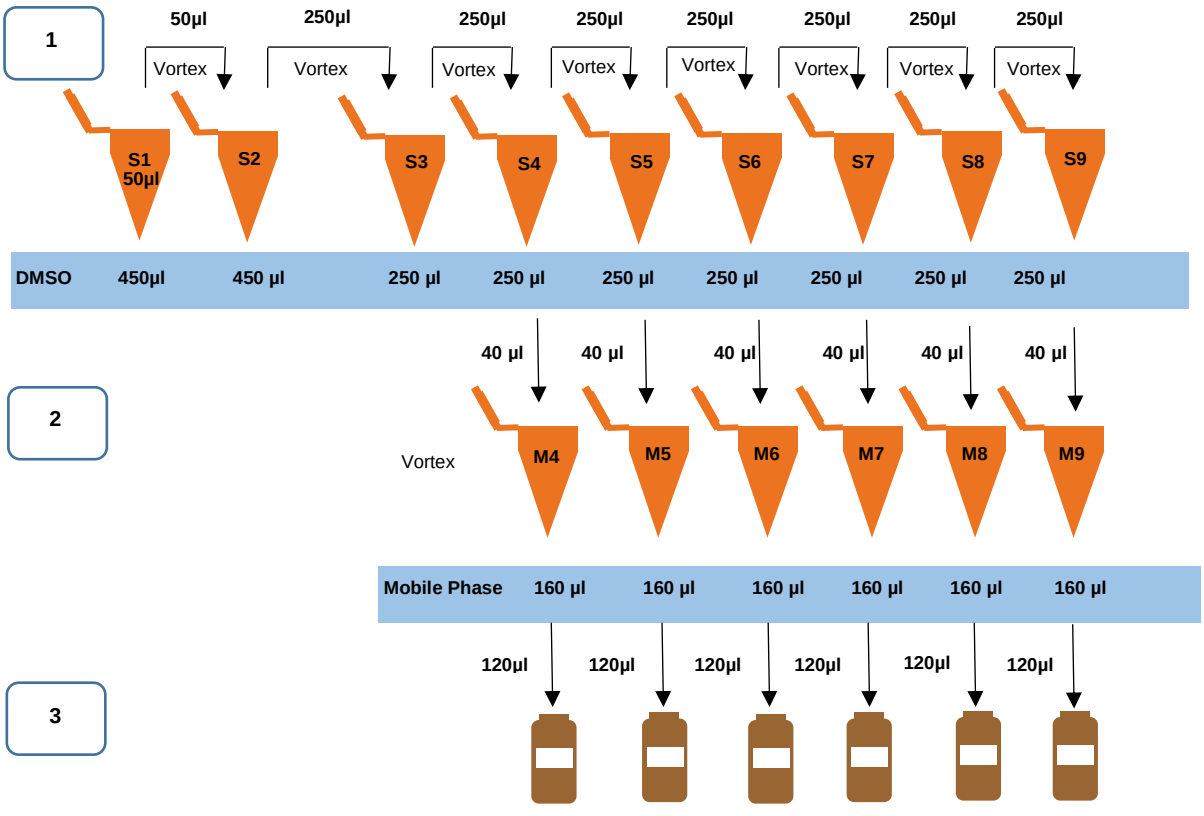

Figure 11: Preparation of the standard dilution

3.3.4 HPLC preparation

Before starting any of the sample preparation steps, it was crucial that certain conditions (table. 4) regarding the machine's status were met in order to ensure delivery of optimal results. The column, as well as the pre-column, were regularly checked for any leakage. Half an hour after the machine was switched on, a preview run was started for about 2 minutes to determine the stability of the baseline (little to no noise, the absence of drifting). Once all the conditions have been met, sample preparation was begun.

Table 4: HPLC configuration for the assessment

Configuration	Optimal Condition
Auto-Sampler temperature	5 °C
Column Oven temperature	35 °C
Injection Volume	20µl
Flow	1 ml/Minute
Pressure	140-170 bar

3.3.5 Sample preparation

The samples were taken out of the freezer and thawed. 70µl were then aliquoted into microtubes followed by centrifuging for 10 minutes at a speed of 14,000 RPM and a temperature of 4°C. Subsequently, 50µl were pipetted from the previously mentioned microtubes and mixed with 200µl mobile phase, followed by mixing with the Vortex for 30 seconds for each microtube and another round of centrifuging. 120µl of the supernatant were pipetted into vials and placed in the autosampler.

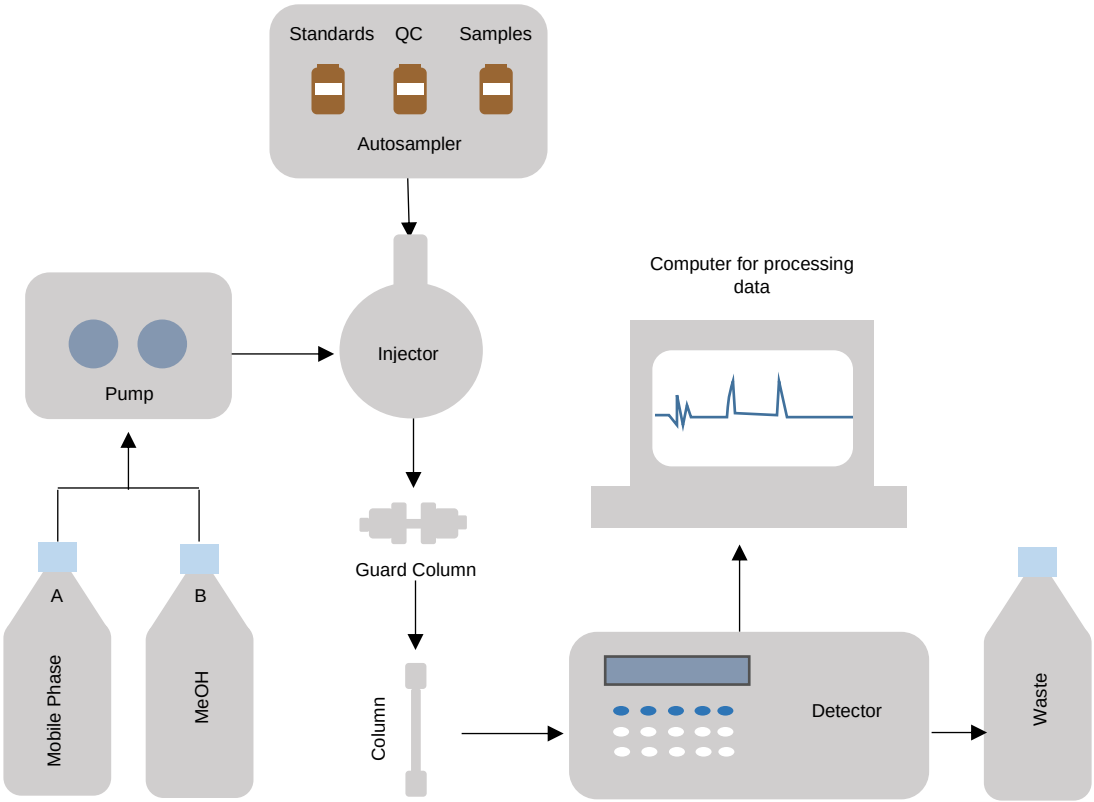

Figure 12: Graphic presentation of HPLC analysis.

Quality control (QC) samples of known origin and concentration were analyzed with each batch of samples following the same procedure. A volume of 80µl of a pre-prepared and frozen serum were mixed with 320µl mobile phase and homogenized using a vortex. Afterward, the microtube was placed in the centrifuge with the rest of the samples and 120µl of the supernatant was pipetted into two vials and placed in the autosampler. The purpose of the control samples is to provide an overall image on the status of the machine and to measure reliability, repeatability, and accuracy (Masson, 2007; Wallner et al., 2013).

3.4 Data interpretation

The program uses the retention time as well as the signal intensity of each individual sample to demonstrate the concentration in the form of a peak. (Chromacademy.com, 2018) Human serum samples show only one peak, Bilirubin IXα, while standards consist of three isomer peaks, Bilirubin IIIα; IXα & XIIIα, with a retention time of approximately 8 minutes (McDonagh and Assisi, 1972).

Figure 13: Sample peak

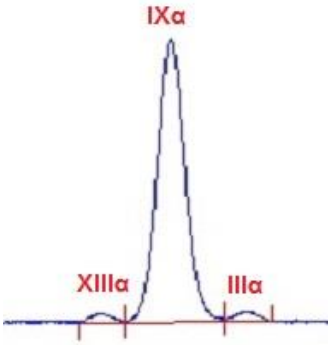

Figure 14: Standard

The measured Area Under the Curve of each peak was copied into a Microsoft Excel folder to be used to determine the concentration using a simple linear function: y=ax+b,

$$Concentration(\mu mol\backslash L) = \frac{AUC\ Bilirubin\ IX\alpha - Intercept}{Slope}$$

3.5 Statistical Analysis

The statistical evaluation of the data was done using IBM SPSS Statistics 25. In order to determine whether the entries were normally distributed, a Kolmogorov–Smirnov test was performed on each variable. The variables that have shown to have a normal distribution were consequently tested for differences across population using the T-Test. On the other hand, the sampled data which were not normally distributed were tested using the Mann–Whitney

U test. A correlations test was then done to determine whether there is an association between UCB and the variables tested. The significance value is shown in the table under “p-value” (probability value) as well as under “significance” with the significance level being <0.05.

The results shown in the tables found in the next section are represented with the mean and the standard deviation without including the median. This was done to allow the reader to better interpret the data. The tests were performed on the entire population as well as on men and women separately.

4. RESULTS

The samples were provided by two different biobanks, as stated in detail in the material and methods section, and were therefore analyzed using SPSS separately. The reason for not merging the data is the difference in some of the available data. While one biobank provided additional blood biomarkers, the other one did not.

4.1 Estonian samples

The Estonian biobank provided 680 samples for analysis. Those who developed CRC were given the term case and those who did not were referred to as control. The number of female participants was 414, exactly half of which were cases and the other half were controls. The remaining 266 were from men; 133 were cases and 133 were controls. The average age of the participants was 67±10.0 years. The youngest was a female who was 29 years old and the oldest was also a female who was 95 years old. UCB was measured at 5.0±3.4 (µmol/l) for cases and 5.1±3.4 (µmol/l) for controls, with a p-value of 0.8 showing no significant difference between the two values. The values of various additional variables can be found in table 5.

Table 5: Descriptive analysis for Estonian Samples

	Case		Control		P-value
	Frequency (%)	Mean±SD	Frequency (%)	Mean±SD	
Unconjugated bilirubin(µmol/l)		5.0±3.4		5.1±3.4	.8
BMI(kg/m^2)		28.5±5.0		28.0±4.6	.2
Age(years)		67.1±10.7		67.1±10.5	.9
Alcohol g/d		3.6±24.8		0.8±3.6	.8
Waist/hip ratio		0.9±0.1		0.9±0.1	
Follow-Up(years)		11±6			
Gender					
Male	133 (20)		133 (20)		
Female	207 (30)		207 (30)		
Smoking Status					.3
Never	217 (32)		212 (31)		
Former	79 (12)		70 (10)		
Smoker	43 (6)		55 (8)		
Unknown	1 (0)		3 (0)		
Alcohol Consumption					.4
Currently	233 (34)		228 (34)		
Former	36 (5)		27 (4)		
Never	62 (9)		75 (11)		
Unknown	9 (1)		10 (1)		
Physical Exercise					.1
Yes	105 (15)		83 (12)		
No	156 (23)		178 (26)		
Unknown	79 (12)		79 (12)		
Use of hormone preparations					.1
Yes	13 (2)		20 (3)		
No	173 (25)		161 (24)		
Meat consumption/week					.09
0 times	18 (3)		10 (2)		
1-2 times	140 (21)		132 (19)		
3-5 times	126 (19)		148 (22)		
6-7 times	55 (8)		45 (7)		
Unknown	1 (0)		5.0 (1)		
Meat products consumption/week					.3
0 times	40 (6)		48 (7)		
1-2 times	121 (18)		104 (15)		
3-5 times	114 (17)		116 (17)		
6-7 times	63 (9)		67 (10)		
Unknown	1.0 (0)		5.0 (1)		
Use of NSAID					.4
Yes	49 (7)		41 (6)		

No	290 (43)	296 (44)	
Unknown	1.0 (0)	3.0 (0)	

*The mean difference is significant <0.05

4.1.2 Gender differences

Bilirubin has been shown to be affected by gender, with serum levels being higher in men than they are in women (Zucker et al., 2004). Therefore, table 6 shows the differences in the concentration of UCB of cases and controls grouped by gender.

Table 6: Bilirubin in men and women divided by case/control (Estonia)

Groups	N	UCB Mean±SD (µmol/l)	Median (µmol/l)	Min-Max (µmol/l)	P-Vlaue
Female	414	4.8±3.4	4.0	1.0-28.0	.1
Case	207	5.0±3.6	4.0	1.0-28.0	
Control	207	4.6±3.1	3.7	1.0-21.0	
Male	266	5.5±3.4	4.6	.05-27.4	.1
Case	133	5.2±3.0	4.6	.05-16.0	
Control	133	6.0±3.7	4.6	1.1-27.4	

*The mean difference is significant <0.05

Analysis of all the female participants, 414 to be exact, revealed the mean value of the concentration of UCB was 4.8±3.4 µmol/l with the lowest concentration of 1 µmol/l and the greatest at 28 µmol/l. Female cases had an average concentration of 5.0±3.6 µmol/l as well as a minimum concentration of 1 µmol/l and a maximum of 28 µmol/l. When compared with the cases, the controls had a slightly lower concentration of UCB (4.6±3.1 µmol/l). The minimum concentration of the controls was identical to the cases, while the maximum was found to be lower (21 µmol/l). The difference in the female population was not significant (P=0.1).

The 266 men who participated in the study had a mean UCB of 5.5±3.4 µmol/l. The lowest concentration recorded was 0.05 µmol/l in the case group while the highest was found in the control group at 27.4 µmol/l. Similar to the female participants, there was no significant difference in the concentration of UCB (P=0.1).

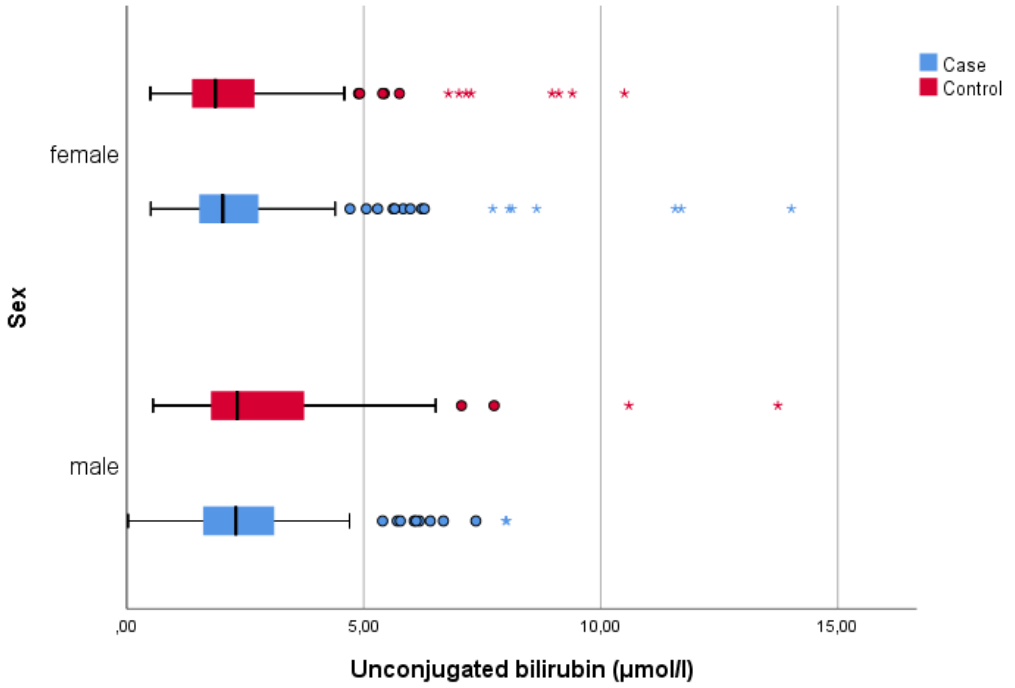

Figure 15: UCB concentrations in cases and controls grouped by gender (Estonia)

The boxplot illustrated in figure 15 shows the median as a straight line in the middle of the box. Female cases had a slightly higher median value of 4.0 µmol/l than that of the controls with a median of 3.7 µmol/l. Outliers are represented by stars with the maximum shown at 28 µmol/l. Male cases and controls had the same value for the median at 4.6 µmol/l with fewer outliers than in the female group.

4.1.3 UCB in variables

Various variables have been selected that could have an impact on the concentration of UCB in cases and controls. Table 7 contains the concentrations amongst participants who had developed CRC and those who had not.

Table 7: UCB concentrations of variables for cases and controls (Estonia)

Variables					
	N	**Case**	**N**	**Control**	**P-value**
Smoking status		**UCB±SD(µmol/l)**		**UCB±SD(µmol/l)**	
Never	217	5.3±3.8	212	5.4±3.7	.7
Former	79	4.8±2.6	70	4.9±2.8	.7
Smoker	43	4.3±2.6	55	4.4±2.7	.8
Alcohol Consumption					
Currently	233	5.1±3.3	228	5.2±3.3	.6
Former	36	4.2±2.0	27	5.0±3.7	.9
Never	62	5.6±4.6	75	5.0±3.6	.2
Physical Activity					
Yes	105	5.4±3.2	83	6.2±4.3	.2
No	156	5.2±3.5	178	4.8±3.0	.3
Meat consumption/week					
0 times	18	7.3±4.8	10	5.1±3.0	.1
1-2 times	140	4.8±3.1	132	5.2±3.7	.7
3-5 times	126	5.2±3.8	148	5.1±3.4	.6
6-7 times	55	4.4±2.4	45	5.1±2.7	.2
Meat products consumption/week**					
0 times	40	5.9±4.2	48	5.2±3.9	.1
1-2 times	121	5.0±3.9	104	5.5±4.1	.2
3-5 times	114	4.9±2.8	116	4.6±2.4	.2
6-7 times	63	4.8±2.9	67	5.4±3.2	.3
Use of NSAID* **					
Yes	49	5.6±4.4	41	4.5±2.4	.1
No	290	5.0±3.2	296	5.2±3.5	.6
BMI (kg/m^2)					
<27.9	156	5.4±4.0	180	5.5±3.8	.9
>27.9	183	4.7±2.7	157	4.7±2.8	.8
BMI (kg/m^2) categorial					
Underweight(<18.5)	2	3.7±0.8	2	2.7±0.5	.3
Normal(18.5-24.9)	74	5.3±3.8	85	5.8±4.5	.6
Overweight(25-29.9)	147	5.2±3.8	136	5.3±3.3	.4

Obese(>30)	110	4.7±2.6	109	4.4±2.4	.2
Age(years)					
<68	168	4.8±3.5	174	4.6±2.8	.5
>68	172	5.2±3.3	166	5.6±3.9	.6

*The mean difference is significant <0.05

** processed meat goods such as sausages and frankfurters

*** Nonsteroidal anti-inflammatory drugs

4.1.4 Association between smoking and UCB

The first subgroup in the smoking status category is “Never” for those who have never smoked. The 217 cases who were non-smokers had an average UCB concentration of 5.3±3.8 µmol/l, and the 212 controls were measured at 5.4±3.7 µmol/l. With the concentrations being so close, the difference was found to be insignificant (P=0.7).

Former smokers in both cases and controls displayed a slightly lower concentration of UCB when compared to those who had never smoked. Former smokers who were also cases had a concentration of 4.8±2.6 µmol/l and the concentration for controls was 4.9±2.8 µmol/l. (P= 0.7).
The last subgroup, smokers, displayed the lowest concentrations of the three groups. The concentration for cases was found to be 4.3±2.6 µmol/l and 4.4±2.7 for controls with a p-value of 0.8.

4.1.5 Association between alcohol consumptions and UCB

The most noticeable difference in concentration for this variable was found to be in the subgroup of those who were former drinkers. The concentration for the cases was 4.2±2.0µmol/l and 5.0±3.7 µmol/l for controls. However, this difference was insignificant at a value of 0.9.
The group who has never drunk had a difference in the average concentration of approximately 6 µmol/l between cases and controls with the latter being lower

(P=0.2). Participants who were regular drinkers barely had any difference between cases and controls (P=0.6).

4.1.6 Association between physical activity and UCB

People in the control group, who exercised on a regular basis, had a higher concentration (6.2±4.3 µmol/l) of UCB than the cases in the same subgroup (5.4±3.2 µmol/l). The opposite was observed in participants who were not physically active: the cases had a higher concentration (5.2±3.5 µmol/l) than the controls (4.8±3.0 µmol/l). The significance level of both subgroups indicates no significant difference.

4.1.7 Association between meat and meat product consumption and UCB

The frequency of meat consumption is shown by the number of times per week meat has been consumed. The first part will discuss the possible association between the consumption of non-processed meat and the concentration of UCB. A concentration of 7.3±4.8 µmol/l was measured in cases who did not consume meat compared to 5.1±3.0 µmol/l in controls of the same subgroup. Very little difference was present across the rest of the groups in this variable, with none having any significant difference.

The frequency of processed meat consumption did not appear to have an impact on the concentration of UCB across cases and controls. None of the significance levels listed in the table for this variable were below the significance level of 0.05.

4.1.8 Association between BMI and UCB

Two different classifications were used for BMI. In the first subclass, participants were categorized into two groups: those whose BMI was under the median and those above it. The median for the Estonian sample population was 27.9 kg/m^2.

The concentration of UCB did not seem to be affected by the BMI in both subcategories.

Based on the BMI classification of the American Cancer Society, BMI was divided into four subgroups, which were then used for the second categorial BMI group. BMI under 18.5 is classified as underweight, 18.5-24.9 is normal, 25-29.9 is overweight, and over 30 is obese (Cancer.org, 2019). 4 participants were classified as underweight, 2 were cases and 2 were controls, which had no significant difference in the concentration of UCB. Moreover, none of the other subgroups had any significant difference in their concentrations.

4.1.9 Age and NSAIDs

The median age of the participants was 68 years. Both age groups, above and under median, showed no significant difference in the distribution of UCB at 0.6 and 0.5, respectively.

The use of NSAIDs did not appear to make a significant difference in the concentration of UCB. Participants who had used them showed no significant difference (P=0.1), as did participants who had not (P=0.6).

4.2 UCB in men and women

In order to examine the association between gender and the UCB concentration, the same variables of table 7 were analyzed for differences across cases and controls after being split by gender. The results are shown in table 8.

The concentration of UCB did not seem to be affected by smoking in men. No significant difference was detected across case and controls in participants who had never smoked, who were former smokers, and who were active smokers.

Women who had never smoked and women who were smokers did not show a significant difference in the UCB concentration between cases and controls,

with p-values of 0.6 and 0.9 respectively. On the other hand, there was a significant difference in the concentration of UCB in women who were former smokers (P=0.035). Former female smokers who had developed CRC had a concentration of 4.7±2.6 µmol/l compared to 3.5±1.7 µmol/l in the controls of the same subcategory.

Alcohol consumption did not appear to influence the UCB concentration in both men and women. In men, the highest concentration was measured in control non-drinkers (6.6±3.7 µmol/l) and the lowest in control former drinkers (4.1±1.9 µmol/l). The highest concentration for women in this category was measured in former drinkers of the control group (6.0±4.9 µmol/l) and the lowest also in the former drinkers of the case group (4.0±1.7 µmol/l).

Table 8: UCB concentration of variables in men and women (Estonia)

Variables										
				Men					Women	
	N	Case	N	Control	P-value	N	Case	N	Control	P-value
Smoking status										
Never	43	5.8±3.4	57	6.4±4.5	.8	16	3.5±1.6	26	3.4±1.8	.6
Former	63	4.8±2.6	47	5.6±3.0	.1	16	4.7±2.6	23	3.5±1.7	.035
Smoker	27	4.8±3.0	29	5.3±3.0	.4	174	5.1±3.8	155	5.0±3.4	.9
Alcohol Consumption										
Currently	109	5.3±3.1	107	6.0±3.8	.1	124	4.9±3.4	121	4.5±2.7	.4
Former	17	4.5±2.3	14	4.1±1.9	.5	19	4.0±1.7	13	6.0±4.9	.5
Never	4	4.8±1.9	10	6.6±3.7	.4	58	5.6±4.7	65	4.7±3.5	.1
Physical Activity										
Yes	57	5.3±3.3	41	7.7±4.8	.001	48	5.5±3.1	42	4.8±3.1	.1
No	53	5.2±3.0	69	5.0±2.7	.6	103	5.2±3.8	109	4.8±3.1	.3
Meat consumption/week										
0 times	5	7.5±2.9	1	9.5±0.0	.6	13	7.2±5.4	9	4.6±2.6	.1
1-2 times	45	5.3±3.1	44	6.1±4.8	.6	95	4.7±3.1	88	4.7±2.9	.9
3-5 times	54	5.4±3.1	69	5.4±3.0	.8	72	5.1±4.2	79	4.9±3.7	.5
6-7 times	29	4.1±2.2	19	6.8±3.1	.001	26	4.8±2.6	26	3.8±1.5	.1
Meat products consumption/week**										
0 times	13	6.9±4.3	13	4.8±2.8	.2	27	5.4±4.1	35	5.3±4.3	.3
1-2 times	43	5.1±3.0	39	6.6±5.1	.2	78	5.0±4.3	65	4.9±3.3	.4
3-5 times	49	5.3±3.0	50	5.4±3.0	.9	65	4.6±2.6	66	4.0±2.0	.1
6-7 times	27	4.2±1.7	31	6.3±2.9	.004	36	5.3±3.5	36	4.6±3.2	.2
Use of NSAID* **										
Yes	11	6.0±3.3	11	5.0±2.8	.4	38	5.7±4.7	30	4.4±2.2	.2
No	122	5.1±3.3	122	5.9±3.8	.06	168	4.8±3.4	174	4.7±3.2	.3
BMI (kg/m^2)										
<27.9	68	5.5±3.3	81	6.1±4.1	.5	88	5.3±4.6	99	5.0±3.5	.4
>27.9	65	4.8±2.6	52	5.6±2.9	.1	118	4.7±2.8	105	4.3±2.7	.2
BMI (kg/m^2) categorial										
Underweight(<18.5)	1	3.1±0.0	NA	NA	NA	1	4.2±0.0	2	2.7±0.4	.6
Normal(18.5-24.9)	30	5.5±3.0	41	6.2±5.3	.7	44	5.1±4.3	44	5.4±3.6	.3
Overweight(25-29.9)	66	5.2±3.2	56	5.8±2.6	.07	81	5.2±4.2	80	4.9±3.6	.5
Obese(>30)	33	4.7±2.4	34	5.4±3.0	.4	77	4.7±2.6	75	4.0±2.0	.06
Age(years)										
<68	70	4.9±2.9	73	5.5±3.2	.3	98	4.8±3.8	101	4.0±2.2	.07
>68	63	5.4±3.0	60	6.4±4.2	.2	109	5.1±3.5	106	5.2±3.7	.7

*The mean difference is significant <0.05

** processed meat goods such as sausages and frankfurters

*** Nonsteroidal anti-inflammatory drugs

Physical activity did not have an impact on UCB in women who exercised (P=0.1) as well as those who did not (P=0.3). Men who were not physically active also did not exhibit any significant difference between the concentrations of UCB in cases and controls. However, physically active men, who belonged to the case group, had a significantly lower concentration (5.3±3.3 µmol/l) than those in the control group (7.7±4.8 µmol/l) at a value of P=0.001.

The frequency of meat consumption did not cause a significant change in the UCB concentration in women. The greatest difference was between cases and controls who had not consumed meat, with the average concentration of the cases at 7.2±5.4 µmol/l and 4.6±2.6 µmol/l for controls. Despite this difference, the p-value showed no significance (P=0.1). In men, meat consumption caused a significant difference (0.001) in the UCB concentration in those who had consumed meat 6-7 times per week. Cases had a much lower average concentration (4.1±2.2 µmol/l) than the controls (6.8±3.1 µmol/l).

Interestingly, the variable “meat product consumption” exhibited the same effect on men and women as did the “meat consumption’ variable. No significant difference was observed in women across all consumption frequencies. Only men who consumed meat products 6-7 times per week exhibited a significant difference (P=0.004).

The use of NSAIDs appeared to have no important influence on the concentration of UCB, with no significant differences between cases and controls in both genders. Age also did not affect the concentration in both age groups, those older than 68 years and those younger.

Both BMI variables, median and categorial, showed no significant difference in any of the subcategories across cases and controls in males as well as in females.

4.3. Correlation between UCB and biomarkers

The last statistical test done on the samples from Estonia was the correlations test in order to determine whether there is a relationship between UCB and the tested variables. The first table, table 9, of this section contains the results of the test for the entire population. In the second table, table 10, are the results of the controls. A third table containing the results for cases can be found in the attachment.

Table 9: UCB concentration correlation with variables (Estonia)

Variable	correlation with UCB	significance P=
BMI	-.085*	.027
Age	.128**	.001
Alcohol (g/d)	.065	.395
Use of hormone	-.085	.103
Use of NSAID	.021	.593

**correlation is significant at the 0.01 level (2-tailed)

*correlation is significant at the 0.05 level (2-tailed)

Table 10: UCB concentration correlation with variables in controls (Estonia)

Variable for controls	correlation with UCB	significance P=
BMI	-.126*	.020
Age	-.136*	.012
Alcohol (g/d)	.127	.229
Use of hormone	-.182*	.014
Use of NSAID	-.034	.537

**correlation is significant at the 0.01 level (2-tailed)

*correlation is significant at the 0.05 level (2-tailed)

4.4 Epic samples

The total number of samples obtained was 688. 149 of these samples came from Sweden. 89 samples were from men, 45 of which developed CRC, while 44 did not. Denmark provided the remaining 539 samples with 329 from male participants and 110 from females. The ratio of case:control was almost 1:1 in samples from both countries. Sweden had 75 cases and 74 controls while Denmark had 270 cases and 269 controls. The average age of participants at the point of blood collection was 57.7±5.0 years, with the youngest participant being 29 years and the oldest 65 years of age. UCB levels exhibited no significant difference between cases and controls (P= 0.7). The results of the descriptive analysis can be found in table 11 along with the differences between the variables in cases and controls.

Table 11: Descriptive analysis for Epic Samples

	Case		Control		P-value
	Frequency (%)	Mean±SD	Frequency (%)	Mean±SD	
Unconjugated bilirubin(µmol/l)		3.6±2.3		3.7±2.4	.7
BMI(kg/m^{2})		26.3±4.0		26.0±4.0	.2
Waist/hip ratio		0.9±0.9		0.8±0.9	.1
Age at blood collection(years)		57.7±5.0		57.7±5.0	.9
Duration of smoking(years)		31.6±12.0		31.4±1.0	.9
Follow-Up(years)		4.5±2		-	
Biomarkers					
ROM(u/ml)		374.6±71.5		376.2±71.1	.7
C-peptide(ng/ml)		6.01±3.5		5.4±3.2	.1
Total adiponectin (myg/ml)		7.8±3.7		8.1±4.0	.4
Hemoglobin A1C(%)		5.8±0.5		5.7±0.5	.9
FRAP(µmol/l)		1101.6±268.4		1118.4±270.1	.4
Hs-CRP(mg/l)		4.0±6.3		3.3±3.4	.5
Gender					.9
Male	210 (30)		208 (30)		
Female	135 (20)		135 (20)		
Country					
Sweden	75 (10)		74 (10)		
Denmark	270 (40)		269 (40)		
Diabetes					.9
No	322 (47)		320 (47)		
Yes	6 (1)		6 (1)		
Don't know	14 (2)		15 (2)		
Unknown	3 (0)		2 (0)		
Smoking Status					.9
Never	121 (18)	121 (18)			
Former	110 (16)	104 (15)			
Smoker	111 (16)	114 (17)			
Unknown	3 (0)	4 (0)			
Cambridge physical activity index					.07
Inactive	46 (7)	43 (6)			
Moderately inactive	102 (15)	106 (15)			
Moderately active	97 (14)	69 (10)			
Active	100 (15)	124 (18)			
Alcohol at recruitment					.7
Never/Seldom	18 (3)	15 (2)			
1 glass/month	0	0			
2-3 glasses/month	95 (14)	97 (14)			
1 glass/week	78 (11)	75 (11)			
2-4 glasses/week	63 (9)	64 (9)			

5-6 glasses/week	63 (9)	73 (11)	
>1 glass/day	24 (4)	15 (2)	
Unknown	4 (1)	3 (0)	
Use of Pill(hrt-ert)			.9
Yes	1 (0)	1 (0)	
No	116 (17)	119 (17)	
Unknown	228 (33)	223 (33)	

*The mean difference is significant <0.05

4.4.1 Gender differences

As with the Estonian samples, table 12 shows differences in UCB between cases and controls in men and women.

Table 12: UCB in men and women divided by case/control (EPIC)

Groups	N	UCB Mean±SD (µmol/l)	Median (µmol/l)	Min-Max (µmol/l)	P-Vlaue
Female	270	3.1±2.0	2.8	0.1-13.3	.3
Case	135	3.0±1.7	2.6	0.1-10.5	
Control	135	3.3±2.0	2.8	0.1-13.3	
Male	418	4.0±2.6	3.5	0.1-22.5	.7
Case	210	4.0±2.6	3.4	0.6-18.0	
Control	208	3.9±2.6	3.5	0.1-22.5	

*The mean difference is significant <0.05

The mean value of UCB for all females was 3.1 µmol/l. when split into the cases and controls, the mean value was 3.0 µmol/l and 3.3 µmol/l respectively. The difference has been shown not to be significant amongst the female population (P=0.3). Males had a mean of 4.0 µmol/l in the average population as well as in cases, while controls had slightly less concentration of 3.9 µmol/l, with no significant difference across cases and controls (P=0.7).

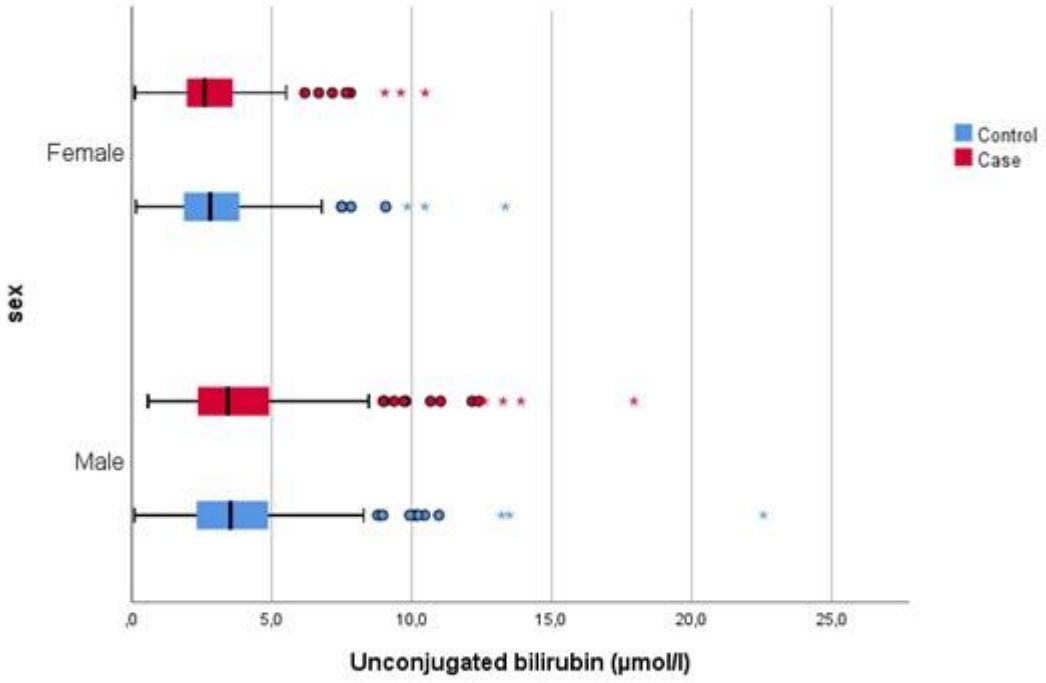

Figure 16: UCB concentrations in cases and controls grouped by gender (EPIC)

A closer look at the boxplot reveals very little difference between medians across cases and controls in both men and women. The median in female cases was 2.6 µmol/l and 2.8 µmol/l for controls, with a maximum value of 13.3 µmol/l shown as an outlier in the control group. The median for male cases was 3.4 µmol/l and 3.5 µmol/l for controls and the highest concentration was 22.5 µmol/l also occurring in the control group.

4.5 Association between UCB and variables

Table 13 shows the concentrations of bilirubin measured across various variables categorized in cases and controls. The goal was to determine whether there is a significant difference between the two groups. The results will be briefly discussed in this section.

Table 13: UCB concentration of variables for cases and controls (EPIC)

Variables		Frequency/UCB±SD (µmol/l)			
	N	Case	N	Control	P-value
Smoking status					
Never	121	3.8±2.0	121	3.9±2.3	.8
Former	110	4.0±2.7	104	4.3±2.8	.1
Smoker	111	2.9±1.6	114	2.8±1.9	.3
Diabetes					
Yes	6	3.4±1.5	6	3.2±1.7	.8
No	322	3.6±2.3	320	3.7±2.4	.8
Physical Activity					
Inactive	46	3.7±2.0	43	3.2±1.7	.1
Moderately inactive	102	3.7±2.5	106	4.0±3.0	.3
Moderately active	97	3.3±2.0	69	3.8±2.3	.1
Active	100	3.8±2.5	124	3.5±2.1	.3
Alcohol Consumption					
Never/Seldom	18	3.3±2.1	15	3.3±1.8	.9
1 glass/month	-	-	-	-	-
2-3 glasses/month	95	3.6±2.1	97	3.6±1.8	.6
1 glass/week	78	3.8±2.3	75	3.7±3.0	.4
2-4 glasses/week	63	3.3±2.1	64	3.5±2.5	.9
5-6 glasses/week	63	4.2±3.0	73	4.1±2.6	.9
>1 glass/day	24	3.0±2.0	15	3.8±1.8	.1
BMI (kg/m^2)					
<25.4	159	4.0±2.8	179	3.7±2.3	.8
>25.4	186	3.3±1.7	164	3.6±2.6	.6
BMI (kg/m^2) categorial					
Underweight(<18.5)	2	2.5±0.7	1	3.0±0.0	1
Normal(18.5-24.9)	137	4.1±3.0	155	3.7±2.2	.6
Overweight(25-29.9)	140	3.4±1.8	140	4.0±2.8	.1
Obese(>30)	57	3.2±1.7	44	3.1±1.7	.4
Age(years)					
<59.2	173	3.7±2.5	169	3.5±2.3	.7
>59.2	172	3.5±2.1	174	3.8±2.5	.5

*The mean difference is significant <0.05

4.5.1 Association between smoking and UCB

Smoking habits were divided into 3 categories: never has smoked, former smoker, and current smoker. Non-smokers who had developed CRC had an average concentration of 3.8 µmol/l compared to 3.9 µmol/l in those who had not (P=0.8). The former smokers case group displayed a slightly lower value of 4.0 µmol/l than that of the controls with 4.3 µmol/l, but this difference is shown to not be significant (P=0.1). The last group in this category is the smokers, with a concentration of 2.9 µmol/l and 2.8 µmol/l for cases and controls, respectively. A P-value of 0.3 indicates an equal distribution of UCB across the sub-group.

4.5.2 Association between diabetes and UCB

Only 12 participants were diabetic, half of which in the case group scoring an average of 3.4 µmol/l UCB, and the other half in the control with a concentration of 3.4 µmol/l. the difference between the diabetic category (P=0.8) was not significant.

4.5.3 Association between physical activity and UCB

Physical activity was divided in accordance with the Cambridge physical activity level index into four subgroups: inactive, moderately inactive, moderately active, and active. Cases in the inactive subcategory showed a slightly higher concentration of 3.7 µmol/l than those of controls with 3.2 µmol/l, but this difference seems to be insignificant. In the moderately inactive group, a value of (P=0.3) indicates no difference in the concentrations between cases, with 3.7 µmol/l, and controls, with 4.0 µmol/l. A value of 3.3 µmol/l for cases and 3.8 µmol/l for controls in the moderately inactive group show no significance difference as well (P=0.1). Controls who were physically active had a slightly lower UCB concentration of 3.5 µmol/l than active cases with 3.8 µmol/l. nevertheless, the difference was not significant (P=0.3).

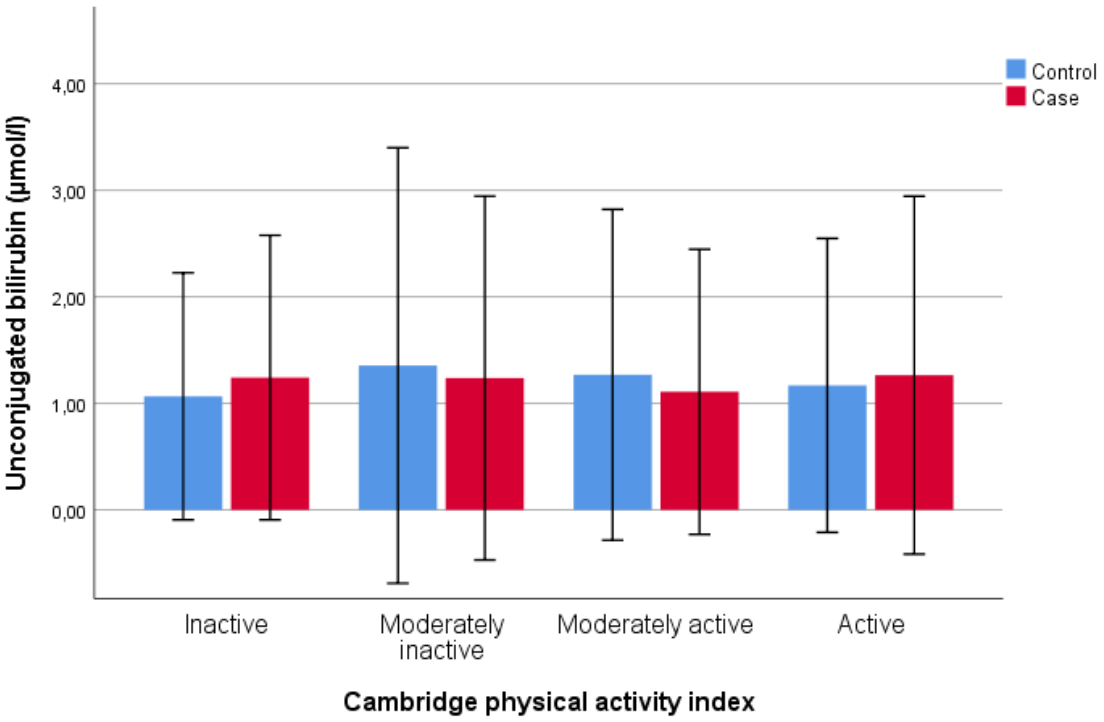

Figure 17: UCB concentrations in cases and controls grouped by physical activity

4.5.4 Association between alcohol consumption and UCB

Alcohol consumption was measured by glass consumed during a certain period of days (month, week, and day). Throughout the entire group, there were no significant differences between cases and controls across each subcategory. The most noticeable can be observed in participants who consumed more than one glass a day with a concentration of 3.0 µmol/l in cases and 3.8 µmol/l in controls. This difference, however, appears to not be significant (P=0.1).

4.5.5 Association between BMI and UCB

The categorization of the BMI variable in this section is identical to the BMI variable of the Estonian samples discussed in more details in the previous section. The median value was calculated at 25.4 kg/m^2. In the group under 25.4, the difference was shown to be insignificant. The same could be said

about the group which BMI was above 25.4. None of the categorical BMI subgroups have shown to have any significant difference across cases and controls.

4.5.6 Association between age and UCB

The categorical age groups were divided, as with the BMI variable, into 2 subgroups: first group for those aged below the median and the second for those aged above the median. The age median was 59.2. There was no significant difference in both subgroups.

4.6 UCB in men and women

Table 14 shows the concentration of UCB across variables divided by cases and controls for each gender. The variable categories, as well as the significance level, are identical to those in the previous table.

Smoking did not seem to influence the concentration of UCB in men. The differences across cases and controls in men who had never smoked, former smokers, and current smokers are insignificant for each subgroup.

In women who are active smokers and who had never smoked, the difference is also insignificant. However, former female smokers who had developed CRC had a considerably lower concentration of 3.2 µmol/l compared to control former smokers with a concentration of 4.3 µmol/l. However, the difference is considered insignificant.

The amount of alcohol consumed by men and women did not seem, to have had an association with the UCB concentrations in both cases and controls.

Table 14: UCB concentration of variables in men and women (EPIC)

Variables										
				Men					Women	
	N	Case	N	Control	P-value	N	Case	N	Control	P-value
Smoking status										
Never	51	4.8±2.6	59	4.4±2.5	.4	70	3.2±1.8	62	3.4±2.0	.5
Former	89	4.4±2.8	77	4.3± 3.0	.7	21	3.2±2.1	27	4.3±2.4	.037
Smoker	67	3.2±2.0	68	3.1±2.1	.5	44	2.6±1.1	46	2.5±1.5	.4
Diabetes										
Yes	6	3.4±1.5	4	3.6±2.0	1	NA	NA	2	2.3±1.1	-
No	191	4.1±2.6	193	4.0±2.6	.5	131	3.0±1.7	127	3.3±2.0	.3
Physical Activity										
Inactive	25	3.8±1.9	28	3.4±1.8	.2	21	3.5±2.2	15	2.8±1.5	.1
Moderately inactive	59	4.4±2.9	54	4.5±3.6	.9	43	2.8±1.7	52	3.6±2.3	.031
Moderately active	63	3.5±2.1	45	4.0±2.5	.3	34	3.0±1.8	24	3.4±2.1	.2
Active	63	4.3±3.0	80	3.8±2.1	.4	37	3.0±1.3	44	3.0±1.8	.3
Alcohol Consumption										
Never/Seldom	8	4.0±2.0	9	3.8±2.0	.5	10	2.7±2.1	6	2.6 ±1.2	.8
1 glass/month	NA	NA	NA	NA	NA		NA	NA	NA	NA
2-3 glasses/month	43	4.2±2.2	5	3.8±1.8	.4	52	3.0±1.9	44	3.3±1.9	.4
1 glass/week	45	4.3±2.6	45	4.3±2.6	.8	33	3.0 ±1.5	42	2.9±1.5	.5
2-4 glasses/week	39	3.5±2.2	40	3.2±2.1	.5	24	3.1±1.8	24	3.8 ±3.0	.5
5-6 glasses/week	48	4.6±3.3	56	4.2±2.7	.5	15	2.8±1.2	17	3.8±2.2	.3
>1 glass/day	23	3.0±2.0	13	4.0±1.9	.1	1	1.1±0.0	2	3.1±2.3	.6
BMI (kg/m^2)										
<25.4	93	4.5±3.1	94	3.9±2.3	.3	66	3.2±2.0	85	3.6±2.2	.6
>25.4	117	3.6±1.9	114	4.0±2.8	.3	69	2.7±1.3	50	2.7±1.6	.7
BMI (kg/m^2) categorial										
Underweight(<18.5)	NA	NA	NA	NA		2	2.5±0.7	1	3.0±0.0	1.0
Normal(18.5-24.9)	78	4.6±3.4	80	4.0±2.4	.3	59	3.3±2.1	75	3.4±2.0	.6
Overweight(25-29.9)	92	3.8±2.0	101	4.2±3.0	.7	48	2.5±1.0	39	3.4±2.5	.1
Obese(>30)	33	3.3±1.8	26	3.4±2.0	.9	24	3.1±1.7	18	2.6±1.1	.3
Age(years)										
<59.2	106	4.1±2.9	103	3.8±2.5	.6	67	3.0±1.6	66	3.2±2.0	.9
>59.2	104	3.9±2.1	105	4.1±2.7	.9	68	2.9±1.8	69	3.4±2.2	.2
Use HRT										
Yes	-	-		-	-	1	2.4±0.0	1	4.0±0.0	1.0
No	-	-		-	-	116	2.9±1.7	119	3.2±2.1	.2

*The mean difference is significant <0.05

The majority of diabetics were men that exhibited no significance difference across cases and controls, with a concentration of 3.4 µmol/l for cases and 3.6 µmol/l for controls. The difference amongst non-diabetic men was also not significant. The total number of females who had DM2 was 2 and both were in the control group. Therefore, testing for differences was not possible for this subgroup. Non-diabetic women did not exhibit a significant difference.

Physical activity did not appear to have an impact on UCB levels in men. All four subcategories displayed differences at non-significant levels.

Women who were physically or moderately active and inactive showed no difference in significance across the case and controls groups. Moderate inactivity did not have a significant difference (P=0.31). In order to further investigate the association of inactivity and UCB, the four groups were merged into two groups: inactive/moderately inactive and moderately active/active and retested. The results Indicated no significant difference in the first group (P=0.3) as well as in the second group (P=0.8).

Male cases, whose weight was below the BMI's median, had a higher concentration of UCB of 4.5 µmol/l compared to 3.9 µmol/l for the male controls. This difference, however, was not significant. Interestingly, the opposite was observed in males whose BMI is greater than 25.4 kg/m^2. Male controls had a higher concentration of 4.0 µmol/l compared to cases with 3.6 µmol/l, with the difference also being insignificant (P=0.3). Similarly, female cases and controls showed no significant difference in the BMI group <25.4 kg/m^2 (P=0.6) as well as the >25.4 kg/m^2 group (P=0.7).

The categorical BMI group for males revealed no men were classified as underweight. The normal subgroup has the most notable difference between cases with a concentration of 4.6 µmol/l and controls with 4.0 µmol/l. This difference was, nonetheless, insignificant. The two remaining groups, overweight and obese, did not have any significant differences as well. A total number of three women were classified as underweight, two in the case group and one in the control group, with an insignificant difference in the concentrations of unconjugated. The concentrations that vary the most were

found to be in the overweight subgroup, cases measured at 2.5 µmol/l and controls at 3.4 µmol/l, but the value of the difference was found to be insignificant. The normal and obese subgroups had no significant differences in their concentrations (P=0.6 and P=0.3, respectively).

The concentration of UCB (4.1 µmol/l) was slightly higher in male cases under the age of 59.2 years when compared to controls, 3.8 µmol/l, with a difference value of P=0.6. The effect was reversed in males older than 59.2 years, where cases had a concentration of 3.9 µmol/l and controls had 4.1 µmol/l. The difference, however, is too small to be significant. Females under the age of 59.2 barely had a difference between cases (3.0 µmol/l) and controls (3.2 µmol/l) with a value of 0.9. The subgroup of women over the age of 59.2 years had a slightly bigger difference between cases (2.9 µmol/l) and controls (3.4 µmol/l), which was also insignificant.

The total number of women who have used hormone replacement therapy were only two, one was a case and had a concentration of 2.4 µmol/l, and the other was a control with a 4.0 µmol/l concentration. Even though the difference is high, the very low number of two women makes this difference statistically insignificant. For women who were not on hormone replacement therapy, the difference was found to be insignificant as well.

4.7 Correlation between UCB and biomarkers

A correlations test was performed to determine whether a correlation exists between UCB and variables. Various biomarkers were also tested for correlation with UCB. The test results can be found in table 15 for the entire EPIC study population, and table 16 includes the results for the control group. A table with the results for the case group can be found in the attachment of this thesis.

Table 15: UCB concentration correlation with variables (EPIC)

Variable	correlation with UCB	significance P=
BMI	-.079*	.038
Waist/hip ratio	.045	.335
Duration of smoking (years)	-.233**	.000
Alcohol at recruitment(g/d)	.044	.249
Biomarkers		
*ROM(u/*ml)	-.233**	.000
C-peptide(ng/ml)	-.017	.702
Total adiponectin (myg/ml)	-.093*	.037
Hemoglobin A1C (%)	-.222**	.000
FRAP(µmol/l)	.069	.094
Hs-CRP(mg/l)	-.191**	.000

**correlation is significant at the 0.01 level (2-tailed)

*correlation is significant at the 0.05 level (2-tailed)

Table 16: UCB concentration correlation with variables in controls (EPIC)

Variable for controls	correlation with UCB	significance P=
BMI	-.069	.204
Waist/hip ratio	.029	.631
Duration of smoking (years)	-.267**	.000
Alcohol at recruitment(g/d)	.063	.242
Biomarkers		
*ROM(u/*ml)	-.161**	.008
C-peptide(ng/ml)	-.018	.779
Total adiponectin (myg/ml)	-.148*	.024
Hemoglobin A1C (%)	-.194**	.004
FRAP(µmol/l)	.052	.393
Hs-CRP(mg/l)	-.197**	.002

**correlation is significant at the 0.01 level (2-tailed)

*correlation is significant at the 0.05 level (2-tailed)

5. DISCUSSION

The purpose of this thesis was to examine whether there is a relationship between UCB and CRC. This was done by analyzing the concentration of bilirubin in samples sent by two different biobanks from Estonia and by the EPIC biobank, which were then tested for differences in men and women. Both genders had data obtained from healthy individuals, controls, and patients who have had CRC, cases.

Men had a higher concentration of UCB than women in data obtained from both biobanks. The gender effect was also observed in two other studies where they measured total serum bilirubin (Zucker et al., 2004; Ioannou et al., 2006).

The results in this thesis have shown no significant differences in the concentration of UCB between cases and controls. Estonian women with CRC had a slightly elevated concentration of 5.0±3.6 µmol/l when compared to women who were healthy with a concentration of 4.6±3.1 µmol/l. The opposite was observed in men, where controls had a concentration of 6.0±3.7 µmol/l and CRC patients had 5.2±3.0 µmol/l. CRC female patients from the EPIC study were shown to have had a lower concentration (3.0±1.7 µmol/l) than the controls (3.3±2.0 µmol/l). On the other hand, male cases of the EPIC study had a slightly higher concentration of 4.0±2.6 µmol/l than healthy participants with a concentration of 3.9±2.6 µmol/l, however all findings are insignificant.

These findings were consistent with the results of a cohort study done by Ioannou et al (Ioannou et al., 2006) aimed to investigate the role of baseline serum bilirubin on the incidence of CRC in the US population. Data from 110 cases of CRC provided by NHANES I were tested in order to determine whether a correlation exists to UCB. This cohort measured total serum bilirubin and any association that UCB could have had with CRC would have been difficult to establish. The role gender plays on the concentration was not taken into consideration, as the analysis was done on the entire population. Additionally, the participants were not asked to fast prior to blood collection.

Contrasting results were reported by Zucker et al. who performed an analysis of data obtained from NHANES III. The study included data of 20,216 participants. They have found a decreased incidence of developing CRC with each 1 mg/dL increase of serum bilirubin. Despite the high number of participants in the study, only 88 had CRC compared to 685 in the study done for this thesis. Additionally, only total serum bilirubin was measured, making it impossible to determine whether the observed effect was caused by conjugated or UCB.

Jirásková et al (Jirásková et al., 2012) also reported a possible protective effect against CRC in individuals who are UGT1A1*28 allele carriers, which plays an important role bilirubin homeostasis. They observed a 20% decrease in CRC risk which was more pronounced in men than in women. As with the previously mentioned studies, the measured biomarker was total serum bilirubin and not UCB. For measuring bilirubin, only 174 cases and 247 controls were included.

Smoking has been shown to be inversely related to serum bilirubin concentration (Schwertner, 1998). In both sets of data, the concentration of UCB was lower in active smokers and former smokers than those who had never smoked. Similar results were reported by a Belgian study which reported a decrease in the concentration of bilirubin in smokers, with the association being more distinct in men (Hoydonck et al., 2001). The exception was the women of the Estonian population, where active smokers had a higher concentration in cases than controls, as well as former and non-smokers. Former female smokers in both populations showed a significant difference in the concentration of UCB between cases and controls. Opposite trends were observed in the subcategory of female smokers: former Estonian smokers with CRC had a higher concentration than the controls, while the Epic former smokers in the controls group had a higher concentration than women who had developed CRC.

BMI was shown to have a negative correlation with the concentration of UCB in participants from both cohorts. Similar results were reported by a Korean study

which investigated the relationship between total serum bilirubin and metabolic syndrome (Choi et al., 2013).

A positive correlation was observed between age and UCB concentration in the Estonian population. The same result was observed in a cohort analysis of the Rancho Bernardo Study where there was a slight increase in UCB with age (Boland et al., 2014).

A major factor contributing to CRC is oxidative stress. Reactive oxygen metabolite (ROM) and ferric reducing ability of plasma (FRAP), were included in the EPIC data and were tested for correlation with UCB. Reactive oxygen metabolite was found to be inversely related to UCB while the ferric reducing ability of plasma did not appear to be significantly related to UCB. The relationship between the two biomarkers and CRC was investigated in a cohort study and found similar results, with the reactive oxygen metabolite being associated with CRC risk and the ferric reducing ability of plasma showing no correlation with CRC (Leufkens et al., 2011).

C-peptide, Hemoglobin A1c, and Adiponectin were additional biomarkers included with the EPIC data used for the diagnosis of DM2. Hemoglobin A1c is used to measure glucose metabolism and gives an accurate measure of the average glucose levels in the blood over a period of 2-3 months. Due to the fact that it is more stable than alternative tests of glucose, it is considered a great marker of DM2. A case-control study investigated the relationship between HbA1c and the aggressive forms CRC and found them to be related, as hyperglycemia is a significant risk factor for that type of cancer (Siddiqui et al., 2008). The analysis done for this thesis showed a negative correlation between UCB and the biomarker Hemoglobin A1c. According to a meta-analysis done on adiponectin, low levels of this biomarker could be a factor contributing to the development of CRC (Lu et al., 2018). The analysis done for this paper has found an inverse correlation between total adiponectin and UCB concentrations.

An important marker for inflammation, high sensitivity CRP, was also amongst the available biomarkers. It is produced in the liver as a defense mechanism against infections, chronic inflammatory conditions, and cancer. CRC patients

have shown to have elevated levels of high sensitivity CRP (Erlinger, 2004). An inverse relationship between bilirubin and high sensitivity CRP was reported in a metabolic syndrome study, indicating a possible role of decreased levels of bilirubin on inflammation (Deetman et al., 2013). An inverse correlation was also detected between high sensitivity C-reactive protein and UCB in the EPIC samples analyzed in this study.

These findings suggest a possibility for UCB to be used as a biomarker for detecting oxidative stress which is, as mentioned previously, an important factor in the development of CRC.

The main organ in which bilirubin is metabolized is the liver. A number of factors could contribute to an increase in UCB levels some of which are liver diseases. That makes bilirubin and bilirubin conjugates excellent biomarkers for the detection of liver diseases and liver dysfunctions (Chowdhury et al, 2013). The data provided for this study did not include information concerning the presence of liver diseases and were therefore unable to exclude patients with liver conditions that could contribute to elevated serum levels of UCB.

Based on the findings of this study, no significant relationship was established between CRC and UCB.

6. CONCLUSION

The results of this paper have shown no significant difference in the concentration of UCB between healthy participants and individuals with CRC.

Lifestyle factors, such as smoking, appeared to have had an effect on the UCB concentration. Participants who were active smokers and those who were former smokers had a lower concentration than those who had never smoked. The same impact was reported in many other studies done on the relationship between levels of bilirubin and smoking (Schwertner, 1998). BMI was another factor that was inversely correlated to UCB in both populations.

A major limitation of this research thesis is the lack of liver biomarker data. This made it impossible to exclude patients with liver diseases that increases the concentration of UCB significantly. Hence, it was not possible to definitely determine the reason behind the elevated concentrations.

Based on the data of this master's thesis, no relationship between the concentration of bilirubin and CRC was identified. The lack of liver biomarkers was crucial in order to be able to distinguish whether higher concentrations were caused by liver conditions or by other factors. Therefore, further research is warranted to investigate the possible relationship between UCB and CRC.

7. SUMMARY

The aim of this thesis was to investigate the possible relationship between UCB and CRC. This was done by analyzing the concentration of UCB of CRC cases and individually matched controls by using high-performance liquid chromatography.

A total number of 1368 samples, 685 CRC cases and 683 individually matched controls, obtained from two different European cohorts, were tested for their UCB concentration. The goal was to investigate the presence of a correlation between UCB and the prevalence of CRC.

The statistical analysis of the data was done using the program SPSS to attempt and find a relationship by comparing the UCB concentrations of CRC patients to those of the controls group. Additional data was provided by the biobanks that could also influence the concentration of UCB such as BMI, smoking status, alcohol consumption, and age. Those variables were additionally tested using a correlations test in order to determine how they relate to the concentration of UCB. Since it was established that men naturally have a higher concentration of UCB, the data were also analyzed separately between men and women to determine the effect of gender.

The results of the data from both biobanks showed no significant difference in UCB levels between cases and controls in both men and women.

8. ZUSAMMENFASSUNG

Das Ziel dieser Arbeit war den möglichen Zusammenhang zwischen nicht konjugiertem Bilirubin und der Inzidenz von Darmkrebs zu untersuchen. Dies erfolgte durch die Analyse der Konzentration von nicht konjugiertem Bilirubin bei Darmkrebspatienten sowie angepasster Kontrollen mittels Hochleistungsflüssigkeitschromatographie.

Insgesamt wurden 1368 Proben aus zwei verschiedenen Biobanken auf ihre Konzentration von unkonjugiertem Bilirubin (UCB) untersucht. 685 der Teilnehmer hatten Darmkrebs entwickelt und 683 waren gesunde Kontrollen. Das Ziel war den Zusammenhang zwischen nicht konjugiertem Bilirubin und der Prävalenz von Darmkrebs zu untersuchen.

Die statistische Analyse der Daten wurde unter Verwendung des Programms SPSS durchgeführt, um eine Korrelation zu untersuchen, wurden die USB-Konzentrationen von Darmkrebspatienten mit denen der Kontrollgruppe verglichen. Zusätzliche Daten, die auch die Konzentration von UCB beeinflussen könnten, wie BMI, Raucherstatus, Alkoholkonsum, oder das Alter wurden von Biobanken zu Verfügung gestellt. Diese Variablen wurden zusätzlich auf Korrelationen getestet. Da festgestellt wurde, dass Männer eine höhere Konzentration an UCB aufweisen, wurden die Daten auch bei Männern und Frauen getrennt analysiert, um den Einfluss des Geschlechts zu berücksichtigen.

Die Ergebnisse der Daten beider Biobanken zeigten keinen signifikanten Unterschied in den UCB Spiegeln zwischen Fällen und Kontrollen, weder bei Männern noch bei Frauen.

9. ATTACHMENTS

Estonia: UCB concentration correlation with variables in cases

Variable	correlation with UCB	significance P=
BMI	-.039	.474
Waist/hip ratio	.004	.944
Alcohol (g/d)	-.009	.935
Use of hormone	-.038	.604
Use of NSAID	.073	.181

**correlation is significant at the 0.01 level (2-tailed)

*correlation is significant at the 0.05 level (2-tailed)

Epic:UCB concentration correlation with variables in cases

Variable	correlation with UCB	significance P=
BMI	-.084	.120
Waist/hip ratio	.060	.326
Duration of smoking (years)	-.196**	.004
Alcohol at recruitment(g/d)	.023	.673
Biomarkers		
*ROM(u/*ml)	-.301**	.000
C-peptide(ng/ml)	-.014	.823
Total adiponectin (myg/ml)	-.041	.501
Hemoglobin A1C (%)	-.251**	.000
FRAP(µmol/l)	.081	.145
Hs-CRP(mg/l)	-.177	.002

**correlation is significant at the 0.01 level (2-tailed)

*correlation is significant at the 0.05 level (2-tailed)

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