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The author of the doctoral dissertation: Natalia Łozińska

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DOCTORAL DISSERTATION

Title of doctoral dissertation: Effect of bile salts and their conjugation on the process of lipolysis

Title of doctoral dissertation (in Polish): Wpływ soli żółciowych i ich konjugacji na mechanism lipolizy

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Supervisor   Signature
dr hab. Christian Jungnickel

Gdańsk, 2024

Załącznik nr 2/2 do Zarządzenia
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DESCRIPTION OF DOCTORAL DISSERTATION

The Author of the doctoral dissertation: Natalia Łozińska

Title of doctoral dissertation: Effect of bile salts and their conjugation on the process of lipolysis

Title of doctoral dissertation in Polish: Wpływ soli żółciowych i ich conjugacji na process lipolizy

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Keywords of doctoral dissertation in Polish: sole żółciowe, trawienie in-vitro, lipoliza, napięcie powierzchniowe, emulsja

Keywords of doctoral dissertation in English: bile salts, in-vitro digestion, lipolysis, interfacial tension, emulsion

Abstract of doctoral dissertation in Polish: Głównym celem rozprawy doktorskiej było wskazanie znaczenia koniugacji soli żółciowych na proces lipolizy, określenie czynników wpływających na zmianę składu soli żółciowych oraz ustalenie procesów kontrolujących szybkość lipolizy. Wyniki eksperymentów (modele trawienia in vitro, badania międzyfazowe) i meta-analizy danych literaturowych połączono w celu określenia najbardziej istotnych czynników wpływających na szybkość procesu lipolizy. Otrzymane wyniki wykazały, że kilka czynników, takich jak antybiotyki, stan chorobowy i skład mikroflory jelitowej, może wpływać na proces trawienia lipidów poprzez działanie soli żółciowych. Wykazano, że sprzężone formy soli żółciowych – taurochlorany sodu zwiększają uwalnianie wolnych kwasów tłuszczowych do znacznie wyższego poziomu niż nieskoniugowane formy soli żółciowych – dezoksycholany sodu, obecnych w naszym przewodzie pokarmowym. Taurochloran sodu wykazał większy potencjał adsorbcji na powierzchni kropli lipidu, zwiększając adsorpcję lipazy i sprzyjając procesowi emulgowania. Co więcej, taurochloran sodu potrzebował mniejszej liczby cząsteczek i stężenia środka powierzchniowo czynnego, aby stworzyć agregaty odpowiedzialne za zbieranie produktów lipolizy z interfazy olejowej. Lipoliza napędzana taurochloranem sodu może osiągnąć większe uwalnianie wolnych kwasów tłuszczowych dzięki szybszemu usuwaniu produktów lipolizy w procesie desorpcji, umożliwiając ciągły proces trawienia lipidów. Wykazano, że lipoliza jest kontrolowana przez stężenie skonjugowanych soli żółciowych poprzez modulację pięciu zidentyfikowanych procesów.

Abstract of the doctoral dissertation in English: The main aim of the PhD dissertation was to indicate the importance of conjugation of bile salts (BS) on the level of lipolysis, determine factors influencing the alteration of BS composition, and establish processes controlling the rate of lipolysis. Experimental results (in-vitro digestion models, interfacial studies) and meta-analysis of literature data were combined to determine the most influential factors affecting the rate of lipolysis. The results demonstrate that several factors such as antibiotics, disease state, and gut microbiota composition may affect the lipid digestion process via the action of BS. Conjugated forms of BS – sodium taurocholate (NaTC) were shown to enhance free fatty acids (FFA) release to a significantly higher level than unconjugated forms of BS – sodium deoxycholate (NaDC) present in our gastrointestinal tract. NaTC showed greater potential to adsorb to the lipid droplet enhancing the adsorption of lipase and promoting the emulsification process. Moreover, NaTC required fewer molecules and surfactant concentration to create aggregates responsible for incorporating lipolysis products from the oil interphase. The lipolysis driven by NaTC could achieve greater FFA release due to faster removal of lipolysis products via the desorption process, allowing the continuous process of lipid digestion. The lipolysis was shown to be controlled by the concentration of conjugated BS by modulation of five identified processes.

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Also, I want to thank my best friend Marianna Tomaszewska for her support and for constantly providing me with an incentive to act.

Dedications

Rozprawę doktorską dedykuję mojemu Tacie.

Table of Contents

Scientific achievements constituting a Doctoral dissertation
"Effect of bile salts and their conjugation on the process of lipolysis"

L.p	Title of scientific publication	MNiSW	IF
A1	**Łozińska, N.,** & Jungnickel, C. (2021). Importance of Conjugation of the Bile Salt on the Mechanism of Lipolysis. *Molecules*, *26*(19), 5764 DOI: 10.3390/molecules26195764	140	4.6
A2	Krupa, Ł., Staroń, R., Dulko, D., **Łozińska, N.,** Mackie, A. R., Rigby, N. M., ... & Jungnickel, C. (2021). Importance of bile composition for diagnosis of biliary obstructions. Molecules, 26(23), 7279. DOI: 10.3390/molecules26237279	140	4.6
A3	**Łozińska, N.**, Maldonado-Valderrama, J., Del Castillo-Santaella, T., Zhou, Y., Martysiak-Żurowska, D., Lu, Y., & Jungnickel, C. (2024). Bile conjugation and its effect on in vitro lipolysis of emulsions. Food Research International, 114255. DOI: 10.1016/j.foodres.2024.114255	140	8.1
	Summary	420	17.954

Scientific achievements that do not constitute a doctoral dissertation

L.p	Title of scientific publication	MNiSW	IF
A4	**Łozińska, N.**, Głowacz-Różyńska, A., Artichowicz, W., Lu, Y., & Jungnickel, C. (2020). Microencapsulation of fish oil–determination of optimal wall material and encapsulation methodology. *Journal of Food Engineering*, *268*, 109730. DOI: 10.1016/j.jfoodeng.2019.109730	140	5.7
A5	Jungnickel, C., & **Łozińska, N.** (2019). Predicting the Environmental Fate of Ionic Liquids, Article Chapter 51-1 , 1-10. DOI: 10.1007/978-981-10-6739-6_51-1	NA	NA

Abbreviations

BA – bile acids
BS – bile salts
BSH – bile salt hydrolase
CMC – critical micelle concentration
FFA–free fatty acids
MSR – molar solubilisation ratio
NaDC – sodium deoxycholate
NaTC – sodium taurocholate
NaGCDC – sodium glycochenodeoxycholate
NaGDC – sodium glycodeoxycholate
PC – primary conjugated
SU – secondary unconjugated

1. Introduction

The increasing problem with obesity in the last decades (recent statistics have shown that the obesity problem increased by up to 50% in Europe (Stival et al. 2022) and 55% in Poland (Rychlik et al. 2022)) highlight the importance of the digestion process, as the controlling factor of calorie uptake. Essential nutrients can be used for energy, repair of cells, and growth, etc are obtained during the digestion process by breaking down food. Absorption of food and the final stage of digestion takes place in the small intestine. One of the main components of our diet is lipids, which are broken done through the process of lipolysis. This process uses BS as the key factor responsible for emulsification and creating micelles which may transport digestion products to our body.

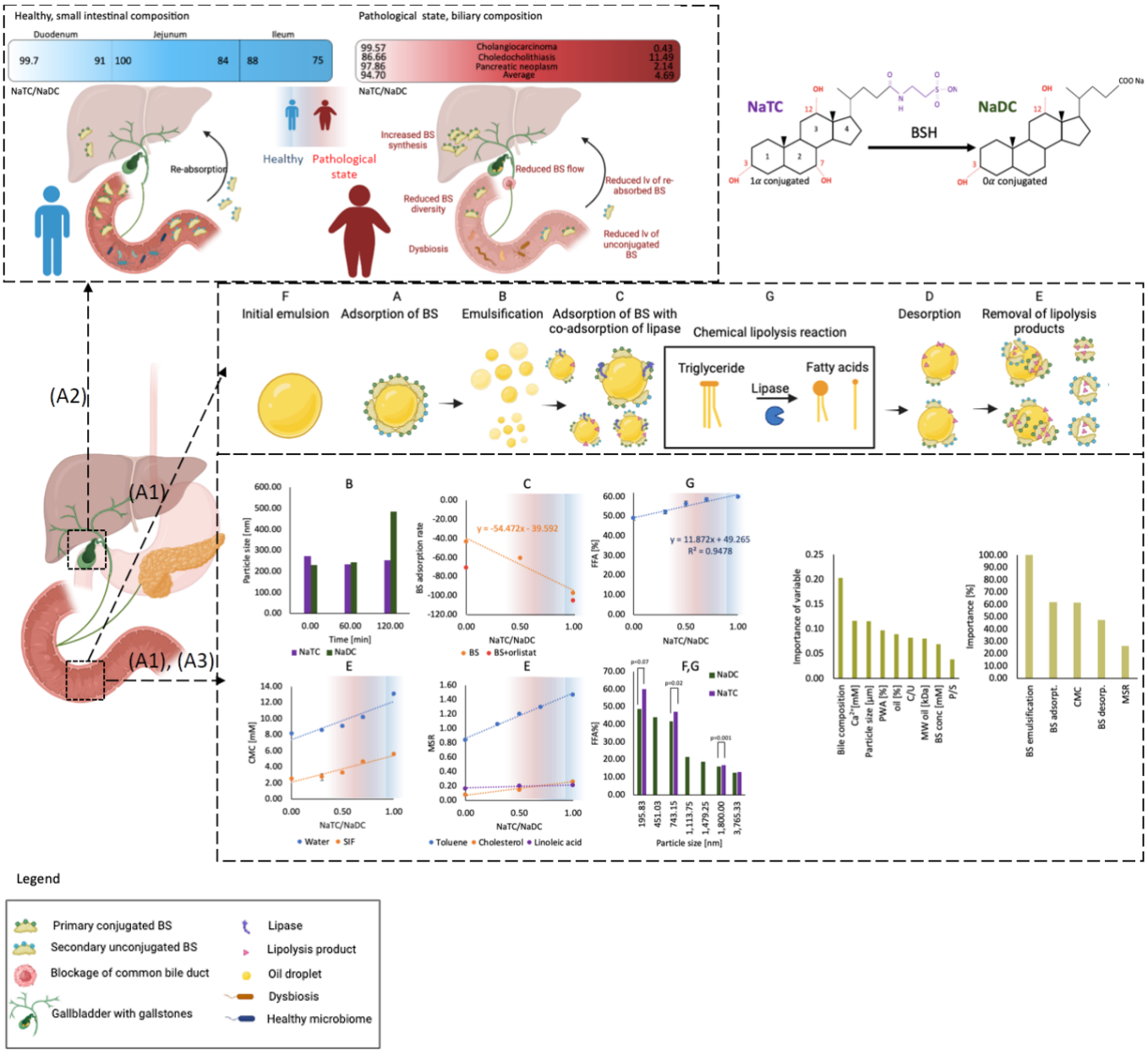


Figure 1 The workflow presents the graphical representation of the results from three scientific articles covering the subject of the dissertation. Publication A1 presents the steps of the lipolysis process: initial emulsion, adsorption of BS and lipase/colipase complex, emulsification process, desorption, and removal of lipolysis products. Each of the steps of lipolysis (A-G) is linked with results from publication A1 and publication A3. The reddish-blue shadow behind the graph shows changes in BS ratio and corresponding alteration of results concerning healthy and pathological states. Publication A2 represents

changes in BS composition in healthy and pathological patients concerning four diseases: cholangiocarcinoma, choledocholithiasis, and pancreatic neoplasm. The composition of BS in pathological patients is disturbed due to the reduction of the flow of BS which results in alteration of gut microflora composition. BS- bile salts, NaTC – sodium taurocholate, NaDC – sodium deoxycholate, CMC – critical micelle concentration, MSR – molar solubilisation ratio, PWA – protein weight average, MW – molecular weight, Ca – calcium, FFA – free fatty acids, C/U – conjugated/unconjugated, P/S – primary/secondary, SIF – simulated intestinal fluid, BSH – bile salt hydrolase.

In our first publication (publication A1) we performed the meta-analysis for three main defined lipolysis parameters: (1) critical micelle concentration (CMC), (2) aggregation number, and (3) molar solubilization ratio (MSR) for four types of BS: Primary conjugated (PC) and unconjugated and secondary conjugated and unconjugated. Our analysis revealed that the type of BS influences the main parameters of the lipolysis process. Further analysis revealed that bacterial transformation in the small intestine results in deconjugation of PC BS into secondary unconjugated (SU) BS. Therefore, representatives of two predominant forms of BS in our gastrointestinal tract were chosen for further analysis.: PC – sodium taurocholate (NaTC) and SU – sodium deoxycholate (NaDC). We used the static pendant droplet technique to screen the difference in interfacial properties of BS. Finally, the in-vitro digestion model was used to check the extent of lipolysis influenced by NaTC and NaDC. Our results indicated that NaTC and NaDC yield significantly different surface activity properties and could influence lipolysis efficiency to a significantly different extent. NaTC was shown to promote the release of free fatty acids (FFA) to a higher extent than NaDC.

Our second publication (publication A2) discussed BS as a disease indicator and also as a factor that is sensitive towards changes in health state. The data collected on the composition of BS during different diseases state: cholangiocarcinoma, choledocholithiasis, pancreatic neoplasm, and stricture have shown significant change concerning the concentration of BS in healthy individuals. The reason for the significant alteration of BS composition was mostly connected with blockage of the flow of BS to the small intestine providing a decreasing concentration of re-absorbed BS and by modulation of molecular receptors increasing BS synthesis. Even though the analysis showed that BS is not specific enough to serve us markers, the results bring attention to the connection between disease development and the possible effect on the lipolysis process due to significant changes in BS concentration. Moreover, the performed analysis revealed that reduced BS concentrations in the small intestine resulted from the development of disease state, and enhanced BS synthesis due to absorption of low concentrations of BS. Normal BS synthesis results in a concentration of BS in the small intestine in the range of 5-10mM (Naso et al. 2019). Increased BS synthesis leads to the formation of excessive concentration of BS, which is correlated with two effects: (1) a greater concentration of conjugated BS in the small intestine may significantly enhance the rate of the lipolysis process which can promote the development of the obesity problem and (2) a formation of excessive concentration of unconjugated BS by intestinal bacteria, which may further disturb BS synthesis and promote development of diseases connected with the toxicity of SU BS.

Finally, our last publication (publication A3) aimed to develop the main outcomes and conclusions from two previous publications: publication A1 and publication A2. First of all the idea of the dominant process during lipolysis was developed including adsorption, co-adsorption of lipase, desorption, formation of micelles, and MSR of lipolysis products. The influence of particle size of initial emulsion in a high range of 200-3800nm was also investigated, to consider the possible variation of delivered food. Results also indicated that the efficiency of previous stages, gastric digestion, also influences the final rate of lipolysis. Moreover, the results showed that with decreasing particle size of the digested emulsion, the efficiency of digestion increases. Duodenum consists of 98% of conjugated BS, it is also where most lipolysis takes place. Conclusions from publication A2 allowed us to consider additional factors influencing lipolysis efficiency, such as the development of disease state, but also the possibility of consuming antibiotics connected with specific diseases and resulting changed BS profile in the small intestine. Therefore, all experiments in publication A3 were performed within the whole range of NaTC/NaDC ratio assuming 0 NaTC/NaDC (100% NaDC) and 1 NaTC/NaDC (100% NaTC). The digestion process was studied by performing experiments covering in vitro digestion models (release of FFA) and surface science (interfacial tension measurements). To determine the most influential parameter controlling the lipolysis process the meta-analysis was performed covering the lipolysis process for single systems (pure BS) and complex systems (BS of various animals). All of the previously collected data, as well as analysis of meta-analysis results, allowed us to consider emulsification as the predominant step. BS by modulating five separate processes have been shown to influence the lipolysis process. Results revealed the possibility of controlling the rate of lipolysis by modulating lipolysis processes.

Modulating the food digestion process is a worldwide challenge. Digestion of the lipid component may be controlled either by altering the food structure or modulating the digestion process. BS in respect to their form may demonstrate different properties, which will influence the final rate of lipolysis and regulate nutrient absorption. The impact of the BS on the lipolysis efficiency is modulated by five processes. The results of publication A1, publication A2 and publication A3, as shown in Figure 1 indicated BS as the agent's modulation of the lipolysis process.

1.1. Digestion of lipids

The research of publication A1 and publication A2 includes the digestion of lipid droplets and the complexity of their evolution during the lipid digestion process. Unravelling the mechanism of lipid digestion gives the potential to modulate the lipolysis process. The research has been focused on lipid digestion, as lipids are common diet components. Humans need to eat as our bodies require sources of energy and building blocks that we cannot provide for ourselves. Digestion is a complex and long process, which starts when we consume food and is responsible for processing material to the form that is useful for our organism. Lipids are an important part of our diet as they are responsible for delivering energy to our body and enable absorption of vitamins A, D, E, and K. They are also widely available and are ingredients of many food products. However, our body cannot just absorb lipids in the form as is delivering to our body. Instead, it needs to be broken down into preliminary parts which we can absorb.The research covered in this work covers small intestinal digestion of lipids, however, the potential impact of digestion in the mouth and stomach were also considered (particle size reduction), as they influence the final efficiency of digestion

1.2. Digestion in the mouth

Consumed food, starts to be digested in the mouth when saliva is released, which is a neutral fluid composed of a mixture of proteins and minerals (Bansil and Turner 2006). The emulsion is destabilized due to the flocculation and coalescence process.(Vingerhoeds et al. 2005; Silletti et al. 2007a, b). During the limited time that food bolus spends in the mouth, the exposure to mastication and temperature results in phase inversion of the emulsion.

1.3. Gastric digestion of lipids

Before small intestinal digestion, food enters the stomach, where it is exposed to the acidic environment created by gastric juices, which results in a decrease in the colloidal stability of the emulsion due to the electrostatic screening of protein. Digestion of lipids in the stomach is driven by gastric lipase which is characterized by high-range activity (pH 3-7) in comparison to intestinal lipase, with an optimal working pH of 6.5 (Hamosh 1990; Carriere et al. 1993; Porter et al. 2007). The optimal activity of gastric lipase was estimated to be around pH 5.4.(Carriere et al. 1993). Therefore digestion of the lipids in the stomach occurs mostly during the first hour, later on, the consumed product may disturb pH level and reduce lipase activity. One hour may not yield enough time to digest lipids, resulting in only partially digested products. The gastric lipase hydrolyses dietary lipids into fatty acids and diglycerides. Gastric hydrolysis yields 5-30% of the total lipid digestion(Armand et al. 1999). Gastric digestion helps to emulsify lipid droplets and, therefore, may enhance the efficiency of further intestinal digestion by increasing the surface area of the lipid droplet. However, gastric hydrolysis yields 5-30% of the total lipid digestion (Golding and Wooster 2010). In the case of emulsions that were tested during my research gastric hydrolysis would not have a significant effect, as the emulsions could be fully digested during the intestinal process. Moreover, partially digested emulsions from the mouth and gastric phase could hide the full action of BS and, therefore were not considered during research.

1.4. Small intestinal digestion of lipids

When food is moved from the stomach to the intestine, pH rapidly increases due to the secretion of alkaline bile juice. Intestinal digestion of lipids is driven by pancreatic lipase(Carriere et al. 1993). The lipolysis process promotes breaking down lipids into FFA and glycerol with the assistance of BS – the key factors responsible for the digestion and absorption of lipids.

The lipolysis process starts when BS is transported to the small intestine. The first role of BS is to improve the adsorption of lipase by increasing the accessibility of the oil droplet. BS acts as an emulsifying agent and increases the surface area of the lipid droplet (Macierzanka et al. 2014). BS are also responsible for displacing the lipolysis material from the oil interface, therefore promoting the adsorption of the lipase(Torcello-Gómez et

al. 2011). BS also play an important role in the transportation of FFA generated during the lipid digestion process to enterocytes where they are absorbed (Maldonado-Valderrama et al. 2011).

BS are multi-tasking biosurfactants, necessary during the digestion process of lipids. Due to its complex and rate-limiting functions during the digestion process, BS were the main interest of our research. Although it was observed that lipolysis is controlled by different parameters acting simultaneously, still there insufficient data to indicate a distinct rate-limiting factor. More detailed research on the relation between lipolysis rate and type of BS should be investigated to understand their contrasting role in this process. The behaviour of the BS is associated with their molecular structure, therefore, understanding the source of their action would give perspective to control the lipolysis process.

1.5. Nature of BS

In the third century, Hippocrates created a concept of the human body being composed of four "humours", consisting of two biles (Guzior and Quinn 2021). The four humours were referred to as *blood*, *phlegm*, *yellow bile*, and *black bile* (Thompson and Turner 1913; Goodacre and Naylor 2020). According to Hippocrates' idea, the body is healthy when humours are balanced and a disease state develops when any of the "humour" is in excess or deficiency (Goodacre and Naylor 2020). Greek physician, Galen, developed the Hippocrates idea by describing personalities with unbalanced "humours". A person with an excessive concentration of *yellow bile* was named choleric and one with an excessive concentration of *black bile* was named melancholic (Goodacre and Naylor 2020). Still, since then we have been trying to understand and we are developing ideas of how the BS ratio influences our health state. The role and importance of BS was more widely understood by discovering the structure of BS.

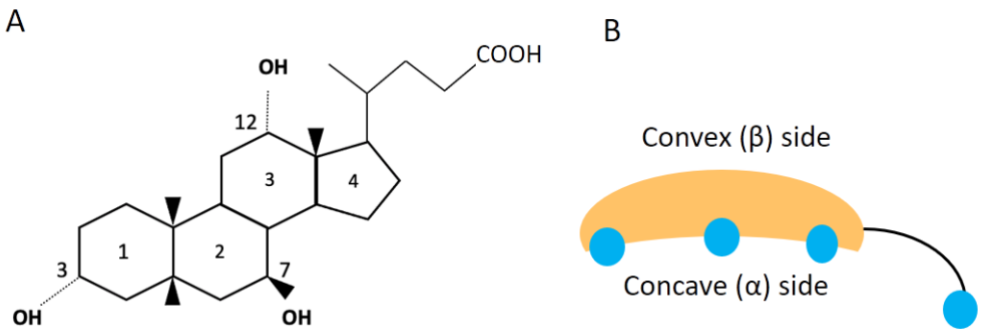


Figure 2 A Structure of BA. Cholic acid is synthesized from cholesterol in the liver and is known as primary unconjugated BS. BS consists of 4 rings. B Planar polarity of BS. The hydrophobic part is located on the convex side (yellow part) consisting of methyl groups and the hydrophilic part is located on the concave side (blue part) consisting of hydroxyl groups. This yields the unique structure of BS, different from standard surfactants.

Over the years the knowledge about BS expanded and the concept of "humour" evolved, and an understanding of the structure and real impact of BS on the human body emerged. The discovery of the chemical structure of BS in 1932 was the breakthrough moment, which enabled further research development (Hofmann and Hagey 2014). BS are surface active, steroid and ionic compounds with an amphiphilic nature (Moghimipour et al. 2015). As shown in Figure 2 A they consist of a steroid skeleton, composed of four rings, three six-carbon rings (1-3) and five carbon rings (4). Differently from traditional surfactants, mostly consist of a polar head and non-polar tail (Holm et al. 2013), BS possessed planar polarity, as shown in Figure 2 B (Warren et al. 2006). The hydrophilic part of BS with hydroxyl groups is located on the concave (α) side and the hydrophobic part with methyl groups is located on the convex (β) side. Different types of BS differ by the number of hydroxyl groups and functional groups, which were shown in publication A1 and publication A3 to have a direct influence on the lipolysis efficiency process. Therefore their concentration and type are of high importance.

1.6. Enterohepatic circulation and alteration of BS structure

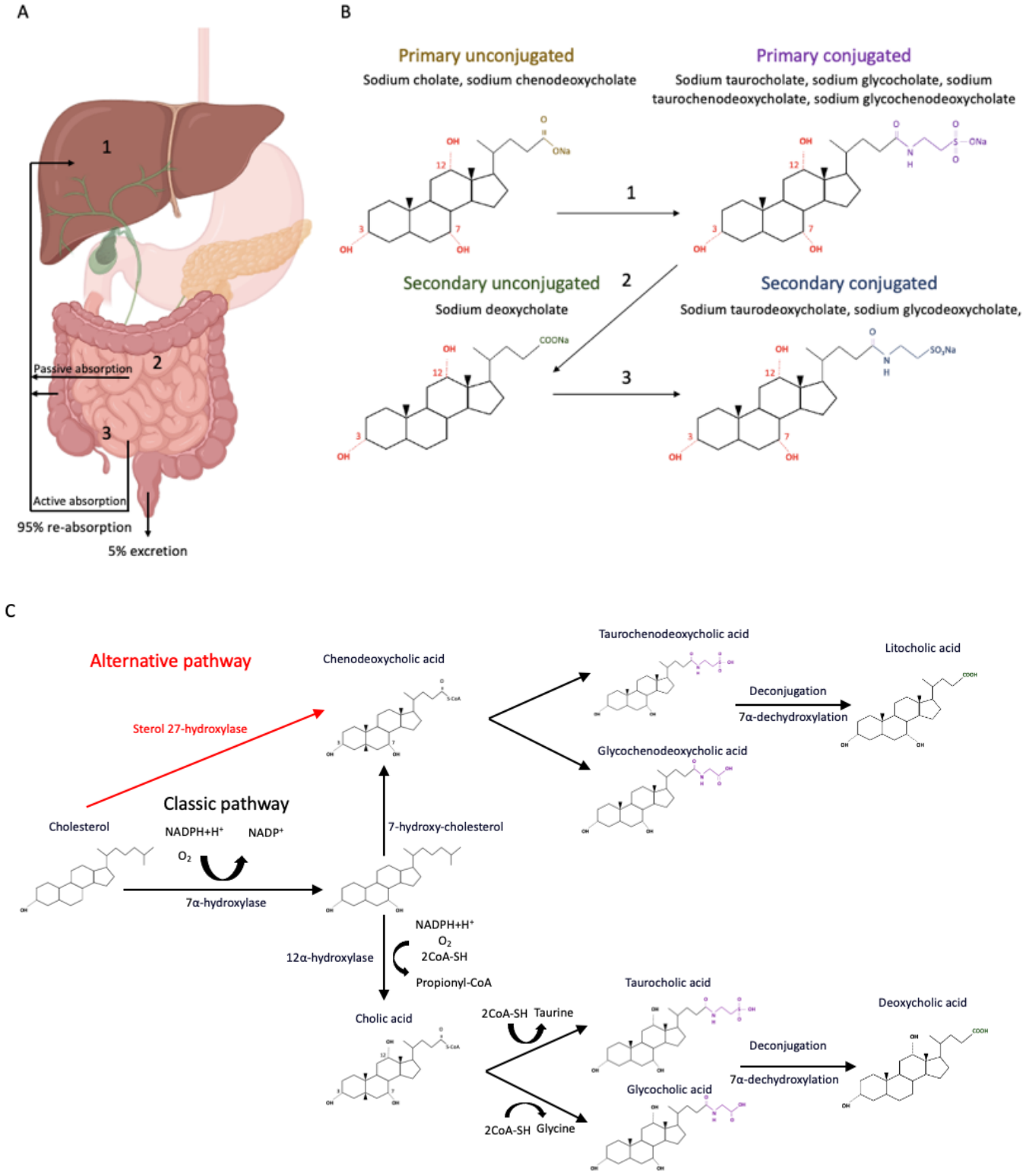


Figure 3 A. Enterohepatic circulation of BS in our gastrointestinal tract (Ridlon et al. 2006). *B. Structures of exemplary four predominant forms of BS in our organism. The number above the arrows corresponds to the transformation processes of BS taking place in marked locations. C. Schematic representation of bile acid synthesis from cholesterol as shown by Moghimipour et al.* (Moghimipour et al. 2015). *Classic pathway represents the formation of cholic acid and chenodeoxycholic*

acid from 7-hydroxy-cholesterol and alternative pathways, knows also as acidic pathways, represent the formation of chenodeoxycholic acid from cholesterol.

The disease state results in changes in BS composition, which in publication A2 was concluded to be an effect of blockage of BS flow. The BS enterohepatic recirculation mechanism controls the synthesis of BS. Increased or reduced concentration of BS synthesis was linked to alteration of lipolysis efficiency. Therefore the proper BS flow and re-absorption are crucial for our organism.
Bile acid (BA) are synthesized from cholesterol in the liver by two possible pathways. Neutral pathways, also known as classical pathways are responsible for synthesizing almost 90% of BA from the liver, while alternative pathways synthesize about 10% of BA. BA either cholic acid or chenodeoxycholic acid are formed from cholesterol, as shown in Figure 3C (Moghimipour et al. 2015). The conjugation of primary BA with glycine or taurine is catalyzed by BA CoA: amino acid N acyltransferase , created respectively by taurocholic acid, glycocholic acid, taurochenodeoxycholic acid and glycochenodeoxycholic acid (Ridlon et al. 2006; Chiang and Ferrell 2018), as shown in Figure 3A number 1. Healthy human bile consists of 75% of glycol-conjugated BS and 25% of tauro-conjugated BS. In the small intestine, BA is transformed into BS due to deprotonation. PC BS is further transformed in the small intestine by bacterial action to create secondary forms, as shown in Figure 3B. The enterohepatic recirculation process controls the flow of BS through our body. Disturbance of the flow of BS, as it was shown in publication A2, leads to a disorder of BS synthesis which is correlated with changed concentration of BS in the small intestine, alteration of the efficiency of the lipolysis process, and development of disease state.

1.7. Effect of changing microbiota on BSH

Intestinal microbiota plays a crucial role in our organism by ensuring a healthy balance (Shreiner et al. 2015). One of its main roles is the deconjugation process of BS. The deconjugation process is known as the removal of the amino acid side chain and as a result, the secondary BS are created (Begley et al. 2005). The formation of secondary BS is catalyzed by bile salt hydrolase (BSH). Most of the bacteria with the ability to promote the deconjugation process are gram-positive bacteria: *Lactobacillus, Enterococcus, Bifidobacterium, Clostridium,* and *Bacteroides spp* (Urdaneta and Casadesús 2017) with the exceptions of two strains of gram-negative Bacteroides (Begley et al. 2006).
There is a strong relationship between BS and gut microbiota composition, as one of the roles of BS is to control gut microbiota composition and the intestinal microflora influence the BS pool size (Sayin et al. 2013). For this reason, the alteration of BS-microflora homeostasis may result in the alteration of BS composition and the development of dysbiosis. Overgrowth of bacterial species not possessing BSH over another one may lead to a change in the ratio of conjugated/unconjugated BS. Therefore, modification of gut microbiota species may influence the concentration of BS. One of the most common reasons for overgrowth the of bacterial species without BSH over another one is antibiotics.

1.8. Antibiotics and gut microbiota

Consumption of antibiotics has become a global trend (Klein et al. 2018). In 2000-2015 using of antibiotics increased up to 65% globally (Nandi et al. 2023). Poland is a country with one of the highest rates of antibiotic consumption (Wojkowska-Mach et al. 2018). Antibiotic utilization was shown to reduce microbial diversity (Ianiro et al. 2020). Results presented by Palleja et al. (Palleja et al. 2018). have shown that consumption of antibiotics by adults results in an increased concentration of *Enterobacteriaceae* and a reduced concentration of *Bifidobacterium* Therefore, it is important to note that antibiotics by themselves do not necessarily decrease the overall number of bacteria but they lead to alteration of their diversity (Duvallet et al. 2017). Bacteria which are sensitive to antibiotics may be eliminated and antibiotic-resistant bacteria can multiply and replace them.
Moreover, BSH activity can be reduced by the administration of antibiotics(Wang et al. 2012). Smith et al. (Smith et al. 2014) have shown the impact of different antibiotic classes on the potential to inhibit BSH. Recent studies have revealed that the concentration of *Lactobacillus*, the main bacteria strain possessing BSH in our small intestine was reduced in the presence of antibiotics (Dumonceaux et al. 2006; Guban et al. 2006). Khodakivskyi et al. (Khodakivskyi et al. 2021) examined the alteration of BSH based on bioluminescence image due to deconjugation of BS. The result revealed the potential of antibiotics to reduce BSH of about 30% of gut microbiota. Furthermore, antibiotics, by changing gut microflora composition, were linked to the development of obesity (Vallianou et al. 2021). Antibiotics are also used as therapeutic agents. Children with malnutrition are treated with *amoxicillin* which results in weight gain (Francis et al. 2023). Studies performed on mice concerning transferring microbiota from obese adult twins to germ-free mice resulted in weight gain of mice (Lange et al.

2016). Antibiotics are external factors, which may influence the composition of the gut microflora and consequently, indirectly influence the BS composition, leading to disturbance of the enterohepatic recirculation system.

1.9. BS and obesity

The research covers the topic of the digestion process of food. However the diet is a key parameter as it may lead to the development of disease, therefore we should control the process of food modulation in our organism. Obesity appears to be a worldwide problem that negatively influences our body development. The diet was considered as a direct factor influencing weight profile. High-fat diet enhances obesity development, while a diet rich in vegetables results in a healthy homeostasis (Sakamaki et al. 2005). Diet was also proven to impact the gut microbiota diversity and complexity, by changing intestinal environmental conditions and influencing the BS composition (Scott et al. 2013).

Except for diet, obesity may be also promoted by changes in microbiota composition, induced for example by taking antibiotics or the development of disease (Li et al. 2021). Antibiotics were considered to reduce the composition of gut microflora and decrease the activity of BSH. (Guban et al. 2006). This reduces the concentration of deconjugated BS and increases the C/U ratio.

Results presented in publication A1 have shown that conjugated BS enhance the lipolysis process to a higher extent than unconjugated BS. The formation of high concentrations of conjugated BS over unconjugated ones may result in the unbalanced process of lipid digestion. Conjugated BS, as it was shown in publication A3, has a faster adsorption rate on the oil droplet than unconjugated BS results in greater surface area and ensures a more effective lipolysis process. Moreover, conjugated BS may more effectively remove lipolysis products through the desorption process than unconjugated BS, which gives more space for another BS to adsorb to the oil droplet and continue the digestion of lipids. Excessive lipolysis rate promoted by conjugated BS may result in the development of obesity. Exorbitant conjugation levels may be considered as a factor contributing to the obesity problem due to the enhanced lipolysis process. Moreover, excessive concentration of conjugated BS may disrupt BS synthesis resulting in reduced BS concentration. Unbalanced BS concentration and disturbed BS synthesis, as shown in publication A2, results in the development of diseases, such as gallstone formation or choledocholithiasis.

Several factors induce changes in BS concentration, by affecting intestinal microbiota, which are responsible for lipolysis efficiency. Therefore, it can be concluded that BS play an important role as a factor controlling the development of obesity by modulating the calorie uptake.

1.10. Role of BS in the lipolysis process

Digestion of lipids takes place in the stomach by hydrolysis of lipids driven by gastric lipase, however, this process was estimated to occur to a limited extent (10-30%). Predominantly (70-90%) lipolysis process takes place in the small intestine due to the hydrolysis of lipids by lipase (Maldonado-Valderrama et al. 2011). Therefore, the research covers the lipid digestion process in the small intestine.

Lipolysis is a well-known and broadly studied process. Its efficiency is mostly measured in the form of FFA releases. It has been previously shown that different compositions of emulsion influence the final FFA release (Wilde et al. 2019). Different forms of BS have also been shown to promote lipolysis efficiency to various extents (Pabois et al. 2020). Previously it was concluded that lipolysis is controlled by three main parameters: 1. Adsorption of BS/lipase to the oil interphase, 2. Emulsification of lipid droplets and 3. Desorption and solubilization of lipolysis products by BS micelles (Golding and Wooster 2010; Bellesi and Pilosof 2021; Łozińska and Jungnickel 2021). Taking into consideration the influence of BS on each aspect of lipolysis, it can be assumed that these parameters are too general. Therefore, based on previous assumptions, there were proposed six separate factors, including five unique parameters (as shown in publication A1 and publication A3) of which lipolysis consists. First (1), BS **adsorb** on the oil interface, promoting the emulsification process, removing surface materials such as proteins or emulsifiers and facilitating adsorption of the pancreatic lipase/colipase. Higher adsorption of the BS on the oil surface may suggest that they can facilitate the lipolysis process by enhancing pancreatic lipase/colipase to adsorb. Moreover, the higher the ability of the BS to break down the fat droplet (**surface tension of oil droplet**) into smaller droplets, the higher the surface area would be available for lipase/colipase to adsorb (2). Next, lipase/colipase promotes the hydrolysis of triglycerides into FFA and monoglycerides, which stay at the interface of the oil droplet, inhibiting further digestion by blocking the contact of lipase/colipase with the oil interface (3). BS create small aggregates called mixed micelles, which act as

vehicles for lipolysis products. BS can incorporate those products, remove them from the interphase and further transport them (4). After solubilizing lipolysis products into their structures BS desorb from the soil surface (5). **CMC** displays the minimum concentration of the substance to create those aggregates. Therefore, a lower CMC of the BS would indicate a lower concentration necessary for starting agglomeration which may be beneficial for lipid digestion. A smaller **aggregation number** of the BS would mean that a lower number of surfactants is required for micelle creation. Thus, within the same concentration of the BS, assuming that conjugated forms of the BS reveal smaller CMC and aggregation numbers, a greater number of the micelle might be created in comparison to unconjugated BS. Moreover, the ability of the BS to incorporate lipolysis products into mixed micelles is evaluated by the **MSR** of the compound. The higher the MSR, the greater amount of the substance would be incorporated, therefore more lipolysis products might be removed from the oil surface, providing more space for lipase/colipase to adsorb. The final rate of the lipolysis will be measured by the FFA released. The greater the number of the FFA released, the higher the extent of the lipolysis may be achieved.

1.11. Adsorption of BS

The ability of the lipase to adsorb to the lipid droplet is assisted by BSs, and therefore the BS adsorption kinetics (Pilosof 2017). First, the ability of the BS to adsorb at the oil/water interface would indicate their potential to remove the surface materials and facilitate lipase adsorption. It is a rate-limiting step influencing the lipolysis efficiency. The number and position of the hydroxyl group were shown to affect the adsorption profile of BS, as shown by Castillo-Santaella et al. (del Castillo-Santaella and Maldonado-Valderrama 2023). The results pointed out that NaTC would yield the highest surface tension at air/water interphase, concerning other investigated BS: NaGCDC and NaGDC, due to its highly hydrophilic nature. The study conducted by Parker et al. (Parker et al. 2014) allowed us to distinguish two groups characterized by different adsorption behaviour. The first group demonstrates reversible adsorption behaviour (NaGDC, NaTDC), while the second group displays a significant degree of irreversibility (NaTC, NaGC, NaGCDC). The study demonstrated that the adsorption behaviour follows the micellization properties. Faster desorption was represented by BS which had low CMC and large aggregation numbers and high CMC and low aggregation numbers promoted irreversibility adsorption.

Secondly, the interaction between the BS-lipase complex may give the information about potential of the BS to change lipase structure. To better understand the influence of the type of BS on the lipolysis process, the conformational structural changes of lipase introduced by BS were investigated, which may further reflect the potential of lipase adsorption. The molecular dynamic simulation performed by Haque et al. (Haque and Prakash Prabhu 2018) revealed the alterations in the interfacial activity of pancreatic lipase. The binding of NaTC to porcine pancreatic lipase resulted in changing the structure of the lipase. Moreover, this interaction prevents the loss of helical structure. The binding of NaTC prevents against conformation and induces an open-conformation (Haque and Prabhu 2016). Open conformation helps lipase to stay active without the co-lipase. Thus, the interaction between BS and lipase complex may induce conformational changes in the lipase, influence the lipase activity and stimulate the lipid digestion process.

Stronger adhesion of the BS may facilitate adsorption of the lipase/co-lipase to the surface of the lipid, which may promote lipolysis. However, reduction of the residence time at the interface can decrease the adhesion of the lipase/colipase but at the same time can facilitate displacement of lipolysis products from the surface. Therefore, the examination of BS behaviour at the interface is a key aspect considering their role in the lipolysis process.

1.12. Emulsification of fat droplet

Lipolysis efficiency was strongly correlated with particle size of the emulsion. Greater particle size was observed to reduce FFA release, which is correlated with a smaller surface area available for BS/lipase complex for the adsorption (Wilde et al. 2019). The smaller particle size of the initial emulsion was shown to promote higher lipolysis efficiency than the emulsion with a bigger particle size (Sarkar et al. 2016). The composition of digested emulsion may also influence the final FFA release due to the interaction of components of emulsions with BS. Wilde et al. (Wilde et al. 2019) examined the effect of phytosterol, a known component to reduce blood cholesterol levels (Dumolt and Rideout 2017) on the lipid digestion process. The results show that phytosterol accumulates at the surface of the oil, reducing space for BS to adsorb at the interphase, therefore reducing FFA release. Recently, plant-based diets gained popularity due to health and environmental concerns and animal welfare (Alcorta et al. 2021). Dietary fibres, the components of plant-based diets, were shown to trap BS in an aggregated structure during the digestion process and reduce the FFA release (Bellesi et al. 2018). The properties of emulsions, both composition and size of emulsion influence the lipid digestion process.

1.13. Micellization of BS

BS forms small aggregates in the aqueous solutions when their concentration exceeds CMC. The formation of micelles allows BS to complete their roles during the lipolysis process. It ensures the solubilization of lipolysis products into their structures and removal them from the oil interphase, which gives a greater surface for the BS/lipase complex to adsorb (Holm et al. 2013). Moreover, micelles play an important role as transport vehicles. Thanks to them lipolysis products can be delivered to our organism, which would be impossible due to their hydrophobic nature.

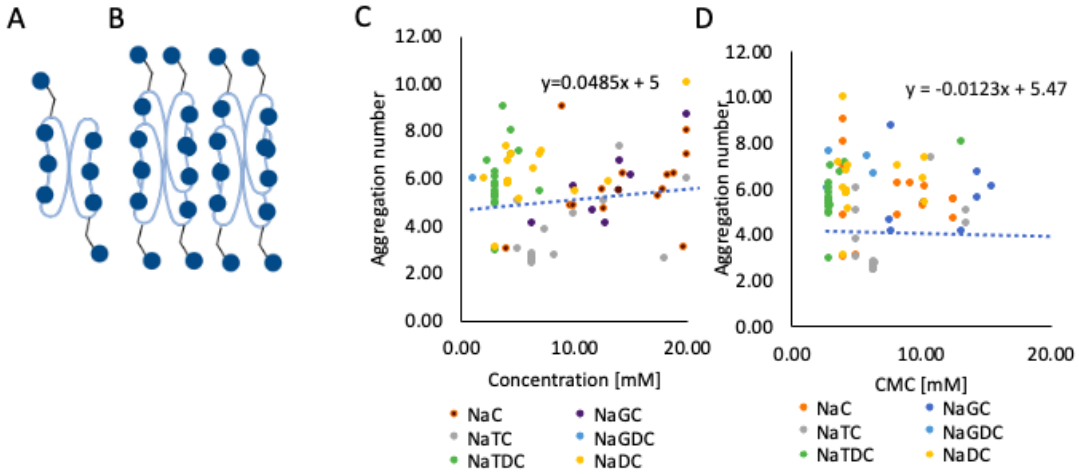


Figure 4 Arrangement of BS micelles into A. Primary structures, resulting from hydrogen interactions. Aggregation number was estimated to vary between 2-10. B. Secondary structures resulting from hydrogen bonding. The aggregation number was estimated to vary between 10-100. The scheme was prepared according to Moghimpour et al. (Moghimipour et al. 2015). *C. Aggregation number in respect to the concentration of different BS. Aggregation number increases with increasing concentration of BS. PC BS (NaTC) had the lowest aggregation number. D. Aggregation number with respect to CMC of BS. The increasing CMC did not result in a decreasing aggregation number. The results of CMC and aggregation number were taken from the meta-analysis. The data of aggregation numbers from C. and D. were previously published by Łozińska et. al.* (Łozińska and Jungnickel 2021). *NaC – sodium cholate, NaTC – sodium taurocholate, NaTDC – sodium taurodeoxycholate, NaGC – sodium glycocholate, NaGDC – sodium glycodeoxycholate, NaDC – sodium deoxycholate, CMC – critical micelle concentration.*

Over the years various techniques have been used to determine CMC (surface tension, dye solubilization, light-scattering, fluorescent, conductivity, and potentiometry). Each of the techniques is characterized by different selectivity and sensitivity. Moreover, CMC depends on BS type. Maestre et al. (Maestre et al. 2014) indicated that the higher number of hydroxyl groups and more hydrophilic character will contribute to higher CMC concentration due to greater water solubility of the molecule. Roda et al (Roda et al. 1983) observed that the CMC values of BS increase with an increasing number of hydroxyl groups. Trihydroxy BS have less hydrophobic character than dihydroxy BS which results in a lower CMC value of dihydroxy BS (Mukherjee et al. 2016). I Partay et al. (Pártay et al. 2007) indicated that the CMC of SC dihydroxy BS – NaDC was smaller than that of PU trihydroxy BS- NaC.

CMC of BS is one of the key parameters controlling the rate of lipolysis. The formation of micelles by BS is preceded by the creation of small aggregates such as dimers and trimers. (Duane and Gilboe 1995) Micelles are not only responsible for solubilizing lipolysis products, therefore allowing them to be removed from the oil interphase during the lipid digestion process but also ensure the safe transport of digested components through our body. Adsorption of BS was mentioned to be the most influential factor in controlling the process of lipid digestion (Macierzanka et al. 2019). However, the attention was also directed to the presence of unadsorbed BS, which inside the mixed micelles could effectively influence the lipolysis processes by solubilizing and removing products from the oil interface (Sarkar et al. 2016).

The formation of micelles of BS has multiple roles during the lipolysis process. It serves as aggregates in which lipolysis products can be incorporated and removed from oil interphase by the desorption process, which influences lipolysis efficiency and it also ensures the safe transport of necessary for our body components. Therefore, the tendency of BS to form micelles was studied by meta-analysis, for all types of BS, and experimental approach, for specific BS, in publication A1 and publication A3.

1.14. Desorption of BS

The ability to desorb from the lipid surface plays an essential role in the lipolysis process (Maldonado-Valderrama et al. 2014). Adsorption-desorption process is a rate-limiting step controlling the rate of the lipolysis. Increasing the surface tension of the surface layer indicates the depletion of the BS and it is desorption from the surface. The study performed by Maldonado-Valderrama et al. (Maldonado-Valderrama et al. 2014) showed different desorption properties of two conjugated forms of BS. While NaGDC fully desorbs from the surface within the whole concentration range, the NaTC tends to form irreversibly adsorbed structures at the interface. Desorption of NaTDC at lower concentrations was linked to reduced lipolysis efficiency (Pabois et al. 2020).
Desorption of BS from the oil interphase is a key parameter ensuring the removal of lipolysis products from the interphase and providing free space for BS/lipase to adsorb and continue the lipid digestion process. Finally, the desorption of BS ensures the incorporation of lipolysis products and delivers them to our bodies.

1.15. The solubilisation function of BS

Lipolysis products are solubilized by BS into created aggregates. BS forms vehicles that facilitate the transportation of lipolysis products. (Pigliacelli et al. 2023). Hofmann and Borgstrom (Hofmann and Borgstrom 1962) performed an ultracentrifugation experiment on human lipid digestion products, where they demonstrated that it consists of an oily phase and a solubilized BS mixed micellar phase. The studies comparing the effect of the ratio of BS and surfactant concentration (cationic, anionic, nonionic) on lipid digestion were performed, showing that the nature of surfactant plays an important role in the lipolysis process (Vinarov et al. 2012).
Formation of the micelles and solubilization of lipolysis end products is known as the process that completes the digestion of lipids. Created micelles transport lipid-digested products by absorption through enterocytes (Leal-Calderon and Cansell 2012). This requires micelles to diffuse through the protective layer of intestinal mucus. The small intestinal mucus is known as a complex colloidal system that protects the intestinal epithelium from exposure to luminal contents by creating a protective layer for the entire intestinal epithelium (Macierzanka et al. 2019). The mucus layer is a natural filter, that prevents epithelium against pathogenic microorganisms and ensures absorption of nutrients, so they can reach enterocytes (Cone 2009). Intestinal mucus is composed of a range of organic compounds, among which are two major ones that create a coherent network: gel-forming biopolymers, MUC2 mucin glycoprotein, and extracellular DNA (Hansson 2012; Macierzanka et al. 2014). The intestinal epithelium is composed of goblet cells that produce and secrete mucin (Zhang and Wu 2020). Viscoelasticity and strength of the gel depend on many factors such as concentration of mucin, DNA, size of pores, and level of entanglement (Macierzanka et al. 2019). Peristaltic movements cause a decreasing thickness of the mucus layer by inducing shear force. Penetration through a thinned mucus layer is possible only by diffusion (Cone 2009). Because lipids are insoluble in water they require transportation by BS in micelles.

1.16. Intestinal absorption

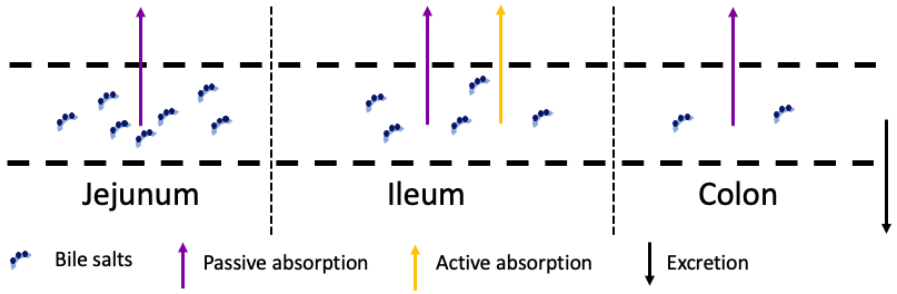


Figure 5 Intestinal absorption of BS. The jejunum and colon allow only for passive absorption, whereas the ileum transport BS through passive and active transportation mechanisms.

At the end of the enterohepatic recirculation mechanism, 5 % of BS is excreted from our body and 95% of BS is transported back to the liver through intestinal absorption. The intestinal transport mechanism of conjugated and unconjugated BS is a crucial process, ensuring the enterohepatic recirculation flow. The BS present in the small intestine can be reuptake by both active transport and passive ionic and nonionic transport mechanisms. Based on the BS features they may be transported in three main ways: by passive absorption in the jejunum, active and passive mode in the ileum, and passive mechanism in the colon, as shown in Figure 5. The active transport plays an essential role in the reabsorption of BS from the ileum. Tyor et al. (Tyor et al. 1971) concluded

that the majority of the BS present in the small intestine is transported back to the liver In the ileum. They also pointed out that limited absorption of BS may appear in other parts of the intestinal tract. The ionized and conjugated form of the BS reduces its absorption ability to the ileum transport mechanism and potential absorption by brush border membrane limits the action of a transporter (Dawson and Karpen 2015). However passive transport system was proved to exhibit good absorption ability for unconjugated or secondary conjugated BS. Passive absorption is based on the concentration gradient rule. Unconjugated BS can be absorbed at any level in the small intestine by passive absorption.
Aldini et al. compared the transport concentration of BS in the jejunum and ileum (Roda et al. 1983; Aldini et al. 1996). The mechanism of absorption for conjugated and unconjugated BS was examined. They noticed that unconjugated BS undergo passive transport in both the jejunum and ileum, the taurocholate was more likely to be absorbed in the ileum by an active transport system, while glycol-conjugated BS possessed an ability to undergo jejunum and ileum passive absorption as well as the ileum active absorption. Glycine-conjugated BS were examined to have a more hydrophobic nature than taurine-conjugated BS (Podda et al. 1990). Moreover, Merkus et al. pointed out that the conjugation of BS reduces the hydrophobicity of the created BS (Merkus et al. 1996). This process ensures maintaining the ionized form of BS, which prevents undergoing the absorption process before the fat absorption (Heaton 1969), which also indicates that the diffusion rate of BS in the small intestine depends on its form. Tyor et al. (Tyor et al. 1971) pointed out that the absorption concentration of the BS depends on the presence of specific microorganisms with the capability to deconjugate BS present in the small intestine. An important aspect is connected with the fact that conjugated BS possess a stronger ability to be absorbed, thanks to their enhanced water solubility, than their deconjugated forms, which are more likely to undergo an excretion process (McHugh et al. 2004).

So far there has been no clear investigation of how individual forms of BS may impact each of the mentioned parameters about lipolysis efficiency. There were performed individual studies of the physiochemical functions of BS, including CMC, aggregation number, solubilization, adsorption and desorption properties (Heuman 1989; Nagadome et al. 2001; Maestre et al. 2014; Maldonado-Valderrama et al. 2014; Mukherjee et al. 2016), as well as physiological functions such as FFA release (Bellesi and Pilosof 2021; Łozińska and Jungnickel 2021). However, based on the existing research, it is impossible to determine the rate-limiting step during the lipolysis process. BS performs its action simultaneously, therefore, investigation of all parameters concerning the lipolysis process may be sufficient for fully understanding the impact of BS on the digestion process. Imbalance in the BS concentration, caused by various diseases, non-healthy diet, environmental stress, etc, may inhibit the lipid digestion process and promote weight imbalance, as well as further development of diseases. Therefore, the investigation of the influence of BS in the lipolysis process was further developed in the publication A1 and may give a perspective to modulate the lipolysis process in a controlled way.

1.17. Physiological function of BS

In addition to their role in the lipolysis process BS are multifunctional biosurfactants. They act as anti-microbial agents in the small intestine, towards gram-positive bacteria (Hagey et al. 2010). The anti-microbial activity was related to oxidative DNA damage, disrupting cell membranes and cellular homeostasis (Moghimipour et al. 2015). BS act as signalling molecules, they regulate activation of G-protein coupled receptor and FXR.and they are responsible for stimulating lipid, glucose and energy metabolism (Da Silva et al. 2013). BS also regulate the secretion of lipoproteins from hepatocytes (Torchia et al. 2001), and colonic mucosal growth and stimulates the proliferation of colonic epithelium (Strauch et al. 2003). BS are responsible for the stimulation of intestinal immunity and regulating immune cells in the mucosa (Keating and Keely 2009; Soroka and Boyer 2014). BS also are responsible for removing toxins and excessive concentrations of cholesterol, preventing the formation of gallstones (Krupa et al. 2021).

1.18. Methods of measuring the lipolysis process

The increasing interest and awareness of the importance of digestion in the human body contributed to the search for a way to measure the effectiveness of the digestion process. To represent physiological conditions during digestion, both in-vitro and in-vivo techniques have been developed. In-vitro static models are commonly used as they may reflect the biochemistry of specific regions of the gastrointestinal tract, are easy to use and cheap. Single compartment pH stat model requires cheap and easily accessible equipment but it does not consider processes in the stomach, for example, gastric emptying, gastric and intestinal phases have to be performed separately and transferring the sample requires pre-conditioning (Lee et al. 2018). To overcome the

limitations two-compartment model can be used as they simulate both the gastric and the intestinal phase and connection by peristaltic pump ensures transportation of gastric medium to the intestinal vessel (Huang et al. 2021). In-vitro static techniques are well-standardized models and allow capturing the individual key parameters but they can not reflect the complexity of the intestinal microbiota, don't mimic the peristaltic movement and do not reflect the shape of the specific organ of the body (Huang et al. 2021). In-vitro dynamic models were developed to overcome multiple limitations of static techniques and ensure the reflection of digestion kinetics of the gastrointestinal tract and reproduction of the gastrointestinal environment. However dynamic models are very complicated, time-consuming, and not standardized and they do not focus on a simple parameter, but rather on the complexity of biochemical changes in the gastrointestinal tract therefore their accessibility is much lower than static models (Mulet-Cabero et al. 2020). Another alternative in-vitro method is a pendant drop surface film balance implemented with multi-subphase exchange(Maldonado-Valderrama et al. 2014). This method uses a single droplet immersed in the oil phase to simulate in-vitro digestion of emulsion. The droplet solution is exchanged with simulated digestive media that mimics the lipolysis process.
Due to the complexity of the process, reflection of lipolysis with a validation level comparable to our digestion system is a huge challenge. Recently, due to increasing interest and awareness of the importance of the digestion process, several in-vivo noninvasive and invasive methods have been developed. Magnetic resonance imaging is a non-invasive technique used to visualise changes in meal composition in the gastrointestinal tract, mainly in the stomach (Mariani et al. 2004). Another technique that allows following the gastric emptying process and uptake of nutrients is stable isotope breath testing with MS. This technique allows following the meal by using specific compounds that depend on the tested material (Golding and Wooster 2010). In-vivo invasive techniques may give a greater range of information. These studies are mainly performed with clinical assessment, for example, blood tests (Degen et al. 2007). The main aim of developing the in-vitro techniques is to ensure the appropriate validation level, reflecting in-vivo actions. In our research, we were using non-invasive techniques, which effectively reflect the complexity of the lipolysis process.

1.18.1. Static in-vitro digestion model

Digestion models are used to measure the efficiency of the lipolysis process, based on the formation of the final product however, they cannot measure the efficiency of each step of the lipolysis. The main advantage of this model is that they are cheap, non-complicated to use have good reproducibility, easy to use. The most popular and widely used model is the Brodkorb model (Brodkorb et al. 2019) based on a systematized in-vitro digestion model. Unfortunately, static models do not reflect the multi-complexity of biochemical reactions, absorption, secretion, peristaltic movements and emptying. We used the Brodhorb model, as its standardized method was created due to over three years of cooperation between the INFOGEST group, allowing results to be easily comparable between each other, to compare the efficiency of the lipolysis process in the presence of two different forms of BS: NaTC and NaDC.

1.18.2. OCTOPUS technique

We used the pendant drop technique which allows us to measure digestion efficiency in a single droplet. The work of OCTOPUS is based on a subphase device that allows to replacement of digestive media. Experiments performed on in-vitro static digestion models allowed us to compare the efficiency of lipid digestion in the presence of different forms of BS by measuring the final FFA release as an indicator of the efficiency of the lipolysis process. These experiments proved that the form of BS modulates lipid digestion and showed that NaTC increases the final FFA release, leaving us with the question – why does NaTC promotes higher FFA release? Lipolysis is a process consisting of cumulative effects. The pendant drop technique was used to determine the continuous evolution (changes in interfacial tension and dilatational modulus) of the interface during the lipolysis process, considering the influence of NaTC and NaDC. BS during the lipolysis process has to adsorb on the surface of the oil to allow lipase and co-lipase to perform the emulsification process and has to also desorb from the oil surface, ensuring removal of lipolysis products from the interface. The desorption process is also important because if the accumulated lipolysis products would not be removed from the oil interface, further digestion would be blocked due to a lack of place for the adsorption of the BS-lipase complex. Therefore, interfacial properties would strongly affect the extent and rate of lipolysis and were further examined in our research.

2. Purpose and scope of the work

The PhD thesis aimed to determine the effect of two predominant forms of BS: NaTC and NaDC on the process of lipolysis by identifying their influence on individual processes of mechanism of lipolysis. Lipolysis mainly takes place in the duodenum, which consists of PC forms of BS, and SU, ones due to the deconjugation process (Corstens et al. 2017).

The scope of the doctoral dissertation includes (1) the identification of predominant forms of BS influencing the lipid digestion process, (2) the investigation the potential of which different BS influence the specific parameter (CMC, N_a, and MSR), (3) determine factors disturbing BS synthesis and its effect on BS composition, (4) establish influence of choledocholithiasis, cholangiocarcinoma and pancreatic neoplasm towards changes in BS composition and its potential to disturb lipid digestion process, (5) identification the processes of lipolysis mechanism, (6) determination the influence of predominant forms of BS in our small intestine: NaTC and NaDC on each process of lipolysis, (7) assessment the impact of BS action on the lipolysis efficiency, (8) identification the rate-limiting process of lipolysis.

The results of the PhD work allowed us to reveal the potential to control the lipolysis mechanism via the action of BS. The influence of BS on each lipolysis process was experimentally measured and the final rate of lipolysis was asses.

3. Discussion of scientific literature results

This part of the work presents the results of the research included in the doctoral dissertation in the form of a series of three original scientific publications on the presented research issues, published in two journals from the JCR list with a total IF = 17.954. A short description of the works is presented below.

3.1. Publication 1 -A1

Łozińska N, Jungnickel C. Importance of Conjugation of the Bile Salt on the Mechanism of Lipolysis. *Molecules*. 2021; 26(19):5764. DOI: 10.3390/molecules26195764.

3.1.1. Objective of research

Publication A1 was focused on meta-analysis and statistical analysis of three parameters covering aggregation properties of BS: (1) micellization properties (CMC, β parameter), (2) aggregation number and (3) MSR.
The second aim of the publication A1 was to determine the influence of two predominantly present forms of BS in our small intestine: PC NaTC and SU NaDC, on digestion efficiency. The goal was to use a standardized, easy method which results will be, in the future, comparable with other experiments. Therefore, the static in-vitro digestion model, according to the Brodkorb protocol, was used to determine the progress of digestion by measuring FFA release.

3.1.2. Reason for undertaking the research problem

The main research problem that encouraged undertaking work on publication A1 was that there was a poor understanding of the relation between BS, aggregation properties (CMC, aggregation number, MSR) and lipolysis efficiency.

The type and concentration of BS determine its effect of action on lipolysis parameters. Alterations of those parameters may impact the role of BS in the lipid digestion process and modulate the final rate of lipolysis. Moreover, the nature of BS influences the processes modulating lipolysis efficiency. Increasing hydrophilicity of BS was correlated with the reduction of CMC, which is contradictory to linear surfactants. The factor responsible for this difference was no of BS-water hydrogen bonds. Therefore, an investigation effect of the simultaneous action of those parameters on the lipolysis process had to be performed.
The meta-analysis of experimental data has revealed that there is a limited number of data points from experiments performed under physiological conditions.
Lipolysis experiments are often performed under various, changeable conditions (type of oil, conc of BS, particle size of emulsion etc.) which limits comparability between each other. Moreover, it could be observed that there is a limited number of studies on the influence of the BS ratio on lipolysis efficiency.

Meta-analysis of CMC of BS showed a high variation of data for a single BS. The results strongly depend on the technique used to determine CMC. Some of the techniques with high sensitivity, allowed to detect the primary CMC and techniques with low sensitivity were only able to detect secondary micelles. The review of the literature data (Roda et al. 1983; Astrup 2001) revealed that dihydroxy BS may form both primary and secondary micelle, while trihydroxy BS mostly forms primary micelles. Dihydroxy BS forms primary micelles at a concentration of n 10-50mM and secondary micelles at a concentration above 100mM (Mishra et al. 2019). The formation of secondary micelles for trihydroxy BS was reported to be above the 300mM (Pártay et al. 2007). For this reason, only experiments that were carried out within the intestinal composition of BS, 10mM (Łozińska et al. 2024) were taken into consideration. This approach allowed to solve the problem with the high variation of CMC data, especially for dihydroxy BS such as NaDC. Moreover, the divergence of the CMC data was also connected with the year of the performed experiments, CMC increased with time, probably due to the increased purity of BS.

Analysis of β of different BS: BS systems revealed two occurring effects within the systems: antagonistic (with high CMC, positive β) and synergistic (with low CMC, negative β). In our research, we assumed the individual effect of BS on lipolysis efficiency taking into consideration only the CMC of the single BS. However, during the digestion process are present different types of BS and we decided to expand our research to two questions: (1) why does the antagonistic effect occur and (2) what is the reason for the synergistic effect? Moreover, the research revealed that the system composed of the PC and the SU BS balances the impact.

The increasing hydrophilicity of BS resulted in lower CMC, which was contradictory to the behaviour of linear surfactants. A similar situation appeared in the case of the BS: BS systems, where both the antagonistic and the synergistic effects could be observed, while systems created by linear surfactants mainly result in synergistic effects. The contradictory behaviour of BS towards linear surfactants resulted from their planar polarity.

To assess the level of difference of influence on the lipolysis process by two BS: NaTC and NaDC, stable and uniform emulsion had to be designed. When an emulsion with high particle size was created the emulsion met the conditions of establishment but the difference of FFA between NaTC and NaDC was very low (2%). When the emulsion with the smaller particle size was tried to be created the final emulsion was uniform with low PDI index. Moreover, the long work of the sonicator with high frequency resulted in the destabilization of the emulsion due to the high temperature of the end of the sonicator. Lower frequencies of work of the sonicator were not efficient enough to create an emulsion with a smaller particle size. Changes introduced at the formation of pre-emulsion – replacing vortexing with homogenization - allowed to decrease in the particle size of the emulsion and obtained a uniform and stable emulsion. Formed emulsion allowed to obtain a difference in FFA release between NaTC and NaDC of 15%.

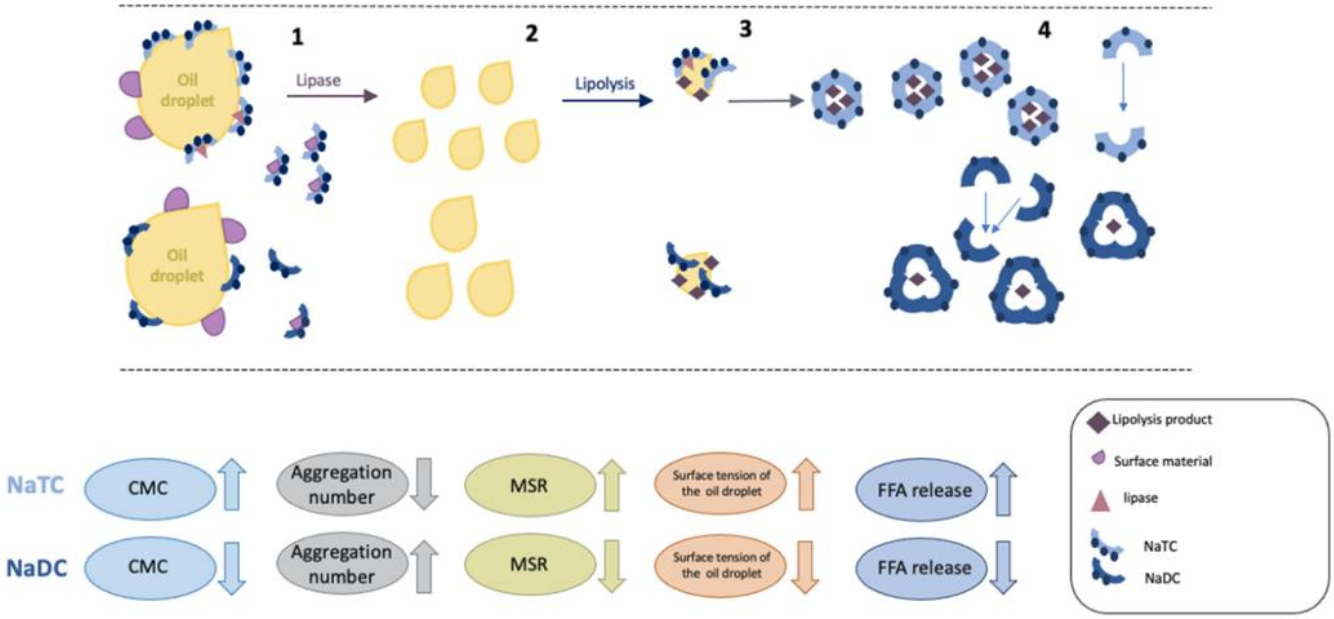
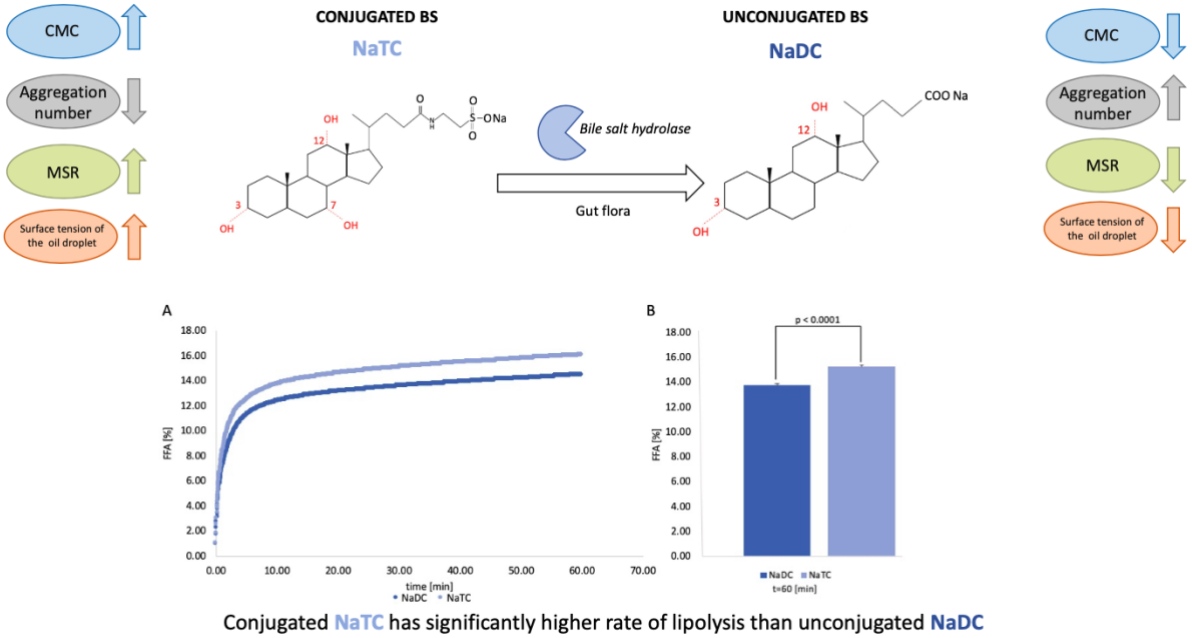


Figure 6 Schematic representation of the role of BS during the lipolysis process. The role of BS can be reflected by its influence on different parameters. The impact of each of the processes influences the final rate of lipolysis. CMC – critical micelle concentration, MSR – molar solubilisation ratio, FFA – free fatty acids, NaTC – sodium taurocholate, NaDC – sodium deoxycholate.

3.1.3. Main outcomes and conclusions

- The deconjugation process affects the physiochemical properties of BS in GIT
- NaDC have lower CMC than NaTC
- NaDC showed greater emulsification properties of oil droplets than NaTC
- The higher hydrophilic character of NaTC allows them to desorb easier from the surface of the emulsion
- $LogK_{OW}$ showed a negative contribution towards MSR
- NaTC enhances FFA release to a higher extent than NaDC.
- The interface activity of NaDC is higher than NaTC, indicating that lipolysis is dominated by other factors

3.1.4. Graphical abstract of publication A1



Conjugated NaTC has significantly higher rate of lipolysis than unconjugated NaDC

3.1.5. Publication A1

Importance of Conjugation of the Bile Salt on the Mechanism of Lipolysis

Natalia Łozińska and Christian Jungnickel

Journal of publication: Molecules

DOI: 10.3390/molecules26195764

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Abstract: We aim to advance the discussion on the significance of the conjugation of bile salts (BS) in our organism. We hypothesize that conjugation influences the rate of lipolysis. Since the rate of lipolysis is a compound parameter, we compare the effect of conjugation on four surface parameters, which contribute to the rate. Since deconjugation is due to gut microbiota, we hypothesize that microbiota may affect the rate of lipolysis. A meta-analysis of literature data of critical micelle concentration, β, aggregation number, and molar solubilization ratio has been performed for the first time. In addition, critical micelle concentration (CMC), interfacial tension, and lipolysis rate measurements were performed. It was found that the unconjugated BS in mixed micelles increases the antagonism between the BS, therefore, increasing the CMC. This correlated with the effect of unconjugated BS on the solubilization capacity of mixed micelles. The collected literature information indicates that the role of the BS and its conjugation in our organism is a key factor influencing the functioning of our organism, where too high levels of unconjugated BS may lead to malabsorption of fat-soluble nutrients. The experimental lipolysis results irrevocably showed that conjugation is a significant factor influencing the rate.

Keywords: bile salts; lipolysis; CMC; aggregation number; MSR

3.1.6. Publication A1 Supporting Information

Importance of Conjugation of the Bile Salt on the Mechanism of Lipolysis

Natalia Łozińska and Christian Jungnickel

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3.2. Publication 2 -A2

Krupa, Łukasz, Robert Staroń, Dorota Dulko, Natalia Łozińska, Alan R. Mackie, Neil M. Rigby, Adam Macierzanka, Aleksandra Markiewicz, and Christian Jungnickel. 2021. "Importance of Bile Composition for Diagnosis of Biliary Obstructions" *Molecules* 26, no. 23: 7279. https://doi.org/10.3390/molecules26237279

3.2.1. Objective of research

The main aim of the research was to determine the importance of BS as the disease indicator. Development of the disease state may change the BS concentration, which may lead to alteration of the BS synthesis, which was hypothesized to result in a reduced health state. Therefore, the main objective was to identify BS as a biomarker for specific diseases. BS undergo specific changes, which allows them to work as disease indicators. The research presented the hypothesized mechanism of changing BS composition concerning the disease state.

3.2.2. Reason for undertaking the research problem

Besides BS's role in the lipid digestion process, they are responsible for stimulating receptors and controlling their synthesis.

The development of gallstones and cholangiocarcinoma may be responsible for the alteration of BS concentration and its synthesis.

There are only a few scientific works that characterized the BS profile in plasma or serum in cholangiocarcinoma and pancreatic neoplasm. Therefore, it was important to analyze those reports and compare them to experimental results for a better understanding of the mechanism under which BS concentration changes.

There is no clear information on how specific diseases alter BS profiles. To reveal new disease indicators, it is valuable to understand the mechanism of changing BS concentration and its ratio

The disease state indirectly induces changes in BS composition, which may consequently influence the rate of the lipolysis process.

Experiments considering different variations of predominant forms of BS in our gastrointestinal tract would be dominant in assessing the efficiency of the lipid digestion process. This would provide us with information on how the disease state and its corresponding BS can influence the lipolysis process.

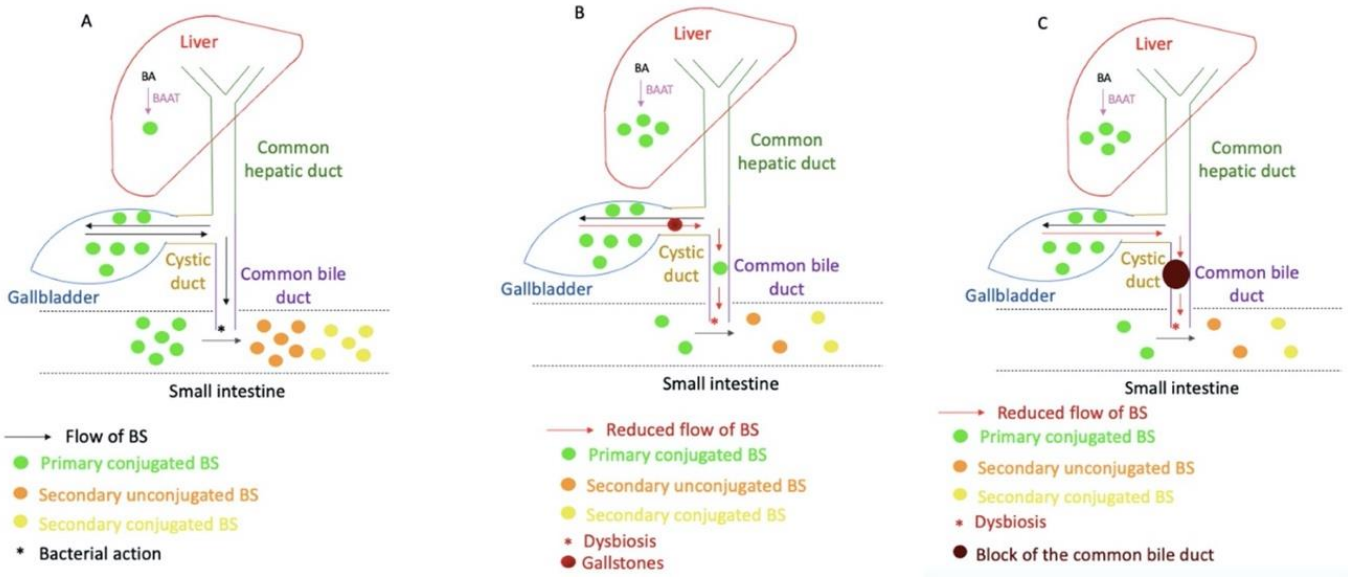

Figure 7 A. The healthy state ensures the normal flow of the BS. B. Formation of gallstones results in reduction of BS flow and dysbiosis which influence the composition of BS in the small intestine. C. Blockage of the common bile duct results in reduced BS flow and promotes dysbiosis in the small intestine. BS – bile salts, BA – bile acids, BAAT - BA CoA: amino acid N-acyltransferase

3.2.3. Main outcomes and conclusions

- BS showed a potential to work as a disease indicator of gallstones cholangiocarcinoma, and choledocholithiasis. However, the changes in BS concentration were not sensitive enough to identify them as disease indicators. We did not have enough data and knowledge to specify if the alteration of BS concentration was only related to the development of disease. A pathogenic state could result in a change of BS concentration but probably BS concentration could be a result of a different factor (for example change in BS synthesis) and therefore result in the development of disease. The process is very complicated and should be investigated in separate research only focused on BS synthesis and concentration.
- Experimental data and literature data collected by meta-analysis of disease state showed to be statistically significant from the reference value of the healthy state, indicating alteration of BS conc with development of disease
- Development of cholangiocarcinoma, choledocholithiasis and pancreatic neoplasm tend to increase the C/U BS ratio from 2.54 in healthy patients to respectively 35.90, 56.03 and 46.45 in experimental data and 135.35, 79.49 and 43.57 of literature data
- The gallstones formed in the gallbladder may reduce the flow of the BS and lead to blockage of the cystic duct and common bile duct leading to dysbiosis in the small intestine and affecting the deconjugation process.

3.2.4. Publication A2

Importance of bile composition for diagnosis of biliary obstruction

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Journal of publication: Molecules

DOI: 10.3390/molecules26237279

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Abstract: The composition of bile of 74 patients with obstruction of the biliary system was determined by HPLC-MS. The samples were collected during an endoscopic retrograde cholangiopancreatography(ERCP) performed on the patients. The concentration of eight bile salts, specifically sodium cholate, sodium glycocholate, sodium taurocholate, sodium glycodeoxycholate, sodium chenodeoxycholate, sodium glycochenodeoxycholate, sodium taurodeoxycholate, and sodium taurochenodeoxycholate, as well as the bile cholesterol were determined. Serum alanine aminotransferase (ALT), aspartate transaminase (AST), and bilirubin were measured before the ERCP. Ratios of conjugated/conjugated, primary/secondary, and taurine/glycine conjugated bile were determined to ease comparison to literature data. Statistical significance was determined by Kruskal Wallis test. ROC curves were determined, and cut-off values were determined using the distance to 0,1. It was found that serum bilirubin was a successful indicator for type of biliary obstruction; it was able to differentiate between choledocholithiasis (>11 µmol/L) and pancreatic neoplasm or cholangiocarcinoma (>59 µmol/L). In addition, it was theorized that the level of conjugated/unconjugated bile leads to and confirms the presence of an obstruction. With lower levels of conjugated/unconjugated bile the possibility for inflammation and this neoplasms increase.

Keywords: bilirubin; conjugated bile; biliary obstruction; pancreatic neoplasm; cholangiocarcinoma; choledocholithiasis

3.3. Publication 3 -A3

Łozińska, N., Maldonado-Valderrama, J., Del Castillo-Santaella, T., Zhou, Y., Martysiak-Żurowska, D., Lu, Y., & Jungnickel, C. (2024). Bile conjugation and its effect on in vitro lipolysis of emulsions. *Food Research International*, *184*, 114255.

3.3.1. The objective of the research

- The main aim of this research was to determine five processes influencing the efficiency of the lipolysis process.
- Set measurable parameters for each process.
- Experimentally measure the changes in each parameter concerning the changing ratio of C/U BS.
- Perform meta-analysis on lipolysis data.
- Determine the most influential process affecting lipolysis efficiency.

3.3.2. Reason for undertaking the research problem

Lack of studies on changing lipolysis parameters concerning changing concentration of conjugated and unconjugated BS.

There is no clear understanding of to what extent changes in lipolysis parameters affect the FFA release from oil emulsion.

MSR is mainly measured for substances not related to the digestion process (PAH, drugs), therefore lipolysis products should be investigated to understand the importance of solubilization properties. MSR may indicate the ability of the BS to incorporate lipolysis products into their aggregates concerning the product type, which is a rate-limiting step influencing the efficiency of lipolysis.

Changes in IT and dilatational modulus for individual BS during the lipolysis process may determine their behaviour at the interphase. For now, the previously performed experiments considered IT changes concerning changes in BS concentration. Therefore, there were very limited results presenting changes in IT measurements at the physiological concentration of BS. Moreover, SU BS was not previously tested, limiting the conclusions only to the behaviour of conjugated forms of BS.

Many data covering the lipolysis experiments exist. However, there are a lot of changing variables that may influence the final result, which makes them hard to compare and uneasy to decide on the most predominant factor. The unification of data and the creation of the lipolysis modelling can give us perspective to foresee the final FFA release by controlling the individual factors of the process. Moreover, it would also allow us to determine the most predominant factor affecting the lipolysis process.

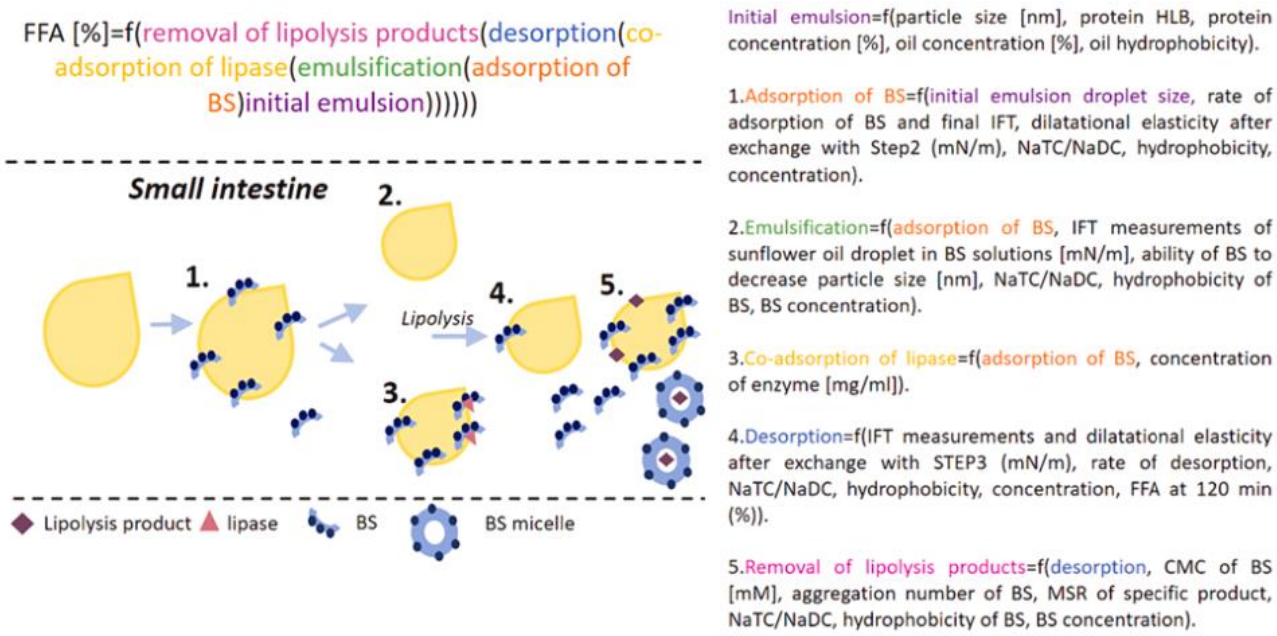
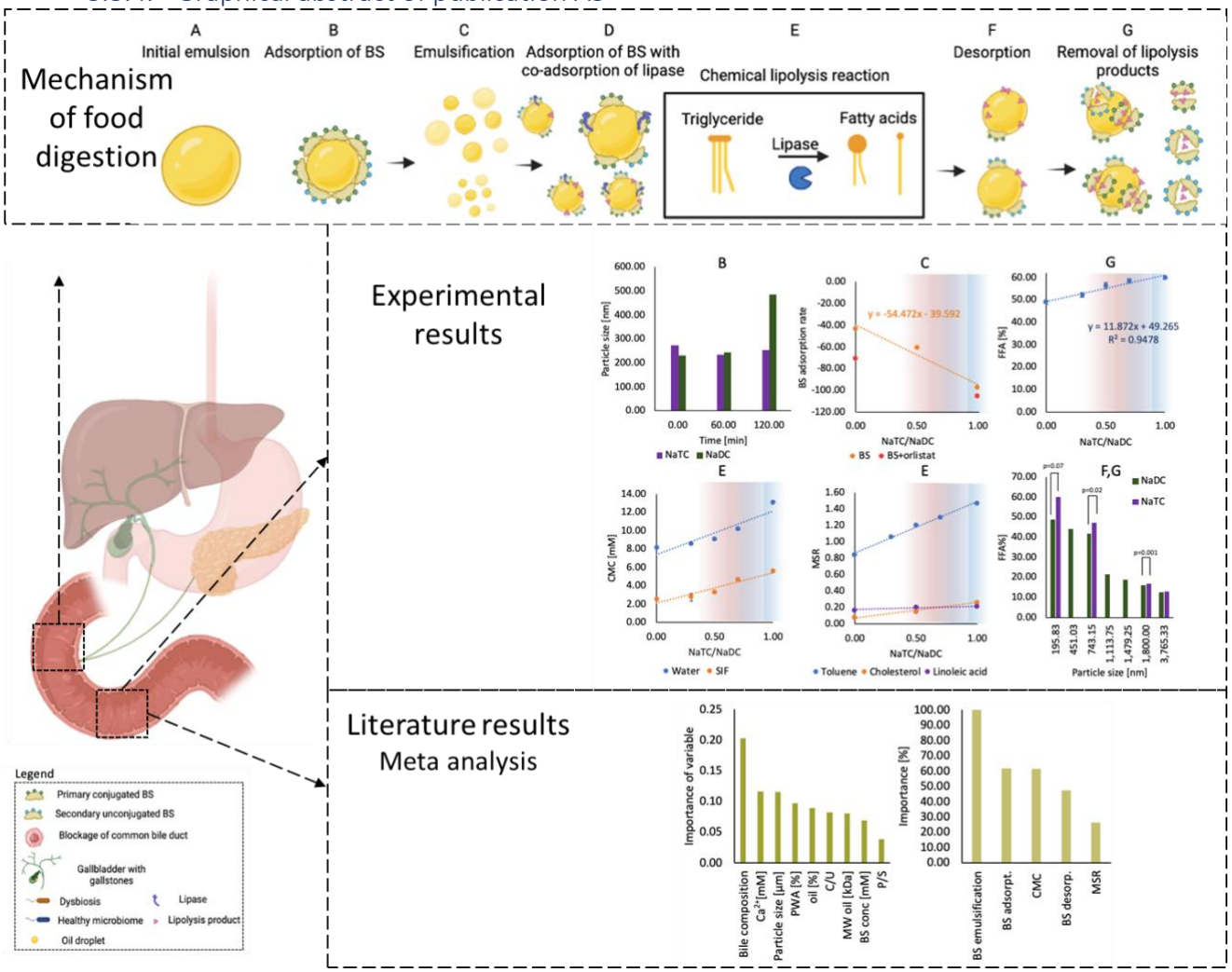


Figure 8 Lipolysis was determined to depend on five dominant processes: 1. Adsorption of BS, 2. Emulsification, 3. Co-adsorption of lipase, 4. Desorption, 5. Removal of lipolysis products. Each of the processes was described as a mathematical function, with parameters influencing its efficiency. Each of the parameters can be experimentally measured concerning the form of the BS. BS – bile salts, IFT – interfacial tension, NaTC – sodium taurocholate, NaDC – sodium deoxycholate, HLB – hydrophilic lipophilic balance, FFA – free fatty acids, CMC – critical micelle concentration, MSR – molar solubilisation ratio.

3.3.3. Main outcomes and conclusions

- Lipolysis efficiency depends on five processes
- NaDC more significantly reduces particle size during the lipolysis process.
- Increasing the concentration of conjugated over unconjugated BS increases interfacial tension and dilatational modulus during the adsorption and desorption step.
- FFA release is enhanced by increasing the concentration of conjugated BS.
- MSR is not affected by conjugation.
- Emulsification is a rate-limiting step of lipolysis

3.3.4. Graphical abstract of publication A3



3.3.5. Publication A3

Bile conjugation and its effect on in vitro lipolysis of emulsions

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Abstract: Bile Salts (BS) are responsible for stimulating lipid digestion in our organism. Gut microbiota are responsible for the deconjugation process of primary conjugated to secondary unconjugated BS. We use two structurally distinct BS and characterize the rate of lipolysis as a compound parameter. A static in-vitro digestion model as well as meta-analysis of literature data has been performed to determine the most influential factors affecting the lipid digestion process.
The results demonstrate that lipolysis of emulsions using conjugated BS (NaTC) enhances the release of FFA compared to deconjugated BS (NaDC*).* These results indicate that conjugation plays an important role in controlling the rate of lipolysis in our organism which can be in turn, tuned by the microflora composition of our gut, ultimately controlling the rate of deconjugation of the BS.

Keywords:Bile salts; lipolysis; in-vitro digestion; lipids; emulsion; interfacial tension; adsorption; desorption

3.3.6. Publication A3 Supporting Information

Bile conjugation and its effect on in vitro lipolysis of emulsions

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4. Additional results

Additional results concerning the action of two other BS were also examined: sodium glycochenodeoxycholate (NaGCDC) and sodium glycodeoxycholate (NaGDC). NaGCDC is the PC BS formed in an alternative pathway, as shown in Figure 3, and NaGDC is the secondary conjugated BS formed after the deconjugation process. Our research aimed to determine the impact of the deconjugation process on lipolysis efficiency, therefore only two BS (NaTC and NaDC) have been chosen as representatives, as their concentration in the small intestine exceeds other BS.

However, NaGCDC differs from NaTC by an additional hydroxyl group, which due to our presented research, may be of great importance in affecting lipolysis efficiency. Moreover, examination of the effect of NaGCDC expands our research of information about the effect of the action of secondary conjugated BS on the lipolysis process.

The aim of performing these additional results was to check if their action has a significant impact in comparison to previously chosen BS: NaTC and NaDC in two main experiments: in-vitro lipolysis of emulsion and in-vitro digestion.

4.1. Methodology

4.1.1. Dilatational rheology

The dilatational rheology on the interfacial layer is measured at the end of each phase by subjecting the droplet to 10 cycles of periodic deformation by injection/extraction volume at 0.1 Hz of measurement frequency (v). The dilatational modulus is calculated from the response of the interfacial tension to the deformation by the following equation: $E = E' + iE'' = \epsilon + iv\eta$ (1)

E' is the storage modulus, which accounts for the elasticity of the interfacial layer (ε), E'' is the loss modulus, which accounts for the viscosity (η) of the interfacial layer, and v is the angular frequency of the applied oscillation. The amplitude of the applied oscillation was set up to < 5% to avoid excessive perturbation of the adsorbed interfacial layer (del Castillo-Santaella et al. 2015). At this oscillation frequency, the interfacial layer displays a mostly elastic response obtaining E'>>E'' in all cases. Hence, only the values of the complex modulus will be reported here and discussed as dilatational elasticity.

4.1.2. In-vitro lipolysis of emulsion

A modified INFOGEST *in-vitro* lipolysis model (Brodkorb et al. 2019) was used to simulate the environmental condition of the duodenum. Specifically, 0.8 mL of the SIF and 0.375 mL of the emulsion were added to the thermostatted vessel. After mixing with a magnetic stirrer (1500 rpm),0.3 mL of 10mM BS (NaTCorNaDC) and 3 µL of 0.3M $CaCl_2$ were pipetted, and the pH was set to 7.0 using 0.1 M HCl. Finally, with the addition of 1.0 mL of freshly prepared pancreatin (75 mg at 80 U/mg), the titration was started. The reaction vessel was continuously stirred and thermostatically controlled to maintain 310.15 K. The extent of the lipolysis was measured by continuous titration with an autotitrator (Cerko Lab *N.* System CLS/M/07/06, Gdynia, Poland) of (FFA) with 0.1 M NaOH. All lipolysis experiments were carried out in duplicate. Experiments were performed according to Łozińska et al. (Łozińska et al. 2024)

4.1.3. In-vitro lipolysis

In-vitro lipolysis of adsorbed protein layers at the oil-water interface was measured in OCTOPUS by sequential adsorption comprising three steps: Step1- protein, Step2- lipolysis: BS, BS + lipase or BS + lipase + inhibitor, and Step 3- desorption: replacement of bulk solution by SIF (Maldonado-Valderrama et al. 2014; Łozińska et al. 2024).

4.2. Results&Discussion

4.2.1. Dilatational modulus

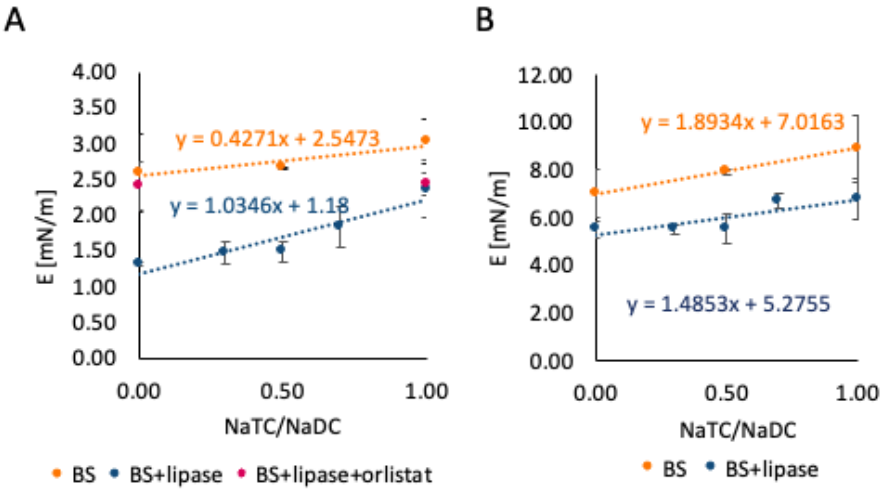

Figure 9 A. Dilatational complex moduli of interfacial layers after an exchange of the adsorbed protein at the interface with one of the following solutions: BS, BS+lipase, BS+lipase+orlistat.Average taken from 10 records + SD. B. Dilatational complex moduli of interfacial layers after an exchange of the previous solution with simulated intestinal fluid. BS – bile salts, NaTC – sodium taurocholate, NaDC – sodium deoxycholate, E - dilatational modulus.

In the presence of lipase, the dilatational modulus increases slightly with the concentration of BS (as shown in *Figure 9* A, again supporting the increased presence of lipolytic products at the interface which is enhanced as the concentration of BS increases. The dilatational modulus of desorption (*Figure 9*B) follows the trend from adsorption results

4.2.2. In-vitro static lipolysis

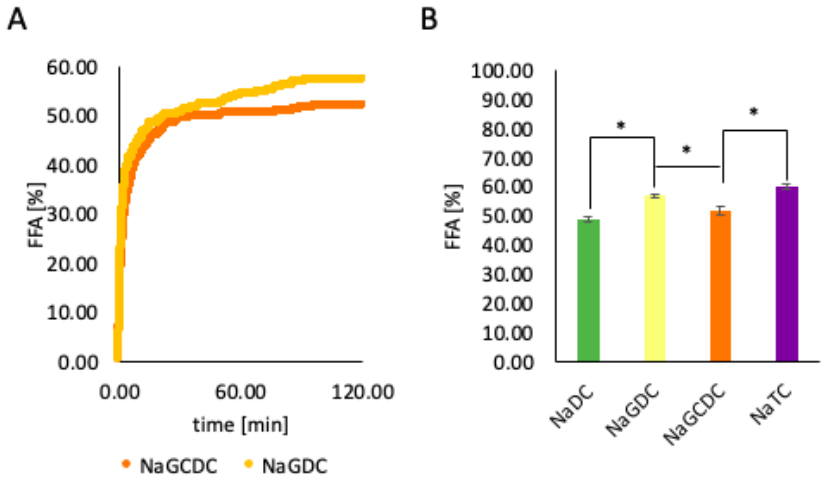

Figure 10 A. In-vitro static digestion experiments of PC NaGCDC and SC NaGDC. B. T-sample t-test was used to calculate the statistical significance of FFA release between Na GCDC and NaGDC. NaGDC has been shown to have statistically higher FFA release (FFA=57.07%) than NaGCDC (FFA=51.79%). NaGCDC – sodium glycochenodeoxycholate, NaGDC – sodium glycodeoxycholate, NaTC- sodium taurocholate, NaDC – sodium deoxycholate, FFA – free fatty acids

The results presented in Figure 10 show that SC NaGDC have greater potential to enhance FFA release during the lipid digestion process than PC NaGCDC. The greater lipolysis efficiency of NaGDC is a result of its greater desorption potential from the oil droplet during the lipolysis process, as shown in Figure 11B. The space left after the removal of lipolysis products from the oil interphase ensures the continuous process of lipid digestion by adsorption of NaGDC and lipase/co-lipase complex. The FFA release differs significantly for PC NaTC and NaGCDC, which means that the conjugation ratio with cholic and chenodeoxycholic has an impact on the lipolysis process. Moreover, the results indicated that SC NaGDC has greater potential to enhance FFA release than SU NaDC, showing the importance of the conjugation process after the action of BSH. As it was shown in our research FFA release from emulsion is connected with interfacial processes.

4.2.3. In-vitro lipolysis

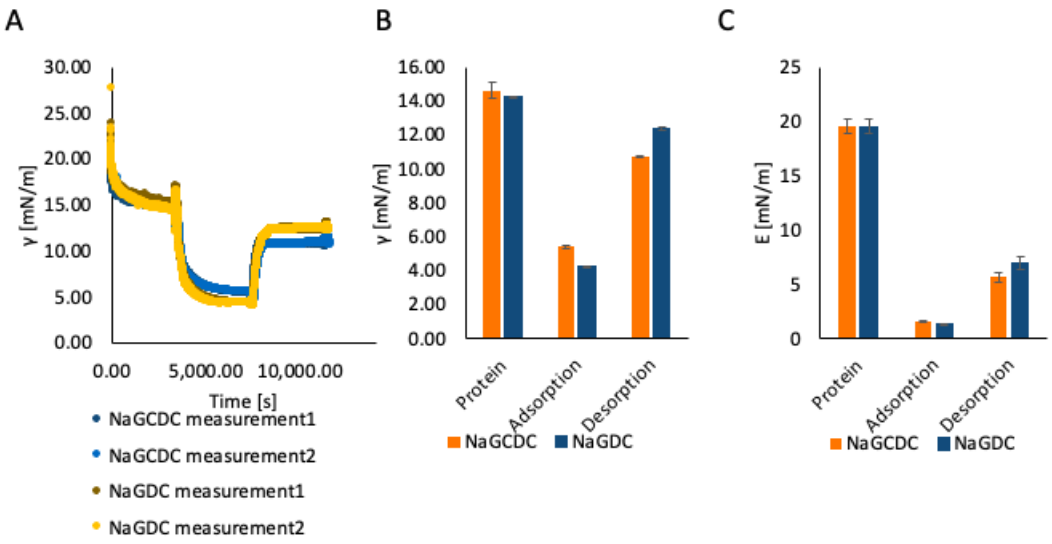

Figure 11 A. FT measurements of in-vitro digestion process performed on OCTOPUS device for different BS: SU sodium glycodeoxycholate (NaGDC) and PC sodium glycochenodeoxycholate (NaGCDC). B. Final (average of last 30 points) IFT have shown that NaGCDC has greater adsorption potential, which means it can more effectively adsorb to the surface of the oil droplet which enhances the adsorption of lipase/co-lipase complex during the lipolysis process. However, NaGDC has shown a greater potential to desorb from the soil surface, which can ensure the effective removal of accumulated lipolysis products from the oil interphase. C. Young modulus of NaGCDC and NaGDC during adsorption and desorption steps. NaGCDC – sodium glycochenodeoxycholate, NaGDC – sodium glycodeoxycholate, γ – interfacial tension. E - dilatational modulus.

The results presented in Figure 11 B show that NaGCDC have a greater ability to adsorb to oil interphase, therefore enhancing lipase/co-lipase complex to adsorb to the oil droplet and start the digestion process. However, the results also indicate that NaGDC faster desorbs from the oil droplet, which plays an important role in ensuring the removal of lipolysis products from the oil interphase, which gives greater space for lipase/co-lipase complex for further adsorption on the lipid droplet and continue lipolysis process.

5. Conclusions

The results of the PhD dissertation revealed the potential of BS to control the lipolysis process. The deconjugation process was shown to be crucial for the efficiency of the lipolysis process. Two predominant forms of BS in the small intestine: PC-NaTC and SU-NaDC were chosen for further analysis concerning lipolysis efficiency.

In the first stage of the research the meta-analysis of phenomenological parameters: was performed to indicate the importance of the concentration of conjugated BS (Łozińska and Jungnickel 2021). The performed analysis of research results aimed to assess the ability to create micelles, responsible for incorporating lipolysis products into their structure, removing them from the oil interphase and further transporting them to our organism (Pabois et al. 2021). The collected results showed that conjugated forms of BS require lower concentrations and fewer molecules than unconjugated BS to form micelles. Moreover, micelles created by PC BS showed a greater ability to incorporate components into their structure. Performed experiments of in-vitro digestion of emulsion of conjugated and unconjugated systems of BS showed the importance of conjugation. For the first time, it was presented that concentration of conjugated BS can modulate lipolysis efficiency.

In the next stage of the research, the analysis of BS composition concerning specific diseases has been performed (Krupa et al. 2021). The development of disease was shown to significantly alter the ratio of conjugated and unconjugated BS. Blockage of the common bile duct reduced the flow of the BS from the gallbladder to the small intestine which resulted in the alteration of the BS synthesis (Dai et al. 2011). Since the composition of BS stimulates BS synthesis, their reduced concentration during re-absorption from the small intestine promotes BS synthesis by an increased activation of the Cholesterol 7-α hydroxylase providing to formation of the excessive concentration of conjugated BS (Kok et al. 2003). Development of disease was indicated as factors directly altering the composition of BS and indirectly modulating the efficiency of the lipolysis digestion process.

The following stage of the research (Łozińska et al. 2024)for the first time revealed that the lipolysis process is modulated by five processes and their efficiency is controlled by the form of BS. Each of the processes was presented as a mathematical function of measurable variables. The rate of lipolysis was shown to be controlled by the conjugation form of BS. Meta-analysis of in-vitro lipolysis experiments was performed revealing the significance of individual factors in the lipid digestion process. Unconjugated forms of BS, NaDC, have been shown to reduce the size of the droplet to a higher extent than conjugated NaTC providing greater efficiency of the emulsification process at the beginning of the lipolysis, later on, NaDC contributes to decreasing efficiency of emulsification, by promoting coalescence of the droplet. NaTC promotes FFA release during the lipolysis process by a greater rate of adsorption and desorption than NaDC. The experimental results and meta-analysis of the lipolysis process allowed us to determine the adsorption process and formation of the micelles as predominant factors influencing lipolysis.

The results of my PhD dissertation revealed the importance of the deconjugation process of BS. Conjugation concentration, for the first time, was shown to regulate the rate of lipolysis and the results have shown the controlled way to modulate lipolysis efficiency by modulating five different processes. The importance of the development of diseases, as a factor disturbing the lipolysis process, was shown to alter the BS composition in our body and dysregulate their function as lipolysis agents.

6. Limitations

6.1. **In-vitro digestion studies** One-compartment static digestion models, that were used in publication A1 and publication A3 are good for determining the endpoint of the digestion process, however, the main limitation of the research is that the experiments performed in the static digestion model do not reflect and include the kinetics and physiology of digestion such as absorption, the response of hormones, no effect of gastric emptying and peristaltic movements (Wang et al. 2021). Moreover, the titration reaction is not specific for lipid digestion. There is no possibility to differentiate between digestion products in the case of a complex food matrix, consisting of proteins or starch, which are also neutralised by an alkaline solution (Zhou et al. 2021). This kind of model also does not consider the conditions and processes in the stomach. The gastric and intestinal phases can be performed separately by using a two-compartment model, however, it also requires pre-conditioning as the results obtained from the gastric phase have to be manually transferred (Huang et al. 2021).

6.2. **Digestion conditions**: Another limitation of performed research is its general focus on the BS function. The in-vitro digestion experiments were performed only in the presence of BS, however, the human intestinal digestion of lipids takes place in the presence of a complex matrix of cholesterol, phospholipids, and a greater number of BS diversity, such as PC–sodium glycocholate, sodium glycochenodeoxycholate, sodium taurochenodeoxycholate, SU – sodium lithocholate and secondary conjugated – sodium glycodeoxycholate, sodium taurodeoxycholate (Sensoy 2021). The performed experiment didn't consider the effect of phospholipids, which also influence final FFA release.

6.3. **Modulation of emulsion:** Digestion studies were performed by using an emulsion of the same type of oil and stabilised by WPI. The studies were limited to the interaction of BS with only one type of emulsion. The effect of the composition and structure of emulsion on the lipolysis extends by decreasing fat absorption or increasing the bioavailability of nutrients (Pabois et al. 2020)was restricted in performed research.

6.4. **BS action:** The BS play an important role in the digestion process, but they are also crucial components as receptor regulators (Da Silva et al. 2013). The performed studies don't cover the influence of changes in BS composition apart from the digestion process. For example, changes induced in the regulation of FXR or functioning of BS synthesis.

6.5. **Digestion conditions** Experiments measuring CMC of BS used a simple and non-invasive micro-titration technique, however, its sensitivity allowed only to determine one CMC, whereas the current more advanced techniques allow for more detailed measurements of primary and secondary BS micelles (Mukherjee et al. 2016).

7. Future perspectives

Future perspectives should focus on increasing the potential to understand the mechanism of lipolysis and to control the lipid digestion process

7.1. **In-vitro digestion studies:** During the digestion process in our organism, there are a lot of factors acting simultaneously, that influence the final rate of digestion (Bauer et al. 2005; Bellesi et al. 2018; Macierzanka et al. 2019), therefore, In-vitro semi-dynamic and dynamic models should be used considering: (a) bioaccessibility of nutrients and passive absorption of digestion products (for example ESIN or ARCOL system), (b) interaction of nutrients and delivery of functional food, (c) action of gut microbiota (for example TIM-1 system providing complex high-density microbiota of animal or human origin), (d) digestive secretion (DGM system) should be used in future studies. Moreover, in-vivo studies should be performed.

7.2. **Digestion conditions:** To expand the opportunity for performing lipolysis under controlled conditions in-vitro digestion studies with the presence of phospholipids, cholesterol and complex BS compositions should be performed.

7.3. **Modulation of emulsion:**: The interaction of BS emulsifiers is considered a key factor in modulating lipolysis (Naso et al. 2019). Therefore, microscopic examination, such as confocal microscopy technique, microscope laser light scattering spectroscopy, of lipid droplets during lipolysis for individual BS and different digestion conditions should be investigated to determine the impact of the interaction of BS with emulsion on final fat digestion efficiency and possible mechanism to control lipolysis rate. Moreover, the effect of more complex emulsions and their effect on final FFA release via interaction with BS should be studied to determine the possible mechanism of interaction of emulsion with BS. The results may give a perspective to design an emulsion that would be digested in a controlled way and would modulate the lipolysis process by enhancing or suppressing FFA release from the emulsion.

7.4. **BS action:** The effect of BS towards BS synthesis via controlling FXR should be more deeply studied to understand the possible perturbation of the lipolysis process from the molecular side. Controlling the BS synthesis appears to be a key solution to reducing the obesity problem (Haeusler et al. 2016). Therefore, uncovering the potential to modulate it would give great value to future studies in finding a solution to the obesity epidemic.

7.5. **Digestion conditions:**: Studies of the activity of BSH should be performed, by using non-invasive methods such as bioluminescent imaging (Khodakivskyi et al. 2021), as the BSH controls the C/U ratio of BS in the small intestine (Bourgin et al. 2021). The increasing activity of BSH would affect in formation of excessive conc of SU BS, which were considered as agents contributing to the development of colon cancer, fat malabsorption and obesity. Moreover, the influence of exogenous parameters, such as antibiotics, should be examined, as they are correlated with the development of disease, decreasing BS composition and diversity and reducing BSH activity (Kronman et al. 2012; Daly et al. 2021). Also, the effect of probiotics and prebiotics should be examined, to check their desirable properties as agents increasing BSH activity.

7.6. **In-vitro digestion studies**: BS are responsible for delivering the essential components to our organism, the disturbance of this process is of great importance and may also be connected with the development of a disease state, such as malabsorption(Montoro-Huguet et al. 2021). Therefore, the absorption potential of digestion end products and BS should be measured, by using the CaCo2 cells - hd29 model, animals' cell lines from piglets or rats, human cell lines or using chambers (ex-vivo models) that use intestinal tissue.

8. Other scientific achievements

8.1. Research internships

POWR.03.05.00-00-Z044/17, 27.03-2022-27.06.2022. University of Granada, Faculty of Science, Department of Applied Physics. Supervisor: Julia Maldonado Valderrama, Associate Professor

BIP (Blended Intensive Programme) at L'Institut Agro - Institut national d'enseignement supérieur pour l'agriculture, l'alimentation et l'environnement (16.06.2023-23.06.2023)

8.2. Other research internships

Internship at Dezhou University, China (08.2018-09.2018)

8.3. Conferences

7th International Conference on Food Chemistry & Technology (FCT 2021), Paris, France 8-10.11.2021, oral presentation.

4th Food Structure and Functionality Forum Symposium 2021, 19-20.10.2021, poster presentation

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