APPENDICES

English summary

Cystic fibrosis (CF) is a life-limiting genetic disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. Significant progress has been made in studying and managing the pulmonary complications of CF, which are the leading cause of death; CFTR modulators, which directly target the defective CFTR protein, convincingly improve pulmonary outcomes. However, the intestinal and hepatic manifestations of CF have received less attention despite their significant impact on quality of life, morbidity and life expectancy. This thesis aimed to address this knowledge gap by furthering our understanding of the pathophysiology of intestinal and hepatic CF manifestations, which can be used to develop targeted treatments for these aspects of CF.

Intestinal manifestations of CF

The first part of this thesis explored some less-well studied intestinal manifestations of CF. In **Chapters 3 and 4**, we characterized the abnormal intestinal absorption of bile acids, fat, and cholesterol in two mouse models of CF, with either complete absence of *Cftr* expression (*Cftr*$^{-/-}$) or with the most common CF-causing *Cftr* mutation (*Cftr*$^{\Delta F508/\Delta F508}$). Our findings shed light on the underlying mechanisms of defective intestinal bile acid, fat and cholesterol absorption and its implications for other organs.

In **Chapters 3 and 4**, we observed increased fecal loss of bile acids in CF mice, similar to what is observed in CF patients. Our findings indicated that the excessive fecal loss of bile acids in CF mice is primarily caused by factors within the intestinal lumen (intraluminal factors) rather than inherent defects within the intestinal cells themselves (enterocytic defects). These factors include the accumulation of mucus on the intestinal brush border and the dehydration of the ileal contents, which could impede the uptake of bile acids by intestinal cells from the ileal lumen. We found that the administration of the hydrophilic laxative polyethylene glycol (PEG), which can reverse mucus accumulation and gut dehydration, significantly improved bile acid reabsorption. Our results did not support a role for enterocytic defects in bile acid absorption, as PEG improved bile acid reabsorption without directly affecting the mRNA expression of the bile acid importer *Asbt*, which was unaltered in CF mice. To gain a better understanding of the mechanism of action of PEG, future studies could measure ileal mucus accumulation, hydration levels of gut contents, and the retention of bile acids in the small intestine.

In **Chapter 4**, we investigated fat malabsorption in CF. By using a pancreatic sufficient CF mouse model, we could study fat malabsorption that is independent of deficient lipolytic enzymes consequent to exocrine pancreatic insufficiency. We found that saturated fatty acids exhibited the highest degree of malabsorption, compared to

unsaturated fatty acids. Saturated fatty acids rely more heavily on the process of micellization with bile acids for efficient reabsorption compared to unsaturated fatty acids. Hence, residual (i.e., independent of pancreatic insufficiency) fat malabsorption in CF likely depends on impaired micellization of fat with bile acids. We found that the hydration of gut contents through the use of PEG resulted in improved fat absorption. This led us to hypothesize that gut dehydration interferes with the proper micellization of fat with bile acids. To further corroborate this hypothesis, future studies could directly measure the extent of micellization of fat with bile acids in CF mice, both with and without PEG treatment.

We also studied cholesterol malabsorption in CF. Cholesterol excreted in feces originates from the diet, bile, and a process known as transintestinal cholesterol excretion (TICE). TICE is a recently discovered pathway through which the intestine eliminates cholesterol via feces. TICE involves the secretion of cholesterol by the intestine and the reabsorption of some of this cholesterol, with the fraction remaining unabsorbed resulting in TICE-derived cholesterol found in feces. In **Chapter 2,** we focused on understanding the role of bile acids in the TICE pathway. Our results revealed that bile acids are essential for the reabsorption of TICE-derived cholesterol, as deficiency of bile acids hindered the reabsorption of cholesterol secreted by the intestine. In **Chapter 4**, we examined intestinal cholesterol handling in CF mice and observed an increase in fecal cholesterol loss, similar to what is observed in CF patients. We established that the origin of elevated fecal cholesterol excretion in CF mice is the TICE pathway. Importantly, we found that treatment with PEG reduced fecal loss of TICE-derived cholesterol, likely by increasing its reabsorption. We therefore hypothesize that PEG treatment improves the micellization of cholesterol with bile acids by enhancing the hydration of intestinal contents and reducing mucus accumulation.

Finally, in **Chapter 3 and 4**, we explored the impact of defective intestinal nutrient handling in CF on intestinal nuclear receptor signaling and other organs. We observed a decrease in the signaling of several nuclear receptors involved in bile acid, lipid, and drug metabolism (RXR, FXR, CAR and PXR) in the CF intestine. Decreased signaling of nuclear receptors involved in nutrient signaling was not attributed to inflammation, but likely to reduced availability of ligands for these receptors due to their diminished intestinal absorption in CF. The consequences of impaired nuclear receptor signaling in CF require further investigation and may include disrupted intestinal barrier function, compromised energy and xenobiotic metabolism, and reduced capacity for liver regeneration after injury.

Our research has provided valuable insight into the underlying causes of residual fat malabsorption despite pancreatic enzyme replacement therapy (PERT) use, as well as bile acid and cholesterol malabsorption in CF. These studies have also begun

to uncover the potential consequences of nutrient malabsorption in CF, which need to be investigated further. Further research should focus on validating our findings in CF patients and exploring potential therapeutic strategies to optimize nutrient absorption and restore intestinal and metabolic physiological processes in CF.

Hepatic manifestations of CF

The second part of this thesis aimed to shed light on the pathophysiology of cystic fibrosis-related liver disease (CFLD), which affects a proportion of CF patients, with liver cirrhosis and portal hypertension being severe manifestations associated with increased mortality. Although ursodeoxycholic acid (UDCA) is commonly used to treat CFLD, there is a lack of experimental evidence supporting its ability to modify disease progression. Unfortunately, the development of targeted treatments is hindered by the lack of pathophysiological understanding of CFLD.

Chapter 6 summarizes our current knowledge on CFLD. The research outlined in **Chapters 7 and 8** explores two hypotheses regarding the development of CFLD: the secretory defect hypothesis and the gut-liver hypothesis.

The secretory defect hypothesis suggests that defective CFTR function in cholangiocytes (the cells lining bile ducts) leads to CFLD. CFTR is responsible for chloride and fluid transport into bile, and its dysfunction is hypothesized to cause bile thickening, stasis, cholestatic injury, and eventually cirrhosis. However, bile duct plugs and histological signs of cholestasis are infrequently observed in CF livers. In **Chapter 4**, we observed that CFTR deficiency did not significantly affect total bile flow, arguing against a major role for CFTR in bile secretion. In **Chapter 7** we rejected the hypothesis that CFTR in cholangiocytes can be activated by bile acid reabsorption via ASBT, as occurs in the intestine.

The gut-liver hypothesis suggests that intestinal factors such as microbial dysbiosis contribute to the development of CFLD. This hypothesis is based on previous findings showing that CFTR-deficient cholangiocytes exhibited an excessive inflammatory response to bacterial-derived lipopolysaccharide (LPS), and that inducing colitis in CF mice worsened liver pathology, while antibiotic treatment reduced its severity. CF patients also exhibit intestinal dysbiosis and increased intestinal permeability, which may facilitate the translocation of bacterial byproducts to the liver. To determine the role of intestinal factors in the development of CFLD, in **Chapter 8** we compared CFTR-KO mice (with liver and intestinal CFTR deficiency) to gut-corrected CFTR-KO mice (CFTR-KO-GC mice, with CFTR expression in the gut but not in the liver). CFTR-KO mice showed increased intestinal permeability and dysbiosis, while CFTR-KO-GC mice did not. Surprisingly, CFTR-KO mice displayed liver inflammation and biliary proliferation, whereas gut-corrected CFTR-KO mice did not exhibit liver pathology at the histological level. Proteomic and transcriptomic analysis of CFTR-

KO mouse livers revealed an immune response against gut-derived pathogens, and antibiotic treatment reduced liver inflammation. These results suggest that abnormal CFTR function in the gut is necessary to induce a CFLD-like phenotype in mice, highlighting gut-derived bacteria as a potential "second hit" in CFLD development. Interestingly, while no liver pathology was observed in gut-corrected CFTR-KO mice at the histological level, transcriptomic analysis revealed a subtle pro-inflammatory phenotype, indicating that CFTR-deficient cholangiocytes exhibit some degree of inflammation at a basal level. Overall, these findings suggest that defective CFTR in cholangiocytes might serve as the 'first hit' in CFLD development, while gut-derived byproducts may act as a 'second hit'. This study highlights the importance of the gut-liver axis in CFLD development in a mouse model of CF. Future studies could focus on identifying the specific gut-related factors that contribute to CFLD and ways to target them.

Several knowledge gaps remain regarding CFLD pathophysiology. Our finding that intestinal factors may play a role in the development of CFLD need to be confirmed in patients. Future research could explore the role of CFTR in the biliary epithelium, particularly its impact on the glycocalyx and barrier function of cholangiocytes. Additionally, the epidemiology and underlying mechanisms of non-cirrhotic portal hypertension in CF patients require further investigation.

Nederlandse samenvatting

Cystic fibrosis (CF) is een levensbeperkende genetische aandoening die wordt veroorzaakt door mutaties in het cystic fibrosis transmembrane conductance regulator (CFTR)-gen. Er is aanzienlijke vooruitgang geboekt in het bestuderen en behandelen van de pulmonale complicaties van CF, die de belangrijkste doodsoorzaak zijn; CFTR-modulatoren, die zich rechtstreeks richten op het defecte CFTR-eiwit, verbeteren de pulmonale complicaties op overtuigende wijze. De intestinale en hepatische manifestaties van CF hebben echter minder aandacht gekregen, ondanks hun significante invloed op de kwaliteit van leven, morbiditeit en levensverwachting. Dit proefschrift richtte zich op deze kennisleemte door ons begrip van de pathofysiologie van intestinale en lever manifestaties van CF te vergroten, wat gebruikt kan worden om gerichte behandelingen voor deze aspecten van CF te ontwikkelen.

Intestinale manifestaties van CF

In het eerste deel van dit proefschrift zijn enkele minder goed bestudeerde intestinale manifestaties van CF onderzocht. In **hoofdstuk 3 en 4** karakteriseerden we de verlaagde intestinale absorptie van galzuren, vet en cholesterol in twee muismodellen van CF, met ofwel volledige afwezigheid van *Cftr*-expressie (*Cftr*$^{-/-}$) of met de meest voorkomende CF-veroorzakende *Cftr*-mutatie (*Cftr*$^{\Delta F508/\Delta F508}$). Onze bevindingen werpen licht op de onderliggende mechanismen van defecte intestinale galzuur-, vet- en cholesterolabsorptie en de implicaties daarvan voor andere organen.

In **hoofdstuk 3 en 4** zagen we een verhoogd fecaal verlies van galzuren in CF muizen, vergelijkbaar met wat wordt waargenomen bij CF patiënten. Onze bevindingen gaven aan dat het overmatige fecale verlies van galzuren bij CF-muizen voornamelijk wordt veroorzaakt door factoren in het darmlumen (intraluminale factoren) in plaats van inherente defecten in de darmcellen zelf (enterocytische defecten). Deze factoren zijn onder andere de ophoping van slijm op de darmborstelrand en de uitdroging van de ileuminhoud, die de opname van galzuren door darmcellen vanuit het ileale lumen zou kunnen belemmeren. We ontdekten dat de toediening van het hydrofiele laxeermiddel polyethyleenglycol (PEG), dat slijmophoping en darmuitdroging kan tegengaan, de galzuurreabsorptie aanzienlijk verbeterde. Onze resultaten ondersteunen een rol van enterocytische defecten in galzuurabsorptie niet, aangezien PEG de galzuurreabsorptie verbeterde zonder directe invloed op de mRNA-expressie van de galzuurimporteur *Asbt*, die onveranderd was in CF-muizen. Om een beter inzicht te krijgen in het werkingsmechanisme van PEG, zouden toekomstige studies de ileale slijmophoping, het hydratatieniveau van de darminhoud en de retentie van galzuren in de dunne darm kunnen meten.

In **hoofdstuk 4** hebben we vetmalabsorptie bij CF onderzocht. Door een CF-muismodel te gebruiken dat pancreassufficiënt is, konden we vetmalabsorptie bestuderen die onafhankelijk is van deficiënte lipolytische enzymen als gevolg van exocriene pancreasinsufficiëntie. We ontdekten dat verzadigde vetzuren de hoogste mate van malabsorptie vertoonden, vergeleken met onverzadigde vetzuren. In vergelijking met onverzadigde vetzuren zijn verzadigde vetzuren voor een efficiënte reabsorptie sterker afhankelijk van het micellisatieproces met galzuren. Vandaar dat resterende (d.w.z. onafhankelijk van pancreasinsufficiëntie) vetmalabsorptie bij CF waarschijnlijk afhankelijk is van een verminderde micellisatie van vet met galzuren. We ontdekten dat hydratatie van de darminhoud door het gebruik van PEG resulteerde in verbeterde vetabsorptie. Dit leidde ons tot de hypothese dat darmuitdroging de micellisatie van vet met galzuren verstoort. Om deze hypothese verder te onderbouwen, zouden toekomstige studies direct de mate van micellisatie van vet met galzuren kunnen meten in CF muizen, zowel met als zonder PEG behandeling.

We onderzochten ook cholesterol malabsorptie in CF. Cholesterol dat wordt uitgescheiden in de ontlasting is afkomstig uit de voeding, gal en een proces dat bekend staat als transintestinale cholesteroluitscheiding (TICE). TICE is een recent ontdekt proces waarbij cholesterol rechstreeks door de darm(cellen) in de ontlasting wordt uitgescheiden. TICE is de balans tussen het door de darm uitgescheidde cholesterol en het heropgenomen deel van dit cholesterol, waarbij het niet-opgenomen deel resulteert in TICE-afgeleid cholesterol in de ontlasting. In **hoofdstuk 2** was het doel om meer inzicht te krijgen in de rol van galzuren in de TICE-route. Onze resultaten toonden aan dat galzuren essentieel zijn voor de reabsorptie van TICE-afgeleid cholesterol, aangezien een tekort aan galzuren de reabsorptie van cholesterol dat door de darm wordt uitgescheiden, belemmerde. In **hoofdstuk 4** onderzochten we de intestinale cholesterolverwerking bij CF-muizen en zagen we een toename in het verlies van cholesterol via de ontlasting, vergelijkbaar met wat wordt waargenomen bij CF-patiënten. We stelden vast dat de verhoogde uitscheiding van fecaal cholesterol bij CF-muizen veroorzaakt wordt door de TICE route. Belangrijk is dat we ontdekten dat behandeling met PEG het fecaal verlies van TICE-afgeleid cholesterol verminderde, waarschijnlijk door de reabsorptie te verhogen. We veronderstellen daarom dat PEG-behandeling de micellisatie van cholesterol met galzuren verbetert door de hydratatie van de darminhoud te verbeteren en slijmophoping te verminderen.

Tot slot onderzochten we in **hoofdstuk 3 en 4** de invloed van een gebrekkige intestinale nutriëntenopname bij CF op de signalering van intestinale nucleaire receptoren en andere organen. We zagen een afname in de signalering van verschillende nucleaire receptoren die betrokken zijn bij galzuur-, lipide- en xenobiotica(medicijnen)metabolisme (RXR, FXR, CAR en PXR) in de CF-darm.

Verminderde signalering van nucleaire receptoren die betrokken zijn bij signalering van voedingsstoffen werd niet toegeschreven aan ontsteking, maar waarschijnlijk aan verminderde beschikbaarheid van liganden voor deze receptoren door verminderde intestinale absorptie bij CF. De gevolgen van verminderde signaaltransductie door nucleaire receptoren bij CF moeten verder onderzocht worden en kunnen bestaan uit een verstoorde barrièrefunctie van de darm, een verstoord xenobiotica- en energiemetabolisme en een verminderde regeneratiecapaciteit van de lever na letsel.

Ons onderzoek heeft waardevol inzicht opgeleverd in de onderliggende oorzaken van resterende vetmalabsorptie, galzuur- en cholesterolmalabsorptie bij CF patiënten, ondanks gebruik van pancreasenzymvervangingstherapie (PERT). Deze onderzoeken hebben ook een begin gemaakt met het blootleggen van de mogelijke gevolgen van malabsorptie van voedingsstoffen bij CF, die verder onderzocht moeten worden. Verder onderzoek moet zich richten op het valideren van onze bevindingen bij CF-patiënten en het onderzoeken van mogelijke therapeutische strategieën om de absorptie van voedingsstoffen te optimaliseren en de darm- en stofwisselingsfysiologische processen bij CF te herstellen.

Levermanifestaties van CF

Het tweede deel van dit proefschrift richtte zich op de pathofysiologie van CF-gerelateerde leverziekte (CFLD), die een deel van de CF-patiënten treft, waarbij levercirrose en portale hypertensie ernstige manifestaties zijn die gepaard gaan met een verhoogde mortaliteit. Hoewel ursodeoxycholzuur (UDCA) vaak wordt gebruikt om CFLD te behandelen, is er een gebrek aan experimenteel bewijs dat het vermogen van UDCA om de progressie van de ziekte te veranderen ondersteunt. Helaas wordt de ontwikkeling van gerichte behandelingen belemmerd door het gebrek aan pathofysiologisch inzicht in CFLD.

Hoofdstuk 6 geeft een samenvatting van onze huidige kennis over CFLD. Het onderzoek in de **hoofdstukken 7 en 8** onderzoekt twee hypothesen met betrekking tot het ontstaan van CFLD: de secretorische defecthypothese en de darm-leverhypothese.

De secretoire defecthypothese suggereert dat een defecte CFTR-functie in cholangiocyten (de cellen die de galwegen bekleden) leidt tot CFLD. CFTR is verantwoordelijk voor het chloride- en vloeistoftransport in de gal en het disfunctioneren van CFTR zou leiden tot galverdikking, stase, cholestatische schade en uiteindelijk cirrose. Galkanaalpluggen en histologische tekenen van cholestase worden echter zelden waargenomen in CF-levers. In **hoofdstuk 4** zagen we dat CFTR-deficiëntie geen significante invloed had op de totale galstroom, wat argumenteert tegen een belangrijke rol van CFTR in de galsecretie. In **hoofdstuk 7**

verwierpen we de hypothese dat CFTR in cholangiocyten geactiveerd kan worden door galzuurreabsorptie via ASBT, zoals in de darm gebeurt.

De darm-lever hypothese suggereert dat darmfactoren zoals microbiële dysbiose bijdragen aan de ontwikkeling van CFLD. Deze hypothese is gebaseerd op eerdere bevindingen die aantoonden dat CFTR-deficiënte cholangiocyten een overmatige ontstekingsreactie vertoonden op van bacteriën afkomstige lipopolysaccharide (LPS), en dat het induceren van colitis bij CF-muizen de leverpathologie verergerde, terwijl behandeling met antibiotica de ernst ervan verminderde. CF-patiënten hebben ook darmdysbiose en een verhoogde darmpermeabiliteit, wat de translocatie van bacteriële bijproducten naar de lever kan bevorderen. Om de rol van darmfactoren in de ontwikkeling van CFLD te bepalen, vergeleken we in **hoofdstuk 8** CFTR-KO muizen (met lever- en intestinale CFTR-deficiëntie) met darmgecorrigeerde CFTR-KO muizen (CFTR-KO-GC muizen, met CFTR expressie in de darm maar niet in de lever). CFTR-KO muizen vertoonden een verhoogde darmpermeabiliteit en dysbiose, terwijl CFTR-KO-GC muizen dit niet vertoonden. Verrassend genoeg vertoonden CFTR-KO muizen leverontsteking en biliaire proliferatie, terwijl darmgecorrigeerde CFTR-KO muizen geen leverpathologie op histologisch niveau vertoonden. Proteoom- en transcriptoom-analyse van de lever van CFTR-KO muizen toonde een immuunrespons tegen pathogenen afkomstig uit de darm aan, terwijl antibioticabehandeling de leverontsteking verminderde. Deze resultaten suggereren dat een abnormale CFTR-functie in de darm noodzakelijk is om een CFLD-achtig fenotype in muizen te induceren, waarbij bacteriën afkomstig uit de darm een potentiële “tweede treffer” zijn in de ontwikkeling van CFLD. Interessant is dat, hoewel er op histologisch niveau geen leverpathologie werd waargenomen bij CFTR-KO muizen met darmcorrectie, transcriptoom-analyse een subtiel pro-inflammatoir fenotype onthulde, wat aangeeft dat CFTR-deficiënte cholangiocyten op basaal niveau een zekere mate van ontsteking vertonen. Over het geheel genomen suggereren deze bevindingen dat een defect CFTR in cholangiocyten de ‘eerste treffer’ zou kunnen zijn in de ontwikkeling van CFLD, terwijl darmafgeleide bijproducten als ‘tweede treffer’ zouden kunnen fungeren. Deze studie benadrukt het belang van de darm-lever as in de ontwikkeling van CFLD in een muismodel van CF. Toekomstige studies zouden zich kunnen richten op het identificeren van de specifieke darmgerelateerde factoren die bijdragen aan CFLD en manieren om deze aan te pakken.

Er zijn nog verschillende hiaten in de kennis over de pathofysiologie van CFLD. Onze bevinding dat darmfactoren een rol kunnen spelen bij de ontwikkeling van CFLD moet worden bevestigd bij patiënten. Toekomstig onderzoek zou zich kunnen richten op de rol van CFTR in het galepitheel, met name de invloed ervan op de glycocalyx en de barrièrefunctie van cholangiocyten. Daarnaast moeten de epidemiologie en onderliggende mechanismen van niet-cirrhotische portale hypertensie bij CF-patiënten verder worden onderzocht.

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About the author

Anna Bertolini was born on February 19th, 1993, in Rovereto, Italy. She grew up with her parents, Raffaella and Vittorio, and her older sister Tania in Arco, a town in the North-Eastern Italian region of Trentino-Alto Adige, renowned for its mild Mediterranean climate and rock climbing. Motivated by her passion for biology, Anna relocated to Civezzano, Italy, from 2007 to 2012 to attend the Istituto Tecnico Biologico high school. It was during these years that Anna immersed herself in her first lab and research experiences, which fostered a blossoming enthusiasm for science. She also spent her summer breaks in Brighton, UK, working as a videogame tester, acquiring proficiency in English, and nurturing her love for travel and diverse cultures. In 2013, Anna commenced her medical training at the University of Groningen, with a focus on global health. The joy of research from her high school experiences prompted her to participate in the Honours College and Junior Scientific Masterclass programs. These educational activities paved the way for her MD/PhD aspirations, and it was during this time that she encountered Maximilian, her partner, marking the beginning of a fulfilling academic and personal journey. Anna met her PhD supervisors, Prof. Henkjan Verkade, Prof. Hans Jonker, and Dr. Frank Bodewes of the Department of Paediatrics at the University Medical Center Groningen, during the Junior Scientific Masterclass programs. Guided by Dr. Marcela Doktorova and Dr. Ivo van de Peppel, Anna acquainted herself with the lab, and her PhD project began to take shape. Throughout her MD/PhD, Anna conducted research for two years at the Department of Paediatrics, University of Groningen, and one year at the Yale Liver Center, Yale University. This thesis represents the result of those years of research. Between research periods, Anna completed medical internships at the Medisch Centrum Leeuwarden, as well as the Department of Genetics, University Medical Center Groningen, and the Department of Paediatric Genetics, University of Utah Hospital in Salt Lake City. Presently, Anna works as a doctor at the psychosis department of the University Center Psychiatry in Groningen.

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