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**Variations in gastrointestinal lipases, pH and bile acid levels
with food intake, age and diseases:
possible impact on oral lipid-based drug delivery systems**

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Abbreviations : API, active pharmaceutical ingredient; BA, bile acids; BSSL, bile salt-stimulated lipase; CCK, cholecystokinin; CEH, carboxyl ester hydrolase; CF, cystic fibrosis; CMC, critical micellar concentration; CP, chronic pancreatitis; DAG, diglycerides; EPI, exocrine pancreatic insufficiency; FFA, free fatty acids; GL, gastric lipase; HCEH/BSSL, human carboxylester hydrolase or bile salt-stimulated lipase; HGL, human gastric lipase; HPL, human pancreatic lipase; HPLRP1, human pancreatic lipase-related protein 1; HPLRP2,

human pancreatic lipase-related protein 2; HV, healthy volunteers; LBF, lipid-based formulations; LC, long chain; LIPF, human gastric lipase gene; MAG, monoglycerides; MC, medium chain; MCT, medium chain triglycerides; MRI, magnetic resonance imaging; NaTDC, sodium taurodeoxycholate; PEG, polyethyleneglycol; PERT, pancreatic enzyme replacement therapy; PL, pancreatic lipase; PLA2, pancreatic phospholipase A2; PLRP2, pancreatic lipase-related protein 2; PPI, proton pump inhibitors; SEDDS, Self-Emulsifying Drug Delivery Systems; SMEDDS, Self-MicroEmulsifying Drug Delivery Systems; TAG, triglycerides;

Abstract

The lipids and some surfactants present in oral lipid-based drug delivery systems are potential substrates for the various lipases involved in gastrointestinal (GI) lipolysis. The levels of these enzymes, together with pH and biliary secretion, are important parameters that condition the fate of lipid-based formulations (LBF) and the dispersion, solubilisation and absorption of lipophilic drugs in the GI tract. Since *in vitro* methods of digestion are now combined with dissolution assays for a better assessment of LBF performance, it is essential to have a basic knowledge on lipase, pH and bile acid (BA) levels *in vivo* to develop relevant *in vitro* models. While these parameters and their variations in healthy subjects are today well documented, *in vivo* data on specific populations (age groups, patients with various diseases, patients with treatment affecting GI tract parameters,...) are scarce and obtaining them from clinical studies is sometimes difficult due to ethical limitations. Here we collected some *in vivo* data already available on the levels of digestive lipases, gastric and intestinal pH, and BAs at various ages and in patients with exocrine pancreatic insufficiency, a pathological situation that leads to drastic changes in GI tract parameters and impacts pharmacological treatments.

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1. Introduction

The lipids present in the oil phase of Self-Emulsifying Drug Delivery Systems (SEDDS), such as triglycerides (TAG), diglycerides (DAG), monoglycerides (MAG) [1], are natural substrates of the various lipases found in the gastrointestinal (GI) tract and they are expected to be digested like dietary lipids [2]. Similarly, the synthetic surfactants with ester bonds present in SEDDS, like polyethyleneglycol (PEG) mono- and di-esters, polysorbates (Tweens[®]), glycerol-polyethylene glycol ricinoleate (Cremophor[®] EL), are also potential substrates for lipolytic enzymes and their enzymatic cleavage has been shown in the course of *in vitro* digestion studies [3-6]. Therefore, most of the SEDDS components can be digested in the GI tract and this can impact the dispersion and solubilisation of lipophilic drugs in various ways [5, 7].

The GI lipolysis process is dependent on various parameters (Figure 1), including (1) the levels of several lipases present in gastric and pancreatic secretions, (2) the pH of action of these enzymes and pH variations occurring in the stomach and small intestine, and (3) the biliary secretion that allows the micellar solubilisation of lipolysis products and lipophilic drugs [2, 8-10]. The bile acids (BA) present in biliary secretion have a dual function as surfactants since they trigger lipolysis by removing lipolysis products (MAG, free fatty acids (FFAs)) from the oil-water interface and they ensure the formation of mixed micelles that drive MAGs and FFAs towards the intestinal epithelium where they are absorbed by enterocytes (Figure 2). These parameters are recognized as the most important ones for simulating *in vitro* the digestion of dietary lipids [11-13] and lipid-based formulations (LBFs) [5, 14, 15], particularly for static digestion models, while additional parameters like gastric emptying rate, gut transit time and gut motility have to be considered for dynamic models [16, 17].

So far, *in vitro-in vivo* correlations are not optimum and this has triggered a collective effort for collecting *in vivo* data to support the development of physiologically relevant *in vitro* digestion models using either static [11] or dynamic methods [16, 18-20]. One can for instance cite two very efficient European Union initiatives, the INFOGEST COST action FA1005 (<http://www.cost-infogest.eu/>) and the UNGAP COST action CA16205 (<https://gbiomed.kuleuven.be/english/research/50000715/50000716/ungap>), aiming at a better understanding of digestive and intestinal absorption processes in the GI tract and the development of standard methods for assessing *in vitro* the digestibility of food and the fate of oral LBFs, respectively. Besides the recognition of the important GI parameters and their variations in healthy subjects, it is also very important to get more knowledge on their variations in specific populations (age groups, patients with various diseases, patients with treatment affecting GI tract parameters,...). Obtaining *in vivo* data on these populations is often more challenging than in healthy adults due to technical and ethical limitations. Here we present a review of some *in vivo* data on digestive lipases, gastric and intestinal pH, and BA levels that are already available for humans at various ages and patients with exocrine pancreatic insufficiency (EPI), a pathological situation encountered in chronic pancreatitis (CP) and cystic fibrosis (CF). These diseases, as well as the immature digestive processes in newborns, provide typical examples of drastic changes in GI tract parameters that can impact the fate of LBFs and pharmacological treatments.

2. The GI digestion of dietary lipids and SEDDS compounds in healthy adults

The enzymatic digestion of lipids or GI lipolysis involves several enzymes produced in the stomach and by the exocrine pancreas [2]. It is an essential step preceding the intestinal absorption of fat and lipophilic micronutrients like vitamins A and E [2]. Gastric (GL) and pancreatic (PL) lipases are the main triacylglycerol hydrolases (EC 3.1.1.3)

involved in the digestion of dietary TAG in adults (Figure 2A) [8, 21]. GL shows a stereopreference for the hydrolysis of the ester bond at position sn-3 of TAG [22], while PL is a sn-1,3-regioselective lipase [23], and their combined actions lead to the transient production of sn-1,2(2,3)-DAG and sn-2-MAG. Under normal adult conditions, the conversion of sn-2-MAG into glycerol is considered as minor, and is not mandatory since both FFA and MAG can be absorbed by enterocytes. Nevertheless, in rats, a substantial release of the fatty acids present at the sn-2 position has been deduced from changes in the fatty acid distribution on the glycerol backbone of chylomicrons TAG compared to dietary TAG [24]. MAG are poor substrates for GL and PL, but two other lipases present in pancreatic secretion, pancreatic lipase-related protein 2 (PLRP2) and carboxyl ester hydrolase/bile salt-stimulated lipase (CEH/BSSL), display a high activity on MAG [3, 4] and can achieve the conversion of MAG into glycerol and FFA (Figure 2A). Their contribution to GI lipolysis is considered to be higher in newborns when PL secretion is still immature and BA levels are lower [25, 26] (see section 6).

Lipolysis of TAG is favoured by emulsification, a process that increases the specific surface area of substrate per unit volume by decreasing the size of lipid droplets and enhances the adsorption of lipases at the oil-water interface [27]. TAG can be already found emulsified in some foods and drinks like milk, seasonings and ice creams, but emulsions can also be formed by peristaltic forces in the stomach and stabilized by amphiphiles like dietary proteins, phospholipids and lipolysis products [28]. However, a reverse situation, i.e. the coalescence of fat droplets, can also occur in the stomach [29-32] and is often ignored. It leads to the creaming of fat in the gastric contents, a phenomenon that is commonly observed upon the collection of gastric samples by intubation in the course of solid-liquid test meal digestion. Indeed, an increased proportion of fat floating on the top of samples can be observed in the late stages of digestion before meal gastric emptying is completed. As a result, the gastric

emptying of dietary fat from solid-liquid test meals is delayed compared to water [33]. Test meal experiments with non-hydrolyzable fat have shown however a parallel emptying of fat and water, which indicates that the slow emptying of dietary fat is not simply due to its coalescence and lower density but also to fat digestion and control of gastric emptying by receptors sensitive to lipolysis products [34]. This hypothesis was made before the identification of receptors of FFA along the GI tract. It is known today that CD36 receptors can sense FFA in the upper part of small intestine [35, 36] and trigger the secretion by I-cells of the GI hormone cholecystokinin (CCK; Figure 2B) [37] that negatively controls gastric emptying rate [38]. The inhibition of lipolysis by the lipase inhibitor Orlistat has allowed a better understanding of these processes [38]. More recent *in vivo* investigations using magnetic resonance imaging (MRI) have confirmed the coalescence of fat droplets and creaming in the stomach depending on the type of emulsions given to volunteers [29-31]. *In vitro* [39] and *in vivo* [32] studies on milk digestion in newborns have also shown the coalescence of milk fat globules during gastric digestion. This process appears partly reversible after long incubation times *in vitro* [40], probably due to the emulsifying properties of digestion products. A recent study combining an *in vitro* digestion approach and MRI in human volunteers has shown how various emulsions could either undergo structuration or coalescence in gastric contents depending on the type of emulsifiers used for preparing these emulsions [41]. While an emulsion stabilized by whey protein isolate showed the faster gastric lipolysis rate and coalescence, another emulsion prepared with nanocrystalline cellulose was not hydrolysable by gastric lipase and no coalescence was observed in gastric contents. The role of gastric lipolysis in the coalescence process was nicely shown under the microscope using a microfluidic device [42]. These studies have also confirmed the essential role of the FFA released during gastric lipolysis on the subsequent release of CCK [41]. This is not astonishing since the I-cells sensing FFA via the CD36 receptor and producing CCK are

localized in the upper part of the duodenum [35]. Thus, gastric lipolysis has several essential functions and impacts: (1) it initiates the GI lipolysis process in the stomach where 10 to 25% of FFA can be released by GL [8], (2) it can promote lipid droplet coalescence, (3) the FFA released in the stomach trigger CCK secretion that further triggers pancreatic and biliary secretions, and control gastric emptying, and (4) the lipolysis products released in the stomach promote the subsequent action of PL [43].

The emulsification of dietary fat becomes highly efficient in the small intestine when the chyme emptied from the stomach is mixed with biliary secretion [44]. Bile acids (BA), cholesterol, phospholipids and proteins are the major constituents of the biliary secretion [45]. In humans, the biliary secretion and pancreatic juice are mixed before they enter into the duodenum, in the so-called Vater ampulla [46]. Once they have reached the small intestine and are mixed with various amphiphiles and lipids, BA are present in mixed micelles and multilamellar structures, as well as adsorbed at the surface of lipid droplets [47]. BA and phospholipids have important function in the presentation of lipid substrates to lipases: they favour the action of the PL-colipase complex by emulsifying TAG and DAG, as well as the action of CEH/BSSL and PLRP2 by the micellar solubilization of their preferential substrates (MAG, phospholipids, galactolipids) [2]. The association of BA and lipolysis products in mixed micelles allows the transfer of FFA and MAG from the oil-water interface to the aqueous phase and then to the intestinal brush border, which allow their absorption by enterocytes (Figure 2B). BA are therefore essential for both lipolysis and the subsequent micellar solubilization of lipolysis products that leads to intestinal absorption.

A better knowledge of the kick-off action of gastric lipolysis is essential to anticipate the fate of SEDDS in the GI tract and this has been overlooked so far [28]. Most LBF have been designed with the idea that their digestion takes place only the small intestine. However, it has now been shown that GL is active on various compounds of LBF, and it shows one of

the highest activities on SEDDS compounds like Labrasol[®] and Gelucire[®] 44/14 compared to the other digestive lipases [3, 4]. The impact of GL on the digestion of representative LBF from the Lipid Formulation Classification System [48, 49] containing either medium (MC) or long (LC) chain TAGs and a range of surfactants has been tested *in vitro* and all these formulations were hydrolyzed by GL [6]. The highest specific activities were measured at pH 4 with the type II (SEDDS) and IIIA MC (SMEDDS) formulations that contain Tween[®]85 or glycerol-polyethylene glycol ricinoleate (Cremophor[®] EL), respectively. The maximum activity on LC formulations was recorded at pH 5 for the type IIIA-LC formulation (SMEDDS) [6]. Since GL can initiate the digestion of LBF in the stomach, a gastric step should be implemented in standardized *in vitro* assays for testing LBF performance, as it is now more common in the area of *in vitro* food digestion [40, 50-53]. Protocols including gastric and duodenal steps, inspired from food digestion [54] and lipase inhibitor testing [55], have been already used for testing the solubilisation of model drugs like Piroxicam and Cinnarizine [56]. Other studies including a gastric step have been performed but using microbial lipases like *Candida antarctica* lipase A [57] and *rhizopus oryzae* [58] as substitutes of GL. Using these *in vitro* models, the future of SEDDS developments might be in the design of formulations non-digestible by GL in order to preserve their emulsified state and Active Pharmaceutical Ingredient (API) dispersion in gastric contents prior to their gastric emptying. Another important point to consider will be the possible impact of the FFA released from LBF in the stomach on the overall sensing of FFA in the upper small intestine and regulation of GI physiology.

3. Changes in gastric and duodenal pH that can impact GL lipolysis of SEDDS

Human GL (HGL) shows a maximum activity at pH 5-5.4 on long chain TAG [59] and is highly stable at pH values ranging from 2 to 7 [60]. Human PL (HPL) presents an

optimum stability and activity at pH 7.0-7.5 in presence of colipase and bile salts, and it is inactivated below pH 3 [61]. These two lipases are therefore tailored for acting in the stomach and small intestine, respectively, under normal adult conditions. Indeed, during a meal, gastric pH raises from 1.0-1.5 (basal fasting conditions) to 5-7 depending on the type of meal ingested and its buffering capacity. It then decreases due to meal dilution by gastric acid secretion before returning to basal conditions after around 3 hours (Figure 3; [8]). At half gastric emptying time, gastric pH is close to 4-5 (Figure 4A; [12, 21, 54, 55, 62]), which is the optimum pH range for the hydrolysis of SEDDS and other LBF by GL [3, 4, 6]. The duodenal pH is less variable and found between 5 and 7, with an average value of 6.25 under normal conditions (Figures 4B and 5A; [21, 54, 55, 62, 63]). Pancreatic lipases (PL, PLRP2 and CEH/BSSL) have an optimum activity on SEDDS in the 6.5 to 7.5 pH range [3, 4] and therefore duodenal pH in healthy adults will favour the lipolysis of SEDDS by these enzymes.

In patients with severe CP, the gastric acid secretion rate is increased during a meal and the gastric pH decreases faster than in healthy adults, reaching values of around 2 after only 60 minutes (Figure 3) [62]. As a result, the mean intragastric pH at half gastric emptying time is close to pH 3 (Figure 4C). The higher acidity of gastric contents emptied from the stomach and the lower pancreatic bicarbonate secretion observed in CP patients also lead to lower duodenal pH values compared to healthy adults (Figure 4D), with a fast decrease during the first hour of digestion and mean pH values of 2-3 for the two following hours (Figure 5A) [62, 63].

In CF patients, a greater gastric acid output combined with a lower pancreatic bicarbonate output also result in an acidic duodenal pH [64]. Postprandial duodenal pH is lower in CF patients than in healthy subjects, especially in the first postprandial hour during which duodenal pH becomes rapidly lower than 6. However, duodenal pH remains above pH 4 for more than 90% of the time on the average [64], what should limit the irreversible

inactivation of PL occurring at low pH ≤ 3 [61]. These changes in duodenal pH, as well as those observed in CP patients, are known to impair the pH-dependent dissolution of acid-resistant enteric-coated pancreatic enzyme preparations given to these patients to improve digestion [64]. Indeed, these preparations have usually a pH threshold for enzyme release at pH 5-5.4 [65]. For these reasons, an additional treatment with proton pump inhibitors (PPI) is often associated with pancreatic enzyme replacement therapy (PERT) to allow a better efficacy of pancreatic enzyme preparations [66]. In patients treated with PPI like lansoprazole, gastric pH remains above 4 (Figure 3) during a meal, while the duodenal pH is kept above 6 and remains close to 7 during the two hours after meal intake [67].

Gastric pH values higher than in normal adults are also observed in premature infants due to immature gastric acid secretion [32, 68-73]. Gastric pH generally ranges from 3.2 to 3.5 under fasting conditions to 6.0 to 6.5 immediately after feeding. It remains higher than pH 5.0 for the first two post-prandial hours and close to pH 4 at the end of gastric emptying (Figure 3; [73]).

It is known that fluctuations in gastric pH may change API ionisation, possibly affecting its dissolution, solubilisation and absorption [74]. One has now to consider that pH fluctuations, such as those listed above, may also impact the fate of SEDDS in the GI tract and in particular their rate of lipolysis by digestive lipases in both the stomach and duodenum.

4. Variations in gastric lipase levels with age and disease

Like pepsinogen [75], GL is produced by the chief cells of the fundic mucosa in humans [76]. In healthy adults, 9 to 25 mg of GL are secreted during a meal [8]. Some adaptation of GL levels in gastric mucosa in response to increased dietary fat has been observed in rabbits [77] but never confirmed in humans so far. GL contribution to TAG lipolysis depends on the type of meal ingested and varies from 10% with a solid-liquid meal

to 25% with a liquid meal in which TAG are emulsified [21, 55, 62]. Intra-gastric lipolysis by GL mainly generates DAG and FFA. The mean basal concentration of GL in gastric juice is close to 100 $\mu\text{g}/\text{mL}$ [12] (which corresponds to 120 U/mL using tributyrin as substrate and the standard assay of GL [59]). GL levels in the stomach are low after the ingestion of a meal due to the dilution of gastric contents but they increase with time and the stimulation of gastric secretions by the meal (Figure 6A) [8, 21]. It has been shown that GL is still present in its active form in the duodenal contents during a meal and can still contribute to lipolysis in the upper small intestine [21]. The maximum concentration of GL in the duodenal contents is close to 20 $\mu\text{g}/\text{mL}$ (Figure 6B).

Concerning the variations in GL levels with age, it has been shown from gastric biopsies that it appears around the 11th week of gestation and increases slowly during the pre- and postnatal development before reaching its adult level in the third month of life (Figures 7 and 8) [78]. *In vivo* digestion studies in premature infants have confirmed that the secretion of GL is well developed at birth with GL and lipolysis levels in gastric contents only slightly lower than those found in adults (Figure 6A) [32, 73]. In adults and under 50 years of age, the average level of GL in gastric mucosa remains high and is around 4,700 U/mg of fresh tissue (Figure 8). GL levels are however found to decrease significantly after 60 years (Figure 8) [76]. The fate of SEDDS in gastric contents might therefore be different in the elderly population.

In patients with CP and severe EPI, GL output is increased 3- to 4-fold and its concentration in both gastric (Figure 6A) and duodenal (Figure 6B) contents is higher than in healthy subjects. Under these conditions and in the absence of PL (see section 5), GL ensures around 30% of the fat digestion normally observed in healthy subjects [62]. Higher preduodenal lipase levels have been also observed in the upper GI tract of CF patients [79]. Previous literature on that topic is however confusing for the new reader in this field because

lipase activity measured in gastric contents is often assigned to lingual lipase rather than GL [80, 81]. It is well known today that human preduodenal lipase encoded by the LIPF gene is only expressed in the gastric mucosa, which is the main origin for the lipase activity measured in the stomach [2, 82]. In both CP and CF patients, GL plays an essential role in fat digestion because of EPI and low levels of pancreatic enzymes, together with the fact that GL is present in duodenal contents at higher levels (Figure 6B). GL is therefore the main lipase to be considered when studying the fate of SEDDS in these patients.

5. Variations in pancreatic lipase levels with food intake, age and disease

In healthy adults, the contribution of PL to the digestion of dietary TAG is close to 75% [2, 21]. In the presence of bile salts, PL requires the presence of a protein cofactor, colipase, to bind at the oil-water interface [83, 84] [85, 86].

By combining the data from several *in vivo* experiments with test meals [2, 8], it has been estimated that the mean duodenal concentration of PL in healthy humans is 328 $\mu\text{g/mL}$, with a maximum of 1,450 $\mu\text{g/mL}$. These values correspond to 2,624 U/mL and 11,600 U/mL, respectively, based on PL standard pHstat assay with tributyrin as substrate [87] and the specific activity of PL (8,000 U per mg of enzyme) under these conditions [21]. Similar values (340 and 1,130 $\mu\text{g/mL}$, respectively) were previously reported by Sternby *et al.* [88], using both an ELISA of PL and the same pHstat assay with tributyrin. Several other studies have reported PL levels in duodenal contents or pancreatic secretion in terms of enzyme activity units (U) but using different assay conditions and therefore, lipase units cannot be compared to those mentioned above, nor converted into $\mu\text{g/mL}$ of active enzyme when details on the lipase assay and specific activities are not available.

The total secretion of PL varies from 88 to 645 mg during a meal [8]. A retrospective analysis of PL outputs reported in several *in vivo* studies with various test meals clearly shows

an increase of PL secretion with the amount of fat in the meal (Figure 9A). It suggests an adaptative response of PL secretion to fat intake in humans, as previously observed with PL and colipase mRNA levels in rats fed with various diets [89, 90]. Thus, the more fat (TAG) in the meal, the more PL is produced. This adaptation is probably mediated via the increased production of FFA that further amplifies the production of CCK and the stimulation of PL secretion (Figure 9A). In line with this finding, the direct intake of FFA instead of TAG in the meal results in a much higher secretion of PL (Figure 9A) [91]. PL secretion in healthy volunteers fed with a homogenized liquid meal is higher (125%) than with a solid-liquid meal containing the same amount of fat (Figure 9B), probably because the lipolysis and FFA levels are higher with the liquid meal [55]. The feedback regulation of pancreatic lipase secretion by FFA and CCK has been demonstrated using the lipase inhibitor Orlistat [92]. Indeed, lipase inhibition leads to lower levels of FFA in duodenal contents, which results in lower levels of CCK in plasma and lower levels of PL secretion. These effects of PL inhibition by Orlistat on PL secretion levels have also been observed in the healthy volunteers who received either liquid or solid-liquid test meals containing the same amount of fat (Figure 9B) [55]. With both meals, the administration of 120 mg Orlistat, either as pure Orlistat powder mixed to the meal or as Xenical formulation of Orlistat, led to a decrease of around 30 % [24 to 37%] of PL secretion. Lipase inhibitors like Orlistat have therefore a double effect in the reduction of GI lipolysis: a direct one due to the inhibition of lipases, and an indirect one due to a reduction in lipase secretion. These studies with various dietary fat amounts and Orlistat highlight the essential role of FFA in the regulation of GI physiology. One has to be aware that FFA released from LBF might have similar effects to those released from dietary fat, although most LBF are designed to be used under fasting conditions.

In recent years, the progresses in the development of *in vitro* models of digestion have led to more physiologically relevant values for PL levels in the intestinal phase of digestion.

A 250 µg/mL concentration of PL, corresponding to 2,000 U/mL using tributyrin as substrate, was chosen in a two-step digestion model simulating the conditions in the duodenum of healthy adults at half gastric emptying [54-56]. The static *in vitro* digestion model proposed by the INFOGEST initiative for testing food digestion also recommends using a PL level of 2,000 U/mL, using the same PL assay and unit definition [11]. In the *in vitro* digestion model developed by the LFCS consortium for testing LBFs, a PL level of approx. 1,000 U/mL (tributyrin as substrate) has been chosen [5], which still remains representative of PL levels in healthy adults.

On the contrary to GL secretion, PL secretion is still immature at birth (Figure 7), with PL outputs representing only 6% of the secretion in children (nine months to 13 years old) over a 50-minute period post-stimulation by pancreozymin (Figure 10) [93]. PL secretory levels are also much lower in children with CF compared to healthy children (Figure 10) [94]. In that case, the rate of pancreatic enzyme secretion is drastically reduced by mucus and the gradual loss of pancreatic acinar cells [95]. A similar situation is observed in adult CP patients with severe EPI, in whom PL secretion during a test meal is almost abolished with less than 2% of the PL outputs measured in healthy adults (Figure 5B) [62, 63]. In the absence of PL, patients present fat malabsorption and steatorrhea. Nevertheless, they can still digest a significant amount of dietary fat (around 30% in the complete absence of PL [62]) and lipolysis in the GI tract results from the action of other lipases. In CP and CF patients, in whom all pancreatic enzymes are deficient, GL is the most important lipase and its secretory levels can be increased [62, 80, 96], as seen in section 4. In newborns, only the secretion of PL is immature (Figure 7), and lipolysis can result from the combined action of GL, CEH/BSSL [97] and PLRP2 [25, 26], although their respective contributions are not known. These lipases are therefore the most important to be considered when testing LBF lipolysis in these specific populations.

6. Variations with age in the contribution of pancreatic lipase-related protein 2 and carboxyl ester hydrolase to lipid digestion

In addition to PL, two pancreatic lipase-related proteins, PLRP1 and PLRP2, are produced by the human pancreas. They show around 70% amino acid identity with the so-called classical PL [98]. PLRP1 is an inactive protein and its physiological role is still unknown [99, 100], while PLRP2 shows a broad substrate specificity and is now well characterized [2, 26, 101].

Pancreatic carboxyl ester hydrolase (CEH; or bile salt-stimulated lipase, BSSL) also shows broad substrate specificity. Like PL and PLRP2, CEH/BSSL is produced in pancreatic acinar cells and found in pancreatic juice [102, 103]. BSSL gene is also expressed in the mammary glands, which leads to the presence of BSSL in human milk [104-108]. This suggests an important role for this enzyme in fat digestion during the lactation period [97, 109]. It has been shown *in vitro* that the combined actions of GL, PL and CEH/BSSL leads to the complete conversion of milk TAG into FFA and glycerol [97]. PLRP2 was not identified yet at the time this study was performed but its potential contribution to fat digestion in newborns has now to be considered [25, 26].

In vitro, both CEH/BSSL [110-112] and PLRP2 [113-118] are able to hydrolyze a large set of substrates, including TAG, DAG, MAG, phospholipids, galactolipids, vitamin and cholesterol esters. However, their substrate preference can be modulated by BAs. Indeed, both enzymes are much more active on substrates dispersed in the form of mixed micelles (MAG, phospholipids, galactolipids) than on emulsified TAG when BA are present [3, 4, 119]. On the contrary to PL, PLRP2 poorly interacts with colipase [26, 113, 115] and can hardly bind to the oil-water interface to hydrolyze TAG when BA compete with lipase and other proteins for adsorption. The maximum specific activity of human PLRP2 on long chain TAG (210 ± 3

U/mg) recorded on olive oil, in presence of gum arabic and colipase at a physiological pH of 6.5, only represents 5-10% of PL activity under similar conditions [116]. It was proposed that PLRP2 cannot be very efficient as a TAG lipase *in vivo* under normal adult conditions and is more important for the digestion of phospholipids [113], galactolipids [115] and MAG [3, 4, 116]. It was shown more recently that human PLRP2 could better bind to the oil-water interface in the presence of free fatty acids but using a much higher colipase to lipase molar ratio than the physiological one (>5 instead of 1) and the maximum specific activity recorded on triolein (around 150 U/mg) was still much lower than that of PL [26].

The substrate specificity and the contribution of PLRP2 and CEH/BSSL to fat digestion can evolve however, depending on the BA levels. Indeed, PLRP2 can better bind to the oil-water interface and hydrolyze TAG in the absence of BA or at low levels of BA below the CMC [115, 116]. Conversely, substrates like phospholipids are not presented under their optimum form to PLRP2 and its phospholipase activity is decreased [120]. CEH/BSSL is also less active on water-dispersed substrates [110, 111, 121] in the absence of BA. This situation is encountered in early life with low levels of BA (Figure 11) and PL (Figure 10), while both PLRP2 and CEH/BSSL gene expression already reaches adult levels at birth (Figure 7) [122]. It is today considered that both PLRP2 and CEH/BSSL have a higher contribution to GI lipolysis in newborns, in addition to GL, when PL secretion is still immature and BA levels are low [13, 25, 26, 123]. Thus, these enzymes undergo an apparent change in their substrate specificity with age, which is worth noticing as an unusual finding in enzymology! This may have an important impact on the fate of LBF depending on age. Considering the contribution of PLRP2 and CEH/BSSL to LBF lipolysis appears even more important today with the possible use of milk as a lipid-based drug delivery system [124].

7. Variations in intestinal bile acid levels with food intake, age and disease

The BA play an essential role in the digestion of fats and absorption of lipolysis products [9, 44], as well as in the solubilization of lipophilic drugs [125-127]. They are involved in the micellar solubilization of FFA and MAG, which ensures the removal of these lipolysis products from the oil-water interface, their dispersion in the aqueous phase of intestinal contents and their diffusion towards the intestinal brush border prior to absorption. Similarly, drugs with a low aqueous solubility are generally well solubilized by BA micelles in the intestine, which allows their transport to the intestinal wall [128]. In the absence of BA secretion, both fat absorption [129] and lipophilic drug bioavailability [130-133] are impaired. Levy *et al.* have shown for instance that the bioavailability of cyclosporin (Sandimmune® or Sandimmune Neoral® formulations) was reduced 5-fold when administered to patients with biliary fistula in the absence of exogenous bile [132]. Similar findings were made in rats when studying the absorption of ER-1258, an anti-cancer drug targeting the estrogen receptor [133]. The bioavailability of ER-1258, dissolved in either medium chain triglyceride (MCT) or MCT-based SMEDDS, was reduced 12-fold and 3-fold, respectively, in bile duct-cannulated rats compared to normal rats. Using sodium taurodeoxycholate (NaTDC) and Danazol as models of bile salt and poorly-water soluble drug, respectively, it has been shown that increasing NaTDC concentration increased the digestion of the most lipophilic LBF and promoted lipid and drug transfer from poorly dispersed oil phases to the aqueous colloidal phase [14]. In general, BA at high concentrations also showed some capacity to reduce drug precipitation [14, 128].

In healthy adults, there is a pool of around 3 g of BA that circulates 4 to 12 times per day in the small intestine [134] and the total amount of BA recovered from duodenal aspiration during a meal (3 hours) is close to the pool value (2.62 ± 2.14 g or 5.52 ± 4.53 mmoles) [63]. In the fasted state, total BA concentration in the small intestine is found in the

2 to 6 mM range [128]. It increases after meal intake and reaches a maximum value of around 15 mM after 30 min (Figure 5C). The intestinal BA concentrations has been reported to be highly variable during meal digestion and found between 0.5 mM [135] and 37 mM [47]. Nevertheless, the postprandial BA levels recorded after 30 to 60 min, which corresponds to the gallbladder emptying [134, 136], are often close to 15 mM [47, 135, 137-139], as recently reported by Humbert *et al.* (14.8 ± 12.7 mM) [63]. After gallbladder emptying, the duodenal concentration of BA decreases and levels off around 3-4 mM after 120 minutes (Figure 5C). In healthy adults, the mean BA concentration in intestinal contents thus remains higher than the CMC of the main BAs present in human biliary secretion, which has been estimated to 4 mM on average [140].

In newborn infants, the analysis of lipids in bile aspirated from the gallbladder demonstrated a significantly lower total lipid content (BA, cholesterol, phospholipids) than in children (33 g/L vs. 91 g/L). The most prominent difference was observed with the BA concentration (43.2 mM vs. 126.7 mM on average) and with the phospholipid content [141]. The variation with age in gallbladder BA concentration (Figure 11) shows that the BA levels of children, similar to those found in adults, are reached after 9 to 30 months of age [141]. The immature secretion of BA in newborns concentrations leads to low intestinal concentrations ranging from 0.41 to 1.48 mM during a test meal [142].

A comparison of postprandial BA levels in healthy adults and CP patients with severe EPI was recently performed using LC-MS/MS analysis of BA [63]. The total BA recovery in the duodenum during a meal is much lower in CP patients and amounts only 18% of BA output in healthy subjects. It results in a lower BA concentration in duodenal contents that remains below the mean CMC value, except for the 30 to 45-min period after meal intake, during which a maximum BA concentration of around 4 mM is observed (Figure 5C). Some previous studies have suggested that the low levels of BA in CP patients results from the

precipitation of glycine-conjugated BA at the low intestinal pH encountered in these patients [143, 144]. Nevertheless, the primary to secondary BA ratio is much higher in these CP patients compared to healthy subjects [63], which rather indicates an alteration of the enterohepatic circulation of BA. Indeed, primary BA are synthesized in the liver while secondary BA are produced in the gut from the conversion of primary BA by the intestinal microbiota. One possible explanation for the low BA levels in CP patients is a malabsorption of BA in the intestine, which would result in a fecal loss of BA and a depletion of secondary BA versus the primary BA newly synthesized in the liver. An impaired delivery of BA in the small intestine due to bile duct obstruction seems however the most plausible explanation since the severe CP patients enrolled in this study showed bile duct stenosis [63].

In CF patients, total bile acid secretion rates are normal in young patients and deteriorate with age, probably because of the development of liver disease [145]. CF patients, at all levels of pancreatic function, have a significantly reduced total BA output compared with control subjects, with delayed appearance of the BA peak resulting from gallbladder emptying [146]. The duodenal BA concentrations are however higher in CF patients than in controls, probably because of the markedly reduced water output observed in these patients [146].

The low levels of BA in these populations can significantly impact the bioavailability of lipophilic drug as observed with cyclosporin administered to patients with biliary fistula in the absence of exogenous bile [132] or ER-1258 administered to bile duct-cannulated rats [133]. Nevertheless, in this later case, the bioavailability of ER-1258 is higher when the drug is dissolved in MCT-based SMEDDS than in MCT. Thus SMEDDS and their compounds can partly compensate for the deficiency in BA, probably by ensuring a better dispersion and solubilization of lipophilic drugs.

The use of SEDDS might be also a step forward in the development of novel drug formulations for the treatment of CP and CF patients. Indeed, the Gelucire 44/14 was found to improve fat absorption (from 70% to 82%, $p < 0.001$) in a rat model with impaired lipolysis (diet containing the lipase inhibitor Orlistat) [147]. It was speculated that, due to its self-micro-emulsification properties, Gelucire 44/14 stabilizes and improves residual lipolytic enzyme activity *in vivo*, which could have a therapeutic value in clinical conditions of fat malabsorption due to impaired lipolysis. Moreover, the combination of SEDDS and porcine pancreatic extracts could further improve PERT in patients with EPI [148]. However, in rats with impaired solubilization of lipolysis metabolites (permanent bile diversion), Gelucire 44/14 did not increase fat absorption nor the absorption kinetics of an isotope-labeled FFA added to the diet [147]. Gelucire 44/14 is therefore efficient for enhancing lipolysis but it is not sufficient to improve lipolysis products solubilisation in absence of bile. The oral co-administration of exogenous BA (deshicated ox bile or synthetic conjugated BA cholylsarcosine) and pancreatic enzymes has been already investigated to improve lipid digestion and absorption in patients with EPI [149-151]. One can speculate that future developments in SEDDS formulation might lead to a combined improvement of both lipid digestion and absorption, and these could be also beneficial for the delivery of lipophilic drugs in patients with EPI or/and BA deficiency.

8. Concluding remarks

We compiled here a sum of *in vivo* data on the levels and variations of GI parameters that are essential for a better understanding of lipid digestion processes occurring in healthy adult subjects and specific populations like newborns and patients with EPI (CF and CP). This knowledge is essential for studying the fate of LBF in the gut, in connection with the dissolution and absorption of lipophilic drugs. We hope it will help developing *in vitro*

models of digestion coupled to dissolution assays that are relevant for these various populations, and this should be beneficial for adapting LBF based on age or pathology and improving their performance. Among key points, one has to take into account the existence of several digestive lipases with various substrate specificities for lipid hydrolysis and levels that vary with food intake, age and disease. GL, PLRP2 and CEH/BSSL have for instance a higher contribution than PL to GI lipolysis in newborns, while gastric lipolysis by GL may be reduced in elderly. GL will be the main digestive lipase to consider in cases of EPI. Moreover, the substrate specificity and the turn-over of digestive lipases can be modulated by BA, the levels of which can also vary with age and disease. This highlights the fact that BA have a central role in both the control of GI lipolysis and the solubilization of lipophilic molecules. Their deficiency observed in newborns, CF and CP patients is therefore critical for the digestion of LBF and drug solubilisation. The use of SMEDDS appears as an interesting track to trigger lipolysis when PL levels are low and to partly compensate for the deficiency in BA in some pathological situations.

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Figure legends

Figure 1: Important parameters from GI secretions influencing the fate of oral lipid-based drug delivery systems in the GI tract. LBF released from gelatine capsules in the stomach can spontaneously form fine emulsions when formulated as SEDDS. Some of the components of these emulsions (glycerides, surfactants with ester bonds) can start to be hydrolyzed in the stomach by gastric lipase (GL), which already leads to dynamic changes of SEDDS dispersions. After gastric emptying, this process proceeds in the small intestine by the action of pancreatic lipolytic enzymes (CEH/BSSL, carboxylester hydrolase or bile salt-stimulated lipase; PL, colipase-dependent classical pancreatic lipase; PLA2, pancreatic phospholipase A2; PLRP2, pancreatic lipase-related protein 2) and bile secretion.

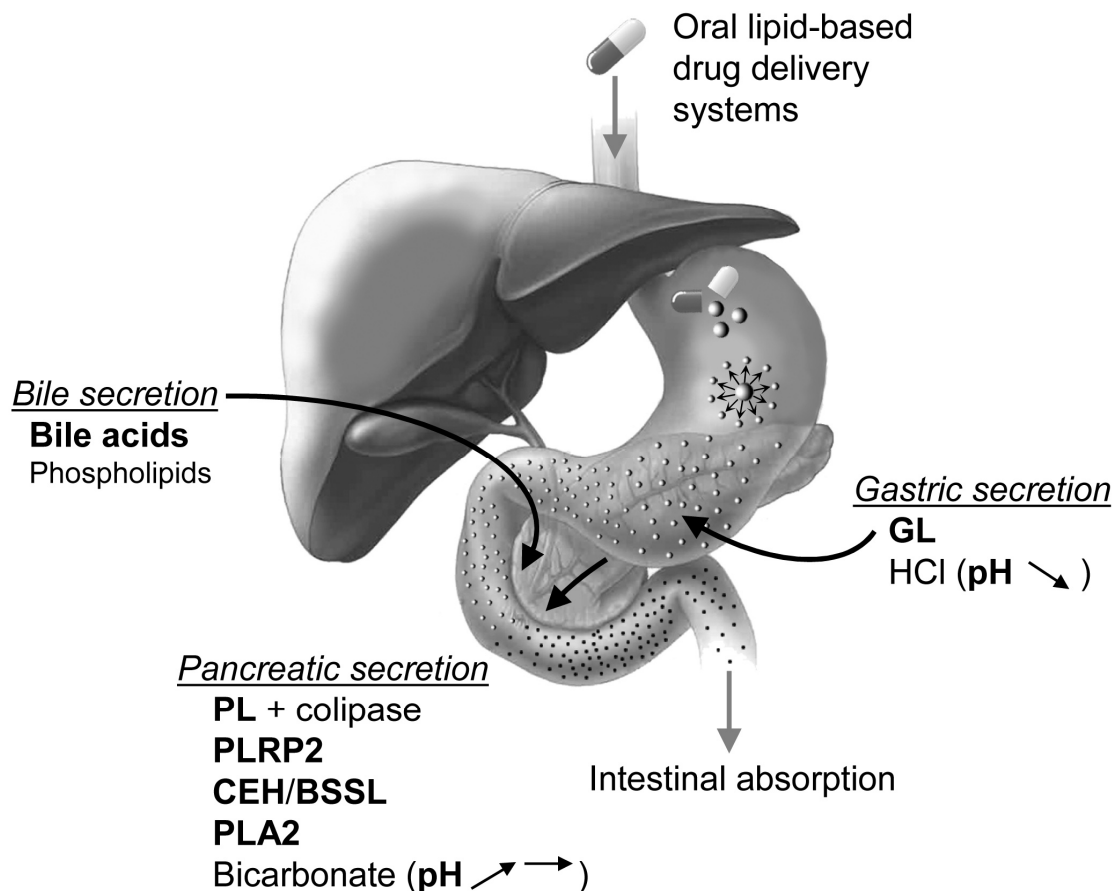


Figure 2: *Chemistry, colloidal aspects and physiological role of GI lipolysis in healthy adults.* The enzymatic cascade leading to the conversion of triglycerides (TAG) into lipolysis products (DAG, diglycerides; MAG, monoglycerides; FFA, free fatty acids; glycerol) involves several lipases and their respective contributions are indicated in panel A and ranked using different sizes for the lipase abbreviations. GL, gastric lipase; PL, classical pancreatic lipase; CEH/BSSL, carboxylester hydrolase or bile salt-stimulated lipase; PLRP2, pancreatic lipase-related protein 2. FFA and MAG produced at the oil-water interface are solubilized by bile acid (BA) via the formation of mixed micelles and further absorbed by enterocytes where they are used for TAG re-synthesis prior to chylomicron assembly and secretion (panel B). FFA sensing by I-cells in the upper part of the small intestine via the CD36 receptor also leads to the production of cholecystokinin (CCK) that can further trigger pancreatic and biliary secretion.

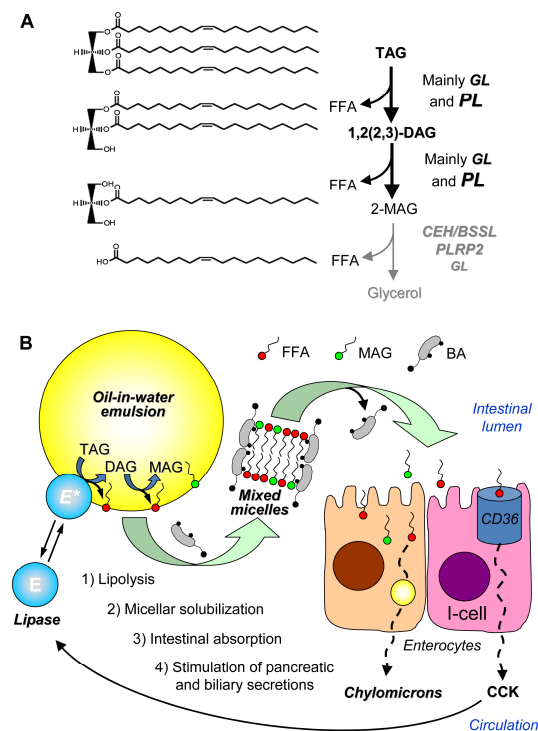


Figure 3: Gastric pH variations during test meals in human adults and newborns. Mean values and standard deviations were calculated from data obtained in experiments performed with a liquid test meal on healthy volunteers (HV; n=30) [8], with a solid/liquid test meal on HV (n=40) [8], with a liquid test meal on HV after administering the proton pump inhibitor (PPI) lansoprazole (n=6) [67], with a solid-liquid test meal on patients with severe chronic pancreatitis (CP; n=15) [62] and premature newborns fed with an infant formula (n=9) [73].

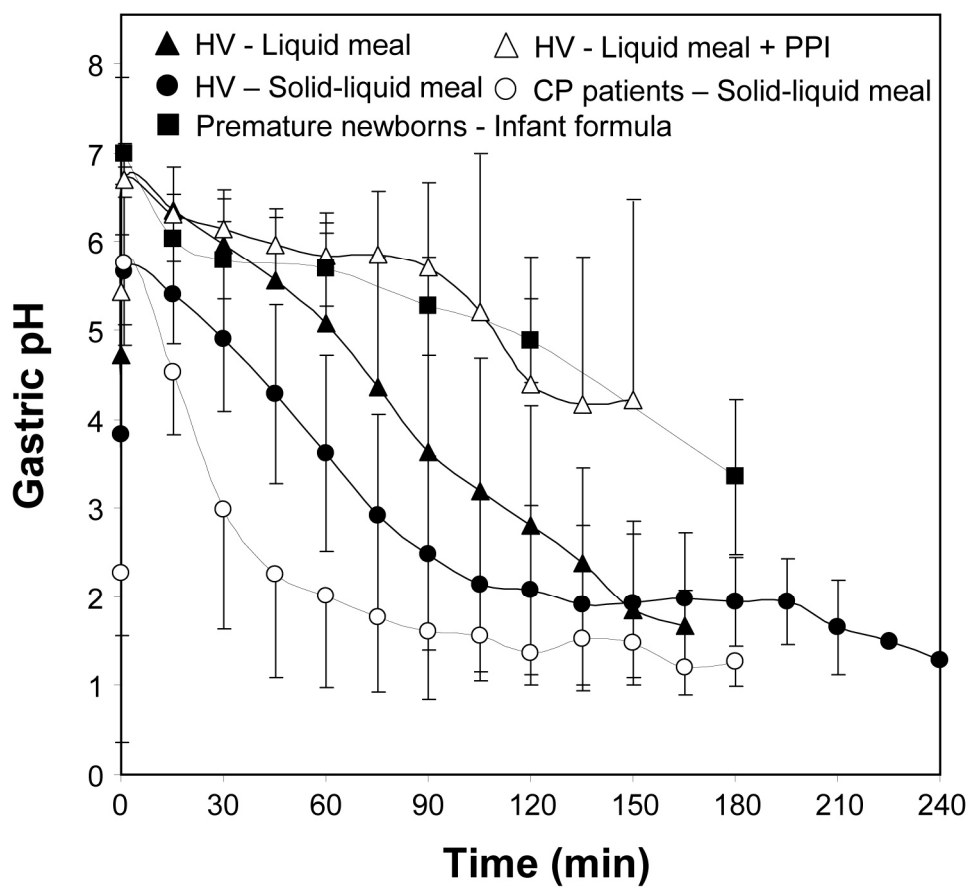


Figure 4. Gastric and duodenal pH variations with gastric emptying in healthy volunteers (HV) (A, B) and severe chronic pancreatitis (CP) patients (C, D) during a solid-liquid test meal. Data in panels A, B, C and D are from 53, 27, 15 and 15 test meal experiments, respectively, previously reported in [12, 21, 54, 55, 62, 63]. In each panel, the mean variation with time was estimated by 3rd order polynomial regression and is shown as a bold line.

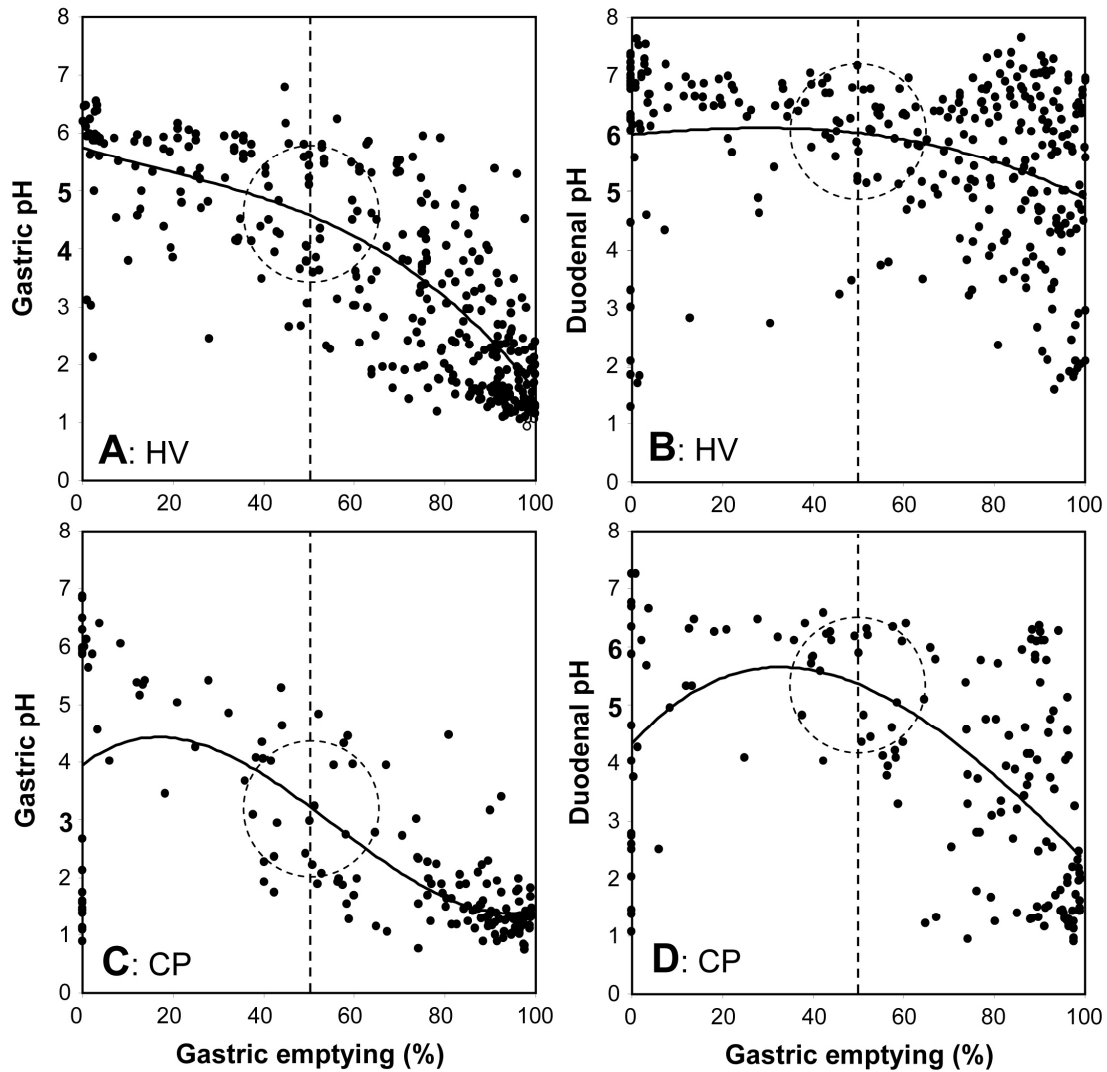


Figure 5: Variations with time in duodenal pH (A), HPL (B) and bile acid (C) concentrations in healthy volunteers (HV) and patients with severe chronic pancreatitis patients (CP). BA, bile acids; HPL, human pancreatic lipase. Values are means \pm SD (n=6 for CP patients and n=6 for HV). In panel B, pancreatic lipase activity was measured using tributyrin as substrate [21, 87] and expressed in international units (1 IU = 1 μ mole of free fatty acid released per min) per mL of duodenal contents at the time of collection. Caution: the lipase units given here cannot be directly compared with those of Figure 7 because assay conditions and substrate are different. In panel C, total BA levels in duodenal contents were measured by LC-MS/MS [152]. The horizontal dotted line indicates the mean critical micellar concentration (CMC) of main BAs finds in human bile (around 4 mM according to [63, 140]). Adapted from original data presented in [63].

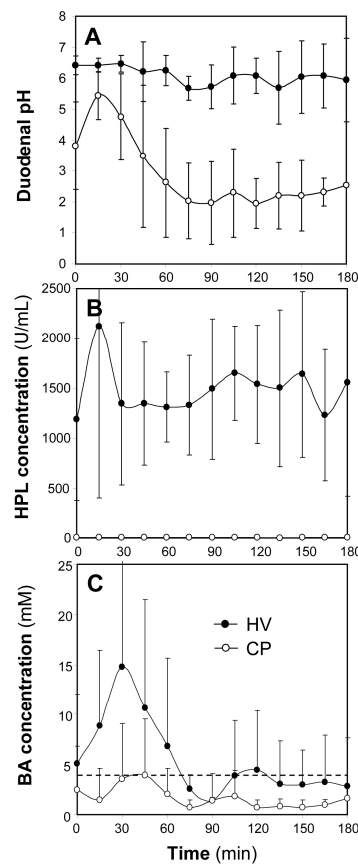


Figure 6: Human gastric lipase levels in gastric (A) and duodenal (B) contents during test meals in healthy adult volunteers (HV), adult patients with severe chronic pancreatitis (CP) and premature infants. In panel A, data for HVs fed with a solid-liquid meal (n=53 subjects; 545 data points) are from [12] and the mean variation with time obtained by polynomial regression is shown as a bold line. Data for adult patients with severe CP and fed with a solid-liquid meal (mean \pm SD; n=7) are from [62]. In panel B, data for HVs (mean \pm SD; n=6) and adult patients with severe CP patients (mean \pm SD; n=7), both fed with a solid-liquid meal, are from [62]. In all these experiments, GL levels were estimated from the measurement of lipase activity in gastric contents collected by intubation, using the same assay with tributyrin as substrate [21, 59]. These levels are expressed in units (U) per mL of gastric contents (1 U = 1 μ mole of free fatty acid released per min), as well as in μ g of active enzyme per mL estimated from the specific activity of pure HGL under these assay conditions (1200 U/mg of enzyme).

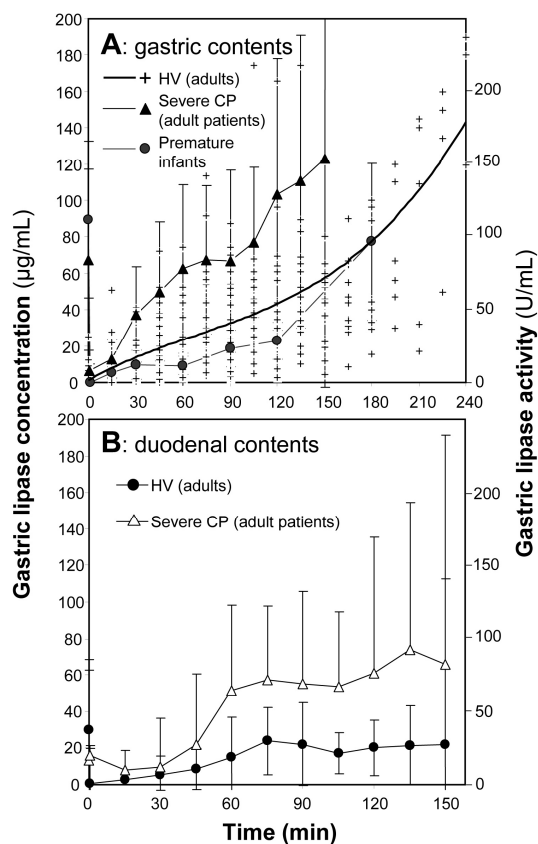


Figure 7: *Ontogeny of GI lipases based on enzyme activity and mRNA levels reported in the literature.* For each lipase, levels as a function of age are expressed as percentage of adult levels and represented graphically. * data deduced from mRNA levels in pancreas and adapted from [122]. HGL, human gastric lipase; HPL, human pancreatic lipase; HPLRP1, human pancreatic lipase-related protein 1; HPLRP2, human pancreatic lipase-related protein 2; HCEH/BSSL, human carboxylester hydrolase or bile salt-stimulated lipase.

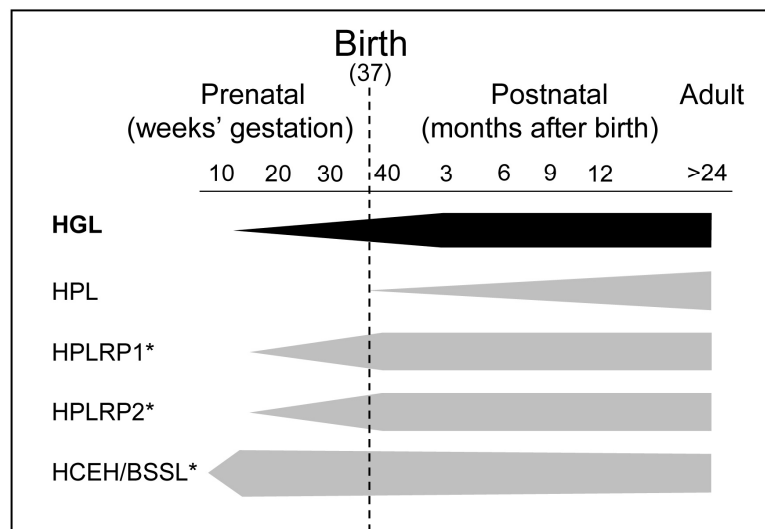


Figure 8: *Human gastric lipase levels in fundic mucosa as a function of age.* Lipase activity was measured on homogenates of biopsies taken in the upper part of the fundic mucosa, using tributyrin as substrate [21, 59], and is expressed as units (U) per mg of fresh tissue. 1 U = 1 μ mole of free fatty acid released per min. Adapted from [78] and [76].

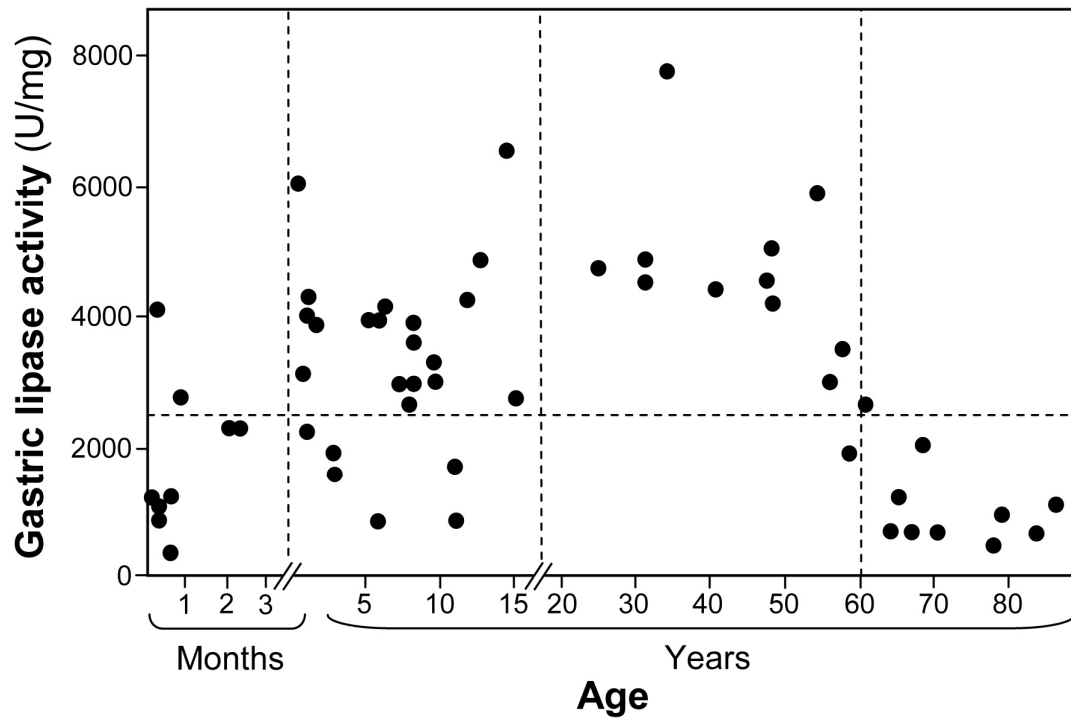


Figure 9: Pancreatic lipase outputs in response to fat contents in test meals (A) and treatment with the lipase inhibitor Orlistat (B). Data in panel A are from [21, 54, 55, 62, 91, 92]. Data in panel B are from [55]. Healthy volunteers were fed either with a complete homogenized liquid test meal or with a solid-liquid test meal, both containing 15 g of fat. The lipase inhibitor Orlistat (120 mg) was administered with the meal, either as pure Orlistat powder mixed to the meal or as Xenical formulation of Orlistat. PL outputs were measured in duodenal contents collected by intubation, using an anti-PL ELISA [55].

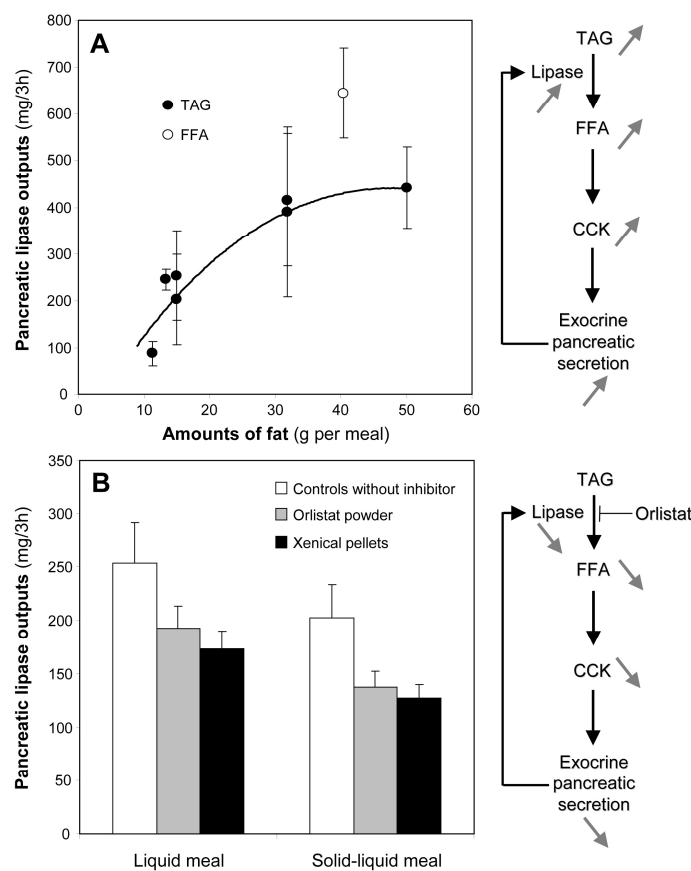


Figure 10: *Pancreatic lipase activity in duodenal juice of preterm or full-term newborns as compared to healthy children and children with cystic fibrosis.* Duodenal juice was collected by intubation using a three-lumen tube with two channels to inflate a proximal occluding balloon in the upper part of duodenum and a second distal one at the Ligament of Treitz, and a central lumen for the continuous aspiration of duodenal juice for a total period of 50 min. After an initial collection of duodenal juice for 10-20 min, exocrine pancreas secretion was stimulated with pancreozymin first and 20 min later with secretin, both administered by intravenous injection. Data at birth were obtained before the first feeding. Lipase activity was measured using olive oil as substrate [153] and expressed in international units (1 IU = 1 μ mole of free fatty acid released per min) per mL of total duodenal juice collected for 50 min in response to pancreozymin and secretin injections, and per kilogram of body weight. Data for preterm (n=16) and full-term (n=8) newborns fed for one month with the same infant formula are from [93]. Data for healthy children (n=12) and children with CF (n=5), with ages ranging from 9 months to 13 years, are from [94]. Caution: the lipase units given here cannot be directly compared with those of Figure 5B because assay conditions and substrate are different.

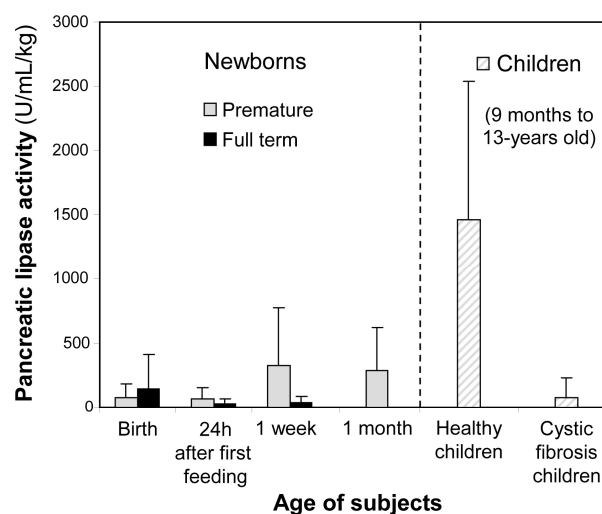


Figure 11: Variation with age in the bile acid concentration in gallbladder bile of infants and children. Adapted from [141].

