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Identification and Molecular Characterization of Monogenetic Defects Leading to Early-Onset Protein-Losing Enteropathy and Inflammatory Bowel Disease

Doctoral Thesis at the Medical University of Vienna for obtaining the academic degree

Doctor of Philosophy

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Declaration

This thesis is written in a cumulative fashion. The work described in this thesis was performed in different laboratories in a collaborative manner. Contributions of each individual co-authors in the manuscripts are described below.

Chapter 3.1 was published by Ozen, Comrie, and Ardy et al. in the New England Journal of Medicine (DOI: 10.1056/NEJMoa1615887). The author of this thesis identified CD55 mutations in families 3 and 5, performed flow cytometry, generated CD55-CRISPR knockout Jurkat cells, performed complement deposition assays on Jurkats and quantified T-cell activation and cytokine production. Ahmet Ozen identified families 1, 2, 4, 6 and 7, performed flow cytometry and complement deposition assays, and performed CD55 rescue on patient T cells. William A. Comrie performed qRT-PCR studies, performed anaphylatoxin and C3d deposition experiments, and quantified cytokine production and analyzed the effects of cytokines and ATRA on HUVEC cells. Cecilia Domínguez Conde and Nina K. Serwas performed variant filtering, Sanger validation and identified the CD55 mutation in family 3. Aaron R. Morawski validated mutations in Patients 1.1 and 2.1. Aaron R. Morawski and Ahmet Ozen discovered and described novel CD55 mutations in Family 6 and Family 7. Aaron R. Morawski performed immunoblotting experiments and screened early onset and very early onset-IBD patients for CD55 expression by flow.cytometry. Ana Krolo performed additional targeted sequencing of CD55. Yu Zhang performed bioinformatics analyses on whole exome sequencing (WES) data. Roderick H.J Houwen, Helen L. Leavis, Buket Dalgic, Omer Faruk Beser, Elif Karakoc-Aydiner, Engin Tutar, Safa Baris, Figen Ozcay, Aysel Unlusoy Aksu, Ayca Kiykim, Zeren Baris, Meltem Gulsan, Deniz Ertem, Sinan Sari and Tulay Erkan took care of and enrolled patients into the study. Ismail Ogulur and Ayca Kiykim enrolled patients, collected samples and screened potential families for CD55 deficiency by flow cytometry. Sinan Sari and Scott B. Snapper provided critical gastroenterologic expertise on discussion. Helen F. Matthews coordinated clinical study protocol and sample collection. Stefania Pittaluga provided histopathologic evaluation of GI biopsy material. Les Folio reviewed radiologic images and provided expertise. Renate Kain performed electron microscopy and immunohistochemistry and provided expertise. Joshua J. McElwee performed/organized WES studies and analyzed data. The author of this thesis, along with Ahmet Ozen, William A. Comrie, Kaan Boztug, and Michael J. Lenardo wrote the initial draft and revised version of the manuscript. Helen C. Su, Kaan Boztug, and Michael J. Lenardo supervised research and data analysis, provided ideas and advice, and edited the manuscript. All authors provided critical input and agreed to the publication.

Chapter 3.2 was published by van Rijn, Ardy, Kuloglu, Härter, and van Haaften-Visser et al in Gastroenterology (DOI: 10.1053/j.gastro.2018.03.040). The author of this thesis performed experiments on materials from families 1-4. Jorik van Rijn and Desiree van Haaften-Visser performed experiments on materials from families 5 and 6. Zarife Kuloglu, Bettina Härter, Hubert P. J. van der Doef, Marliek van Hoesel, Aydan Kansu, Meryem Keçeli Basaran, Neslihan Gurcan Kaya, Aysel Ünlüsoy Aksu, Buket Dalgıç, Figen Ozcay, Zeren Baris, Andreas R. Janecke, and Roderick H. J. Houwen cared for the patients in their respective hospitals. Marini Thian and Ana Krolo performed experiments on families 3 and 4. Anke H. M. van Vugt, Freddy T. M. Kokke, Edwin C. A. Stigter, Klaske D. Lichtenbelt, Maarten P. G. Massink, Karen J. Duran, Joke B. G. M Verheij, Dorien Lugtenberg, Peter G. J. Nikkels, Henricus G. F. Brouwer, Henkjan J. Verkade, René Scheenstra, Bart Spee, Edward E. S. Nieuwenhuis, Paul J. Coffer, Gijs van Haaften performed experiments for families 5 and 6. Renate Kain provided histopathology expertise. Thomas Müller, Sabine Middendorp, and Kaan Boztug supervised the project, provided financial support, and wrote the manuscript together with the author of this thesis. All authors provided critical input and agreed to the publication.

The author of this thesis is a shared first author in both publications and will be the only individual using these manuscripts for the purpose of a dissertation for the Medical University of Vienna. All chapter of the thesis is written by the author. Dr. Kaan Boztug provided critical input to the writing of the thesis.

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Reprint permissions for all figures and manuscripts were obtained.

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Abstract

Rare diseases of the gastrointestinal tract allow for further insight into essential and important players of gut homeostasis. In many cases, the disease arises from an aberration in a single gene. Identifying novel monogenetic defects as well as understanding the underlying molecular mechanism of disease will allow us to gain further knowledge regarding important hubs of human biology that could lead to a personalized therapy.

Through international collaborations with various research laboratories and research hospitals, we identified and molecularly characterized two monogenic diseases in patients that presented with early-onset protein-losing enteropathy (EO-PLE) and early-onset inflammatory bowel disease (EO-IBD).

Firstly, we identified a total of 11 patients from 8 families suffering from EO-PLE and bowel inflammation. We identified 5 distinct homozygous mutations in the gene called *CD55*, encoding for decay accelerating factor (DAF) or CD55. CD55 is an important regulator of the complement system and is ubiquitously expressed in all cells. We could show that the mutations led to a complete lack/severe reduction of protein expression on patient cells. This in turn led to increased complement activation and deposition as shown by increased C3d binding. We showed that reconstitution with wildtype CD55 resulted in a reversion of the complement deposition phenotype. Lastly, we showed that CD55 deficiency led to increased production of anaphylatoxin C5a in vitro which can be reversed using Eculizumab, a humanized monoclonal antibody against complement protein C5.

In the second study, we identified 10 patients from 6 families with deleterious mutations in the gene encoding for diacylglycerol-acyltransferase 1 or DGAT1. The mutations resulted in a lack of or severe reduction of protein expression. We could show that DGAT1 is important in the context of triglyceride metabolism in gut epithelial cells, as healthy controls highly express DGAT1 in gut epithelial cells. We show that DGAT1-deficient cells fail to form lipid droplets, as seen by their lack of granularity or NileRed/LD540 staining in a flow-cytometry based assay upon addition of oleic acid. Interestingly, DGAT1 or DGAT2 overexpression in patient fibroblasts is able to rescue the aberrant lipid metabolism. Lastly, we show that DGAT1-deficient cells undergo lipotoxic cell death upon treatment with oleic acid, showcasing the importance of DGAT1 in lipid metabolism of the gastrointestinal tract.

Altogether, we identified and molecularly characterized 2 distinct monogenic defects leading to EO-PLE and EO-IBD, which expanded our knowledge on the importance of the complement system as well as intestinal lipid metabolism in the context of GI tract homeostasis. Additionally, achieving a genetic diagnosis for these patients and additional uncharacterized patients will allow clinicians to provide patients will allow clinicians to provide personalized, genome-informed therapy.

Deutschsprachige Zussamenfassung

Die Erforschung seltener Erkrankungen des Gastrointestinaltrakts erlaubt Einsichten in essentielle Faktoren der Darmgesundheit. In den meisten Fällen liegen diesen Erkrankungen Veränderungen eines einzelnen Gens zugrunde. Die Identifikation noch unbekannter Gendefekte und derer molekularer Mechanismen führt hierbei nicht nur zu neuen humanbiologischen Erkenntnissen, sondern kann in einigen Fällen auch in der personalisierten Medizin Anwendung finden.

In internationalen Kollaborationen mit Forschungsgruppen und Kliniken entdeckten wir zwei monogenetische Erkrankungen in Patienten mit früh einsetzender Proteinverlust-Enteropathie und früh einsetzender chronisch entzündlicher Darmerkrankung.

In 11 Patienten aus 8 Familien identifizierten wir 5 verschiedene Mutationen in einem Gen namens *CD55*, welches den decay accelerating factor (DAF) oder CD55 kodiert. CD55 ist ein universales Regulationsprotein des Immun-Komplementsystems. In unserer Studie konnten wir zeigen, dass die Mutationen eine Reduktion oder die komplette Abwesenheit des Proteins verursachen, was wiederum eine überaktive Komplement-Aktivierung zur Folge hat und durch erhöhte Bindung des Faktors C3d belegt werden konnte. Rekonstitution mit dem Wildtyp-Protein resultierte in wiederhergestellter Unterdrückung des Komplementsystems. Wir konnten weiters zeigen, dass CD55-Defizienz in der Überproduktion von Anaphylatoxin C5a resultiert, welche durch Zugabe von Eculizumab, eines humanisierten monoklonalen Antikörpers gegen Komplementfaktor C5, blockiert werden konnte.

In einer zweiten Studie untersuchten wir 10 Patienten von 6 Familien und identifizierten Mutationen in einem Gen, das die Diacylglycerol-Acyltransferase 1 or DGAT1 kodiert. Auch hier fanden wir Reduktion oder die komplette Abwesenheit des (mutierten) Proteins. Wir konnten in weiterer Folge zeigen, dass DGAT1 im Triglyzerid-Metabolismus der Epithelzellen des Darms eine Rolle spielt. DGAT1-defiziente Zellen konnten im Laborexperiment keine Lipid-Tröpfchen nach Zugabe von Oleinsäure bilden, was wir mittels Durchflusszytometrie und NileRed/LD540 Färbung untersuchten. Die Überexpression von DGAT1 oder DGAT2 in Patientenzellen, hingegen, konnte diesen Defekt des Lipidmetabolismus ausgleichen. Weiterhin konnten wir nachweisen, dass DGAT1-defiziente Zellen durch Zugabe der Fettsäure ein lipotoxisches Zelltod-Programm initiieren. In dieser Studie zeigten wir die Rolle von DGAT1 im Fettstoffwechsel des humanen Gastrointestinaltrakts.

Die Identifizierung und Charakterisierung zweier monogenetischer Erkrankungen als Ursache für EO-PLE und EO-IBD konnte hiermit zwei fundamentale Mechanismen des Komplementsystems bzw. des Lipidmetabolismus für die Darmgesundheit aufzeigen. Letztlich gelang es, für die untersuchten als auch zukünftige Patienten eine genetische Diagnose und eine personalisierte, Genom-informierte Therapiemöglichkeit aufzuzeigen.

Publications arising from this thesis

Ozen A*, Comrie WA***, Ardy RC*,** Domínguez Conde C, Dalgic B, Beser ÖF, Morawski AR, Karakoc-Aydiner E, Tutar E, Baris S, Ozcay F, Serwas NK, Zhang Y, Matthews HF, Pittaluga S, Folio LR, Unlusoy Aksu A, McElwee JJ, Krolo A, Kiykim A, Baris Z, Gulsan M, Ogulur I, Snapper SB, Houwen RHJ, Leavis HL, Ertem D, Kain R, Sari S, Erkan T, Su HC, Boztug, K[#], and Lenardo, MJ[#]. (2017) CD55 Deficiency, Early-onset Protein Losing Enteropathy, and Thrombosis. *N Engl J Med* **377**:52-61

van Rijn JM*, **Ardy RC*,** Kuloğlu Z*, Härter B*, van Haaften – Visser DY*, van der Doef HPJ, van Hoesel M, Kansu A, van Vugt AHM, Thian M, Kokke FTM, Krolo A, Başaran MK, Kaya NG, Aksu AÜ, Dalgıç B, Ozcay F, Baris Z, Kain R, Stigter ECA, Lichtenbelt KD, Massink MPG, Duran KJ, Verheij JBGM, Lugtenberg D, Nikkels PGJ, Brouwer HGF, Verkade HJ, Scheenstra R, Spee B, Nieuwenhuis EES, Coffer PJ, Janecke AR, van Haaften G, Houwen RHJ, Müller T[#], Middendorp S[#], and Boztug K[#]. (2018) Intestinal Failure and Aberrant Lipid Metabolism in Patients with DGAT1 Deficiency. *Gastroenterology*. **155(1)**:130-143.e15

* indicates shared first authorship
[#] indicates shared last authorship

Abbreviations

AA – Amino acid
AD – Autosomal dominant
aHUS – Atypical hemolytic-uremic syndrome
AR – Autosomal recessive
ATP – Adenosine triphosphate
CD – Crohn's disease
CDD – Congenital diarrheal disorders
CF – Cystic fibrosis
CGD – Chronic granulomatous disease
cGMP – cyclic guanosine monophosphate
CHAPLE – CD55 deficiency with Hyperactivation of complement, angiopathic thrombosis and protein-losing enteropathy
CNV – Copy number variation
CTE – Congenital Tufting Enteropathy
DAG – Diacylglycerol
DAF – Decay accelerating factor
DGAT – Diacylglycerol acyltransferase
DNA – deoxyribonucleic acid
EO – Early-onset
EGF – Epidermal growth factor
ER – endoplasmic reticulum
FAT – Fatty acid translocase
GI – Gastrointestinal
GPI – glycophosphatidylinositol
GWA – Genome-wide association
HSCT – Hematopoietic stem cell transplantation
IBD – Inflammatory bowel disease
IBDu – Inflammatory bowel disease (unclassified)
IEI – inborn error of immunity
IL – Interleukin
kDA – kilodalton
MAC – Membrane attack complex
MVID – Microvillous inclusion disease
NADPH – Nicotinamide adenine dinucleotide phosphate
NOD – Nucleotide-binding oligomerization domain-containing protein
NGS – Next generation sequencing
PLE – Protein-losing enteropathy
PNH – Paroxysmal nocturnal hemoglobinuria
ROS – Reactive oxygen species
SECIS – Selenocysteine insertion sequence
SLC – Solute carrier
SLE – Systemic lupus erythematosus
TCR – T cell receptor
TG – Triglyceride
THE – Tricohepatoenteric syndrome
TLR – Toll-like receptors
UC – Ulcerative colitis
WES – Whole exome sequencing
WGS – Whole genome sequencing
VEO – Very-Early Onset
XLR – X-linked recessive

1. Introduction

1.1 Human genetics

The current consensus within the scientific community is that the human genome is made up of approximately 3 billion nucleotides, either found within the nuclear envelope in a cell or the mitochondria (Venter *et al*, 2001; Lander *et al*, 2001). In the nucleus, these nucleotides are compacted into 22 autosomes (chromosomes that are not the sex chromosome X or Y) and 2 allosomes or sex chromosomes: X and Y. In healthy individuals, each diploid nucleus will contain a total of 46 chromosomes: 44 autosomes and an XY in males and XX in females.

The coding regions of the human genome - also called the exonic regions - has been approximated to make up about 3% of the total human genome (Dunham *et al*, 2012). An estimated number of 21.000 protein coding genes have been proposed to be located within these coding regions (Dunham *et al*, 2012). In the context of autosomes, each gene is encoded by 2 copies found on the two alleles of homologous chromosomes. In the study of human population genetics, the prevalence of an allele in many cases is inversely correlated with its effect size. Deleterious alleles are seldom inherited due to its effects on the fitness of an organism. Therefore, common, non-deleterious variants can be found in many individuals. Rare deleterious variants (where the prevalence of the less abundant allele accounts for less than one percent of the observed allele 0.01) are less prevalent due to the lack of genetic drift (Manolio *et al*, 2009). This results in the identification of rare, deleterious but not lethal alleles in various congenital rare diseases (Figure 1).

A variety of human diseases manifests when errors in the genetic code happen. In the case of monogenic disorders, this refer to diseases that occur when an aberration in a single gene results in the occurrence of disease. As of 2019, an estimated 7.000 diseases have been attributed to a single gene defect (Slade *et al*, 2018). These mutations can occur in any gene within the genome, therefore affecting a variety of pathways and present with a multitude of disease phenotypes (Blencowe *et al*, 2018). These monogenic diseases are seldom found in the population - deeming them rare diseases - which is defined in the European Union as a disease that affects less than 1 per 2000 person (Slade *et al*, 2018).

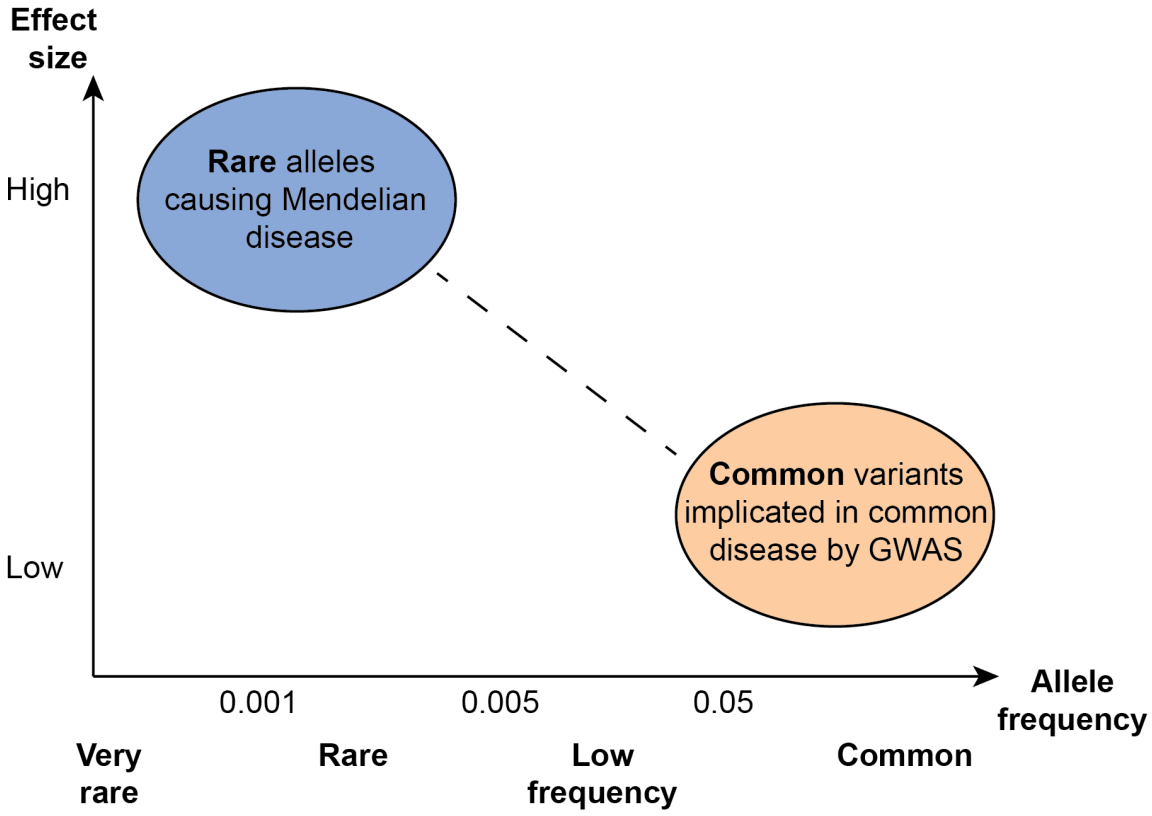

Figure 1. Graph of allele effect size against allele frequency found in a population, showing rare alleles causing Mendelian disease have a high effect size. Reprinted by permission and adapted from Springer Nature: Nature, “Finding the missing heritability of complex diseases” Manolio et al. (Copyright) 2009.

1.1.1 Modes of inheritance

Different modes of inheritance exist in genetically inherited Mendelian disorders. The four main modes of inheritance that can be found in Mendelian-inherited diseases are as follows: autosomal dominant (AD), autosomal recessive (AR), X-linked recessive (XLR), and X-linked dominant (XLD) (Figure 2). In AD diseases, inheriting a single faulty copy of a gene will result in the manifestation of disease. In cases of AD diseases, usually one of the parents is affected and passed on the mutated allele. Though not strictly inherited, AD diseases can also occur when a *de novo* mutation that occurred during germ cell formation are passed on to the offspring. AR diseases occur when an individual inherits two faulty copies of a gene from each parent, where the parents are more often than not healthy. In AR diseases, people who have one healthy allele and one mutated allele are also called heterozygotes or carriers. XLR diseases usually affect male offspring as they only possess a single copy of the X chromosome, whereas females who inherit one healthy X chromosome and a mutated X

chromosome tend to be healthy. Lastly, rare XLD cases occurs when the phenotype is driven by a dominant mutant allele inherited through the X chromosome. In this case, the offspring has a 50% chance of being affected regardless of their sex.

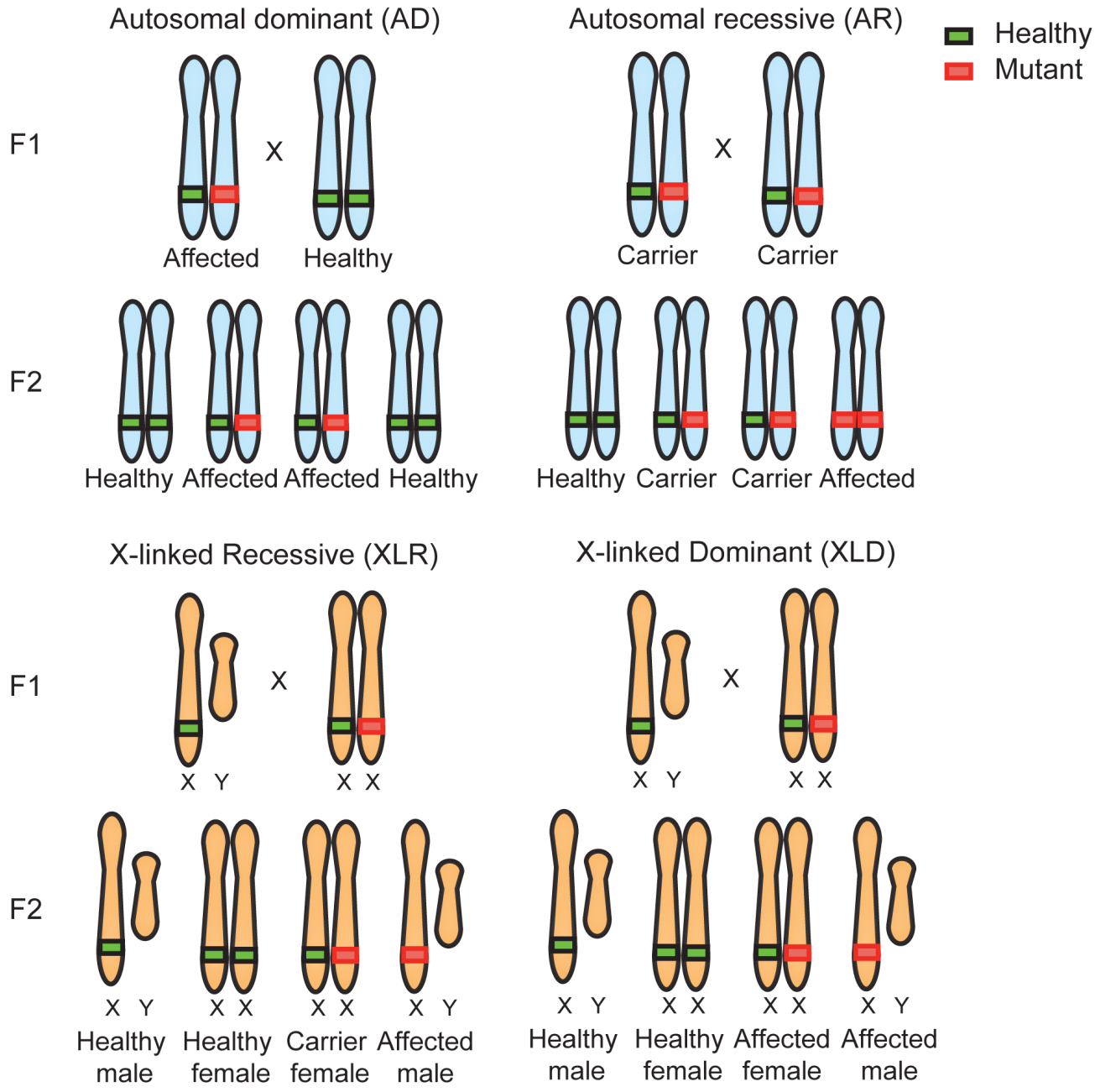

Figure 2. Different modes of Mendelian inheritance: autosomal dominant (AD), autosomal recessive (AR), X-linked recessive (XLR), and X-linked dominant (XLD). Green band represents healthy allele, red band represents affected allele.

1.2 Gastrointestinal system – digestion and absorption

The gastrointestinal (GI) system is an essential component of human biology that is made up of various organs that work in a concerted fashion to ensure its functionality and homeostasis (Grand *et al*, 1976). A short focus on the process of nutrient digestion and absorption - one of the most important function of the GI tract - will be explored in this chapter.

1.2.1 Start of digestion process – mechanical digestion

As humans are not able to produce energy intrinsically, they require outside source of energy in the form of food in order to survive. This energy mostly come from the three main components of digestible food: carbohydrates, proteins, and fats (Carreiro *et al*, 2016). Digestion of these three main components begins at different points of the GI tract. In this chapter, an overview of physical and chemical digestion of the three main macronutrients will be further discussed.

Mechanical digestion is an important process that is required for proper digestion of food. For humans, mechanical digestion may include the step of cooking whereby the molecular structure of the macromolecules is changed through heat or other methods of cooking. The section where human anatomy plays a role begins in the mouth when food products are physically broken down into smaller pieces by the process of mastication (Slavicek, 2010). Humans, like other omnivorous species in the animal kingdom, have developed specialized teeth to mechanically process food that they digest. This physical act allows for ease of swallowing food and increases the surface area available for enzymatic digestion. When the food (also called the bolus) reaches the stomach, further mechanical digestion occurs as the stomach churns through the action of the inner oblique muscles (Kong & Singh, 2008).

1.2.2 Carbohydrate digestion and absorption

Carbohydrates should make up the bulk of calories that is consumed by humans, providing somewhere between 45-65% of daily caloric intake (Slavin & Carlson, 2014). Each gram of carbohydrate is generally accepted to provide 4 kilocalories of energy (WHO/FAO, 2003). It is a biomolecule consisting of carbon, hydrogen, and oxygen atoms. Carbohydrates consists of 3 main forms: monosaccharides, disaccharides, and polysaccharides (Slavin & Carlson, 2014). Monosaccharides are the simplest forms of carbohydrates as they are unable to be further processes into smaller components. Some examples of monosaccharides include glucose, fructose, and galactose where all these moieties share the same chemical formula of $C_6H_{12}O_6$ but differ in the arrangements of these atoms (Slavin & Carlson, 2014). When 2 monosaccharides are linked through a dehydration reaction, they form a disaccharide with a

glycosidic bond between the two monosaccharides. When longer chains of monosaccharides are formed, they are called polysaccharides. Starch and cellulose constitute most of the polysaccharides in plant products for human consumption (Ghazarian *et al*, 2011; Lapis *et al*, 2017).

The end goal of carbohydrate digestion is to completely breakdown complex carbohydrates into monosaccharides - a form that can be easily absorbed by the GI tract. Carbohydrate digestion begins in the mouth, with the breakdown of complex carbohydrates by alpha-amylases found in the saliva(Valdez & Fox, 1991). This enzyme is produced by the acinar cells of the submandibular and parotid gland. Alpha-amylases function best in a neutral pH range and breaks down complex carbohydrates/dietary starch into maltose and polysaccharides in the mouth, continuing to do so until the food reaches the stomach (Rosenblum *et al*, 1988). The change in pH renders the salivary amylase non-functional. Minimal carbohydrate digestion occurs in the stomach due to its acidic environment (Rosenblum *et al*, 1988). Digestion of starch continues in the small intestine upon the release of pancreatic amylase into the duodenum (Butterworth *et al*, 2011). Breakdown of oligosaccharides and disaccharides into its simpler components occur in the brush border area of the microvilli due to the local concentration of disaccharidases produced by the epithelial cells (Holmes & Lobley, 1989). These cells produce enzymes such as maltase-glycoamylases, sucrose-isomaltase, and trehalase that hydrolyzes disaccharides into their monosaccharide components (Galand, 1989).

Dietary carbohydrate has to be digested fully into monosaccharides before it can be absorbed by the intestinal epithelial cells to be transported into the liver through the hepatic portal vein prior to being further processed (Wright *et al*, 2003). Glucose and galactose molecules are absorbed in the small intestine through the action of the sodium-glucose cotransporter SGLT-1, encoded by the gene *SLC5A1* (Lehmann & Hornby, 2016). The channel works by establishing a gradient of sodium using adenosine triphosphate (ATP) molecules to pump sodium out into the lumen. Using more ATP, glucose is moved along with sodium molecules against its concentration gradient into the enterocyte. Fructose is absorbed into the enterocytes through the GLUT5 channel by facilitated diffusion (Douard & Ferraris, 2008). These monosaccharides are then transported into the circulation through the basolateral membrane of the enterocyte, facilitated by the glucose transported GLUT2 in an energy-independent manner (Thorens, 2015). The regulation of glucose homeostasis in the body is then controlled by various endocrine hormones to ensure healthy glucose levels in the bloodstream (Aronoff *et al*, 2004).

1.2.3 Protein digestion and absorption

Another major component of food that is important for human life is protein. Like carbohydrates, proteins are macromolecules made up of smaller components called amino acids. Each gram of protein provides 4 kilocalories of energy, similar to carbohydrates (WHO/FAO, 2003). Amino acids (AA) are organic compounds with a carbon backbone that contains one amine functional group, one carboxyl functional group, and a side chain that determines their property (Wu, 2009). However, these amino acids are joined together by a special moiety called the peptide bond in which the amine group of one AA and the carboxyl group of another AA form a bond together through a dehydration process. Polymers of amino acids joined together is called a polypeptide. Polypeptide chains can then form secondary and tertiary structures and create complex protein molecules (Berg *et al*, 2002).

There are more than 500 naturally occurring amino acids (Wagner & Musso, 1983). However, only 21 of these are proteinogenic which means that they are able to be incorporated biochemically to form polypeptides. 20 of the proteinogenic amino acids are in the standard genetic code, while selenocysteine can be incorporated into amino acids chains through the selenocysteine insertion sequence (SECIS) elements (Schmidt & Simonović, 2012). Of the 20 amino acids encoded in the human genome, humans are able to synthesize 12 from other amino acids or intermediary metabolic molecules (Blanco & Blanco, 2017). 9 of these amino acids are called essential amino acids as they need to be consumed through food products. The nine essential amino acids are as follows: valine, tryptophan, threonine, phenylalanine, methionine, lysine, leucine, histidine, and isoleucine (Young, 1994).

Chemical digestion of proteins begins in the stomach. Stomach acid plays two important roles in the context of protein digestion (Ramsay & Carr, 2011). On one hand, the low pH results in the unfolding of complex protein structures and breaks down protein aggregates that are formed. On the other hand, the acidity is required to activate the first enzyme for protein digestion(Ramsay & Carr, 2011). Active endopeptidase pepsin is secreted into the stomach by chief cells in the form of a proenzyme called pepsinogen, which gets activated upon mixing with the hydrochloric acid of the gastric juice. Pepsinogen undergoes a conformation change and autocatalyze its activation through cleavage of 44 amino acid residues, forming pepsin (Kageyama, 2002).

The chyme is then moved into the small intestine, where majority of protein digestion occurs. At this time, the pancreas releases additional proteases: chymotrypsin, trypsin, elastase, and carboxypeptidase (Hart & Conwell, 2017). These enzymes catalyze additional hydrolysis of peptide bonds that continues protein digestion into its smaller molecules of 1-3 amino acids in

length that can then be absorbed by the intestinal lining. As with carbohydrates, the final stages of protein digestion occurs on the brush border of the small intestine (Tobey *et al*, 1985). Here, a variety of membrane bound dipeptidases and tripeptidase further break down 2 or 3 amino acid peptides into its single amino acid components to be transported into the gut epithelial (Tobey *et al*, 1985).

The absorption of amino acids and short chain peptides require the process of active transport through several solute carriers that are present on the apical side of the small intestine epithelium (Holmes & Lobley, 1989). A variety of carriers co-transport one amino acid and another product (for example sodium) into the gut epithelium, one of the important ones being the oligopeptide transport Pept-1 encoded by the *SLC15A1* (Adibi, 1997). In cases of dipeptide and tripeptide transport, these molecules are then further digested in the cytoplasm to its amino acid components prior to being moving into blood circulation via diffusion. For example, LAT1 is a heterodimer composed of SLC3A2 and SLC7A5 that preferentially transports aromatic and branched chain amino acids into the gut epithelial in conjunction with a Na^+ molecule (Bröer, 2008). These amino acids molecule then move along their concentration gradient into the interstitial spaces next to the basolateral membrane of the epithelial and enter the circulation before being transported and processed elsewhere in the body.

1.2.4 Lipid digestion and absorption

Lipids constitute a group of biomolecules that is only soluble in a non-polar solvent. It is essential for human life as it is involved in a variety of cellular processes including energy storage, various signaling axis, and importantly for compartmentalization and separation of different organelles (Fahy *et al*, 2011). Lipids can be categorized into different classes depending on the moieties on them. In the context of dietary fat, most fat consumption is made up of triglycerides (TG), where a single molecule of glycerol carries 3 fatty acid chains which can be of differing lengths and conformation (Forouhi *et al*, 2018). TG can be made up of a variety of fatty acids ranging from polyunsaturated fatty acids found in most plant-based foods to saturated fatty acid which is found in abundance in animal products (Adibi, 1997). Other lipid moieties present in food includes phospholipids (which makes up cellular membranes) and cholesterol and its ester derivatives. Dietary fat has been shown to provide the human body with the highest amount of energy, amassing approximately 9 kcal of energy per gram (Rolls, 2017).

Lipid digestion begins in the mouth, with lingual lipases being released with saliva to kickstart the process (Liao *et al*, 1984). Lipases catalyzes the hydrolysis of medium and long chain triglyceride releasing molecules of free fatty acid. Lingual lipases catalyze the breakdown of triglycerides into diacylglycerol (DAG) and a free fatty acid in the first instance. Lingual lipase and gastric lipase are examples of acidic lipases that has an optimum working pH in the acidic range (Liao *et al*, 1984). This activity is especially important in the context young neonates with insufficient pancreatic development, or in diseases such as cystic fibrosis with compromised pancreatic function. These lipases perform a substantial amount of TG hydrolysis prior to the chyme being emptied into the small intestine. Importantly, the churning action in the stomach allows for the formation of emulsion droplets, which increases the surface area at which these lipids can be digested.

In the small intestine, the emulsion within the chyme encounters lipases and bile salts that are released by the pancreas and gall bladder into the duodenum. Bile acids and bile salts are synthesized in the liver, and are amphiphipatic molecules consisting of a hydrophobic side and a hydrophilic side (DeNigris *et al*, 1988). Bile acid and bile salts stabilizes small lipid emulsions and increase their surface area for a more efficient lipid digestion. These bile salts are then reabsorbed in the small intestine and recycled. Pancreatic lipases, with the help of a coenzyme called colipase, can attach themselves into lipid emulsions and micelles and catalyze the hydrolysis of TG and DAG in the sn-1 and sn-3 position (Hofmann, 1999). In addition to pancreatic lipase, another enzyme called cholesterol esterase is secreted by the pancreas and performs hydrolysis of fat molecules with broad specificity. Phospholipids from food or biliary secretions are broken down by phospholipase A1 and phospholipase A2 into its glycerol and fatty acid components (Lowe, 1997).

Lipid absorption occurs in the small intestine both through active processes involving proteins and through passive diffusion (Mansbach, 1990). Free fatty acids are present in high concentrations near the small intestine epithelium and can passively diffuse through the cell membrane and into the enterocyte. Fatty acid translocase FAT, encoded by the *CD36* gene has been shown to be highly expressed in enterocytes, and is implicated to be important in the uptake of fatty acids important for the formation of chylomicrons (Hussain, 2014). The absorbed fatty acid then needs to be reformed into neutral lipid TG and repackaged into chylomicrons before leaving the gut epithelium through the lacteal and into the lymphatic system to be further processed in other parts of the body.

1.3 Congenital Diarrheal Disorders

Congenital diarrheal disorders (CDD) refer to group of diseases where patients suffer from early-onset enteropathies that can often be attributed to a single gene defect that are inherited in an autosomal recessive fashion (Canani *et al*, 2015). As the name suggests, CDD patients typically present with chronic, severe diarrhea within the first few weeks in life and suffer from symptoms ranging from dehydration due to fluid loss to complete dependence on parenteral nutrition as a result of intestinal failure (Canani *et al*, 2015). Some patients suffer from generalized edema due to the protein-losing enteropathy (PLE), resulting in the accumulation of fluid in peripheral tissues (Levitt & Levitt, 2017). The heterogeneity of clinical manifestations can be attributed to the different gene defects that can manifest as CDD. Additionally, different mutations in the same gene might result in different functionalities, further contributing to heterogeneity. It was proposed that these disease entities can be preliminarily categorized into the four categories: defects in absorption and transport of electrolytes and nutrients, defects in enterocyte structures, defects in enteroendocrine cell differentiation, and defects in immune-related homeostasis (Canani *et al*, 2015). However, defects leading to aberrant lymphatic vessels as well as endothelial integrity should also be included as a category of CDD. In the following chapter, previously described defects leading to CDD and their accompanying molecular mechanism will be further explored.

1.3.1 Definition and types of diarrhea

Diarrhea is briefly defined as an increase in stool frequency, liquidity, and/or volume (Sweetser, 2012). The main pathological process aberrant that leads to the onset of diarrhea is the inability of the gastrointestinal tract to fully absorb water from its luminal content (Sweetser, 2012). Various pathomechanisms resulting in the common phenotype of diarrhea have been identified and these include secretory, osmotic, inflammatory, and altered motility. Briefly, secretory diarrhea usually results from aberrant electrolyte transport that results in decreased water absorption (Camilleri *et al*, 2017). Osmotic diarrhea arises from osmotic gradient generated by unabsorbed substances other than electrolytes in the intestinal lumen, resulting in water from the plasma to be pulled into the lumen. It has been observed that secretory diarrhea tends to persist through fasting, while osmotic diarrhea might resolve with the prevention of food intake (Terrin *et al*, 2012). Inflammatory diarrhea occurs when there is a damage to the epithelial barrier, allowing for the movement of blood and serum into the intestinal lumen. In addition, inflammatory diarrhea often results in the destruction of highly absorptive epithelium, further disturbing the absorption homeostasis (Sweetser, 2012). Lastly, altered motility of ingested food in the lumen results in the disruption of homeostatic absorption and secretion of water, culminating in the manifestation of diarrhea (Terrin *et al*, 2012). Of

note, it is common that more than one mechanism of diarrhea onset to occur simultaneously in disease.

1.3.2 Defects in absorption and transport of electrolytes and nutrient

Various inborn errors related to protein or enzymes that are involved in the digestion or absorption of macromolecules and electrolytes have been attributed to CDD, showcasing the importance of the process of digestion in GI homeostasis. In the following section, descriptions of previously described deficiencies involved in defective absorption and transport of electrolytes and nutrient as well as their accompanying molecular mechanism will be discussed.

1.3.2.1 Defects in carbohydrate digestion and absorption

Defective digestion and absorption of carbohydrates happens when mutations that give rise to defective enzymatic or transport function occur. These defects result in osmotic diarrhea due to the accumulation of undigested and/or unabsorbed polysaccharides. Interestingly, a way to diagnose carbohydrate malabsorption includes measurement of lactic acid in patient stool due to increased bacterial fermentation of luminal saccharides (Terrin *et al*, 2012). Examples of mutations in brush-border enzymes that is involved in carbohydrate processing includes congenital lactase deficiency, congenital sucrase-isomaltase deficiency, congenital maltase-glucoamylase deficiency, and trehalase deficiency. In addition, mutations in monosaccharide transport proteins such as *SLC5A1* encoding for the glucose transporter sodium-glucose linked transporter SGLT1 or facilitated glucose transported GLUT2 has also been shown to cause CDD.

In lactase deficiency, patients suffer from osmotic diarrhea due to an increase of undigested lactose that remain in the lumen upon ingestion. These patients were found to have mutations in the coding region of the lactase *LCT* gene that enzymatically cleave lactose into glucose and galactose. Interestingly, the first genetic mutation in lactase deficiency was found in a cohort of Finnish patients, where some shared a founder mutation c.4170T>A that resulted in an early stop codon (Kuokkanen *et al*, 2006). Congenital sucrase-isomaltase deficiency was first identified in the 1960, where 3 patients showed intolerance to sucrose but not to other carbohydrates such as glucose or fructose (Weijers *et al*, 1960). Since then, various mutations that renders the protein functionless or prevents its transport and secretion have been identified (Cohen, 2016). Congenital maltase-glucoamylase deficiency is another brush-border enzyme deficiency where patients suffer from diarrhea due to ineffective digestion of carbohydrates. However, no loss-of-function mutations in the gene encoding this protein

(*MGAM*) has been identified, pointing towards a defect in the regulation of secretion of this protein (Nichols *et al*, 2002). This deficiency highlights the presence of CDD without an underlying genetic cause. Lastly, trehalase deficiency was first found in patients that are not able to process trehalose, a polysaccharide highly abundant in food items such as mushrooms (Madzarovova Nohejlova, 1973). However, as with congenital maltase-glucoamylase deficiency, no genotype-phenotype correlation has been performed for trehalase deficiency.

Accumulation of monosaccharides happen when mutations in their respective transporters that renders them non-functional occur. This is the case in patients that suffer from CDD who harbors mutations in the genes *SLC5A1* or *SLC2A2*, which encodes for 2 important monosaccharide transporters SGLT1 and GLUT2 respectively. The first identified SGLT1 mutation was described in 1991, where a missense mutation p. Asp28Asn resulted in a complete loss-of-function of its glucose transport capability (Turk *et al*, 1991). Homozygous *SLC2A2* mutations have been identified in patients with Fanconi-Bickel syndrome, in which patients suffer from nephropathy and impaired utilization of monosaccharides glucose and galactose where some patients presented with diarrhea, presumably due to the accumulation of GLUT2 substrate in the gut lumen (Santer *et al*, 1997).

1.3.2.2 Defects in protein digestion and absorption

Amino acid digestion and absorption plays a pivotal role in human growth, and therefore defects in these processes can be fatal. Numerous amino acid malabsorption diseases have been previously described in the context of renal reabsorption, but few cases of intestinal amino acid processing defects have been described (Bröer & Palacín, 2011). Two known genetically-defined defects that results in early-onset osmotic diarrhea include enterokinase deficiency and lysinuric protein intolerance (Canani *et al*, 2015).

Enterokinase, also known as enteropeptidase, is a transmembrane enzyme found in the brush border that is responsible for hydrolysis of peptide bonds found on polypeptides especially activating various other proenzymes in the duodenum (Kitamoto *et al*, 1994). Patients with enterokinase deficiency suffer from failure to thrive and chronic diarrhea, purportedly attributed to the accumulation of undigested polypeptides due to lack of activation of other enteric peptidases. In 2002, stop-gain mutations in the gene encoding for the proenteropeptidase (*TMPRSS15*) was identified in patients with reported enterokinase deficiency, cementing the genetic etiology of the disease (Holzinger *et al*, 2002). In lysinuric protein intolerance, patients suffer from generalized failure to thrive and some suffer from osmotic diarrhea. These patients were found to have decreased absorption of cationic amino acid in the intestines due to loss-

of-function mutations in the gene *SLC7A7* encoding for the cationic amino acid transporter of the same name (Torrents *et al*, 1999; Borsani *et al*, 1999).

1.3.2.3 Defects in fat digestion and absorption

Defects in fat digestion and absorption do not only include dietary fat, but also defects in absorption of factors involved in fat digestion such as bile acids and other lipoproteins. Patients with defects in fat digestion and absorption might experience steatorrhea, in which fat molecules can be detected in their stool (Di Sabatino *et al*, 2014). Currently, no known mutations in lipases secreted into the gut lumen has been identified but a study of a patient with congenital pancreatic lipase deficiency has been previously described (Figarella *et al*, 1980). However, mutations in the chloride channel *CFTR*, as well as *MTTP*, *APOB, and SAR1B* involved in formation and transport of lipoproteins have been shown to cause fat malabsorption. In addition, mutations in bile acid transporter *SLC10A2* also result in CDD termed primary bile acid diarrhea.

Mutations in the cystic fibrosis transmembrane conductance regulator *CFTR* gene has been extensively studied in patients with cystic fibrosis (CF), whose main clinical manifestations involve severe pancreatic insufficiency and recurrent lung infections (Rey *et al*, 2019). The mutations in the chloride channel CFTR renders the protein functionless, which results in the disturbed homeostasis of chloride ion that has pleiotropic effect on sodium as well as water homeostasis in mucus-producing cells among others. CF patients experience pancreatic insufficiency as the pancreas is not able to secrete enough enzymes to deal with the process of dietary fat digestion (Rey *et al*, 2019). Lack of water secretion in pancreatic ducts results in the production of thick mucus which blocks these ducts and preventing their exocrine function.

Mutations in proteins involved in lipid packaging and transport have also been associated with CDD that stems from defects of fat absorption. *MTTP* encodes for the microsomal triglyceride transport protein large subunit, while *APOB* encodes for apolipoprotein B (Davidson & Shelness, 2000). These two proteins are present in absorptive intestinal epithelial cells, and are essential to form apoB-containing lipoproteins that is important in the context of lipid transport within the human body (Davidson & Shelness, 2000). Patients with this lipid packaging defects experience poor fat absorption, which results in failure to thrive as well as fat-soluble vitamin deficiencies and neurological presentation amongst others (Young *et al*, 1988; Sharp *et al*, 1993). Patients with chylomicron retention disease also suffer from fat malabsorption and fat-soluble vitamin deficiency. These patients were found to harbor mutations in the gene *SAR1B*, a small GTPase that was shown to regulate chylomicron

transport within cells (Jones *et al*, 2003). These gene defects cemented the importance of lipid packaging and transport in the gastrointestinal tract as an essential process to gut homeostasis.

Bile acids are amphipathic molecules that binds to dietary fats to form micelles, allowing for the efficient emulsification and increase of surface area for digestion of fat molecules (Chiang, 2017). Bile acid homeostasis is a highly regulated process and approximately 95% of secreted bile acid is reabsorbed and transported back into the liver in a healthy individual (Chiang, 2017). Patients with primary bile acid malabsorption suffers from chronic watery diarrhea, excess fecal bile acids, as well as steatorrhea (Pattni & Walters, 2009). These patients were shown to have mutations in *SLC10A2*, which encodes for a sodium/bile acid transported that is important in the reabsorption of bile acids to be returned into the liver (Oelkers *et al*, 1997). The loss-of-function mutation led to a malabsorption of fat and accumulation of lipid in the GI tract, leading to CDD.

1.3.2.4 Defects in electrolyte transport

The relationship between various electrolytes and water results in the complex regulation of osmolarity in various biological context. This is especially important in the GI tract as it is the first area in the human body to absorb water. Numerous proteins tightly regulate this important process and mutations in channels that regulate electrolyte transport result in CDD (Canani *et al*, 2015). This is showcased in diseases caused by mutations in genes encoding electrolyte channels such as *SLC26A3*, *SLC9A3*, and *SLC39A4* causing CDD. In addition, regulators of these channels such as *GUCY2C* and *SPINT2* have also been found to play a role in CDD as mutations in these regulators result in patients suffering from diarrhea.

SLC26A3 encodes for a chloride anion exchanger that mediates the exchange of chloride ion from the gut lumen and bicarbonate ion from the gut epithelium (Wedenoja *et al*, 2011). Mutations in this gene was first identified in patients that suffer from congenital chloride diarrhea in which these patients suffer from secretory watery diarrhea due to increased fecal chloride concentration (Höglund *et al*, 1996). *SLC9A3* encodes from the protein NHE3, a sodium/hydrogen exchanger that regulates the absorption of sodium ions across renal and intestinal epithelia (Gurney *et al*, 2017). Patients with congenital sodium diarrhea suffer from secretory watery diarrhea due to the accumulation of sodium ions in the gut lumen, a result of loss-of-function mutations in *SLC9A3* (Janecke *et al*, 2015). Patients with acrodematitis enteropathica suffers from secretory diarrhea and skin inflammation. It was shown that these patients harbor mutations in the gene *SLC39A4*, a zinc transporter that is expressed in the

bowel and regulates zinc ion uptake (Küry *et al*, 2002). Mutations in these transporters involved in ion homeostasis highlights the importance of electrolyte balance for GI health.

The regulation of these transported by other proteins have also been shown to be important in the context of CDD. GUCY2C encodes for the intestinal guanylate cyclase 2C that regulates the activity of both chloride channel CFTR and sodium transporter through its production of cyclic guanosine monophosphate (cGMP) (Rappaport & Waldman, 2018). Activating mutations in GUCY2C were first reported in a large family suffering from mild familial diarrheal syndrome predisposed to small bowel obstruction and esophagitis (Fiskerstrand *et al*, 2012). In GUCY2C gain-of-function mutation, increased cGMP production led to overactivation of the CFTR chloride channel, expulsing chloride and thus water into the gut lumen resulting in secretory diarrhea. Patients with *SPINT2* mutations were shown to present with symptoms of congenital chloride diarrhea, but the exact mechanism of how *SPINT2* loss-of-function mutations affect chloride channels have yet to be determined (Heinz-Erian *et al*, 2008).

1.3.3 Defects in enterocyte structures

The gut epithelium is a highly structured tissue which performs various functions, one of which is barrier formation. Their barrier function depends on the integrity of their architecture and their cell-cell contact. As expected, enteropathies occur when this epithelial tissue does not form correctly. Examples of CDD involving defects in enterocyte structure include microvillous inclusion disease (MVID), congenital tufting enteropathy (CTE), and tricohepatoenteric syndrome (THE). Mutations that cause these disease entities result in disrupted epithelial architecture, either through altered cell adhesion, defects in polarity, or as of yet unknown mechanism.

1.3.3.1 Congenital tufting enteropathy (CTE)

CTE is aptly named due to the presence of ‘tufts’ where epithelial disorganization results in the crowding of cells (Goulet *et al*, 2007). These patients suffer from intractable vomiting and diarrhea that often leads to irreversible intestinal failure (Goulet *et al*, 2007). As with other CDD patients, they also suffer from malnutrition and failure to thrive. Two genetic defects have been attributed to CTE: mutations in *EPCAM* and *SPINT2*. The first genetic aberration in patients with CTE was first identified in 2008 where mutations in the gene *EPCAM* was found in a series of families with CTE (Sivagnanam *et al*, 2008). The mutations identified resulted in reduced EPCAM protein expression in patient biopsies as compared to healthy control, and the biopsies showed disrupted villus architecture in the small intestine (Sivagnanam *et al*, 2008). Additionally, a mutation in *SPINT2* was also identified in two patients that suffered from

CTE, further adding to the complexity of the regulation of epithelial barrier and gut homeostasis by this gene (Sivagnanam *et al*, 2010; Slae *et al*, 2013).

1.3.3.2 Microvillous inclusion disease (MVID)

In MVID, gross morphology defects can be observed upon electron microscopy or histological staining of patient GI biopsy (Ruemmele *et al*, 2006). The disease is named due to the characteristics of the enterocytes seen upon ultrastructural analysis: atrophy of microvilli and inclusion bodies in mature enterocyte (Ruemmele *et al*, 2006). Two recently described genetic aberration that leads to MVID are *MYO5B* and *STX3* mutations. Mutations in the gene encoding for myosin 5B was found in a cohort of AR MVID patients (Müller *et al*, 2008). Myosin VB is an actin-based motor protein important trafficking molecule that dictates apicobasal polarity of gut epithelial cells through proper trafficking of membrane proteins (van der Velde *et al*, 2013). Staining of gut segments from MYO5B-deficient for transferrin receptor (TfR) show apical mislocalization of TfR, suggesting an epithelial polarity defect (Müller *et al*, 2008). Mutations in *STX3* encoding for the protein syntaxin 3 was identified in MVID patients that were negative for MYO5B mutations (Wiegerinck *et al*, 2014). Syntaxin 3 is important in fusion of apical vesicles to the apical membrane. Thus, loss-of-function mutation in STX3 resulted in defective apicobasal polarity which results in MVID.

1.3.3.3 Tricohepatoenteric syndrome (THE)

THE is named after the clinical presentation of the patients: wooly hair, facial dysmorphism, intractable diarrhea that presents early on in life as well as hepatic involvement (Fabre *et al*, 2007). To date, 2 genetic mutations have been identified to cause THE: *TTC37* and *SKIV2L*. Patients with TTC37 deficiency presented with heterogenous clinical presentation including immunodeficiency and distinct facial dysmorphism (Hartley *et al*, 2010). The first study showed that jejunal biopsies from patients showed mislocalization of various transporters including the important sodium transporter NHE2 and NHE3, which might cause the onset of diarrhea in these patients (Hartley *et al*, 2010). Additionally, mutations in *SKIV2L* were found in THE patients who were negative for *TTC37* mutations (Fabre *et al*, 2012). *SKIV2L* encodes for an RNA helicase potentially taking part in antiviral immunity by blocking translation of mRNA lacking a poly A tail (Eckard *et al*, 2014). However, the direct molecular mechanism of *TTC37* and *SKIV2L* loss-of-function mutations in intiating CDD is still elusive.

1.3.4 Defects in enteroendocrine cell differentiation

Enteroendocrine cells play an important part of GI homeostasis as they coordinate processes such as insulin secretion and appetite by secreting various gut hormones (Müller *et al*, 2008).

In mice, these cells originate from Lgr5+ stem cells, much like other differentiated epithelial cells (Gribble & Reimann, 2019). Important transcription factors required for their differentiation include *Math1*, *Neurog3*, and *NeuroD* (May & Kaestner, 2010). Several genetic mutations leading to dysregulation of enteroendocrine cells have been identified in patients that suffer from CDD. These include mutations genes such as *NEUROG3*, *ARX,* and *PCSK1*.

An example of defects in enteroendocrine cell differentiation in humans was observed in patients with mutation in the human equivalent of mice Ngn3, neurogenin-3 (*NEUROG3*) (Wang *et al*, 2006). Patients with homozygous mutations in *NEUROG3* presented with vomiting, diarrhea, and general nutrient malabsorption. Upon immunohistochemical staining of their small bowel biopsies, NEUROG3-deficient patients showed reduced number of chromogranin A+ neuroendocrine cells (Wang *et al*, 2006). These patients also suffer from insulin-dependent diabetes mellitus as NEUROG3 is important for the development of β-cells in pancreatic islets (Gradwohl *et al*, 2000). *ARX* encodes for the important transcription factor Aristaless related homeobox that has been extensively studied in the context of central nervous system, the pancreas, and other enteroendocrine cells involved in hormone secretion (Gécz *et al*, 2006). Patients with *ARX* mutations was described to suffer from mental retardation (Kitamura *et al*, 2002). The GI tract involvement in the disease includes occasional congenital diarrhea, presumably due to the dysregulation neurogenin-3 and various enteroendocrine hormone signaling (Kitamura *et al*, 2002). Lastly, *PCSK1* encodes for proprotein convertase, a protein that is involved in the proteolytic cleavage of various prohormones including insulin and glucagon. Patients with *PCSK1* loss-of-function mutations have been identified to present with a clinically heterogenous manifestations that includes obesity, malabsorptive diarrhea and various endocrine dysfunction (Stijnen *et al*, 2016). Their Gi involvement can be attributed to the lack of enteroendocrine hormone processing and dysregulation that leads to CDD.

1.3.5 Defects in lymphatic architecture and function

The lymphatic system is highly intertwined with the GI system for various functions related to human physiology (Randolph *et al*, 2017). It is especially important in the context of digestion and absorption of nutrients as it helps in the transport of digested dietary fat in the form of chylomicron to the liver for further processing (Cifarelli & Eichmann, 2019). Therefore, the regulation of lymphatic architecture and function plays a central role in CDD, highlighted by the presence of disease upon their dysregulation. These include classical enteropathies such as Hennekam lymphangiectasia-lymphedema syndrome and Waldmann's disease. These patients suffer from EO-PLE, with hallmark clinical manifestations such as hypoalbuminemia

and hypogammaglobulinemia. Genetic mutations related to lymphatic architecture include mutations in the genes *CCBE1, FAT4,* and *ADAMTS3*.

Patients with Hennekam lymphangiectasia-lymphedema syndrome suffers from generalized lymphatic aberrations that can involve the GI tract, pericardium, and limbs. Their GI tract manifestation includes intestinal lymphangiectasia, which is the enlargement of the lymphatic vessels that led to the loss of serum protein albumin and gammaglobulins through the intestine (Hennekam *et al*, 1989). The first mutation identified to cause this disease was in the gene *CCBE1*, encoding for the collagen and calcium-binding EGF-domain containing protein 1 (Alders *et al*, 2009). It was shown that morpholino knockdown of *ccbe1* in zebrafish resulted in altered lymphatic vessel development (Alders *et al*, 2009). It was later found that this syndrome can also be caused by mutations in the gene *FAT4,* encoding for the FAT atypical cadherin 4 (Alders *et al*, 2014). However, the molecular mechanism for FAT4 deficiency remains elusive. Lastly, 2 patients from a western European origin suffered from congenital lymphedema and PLE were identified to have compound heterozygous mutations in a gene called A*DAMTS3*. It encodes for the A disintegrin and metalloproteinase with thrombospondin motifs 3 protein, an important processing molecule (along with CCBE1) of lymphangiogenic signaling molecule VEGF3 (Brouillard *et al*, 2017).

1.3.6 Defects in endothelial integrity

Endothelial cells provide a barrier between various tissues and the circulatory system. A novel syndrome of severe EO-PLE presenting with hypoproteinemia, hypoalbuminemia and hypertriglyceridemia was identified in patients with mutations in the gene *PLVAP*, encoding for protein plasmalemma vesicle-associated protein (Elkadri *et al*, 2015). It was shown that the truncating mutation resulted in the lack of diaphragm forming on fenestrae of endothelial cells in fenestrated capillaries. This results in the leakage of plasma protein in the intestine, culminating in the loss of plasma proteins through the GI tract. These patients suffered from

1.3.7 Defects in immune-related homeostasis

The GI tract acts as a center for immunological activity in the body, maintaining a homeostatic balance between fighting harmful microbes ingested during food ingestion while at the same time dampening responses to commensal microbiota (Mason *et al*, 2008). The immune system and its various cellular components play an integral role in the context of GI homeostasis. Therefore, aberrations that affect immune cells might result in the loss of control of GI homeostasis and result in CDD. A more in-depth insight into defects in immune-related homeostasis will be discussed in the next section.

1.4 Inflammatory Bowel Disease (IBD)

Inflammatory bowel disease (IBD) is an umbrella term for the uncontrolled immune response in the gastrointestinal tract. Two main forms exist, namely ulcerative colitis (UC) or Crohn's disease (CD) (Abraham & Cho, 2009). The two are differentiated according to the localization and pattern of the affected tissues. In UC, the inflammation is mainly restricted to the mucosa and partially submucosal lining of the colon and rectum in an uninterrupted fashion. CD can affect any region of the intestine in patches, and the inflammation can occur deeper in the intestinal tissue. Moreover, CD might have extra-GI symptoms caused by increased risk of fistulization (Wang *et al*, 2006). A third subset of IBD, termed IBD unspecified (IBDu), does not manifest as typical UC or CD but can progress to either form. It has been noted that pediatric patients tend to present with clinical manifestations that categorizes them into this IBDu classification (Rosen *et al*, 2015). In general, patients present with abdominal pain, diarrhea, and rectal bleeding that can be accompanied by weight loss, fever, and growth retardation (Abraham & Cho, 2009)(Cho & Abraham, 2007). In pediatric patients (aged <17), the incidence of CD is higher than UC (Rosen *et al*, 2015). In addition to that, patients diagnosed with CD was observed to have a more severe and aggressive disease progression (Van Limbergen *et al*, 2008).

1.4.1 Genetics of adult-onset IBD

Numerous factors have been attributed to the onset of inflammatory bowel disease in adult patients. Genetic factors have been extensively studied in monozygotic twin and genome-wide association (GWA) studies. Monozygotic twins, who share identical genetic make-up, are significantly more likely to be concordant for both UC and CD. The rate differs between UC and CD, with monozygotic twins with CD having a concordance of 20-50% depending on the study while monozygotic twins with UC have a concordance rate of 14-19% (Halme *et al*, 2006). However, the less-than-expected concordance rate highlights the importance of environmental contribution to the pathogenesis of IBD (Ruel *et al*, 2014). GWA studies utilizes large population-based datasets to identify contributions of certain genomic loci to the onset of a disease by comparing genetic variations in healthy controls versus patients. In-depth GWA studies have identified more than 200 risk loci associated with IBD (Momozawa *et al*, 2018). These loci contain single nucleotide polymorphisms (SNP) that can lie within an intron or within an exon of its associated gene. A number of biological processes that is important in IBD pathogenesis have been identified through GWA, including the NOD2 signaling axis, autophagy, IL-23/IL-17 signaling axis, and numerous immune-related signaling axis (Jostins *et al*, 2012; Liu *et al*, 2015; de Lange *et al*, 2017; Huang *et al*, 2017). Additionally, GWAS loci in genes that function of epithelial barrier were also identified (McCole, 2014). Interestingly,

these loci result in mostly weak associations, as the SNP within them are only predicted to alter protein expression and not necessarily function due to the fact that the lie on regulatory regions (Verstockt *et al*, 2018).

1.4.2 Early-onset IBD – a type of CDD

Patients with early-onset IBD (EO-IBD) is defined as those whose symptoms begin at an age of less than 10 years old (Meddens *et al*, 2019). This group is also known as the A1a group in the Paris classification of IBD (Levine *et al*, 2011) (Figure 3). A subset of these patients are considered very-early-onset IBD (VEO-IBD), in which clinical manifestations of IBD occur before the age of 6 (Levine *et al*, 2011) (Figure 3). These patients usually present with very severe clinical course of disease that is often refractory and resistant to therapy (Moran *et al*, 2015). Patients who present with symptoms within their first year of life are termed infantile IBD (Figure 3). It has been observed that these group of EO/VEO/infantile-IBD patients present with a different clinical manifestations compared to adult-onset IBD patients(Shim & Seo, 2014). Due to the early onset of the disease, there has been increasing interest in the genetics of VEO-IBD patients (Shim, 2019). The incidence of pediatric IBD has been increasing globally over the past decades, showcasing the importance of further understanding the contributions of various factors in the onset of this disease (Benchimol *et al*, 2011). Therefore, EO/VEO/infantile-IBD is a CDD that occur due to defects in immune-related homeostasis.

1.4.3 Early-onset IBD and mutations in *IL10R*

The first loss-of-function mutations affecting either subunit of the IL10 receptor (*IL10RA/IL10RB*) were first identified in 2009 as being one of the monogenetic aberrations leading to EO-IBD (Glocker *et al*, 2009). Patients with *IL10RA/IL10RB* and more recently *IL10* mutations present within the first year of life with intractable bloody diarrhea, perianal abscesses, and gut mucosal immune infiltrates reminiscent of enterocolitis (Glocker *et al*, 2010). The molecular pathology involves the lack of anti-inflammatory IL-10 signaling that centers around the transcription factor STAT3 to dampen TNF-mediated inflammation. This study showcases that IL10 signaling is a key factor that is necessary for the maintenance of gut immunohomeostasis. Recently, IL-1β signaling has been shown to also be involved in the pathomechanism of IL10R deficiency. Patients with IL-10 receptor deficiency experienced clinical improvement upon treatment with anakinra, the IL-1 receptor antagonist (Shouval *et al*, 2016). Patients with IL10/IL10RA/IL10RB deficiency experience clinical remission upon hematopoietic stem cell transplantation (HSCT), further highlighting the key role of the immune system in the pathology of IL10R deficiency (Engelhardt *et al*, 2013).

1.4.4 Other examples of monogenic mutations leading to EO-IBD

Since the identification of monogenic mutations in *IL10*/*IL10RA*/*IL10RB*, the race to identify additional genetic lesions that lead to EO-IBD/VEO-IBD and therefore genetically diagnose patients began. Of note, a few monogenic defects present with a predominantly IBD phenotype, including *ADAM17* and *IL21* deficiencies (Blaydon *et al*, 2011; Salzer *et al*, 2014). This is in contrast to a broader group of inborn error of immunity (IEI), in which patients might present with an IBD-like symptoms as the first clinical manifestation which underlies immune dysregulation (Bousfiha *et al*, 2018). In 2014, more than 50 monogenic defects have been identified to result in EO-IBD with varying penetrance per gene defect (Figure 4) (Uhlig *et al*, 2014). Some examples of these monogenic defects will be highlighted in the following paragraphs.

Patients with chronic granulomatous disease (CGD) suffer from genetic defects in components that are required to form reactive oxygen species (ROS), an important component in antimicrobial defense. As a result, these patients suffer from recurrent bacterial and fungal infections. A large proportion of patients suffer from intestinal granulomas, diarrhea, bloody stool, and abscess formations. CGD patients with a genetic diagnosis have been shown to harbor mutations in the NADPH Oxidase complex including genes such as *CYBA*, *CYBB*, *NFC1*, *NCF2*, and *NCF4* (Arnold & Heimall, 2017).

A large proportion of patients with monogenetic defects in immune dysregulation have been observed to suffer from enteropathies that resemble IBD. For example, patients with mutations in the gene *FOXP3* encoding for the important transcription factor FOXP3 suffers from various clinical manifestation related to immune dysregulation due to the lack of development of CD4+ CD25+ regulatory T cells that requires the transcription factor (Wildin *et al*, 2001; Bennett *et al*, 2001). Another important example of patients that suffer from EO-IBD due to immune dysregulation are patients with mutations in the *LRBA* and *CTLA4* genes (Lo *et al*, 2015; Serwas *et al*, 2015; Schubert *et al*, 2014). Interestingly, the penetrance of GI clinical manifestation in patients with LRBA and CTLA4 deficiency is not 100%, highlighting the importance other factors that contribute to the GI phenotype.

An interesting gene defect that affects both immune cells and gut epithelial cells, thereby resulting in a systemic disease affecting multiple organs was first seen in patients with TTC7A deficiency. Patients with TTC7A deficiency was first described in patients with multiple intestinal atresia and combined immunodeficiency (Samuels *et al*, 2013; Bigorgne *et al*, 2014; Lemoine *et al*, 2014; Chen *et al*, 2013). Further reports confirmed the clinical presentation of

VEO-IBD in some patients with TTC7A deficiency, in particular highlighting the role of TTC7A in the context of the gut epithelial polarity (Avitzur *et al*, 2014; Neves *et al*, 2018).

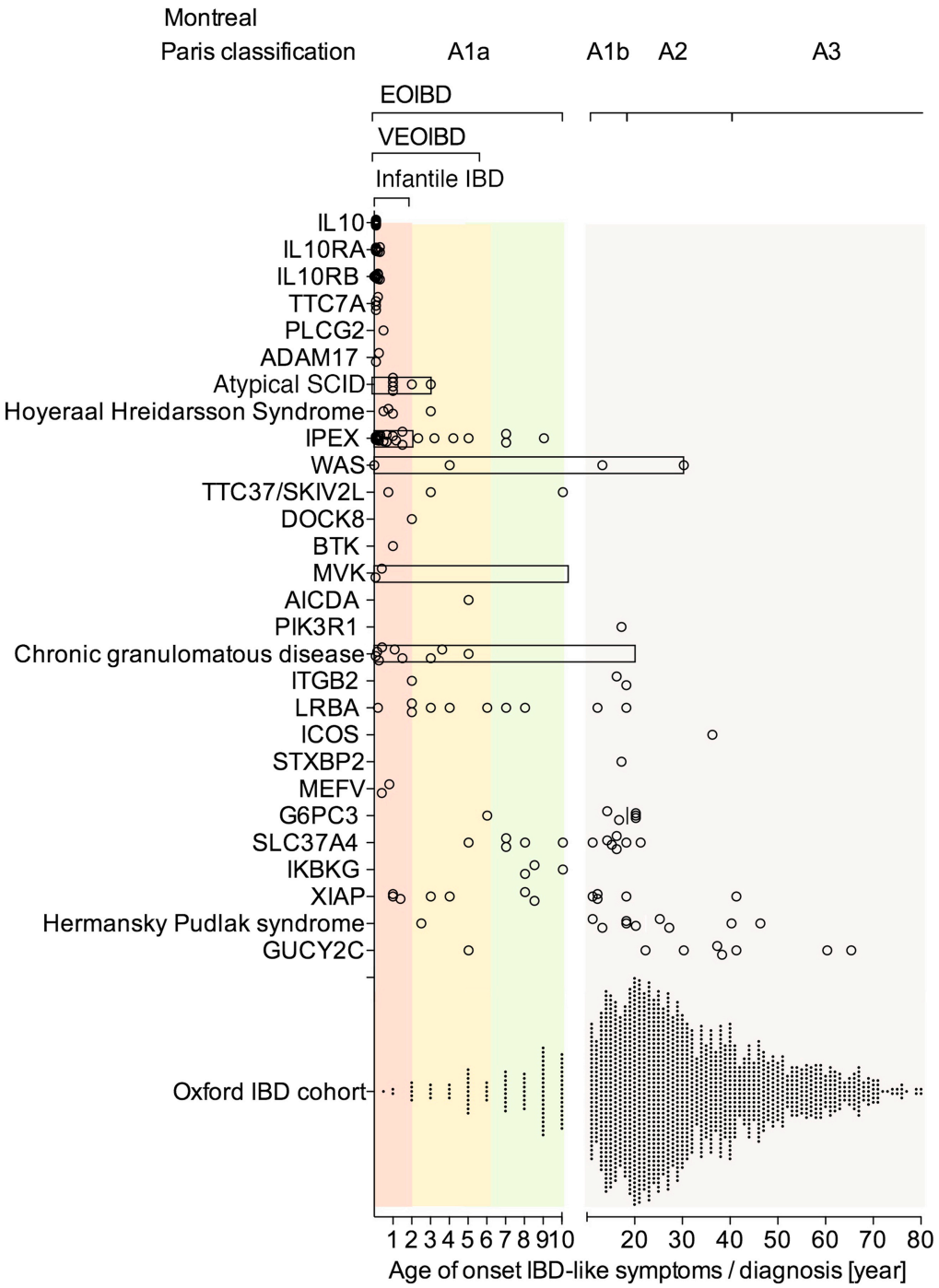

Figure 3. List of some syndromic and monogenic defects with their corresponding age of onset. Figure taken from Uhlig et al. "The Diagnostic Approach to Monogenic Very Early Onset Inflammatory Bowel Disease" Gastroenterology (2014) doi:10.1053/j.gastro.2014.07.023. Image reprinted using permission under the Creative Commons Attribution- Non Commercial - No Derivatives License (CC BY NC ND)

1.5 Technological advancement

1.5.1 Next Generation Sequencing (NGS) for diagnostic purposes

Next generation sequencing (NGS) allows for high-throughput genomic investigations for patients with suspected genetic disease. In diagnostic centers dealing with early-onset diseases, three major types of high throughput deoxyribonucleic acid (DNA) sequencing are used based on the necessity and availability: targeted panel sequencing, whole exome sequencing (WES), and whole genome sequencing (WGS).

Targeted panel sequencing makes use of hybrid capture technology to capture exons of a pre-selected set of genes predetermined by the user (Rehm, 2013). This method has the advantage of screening for mutations in coding region of known disease-causing genes that present with a specific phenotype. This has been highly successful in monogenic diseases such as primary immunodeficiencies, where a known group of mutations in previously described genes have been identified to cause disease. An advantage of panel sequencing is the fact that the data generated is less complex compared to WES and WGS, making it easier to interpret. Moreover, there are less chances of incidental findings being identified.

Another option of NGS method to use for clinical diagnostic is WES. In this case, hybrid capture technology is again used to capture exons of almost all of protein coding genes, which makes up approximately 2-3% of the human genome (Warr et al, 2015). Through this method, a more exploratory nature of genetic diagnostic can be undertaken. Researchers are first able to look for mutations in genes that are known to be disease-causing in cases where the patient phenotype points towards a potential genetic diagnosis. If there were no mutations identified, further look into the other variants identified might result in the discovery of a novel genetic etiology of the disease. This method provides a good cost-benefit ratio for diagnostics purposes (Warr et al, 2015).

In the context of CDD, the use of targeted panel sequencing was proved to be useful in genetically diagnosing EO-IBD patients and patients suffering from chronic diarrhea (Kammermeier *et al*, 2014; Petersen *et al*, 2017). Therefore, targeted panel sequencing is a viable option to ensure rapid diagnosis of CDD patients. Genetic diagnosis in CDD patients can be of utmost importance, especially in cases where bone marrow transplantation can be curative such as in the case for IL10R deficiency (Engelhardt *et al*, 2013).

1.5.2 Gut organoid technology

Organoid technology has dramatically advanced in the past few years, moving cell culture into 3-dimensional, spatially arranged models which can be easily derived from various starting materials (Rossi *et al*, 2018). This method allows for culturing of notoriously difficult materials which includes the brain, the liver, and various portions of the GI tract. The first report on long-term culturing and growth of gut organoids was first published in 2009, highlighting the importance of *Lgr5*+ stem cells in the small-intestinal crypts of mice (Sato *et al*, 2009). The authors were able to show that by embedding these Lgr5+ stem cells in Matrigel and providing them a set of growth factors (Epidermal growth factor (EGF), R-spondin, and Noggin), they recapitulate *in vivo* organization of the gut epithelial expansion and form gut organoids with villus-like structures. This findings were then recapitulated in human-derived samples, allowing researchers to continuously culture gut epithelial cells derived from the colon, Barrett's epithelium as well as adenoma and adenocarcinoma samples (Sato *et al*, 2011).

Gut organoid technology allowed researchers to study diseases in conjunction with patient genomic information which has been especially useful in the context of personalized medicine. For example, genomic data from healthy and cancer tissues of colorectal carcinoma patients were combined with drug screening of patient-derived gut organoids to obtain genomics-informed drug sensitivity assays for personalized medicine (Van De Wetering *et al*, 2015). Another study generated rectal organoids derived from patients suffering from cystic fibrosis (CF) whose mutations in the *CFTR* gene were genetically characterized (Dekkers *et al*, 2016). The study showed that these rectal organoids can be used to characterize patient response to known CFTR modulators, and that this technology can prove to be useful in characterizing mutations of unknown significance and their response to CFTR-modulating drugs in CF patients (Dekkers *et al*, 2016).

Gut organoids have also been shown to play an important role in dissecting molecular mechanisms of monogenic CDDs. Gut organoids were used to show cellular defects related to proliferation, differentiation, and altered apicobasal polarity in TTC7A deficiency (Bigorgne *et al*, 2014). This seminal study showed that the apicobasal polarity defect can be reversed using a ROCK inhibitor Y-27632 on an in vitro gut organoid culture. Gut organoids have also been used to model epithelial-specific defects, such as in the case of *EPCAM* mutations leading to CTE where patient mutation was shown to have various cellular defects when expressed in gut organoids that recapitulated patient phenotype (Das *et al*, 2019). Gut organoids have also been used to study EO-IBD, where organoid-derived DNA methylome and transcriptome can be used to differentiate disease outcome and subtypes (Howell *et al*, 2018).

2. Aims of thesis

The aims of this thesis were to: 1) identify the underlying genetic cause and 2) unravel the molecular mechanism of the pathophysiology of monogenic disorders resulting in early-onset protein-enteropathy (PLE) and/or early-onset inflammatory bowel disease (IBD).

Aims above were achieved using next generation sequencing methods and various molecular biology techniques. The identification of novel monogenic mutations leading to EO-PLE and EO-IBD will allow us to precisely dissect genotype-phenotype relationships and allow us to propose a potential precision medicine-based therapy that corresponds to the genetic defect. It will also allow us to pinpoint critical proteins that are essential for the homeostasis of the GI tract.

3. Results

The following section consists of the two manuscripts that resulted from the research done during the period of this thesis. The first manuscript is titled “CD55 Deficiency, Early-onset Protein Losing Enteropathy and Thrombosis” and is published in the New England Journal of Medicine (DOI: 10.1056/NEJMoa1615887). The second manuscript is titled “Intestinal Failure and Aberrant Lipid Metabolism in Patients with DGAT1 deficiency” published in the journal Gastroenterology (DOI: 10.1053/j.gastro.2018.03.040). The author of this thesis is a shared first author in both manuscripts.

3.1 CD55 Deficiency, Early-Onset Protein-Losing Enteropathy and Thrombosis

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ORIGINAL ARTICLE

CD55 Deficiency, Early-Onset Protein-Losing Enteropathy, and Thrombosis

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ABSTRACT

BACKGROUND

Studies of monogenic gastrointestinal diseases have revealed molecular pathways critical to gut homeostasis and enabled the development of targeted therapies.

METHODS

We studied 11 patients with abdominal pain and diarrhea caused by early-onset protein-losing enteropathy with primary intestinal lymphangiectasia, edema due to hypoproteinemia, malabsorption, and less frequently, bowel inflammation, recurrent infections, and angiopathic thromboembolic disease; the disorder followed an autosomal recessive pattern of inheritance. Whole-exome sequencing was performed to identify gene variants. We evaluated the function of CD55 in patients' cells, which we confirmed by means of exogenous induction of expression of CD55.

RESULTS

We identified homozygous loss-of-function mutations in the gene encoding CD55 (decay-accelerating factor), which lead to loss of protein expression. Patients' T lymphocytes showed increased complement activation causing surface deposition of complement and the generation of soluble C5a. Costimulatory function and cytokine modulation by CD55 were defective. Genetic reconstitution of CD55 or treatment with a complement-inhibitory therapeutic antibody reversed abnormal complement activation.

CONCLUSIONS

CD55 deficiency with hyperactivation of complement, angiopathic thrombosis, and protein-losing enteropathy (the CHAPLE syndrome) is caused by abnormal complement activation due to biallelic loss-of-function mutations in *CD55*. (Funded by the National Institute of Allergy and Infectious Diseases and others.)

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Genetic inquiry has contributed to our understanding of gastrointestinal diseases, associating at least 64 genes with early-onset or very-early-onset inflammatory bowel disease.[1] Deleterious gene variants affect the intestinal epithelial barrier, phagocytosis processes, immune regulation, and inflammation. Protein-losing enteropathy, or gastrointestinal protein wasting causing hypoproteinemia, edema, and pleural and pericardial effusions, has also been linked to monogenic disorders.[2] A loss-of-function variant in *PLVAP* (encoding plasmalemma vesicle associated protein) that disrupts endothelial fenestrated diaphragms and compromises barrier integrity is associated with severe protein-losing enteropathy.[3] This condition can develop secondarily from systemic conditions that arrest lymph flow, such as congestive heart failure, or directly from gastrointestinal mucosal damage or impaired lymph drainage from primary intestinal lymphangiectasia (also known as Waldmann's disease).[2,4] Although primary intestinal lymphangiectasia can be a component of multisystemic genetic syndromes, including the Hennekam syndrome (caused by biallelic loss-of-function variants in *CCBE1* or *FAT4*), the mechanisms of nonsyndromic primary intestinal lymphangiectasia and protein-losing enteropathy remain largely unknown.[5,6] Here, we define the molecular and clinical features of an autosomal recessive syndrome of early-onset protein-losing enteropathy characterized by primary intestinal lymphangiectasia, bowel inflammation, and thrombotic events.

METHODS

STUDY PARTICIPANTS

We enrolled 10 patients who were based in Turkey and 1 who was based in the Netherlands, along with their healthy parents and siblings when available. The 11 patients were from eight families, all of whom were of Moroccan, Syrian, or Turkish ancestry. Patients 3.1, 5.1, and 5.2 were assessed at the Ludwig Boltzmann Institute for Rare and Undiagnosed Diseases in Vienna and were from Istanbul or Ankara, Turkey. The remaining patients were all assessed at the National Institute of Allergy and Infectious Diseases, National Institutes of Health, in Bethesda, Maryland, and were from Turkey, Syria, or Morocco. The ages of the patients ranged from 3 to 23 years as of February 2017. Patients were initially identified on the basis of a diagnosis of persistent protein-losing enteropathy and were included in the study after the identification of biallelic loss-of-function variants in *CD55*. For details regarding individual patients, see the Supplemental Patient Clinical Histories section in the Supplementary Appendix, available with the full text of this article at NEJM.org. All the study participants provided written informed consent for approved protocols at their respective institutions, with consent for minors being provided by the parents.

GENETIC AND FUNCTIONAL ANALYSIS

We performed whole-exome sequencing in the index patients and Sanger sequencing of *CD55* in the other participants. Complement assays were performed before and after lentiviral CD55 reconstitution. Alexion Pharmaceuticals provided reagents. Details are provided in the Supplementary Appendix.

STATISTICAL ANALYSIS

We used the Mann–Whitney U test or Student's t-test to assess the significance between nonpaired samples and the Wilcoxon matched-pairs signed-rank test or a two-tailed paired t-test for the paired samples. Two-tailed P values of less than 0.05 were considered to indicate statistical significance. Statistical comparisons were made with the use of GraphPad Prism software, version 7.0a.

RESULTS

CLINICAL PHENOTYPE

We investigated 11 patients and 2 deceased relatives with a history of protein-losing enteropathy that was characterized by early-onset gastrointestinal symptoms, edema, malnutrition, hypoalbuminemia, and hypogammaglobulinemia, from eight consanguineous families with unaffected parents (Table 1 and Fig. 1A, and Fig. S1 in the Supplementary Appendix). Hypoproteinemia was always present, with minor variations, and, together with edema and gastrointestinal symptoms (abdominal pain, vomiting, and diarrhea), was alleviated by albumin infusion (Fig. 1B). Chronic malabsorption caused micronutrient deficiencies of iron, ferritin, calcium, magnesium, folate, and vitamins D and B_{12} as well as anemia and growth retardation (Table 1). These condi-

Table 1. Demographic and Clinical Characteristics of 11 Patients with the CHAPLE Syndrome.*

Characteristic	No. of Patients
Sex	
Female	6
Male	5
Age at presentation <2 yr	8
Manifestations of gastrointestinal disease or inflammatory bowel disease	
Chronic or recurrent diarrhea	8
Abdominal pain	4
Vomiting	6
Features of protein-losing enteropathy	
Hypoalbuminemia	10
Hypogammaglobulinemia	11
Facial or extremity edema	9
Confirmed primary intestinal lymphangiectasia or Waldmann's disease†	5
Malabsorption features	
Growth retardation	8
Anemia	9
Vitamin or micronutrient deficiency‡	11
Features of thrombotic disease§	
Thrombocytosis	2
Thrombosis	3
Endoscopic findings†	
Mucosal ulcer	4
Lymphoid infiltrates in mucosa	6
Recurrent lung infection	5
Additional features	
Hypothyroidism¶	3
Arthritis or arthralgia	2
Finger clubbing	5

* The CHAPLE syndrome comprises CD55 (decay-accelerating factor) deficiency with hyperactivation of complement, angiopathic thrombosis, and protein-losing enteropathy.
† Two patients did not undergo endoscopic assessment because they did not have gastrointestinal symptoms.
‡ The micronutrients assessed included serum vitamin B_{12}, vitamin D, folate, iron, ferritin, zinc, calcium, and magnesium.
§ Thrombi were located in the deep veins in the abdominal sites, including the mesenteric and hepatic veins, sometimes with extension to the inferior vena cava and heart, and leading to pulmonary embolism.
¶ Tests for antithyroglobulin and anti–thyroid peroxidase antibodies were negative.

tions were improved by means of vitamin and micronutrient supplementation, a protein-rich diet with medium-chain triglycerides, albumin (administered as an intravenous infusion), and blood transfusions. Details are provided in Table S1, Figures S1 and S2, and the Supplemental Patient Clinical Histories section in the Supplementary Appendix.

Histopathological assessment of intestinal-biopsy samples or resections revealed extensive lymphangiectasia, verified by lymphatic endothelial markers, which, together with the patients' young age, suggested the diagnosis of primary intestinal lymphangiectasia (Table 1 and Fig. 1C).[2,4] Transmission electron microscopy of the duodenal-biopsy sample obtained from Patient 6.1 showed lymphatic dilatation, but, unlike in persons with PLVAP deficiency, we found normal capillary architecture. Surgical removal of the lymphangiectatic segments in Patients 2.1 (who had partial bowel obstruction), 5.1, and 5.2 ameliorated clinical symptoms and protein-losing enteropathy (although Patient 5.2 had a relapse), which raised the possibility of a causal relationship. Some patients had bowel inflammation (similar to that seen in persons with inflammatory bowel disease), exudates, and lymphocytic infiltrates without intestinal thromboses (Table 1 and Fig. 1D). Radiologic examinations revealed bowel-wall edema, thickening, or both in Patients 1.1, 2.1, and 6.1. Thus, protein-losing enteropathy and micronutrient deficiencies are probably caused by primary intestinal lymphangiectasia exacerbated by bowel inflammation. Details are provided in Table S1 and Figures S3 and S4 in the Supplementary Appendix.

Five patients had recurrent respiratory infections associated with hypogammaglobulinemia (Table 1 and Fig. 1B). Major immunologic cell subsets and antibody production were normal. Patient 1.1 had concomitant homozygous gene variants in *CD21*, and Patient 5.1 in *CD27*. In a finding consistent with the confirmed CD21 deficiency, Patient 1.1 had decreased class-switched IgD–CD27+ memory B cells.[7] Patient 5.1 had subclinical persistent Epstein–Barr virus infection (viral RNA level, 420 copies per milliliter) and, at the time of this writing, is being monitored closely, because CD27 deficiency increases the risk of lymphoproliferative disease driven by the Epstein–Barr virus.[8] Intravenous immune globulin reduced the frequency and severity of respiratory infections in Patients 1.1 and 2.1. Details are provided in Tables S1 and S3, Figures S2A and S5, and

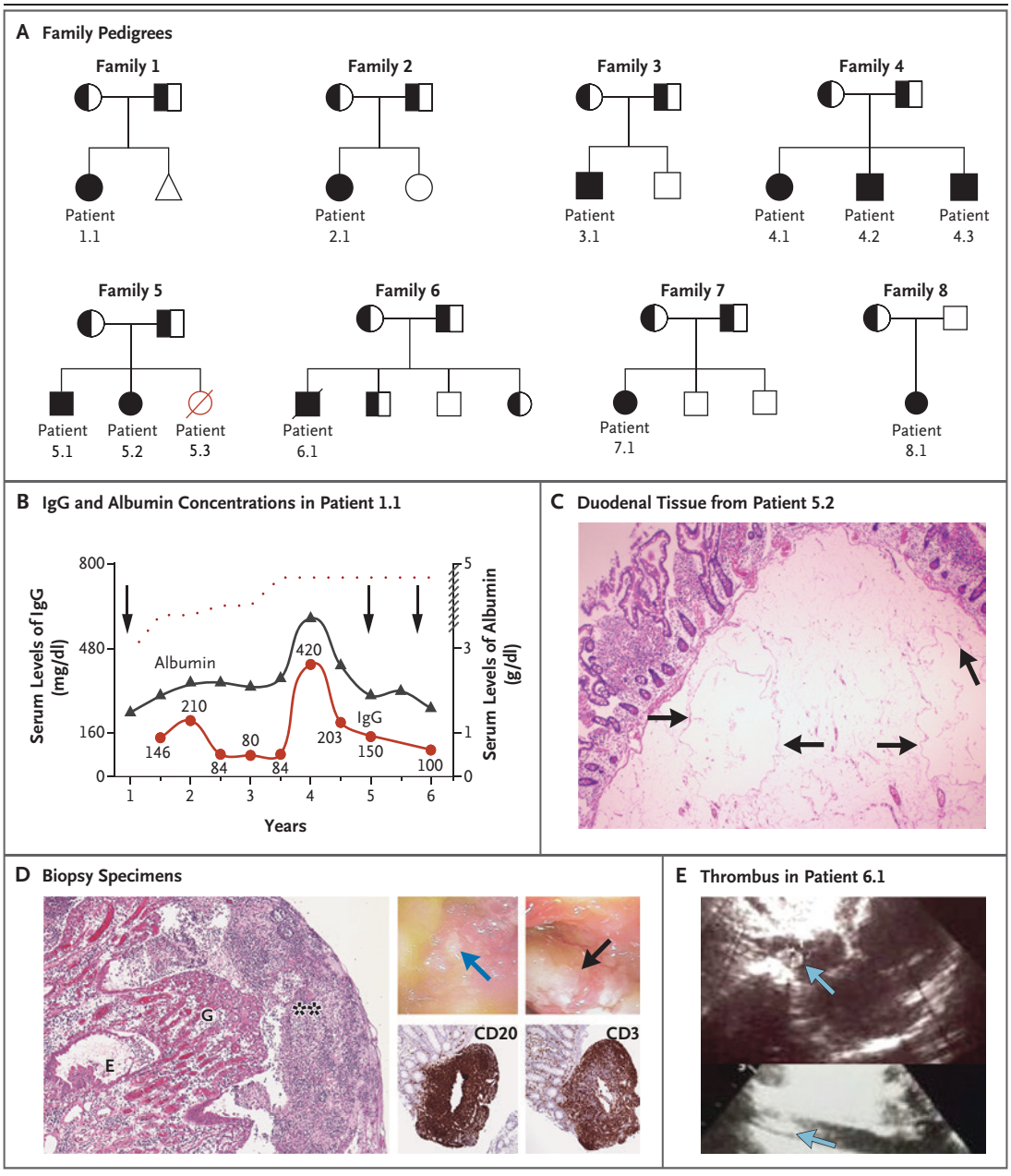
the Supplemental Patient Clinical Histories section in the Supplementary Appendix.

Three patients had severe thrombotic vascular occlusion (Table 1). Thrombi developed in the inferior vena cava, right atrium, and pulmonary arteries of Patient 6.1, causing arteriovenous malformations (Fig. 1E). Transmission electron microscopy of duodenal-biopsy samples revealed malformed erythrocytes binding abnormally to capillary walls and transmural migration. Patient 5.1 had multiple thromboses in the mesenteric and hepatic veins, heart, and cerebral veins that were associated with an intracranial hemorrhage. Thrombosis was unresolved, despite surgical removal of blood clots and anticoagulation. The Budd–Chiari syndrome developed in Patient 8.1, presumably owing to hepatic-vein thrombosis. Examination of extended family histories uncovered two additional patients, Patients 4.4 and 5.3, who had died before genotyping from thrombotic events associated with protein-losing enteropathy, lymphangiectasia, and malnutrition. Patient 6.1 died from pulmonary embolism during the course of the study. Hence, the natural history of the disease includes early death related to severe thrombotic events. Details are provided in Table S1, Figures S1 and S6A through S6D, and the Supplemental Patient Clinical Histories section in the Supplementary Appendix.

LOSS-OF-FUNCTION MUTATIONS IN *CD55*

Whole-exome sequence analysis in Patients 1.1, 2.1, 3.1, and 5.1 revealed novel homozygous variants in the gene encoding the complement regulatory protein CD55 (encoding for decay-accelerating factor) (Fig. 2A, and Table S3 and Figs. S1 and S7A in the Supplementary Appendix).[9] These variants segregated recessively with disease, and heterozygous persons were unaffected. These variants were not present in the Exome Aggregation Consortium (ExAC) database and were predicted by bioinformatic analysis to be deleterious (Table S4 in the Supplementary Appendix). Further screening of 640 patients with inflammatory bowel disease (some with early-onset or very-early-onset disease) and a cohort of 239 samples obtained from Turkish persons revealed only 1 person heterozygous for a *CD55* variant that was predicted to be only moderately deleterious (c.107delTGCCCGCGGCGC; Combined Annotation–Dependent Depletion [CADD] score, 13.23), which indicates that *CD55* loss-of-function variants are rare. Screening of the ExAC database of 60,000 unrelated persons revealed 53 persons who were heterozygous for loss-of-function *CD55* variants and 1 who was homozygous for a loss-of-function *CD55* variant, possibly from a cohort of persons with inflammatory bowel disease, although a lack of informed consent prevented us from identifying and contacting this person. *CD55* had a probability of loss-of-function intolerance (pLI) score of 0.0, which indicates that heterozygous loss-of-function mutations are likely to be benign.[10] Screening of additional patients with early-onset protein-losing enteropathy revealed 6 patients who were homozygous for *CD55* loss-of-function variants in Families 4, 6, 7, and 8.

Patients 1.1 and 7.1 were homozygous for a dinucleotide deletion and a 4-nucleotide insertion at nucleotide positions 149 and 150. Patients 2.1, 3.1, 5.1, and 5.2 were homozygous for a single-nucleotide deletion in *CD55* at position 109. Patient 8.1 was homozygous for a single-nucleotide insertion at position 367. All three variants resulted in a frameshift and were predicted to cause premature termination of *CD55* messenger RNA (mRNA) translation. The variant common to Families 1 and 7 and the variant common to Families 2, 3, and 5 led to mRNA nonsense-mediated decay. In Family 4, a novel homozygous missense mutation in *CD55* encodes a cysteine-to-serine substitution in the fourth short consensus repeat domain (c.800G→C, p.Cys267Ser); the wild-type Cys267 disulfide bond with Cys225 is presumably disrupted by the substituted serine at residue 267.[11] In Patient 6.1, a variant disrupting an exon 3 splice acceptor site probably caused alternative splicing. In all the patients, CD55 protein expression was lost, with only Patient 6.1 having minor residual expression (Fig. 2B, and Fig. S7D in the Supplementary Appendix). We observed that CD55 was normally expressed on capillary endothelial cells in the basal submucosa and lamina propria, the brush-border columnar epithelium, and infiltrating lymphocytes and was absent in tissues (Fig. 2C). These variants have strong (in Patient 6.1) or very strong (in all our other patients) evidence of pathogenicity, according to the guidelines of the American College of Medical Genetics and Genomics.[12] Altogether, we identified five distinct homozygous, novel, loss-of-function *CD55* variants in nine patients from Turkey, one from Syria, and one



from Morocco (Fig. 2A). Details are provided in Figures S1, S6E, and S7 in the Supplementary Appendix.

COMPLEMENT ACTIVATION ON CD55-DEFICIENT CELLS

CD55 is attached to the surface by a glycosylphosphatidylinositol moiety and inhibits complement activation by destabilizing and preventing the formation of C3 and C5 convertases, which prevents complement damage.[9] We therefore tested whether CD55 deficiency accelerated complement activation.[13,14] After incubation with human serum, we observed increased C3 fragment deposition on patients' CD4+ T-cell blasts by staining for an epitope common to C3, C3b, and C3d, which was increased by means of stimulation of the classical pathway by coating the cells with mouse IgG1 (Fig. 3A and 3B). We did not detect the C3b fragment (data not shown), which suggests rapid degradation, possibly by factor I and cofactor activity.[15] CD55 reconstitution reduced complement

Figure 1 (facing page). Clinical Presentation of Eight Families with Familial Early-Onset Protein-Losing Enteropathy.

Panel A shows the pedigrees of the eight families. Affected persons who were homozygous for the mutant allele are indicated by solid symbols, heterozygous persons by half solid symbols, unaffected persons by open symbols, and an affected person with an unknown genotype by an open red symbol. Circles indicate female persons, and squares male persons; the triangle in Family 1 represents a miscarriage, and the slash in Family 5 indicates a person who had died. Patient 6.1 died during the course of the study (indicated by a slash). Affected members of the extended family are shown in Figure S1 in the Supplementary Appendix. Panel B shows the serum levels of IgG in relation to serum albumin concentrations as a function of age in years in Patient 1.1. The age-specific lower cutoff value for IgG is indicated by the red dotted curve, and the reference value for the albumin concentration is more than 3.5 g per deciliter (indicated by the dashed section on the right y axis). Each arrow denotes an episode of pneumonia. Panel C shows sections of resected duodenal tissue (hematoxylin and eosin staining) obtained from Patient 5.2, with markedly dilated lymphovascular spaces within the submucosa (arrows). Panel D shows sections from surgically resected material of the small intestine (hematoxylin and eosin) showing ulceration covered by fibrin with dense granulocytic infiltrate (left panel, double asterisks), granulation tissue (G) with edema in the lamina propria, and reactive epithelial changes (E) in Patient 2.1. Panel D also includes endoscopy photographs showing a mucosal ulcer (blue arrow) and exudate (black arrow) in the terminal ileum of Patient 1.1 (right side, top) and histopathological assessments of a biopsy sample obtained from the ileum of Patient 2.1 and immunohistochemical analysis showing the presence of B cells (CD20) and T cells (CD3) within lymphoid nodules (right side, bottom). Panel E shows an echocardiographic image from Patient 6.1 with thrombus in the right atrium (top, arrow) and inferior vena cava (bottom, arrow).

deposition on T cells in patients (Fig. 3C). Immunohistochemical analysis of duodenal-biopsy samples revealed in vivo terminal complement activation (membrane attack complex [C5b-9]) in submucosal arterioles (Fig. 3D). Details are provided in Figure S7A and S7B in the Supplementary Appendix.

EXCESSIVE PRODUCTION OF INFLAMMATORY CYTOKINES BY CD55-DEFICIENT T CELLS

Complement proteins can provide costimulatory and differentiation signals to T cells by means of either CD46-mediated C3b sensing or anaphylatoxin receptors.[16-20] Studies in knockout mice have confirmed a role for Cd55 in adaptive immune regulation, with *Cd55*$^{-/-}$ mice producing more interferon-γ and less interleukin-10 in autoimmune models.[21,22] CD4+ T cells from patients produced an increased level of tumor necrosis factor (TNF), reduced the interleukin-10 level (with a normal interferon-γ level), and had normal proliferation after T-cell–receptor engagement. Dual inhibition of the anaphylatoxin receptors C3aR and C5aR1 decreased TNF overproduction to control levels, primarily owing to the inhibition of C5aR1. Anaphylatoxin inhibition did not increase the interleukin-10 level, which suggests that this defect in interleukin-10 production is independently regulated.[21] Inflammatory cytokines, including TNF, could instigate the severe thrombophilia in patients with CD55 deficiency by reducing thrombomodulin and augmenting tissue-factor expression on endothelial cells.[23] We found that TNF and interferon-γ induced procoagulatory decreases in thrombomodulin and increases in tissue factor. CD55 expression increased in human umbilical-vein endothelial cells after TNF treatment, which suggests that CD55 limits complement-mediated damage during inflammation.

CD55 can convey a costimulatory signal for T-cell activation and the production of interleukin-10, a cytokine that is inhibitory to intestinal inflammation.[24,25] We found that patients' cells showed impaired proliferation and interleukin-10 production in response to an agonistic anti-CD55 antibody or recombinant CD97 together with T-cell–receptor stimulation. Details are provided in Figure S8 in the Supplementary Appendix.

IN VITRO INHIBITION OF COMPLEMENT BY ECULIZUMAB FORMULATION

Finally, we investigated whether clinically available complement inhibitors could prevent the enhanced activation in samples obtained from patients. We observed that C5a production, which was elevated on incubation with patients' cells, was abrogated by coincubation with an experimental formulation of eculizumab, a complement-inhibitory therapeutic agent that is used to treat paroxysmal nocturnal hemoglobinuria (PNH) and the atypical hemolytic–uremic syndrome (Fig. 4).

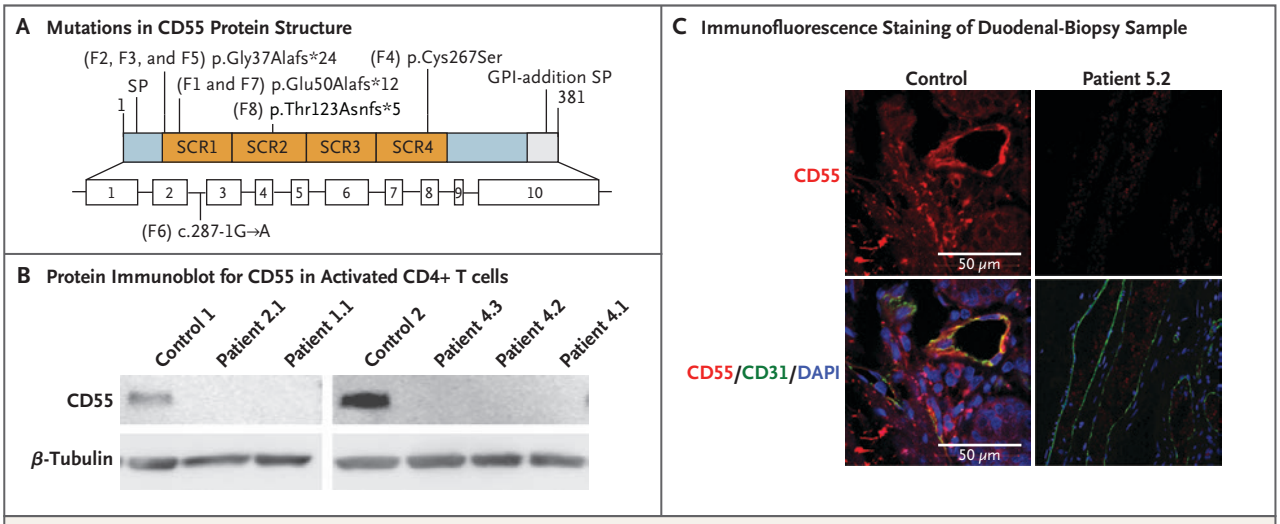


Figure 2. Variants in CD55 Leading to Loss of Protein Expression.

Panel A shows the identified mutations in CD55 leading to loss of protein expression, including the four short consensus repeat (SCR1 through SCR4) domains (top), and exon structure (bottom). The eight families are indicated as F1 through F8. GPI denotes glycosylphosphatidylinositol, and SP signal peptide. Panel B shows a protein immunoblot for CD55 in activated CD4+ T cells in two healthy controls and five patients. We used β-tubulin staining as a loading control. Panel C shows indirect immunofluorescence staining of duodenal-biopsy samples obtained from Patient 5.2 and a healthy control, with staining for CD55 (red), the endothelial marker CD31 (green), and 4′,6-diamidino-2-phenylindole (DAPI, blue).

DISCUSSION

We define a genetic syndrome comprising CD55 deficiency with hyperactivation of complement, angiopathic thrombosis, and protein-losing enteropathy (the CHAPLE syndrome). Protein-losing enteropathy is probably caused by primary intestinal lymphangiectasia, intestinal inflammation, and possibly thromboses (Fig. S9 in the Supplementary Appendix).[2,4]

Complement is a system of interacting proteins that provides host defense by destroying microbes and modulating immunity with soluble anaphylatoxins governed by multiple regulators, including CD55.[9] Genetic variants that increase complement activation cause PNH, the atypical hemolytic–uremic syndrome, C3 glomerulopathy, and age-related macular degeneration (Fig. S7A in the Supplementary Appendix).[26-28] PNH results from somatic mutations that disable the glycosylphosphatidylinositol anchor that tethers CD55 and CD59 to the cell surface, leading to complement-mediated hemolysis and thrombosis.[29-31] Heterozygous germline loss-of-function variants affecting C3, factor H, factor I, or CD46 trigger the atypical hemolytic–uremic syndrome by inducing complement-mediated damage to glomerular microvascular endothelial cells, hemolysis, and kidney failure.[32] These genetic defects also cause complement-mediated retinal damage and age-related macular degeneration.[33] Unlike PNH or the atypical hemolytic–uremic syndrome, isolated CD55 deficiency causes early-onset protein-losing enteropathy owing to primary intestinal lymphangiectasia and bowel inflammation. We found that CD55 is up-regulated by retinoic acid, which is highly concentrated in the gut from the diet (Fig. S8G in the Supplementary Appendix). Persons with the CHAPLE syndrome and PNH are at increased risk for thrombosis, and thrombotic microangiopathy develops in patients with the atypical hemolytic–uremic syndrome. These findings indicate cross-regulation of the complement and coagulation cascades.

CD55 deficiency has been previously found in persons with sporadic gastrointestinal abnormalities who do not have Cromer blood group red-cell antigens (the Inab phenotype).[34-37] The Inab phenotype can be transient (as has been observed in three cases) or persistent (as has been observed in nine cases)[34-37] and is sometimes associated with gastrointestinal disease that has been variously diagnosed as Crohn's disease, capillary angioma, protein-losing enteropathy with intes-

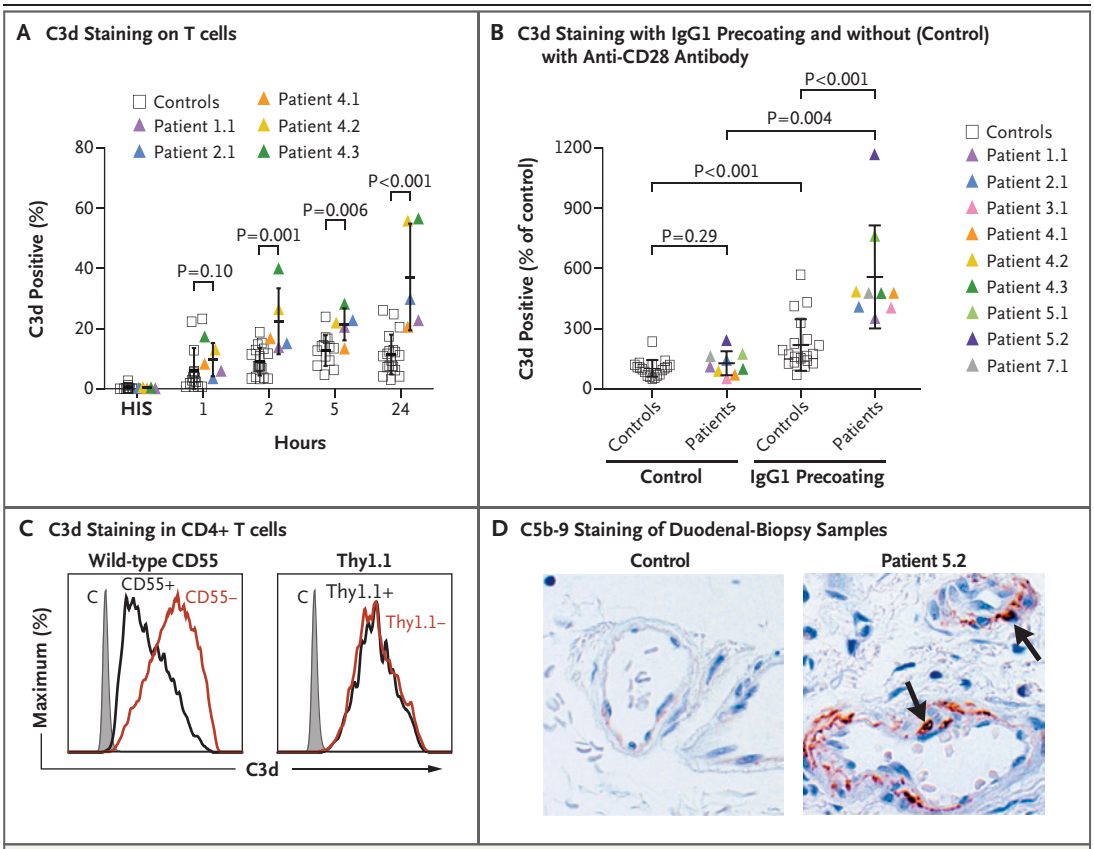


Figure 3. Loss of CD55 and Increased Complement Deposition.

Panel A shows pooled analyses of C3d staining on T cells obtained from healthy controls and from five patients with CD55 deficiency after incubation with medium (pH, 7.4) containing pooled normal human serum for the times indicated. The middle lines of the I bars indicate mean values, and the I bars ±1 SD. A t-test was used to assess the significance of the between-group difference in C3d positivity for the 5-hour incubation, and the nonparametric Mann–Whitney U-test was performed to assess the significance for the difference in the conditions at 1 hour, 2 hours, and 24 hours. HIS denotes heat-inactivated serum. Panel B shows the pooled analysis of C3d staining with or without IgG1 precoating with an anti-CD28 antibody to activate the classical pathway. Each color-coded data point for the patients represents the average of at least three repeated measurements in a single patient. A Mann–Whitney U-test was performed for between-group comparisons, and the Wilcoxon matched-pairs signed-rank test was applied to comparisons of the same group with or without treatment. Panel C shows C3d deposition on CD4+ T cells obtained from Patient 1.1 that were reconstituted with wild-type CD55 (left) or the control marker Thy1.1 (right) and then incubated with serum for 24 hours. The values for the isotype control (C, shown in gray) indicate samples that were not incubated with serum, the black line indicates samples transduced with CD55 or Thy1.1, and the red line indicates nontransduced control cells. Panel D shows C5b-9 staining of duodenal-biopsy samples obtained from a healthy donor and Patient 5.2. Arrows indicate sites of C5b-9 deposition.

tinal tumor, and food intolerance. Three loss-of-function variants were identified (Fig. S7G in the Supplementary Appendix), although no definitive correlation with disease was made. Also, exacerbated dextran sulfate sodium–induced colitis develops in Cd55-deficient mice, which is consistent with the intestinal disease seen in our patients, as does T-cell–mediated autoimmunity in autoimmune models, probably owing to immunoregulatory abnormalities that are similar to those seen in our patients.[21,22,38] Only one patient with the CHAPLE syndrome presented with autoimmunity in the form of polyarthritis, and none had inflammatory markers or elevated levels of cytokine in the blood.

The disease in our patients showed variable expressivity, which was potentially attributable to background genetics, diet, microbiome composition, or other influences. Conventional treatments were only transiently effective, although

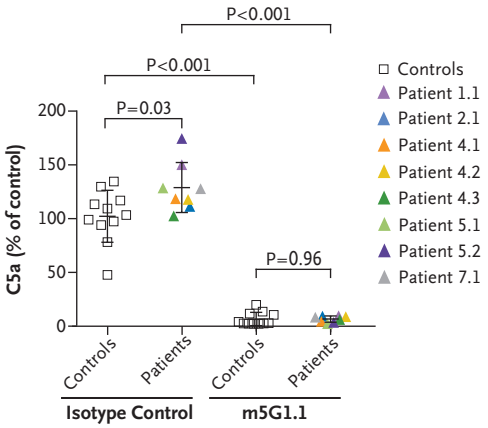


Figure 4. Effect of Eculizumab on C5a Production on Patients' T Cells.

Shown are the levels of C5a in supernatants of CD4+ T-cell cultures, color-coded according to patient, after 2 hours of incubation with 10% normal human plasma and 10 μg per milliliter of either isotype control or C5 inhibitory (m5G1.1) antibodies. Each triangle represents the average of at least three repeated measurements in a single donor or patient. The middle lines of the I bars indicate the sample mean, and the I bars ±1 SD. A two-tailed unpaired t-test with Welch's correction was performed for comparisons between the control group and the group of patients, and the two-tailed paired t-test was applied to comparisons of the same group with or without treatment.

more sustained benefit occurred after the resection of lymphangiectatic intestinal segments. Recurrent infections were responsive to intravenous immune globulin. Eculizumab, which suppressed C5a production on patients' cells, warrants further investigation as a potential treatment of the CHAPLE syndrome.[39] In line with this, work by Kurolap et al., published in this issue of the *Journal*,[40] shows that eculizumab therapy in a family with the CHAPLE syndrome resulted in attenuated protein-losing enteropathy and reduced bowel-movement frequency within 100 days after the initiation of therapy.

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Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

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APPENDIX

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3.2 Intestinal failure and aberrant lipid metabolism in patients with DGAT1 deficiency

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Intestinal Failure and Aberrant Lipid Metabolism in Patients With DGAT1 Deficiency

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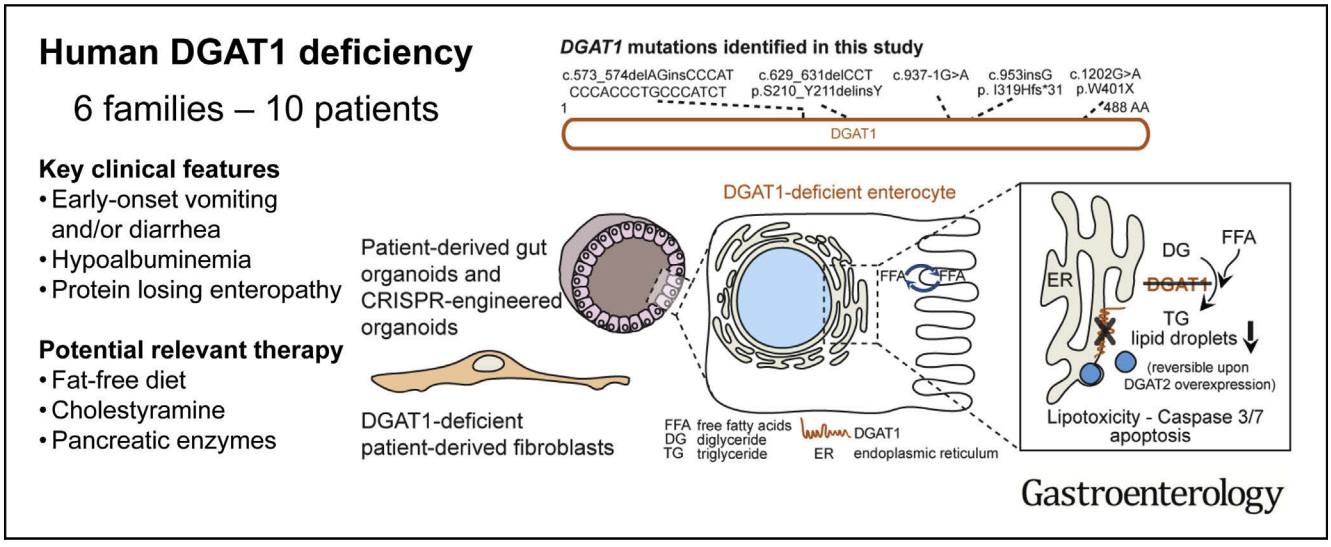

BACKGROUND & AIMS: Congenital diarrheal disorders are rare inherited intestinal disorders characterized by intractable, sometimes life-threatening, diarrhea and nutrient malabsorption; some have been associated with mutations in *diacylglycerol-acyltransferase 1* (*DGAT1*), which catalyzes formation of triacylglycerol from diacylglycerol and acyl-CoA. We investigated the mechanisms by which DGAT1 deficiency contributes to intestinal failure using patient-derived organoids. **METHODS:** We collected blood samples from 10 patients, from 6 unrelated pedigrees, who presented with early-onset

severe diarrhea and/or vomiting, hypoalbuminemia, and/or (fatal) protein-losing enteropathy with intestinal failure; we performed next-generation sequencing analysis of DNA from 8 patients. Organoids were generated from duodenal biopsies from 3 patients and 3 healthy individuals (controls). Caco-2 cells and patient-derived dermal fibroblasts were transfected or transduced with vectors that express full-length or mutant forms of DGAT1 or full-length DGAT2. We performed CRISPR/Cas9-guided disruption of *DGAT1* in control intestinal organoids. Cells and organoids were analyzed by immunoblot, immunofluorescence, flow cytometry, chromatography, quantitative real-time polymerase chain reaction, and for the activity of caspases 3 and 7. **RESULTS:** In the 10 patients, we identified 5 bi-allelic loss-of-function mutations in *DGAT1*. In patient-derived fibroblasts and organoids, the mutations reduced expression of DGAT1 protein and altered triacylglycerol metabolism, resulting in decreased lipid droplet formation after oleic acid addition. Expression of full-length DGAT2 in patient-derived fibroblasts restored formation of lipid droplets. Organoids derived from patients with *DGAT1* mutations were more susceptible to lipid-induced cell death than control organoids. **CONCLUSIONS:** We identified a large cohort of patients with congenital diarrheal disorders with mutations in *DGAT1* that reduced expression of its product; dermal fibroblasts and intestinal organoids derived from these patients had altered lipid metabolism and were susceptible to lipid-induced cell death. Expression of full-length wildtype DGAT1 or DGAT2 restored normal lipid metabolism in these cells. These findings indicate the importance of DGAT1 in fat metabolism and lipotoxicity in the intestinal epithelium. A fat-free diet might serve as the first line of therapy for patients with reduced DGAT1 expression. It is important to identify genetic variants associated with congenital diarrheal disorders for proper diagnosis and selection of treatment strategies.

Keywords: CDD; Genomic; PLE; 3-D Culture Model.

WHAT YOU NEED TO KNOW

BACKGROUND AND CONTEXT

Mutations in *DGAT1* have recently been identified in patients with congenital diarrheal disorders (CDDs), but the underlying molecular pathomechanisms have remained largely elusive.

NEW FINDINGS

The authors identified 10 patients with DGAT1 deficiency representing the largest cohort to date, linking gut epithelial lipid metabolism and lipotoxicity to CDD; and rescued aberrant lipid metabolism with isoenzyme DGAT2.

LIMITATIONS

Although the authors show exogenous DGAT1 or DGAT2 expression or proteasome inhibitors may overcome defects, future studies may need to address how that knowledge can be translated to targeted therapies.

IMPACT

The authors highlight the importance of identifying the genetic defect in patients with CDD, and showcase further use of gut organoid technology to study rare diseases of the gastrointestinal tract.

Congenital diarrheal disorders (CDDs) are a group of rare inherited intestinal disorders that are characterized by intractable, sometimes life-threatening, diarrhea and nutrient malabsorption. CDDs can be classified based on their aberrations in absorption and transport of nutrients and electrolytes, enterocyte differentiation and polarization, enteroendocrine cell differentiation, or dysregulation of the intestinal immune response.[1] Congenital protein-losing enteropathy (PLE) is a type of CDD that is characterized by increased protein loss from the gastrointestinal (GI) system. Patients with PLE often suffer from hypoproteinemia, fat malabsorption, fat-soluble vitamin deficiencies, and malnutrition. Recently, we have identified germline loss-of-function mutations in *CD55* as a major monogenic etiology for congenital PLE.[2]

Previously, mutations in the gene encoding diacylglycerol-acyltransferase 1 (DGAT1) were found to underlie a syndrome of diarrhea and congenital PLE.[3–6] DGAT1 and its isozyme DGAT2 (encoding for diacylglycerol-acyltransferase 2) are responsible for the conversion of diacylglycerol (DG) and fatty acyl-CoA to triacylglycerol (TG) in humans.[7,8] TG is the main energy substrate stored in human adipose tissue, is essential for milk production in the mammary gland, and is part of the very low-density lipoprotein–mediated transport of lipids to peripheral tissue.[9,10] In the human small intestine, DGAT1 is the only highly expressed enzyme, whereas DGAT2 is mainly expressed in the liver.[3,11] In enterocytes, TG is stored in lipid droplets or packaged into chylomicrons before transport into the lymphatic system.[8,12,13]

The pathomechanism responsible for intestinal failure and PLE in DGAT1 deficiency has remained unclear. Through next-generation sequencing, we identified 10 additional patients from 6 unrelated pedigrees with 5 different, novel bi-allelic mutations in *DGAT1* leading to severe, sometimes fatal course of PLE and fat intolerance. We took this unique opportunity to further shed light on the fundamental pathomechanisms of human DGAT1 deficiency.

Materials and Methods

Study Approval

The study was approved by the responsible local ethics committees (Ethics Commission of the Medical University of

*Authors share co-first authorship; § Authors share co-senior authorship.

Abbreviations used in this paper: B-LCL, B lymphoblastoid cell line; BSA, bovine serum albumin; CDD, congenital diarrheal disorder; cDNA, complementary DNA; DG, diacylglycerol; DGAT1, diacylglycerol-acyltransferase 1; hSI-EM, human small intestine expansion medium; FFA, free fatty acid; GI, gastrointestinal; OA, oleic acid; PB, PiggyBac transposon; PBS, phosphate-buffered saline; PLE, protein-losing enteropathy; sgRNA, single-guide RNA; SSC, Side Scatter; TG, triacylglycerol; WT, wild-type.

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Vienna and Institutional Review Board of the University Medical Center Utrecht). All participants provided written informed consent for the collection of samples and subsequent analysis.

DNA Sequencing

Whole-exome sequencing was performed on patients as previously described.[14,15] Targeted panel sequencing was performed as previously described.[16] Conventional Sanger sequencing was performed for validation and segregation analysis of variants.

Cell Culture

Organoids were generated from duodenal biopsies that were obtained from 3 healthy controls and 3 patients during duodenoscopy for diagnostic purposes, as described in detail in the Supplementary Materials and Methods. The healthy controls were patients suspected of celiac disease or inflammatory bowel disease, who did not show abnormalities on endoscopic and histological examinations.

Caco-2 cells and patient-derived fibroblasts were cultured in Dulbecco's modified Eagle's medium with/without GlutaMax and high glucose (Life Technologies, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (GE Health Care, Little Chalfont, UK), 100 U/mL penicillin (Gibco, Waltham, MA), 100 μg/mL streptomycin (Gibco), and 1 mM sodiumpyruvate (Gibco) at 37°C and 5% CO_2. Patient-derived Epstein-Barr virus B lymphoblastoid cell line (B-LCL) was maintained in RPMI 1640 with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin (Gibco), and 100 μg/mL streptomycin (Gibco).

CRISPR/Cas9 Knockout of DGAT1

Plasmid constructs for the expression of DGAT1 single-guide RNAs (sgRNA) and Cas9 nuclease were generated as previously described and outlined in Supplementary Material and Methods.[17] Three DGAT1 sgRNAs targeting exon 7 and 1 sgRNA targeting intron 6-7 were designed. Two different sgRNA (mix)-plasmids were used for transfection: sgRNA#2 and a mix of sgRNA#6, sgRNA#7, and sgRNA#8 (sgRNA#678). Hygromycin-resistance was achieved by co-transfection with the PiggyBac (PB) Transposon System (plasmids PB-Hygromycin and PB-Transposase were kindly provided by Bon-Kyoung Koo).

Transfection of healthy intestinal organoids was performed by electroporation, as described previously[18] and extensively in Supplementary Materials and Methods.

Lipid Droplet Assays

Oleic acid (OA) was conjugated to bovine serum albumin (BSA) as described in Supplementary Materials and Methods. Organoids were grown in expansion medium (EM) on black clear-bottom 96-well imaging plates (Corning Life Sciences, Corning, NY). On day 6, the organoids were incubated with 1 mM OA/BSA for 17 hours in presence or absence of 0.1 μM DGAT1 inhibitor (AZD 3988; Tocris, Bristol, UK). Organoids were then fixed in 4% formaldehyde for 30 minutes at room temperature. Cells were washed in phosphate-buffered saline (PBS) and stained with 0.025 mg/mL LD540[19] and 4′, 6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, St Louis, MO) in PBS for 15 minutes at room temperature in the dark. Cells were washed and stored in PBS. Imaging of the organoids was performed using a Leica (Wetzlar, Germany) SP8X laser-scanning confocal microscope outfitted with a white light laser. The acquired stacks were processed and analyzed with Fiji/ImageJ (National Institutes of Health, Bethesda, MD)[20,21]; shown are maximum projections of approximately 15-μm stacks.

For flow cytometry analysis, organoids were grown and treated with OA/BSA in the same manner as for the confocal analysis. After overnight incubation with 1 mM OA/BSA, the cells were harvested by pipetting and dissociated using TrypLE Express (ThermoFisher, Waltham, MA) until single cells were acquired. The cells were then fixed and stained in the same manner as for the confocal analysis and assayed using a BD FACS Canto II (BD Biosciences, San Jose, CA).

Fibroblasts were seeded at 6×10^4 cells in a 6-well plate and allowed to adhere overnight. Cells were then treated with OA positive control from the Lipid Droplet Fluorescence Assay (#500001; Cayman Chemical, Ann Arbor, MI) at 1:4000 dilution for 24 hours. Cells were fixed and stained according to the manufacturer's protocol and assayed using BD FACS Fortessa (BD Biosciences, Franklin Lakes, NJ).

Cloning and Retrovirus Production

DGAT1 (ENST00000528718.5) and *DGAT2* (ENST00000228027.11) complementary DNAs (cDNAs) were amplified by polymerase chain reaction from HEK293 cDNA library and cloned into pDONR221 using BP reaction according to the manufacturer's protocol (Thermo Fisher). LR reaction was performed into pFMIG for wild-type (WT) DGAT1 and DGAT2 with N-terminal Streptavidin-HA tag.

Quantitative Real-Time Polymerase Chain Reaction

RNA was isolated from Caco-2 cells or organoids grown in either EM or differentiation medium (DM) for 5 days, used to synthesize cDNA by using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) and amplified with SYBR green supermix (BIO-Rad) in a Light Cycler96 (BIO-Rad) according to the manufacturer's protocol. Details on analysis and primers are given in Supplementary Materials and Methods.

Western Blotting

Cell lysates were generated and Western blotting was performed as described in the Supplementary Materials and Methods.

Thin Layer Chromatography

Organoids were grown in EM for 7 days and then prepared for Folch extraction as described previously[22] and in the Supplementary Materials and Methods. Thin layer chromatography was performed by spotting the isolated lipid phase on aluminium-backed silica plates (Merck Millipore, Burlington, MA). As reference samples 1,3-dipentadecanoin (DG, C15:0/-/C15:0), tripentadecanoin (TG, C15:0/C15:0/C15:0) and triheptadecanoin (TG, C17:0/C17:0/C17:0) were included. The plates were then developed in a mobile phase of hexane:diethylether:acetic acid (60:15:2). The lipid bands were

visualized by spraying the plates with a solution of 10% $CuSO_4$ (wt/vol) in 10% H_2SO_4 (vol/vol) and subsequently heating the plates to 120°C for 30 minutes, as described previously[23] to calculate DG/TG ratios, band intensities were quantified by Fiji/ImageJ.[20,21]

Lipotoxicity Assays

For the propidium iodide staining, organoids were grown and incubated with varying concentrations of OA as described for the lipid droplet confocal assay. The organoids were then washed with Hank's balanced salt solution (HBSS) (Gibco), and stained with Hoechst 1 μg/mL (Sigma-Aldrich) and propidium iodide 0.1 mg/mL (ThermoFisher) in HBSS at room temperature for 15 minutes. Organoids were imaged by an inverted Olympus IX53 epifluorescence microscope (Tokyo, Japan).

For the Caspase-Glo 3/7 assay (Promega, Madison, WI), organoids were grown on TC-treated 96-well plates (Greiner, Kremsmunster, Austria) and incubated with OA as was done for the propidium iodide staining. Organoids were washed and resuspended in HBSS and transferred to a white-walled 96-well plate (Greiner). The assay was performed according to the manufacturer's protocol and luminescence was measured on a Tristar 2 luminometer (Berthold Technologies, Oak Ridge, TN).

Statistical Analysis

Data are presented as mean ± SD. Experiments were performed with a minimum of 3 replications. Statistical significance was determined at $P \leq .05$ using 2-way analysis of variance with Tukey's multiple comparison test, a Mann-Whitney U test, or a Student t test where appropriate. Significance is indicated as $P \leq .05$ (*), $P \leq .01$ (**), or $P \leq .001$ (***) or $P < .0001$ (****).

Results

Clinical Phenotype

We investigated 10 patients from 5 consanguineous families and 1 family of unknown consaguinity with unaffected parents. All of the patients had a history of intestinal failure due to congenital diarrhea and/or vomiting, resulting in failure to thrive. (Figure 1*A*, Table 1, Supplementary Table 1, see Supplementary Materials and Methods for

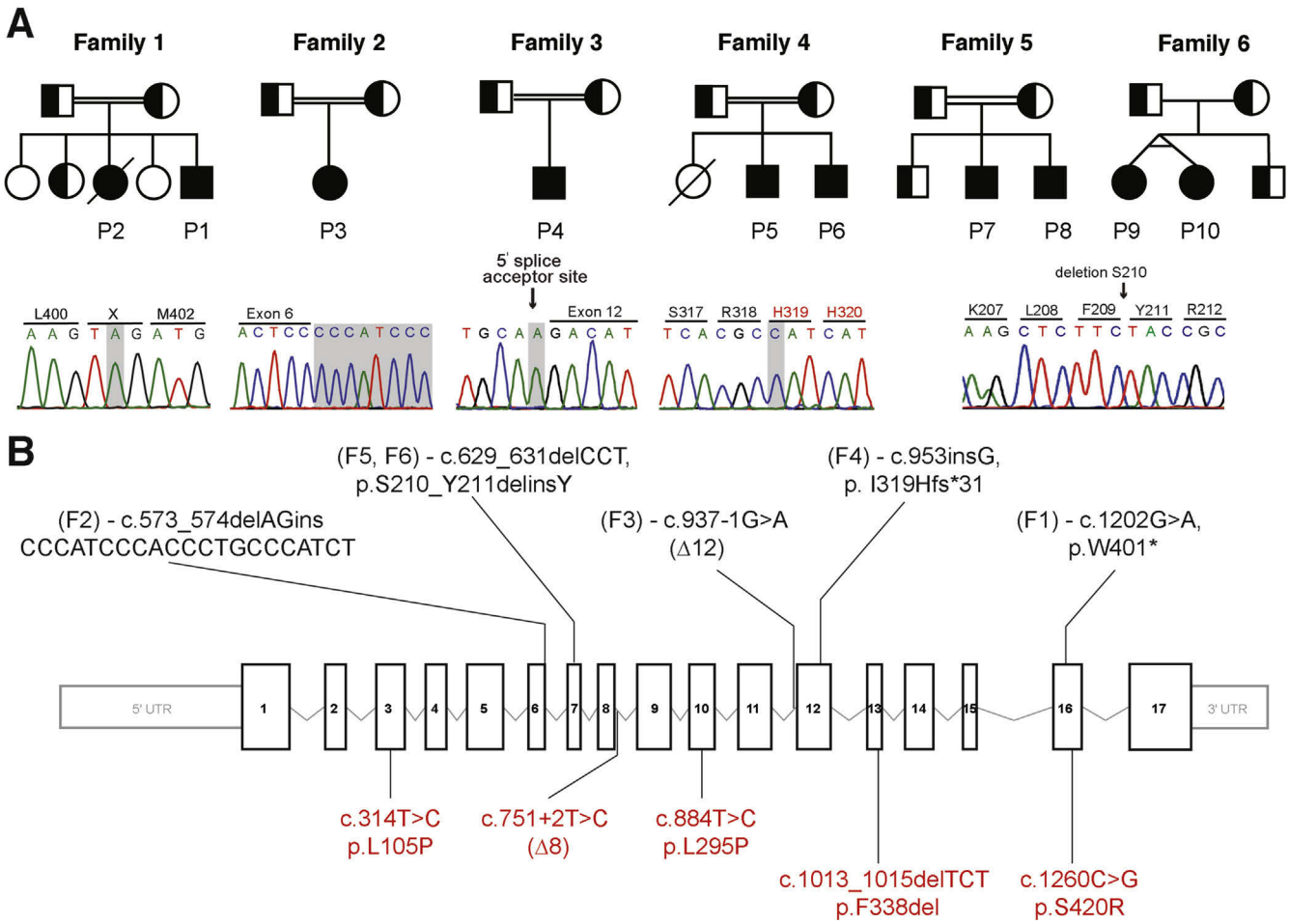

Figure 1. Pedigrees, mutations, and genetic location of 6 families with *DGAT1* deficiency. (*A*) Pedigrees of families with DGAT1 deficiency and chromatograms showing mutation in affected patients. Filled shapes indicate affected individuals, half-filled are heterozygous for mutation indicated, and empty shapes indicate WT. (*B*) Exonic scheme of *DGAT1* showing mutations identified in this study in *black* and previously identified mutations in *red*.[3–6]

Table 1. Patient Characteristics

Patient ID	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
Demographics										
Current age and gender	4.5 y, M	Deceased, F	2 y, F	8 y, M	2 y, M	6 y, M	14 y, M	17 y, M	10 y, F	10 y, F
Country of origin and ethnicity	Turkey, Turkish	Turkey, Turkish	Turkey, Turkish	Turkey, Turkish	Turkey, Turkish	Turkey, Turkish	The Netherlands, Caucasian	The Netherlands, Caucasian	The Netherlands, Caucasian	The Netherlands, Caucasian
Age of clinical onset	Birth	Birth	3 wk	2 mo	40 d	2.5 mo	First month	First month	Birth	Birth
Disease manifestations										
Failure to thrive	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes
Vomiting	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Diarrhea	Yes, fatty	Yes	Yes	Yes	Yes, bloody and watery	Yes, watery	No	No	Yes	Yes
Hypoalbuminemia	Yes	Yes	Yes	Yes	Yes	Yes	No	No	Yes	Yes
Hypogammaglobulinemia	Yes	Yes	Yes	Yes	Yes	Yes	ND	ND	Yes	Yes
Edema	Yes	Yes	Yes	No	No	No	No	No	No	No
GI examinations (endoscopy and imaging)	Normal	Normal	Normal	Normal	ND	Normal	Normal	Normal	Normal	Normal
Hematoxylin-eosin and electron microscopy	Normal	Normal	Duodenal enterocytic lipid accumulation, microvilli are shortened and rarefied	Normal	ND	Focal vacuolization at one area and partially blunted villi	Normal histology (on fat-free diet)	Normal histology (on fat-free diet)	Misdiagnosis of atypical MVID based on CD10 positive globules on LM and laterally located microvilli on EM	Misdiagnosis of atypical MVID based on CD10 positive globules on LM and laterally located microvilli on EM
Extra-GI manifestations	Recurrent infections, otitis media	Recurrent infections	Corneal cystine crystal accumulation and intermittent metabolic acidosis	ND	Hepatomegaly and jaundice	Recurrent infections	Gilles de la Tourette syndrome	Gilles de la Tourette syndrome	Recurrent infections	Recurrent infections
Clinical course										
Treatments	Fat-free formula and MCT, albumin infusions	Albumin infusions	Albumin, TPN	Cholestyramine	Creon pancreatic lipase, hydrolyzed formula	Creon pancreatic lipase	Monthly infusion of Intralipid and Omegaven suppletion of lipid-soluble vitamins	Monthly infusion of Intralipid and Omegaven suppletion of lipid-soluble vitamins	TPN and small bowel transplantation	TPN, recently started with fat-free formula
Outcome	Asymptomatic and normal growth with low-fat diet + MCT oil + fat-free formula	Patient passed away at 6 mo due to sepsis	Stool frequency once a day with Basic F formula feeding	Reduced stool volume and frequency on cholestyramine treatment	Stool frequency reduced on Creon treatment; weight and height are still below 3rd percentile	Stool frequency reduced on Creon treatment; after 2 y, symptoms resolved spontaneously	Enteral feeding without fat	Enteral feeding without fat	Tolerates enteral feeding, but still stunted	Tolerates enteral feeding, but still stunted

EM, electron microscopy; F, female; GI, gastrointestinal; LM, light microscopy; M, male; MCT, medium-chain triglyceride; MVID, microvillus inclusion disease; ND, not determined; TPN, total parenteral nutrition.

clinical details). These 10 patients come from 4 Turkish families originating from Turkey and 2 Caucasian families from The Netherlands. In summary, 8 of 10 cases showed early-onset PLE characterized by hypoalbuminemia, hypogammaglobulinemia, and intractable diarrhea, with 1 patient developing marked steatorrhea. Patients 7 and 8 presented with severe vomiting only, which resulted in failure to thrive in the older sibling. Food containing fat induced abdominal pain and vomiting soon after ingestion and serum lipid profiles of the 10 patients were variable (Supplementary Table 2). Some patients had normal levels of serum TG, whereas 1 patient exhibited hypertriglyceridemia. Most showed a reduced level of high-density lipoprotein, with normal levels of low-density lipoprotein, very low-density lipoprotein, and cholesterol.

Endoscopy was performed on patients 1 to 3 and 7 to 10, which showed no macroscopic abnormalities of the duodenum and colon (Supplementary Figures 1 and 2). Histopathology of a duodenal biopsy from patient 1 showed marked flattening of the villi (Figure 2 and Supplementary Figure 2) and patient 3 showed marked shortening of the villi (Supplementary Figure 1*A*), Electron microscopy of a duodenal biopsy of patient 3 showed lack of microvilli (Supplementary Figure 1*A*). Patients 7 and 8 showed normal pathology, although the biopsies were taken under fat-restricted diet (Supplementary Figure 1*B*). Duodenal

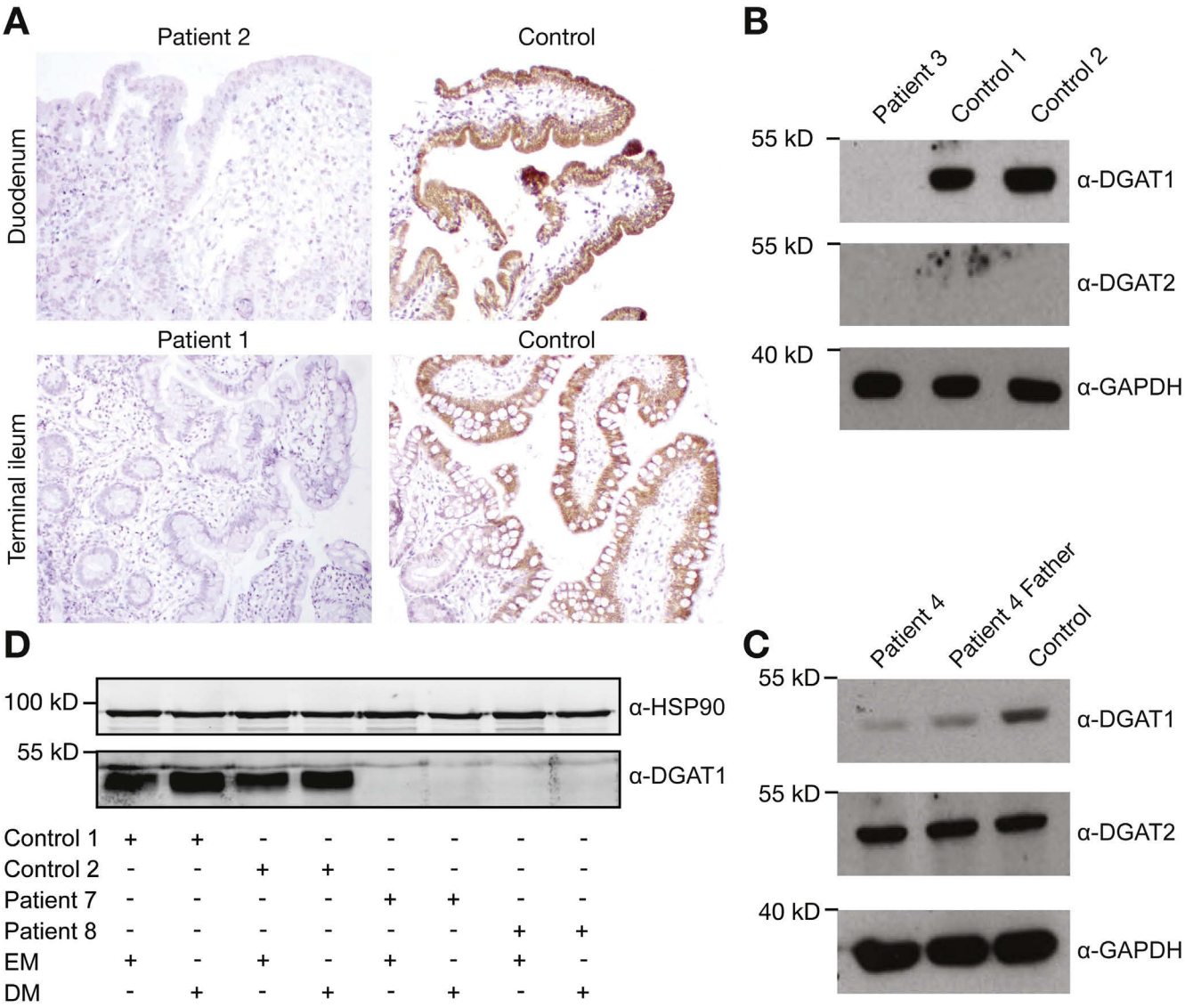

Figure 2. DGAT1 protein expression in patient-derived material. (*A*) Immunohistochemistry of DGAT1 in control and patient 1 and patient 2 ileal and duodenal biopsy, respectively. (*B*) Western blot showing lack of DGAT1 and DGAT2 in patient 3 fibroblast lysate, but normal expression of DGAT1 in healthy control fibroblasts. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. (*C*) Western blot showing lower expression of potentially nonfunctional DGAT1 protein and normal DGAT2 protein level from Epstein-Barr virus–derived B lymphoblastoid cell line of patient 4, a parent, and a healthy control. GAPDH was used as a loading control. (*D*) Western blot for DGAT1 protein expression in undifferentiated (EM) and differentiated (DM) organoids from 2 healthy controls and patients 7 and 8. HSP90 was used as loading control. Results are representative of 3 independent experiments.

biopsies from patient 10 showed lateral microvilli and cytoplasmic CD10 staining (Supplementary Figure 1*C*), which led to the misdiagnosis of atypical microvillus inclusion disease.[22]

Eight patients received various treatments that led to resolution or significant improvement of the GI symptoms. Most patients were placed on a fat-restricted diet, which alleviated their GI symptoms. Addition of medium-chain TG was tolerated, and some patients were infused with intravenous essential fatty acid supplements Intralipid and Omegaven and fat-soluble vitamins. Patient 4 received cholestyramine and patients 5 and 6 received pancreatic lipase due to low fecal elastase level, both of which reduced daily stool frequency. Patients 9 and 10 are twins and patient 9 received an intestinal transplant. The various treatments and outcomes are summarized in Supplementary Table 3.

Some patients developed extra-GI manifestations. In patients 1, 2, and 6, recurrent episodes of unspecific infections were recorded. Patients 9 and 10 had recurrent episodes of septicemia due to catheter-related blood stream infections. None of our patients showed any pattern of unusual or opportunistic infections that might be overtly associated with fecal loss of immunoglobulins or lymphopenia. Treatment of infections was successful with antibiotics, antifungal drugs, or prophylactic intravenous immunoglobulin, as outlined in Supplementary Table 3. Patient 5 had hepatomegaly and jaundice, and liver biopsy revealed fibrosis and cholestasis. Patients 7 and 8 were diagnosed with Gilles de la Tourette syndrome and were treated with dexamphetamine.

Identification of Novel DGAT1 *Mutations*

Whole-exome sequence analysis was performed in patients 1, 3, 7, 8, 9, and 10, while targeted panel sequencing was performed for patients 4 and 5. Variants in affected siblings (patients 2 and 6) were identified using conventional Sanger sequencing. Collectively, we identified 5 novel homozygous variants in the gene *DGAT1* (Mendelian Inheritance in Man: 604900, GenBank: NM_012079.5). These variants segregated with the disease under the assumption of autosomal recessive inheritance, with heterozygous carriers being unaffected (Figure 1*A*). The variants are neither present nor reported as rare heterozygous variants in the gnomAD database,[24] and were predicted to be deleterious using Combined Annotation Dependent Depletion[25] prediction tool (Supplementary Table 1).

In family 1, we identified a homozygous nonsense mutation at amino acid 401 leading to an early stop codon (c.1202G>A, p.W401X). In family 2, we identified a homozygous insertion deletion (c.573_574delAGinsCCCATCCC ACCCTGCCCATCT) in exon 6 of *DGAT1*. In family 3, we identified a homozygous splice site acceptor mutation (c. 937-1G>A) preceding exon 12. In family 4, we identified a homozygous single base-pair insertion, leading to a frameshift and early stop codon (c.953insG, p.I319Hfs*31) in exon 12. In families 5 and 6, we identified a homozygous 3 base-pair deletion (c.629_631delCCT, p.S210_Y211delinsY) in exon 7 (Figure 1, Supplementary Figure 3). Altogether, we identified 5 novel disease-causing homozygous mutations in *DGAT1* in 6 patients of Turkish origin and 4 of Dutch origin.

Consequences of DGAT1 *Mutations*

We proceeded to study the consequences of *DGAT1* mutation on available material. Immunohistochemistry on GI biopsies from patient 1 and patient 2 showed a lack of DGAT1 protein expression specifically in the epithelium of the duodenum, ileum, and colon (Figure 2*A* and Supplementary Figure 2), whereas DGAT1 protein was not detected in the gastric mucosa of control or patient material (Supplementary Figure 2). In patient 3, reverse-transcription polymerase chain reaction of cDNA from patient-derived fibroblast showed aberrant splicing (Supplementary Figure 4), which ultimately led to undetectable protein expression on Western blot (Figure 2*B*). In patient 4, Epstein-Barr virus–derived B-lymphoblastic cell line showed a highly reduced expression of DGAT1 (Figure 2*C*). Intestinal organoids derived from patients 7 and 8 showed normal mRNA levels on differentiation (data not shown), but protein was absent (Figure 2*D*). Similar data were obtained from patient 9 (data not shown). Together, we show that all the novel *DGAT1* mutations identified led to aberrant protein expression.

To confirm that the *DGAT1* c.629_631delCCT mutation specifically leads to reduced DGAT1 protein levels without affecting mRNA levels, Caco-2 cells were stably transfected with Flag-DGAT1 WT or Flag-DGAT1 c.629_631delCCT. Indeed, *DGAT1* mRNA expression was similar in both cell lines (Supplementary Figure 5*A*), but DGAT1 protein was not detectable when *DGAT1* was mutated (Supplementary Figure 5*B*). Incubation with the proteasome inhibitor MG132 shows that the loss of protein is at least partially due to proteasomal degradation of the mutant DGAT1 (Supplementary Figure 5*B*). To further investigate the increased proteasomal degradation of DGAT1 c.629_631delCCT, we determined the level of ubiquitination of the mutant protein. Therefore, Caco-2 cells were co-transfected with His-ubiquitin and Flag-DGAT1 WT or Flag-DGAT1 c.629_631delCCT and treated with MG132. A ubiquitin pulldown assay showed that ubiquitination of Flag-DGAT1 c.629_631delCCT was increased compared with ubiquitination of Flag-DGAT1 WT (Supplementary Figure 5*C*).

Loss of DGAT1 Leads to Aberrant Lipid Metabolism

Free fatty acids (FFAs) can be processed for energy production through beta oxidation, or stored in the form of lipid droplets on its incorporation into TG. In enterocytes, this lipid droplet formation is required for the transport of long-chain FFAs into chylomicrons, before being excreted across the basolateral membrane of enterocytes. Recently, it was shown that *DGAT1* mutant fibroblasts accumulated less lipid droplets when incubated with OA, an 18-carbon FFA.[5] We hypothesized that DGAT1 deficiency will lead to aberrant lipid droplet formation in patient-derived cells.

We performed a staining for LD540, which binds neutral lipids,[19,26] on intestinal organoids from patients 7, 8, and 9 after 16 hours incubation with BSA-coupled OA. Using fluorescence imaging, we observed an increase in lipid droplet formation in healthy control organoids, which was significantly reduced in DGAT1 mutant patient-derived organoids

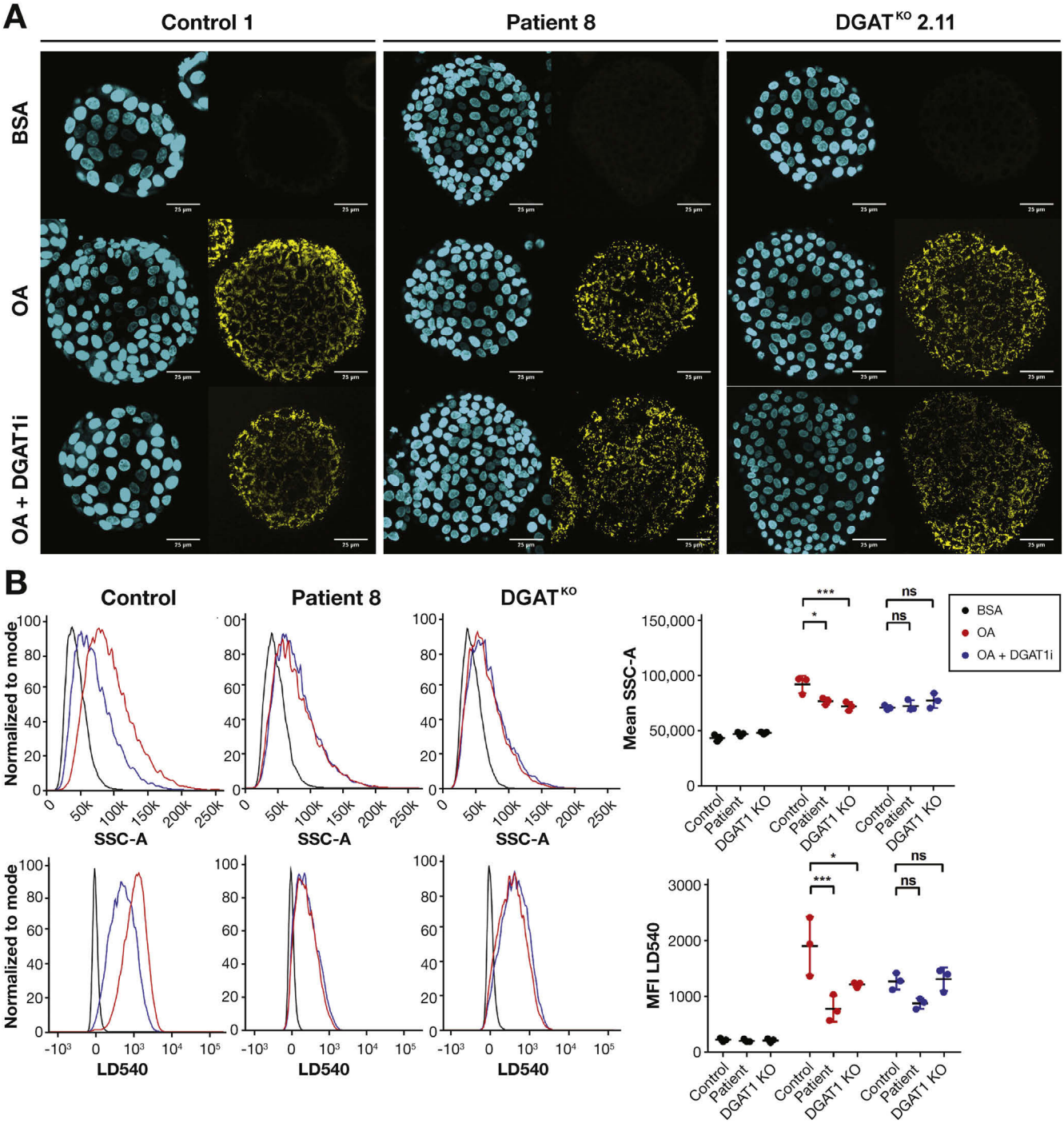

Figure 3. Loss of DGAT1 results in decreased lipid droplet formation in organoids. (*A*) Immunofluorescent images of 4′,6-diamidino-2-phenylindole (DAPI) (blue) and LD540 (yellow) staining of organoids from healthy control, *DGAT1* mutant patient 8 (P8) and *DGAT1*^{KO} organoids after 17-hour incubation with vehicle control (BSA), 1 μM OA, or 1 μM OA + 0.1 μM DGAT1 inhibitor (OA+DGAT1i). Representative images of 3 healthy controls, 3 patients (patients 7–9), and 3 CRISPR/Cas9 genome-edited *DGAT1*-knock-out (*DGAT1*^{KO}) organoids. (*B*) Representative histograms of SSC and LD540 staining in organoids from controls, patients, and *DGAT1*^{KO} organoids as described in (*A*). Upon OA stimulation, control organoids accumulate lipid droplets and show increased SSC and LD540, which was severely reduced in patient-derived and *DGAT1*^{KO} cells. Mean fluorescence intensity (MFI) of SSC and LD540 was plotted for n = 3 per group. Statistical analysis was done using a 2-way analysis of variance with Tukey's multiple comparison test. Mean ± SD is indicated; * $P \leq .05$, *** $P \leq .001$.

from patients 7, 8, and 9 and *DGAT1* knockout (*DGAT1*^{KO}) organoids that were generated using CRISPR/Cas9 genome editing for *DGAT1* in healthy control organoids (Figure 3*A* and Supplementary Figure 6 and Supplementary Table 4). Formation of lipid droplets was at least partially dependent on DGAT1, as the use of a selective DGAT1 inhibitor (DGAT1i) AZD 3988 on healthy control organoids resulted in a reduction of lipid droplet accumulation as shown by decreased LD540 staining (Figure 3*A*). This was further confirmed and quantified using a flow cytometry–based assay, in which healthy control organoids showed an increased granularity due to the accumulation of lipid droplets (side scatter area, SSC-A) and increased LD540 staining on incubation with OA. Absence of DGAT1 (patients or KO) or DGAT1 inhibition caused a significant reduction in granularity and LD540 staining compared with healthy controls (Figure 3*B*).

In addition, we incubated normal donor and patient 3–derived fibroblasts with OA and quantified lipid droplet formation by flow cytometry. Normal donor fibroblasts accumulated lipid droplets on incubation with OA, which was shown by increased granularity (SSC-A) and Nile Red staining, a dye that binds to neutral lipids such as TG.[27] In contrast, patient-derived dermal fibroblast from patient 3 failed to accumulate lipid droplets, as they showed a lack of granularity (Figure 4*A*) and Nile Red staining (Figure 4*B*). In addition, we show that this lack of lipid droplet formation

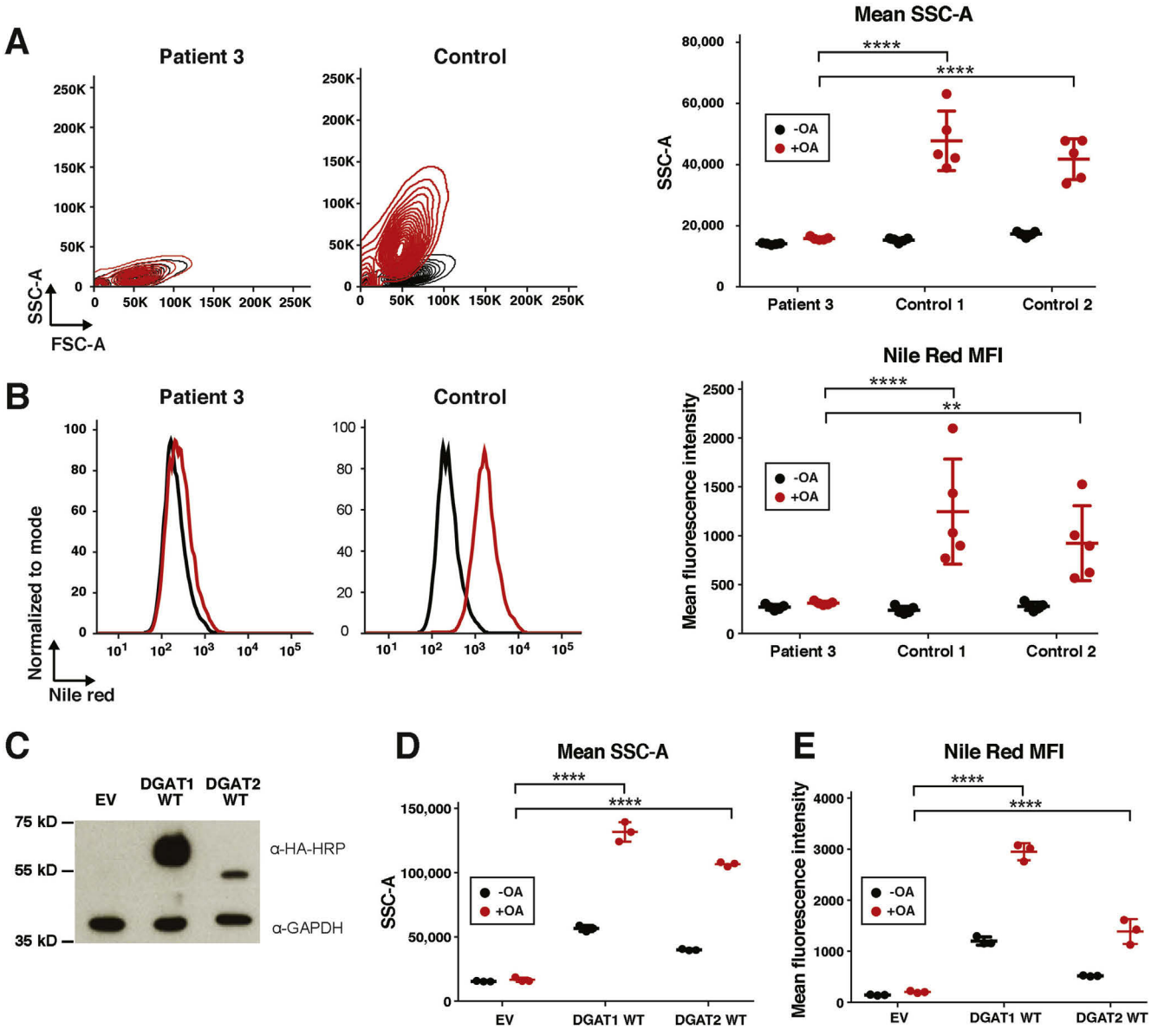

Figure 4. Loss of DGAT1 results in decreased lipid droplet formation in fibroblasts. (*A*) *Left*: Representative contour plots for forward (FSC-A) and SSC-A of patient 3 and 2 control fibroblasts with and without OA addition. *Right*: Mean SSC-A of 5 technical replicates. (*B*) *Left*: Representative histogram of Nile Red mean fluorescence intensity (MFI) of patients 3 and 2 control fibroblasts with and without OA addition. *Right*: MFI of 5 technical replicates. (*C*) Western blot showing retroviral-mediated delivery of exogenous DGAT1 and DGAT2 on patient 3 fibroblasts. (*D*) Mean SSC-A and (*E*) MFI of Nile Red staining on patient 3 fibroblasts reconstituted with empty vector (EV), WT DGAT1, or WT DGAT2. Statistical analysis was done using 2-way analysis of variance with Tukey's multiple comparison test. Mean ± SD is indicated; $^{**}P \leq .01$ or $^{****}P < .0001$.

was not detected in B-LCL derived from patient 4 (Supplementary Figure 7), presumably due to the expression of DGAT2 in this cell type (Figure 2).

WT DGAT1 *and* DGAT2 *Rescues Lipid Droplet Formation in DGAT1 Deficiency*

We reconstituted fibroblasts from patient 3 with WT DGAT1 and DGAT2 protein using a retroviral delivery system. We successfully reconstituted DGAT1 protein expression, and overexpressed DGAT2 in these cells (Figure 4*C*). The DGAT1-reconstituted fibroblasts were able to incorporate OA into lipid droplets as seen by the increased granularity and increased Nile Red fluorescence (Figure 4*D* and *E*). The phenotype was also rescued by overexpression of DGAT2, resulting in restored granularity and Nile Red staining on addition of OA (Figure 4*D* and *E*). We concluded that reconstitution of DGAT1 rescues the altered lipid metabolism phenotype, and that DGAT2 can partially rescue altered lipid droplet formation in *DGAT1*-deficient fibroblasts.

Loss of DGAT1 Specifically Inhibits TG Formation

To substantiate the specificity of DGAT1 deficiency for causing aberrant TG formation, we performed thin layer chromatography to measure the levels of TG and DG in organoids derived from 3 healthy controls and patients 7, 8, and 9 (Figure 5*A*). By quantification of respective DG and TG band intensities, we determined that the TG/DG ratio in healthy control organoids was significantly higher compared with patient-derived organoids (Figure 5*B*). These results indicate a DGAT1-dependent loss of TG synthesis in patient-derived intestinal organoids, whereas levels of DG were comparable.

Loss of DGAT1 Results in Increased Sensitivity to Lipid-induced Toxicity

Most DGAT1-deficient patients reported in this study suffered from PLE after ingestion of dietary lipids, which can be the result of either intestinal mucosal injury or lymphatic abnormalities.[28] To assess whether exposure to lipids leads to mucosal injury in DGAT1-deficient patients, we performed lipotoxicity assays in healthy control and DGAT1-deficient organoids. We incubated the organoids with varying concentrations of BSA-coupled OA in EM and assessed cell death by brightfield microscopy and propidium iodide staining (Figure 6*A*). Remarkably, we observed 100% cell death at 4 mM OA in patient-derived organoids, whereas cell death was still almost absent at 6 mM OA in control organoids. To further quantify these findings, we determined lipid-induced caspase-mediated cell death using a Caspase-Glo 3/7 assay. We show that DGAT1-deficient cells are more sensitive to lipotoxic stress compared with healthy control organoids and undergo programmed cell death on treatment with OA (Figure 6*B*). By nonlinear regression analysis, the median lethal dose was determined to be approximately 7 mM OA for healthy control organoids and 4 mM OA for patient-derived organoids ($P < 0.001$). Overall, these results indicate that DGAT1-deficient organoids are more susceptible to lipid-induced toxicity, which may reflect the clinical feature of PLE in DGAT1-deficient patients that occurs on ingestion of fat.

Discussion

Lipid metabolism is an important physiological function within the human body that includes the digestion and absorption of lipid products from food. Inborn errors of lipid

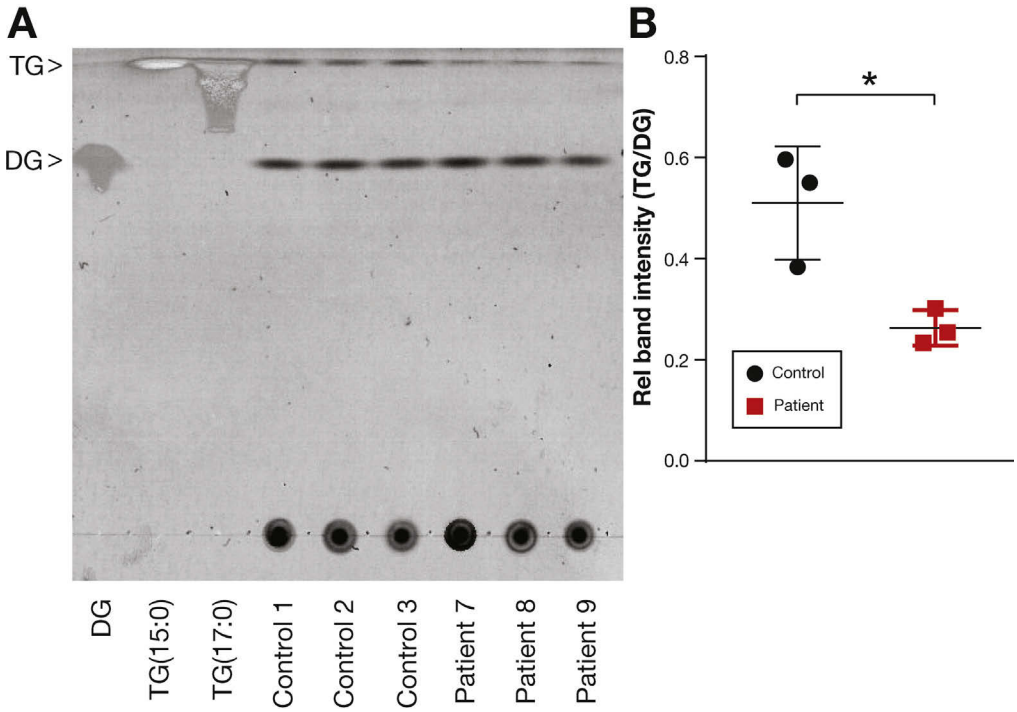

Figure 5. Loss of DGAT1 results in decreased triglyceride (TG) formation. (*A*) Organoids from 3 healthy controls and patients 7, 8, and 9 were grown in EM for 7 days. Lipid extracts were isolated and run by thin layer chromatography to determine levels of diacylglycerol (DG) and triglyceride (TG). Relative positions were indicated by reference samples for DAG and TG. (*B*) Ratio of intensity of TG over DAG for each sample. Statistical analysis was done using a Mann-Whitney U test. Mean ± SD is indicated, $^*P < .05$.

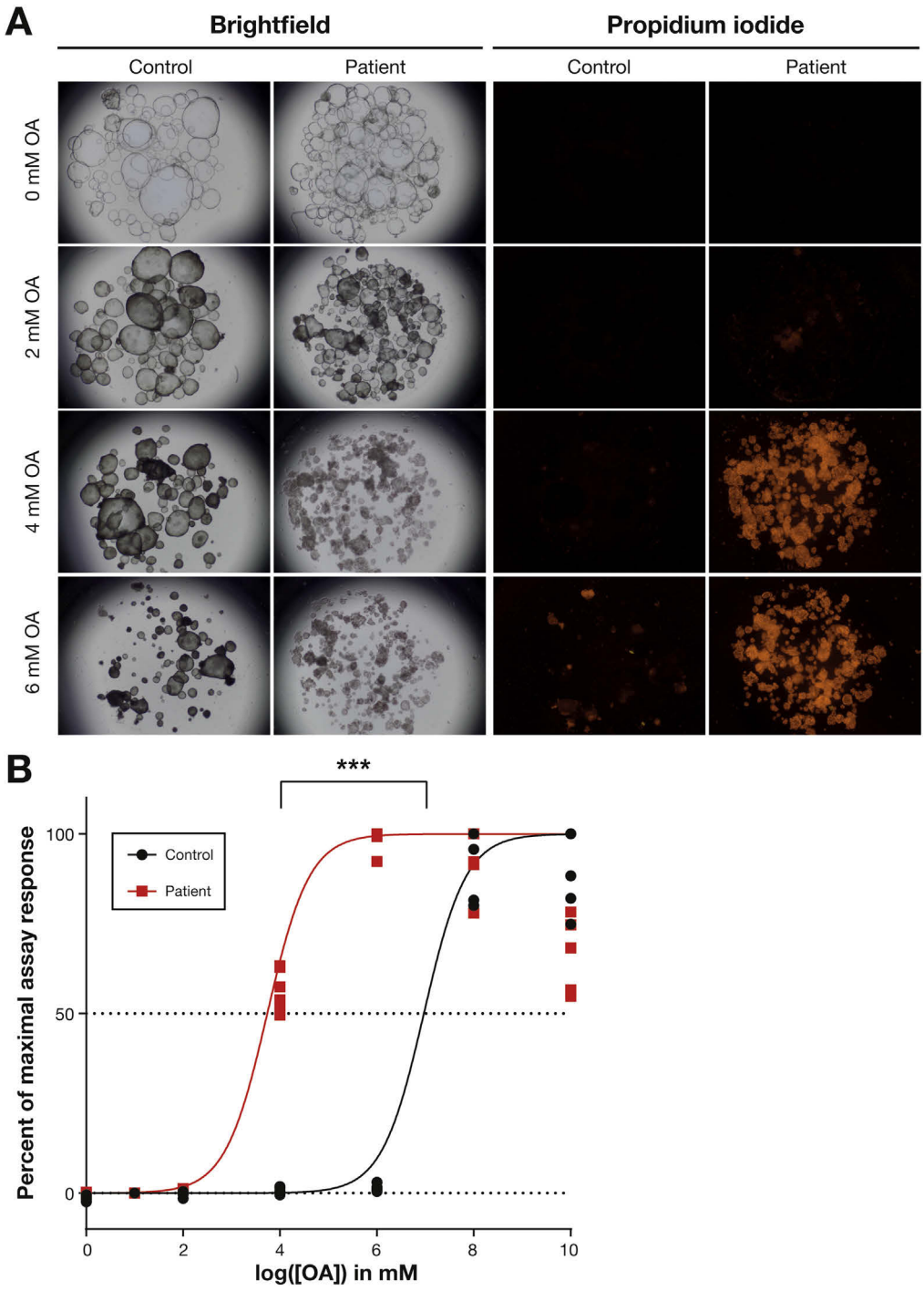

Figure 6. Loss of DGAT1 results in increased sensitivity to lipotoxic stress. Organoids of 3 healthy controls and 3 patients (7–9) were grown in EM and incubated overnight with a range of oleic acid (OA) concentrations. (*A*) Representative images showing brightfield and propidium iodide staining for cell death of the organoids after incubation with OA. (*B*) Caspase-Glo 3/7 assay for apoptotic cells after incubating organoids with OA. Samples were normalized for vehicle control values and the maximum value for each sample was set to 100% assay response. Median lethal dose (LD_{50}) was calculated by regression analysis. Statistical analysis on LD_{50} values was done using a Student's *t* test, $^{***}P \leq .001$.

metabolism can result in a wide range of symptoms, from neurological impairment to hypertriglyceridemia. Recently, lipid metabolism disorders have been linked to CDD, such as in the case of Niemann-Pick disease type C with inflammatory bowel disease due to impaired autophagy.[29] In the case of cytoplasmic TG metabolism disorders, none of the other deficiencies have been reported to develop any GI phenotype.[30]

Previous studies on DGAT1 deficiency have identified a total of 3 distinct homozygous and 2 compound heterozygous mutations in *DGAT1*.[3–6] These patients suffered from severe congenital diarrhea and PLE, clinical features that are shared with most of our patient cohort. Although this shared phenotype further confirms the involvement of DGAT1 in intestinal failure, limited functional data or potential therapeutic options have been reported thus far.

Currently, there is no genotype-phenotype correlation in DGAT1 deficiency, as patients develop varied clinical history ranging from complete resolution of GI symptoms to a lethal course of disease. As previously described and also observed in our study, discordant phenotypes associated with identical mutations in siblings and in different,

unrelated families are of interest. Whether this phenomenon, as well as the novel clinical features, such as normal serum TG and hepatic involvement, observed in our patients indeed extend the spectrum of the clinical phenotype of DGAT1 deficiency, or are unspecific secondary effects of treatment, cannot be inferred from our study. By analogy, the unsatisfactory clinical outcome described for patient 9 after the small bowel transplantation may be attributed to known posttransplant complications rather than unsuccessful correction of the DGAT1 deficiency in the intestine.

To provide further insight into the pathomechanism of DGAT1 deficiency, we have produced cell models that recapitulate an altered lipid metabolism in vitro. Here, we show that patient-derived organoids can recapitulate the molecular pathomechanism of DGAT1 deficiency. We have demonstrated aberrant lipid metabolism as evidenced by reduced lipid droplet and TG formation on incubation with OA in both patient-derived organoids and fibroblasts. In addition, we show for the first time that DGAT1-deficient cells are more susceptible to lipid-induced toxicity, which provides a plausible explanation for the clinical PLE symptoms in DGAT1 deficiency and possibly other forms of PLE, such as primary intestinal lymphangiectasia.[31] The fact that dietary fat restriction can even restore normal fecal protein clearance in PLE further supports the concept that cellular lipotoxicity may be one of the driving forces for ongoing fecal protein loss in untreated PLE. Whether this lipotoxicity-induced enterocyte dysfunction is caused by endoplasmic reticulum stress or induced autophagy, which has been implicated in lipid metabolism disorders such as Niemann-Pick disease type C with inflammatory bowel disease,[29] remains to be determined. Moreover, elucidation of the precise role of DGAT1 in common lipotoxicity-related diseases, such as type 2 diabetes, nonalcoholic fatty liver disease, and metabolic cardiomyopathy, may be the first step toward new therapy strategies for obesity-related disorders.

Of note, the novel technology of patient-derived intestinal organoids provided an unprecedented look into pathobiology of the cells in the GI tract. In the future, a more systematic use and collection of organoids from potential DGAT1 deficiency and other patients with intestinal failure will allow for better dissection of the disease mechanism involving the gut epithelium.

As described in this study and in previous literature, DGAT1-deficient patients usually do well on a fat-restricted diet. An early introduction of such a diet might even have prevented the development of a full-blown PLE in patients 7 and 8. In addition, the administration of short chain fatty acid proves to be a good supplement to the diet for some patients. Intravenous administration of essential fatty acid was also well-tolerated, presumably as it bypasses absorption through the gut epithelium as well. Whether patients benefit from novel ways of treatment with available drugs, such as cholestyramine and pancreas enzymes, as empirically applied for patient 4 and patients 5 and 6, respectively, would have to be attempted and evaluated in additional patients and in carefully designed trials. Before treatment, neither diagnosis of bile acid diarrhea nor exocrine pancreas insufficiency was formally tested or firmly established, because the observation of slightly decreased fecal elastase levels may be unspecific due to fecal dilution in severe chronic diarrhea. Although the course of disease evidently can be mild, early diagnosis of DGAT1 remains vital, as all patients were severely ill during the first few months of life and thus far one patient suffered from a lethal course of disease. Additionally, the impact on the quality of life during chronic treatment (monthly infusions or through a central line that includes risks of infections) and the variable genotype-phenotype relationships of DGAT1 patients should not deter the search for new therapeutic options for these patients.

In pursuit of possible therapeutic strategies, we determined that proteasome inhibitors could provide a potential therapeutic option in case of proteasome-mediated protein degradation, as shown for the mutation in families 5 and 6 (Supplementary Figure 4). In addition, we determined that DGAT2 might be able to compensate for the lack of DGAT1 function. DGAT2 is an isozyme of DGAT1 that does not share any homology in protein sequence or domains.[11] However, DGAT2 shares functional characteristics with DGAT1, catalyzing the formation of TG from DG and fatty acyl-CoA in the liver. In this study, we show that DGAT2 might be able to compensate for this phenotype, as shown by OA addition on DGAT2-expressing B-LCL and exogenous expression of DGAT2 in DGAT1-deficient fibroblasts. A single heterozygous, autosomal dominant *DGAT2* mutation has been described in a family with Charcot-Marie-Tooth syndrome,[32] but no GI involvement was reported, possibly because DGAT2 is not highly expressed in the human intestine.[3] Therapeutic strategies to induce DGAT2 expression might potentially provide an additional, viable treatment strategy in DGAT1 deficiency.

Previous studies performed with *Dgat1*$^{-/-}$ mice implicated a beneficial role of DGAT1 inhibition in obesity through changing metabolic landscapes.[33–35] These studies resulted in the development of DGAT1 inhibitors for use in human obesity.[36] However, participants in a clinical trial of DGAT1 inhibitor Pradigastat developed side effects of diarrhea and nausea,[37,38] similar to the phenotype of DGAT1 deficiency. D*gat1*$^{-/-}$ mice did not develop a GI phenotype, presumably due to the intestinal expression of DGAT2 and diacylglycerol transacylase, which might compensate for the lack of DGAT1.[39] Our data suggest that in humans, lack of TG formation and packaging is detrimental in the context of GI epithelium, and that a serious note of caution on the clinical use of DGAT1 inhibitors should be provided. This finding accentuates the lack of available knowledge of intestinal lipid uptake and metabolism, and emphasized how the use of clinically relevant in vitro systems can help to fill this gap.

In summary, we here described a large cohort of DGAT1-deficient patients, and extended our knowledge of the pathomechanism of DGAT1 deficiency. Our findings expand the differential diagnosis of vomiting and congenital diarrhea in neonates. Clinicians should maintain a high suspicion for DGAT1 deficiency in cases of unexplained vomiting, especially when associated with failure to thrive and PLE,

and could consider a fat-free diet with supplementation of essential fatty acids and fat-soluble vitamins as a first line of therapy. In conclusion, the findings described in this article show that *DGAT1* mutations not only cause congenital diarrhea and PLE, but are also linked to fat intolerance.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at https://doi.org/10.1053/j.gastro.2018.03.040.

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Conflicts of interest
The authors disclose no conflicts.

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Supplementary Clinical Description of the 10 Patients

Patient 1 presented with watery, nonbloody, nonmucoid diarrhea that commenced immediately after birth. He was severely dehydrated; breast milk was discontinued and changed to lactose-free formula, but diarrhea persisted. A diagnosis of microvillus inclusion disease (MVID) was considered after endoscopic biopsy. At 25 days of age, patient 1 was admitted to another hospital, where he required recurrent albumin infusions (AIs) to correct the hypoalbuminemia. Immunological tests were normal except for slight decrease in immunoglobulin (Ig)G and IgM. Fat-soluble vitamins A, D, and E levels were low. Stool steatocrit was positive. Infection, food allergy, cystic fibrosis, and prohormone convertase deficiency had been excluded by appropriate laboratory tests. A second endoscopy and colonoscopy were performed, and the previous endoscopic biopsy was reanalyzed. Congenital enteropathies (Tufting enteropathy, MVID, inflammatory bowel disease, autoimmune enteropathy, and food-protein–induced enteropathy) were excluded. Treatment with amino acid–based formula was not effective, but a slight clinical improvement was observed with basic casein hydrolysate and galactomin 19 formula. He was discharged at 4.5 months of age with some weight gain. Diarrhea and vomiting persisted after his discharge at home, and 2 weeks later, he was referred to the current attending physician for dehydration, weight loss (470 g over 15 days), severe hypoalbuminemia (1.8 g/dL), hyponatremia (130 mEq/L), and hypokalemia (3 mEq/L). On admission, he was malnourished (weight 3680 g [<3%], height 58.5 cm [3%]) and severely dehydrated. Fluid replacement therapy, AI, and total parenteral nutrition (TPN) were started. Laboratory tests showed low serum IgG 1.53 g/L (3.04–12.31), total cholesterol 63 mg/dL, high-density lipoprotein (HDL) 25 mg/dL, low-density lipoprotein (LDL) 12 mg/dL, very low-density lipoprotein (VLDL) 27 mg/dL, triglyceride 135 mg/dL, and vitamin B12 101 ng/mL and steatorrhea was observed. Workup included extensive infectious, immunologic, and hormonal studies, all of which were negative or normal. Upper gastrointestinal endoscopy and ileocolonoscopy, biopsies, and electron microscopy were normal. During his hospitalization, bulky, watery, and greasy diarrhea was observed, and Sudan stain of stool continuously revealed massive droplets of fat. Basic-F (fat-free formula [FFF]) was started for fat malabsorption, and within 3 weeks, a dramatic improvement and subsequent complete resolution of his symptoms was observed and further TPN was not needed. Extensively hydrolyzed formula rich in medium-chain triglyceride (MCT) was gradually added to the FFF. He was discharged at 7 months of age with a combination of these formulas. During the follow-up, solid food with daily MCT oil supplementation was commenced gradually. The child exhibited elevated fasting triglyceride level (207 mg/dL) at 8 months of age, which was decreased with adding omega-3 fatty acids. Due to recurrent episodes of vomiting, cough, and otitis media during follow-up, long-term prophylactic antibiotic with sulfamethoxazole-trimethoprim was added at 12 months of age. He is currently 4 years and 4 months old, with normal growth and development with the combination of low-fat–age-appropriate diet, MCT supplementation, and FFF.

Patient 2, a sibling of patient 1, also presented with nonbilious vomiting and watery, nonbloody, nonmucoid diarrhea 2 to 3 times a day and required repeated hospitalizations for dehydration due to diarrhea. At 5 months, she was referred to a tertiary hospital due to failure to thrive, peripheral edema, and severe hypoalbuminemia (1.8 g/dL, reference 3.2–5.0 g/dL). The stool was negative for leukocytes. Standard laboratory tests, including urinalysis; acute-phase reactants; serum IgA, IgM, and IgE; serum and urine amino acids; artery blood gases; tandem mass spectroscopy; and thyroid function tests were normal. Serum IgG level was slightly reduced (204 mg/dL, reference 304–1230 mg/dL). Serum lipid profile was normal. Triglyceride level was 84 mg/dL (reference <150 mg/dL), total cholesterol 62 mg/dL (reference <170 mg/dL), LDL 28 mg/dL (reference <110 mg/dL), HDL 18 mg/dL (reference 40–60 mg/dL), VLDL 16 mg/dL (reference <30 mg/dL). Upper gastrointestinal endoscopy and biopsy were normal. She required recurrent AIs to correct the hypoalbuminemia and died at 6 months of age due to sepsis.

Patient 3, a female infant with lack of dysmorphic features, was born via vaginal delivery at 39 weeks of gestation following an uneventful prenatal history, weighing 3500 g, measuring 48 cm body length and 35 cm head circumference at birth. The healthy Turkish parents were not aware of a consanguineous relationship but share geographical origin. This small village was founded by the children of 3 siblings, collectively indicating parental consanguinity. A sister of the patient's father died at the age of 11 months from vomiting and diarrhea. Apart from that, family history was inconspicuous. Because of watery diarrhea (7 times a day, 150 g/kg per day) starting at the age of 3 weeks, weight loss, and severe dystrophy, she was admitted to a pediatric clinic weighing 3400 g. Parenteral nutrition was immediately started and the patient was also continuously fed through a nasogastric catheter. Stool analyses showed normal values for α1 antitrypsin (13 mg/dL, reference 90–200 mg/dL), fecal elastase (>500 μg/dL, reference >200 μg/g), stool sodium (66 mmol/L, reference 20–25 mEq/L) and potassium (31.7 mmol/L, reference 50–75 mEq/L), excluding congenital sodium and chloride diarrhea. Serum bile acids were mildly increased (38,9 μmol/L, reference <10 μmol/L), amylase 7 U/L (reference <30 U/L) and lipase 13 U/L (reference 13–60 U/L) were normal. Unfortunately, no information on fecal bile acid levels is available. The patient exhibited hypoproteinemia (3.5–4.7 g/dL, reference 5.7–8.7 g/dL) and hypoalbuminemia (2.2–2.7 g/dL, reference 3.2–4.7 g/dL). Metabolic screening was without pathological findings. Stool cultures as well as polymerase chain reaction (PCR) for adenovirus, rotavirus, and enterovirus in the stool were negative. Serum immunoglobulin levels were normal. Food mix allergen-specific IgE in serum was negative. A gastroduodenoscopy performed at the age of 3 months showed duodenal microvillus atrophy. Light microscopy analysis of the biopsy of the

duodenum excluded enteric anendocrinosis. Electron microscopy revealed a deteriorated integrity of the microvilli, and no microvillus inclusions (Supplementary Figure 1). The patient continuously failed to thrive, and at the age of 11 months, kidney stones of several millimeters in diameter were found, along with lower-extremity edema, corneal cystine crystal accumulation, and metabolic acidosis. Renal function tests and urine analyses were repeatedly normal, and leukocyte cysteine levels were normal with 0.047 nmol/mg protein (0–0.3 nmol/mg). The patient received 1 g/kg albumin every other day due to protein-losing enteropathy and decreasing albumin values. She was fed via a nasogastric tube on elemental and semi-elemental formula. Additionally, parenteral nutrition (protein 2 g/kg per day and lipid 1 g/kg per day) was administered. A second endoscopy of the upper gastrointestinal tract was performed due to persisting diarrhea. Because milky deposits (lipid accumulation) were seen in the duodenum, chylomicron retention disease and abetalipoproteinemia were considered. The patient's triglyceride levels were increased (up to 370 mg/dL [0–150 mg/dL]), whereas total cholesterol was in the normal range (96 mg/dL [0–200 mg/dL]) and HDL and LDL were decreased (HDL 9.5 up to 22 mg/dL [40–60 mg/dL], LDL 23 mg/dL [60–130 mg/dL]). The patient's parents' lipid profiles were normal.

At this point, whole-exome sequencing established the diagnosis of a congenital protein-losing enteropathy due to DGAT1 mutation. Subsequently, oral MCT administration was started. Parenteral nutrition support continued. Subsequent to observing hypocalcemia, secondary hyperparathyroidism was diagnosed (parathyroid hormone: 312 pg/mL, reference 15–65 pg/mL, 25-hydroxyvitamin D 18 ng/mL, reference 25–80 ng/mL, calcium 6.5 mg/dL, reference 8.7–10.4 mg/dL), and vitamin D and calcium supplementation was initiated. The patient is now 13 months old. The stool amount declined from 150 g/kg per day during the first application to 100 g/kg per day in the past 3 months. The stool frequency decreased from 7 times a day to 3 to 4 times a day. She weighs 3850 g (<3 percentile), is 57 cm (<3 percentile) long, and has a head circumference of 38 cm (<3 percentile). She vomits 3 to 4 times every day, has extreme flatulence and abdominal distention. Parenteral nutrition support continues. For this purpose, the patient is hospitalized for 3 to 4 weeks and then she has a maximum break of 1 week without parenteral nutrition. Her psychomotor development is compatible with 3 to 4 months.

Targeted gene sequencing had excluded infantile nephropathic cystinosis, and whole-exome sequencing excluded microvillus inclusion disease and variants in other known genes causing isolated and syndromic forms of congenital diarrheas.

Patient 4 was referred to the attending physician due to intractable diarrhea, which started when he was 2 months old. Intestinal and colonic endoscopic and histopathological investigations were normal. Infectious, metabolic disorders, malignancies, cystic fibrosis, and congenital glycosylation defect were excluded. Longitudinal measurement of lipid profile showed normal levels of total cholesterol, LDL, HDL, VLDL, and triglyceride. Serum immunoglobulin levels were normal. He had marked clinical improvement on treatment with cholestyramine, which resulted in the reduction of stool volume and frequency. At the latest follow-up, the patient weight is at 9.5% and height is at 4.5% of Turkish boys. He does not have any vomiting or diarrhea and is still on cholestyramine treatment.

Patient 5 was born at term to consanguineous parents. He was hospitalized at 40 days of age because of vomiting, bloody diarrhea, and failure to thrive. He was started on amino acid–based formula due to a suspected cow milk allergy. At the age of 5 months, abdominal and cranial ultrasonography were normal and upper gastrointestinal endoscopy and biopsy revealed nonspecific results. He had low IgA, IgM, and IgG levels. At a follow-up, the patient was found to have low serum albumin (2.2g/dL, normal 3.2 g/dL) and total protein. At 1 year of age, he was referred to the current attending physician because of hepatomegaly and jaundice for a liver biopsy. He is below the third percentile in height and weight. Physical examination revealed abdominal distention, cutis marmoratus, 8 cm length of liver below the right costal margin. Laboratory assessment of liver function and immunoglobulin levels were as follows: aspartate aminotransferase 240 U/L, alanine aminotransferase 111 U/L, γ-glutamyltransferase 335 U/L, total/direct bilirubin levels 2.95/2.05 mg/dL, serum IgA 1.59 g/L, IgM 1,14 g/L, IgG 9.14 g/L. He has watery stools 4 to 5 times per day. He also has milk protein intolerance (bloody diarrhea occurs after formula feeding). Liver biopsy revealed hepatocanalicular and ductular cholestasis, paucity of bile ducts, mixed type hepatosteatosis (10%), porto-portal fibrosis, and fibrotic activity of 3/6. His daily stool frequency had reduced with the administrations of pancreatic enzymes (Creon), but his weight and height are below the third percentile.

Patient 6, a sibling of patient 5, was admitted to hospital when he was 2.5 months of age because of watery diarrhea (at least 10 times a day). He presented hyponatremia, hypochloremia, hypoalbuminemia, hypocalcemia. Sweat chloride test was normal. His daily stool frequency had reduced with the administrations of pancreatic enzymes (Creon), but he had recurrent infections and was hospitalized. He was also placed on amino acid–based formula. Endoscopy and colonoscopy was normal at the age of 2 years. Duodenum biopsy revealed focal vacuolization at one area and partially blunted villi. Colon biopsy was normal. Liver profile: aspartate aminotransferase 149 U/L, alanine aminotransferase 56 U/L γ-glutamyltransferase 70 U/L. Total serum protein was normal (4.38 g/dL), but low albumin level (2.59 g/dL) was observed. He has low IgA serum level but normal IgG and IgM levels. Free thyroxine–thyroid stimulating hormone levels were normal. His lipase and amylase levels were within normal range (13/46 U/L respectively). Lipid profile revealed low HDL 21 mg/dL (40–60 mg/dL), LDL 14 mg/dL (<130 mg/dL), VLDL 36 mg/dL (<40 mg/dL), triglyceride 184 mg/dL (<200 mg/dL), and normal total cholesterol 59 mg/dL (<200). Vitamin E level was deficient, but vitamin A and D levels were normal. He had bilateral nephrocalcinosis on abdominal ultrasonography. Urine organic acid analysis and serum

amino acid chromatography were normal. After 2 years of age, his symptoms improved spontaneously, and he was lost to follow-up afterward. His weight is now 16 kg (<third percentile), height 104 cm (<third percentile).

Family 5 is a consanguineous Dutch family, with the parents sharing ancestors 7 generations ago, and consists of 3 brothers of which 2 were affected (patients 7 and 8). The oldest boy (patient 8) presented in the first month of life with vomiting and failure to thrive. Physical examination showed a dystrophic boy without further abnormalities, and a normal serum albumin (4.35 g/dL). A scintigraphy was performed, which showed delayed gastric emptying. Extensive additional research did not reveal a diagnosis. Initially the patient was treated with TPN, which reversed the symptoms. By experiment, a fat-restricted diet was introduced at the age of 7 months, which enabled enteral feeding again. Incidental ingestion of low amounts of fat causes abdominal pain and vomiting within 1 hour after ingestion. The boy currently receives bimonthly infusions of Intralipid and Omegaven and supplementation of fat-soluble vitamins, leading to normal plasma levels of fat-soluble vitamins and fatty acids, except for linoleic acid, which is just below normal. He was diagnosed with Gilles de la Tourette syndrome at the age of 7 years, which was treated with dexamphetamine. The younger brother (patient 7) presented with vomiting in the first days of life, which was reversed after immediate introduction of a fat-restricted diet. Like his brother, he is supplemented with fat-soluble vitamins and monthly infusions of Intralipid and Omegaven, leading to normal plasma levels of fat-soluble vitamins and fatty acids, except for marginally decreased levels of linoleic acid. He was also diagnosed with Gilles de la Tourette syndrome.

Family 6 is a Dutch family of unknown consanguinity, which consists of a monozygotic female twin pair and a male sibling of which the twin pair was affected, patients 9 and 10. They were born after a pregnancy of 34 6/7 weeks. Patient 9 had a birth weight of 2540 g and a bilateral clubfoot. During the first week of life, she developed severe watery diarrhea on bottle feeding. This subsided after a short period with TPN after which she tolerated full enteral feeding on Neocate for some months. However, the diarrhea returned and was accompanied by severe vomiting, also after small amounts of oral feeding. Therefore, enteral feeding was stopped and she was totally dependent on TPN. Protein-losing enteropathy (PLE) was present with a high fecal alpha-1-antitrypsin (9.1 mg/g feces, reference 0.0–1.1 mg/g feces) and a low serum albumin (23.8 g/L, reference 36–42 g/L). Microscopy of the small intestinal mucosa showed minimal villus atrophy, with generally a normal brush border on periodic acid-Schiff staining, but with unspecific CD10-positive globules in the cytoplasm of the enterocyte. As numerous microvilli were seen on the lateral membranes of the enterocyte, a misdiagnosis of variant microvillus inclusion disease was made. Given this diagnosis, and the dependence on TPN, she received a small bowel transplantation at the age of 3.5 years. After the small bowel transplantation, TPN was stopped and polymeric enteral nutrition was tolerated. Her clinical course after small bowel transplantation was complicated by post transplantation lymphoproliferative disease, autoimmune problems, and recurrent (pulmonary) infections. Currently, she is malnourished and still stunted despite a high caloric diet with a polymeric formula for which we started additional parenteral nutrition. After small bowel transplantation, it is sometimes difficult to unequivocally define the origin of failure to thrive, which could be related to at least partially ongoing disease, side effects of immunosuppression, or difficulties in reestablishment of sufficient enteral nutrition necessary to promote catch-up growth. Clinically, we do not have hints for persisting PLE following partial correction of intestinal DGAT1 deficiency due to isolated small bowel transplantation, and intestinal biopsies did not show signs of chronic rejection.

The identical twin sister, patient 10, had a birth weight of 1745 g without any congenital abnormalities. She had the same clinical course as her sister. She also developed severe watery diarrhea on bottle feeding in the first week of life, which subsided on TPN. Subsequently she was fed with Neocate for some months. As her twin sister, she presented with watery diarrhea and vomiting at the age of 5 months, for which enteral feeding was stopped and she was totally dependent of TPN. Results of laboratory investigations were very similar to her sister, with a PLE and an intestinal histology that suggested the diagnosis of atypical MVID. She was also put on the waiting list for small bowel transplantation. However, during the waiting list period (and after the transplantation of her sister) enteral feeding was successfully reintroduced with carbohydrates (first) and proteins (secondly), whereas the introduction of fats was complicated by motility problems (vomiting). At first, she did not tolerate MCT ingestion at all (vomiting directly after ingestion), but MCT was tolerated within months. Long-chain triglycerides were tolerated only in small amounts and oral supplementation of docosahexaenoic acid and amino acids were tolerated. After 4 months, she was not dependent on TPN anymore and she was taken off the waiting list for small bowel transplantation. Her diet consisted of a mixture of Hydrolyzed Whey Protein/Maltodextrin mix, Fantomalt, Liquigen, Calogen, Phlexy-vits, and Key Omega. We tried to switch to a standard oligomeric feeding formula after 2 years, but she did not tolerate this. Although she tolerated enteral feeding, she was still stunted and PLE was still present. For this reason, we recently started a fat-free diet with enteral supplementation of fat-soluble vitamins and monthly intravenous infusions of SMOF lipids.

Supplementary Materials and Methods

Cell Culture

Crypts were isolated from biopsies as described previously[1] and resuspended in medium without growth factors (GF), consisting of Advanced Dulbecco's modified Eagle's medium/F12 (Gibco, Waltham, MA), 100 U mL−1 penicillin-streptomycin (Gibco), 10 mM HEPES (Gibco), and Glutamax (Gibco). Matrigel (Corning, Tewksbury, MA) was added to a final concentration of 70% and plated on prewarmed cell

culture 24-well plates (Corning). After matrigel polymerization, organoid culture medium (human small intestine expansion medium [hSI-EM]) was added consisting of GF-medium, 50% Wnt-conditioned medium, 20% Rspondin-1-conditioned medium, 10% Noggin-conditioned medium, 50 ng/mL murine epidermal growth factor (PeproTech, Rocky Hill, NJ), 10 mM nicotinamide (Sigma), 1.25 mM N-acetyl (Sigma), B27 (Gibco), 500 nM TGF-α inhibitor A83–01 (Tocris, Bristol, UK), 10 μM P38 inhibitor SB202190 (Sigma), and 100 μg/mL Primocin (InvivoGen, San Diego, CA). During experiments, Primocin was left out. Organoids were cultured in 37°C and 5% CO_2 and medium was refreshed every 2 to 3 days. The organoids were passaged 1:3 to 1:6 every week after mechanical disruption or 1:10 to 1:20 as single cells (Trypsin/EDTA or TrypLE Express). For single cells, 10 μM ROCK inhibitor Y-27632 (Abcam, Cambridge, UK) was added for the first 2 to 3 days of the culture. To induce differentiation, organoids were cultured in differentiation medium (hSI-DM, which is hSI-EM lacking WNT3A, nicotinamide, and SB202190) for 5 days, as described previously.[1]

Immunohistochemistry

Biopsies obtained from patients and siblings were either freshly or previously mounted onto glass slides. Staining was done using a standard immunohistochemistry protocol with the Lab Vision UltraVision LP Detection System: HRP (horseradish peroxidase) Polymer (Ready-To-Use) (TL-060-HL; Thermo Scientific, Waltham, MA) DGAT1 protein was detected using a rabbit polyclonal IgG (H-255; Santa Cruz Biotechnology, Inc., Dallas, TX) at 1:400 dilution.

CRISPR/Cas9 Knockout of DGAT1

Corresponding DNA-oligos were ligated into the plasmid vector pSpCas9(BB) (Addgene PX459). Before transfection, the organoids were primed by removing WNT3A and Rspondin-1 and addition of 5 μM CHIR99021, 1.25% dimethyl sulfoxide, and 10 μM Y-27632 (ROCK inhibitor) to the culture medium as described previously.[2] For transfection, 1×10^6 single cells in 80 μL BTXpress buffer (BTX Genetronics, Holliston, MA) were mixed with 10 μL DGAT1 sgRNA construct plasmid (1 μg/μL), 7.2 μL PB-Hygromycin plasmid (1 μg/μL), and 2.8 μL PB-Transposase plasmid (1 μg/μL). Transfection was performed in a NEPA21 Electroporator with settings as described previously.[2] After transfection, priming medium was used for 5 days before replacement by normal hSI-EM and selection with Hygromcyin B (100 μg/mL; Invitrogen, Carlsbad, CA). The organoids were passaged 1:4 by mechanical disruption at day 9. Single organoids were picked 16 days after transfection, disrupted into single cells by trypsinization with TripLE, and scaled up as clonal organoid cell lines. Several clones per transfection were screened for DGAT1 protein expression by Western blot (Supplementary Figure 6*C*). Organoids transfected with sgRNA#2 achieved 10/12 $DGAT1^{KO}$ clones, whereas organoids transfected with sgRNA#678 achieved 3/11 $DGAT1^{KO}$ clones. Finally, 3 $DGAT1^{KO}$ clones were further characterized by genotyping (Data not shown). Clone $DGAT1^{KO}$ #2–11 showed a deletion of 280 base pairs (bp) on both alleles (NG_034192.1; 13347–13627). Clone $DGAT1^{KO}$ #678–02 showed a deletion of 83 bp on both alleles (NG_034192.1; 13487–13570). Clone $DGAT1^{KO}$ #678–07 showed a deletion of 83 bp on one allele (NG_034192.1; 13487–13570); the other allele has a deletion of 14 bp (NG_034192.1; 13485–13499) and an insertion of 1 bp (NG_034192.1; T between 13486–13487) (Supplementary Figure 6*B*).

Reverse-Transcriptase Polymerase Chain Reaction

Protein synthesis inhibitor puromycin in a concentration of 20 mg/12 mL was added to patient 3 and control fibroblast culture 4 hours before harvesting for total RNA extraction. Total RNA was extracted using the Qiagen RNAeasy mini kit (Qiagen, Hilden, Germany). RNA was reverse transcribed into complementary DNA (cDNA) by a standard protocol using M-MLV reverse transcriptase (Promega, Madison, WI). cDNA was PCR-amplified using GoTaq polymerase and buffer (Promega) in 35 cycles of 20 seconds at 95°C, 30 seconds at 60°C, and 30 seconds at 72°C in addition to a 7-minute final extension. The amplicons were cleaned using Microcon centrifugal filter for agarose gel–excised PCR products, and subsequently sequenced using forward (gtaaaacgacggccagtGTGGACCC CATCCAGGTGG) and reverse (caggaaacagctatgacTGAGCCA GATGAGGTGATTGG) primers and run on an agarose gel.

Quantitative Real-Time Polymerase Chain Reaction

RNA was isolated from Caco-2 cells or organoids grown in either EM or DM for 5 days using the RNeasy Mini kit (Qiagen) or TRIzol LS Reagent (Invitrogen), respectively, according to the manufacturer's protocol. cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) and amplified using SYBR green supermix (Bio-Rad) in a Light Cycler96 (Roche, Basel, Switzerland) according to the manufacturer's protocol. The comparative Ct method was used to quantify the data. The relative quantity was defined as $2^{-\Delta\Delta Ct}$. In organoids and Caco-2 cells, *HP1* and *HPRT1* were used as housekeeper genes, respectively. *Sucrase-isomaltase (SI)* and *LGR5* were used to determine the stem cell vs differentiation status of organoids. Primers used were *DGAT1*-forward (FW): cgacgtggagccgc, *DGAT1*-reverse (RV): gctcaagatcagcatcacca, *HP1*-FW: cccacgtcccaagatggat, *HP1*-RV: ctgatgcaccactcttctggaa, *SI*-FW: ggacactggcttggagacaac, *SI*-RV: tccagcgggtacagagatgat, *LGR5*-FW: gaatcccctgcccagtctc, *LGR5*-RV: attgaaggcttcgcaaattct, *HPRT1*-FW: tgacactggcaaaacaatgca, *HPRT1*-RV: ggtccttttcaccagcaagct.

Western Blotting

Caco-2 cells or organoids were lysed in Laemmli buffer (0.12 mol/L Tris-HCl pH 6.8, 4% sodium dodecyl sulfate [SDS], 20% glycerol, 0.05 μg/μL bromophenol blue, 35 mmol/L β-mercaptoethanol) and incubated at 100°C for 5

minutes. Fibroblasts were lysed in RIPA buffer (50 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton-X100, 2 mM EDTA, 1% sodium deoxycholate, 50 mM NaF, 10 mM NaO_3V_4) supplemented with protease inhibitor cocktail (1:200, P8340; Sigma-Aldrich, St Louis, MO) then supplemented with Laemmli buffer. The protein concentration was measured by performing a Lowry or Bradford protein assay. Equal amounts of proteins were separated by SDS–polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane (Merck Millipore, Billerica, MA), blocked with 5% milk protein in TBST (0.3% Tween, 10 mM Tris-HCl pH 8, and 150 mM NaCl in H_2O) and probed with primary antibodies. The membranes were washed with TBST and incubated with appropriate secondary antibodies. Immunocomplexes were detected using the LI-COR (Lincoln, NE) Odyssey. Caco-2 cells transfected with Flag-DGAT1 c.629_631delCCT were left untreated or treated with 2 μM MG132 (Cayman Chemicals, Ann Arbor, MI) for 16 hours.

The primary antibodies used were mouse anti-Flag M2 (1:3000; F3165; Sigma-Aldrich), rabbit anti-DGAT1 (1:1000; ab181180; Abcam), rabbit anti-DGAT1 (1:1000, sc-32861; Santa Cruz Biotechnology), rabbit anti-DGAT2 (1:1000, bs-12998R; Bioss Antibodies, Woburn, MA), mouse anti-GAPDH (1:3000, sc-32233; Santa Cruz Biotechnology), anti-HA-HRP conjugate (1:3000, H6533; Sigma-Aldrich), and rabbit anti-HSP90 (1:10000; kindly provided by Professor L.J. Braakman). The secondary antibodies used were donkey anti-mouse IgG IRDye 680 (1:10000; 926-32222; LI-COR) and donkey anti-rabbit IgG IRDye 680RD (1:10000; 926-68073; LI-COR), goat anti-mouse IgG-HRP (1:15000, 554002; BD Biosciences), and goat anti-rabbit IgG-HRP (1:15000, 172-1019; Bio-Rad).

Lipid Droplet Assays

Oleic acid (OA) was conjugated to bovine serum albumin (BSA) by heating the OA in phosphate-buffered saline (PBS) to 70°C for 1 hour. The OA suspension was then thoroughly vortexed and slowly added to a solution of fatty acid–free BSA in PBS kept at 37°C in a molar ratio of OA:BSA (5:1) to a final stock concentration of 10 mM OA and 2 mM BSA.

Thin Layer Chromatography

Folch extraction was performed by harvesting organoids, washed in PBS and spun down, and the cell pellets were frozen in ice-cold methanol on dry ice to stop cell metabolism. Last, the cell pellets were homogenized using a bullet blender, using glass bullets of 0.1-mm diameter for 2 minutes. Folch extraction was performed similar to the original protocol.[3] In short, chloroform and dH_2O were added to the cell lysate in methanol in a ratio of methanol:chloroform:water (1:2:1). The mixture was vortexed thoroughly, and incubated for 10 minutes in a shaking heat block at 37℃. Afterward, the lysates were spun down at 20,000 relative centrifugal force for 5 minutes to achieve a phase separation. The bottom phase (chloroform/methanol) was carefully collected and evaporated at 40℃ under flow of nitrogen. The residual fatty precipitate was dissolved in chloroform:methanol (2:1) and collected in glass vials.

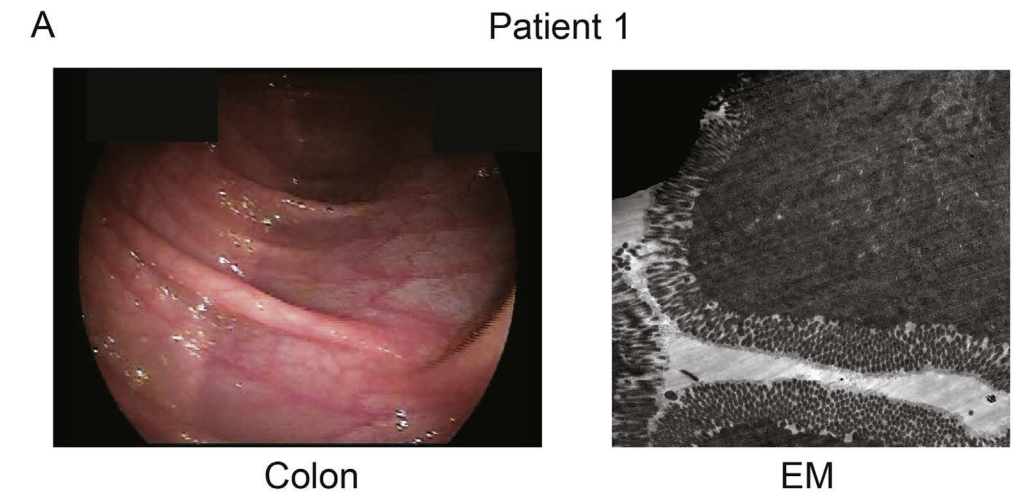
Ubiquitin Pulldown Assay

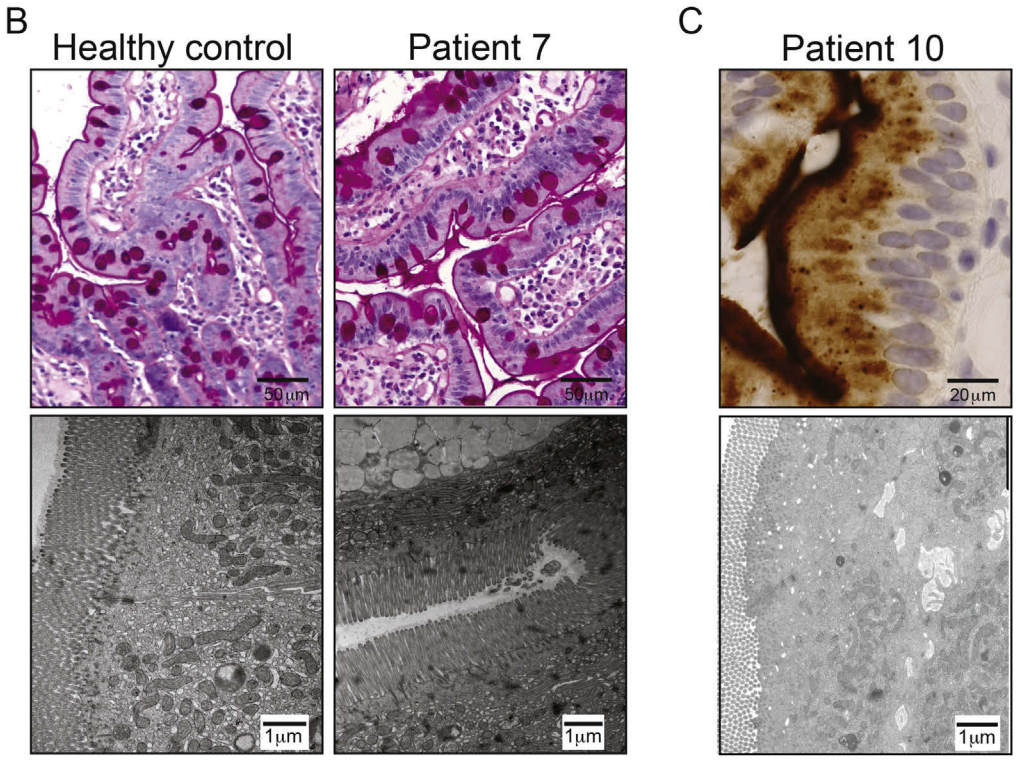
Caco-2 cells were grown in 10-cm dishes and co-transfected with 3 μg pcDNA3, Flag-DGAT1 WT, or Flag-DGAT1 c.629_631delCCT and 3 μg His-ubiquitin. MG132 (2 μM) was added 32 hours posttransfection and the cells were incubated at 37°C for 16 hours. After incubation with His-ubiquitin, transfected cells were once washed with PBS and lysed in lysis buffer pH 8.0 containing 8 M urea (GE Healthcare, Little Chalfont, UK), 7 mM NaH_2PO_4 (Merck, Whitehouse Station, NJ), 100 mM Na_2HPO_4 (Merck), 10 mM Tris-HCl pH 8.0 (Roche), 0.2% Triton-X100 (Sigma-Aldrich), 10 mM imidazole (Sigma-Aldrich), and 5 mM N-ethylmaleimide (Sigma-Aldrich); 10 mM β-mercaptoethanol (Sigma-Aldrich) and Ni-NTA agarose beads (Qiagen) were added and the cells were tumbled for 2 hours at room temperature. The beads were washed twice in buffer pH 8.0, 2 times in buffer pH 6.3 (12 mM urea, 100 mM NaH_2PO_4, 30 mM Na_2HPO_4, 15 mM Tris-HCl pH 6.3, 0.3% Triton X-100, 30 mM imidazole), and once in wash buffer (100 mM NaCl [Sigma-Aldrich], 20% glycerol [Sigma-Aldrich], 20 mM Tris-HCl pH 8.0, 2 mM dithiothreitol [GE Healthcare] and 10 mM imidazole). Sample buffer (0.12 M Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 0.05 μg/μL bromophenol blue and 35 mM β-mercaptoethanol) was added. The samples were incubated at 100°C for 5 minutes and subjected to Western blot.

Supplementary References

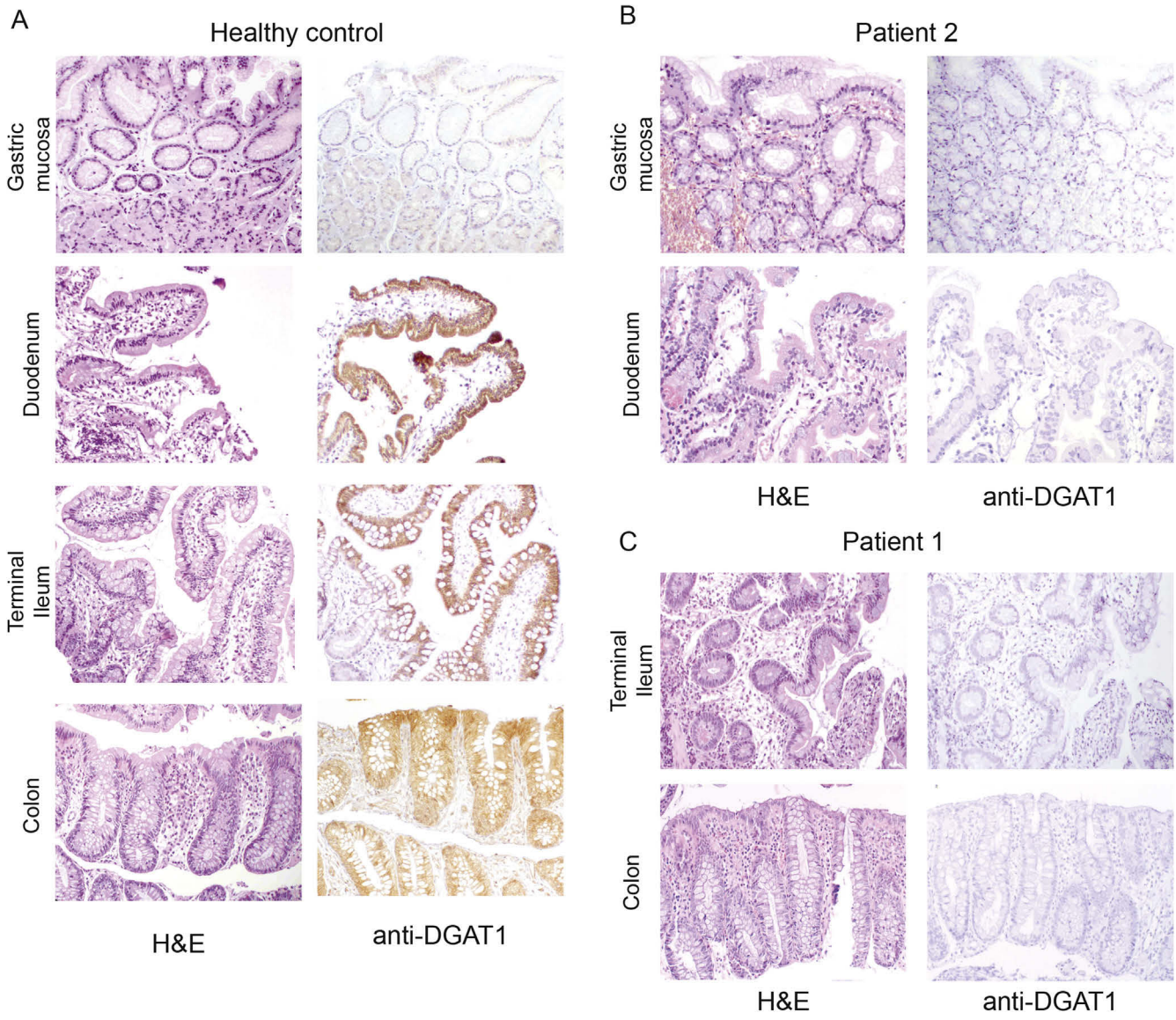
1. **Wiegerinck CL, Janecke AR, Schneeberger K**, et al. Loss of syntaxin 3 causes variant microvillus inclusion disease. Gastroenterology 2014;147:65–68.e10.
2. **Fujii M, Matano M**, Nanki K, et al. Efficient genetic engineering of human intestinal organoids using electroporation. Nat Protoc 2015;10:1474–1485.
3. Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 1957;226:497–509.

Author names in bold designate shared co-first authorship.

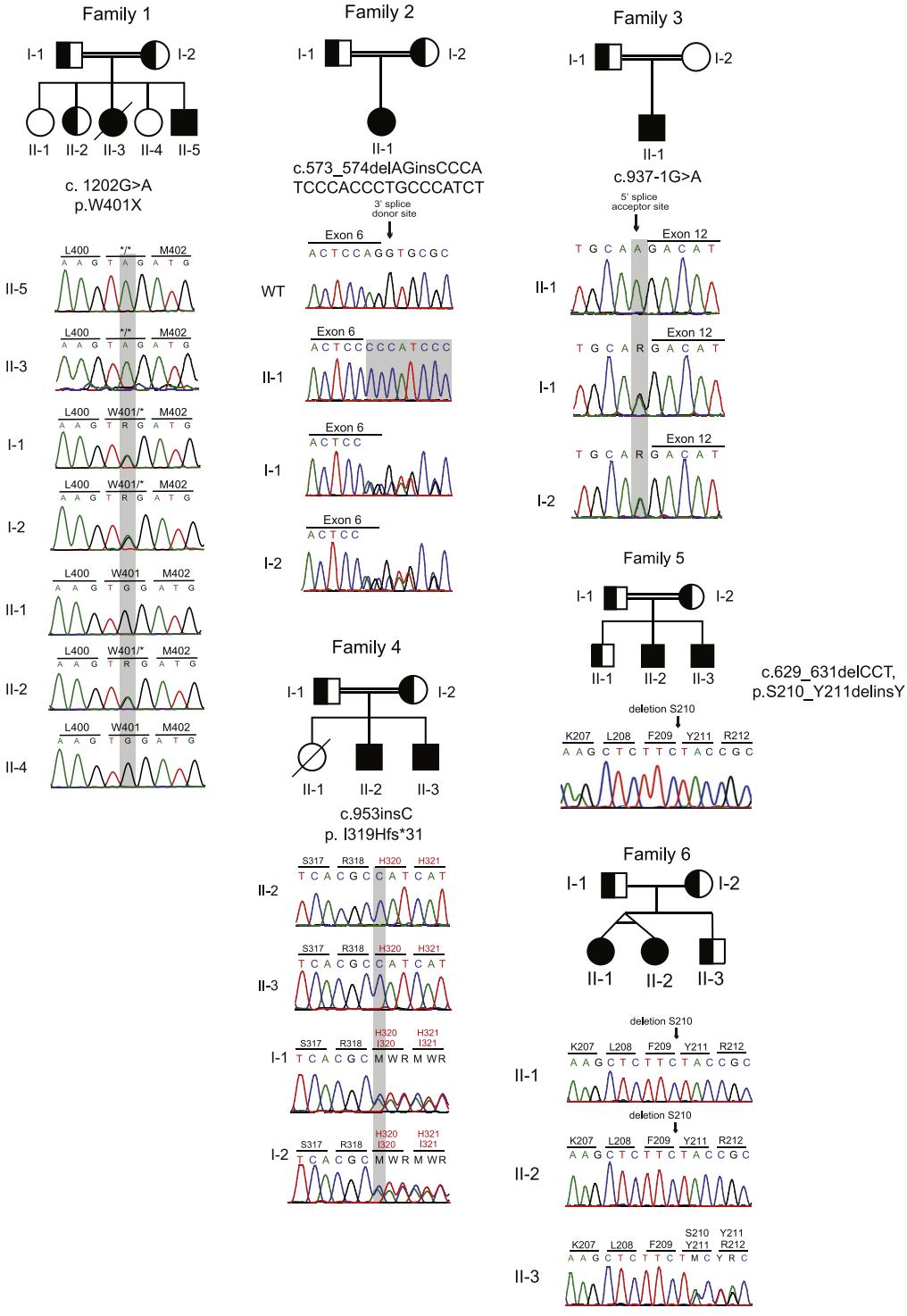




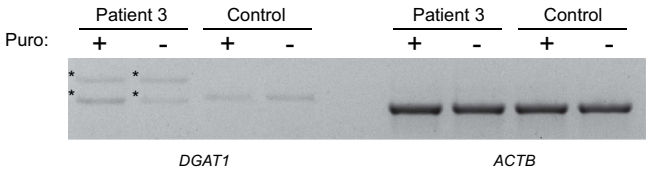
Supplementary Figure 1. Histologic and endoscopic characteristics of intestines of DGAT1-deficient patients. (*A*) Colonoscopy (*left*) and electron microscopy (EM, *right*) revealed normal intestinal structures in patient 1. (*B*) Periodic acid-Schiff and EM of patient 7 were obtained during fat-free diet and do not show abnormalities. (*C*) Patient 10 shows cytosolic CD10 staining and lateral microvilli on EM.



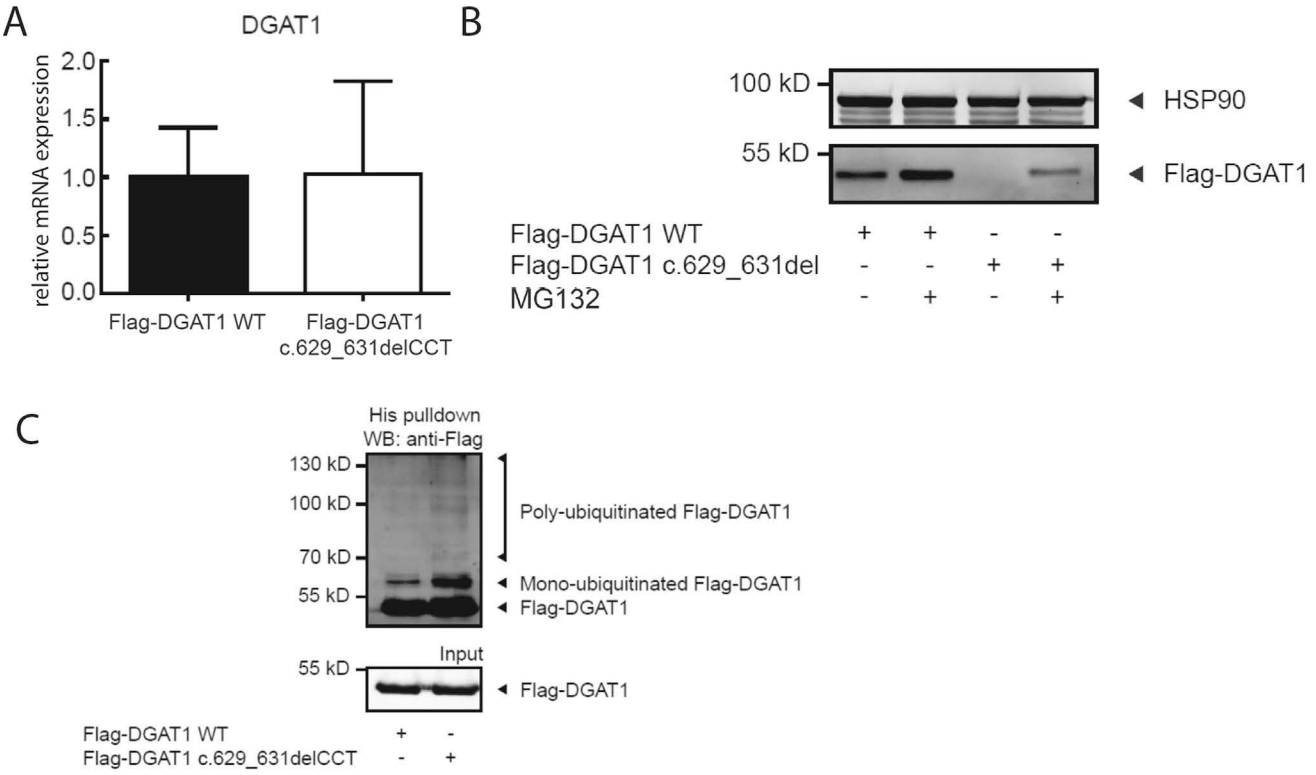
Supplementary Figure 2. Hematoxylin-eosin (H&E) and immunohistochemical staining for DGAT1 in (*A*) healthy controls, (*B*) patient 2, and (*C*) patient 1.



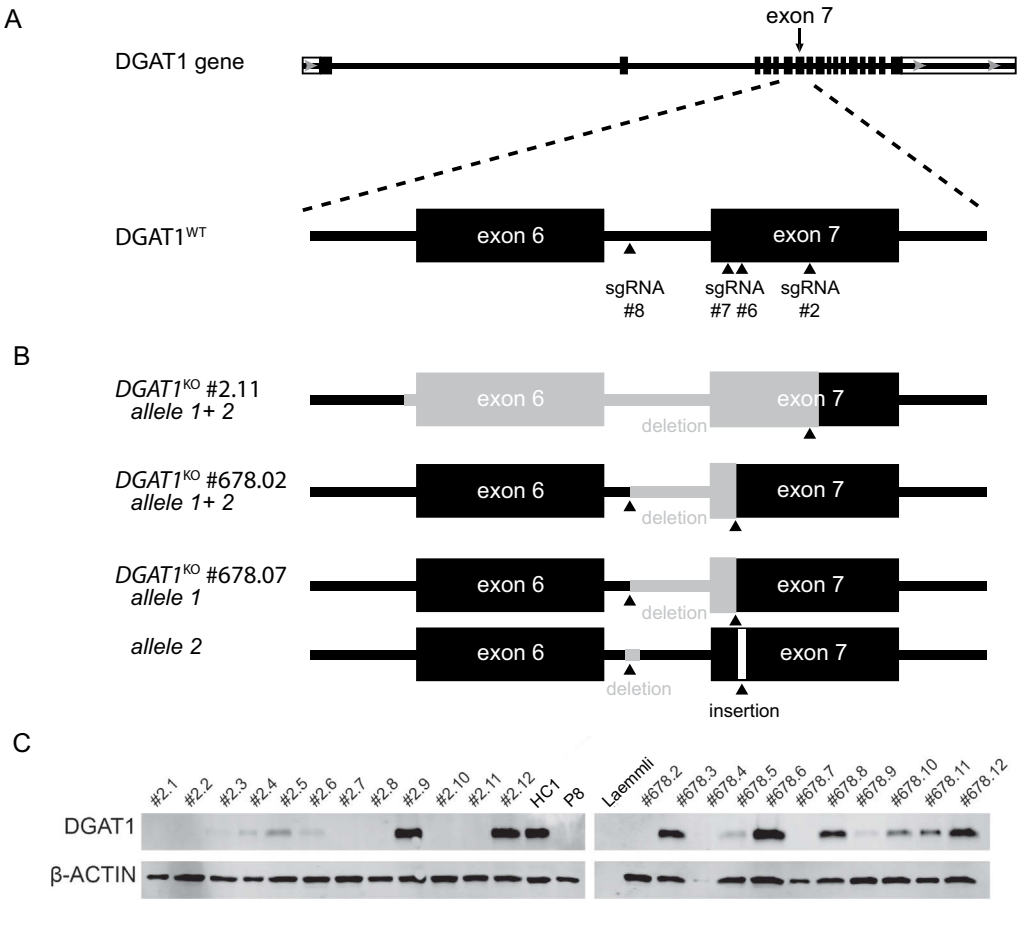
Supplementary Figure 3. Family pedigrees and Sanger sequencing histograms of DGAT1 deficiency. Filled shapes indicate affected individuals, half-filled are heterozygous for mutation indicated, and empty shapes indicate wild type.



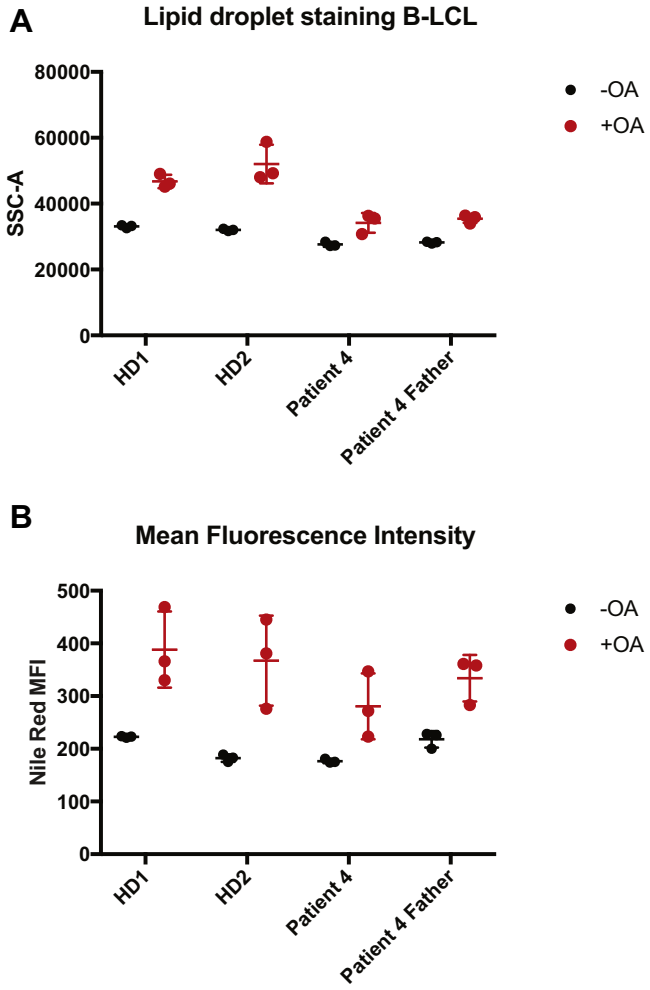
Supplementary Figure 4. Defective mRNA splicing in patient 3. (*A*) Gel showing aberrant *DGAT1* transcripts in mRNA isolated from patient 3 fibroblasts with and without a 4-hour puromycin treatment. * denotes aberrant transcript. *ACTB* was used as control.



Supplementary Figure 5. *DGAT1* c.629_631delCCT results in increased proteasomal degradation of DGAT1. Caco-2 cells were stably transfected with indicated constructs. (*A*) qRT-PCR analysis of *DGAT1* mRNA expression, relative to *HPRT1,* in Caco-2 cells stably transfected with indicated constructs. Average and standard deviation of 3 independent experiments are shown. (*B*) Transfected Caco-2 cells were untreated or treated with 2 μM MG132 for 16 hours. Protein expression was determined by Western blot analysis using anti-Flag and anti-HSP90 antibodies. Results are representative of 3 independent experiments. (*C*) Caco-2 cells were transiently transfected with His-ubiquitin and indicated constructs and treated with 2 μM MG132 for 16 hours. Ubiquitination of DGAT1 was determined by a His pulldown and subsequent Western blot analysis using anti-Flag antibodies. Results are representative of 3 independent experiments.



Supplementary Figure 6. Generation of *DGAT1* knockouts in healthy human intestinal organoids. (*A*) Targeting strategy for the generation of *DGAT1* knockout organoids using CRISPR/Cas9 genome editing. (*B*) Schematic view of insertion-deletion mutations of 3 *DGAT1*^{KO} clones. *Gray areas* represent deleted sequences from the WT *DGAT1* gene. (*C*) Western blot analysis of DGAT1 expression in *DGAT1*^{KO} organoids compared with healthy control 1 (HC1) and patient 8 (P8). Actin, loading control.



Supplementary Figure 7. Lipid droplet staining in Epstein-Barr virus–derived B-lymphoblastic cell line (EBV B-LCL) of patient 4 with and without OA. (*A*) Mean SSC-A and (*B*) Nile Red mean fluorescence intensity (MFI) staining of lipid droplet stained EBV B-LCLs.

Supplementary Table 1. Genetic Description of DGAT1-deficient Patients in This Study and in Previous Reports

Patient	Position Chr. 8	Reference	Alternate	*DGAT1* mutation	DGAT1 mutated protein	CADD score
1	145540731	C	T	c.1202G>A	p.W401X	38
2	145540731	C	T	c.1202G>A	p.W401X	38
3	145542123	CCT	CAGATGGGCAGGG TGGGATGGG	c.573_574delAGinsCCCATCCC ACCCTGCCCATCT	-	25.7
4	145541252	C	T	c. 937-1G>A	-	24.9
5	145541233	T	TG	c.953insC	p. I319Hfs *31	35
6	145541233	T	TG	c.953insC	p. I319Hfs *31	35
7	145541968	TAGG	T	c.629_631delCCT	p.S210_Y211delinsY	18.9
8	145541968	TAGG	T	c.629_631delCCT	p.S210_Y211delinsY	18.9
9	145541968	TAGG	T	c.629_631delCCT	p.S210_Y211delinsY	18.9
10	145541968	TAGG	T	c.629_631delCCT	p.S210_Y211delinsY	18.9
Haas et al.	145541756	A	G	c.751+2T>C	p.A226_R250del	22.9
Stephen et al. (Fam 1)	145541756	A	G	c.751+2T>C	p.A226_R250del	22.9
Stephen et al. (Fam 2)	145541457	A	G	c.884T>C	p.L295P	31
Gluchowski et al.	145542706	A	G	c.314T>C	p.L105P	29.3
Ratchford et al.	145541074	TAGA	T	c.1013_1015delTCT	p.F338del	22.3
	145540567	C	G	c.1260C>G	p.S420R	31

CADD, combined annotation dependent depletion; Chr, chromosome.

Supplementary Table 2. Serum Lipid Values of DGAT1-deficient Patients in This Study

Patient	Age on test date	Triglyceride (*mg/dL*)	HDL (*mg/dL*)	LDL (*mg/dL*)	VLDL (*mg/dL*)	Cholesterol (*mg/dL*)
1	5 mo	135 (<150)	25[a] (40–60)	12 (<110)	27 (<30)	63 (<170)
2	5 mo	84 (<150)	16[a] (40–60)	28 (<110)	16 (<30)	62 (<170)
3	11 mo	370[a] (<200)	9.5[a] (40–60)	23 (<110)	ND	96 (<170)
4	8 y	123 (<180)	Normal	110[a] (<110)	ND	181[a] (<170)
5	ND	ND	ND	ND	ND	ND
6	2 y	184 (<200)	21[a] (40–60)	13 (<110)	36[a] (<30)	59 (<170)
7	9 y	142 (<180)	32[a] (40–60)	ND	ND	139 (<170)
8	12 y	88 (<180)	30[a] (40–60)	ND	ND	112 (<170)
9	22 mo	124 (<180)	24[a] (40–60)	ND	Normal	139 (<170)
10	20 mo	165 (<180)	ND	ND	ND	128 (<170)

NOTE. Parentheses indicate normal values.
HDL, high-density lipoprotein; LDL, low-density lipoprotein; ND, not determined; VLDL, very low-density lipoprotein.
[a]Indicates abnormal value

Supplementary Table 3. Compilation of Clinical Characteristics and Specific Treatment of DGAT1-deficient Patients in This Study

Patient ID	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
Recurrent infections	Yes	Yes	No	No	No	Yes	No	No	Yes	Yes
Type of infections	Upper respiratory tract infections	NA	Central line–related fungal infection	NA	NA	Upper respiratory infections, recurrent otitis media	NA	NA	(1) Sepsis: *Klebsiella* and *Streptococcus* (2) Sepsis: *Escherichia coli*	(1) NA (2) Sepsis: *Klebsiella* (3) Sepsis: *Bacillus cereus* (4) Sepsis: *Staphylococcus aureus*
Treatment for infectious episodes	Antibiotics IV or PO	NA	Amphotericin B IV	NA	Prophylactic immunoglobulins IV	Antibiotics PO	NA	NA	Antibiotics IV	Antibiotics IV
Response to treatment for recurrent infection	Well responded	NA	NA	NA	Well responded	Well responded	NA	NA	Well responded	Well responded
Signs of liver disease	NA	NA	NA	Elevated liver enzymes	Jaundice and hepatomegaly. Elevated liver enzymes. Liver biopsy: hepatocanalicular and ductular cholestasis, paucity of bile ducts, mixed type; hepatosteatosis (10%), porto-portal fibrosis and fibrotic activity of 3/6.	Elevated liver enzymes	NA	NA	Elevated liver enzymes and jaundice. Toxic liver injury due to effects of long-term treatment with antifungal drugs (confirmed by liver biopsy).	Elevated liver enzymes and jaundice. Liver biopsy normal.
Fecal elastase 1 levels (normal range: $>$ 500 μg/g)	ND	NA	ND	NA	130 μg/g	ND	NA	NA	$>$500 μg/g	$>$500 μg/g
Serum albumin level before treatment (normal range: $>$3.2 g/dL)	1.8 g/dL	1.8 g/dL	2.2 g/dL	2.8 g/dL	2.2 g/dL	2.59 g/dL	NA	4.35 g/dL	2.38 g/dL	3.2 g/dL
Fecal-alpha-1-antitrypsin level (normal range: $<$5 mg/g)	NA	ND	Low	ND	NA	NA	NA	NA	9.1 mg/g	7.6 mg/g
Specific treatment	Fat-free formula and medium chain triglyceride Albumin IV	Albumin IV	Albumin IV, TPN Fat-free formula and supplementation of medium chain triglyceride	Cholestyramine	Creon hydrolyzed formula	Creon	Monthly infusion of Intralipid and Omegaven supplementation of lipid-soluble vitamins	Monthly infusion of Intralipid and Omegaven supplementation of lipid-soluble vitamins	TPN and small bowel transplantation	TPN and fat-free formula

Supplementary Table 3. Continued

Patient ID	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
Clinical course after treatment	Significant improvement	Deceased	Significant improvement of diarrhea	Significant improvement; diarrhea stopped after treatment	Mild improvement. His daily stool frequency had reduced with the administrations Creon, but his weight and height are below 3rd percentile.	No improvement of failure to thrive, but significant improvement of diarrhea. After 2 years of age, his symptoms improved spontaneously. His weight is now 16 kg (3rd–10th percentile), height 104 cm (3rd percentile)	Significant improvement	Significant improvement	Completely resolved PLE. Tolerates polymeric enteral feeding. Still stunted and underweight by causes secondary to intestinal transplantation and several autoimmune problems.	NA

IV, intravenous; NA, not available; ND, not done; PLE, protein-losing enteropathy PO, per os; TPN, total parenteral nutrition.

Supplementary Table 4. sgRNAs Used to Target *DGAT1* for CRISPR/Cas9 Gene Editing

sgRNA ID	Core sequence DNA	Position DGAT1 gene	Predicted cleavage[a]
sgRNA#2	GGCACCATGAGTTGACGTCG	Exon 7	13621-13622 (AC)
sgRNA#6	TGTGCGCCATCAGCGCCAGC	Exon 7	13570-13571 (TG)
sgRNA#7	GTGCGCCATCAGCGCCAGCA	Exon 7	13569-13570 (CT)
sgRNA#8	GTGGCTGGCCCGAGACAGAT	Intron 6–7	13486-13487 (CT)

sgRNA, single-guide RNA.
[a]According NCBI Reference Sequence: NG_034192.1; *Homo sapiens* diacylglycerol O-acyltransferase 1 (DGAT1).

4. Discussion

Previously identified monogenetic defects that leads to CDD allowed an in-depth and molecularly mechanistic study of important regulators in the homeostasis of the GI tract. The work in this thesis described two similar diseases that resulted from monogenetic aberration in two different genes. Patients with CD55 deficiency with Hyperactivation of complement, angiopathic thrombosis and protein-losing enteropathy (CHAPLE) syndrome and DGAT1 deficiency presented with early-onset protein-losing enteropathy resulting in low serum protein levels as a result of the disease. However, the two differ in the pathomechanism that resulted in the onset of disease.

4.1 CD55 deficiency and the complement system

The complement system is an evolutionarily conserved innate immune response that is important to help in the removal of microbes and dying cells within the body (Howell *et al*, 2018). Complement proteins are mostly synthesized in the liver and are present as inactive precursors in blood (Noris & Remuzzi, 2013). However, many other cells types, including immune cells, can produce complement proteins locally (Noris & Remuzzi, 2013). Three main activation pathways have been described: classical pathway, alternative pathway, and lectin pathway (Figure 6). These activation pathways kickstart a cascade of complement activation that ends in the accumulation of terminal complement complex C5b-9 which results in the formation of membrane attack complex (MAC) on the surface of microbes or apoptotic cells (Lubbers *et al*, 2017). These are then cleared by phagocytic cells and processed and broken down. The complement pathway also promotes inflammation by releasing anaphylatoxins such as C3a and C5a that facilitates activates other immune cells to allow a pro-inflammatory environment for efficient bacterial clearance (Noris & Remuzzi, 2013).

The alternative pathway is responsible for the so-called “tick-over” mechanism, in which a low-level complement activation is observed due to the breakage of thioester bond in C3 by a water molecule (Lubbers *et al*, 2017). To prevent complement activation on autologous healthy cells, complement regulatory proteins are present in the blood stream or on the surface of these cells to prevent activation of complement cascade and accumulation of MAC. Proteins such as complement factor H, complement factor I, CD46, CD55, and CD59 belong to this group of complement regulatory proteins (Noris & Remuzzi, 2013).

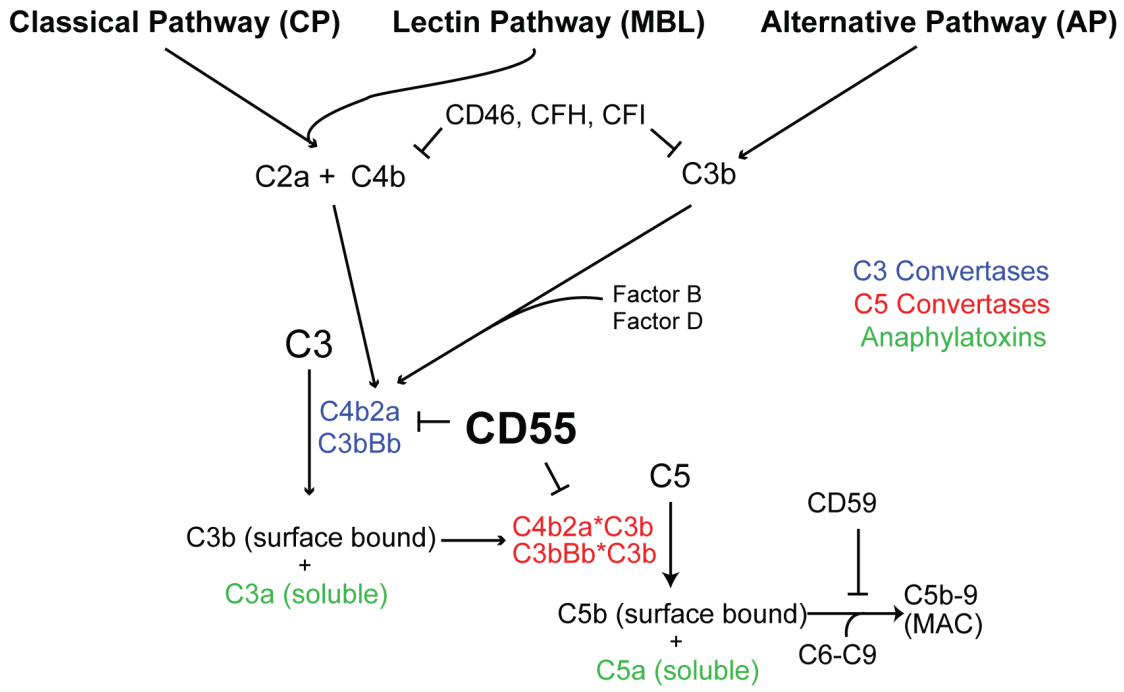


Figure 4. Overview of the complement pathway. Adapted from New England Journal of Medicine, Ozen, Comrie, Ardy et al., CD55 Deficiency, Early-Onset Protein-Losing Enteropathy and Thrombosis 377,52-61 Supplementary Materials. Copyright © (2017) Massachusetts Medical Society. Reprinted with permission.

CD55 encodes for a protein called complement decay-accelerating factor (DAF) or CD55, a protein that was identified as one of the glycophosphatidylinositol-anchored complement regulatory proteins that is present on hematopoietic and non-hematopoietic cells (Zipfel & Skerka, 2009). CD55 binds to complement factor C3 and C5 convertases (C3b complexes and C5b complexes respectively) and prevent amplification of complement preventing their convertase function and by accelerating their decay (Figure 6) (Lublin & Atkinson, 1989). This prevents the formation of MAC on self-cells and prevents autologous cell lysis. This function is mediated through its four Short Consensus Repeat (SCR)/Sushi domains (Lukacik *et al*, 2004). CD55 also functions as the adhesion or invasion receptor for some viruses such as the coxsackievirus B3, coxsackievirus A21, and enterovirus 70 (Bergelson *et al*, 1995; Shafren *et al*, 1997; Karnauchow *et al*, 1996).

CD55 was observed to bind to CD97, a 7 transmembrane molecule that is part of the adhesion GPCR family. Through this interaction, a more recently appreciated role of CD55 is its function as a coreceptor in T cell receptor (TCR) signaling and regulation of T regulatory 1 cells (Tr1) studied in an in vitro system (Sutavani *et al*, 2013; Capasso *et al*, 2014). Naïve CD4+ T cells become IL-10 producing induced T regulatory type 1 cells upon engagement of the TCR in

conjunction with CD55 co-stimulation, either through anti-CD55 antibodies or by its natural ligand CD97 in an in vitro system (Capasso *et al*, 2014).

The important role of complement in diseases is highlighted by the discoveries of complementopathies in which a defective control of the complement system results in an array of diseases. For example, mutations in *C1Q* genes have been found to cause systemic lupus erythematosus (SLE)-like syndrome, owing to the overactivation of the classical pathway and the lack of clearance of apoptotic bodies (Botto *et al*, 1998). Germline mutations in *CD46* and *CD59* have been associated with atypical hemolytic uremic syndrome (aHUS) while somatic mutations of genes involved in the glycophosphatidylinositol (GPI) anchor processing of CD55 and CD59 for surface expression has been found to underlie paroxysmal nocturnal hemoglobinuria (PNH) (Caprioli *et al*, 2006; Takeda *et al*, 1993).

Prior to the work done as part of this thesis, germline mutations in CD55 had only been identified in a handful of individuals that was designated as having the rare Inab blood type (Yazer *et al*, 2006). These individuals lack CD55 expression on the surface of their erythrocyte. However, the association between CD55 deficiency and EO-PLE/EO-IBD was not established. Some patients did suffer from gastrointestinal symptoms such as gastrointestinal reflux disease and PLE but there were too few individuals to draw a conclusion (Yazer *et al*, 2006).

The importance of complement regulation is also seen in the effective use of complement inhibitors in complement-mediated diseases such as myasthenia gravis and PNH. Eculizumab, a monoclonal antibody targeted against complement protein C5, was shown to be effective in preventing hemolysis in patients with PNH and aHUS (Hillmen *et al*, 2006; Legendre *et al*, 2013). Based on these results, we hypothesized that this treatment will also be effective in the context of CD55 deficiency, where we showed an overproduction of anaphylatoxin C5a in serum-treated CD55-deficient cells. We showed that an in vitro formulation of eculizumab is able to reduce C5a production (Figure 4 of manuscript in Chapter 3.1). Concurrently, a report of compassionate use of eculizumab in a family with CD55 deficiency was published in the same issue of the journal (Kurolap *et al*, 2017). Eculizumab proved to be effective in reducing bowel movements and normalizing serum albumin concentration in patients with CD55 deficiency (Kurolap *et al*, 2017). These evidences pointed towards the role of C5a in the pathomechanism of CD55 deficiency. Additionally, a novel formulation of a C5-blocking antibody named Ravulizumab has been recently shown to be as effective as eculizumab in PNH patients (Lee *et al*, 2019; Kulasekararaj *et al*, 2019). This formulation is administered every 8 weeks as compared to eculizumab's biweekly dosage and

is therefore improving patients' quality of life. Ravulizumab should also be considered for patients with CD55 deficiency as a treatment option.

Some CD55-deficient patients suffer from primary intestinal lymphangiectasia, in which the lymphatic vessels in the intestine are dilated which results in the loss of lymph fluid into the GI tract (Vignes & Bellanger, 2008). The role of CD55 in the homeostasis of lymphatic vessels was not explicitly determined in the study performed for this thesis. As CD55 is ubiquitously expressed by every cell in the body, it would be interesting to further explore the role of CD55 deficiency in the context of local complement production in the lymphatic microenvironment.

4.2 DGAT1 deficiency in the context of lipid metabolism

Lipids make up an important component of biology and life, and the study of lipid biology spans from basic biophysical structures of micelles to the effect of a high-lipid diet in obesity and Type 2 diabetes. Lipid metabolism refers to the processes involved in production and breakdown of lipid molecules. This is important in the context of cellular functions, from making membranes to providing signaling molecules for cellular functions. Its importance is further highlighted by the complex biochemical processing and the sheer number of enzymes that has evolved to regulate these processes (Van Meer *et al*, 2008).

Human mutations in proteins related to lipid metabolism present as a heterogenous group of diseases potentially affecting multiple organs. Some examples include a group of diseases called coenzyme A dehydrogenase deficiencies whereby mutations in enzymes involved in the breakdown of fatty acids for energy results in symptoms such as lethargy and liver dysfunction (Matern & Rinaldo, 1993; Rakheja *et al*, 2002). Another example is Tay-Sachs disease where mutations in *HEXA* gene encoding for hexosaminidase leads to a disorder of toxic glycosphingolipid accumulation (Okada & O'Brien, 1969). Patients with Tay-Sachs suffer from neurological manifestations due to the destruction of neurons in the brain and spinal cord. Previous studies on human mutations in *DGAT1* were described, but the underlying molecular mechanism for the onset of EO-PLE was not critically understood (Haas *et al*, 2012; Stephen *et al*, 2016; Gluchowski *et al*, 2017; Ratchford *et al*, 2018).

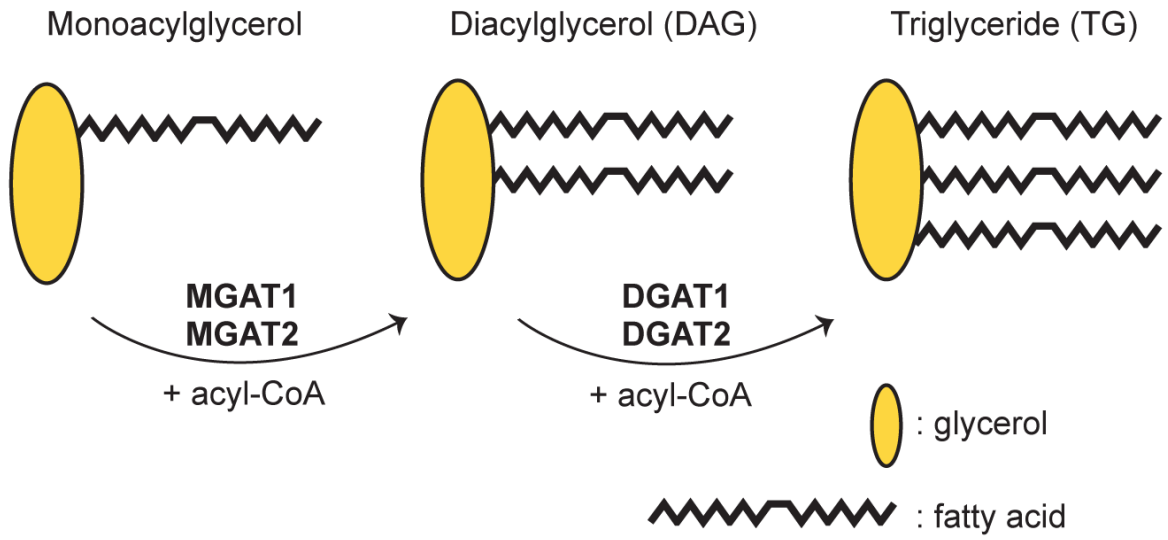


Figure 5. Simplified scheme of triglyceride formation. MGAT; monoacylglycerol-acyltransferase, DGAT;diacylglycerol-acyltransferase.

DGAT1 encodes for diacylglycerol-acyltransferase 1, a 55-kilodalton (kDa) transmembrane enzyme located in the endoplasmic reticulum (ER) responsible for the terminal step of triglyceride formation from diacylglycerol (DAG) and an acyl-CoA molecule (Figure 7) (Cheng *et al*, 2001). Diacylglycerol-acyltransferase 2 or DGAT2 shares the same molecular enzymatic function, but does not share any sequence or structure similarity to DGAT1 (Yen *et al*, 2008).

In the work done, we showed that DGAT1-deficient cells are not able to form TG as they lack neutral lipid staining upon addition of oleic acid into the growth media. This leads to the question of the fate of these fatty acid molecules in a DGAT1-deficient environment. Two potential mechanisms can be proposed: 1) these fatty acid molecules are actively removed from the cell through expulsion from a solute transporter or more likely 2) these molecules are broken down through beta and omega oxidation. To test this hypothesis, unbiased lipidomics approaches such as tracing experiments should be performed in order to delineate the fate of these fatty acid molecules in DGAT1-deficient cells (Thiele *et al*, 2019). As lipid regulation is a highly controlled process and could be coregulated, it would be interesting to see the homeostatic levels of various lipid moieties in DGAT1 deficiency and the resulting physiological changes through this altered lipid metabolism (Köberlin *et al*, 2015).

DGAT1 knockout mice (*Dgat1*$^{-/-}$) are resistant to diet-induced obesity, providing an interesting target for human obesity (Smith *et al*, 2000). Several DGAT1 inhibitors have been synthesized and have been proven to be efficacious in improving metabolism-related clinical manifestations (such as body weight, insulin insensitivity, and hepatic steatosis) in diet-induced obese mice (Cao *et al*, 2011). An attempt to use this inhibitors in humans failed due

to severe GI adverse events (AE), of note the severe diarrhea that these obese volunteers experience upon use of DGAT1 inhibitors (Denison *et al*, 2014). However, patients with familial chylomicronemia syndrome given different dosing of DGAT1 inhibitor pradigastat only reported mild GI AE (Meyers *et al*, 2015). The similarity between germline DGAT1 deficiency and induced deficiency due to DGAT1 inhibition showcase the importance of triglyceride metabolism in GI homeostasis. Patients with DGAT1 deficiency seems to be clinically stable with a fat-dree diet, pointing towards the idea that DGAT1 inhibitors might still prove useful in the right context of dosage and dietary fat intake control for use in obesity studies.

A potential therapy that we proposed in the manuscript involved the upregulation of DGAT2 enzymes in patients' gut epithelial cells as it is not expressed under normal conditions (Haas *et al*, 2012). A potential avenue that will require extensive testing is the use of epigenetic drugs to change the epigenetic marks around DGAT2 in patients' gut epithelium. An example of this is the use of EZH2 inhibitors that upregulate autophagy related proteins in a cancer cell line system (Hsieh *et al*, 2016). However, finding a long-lasting and specific drug that targets the DGAT2 locus might prove to be the biggest hurdle. Exploring the option of using CRISPR-mediated activation systems might be an additional interesting aspect in this context. CRISPR-activators (CRISPRa) makes use of a transactivation domain of the Herpes simplex virus to upregulate a certain gene (Mali *et al*, 2013), which has been recently shown to work in a haploinsufficient obesity mouse model (Matharu *et al*, 2019). Naturally, more in-depth research regarding efficacy and safety in humans will need to be undertaken prior to its use.

In our study, we highly utilized patient-derived gut organoids in order to fully characterize the underlying pathomechanisms of DGAT1 deficiency. This tool was highly informative as it confirmed that the lipotoxicity experienced by DGAT1-deficient cells was indeed in the context of the epithelium. This model system allowed us to study the importance of DGAT1 in GI epithelium lipid metabolism network. A systematic biobanking of genetically uncharacterized CDD patient-derived biobank may allow us to further study the contribution of novel monogenetic defects that affects the gut epithelium. In addition, gut organoid technology will allow us to further apply reductive methods to study the direct relationships between microbiota, various immune cells, and the gut epithelial with each other. Additionally, targeted panel sequencing was performed to identify the underlying DGAT1 mutations in two of the families included in this study. This highlights the importance of identifying novel genetic defects underlying CDDs, and to include them in diagnostics panels for rapid genetic diagnosis.

5. Summary and outlook

In summary, the work done and shown in this thesis resulted in the identification and molecular characterization of the pathomechanisms involved in patients with monogenic mutations in *CD55* and *DGAT1*. The work done in this thesis continued to showcase the importance of identifying key molecular hubs involved in GI tract homeostasis and adds to the current knowledge of diseases involved in the regulation of the complement system and gut lipid metabolism and processing. In addition, the work done also highlighted the importance of timely and correct genetic diagnosis for patients with a potential or suspected monogenic disease as it provides clinicians the potential to prescribe personalized therapy for the corresponding gene defects. Identifying additional patients and mutations in these two genes may prove useful for a more thorough study of genotype-phenotype correlation and response to therapy.

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7. Curriculum Vitae

Rico Chandra Ardy

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Education

Medical University of Vienna 2014-Present
Cell Communication in Health and Diseases Doctoral Program Predoctoral Fellow
Supported by an awarded DOC Fellowship from the Austrian Academy of Sciences

Imperial College London, Faculty of Medicine, Department of Surgery and Cancer 2012 – 2013
Cancer Biology Master of Research (MRes Pass with Merit)

University of California, Los Angeles UCLA) Department of Ecology and Evolutionary Biology 2010 - 2012
Biology Bachelor of Science (B.S. Cum Laude with Highest Departmental Honors)

Los Angeles Pierce College 2008-2010
General Biology Transfer coursework

Research Experience

Boztug Laboratory PI: Dr. Kaan Boztug Sep 2014 – Present
Molecular immunology, genetics, rare diseases of the gastrointestinal tract
Ludwig Boltzmann Institute for Rare and Undiagnosed Diseases (LBI-RUD) and Research Center for Molecular Medicine of the Austrian Academy of Sciences (CeMM), Austria, Vienna

Bakal Laboratory PI: Dr. Chris Bakal Apr 2013 – Jul 2014
High-throughput imaging and siRNA screen, cancer biology, NF-kB signaling
Dynamical Cell Systems, Division of Cancer Biology, Institute of Cancer Research, London.

Ali Laboratory PI: Dr. Maruf Ali Nov 2012 - Apr 2013
Protein purification and crystallization, small scale bacterial work
Department of Molecular Biosciences, Imperial College London

Sack Laboratory PI: Dr. Lawren Sack Jan 2011 – Jun 2012
Plant physiology, database management
Department of Ecology and Evolutionary Biology, UCLA

Professional Experience

Scientific Officer Oct 2013 – Jul 2014
Bakal Lab, Institute of Cancer Research, London

Resident Assistant Sep 2011 – Jun 2012
UCLA Office of Residential Life (ORL)

Leadership Experience

CeMM Research Center for Molecular Medicine — Dec 2014 – Dec 2015
Position: 1st Year Students Representative

Imperial College London Cancer Biology Master of Research — Sep 2012 – Sep 2013
Position: Head Course Representative

UCLA ORL Academic Development Committee — Jan 2012 –Jun 2012
Position: Undergraduate Student Representative

Relevant Publications

- Dominguez Conde C*, Yuce Petronzcki Ö*, Baris S*, Willman n KL*, (…) **Ardy RC**, (…), and Boztug K. Polymerase δ deficiency causes syndromic immunodeficiency with replicative stress. *J Clin Invest. pii: 128903* (2019)
- Serwas NK*, Hoeger B*, **Ardy RC**, (…), and Boztug K**.** Human DEF6 deficiency underlies an immunodeficiency syndrome with systemic autoimmunity and aberrant CTLA-4 homeostasis. *Nat Commun.* **10(1)***:3106* (2019)
- Spencer S*, Köstel Bal S*, Egner W*, Allen HL*, Raza SI*, (…), **Ardy RC**, (…), Boztug K#, Milner JD#, and Thaventhiran JED#. Loss of the interleukin-6 receptor causes immunodeficiency, atopy, and abnormal inflammatory responses. *J Exp Med.* epub ahead of print (2019)
- Pazmandi J, Kalinichenko A, **Ardy RC**, and Boztug K. Early-onset inflammatory bowel disease as a model disease to identify key regulators of immune homeostasis mechanisms. *Immunol Rev.* **287(1)**:162-185 (2019)
- van Rijn JM*, **Ardy RC*,** Kuloğlu Z*, Härter B*, van Haaften – Visser DY*, (…), Müller, T#, Middendorp, S#, and Boztug, K#. Intestinal failure and aberrant lipid metabolism in patients with DGAT1 deficiency. *Gastroenterology*. **155(1)**:130-143.e15 (2018)
- Pfajfer L*, Mair NK*, (…), **Ardy RC**, (…), Dupre L#, and Boztug K#. Mutations affecting the actin regulator WD repeat–containing protein 1 lead to aberrant lymphoid immunity. *J Allergy Clin Immunol.* **142(5)**:1589-1604 (2018)
- Ozen A*, Comrie WA*, **Ardy RC*,** (…), Boztug K#, and Lenardo MJ#. CD55 deficiency, early-onset protein losing enteropathy, and thrombosis. *N Engl J Med.* **377**:52-61 (2017)
- Sero JE*, Sailem HZ*, **Ardy RC**, Almuttaqi H, Zhang T, and Bakal C. Cell shape and the microenvironment regulate nuclear translocation of NF-κB in breast epithelial and tumor cells. *Mol Syst Biol.* **11**:790 (2015)
- Yin Z, Sailem H, Sero J, **Ardy R**, Wong STC, and Bakal C. How cells explore shape space: A quantitative statistical perspective of cellular morphogenesis. *Bioessays.* **36(12)**: 1195-1203 (2014)

* indicates shared first authorship, # indicates shared last authorship

Selected Meetings/Congresses/Conferences

- **Ardy RC,** Ozen A, WA Comrie, JM van Rijn, Kuloglu Z, Härter B, van Haaften–Visser DY et al. Keystone Symposium “From Rare to Care: Modeling and Translation of Rare Diseases”, Vienna, Austria 2018 (Poster presentation)
  “Unraveling molecular mechanisms of monogenic congenital diarrheal diseases: CHAPLE syndrome and DGAT1 deficiency”

- van Rijn JM*, **Ardy RC*,** Kuloglu Z*, Härter B*, van Haaften–Visser DY* et al. 13th Annual Young Scientist Association Symposium of the Medical University of Vienna 2018 (Best Oral Presentation)
  "Intestinal failure, recurrent infections, and aberrant lipid metabolism in patients with DGAT1 deficiency"
- van Rijn JM*, **Ardy RC*,** Kuloglu Z*, Härter B*, van Haaften–Visser DY* et al. 35th Annual Meeting of the Working Group for Pediatric Immunology 2018, Innsbruck, Austria (Best abstract for oral presentation)
  "Intestinal failure, recurrent infections, and aberrant lipid metabolism in patients with DGAT1 deficiency"
- Ozen A*, Comrie WA* and **Ardy RC*** et al. 50th Annual European Society for Pediatric Gastroenterology, Hepatology, and Nutrition (ESPGHAN) 2017, Prague, Czech Republic (Oral presentation)
  "Early onset protein losing enteropathy, bowel inflammation, and thrombosis in patients with complement regulator deficiency"
- Ozen A*, Comrie WA* and **Ardy RC*** et al. 17th Biennial Meeting of the European Society for Immunodeficiencies 2016, Barcelona, Spain (Late breaker oral presentation)
  "Early onset protein losing enteropathy, bowel inflammation, and thrombosis in patients with complement regulator deficiency"
- **Ardy RC** et al. 9th Annual Bridge the Gap Symposium, Cell Communication in Health and Diseases Doctoral Program 2016, Vienna, Austria (Best poster award)
"Identification of monogenic causes of chronic early-onset diarrhea using next generation sequencing"
- **Ardy RC** et al. 9th Annual Young Scientist Association Symposium of the Medical University of Vienna 2015, Vienna, Austria (Poster presentation)
"Identification of monogenic causes of chronic early-onset diarrhea using next generation sequencing"
- **Ardy RC** et al. 32nd Annual Meeting of the Working Group for Pediatric Immunology 2015, Freiburg, Germany (Oral presentation)
"Identification of monogenic causes of chronic early-onset diarrhea using next generation sequencing"

* indicates shared first authorship

HONORS AND AWARDS

35th Annual Meeting of the Working Group for Pediatric Immunology 2018 Travel Grant	May 2018
DOC Doctoral Fellowship Award of the Austrian Academy of Sciences	Mar 2016 – Feb 2019
UCLA ORL Academic Integration Award	Jun 2012
UCLA College Honors	Sep 2010 – Jun 2012
UCLA Dean's Honors List	Sep 2010 – Jun 2011
Pierce College Shari Heller Scholarship	Jun 2010
Pierce College Organic-Biochemistry Award	Jun 2010
Pierce College Honors Program	Sep 2008 – Jun 2010