Moonlighting complement inhibitors and their link to human diseases

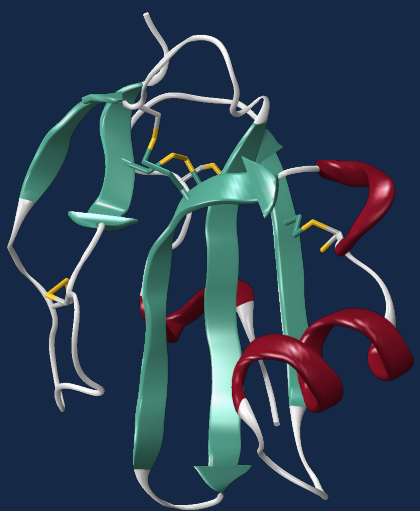
Ekström, Alexander

2025

Document Version:
Publisher's PDF, also known as Version of record

Moonlighting complement inhibitors and their link to human diseases

ALEXANDER EKSTRÖM
DEPARTMENT OF TRANSLATIONAL MEDICINE | FACULTY OF MEDICINE | LUND UNIVERSITY



Moonlighting complement inhibitors and their link to human diseases

Moonlighting complement inhibitors and their link to human diseases

Alexander Ekström

LUND
UNIVERSITY

DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Medicine at Lund University to be publicly defended on the 28th of February at 09.00 in Medelhavet, Wallenberg laboratory, Malmö, Inga Marie Nilssons gata 53

Faculty opponent
Group leader Monica Olcina del Molino, DPhil
Department of Oncology, University of Oxford, UK

Organization: LUND UNIVERSITY, Faculty of Medicine, Department of Translational Medicine, Division of Medical Protein Chemistry

Document name: Doctoral dissertation **Date of issue:** 2025.02.28

Author: Alexander Ekström **Sponsoring organization:**

Title: Moonlighting complement inhibitors and their link to human diseases

Abstract:

Breast cancer and type 2 diabetes are major health concerns worldwide. This thesis has focused on novel non-canonical roles of two complement inhibitors, which led to the discovery of new mechanisms and factors at play in these diseases. The sushi domain-containing protein 4 (SUSD4) was previously portrayed as a breast cancer suppressor, but no mechanism was identified. In paper I, using a syngeneic mouse model, we found further support for a tumour-suppressive effect of SUSD4. In triple-negative breast cancer cells, we discovered that SUSD4 interacts with growth factor receptors and promotes autophagy. Our results also indicate a plausible role for SUSD4 in epidermal growth factor receptor trafficking.

CD59, another complement inhibitor, plays an important non-canonical role in mediating insulin secretion. However, how this surface-anchored protein gains access to the cytoplasm had not been ascertained. In paper II, we identified novel intracellular splice forms of CD59 in both humans and mice that rescue the impaired insulin secretion in CD59-deficient β-cells. In line with this, the isoforms were found to interact with key components of the exocytotic machinery. Our results also indicate a potential link between the CD59 isoforms and the pathogenesis of type 2 diabetes. The role of CD59 in insulin secretion was, in paper III, further explored in a mouse model lacking critical exons of each of the two CD59 genes present in mice. However, the CD59-deficient mice did not exhibit impaired blood glucose homeostasis, and no defect in glucose-stimulated insulin secretion from isolated pancreatic islets was observed. Onwards, we identified a gene product in the CD59-deficient mice composed of the remaining exons of the two CD59 genes spliced together. When expressed in a β-cell line, this gene product could mediate insulin secretion in the absence of CD59, explaining the lack of a phenotype in the mouse model.

Keywords: Complement system, SUSD4, CD59, Diabetes, Type 2 diabetes, Pancreatic islets, Insulin secretion, Breast cancer, Autophagy, EGFR

Classification system and/or index terms (if any) Supplementary bibliographical information

Language: English **Number of pages**: 146

ISBN: 978-91-8021-672-2 **ISSN and key title**: 1652-8220

Recipient's notes

Price Security classification

Signature: Date: 2025-01-10

Moonlighting complement inhibitors and their link to human diseases

Alexander Ekström

LUND
UNIVERSITY

Cover illustration: High-resolution structure of human CD59 and a computed structure model of sushi domain-containing protein 4 generated using AlphaFold. The structures have been modified by Alexander Ekström for artistic purposes.

CD59: Obtained from RCSB Protein Data Bank (CC0 1.0)
PDB ID: 2J8B
DOI citation: Leath, K.J., Johnson, S., Roversi, P., Morgan, B.P., Smith, R.A.G., Lea, S.M. High resolution structure of human CD59 (2006) https://doi.org/10.2210/pdb2J8B/pdb

Literature citation: Leath KJ, Johnson S, Roversi P, Hughes TR, Smith RA, Mackenzie L, et al. High-resolution structures of bacterially expressed soluble human CD59. Acta Crystallogr Sect F Struct Biol Cryst Commun. 2007;63(Pt 8):648-52. DOI: https://doi.org/10.1107/S1744309107033477

SUSD4: Obtained from RCSB Protein Data Bank (CC0 1.0)
AlphaFold DB: AF-Q5VX71-F1

Varadi M, Anyango S, Deshpande M, Nair S, Natassia C, Yordanova G, et al. AlphaFold Protein Structure Database: massively expanding the structural coverage of protein-sequence space with high-accuracy models. Nucleic Acids Research. 2021;50(D1):D439-D44

Jumper J, Evans R, Pritzel A, Green T, Figurnov M, Ronneberger O, et al. Highly accurate protein structure prediction with AlphaFold. Nature. 2021;596(7873):583-9

Faculty of Medicine

Department of Translational Medicine

ISBN 978-91-8021-672-2

ISSN 1652-8220

Printed in Sweden by Media-Tryck, Lund University

Lund 2025

Just because they say it's impossible
doesn't mean you can't do it

- Roger Bannister

Table of Contents

List of papers included in the thesis

I. Papadakos K.S., **Ekström A**., Slipek P., Skourti E., Reid S., Pietras K., Blom A.M. (2022). Sushi domain-containing protein 4 binds to epithelial growth factor receptor and initiates autophagy in an EGFR phosphorylation independent manner. Journal of Experimental & Clinical Cancer Research, 41:363. Doi: 10.1186/s13046-022-02565-1.

II. Golec E., **Ekström A**., Noga M., Omar-Hmeadi M., Lund P-E., Villoutreix B.O., Krus U., Wozniak K., Korsgren O., Renström E., Barg S., King B.C., Blom A.M. (2022). Alternative splicing encodes functional intracellular CD59 isoforms that mediate insulin secretion and are down-regulated in diabetic islets. Proceedings of the National Academy of Sciences of the United States of America (PNAS), 119(24) e2120083119. Doi: 10.1073/pnas.2120083119.

III. **Ekström A**., Villoutreix B.O., Halperin J., Renström E., Blom A.M*., King B.C*. (2024). CD59 double knockout mice express a CD59ba hybrid fusion protein that mediates insulin secretion. The FASEB Journal, 38(21) e70156. Doi: 10.1096/fj.202401808R.
* Contributed equally

Abstract

Breast cancer and type 2 diabetes are major health concerns worldwide. This thesis has focused on novel non-canonical roles of two complement inhibitors, which led to the discovery of new mechanisms and factors at play in these diseases. The sushi domain-containing protein 4 (SUSD4) was previously portrayed as a breast cancer suppressor, but no mechanism was identified. In paper I, using a syngeneic mouse model, we found further support for a tumour-suppressive effect of SUSD4. In triple-negative breast cancer cells, we discovered that SUSD4 interacts with growth factor receptors and promotes autophagy. Our results also indicate a plausible role for SUSD4 in epidermal growth factor receptor trafficking.

CD59, another complement inhibitor, plays an important non-canonical role in mediating insulin secretion. However, how this surface-anchored protein gains access to the cytoplasm had not been ascertained. In paper II, we identified novel intracellular splice forms of CD59 in both humans and mice that rescue the impaired insulin secretion in CD59-deficient β-cells. In line with this, the isoforms were found to interact with key components of the exocytotic machinery. Our results also indicate a potential link between the CD59 isoforms and the pathogenesis of type 2 diabetes. The role of CD59 in insulin secretion was, in paper III, further explored in a mouse model lacking critical exons of each of the two CD59 genes present in mice. However, the CD59-deficient mice did not exhibit impaired blood glucose homeostasis, and no defect in glucose-stimulated insulin secretion from isolated pancreatic islets was observed. Onwards, we identified a gene product in the CD59-deficient mice composed of the remaining exons of the two CD59 genes spliced together. When expressed in a β-cell line, this gene product could mediate insulin secretion in the absence of CD59, explaining the lack of a phenotype in the mouse model.

Abbreviations

MBL	Mannose-binding lectin
MASP-1	MBL-associated serine protease 1
MASP-2	MBL-associated serine protease 2
MAC	Membrane attack complex
C3aR	C3a receptor
C5aR1	C5a receptor 1
CR	Complement receptor
C4BP	C4 binding protein
CSMD1	CUB and sushi multiple domain protein 1
RCA	Regulators of complement activation
CCP domain	Complement control protein domain
GPI anchor	Glycosylphosphatidylinositol anchor
SUSD4	Sushi domain-containing protein 4
mTOR (mTORC1)	mammalian target of rapamycin (complex 1)
ER	Endoplasmic reticulum
PNH	Paroxysmal nocturnal haemoglobinuria
TNFα	Tumour necrosis factor-α
NF-κB	Nuclear factor kappa B
IFNγ	Interferon-γ
IL	Interleukin
EGFR	Epidermal growth factor receptor
T1D	Type 1 diabetes
T2D	Type 2 diabetes
IAPP	Islet amyloid polypeptide

SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
IRS	Insulin response substrate
PKC	Protein kinase C
HLA	Human leukocyte antigen
SAID	Severe autoimmune diabetes
SIDD	Severe insulin-deficient diabetes
SIRD	Severe insulin-resistant diabetes
MOD	Mild obesity-related diabetes
MARD	Mild age-related diabetes
LADA	Latent autoimmune diabetes
MODY	Maturity-onset diabetes of the young
AGE	Advanced glycation end product
HER2	Human epidermal growth factor receptor 2
PTEN	Phosphatase and tensin homolog
TSC2	Tuberous sclerosis 2
PI3K	Phosphatidylinositol 3-kinase
PIP_2	Phosphatidylinositol-4,5-biphosphate
PIP_3	Phosphatidylinositol-3,4,5-triphosphate
ROS	Reactive oxygen species
ULK1	Unc-51-like kinase 1
AMPK	AMP-activated kinase

Introduction

Breast cancer and type 2 diabetes are two major societal burdens globally, both from a public health perspective and an economic perspective. Even without distinguishing between the sexes, breast cancer in women is one of the most common types of cancer and among the leading causes of cancer-related deaths. As such, if only accounting for women, breast cancer represents a significant health concern in our society. Conversely, type 2 diabetes is a public health issue for men and women alike. Hundreds of millions of people worldwide are estimated to have type 2 diabetes. This metabolic disease may progress slowly, but it is associated with devastating complications. Although many treatment options are available for both of these diseases, there is a strong need for new and better alternatives. Each year, breast cancer and type 2 diabetes claim countless lives worldwide, signifying the need for improved care of these patients. In order to develop better treatments and one day conquer these diseases, a better understanding of cellular processes and disease mechanisms at play is needed.

The complement system constitutes a humoral branch of the innate immune system comprised of proteins. It is traditionally viewed as a circulating surveillance system functioning as a first line of defence against intruding pathogens. However, many non-canonical functions of complement proteins have been discovered. Albeit still somewhat controversial, an emerging field of research is the roles and functions of intracellular complement.

This thesis has focused on non-immune-related, non-canonical functions of two complement inhibitors, SUSD4 and CD59, that generally reside on the cell surface and protect against complement-mediated self-harm. A potential breast cancer suppressive effect of SUSD4 had previously been described, but the underlying mechanism had not been addressed. Concerning CD59, a novel intracellular role in pancreatic β-cells whereby CD59 functions in insulin secretion had previously been identified in a β-cell line. However, how this cell surface-anchored protein gains access to the exocytotic machinery to function in insulin secretion was not delineated. The aim of this thesis was thus to identify and characterise the role of SUSD4 in breast cancer cells and to further investigate the role of CD59 in β-cells. This included investigating the novel role of CD59 in insulin secretion *in vivo* using a mouse model. Regarding SUSD4, a new role in breast cancer cells was identified, whereby it promotes autophagy. The investigation related to CD59 led to the

discovery of new proteins involved in insulin secretion. This thesis, thereby, contributed to an increased understanding of cellular processes that are relevant in the context of breast cancer and type 2 diabetes.

The topics of this thesis consequently include the complement system, cancer, autophagy, and diabetes, each of which is a massive topic on its own. Covering all of these in a thesis has been challenging, as one can write entire books about each of these topics alone. Nonetheless, an effort has been made to provide an overview of each of them and to compile sufficient background information for the three publications that are part of this thesis. Beginning with the complement system, each of the four major topics of this thesis will be introduced and described. Where relevant, a description of how these topics are interrelated will also be provided. At the end, a summary of the major findings will be given for each publication. Finally, both the findings of this thesis, along with future perspectives, will be discussed.

The complement system

Introduction

The complement system is a vital humoral component of the innate immune system and constitutes the first line of defence against intruding pathogens. The history behind its discovery dates back to the end of the 19th century. Early observations made already in 1874 showed that microorganisms introduced into the bloodstream were rapidly and effectively cleared from the circulation. Later studies demonstrated a cell-independent bactericidal activity of serum that was retained outside the body. This bactericidal component of serum was 1888 shown to be heat-labile by George Nuttall (1). Hans Ernst August Buchner first coined the term "alexin", which means "to ward off" in Greek, when referring to this heat-labile serum component. Jules Bordet built on the idea of a humoral bactericidal blood component and identified the presence of both the heat-labile alexin and a heat-stable component that he referred to as a "sensitiser". He further demonstrated the dependence of both alexin and the heat-stable sensitisers (now known to be antibodies) for immune lysis to occur (2). The term alexin was later replaced when Paul Ehrlich introduced the term "complement", which referred to the heat-labile serum component's ability to complement antibodies, or "amboceptors", as he called them (1). For his work related to the complement system, Jules Bordet received the Nobel Prize in Physiology or Medicine in 1919 (3).

Note that this thesis will adhere to the new recommendations with regard to nomenclature. As such, the larger enzymatically active C2 cleavage fragment will be referred to as C2b (4).

Activation and function of the complement system

More than a century of research later, significant progress in the field of complement has been achieved. It is now recognised as an intricate network of proteins with vital physiological effector functions. The complement system comprises more than 30 different proteins, including both circulating and cell surface-associated components. Conserved evolutionarily, the complement system constitutes a humoral branch of our innate immune system and plays an important role in immune surveillance. The complement system can be activated via three distinct pathways (**Figure 1**), each described separately below. While having different triggers, all three pathways converge on the cleavage and activation of the central component C3. Upon activation, the system will propagate in a cascade-like manner as the proteolytic complement proteins cleave and activate each other in a hierarchical order. Although capable of modulating adaptive immune responses, the traditional roles of the complement system lie in inflammation, opsonisation and direct killing of intruding pathogens. As more elaborately explained later, some of the cleavage fragments generated during the cascade function as inflammatory mediators, while others can opsonise pathogens to facilitate phagocytosis by immune cells. Additionally, the terminal pathway allows for the direct killing of pathogens through the generation of pores in their surface (5).

The classical pathway

The classical pathway was the first complement activation pathway to be identified based on the observation of a concerted action of the heat-labile and heat-stabile components of serum. Although others have now been described, the prototypical classical pathway activator remains immunoglobulins, more specifically, the IgG and IgM subclasses of immunoglobulins (6). The classical pathway is initiated when inactive, circulating complement component 1 (C1) recognises and binds to the Fc portion of antibodies bound to their antigens (7). C1 is an oligomeric protein complex in which subunits are associated in a Ca^{2+}-dependent manner (8). It is composed of one C1q subunit, two C1r subunits and two C1s subunits. While C1r and C1s are serine proteases responsible for the subsequent propagation of the complement cascade, the initial recognition of molecular triggers is mediated by C1q (9, 10). C1q is often described as assuming a bouquet-like structure where each of its six globular domains is linked to a collagen-like stem (9, 11). While a single IgM molecule is sufficient for C1q binding and complement activation, multiple adjacently bound IgG molecules are needed (8, 12). Although immunoglobulins are the principal activators of the classical pathway, C1q can recognise a wide range of molecules, leading to antibody-independent activation of the classical pathway. In addition to C reactive protein and pathogen-associated molecular patterns such as lipopolysaccharides (13), C1q can also recognise danger signals and damage-

associated molecular patterns presented by dying cells (8), such as DNA (14, 15), annexins (16), phosphatidylserine (17), calreticulin (18, 19), and glyceraldehyde-3-phosphate dehydrogenase (20).

Following C1q binding to its target, a consequential conformational change leads to the autoactivation of the serine protease C1r. Once activated, C1r will, in turn, cleave and activate C1s (21). This enables C1s to subsequently cleave C4 into C4a and C4b and C2 into C2a and C2b (7). C4b and C2b will then assemble close to the C1 complex and form the classical pathway C3 convertase (8). This convertase, C4b2b, will then cleave C3 into C3a and C3b, propagating the cascade further (2). Subsequent steps of the complement cascade are described later on.

The lectin pathway

Apart from the recognition step and the molecular triggers, the lectin pathway shares many of the steps and characteristics of the classical pathway (8). Molecular triggers for lectin pathway activation include pathogen-associated molecular patterns as well as damage-associated molecular patterns (22). Lectin pathway initiators recognise, for instance, carbohydrate moieties present on the surface of pathogens and dying cells (8, 23). The initiation of the lectin pathway is mediated by mannose-binding lectin (MBL), collectins (CL-10 and CL-11) and ficolins 1-3 (22). Albeit differences at the C-terminus, these proteins contain a collagenous N-terminus region just like C1q. Furthermore, similar to the Ca^{2+}-dependent association of C1q with the serine proteases C1r and C1s, the lectin pathway recognition molecules MBL, ficolins, and collectins associate with MBL-associated serine proteases 1 and 2 (MASP-1 and MASP-2) in a Ca^{2+}-dependent manner (8). MASP-1 and MASP-2 are functionally paralleling the role of C1r and C1s in the classical pathway (5). Depending on the degree to which it is oligomerised, MBL can form a complex with both MASP-1 and MASP-2 but is typically associated with one or the other (24). Upon binding of MBL to a target surface, the associated serine protease MASP-1 will undergo autoactivation (23). This enables it to subsequently activate MASP-2 present either within the same complex or in a complex in close vicinity (24). Worth noting is that MASP-2 can itself undergo autoactivation (25). However, MASP-2 activation is mainly mediated by MASP-1, and activation of MASP-1 is required for full activation of the lectin pathway (26). Both MASP-1 and MASP-2 can cleave C2 into C2a and C2b, but only MASP-2 is capable of cleaving C4, demonstrating the importance of its activation. Following the MASP-mediated cleavage of C2 and C4, the C3 convertase C4b2b is formed (27). This C3 convertase is identical to the one formed by the classical pathway, and all subsequent steps leading up to the terminal pathway are the same (8).

Map19, Map44 and MASP-3 are homologs of MASP-1 and MASP-2. They can bind both ficolins and MBL, but they lack the enzymatic activity needed to propagate the cascade. As such, they are considered negative regulators of the lectin pathway (8).

However, MASP-3 can reportedly cleave and activate pro-factor D, which suggests a role in the alternative pathway (28). Additionally, the soluble form of another collectin (CL-12) can reportedly induce complement activation via the alternative pathway (29).

The alternative pathway

In contrast to the trigger-dependent activation of the classical and lectin pathways by recognition of various molecules, activation of the alternative pathway can occur absent such molecular triggers. The alternative pathway is constantly active at low levels owing to the so-called "tick-over" mechanism, which entails the spontaneous hydrolysis of an intrinsic thioester bond of C3 (5, 7). This hydrolysis causes a conformational change, leading to the formation of C3(H_2O), which, similar to C3b, is capable of binding factor B (7). When bound to C3(H_2O), factor B can be cleaved by factor D, leading to the generation of Bb and Ba. While Ba is released, Bb remains associated with C3(H_2O), and together, they constitute the initial C3 convertase of the alternative pathway. Similar to the C3 convertase of the classical and lectin pathways, C3(H_2O)Bb is capable of cleaving C3 to generate C3a and C3b. The newly generated C3b can then bind factor B, and through factor D-mediated cleavage, the principal C3 convertase of the alternative pathway is formed (5). This C3bBb complex allows for full activation of the alternative pathway, both on C3b-bound surfaces and in the fluid phase (7). Similar to the other C3 convertases, C3bBb will cleave C3 to generate additional C3b molecules that can form additional C3 convertases in the presence of the serine proteases factor B and factor D. This constitutes a positive feedback loop wherein the system amplifies its own activation and C3b generated from either pathway can be utilised (7). The rate-limiting step is considered to be the enzymatic activity of factor D (30). In addition to providing an amplification loop for complement activation, the alternative pathway can also propagate the cascade further (6). C3b, generated through the cleavage of C3, can associate with the C3bBb complex to form the alternative pathway C5 convertase C3bBbC3b (6). This triggers the terminal complement pathway, which is described in the next subsection. The constitutive activation of the alternative pathway, together with the amplification loop, enables a rapid and robust response in times of need (7). However, it requires a strict regulation to avoid self-harm (5).

The alternative pathway convertase, C3bBb, is an unstable complex with a short half-life of only about 90 seconds (31). Properdin is expressed and secreted by various immune cells, including T cells (32) and mononuclear phagocytes (33). It is the only known positive regulator of the complement system (34), and it acts by stabilising the alternative pathway C3 convertase, thus preventing its decay (35). However, an additional role for properdin in triggering alternative pathway activation has also been found. By binding to specific targets, properdin is able to direct alternative pathway activation (34, 36, 37). Both C3b and the analogous

$C3(H_2O)$ molecule can also provide a platform for local alternative pathway activation by binding to cell surfaces (8).

The terminal pathway

Via the three activation pathways described, the complement cascade is initiated. In contrast to the distinct modes of initiation, all three pathways converge on a shared terminal pathway (**Figure 1**). All three pathways lead to the formation of a C3 convertase, C3bBb for the alternative pathway and C4b2b for the classical and lectin pathways. These C3 convertases will cleave C3 into C3a and C3b, the latter participating in the formation of a C5 convertase, which is the starting point for the terminal pathway. The C5 convertases C4b2b3b and C3bBbC3b cleaves C5 into C5a and C5b (5), with the latter acting as a scaffold for the formation of a multimeric complex known as the membrane attack complex (MAC) (38). The removal of C5a leads to the exposure of a C6 binding site on C5b (5). C5b is stabilised by the binding, which also causes C6 to undergo conformational changes (39). Subsequently, C7 is recruited to the C5b-6 complex, leading to the exposure of a lipophilic site that allows the complex to associate with the lipid bilayer of the plasma membrane (5, 40). Next, C8 is recruited to the complex, and it is responsible for the initial penetration of the membrane (38). Composed of the three subunits α, β and γ, C8 binds to the C5b-7 complex through its β subunit (41). This allows C8α to penetrate the membrane, enabling the recruitment of C9 (42). Following the incorporation of C9, additional C9 monomers will be recruited to the complex, and subsequent C9 polymerisation will cause a pore to be formed in the target membrane (5). The C5b-9 complex, known as MAC, can form pores as large as 10 nm in diameter (5, 38). The rate-limiting step in MAC formation is the incorporation of the first C9 molecule, as the ensuing oligomerisation is energetically favourable (43, 44). The pore-forming C9 oligomer comprises 12-18 C9 monomers (45, 46); however, a recent report suggests that it might be up to 22 (47). Through the formation of pores in the surface of target cells, the MAC causes osmotic cell lysis, leading to cell death (44).

Worth noting is that functions other than promoting cell lysis have been described for the MAC. In cells resistant to MAC-mediated lysis, the MAC can instead induce and affect intracellular signalling pathways, promote the release of inflammatory cytokines, or cause inflammasome activation. Even MAC precursors such as C5b-7 have been found to affect intracellular signalling events. In addition to complement inhibitors preventing its formation, certain cells can endocytose plasma membrane-associated MAC (44).

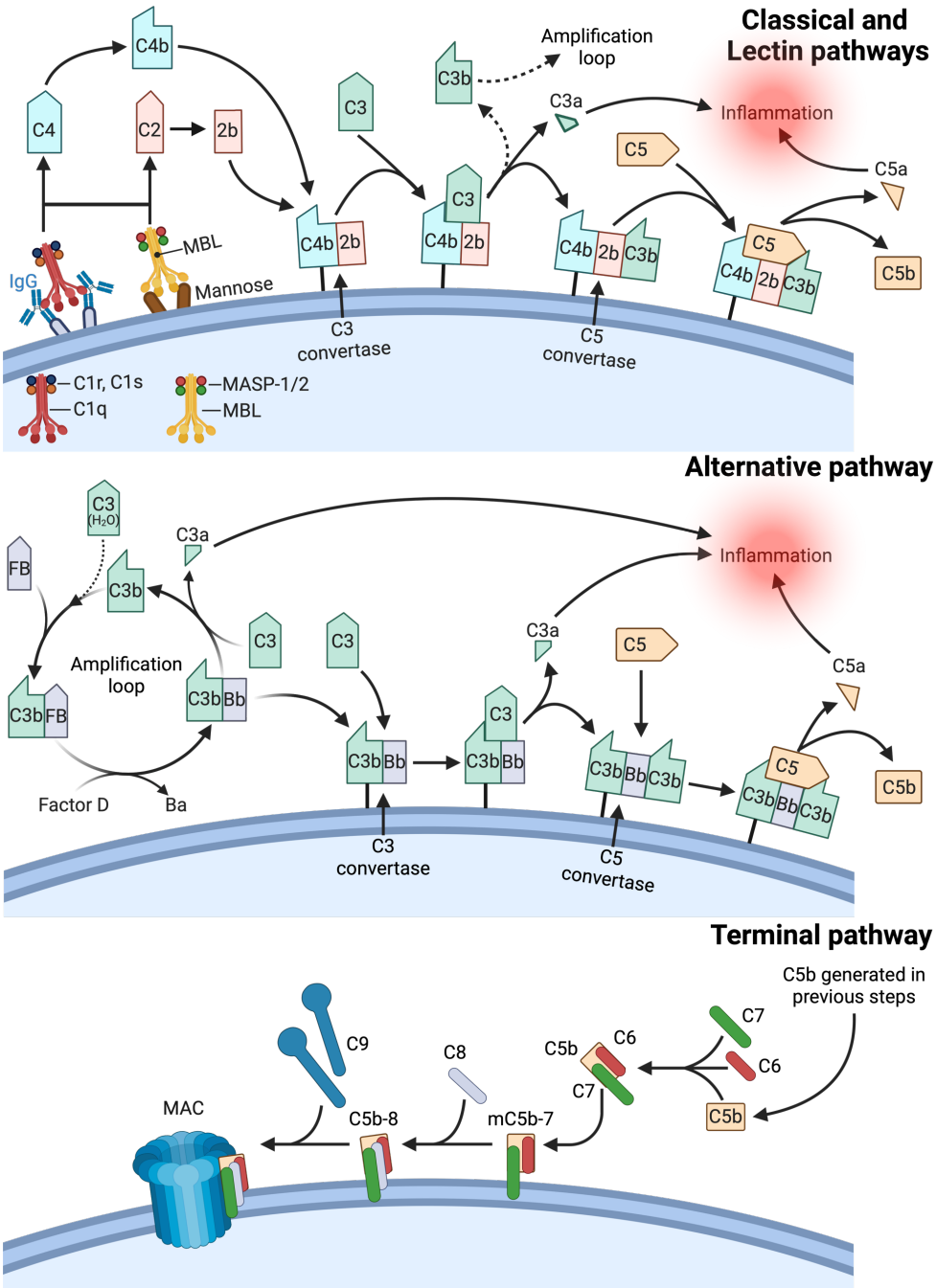


Figure 1. Simplified overview of the complement system's activation pathways. The terminal pathway leading to the formation of the MAC is illustrated at the bottom.

Inflammation and opsonisation

In addition to direct killing through MAC-mediated cytolysis, the complement system also has two other critical immunological functions, namely opsonisation and inflammation. These two functions are mediated by the various cleavage products generated during the complement cascade. The smaller fragments resulting from the cleavage of C4, C3 and C5 are denoted with a suffixed "-a" and function as potent mediators of inflammation (5). They are referred to as complement anaphylatoxins due to their proinflammatory properties. However, the inclusion of C4a in this group is debated as the activity of C4a as an anaphylatoxin is limited compared to C3a and C5a. Additionally, a specific C4a receptor is yet to be identified, and it is unable to activate known anaphylatoxin receptors (48). C3a and C5a mediate their functions by acting on the anaphylatoxin receptors, of which three have been identified, one for C3a (C3aR) and two for C5a (C5aR1 and C5L2). All of them are expressed by myeloid cells, but their expression has also been observed in many other cell types. C3aR and C5aR1 are, for example, also expressed by endothelial cells (49). The proinflammatory effects of complement anaphylatoxins are many but include vasodilation, vascular permeabilisation, chemotaxis and leukocyte recruitment, promoting histamine release from mast cells, and inducing respiratory burst in neutrophils and macrophages (5, 49). It is worth noting that the proinflammatory role of C5L2 is debated, and some reports indicate a more regulatory role (49).

In contrast to the anaphylatoxins, other cleavage fragments opsonise targets and facilitate phagocytosis and immune clearance. The opsonins are recognised by complement receptors (CR) 1-4 as well as CRIg, which belongs to the immunoglobulin superfamily (5). CR1 is expressed by, for example, erythrocytes and myeloid immune cells. It binds C1q, C4b and C3b, but also the C3b cleavage fragments iC3b and C3dg, which are generated by factor I mediated cleavage of C3b, as described later. Opsonin recognition by CR1 promotes phagocytosis, but it can also lead to the production of proinflammatory cytokines. In the case of erythrocytes, CR1 facilitates immune complex clearance (50). Contrastingly, CR2 is mainly expressed by B lymphocytes and plays a role in their activation. It recognises the C3b degradation products iC3b, C3d and C3dg (5). CR3 and CR4 are expressed by immune cells of the myeloid lineage and play an important role in phagocytosis (8). However, they also affect processes such as migration, adhesion and leukocyte trafficking (5). While both of them recognise iC3b, C3d is only recognised by CR3, and C3c only binds CR4 (8). CRIg can be found on tissue-resident macrophages, such as Kupffer cells, and is involved in phagocytosis and clearance of immune complexes by binding to C3b and iC3b (51).

Inhibitors of the complement system

The potent effector functions and the cascade-like nature of the complement system create a need for stringent regulatory mechanisms to avoid self-harm. Several complement inhibitors have been identified, which indirectly provide the system with the ability to distinguish healthy host cells from intruding pathogens. Complement is kept in check through the actions of these inhibitors, and host cells are protected from complement-mediated damage. Aberrant activation and dysregulation of complement have been implicated in the pathogenesis of a multitude of diseases. Both membrane-bound and fluid-phase inhibitors of the complement system are present; these will be described here. The two complement inhibitors SUSD4 and CD59 are at the centre of this thesis and will be described in greater detail further on.

Regulators of complement activation

A gene cluster harbouring the genes encoding several functionally and structurally related complement inhibitors is present on chromosome 1. This gene cluster and group of encoded proteins is known as "Regulators of complement activation", or RCA. A structural commonality among these proteins is the presence of a conserved amino acid motif known as complement control protein domain (CCP) or sushi domain. Each such CCP domain comprises 60-70 amino acids, including four highly conserved cysteine residues that form two internal disulphide bridges within the domain (1, 52). Each CCP domain also contains an additional 10-18 conserved amino acid residues (1). Each RCA protein is composed of several such CCP domains located in tandem, ranging from four (CD55 and CD46) to 59 (C4BP) (52, 53). Owing to the presence of the CCP domains, a common functional feature of the RCA proteins is their ability to bind C3b and C4b (1, 52). Worth noting, however, is that the presence of CCP domains is not exclusive to RCA proteins (53).

Factor H

Factor H is a soluble complement inhibitor and a member of the RCA family. It comprises 20 CCP domains and can inhibit complement both on cell surfaces and in the fluid phase. It plays an important role in regulating the alternative pathway and its amplification loop (54). Factor H has three regulatory functions. First, by acting as a cofactor for factor I (described later), factor H promotes the cleavage of C3b into C3f and iC3b, which lacks enzymatic activity (52). Second, through competitive binding of C3b, factor H can prevent factor B from binding C3b and, thus, inhibit the formation of the C3 and C5 convertases of the alternative pathway. Third, factor H can also accelerate the decay of alternative pathway convertases by dissociating factor Bb from the enzymatic complex (54).

C4BP

C4b-binding protein (C4BP) is a fluid-phase complement inhibitor of the RCA family and contains 59 CCP domains (52). C4BP plays an inhibitory role in the regulation of both the classical and lectin complement pathways. C4BP can both accelerate the decay of the C3 convertase C4b2b and inhibit its formation by sequestering newly formed C4b (55). Additionally, through its cofactor activity for factor I, C4BP can promote the cleavage of C4b into C4c and C4d (1).

CD55

CD55, also known as decay-accelerating factor (DAF), is a glycosylphosphatidylinositol (GPI)-anchored complement inhibitor. It contains four of the CCP domains characteristic of RCA proteins. CD55 is widely expressed and present on almost all cells. As implied by its alternative name, CD55's regulatory function lies in the ability to accelerate the decay of convertases. CD55 can accelerate the decay of both C3- and C5 convertases of all complement activation pathways. Anchored to the cell surface, CD55 can only disrupt convertases present on the same cell; it cannot affect convertases present on other cells (1, 52).

Similar to CD59 (described later), the gene encoding CD55 has undergone duplication in mice, leading to the existence of *Daf1* and *Daf2*. While *Daf1* is widely expressed, *Daf2* expression is restricted to the testis (56).

CD46

CD46, also known as membrane cofactor protein, is a ubiquitously expressed complement regulator present on almost all cells except for erythrocytes (1, 56). Unlike CD55, CD46 does not accelerate the decay of convertases, but its activity can be considered to complement the role of CD55 (1, 52). CD46 acts as a cofactor for the serine protease factor I (described later), which cleaves C3b into iC3b and C4b into C4c and C4d (1). Similar to CD55, CD46 is a membrane-bound RCA protein containing four CCP domains (52). However, CD46 is a transmembrane protein and not a GPI-anchored protein (56). Like CD55, it can only provide protection to the cell where it is attached (1).

In mice, the expression of CD46 is limited to the testes. However, mice possess a functionally related transmembrane regulator called Crry. This complement regulator is ubiquitously expressed and has both CD55- and CD46-like activities (56).

CR1 (and CR2)

Anchored via its transmembrane domain, CR1 is a membrane-bound complement inhibitor of the RCA family, and it is composed of 30 CCPs (56). CR1 is mainly expressed by circulating cells, and it has a multitude of functions (52). CR1 can bind C1q, C3b, iC3b and C4b and is involved in the regulation of both the classical and

alternative pathways. By binding C4b and C3b, it accelerates the decay of both classical and alternative pathway C3- and C5 convertases by displacing C2b or Bb from the convertase complexes. CR1 also has cofactor activity and promotes factor I-mediated cleavage of C4b into C4c and C4d and C3b into iC3b. It also enables further factor I-mediated iC3b cleavage into C3dg and C3c (57).

Furthermore, CR1 also functions in the clearance of immune complexes. C3b or C4b coated immune complexes can be bound by erythrocytes through CR1. The immune complexes are then transferred to the liver and spleen, where resident macrophages clear them from the circulation (58). Additionally, CR1 present on phagocytic immune cells promotes phagocytosis of C3b-opsonised bacteria, for instance (52).

CR2 is encoded by a gene present within the RCA gene cluster; however, it does not possess any complement regulatory function (52). Although it binds C3 cleavage fragments such as C3dg and C3d, it is mainly expressed by and has a role in B cells (59).

Other complement inhibitors

Factor I

Factor I is a serine protease present in the circulation in its active form (52, 60). The function of factor I has been touched upon in the description of other complement inhibitory proteins. Factor I is involved in the regulation of all complement activation pathways as it is capable of cleaving and inactivating both C3b and C4b (60). Factor I-mediated cleavage of C4b results in the generation of C4c and C4d, while cleavage of C3b leads to the generation of iC3b and C3f. However, factor I-mediated cleavage of these complement fragments is dependent on the presence of cofactors. The cofactor activity of C4BP promotes C4b cleavage, while the cofactor activity of factor H promotes C3b cleavage. The cofactor activity of CR1 and CD46 can facilitate factor I-mediated cleavage of both C3b and C4b. However, CR1 is unique in the sense that it can also induce iC3b cleavage into C3dg and C3c (52).

Clusterin

Clusterin, also known as apolipoprotein J, is a glycosylated complement inhibitor present in the plasma. It functions by inhibiting the formation of the terminal complement complex, i.e., the MAC. Clusterin can bind to a common structural motif present in C7, C8β, and C9 that becomes exposed during the encompassing conformational changes of MAC assembly. Clusterin thus inhibits MAC formation and gives rise to soluble inactive terminal complexes (61).

Vitronectin

Vitronectin, also known as S protein, is, similar to clusterin, an inhibitor of the terminal complement pathway. Vitronectin binds the C5b-7 complex and prevents membrane insertion and further assembly into a complete MAC. Furthermore, vitronectin is also capable of binding soluble C5b-7, C5b-8 and C5b-9 complexes. When bound to soluble C5b-7, the recruitment of C8 and C9 is still permitted, yet the soluble complexes bound by vitronectin are haemolytically inactive. The binding of vitronectin also facilitates the clearance of these complexes (62).

C1-inhibitor

C1-inhibitor is a plasma-borne serine protease inhibitor that functions not only in the complement system but also in the coagulation system and contact system. C1-inhibitor is capable of inhibiting both the classical and lectin pathways of complement activation by targeting C1r and C1s, as well as MASPs 1 and 2 (63). C1-inhibitor can be said to function as a decoy as it exposes a reactive loop that mimics the serine proteases' substrates. The cleavage of C1-inhibitor by the serine proteases leads to an irreversible binding between the protease and the inhibitor (52, 63). C1-inhibitor thus acts as a suicide inhibitor of the proteases. In the context of C1 inhibition, the action of C1-inhibitor leads to a dissociation of the C1 complex (52). C1-inhibitor can also interact with the C1r zymogen, precluding its autoactivation. Following the recognition and binding of the C1 complex to an activator, such as immunoglobulins, the C1-inhibitor dissociates, which allows for C1r autoactivation (64).

CSMD1

The human CUB and Sushi Multiple Domain protein 1 (CSMD1) is a transmembrane protein with complement inhibitory properties. The extracellular portion of CSMD1 comprises a continuous string of 15 CCP domains followed by 14 CUB domains, each interspersed with one CCP domain. The complement inhibitory function of CSMD1 is two-fold. It can both act as a cofactor for factor I, promoting the cleavage of C3b and C4b, and it can interact with C7, prohibiting its recruitment to C5b-6 in the process of MAC-assembly (65).

Carboxypeptidase N

The zinc metalloprotease carboxypeptidase N is produced in the liver and released into the circulation. Its function in the complement system is to inactivate anaphylatoxins. As described previously, complement activation leads to the generation of complement cleavage fragments, including the anaphylatoxins C3a and C5a. These are potent inflammatory mediators exerting their functions by interacting with their respective receptors. Carboxypeptidase N can enzymatically remove arginine residues present at the carboxy-terminal of C3a and C5a, causing a 10-100-fold decrease in their activity. The resulting C3a-desArg is unable to

interact with the C3aR, while the ability of C5a-desArg to bind the C5aR is dramatically reduced (66).

Sushi domain-containing protein 4

Sushi domain-containing protein 4 (SUSD4) belongs to a family of diverse transmembrane proteins composed of six members. SUSD1-6 are encoded by genes present on distinct chromosomes, and their tissue expression patterns differ from each other. Despite containing different functional domains, a commonality between them is the presence of a transmembrane domain and one or more CCP domains. While SUSD1 contains two CCP domains, SUSD2, SUSD3, SUSD5 and SUSD6 all contain only a single CCP domain (67).

In humans, SUSD4 is encoded by a gene present on chromosome 1q41. The gene is composed of eight exons, and as a result of alternative splicing, it is predicted to encode two isoforms named SUSD4a and SUSD4b (67). SUSD4a is a membrane-bound 49 kDa protein containing a transmembrane domain in addition to four CCP domains (68). It also contains a cytoplasmic portion in which two phosphorylation sites are present (69). The presence of these phosphorylation sites has been suggested to confer a role in cellular signalling, and they have already been shown to affect certain functions of the protein (67, 69). Contrastingly, SUSD4b lacks a transmembrane domain and is a 27 kDa soluble protein composed of three CCP domains as well as a portion of unascertained homology. Both isoforms, however, harbour several potential N-glycosylation sites (68).

Although tagged and recombinant SUSD4b could be successfully expressed and purified from a Chinese hamster ovary cell line or human Freestyle 293-F to be used for complement deposition assays (68), the wild type protein remains to be detected in tissues. Additionally, SUSD4b could not be detected at protein level when expressed in breast cancer cell lines (70). As such, SUSD4 will henceforth refer to SUSD4a unless otherwise specified.

At the primary structure level, the SUSD4 protein is highly conserved across species. The homology between human SUSD4 and mouse or zebrafish SUSD4 is 95- and 63 percent, respectively. In mice, SUSD4 has been detected at protein level in tissues such as testes and spinal cord. The highest expression, however, was seen for the brain and eyes (71). At mRNA level in humans, both SUSD4a and SUSD4b were found to have a fairly broad tissue expression. While the brain and oesophagus were the primary sites of SUSD4a expression, SUSD4b was found to be highly expressed in the heart and ovaries, in addition to the brain and oesophagus (68).

SUSD4 as a complement inhibitor

An initial study exploring the complement regulatory activity first described SUSD4 as a potentiator of the alternative pathway. Onwards, they showed that SUSD4 is

able to bind C3b (71). However, this study was later disputed, and a new inhibitory role was presented instead. The subsequent study showed that membrane-bound SUSD4a could inhibit C3b deposition by both the classical and alternative pathways. Furthermore, recombinantly expressed and purified SUSD4b was found to inhibit both the classical and lectin pathways of complement activation by inhibiting C3 convertase formation. It did not, however, exhibit any decay-accelerating activity. Moreover, SUSD4b was found to be able to bind both C1q and the C1 complex, but it did not affect the deposition of either C1q or MBL. Additionally, the presence of SUSD4b prevented C1s-mediated cleavage of C2 and inhibited the deposition of C4b and C3b by both the classical and lectin pathways (68).

Worth noting is that, in accordance with the general notion that a minimum of three CCP domains is needed for complement inhibitory activity, no complement regulatory function has been described for the other members of the SUSD family (67).

Pathology

The 1q41q42 microdeletion syndrome manifests with symptoms such as delayed development, facial dysmorphia, seizures, short stature and diaphragmatic hernia. A fraction of those affected will present with Fryns syndrome (72), which is typically a lethal syndrome during the neonatal phase (71). In addition to the symptoms already described for the 1q41q42 microdeletion, those who survive with Fryns syndrome further present with symptoms such as hypoplasia of distal limbs and anophthalmia (71). When analysing the smallest region of overlap in individuals with the 1q41q42 microdeletion, SUSD4 was one of five candidate genes identified, thus linking it to Fryns syndrome (73). In line with a potential link between SUSD4 and Fryns syndrome, the silencing of SUSD4 in zebrafish embryos led to increased mortality and developmental delays (71).

Other roles of SUSD4

In contrast to zebrafish, SUSD4 knockout mice are reportedly viable and growing normally. However, SUSD4 knockout mice displayed impaired coordination and motor function. Additionally, behavioural assessments found that mice lacking SUSD4 exhibited less exploratory- and more anxiety-like behaviour than wild type mice. Although no learning- or memory disabilities were identified in that study (74), a later publication addressed and evidenced such impairments (69). Furthermore, an abnormal morphology was observed for neuronal cells in both the hippocampus and cerebellum of SUSD4 knockout mice. Interestingly, SUSD4 was found to be expressed at a relatively high level in neuronal cells in these regions. The hippocampus and cerebellum are linked to anxiety and motor control, respectively. Moreover, microglia cells play a role in synaptic pruning, a process that is facilitated by complement factors, such as C1q. In addition to indications of

increased microglial activation in SUSD4 knockout brains, both increased levels of C1q and increased deposition of C1q at synapses have been observed. Together, these results suggest that the process of synaptic pruning might be dysregulated in the absence of SUSD4 (74).

SUSD4 has also been implicated in the long-term synaptic plasticity of cerebellar Purkinje cells in mice. The induction of long-term potentiation at Purkinje cell synapses was promoted in SUSD4 knockout mice, while the induction of long-term depression was impaired. The regulation of AMPA receptors at excitatory synapses is a dynamic process that involves endocytosis following their activation. Subsequent recycling to the surface results in long-term potentiation, while long-term depression is the result of endolysosomal targeting and degradation. Whether neurotransmitter receptors are recycled or degraded needs to be properly regulated in order to maintain synaptic plasticity and ensure proper memory and learning. SUSD4 has been found to interact with both an AMPA receptor subunit, GluA2, and a ubiquitin ligase known for ubiquitinating GluA2 AMPA receptors, thus promoting their degradation. Consequently, it has been suggested that in cerebellar synapses, SUSD4 plays a role in synaptic plasticity and long-term depression by targeting GluA2 AMPA receptors for endolysosomal degradation. Interestingly, the two phosphorylation sites in the cytoplasmic domain of SUSD4 were shown to be functionally important for the interaction with the ubiquitin ligase (69).

Moreover, it has been suggested that SUSD4 potentially could serve as a prognostic marker in various types of cancer. When comparing the expression of SUSD4 in tumour tissues with that in normal tissues, SUSD4 was found to be upregulated in some tumour types while downregulated in others. Additionally, depending on the type of cancer, either a high or low expression of SUSD4 was associated with a poor prognosis for patients (75). This also fits well with the general view that in cancer, the role of complement is context-dependent (76). Moreover, the knockdown of SUSD4 in colorectal cancer cell lines resulted in impaired proliferation. In a cell line-derived xenograft model, the expression of SUSD4 resulted in increased tumour size compared to tumours devoid of SUSD4. Furthermore, the silencing of SUSD4 in a colorectal cancer cell line led to a downregulation of JAK3 but an upregulation of several other JAK/STAT pathway genes. This pathway has been linked to processes such as proliferation, apoptosis and migration (75).

Additionally, when analysing RNA sequencing data of peripheral blood mononuclear cells (PBMCs) from patients who received a renal transplant and were treated with two different immunosuppressive agents, SUSD4 was one of four identified genes clearly distinguishing the two groups. All four genes, including SUSD4, were found to be significantly downregulated in PBMCs from patients treated with an inhibitor of mammalian target of rapamycin (mTOR) compared to PBMCs from patients treated with a calcineurin inhibitor. The mTOR inhibitor also caused a downregulation of SUSD4 in PBMCs *in vitro* (77).

CD59

Note that when stating amino acid positions, the 25 amino acid N-terminal signal sequence is included with the starting methionine, thus being residue number 1. The position in the mature protein is given in parentheses.

Gene, structure and distribution

CD59, also known as protectin, is structurally related to a superfamily of proteins that includes urokinase-type plasminogen activator receptor (uPAR) and lymphocyte antigen 6 proteins (Ly6). These are GPI-anchored proteins that share a structural motif known as the three-fingered protein domain (78), which is composed of five β-strands and three highly conserved disulphide bonds (79). CD59 itself contains three β-sheets and an α-helix. However, lower-resolution crystal structures also suggested two short β-sheets at the N-terminus, but these were not present at higher resolution. Additionally, a second small α-helix could be identified at a higher resolution (80). CD59 also contains five disulphide bridges (81, 82).

In humans, the *CD59* gene is located on chromosome 11 (52). Initial reports concerning the span of the gene ranged from 20- to >27 kb. Four exons were then identified. The 45 nucleotides encoded by exon 1, as well as the first 18 nucleotides of exon 2, together constitute the 5´untranslated region. Exon 2 also codes for 22 amino acids that, together with the first three amino acids encoded by exon 3, make up the N-terminal signal peptide of the immature protein. Exon 3 also encodes for an additional 31 amino acids, while exon 4, in addition to the 3´untranslated region, also encodes the remaining 72 amino acids of the protein (83, 84). A fifth alternatively spliced exon was later identified between exons 1 and 2, but even when transcribed, it did not affect the protein structure (52). A new report describes the gene as spanning around 33.5 kb and containing seven exons where exons 5-7 are encoding the CD59 protein (85).

The initially translated single-chain immature CD59 comprises 128 amino acids, of which 25 constitute the N-terminal signal sequence. A C-terminal motif comprising 26 amino acids that signals for GPI-anchor attachment is also present in the immature protein (85). The N-terminal signal sequence of immature GPI-anchored proteins directs the protein to the endoplasmic reticulum (ER) but is removed following the translocation of the protein. In the ER, the signal sequence for GPI-anchor attachment at the C-terminus will be recognised, cleaved off and replaced with a GPI-anchor by means of a GPI transamidase. Once the processing is finished, the GPI-anchored proteins are transferred to the plasma membrane via the Golgi, where additional processing may occur (86). The mature CD59 protein contains 77 amino acids with the GPI-anchor attached to Asn102 (Asn77 excluding the N-terminal signal peptide) (85).

CD59 is an 18-23 kDa protein that is expressed by almost all cells (52, 87). Erythrocytes are estimated to express around 25,000 molecules of CD59 per cell,

while an even higher expression can be seen for nucleated cells (52). CD59 is a heavily glycosylated protein, as suggested by its detection at a molecular mass of 12 kDa following enzymatic removal of glycans (52). It carries an N-linked glycan group of 4-6 kDa at Asn43 (Asn18 in the mature protein) (85). The N-linked glycan group was found to be highly diverse when assessed in erythrocytes; over 120 forms have been identified. CD59 may also carry O-linked glycans at Thr76 and/or Thr77 (Thr51 and Thr52 in the mature protein) (88).

Complement inhibitory function

The last defensive line against complement-mediated cytolysis is conferred by CD59. It is the sole inhibitor of the multimeric complex formed during the terminal complement pathway that is present at the cell surface. GPI-anchored at the cell surface, CD59 serves to protect host cells from complement-mediated cell death by inhibiting the formation of the MAC. It acts as a suicide inhibitor, incorporating itself into the assembling complex (89). CD59 can interact and form stable complexes with both C5b-8 and C5b-9. CD59 prevents MAC formation by inhibiting C9 recruitment and polymerisation. CD59 is capable of interacting with both C8 and C9. It binds to the α-chain of C8 as well as the C9b domain of the C9 molecule. The binding site of C9 is contained within the α-chain of C8 (90). Following their recruitment to the assembling MAC, both C8 and C9 undergo conformational changes, leading to the formation of transmembrane structures. CD59 inhibits the proper unfolding of particular domains within C8 and C9, thereby preventing membrane penetration and cell damage. Based on cryo-electron microscopy analysis, it was recently suggested that the CD59-inhibited immature C5b-9 complex may contain up to a few C9 copies. In the same study, a model was proposed wherein CD59 inhibits further C9 polymerisation by maintaining C9 in a transitional state that prevents stable binding of additional C9 molecules (89).

CD59 also exists in a soluble form that can be detected in bodily fluids such as urine and plasma. Although the binding capability for the MAC is retained, the ability to inhibit the MAC is impaired. The absence of the lipid moiety in the GPI anchor precludes its incorporation into membranes, thus limiting its ability to inhibit complement (91).

A mutational analysis assessing the importance of particular amino acid residues led to the identification of key amino acids that are thought to be part of the active site of CD59. By means of amino acid substitution mutations, Trp65 (Trp40), Arg78 (Arg53) and Glu81 (Glu56) were found to be important for the MAC-inhibitory function of CD59. Cells expressing mutant CD59 with amino acid substitutions at either of these sites were not protected from complement-mediated cytolysis. A fourth important residue, Asp49 (Asp24), was also identified. A substitution at this site also abrogated the protection against complement-mediated lysis. However, the N-glycosylation of CD59 was found to be inessential. By mutating the Asn43 (Asn18), a non-glycosylated mutant variant of CD59 was expressed. This mutant

was fully capable of inhibiting complement. Additionally, the mutant was recognised by various antibodies targeting CD59, suggesting that the protein structure was not affected by the absence of the N-linked glycan group (92).

Other functions of CD59

Several studies have been conducted addressing a potential role for CD59 in T cell biology and activation. Albeit controversial, two initial studies first suggested a direct interaction between CD59 and CD2 (93, 94), which plays a costimulatory role in T cell activation (95). However, others have disputed this as they were unable to replicate the findings (96, 97). Other studies have, however, indicated a role for CD59 in T cell stimulation in a manner that involves both CD2 and CD58 (98, 99). Another study has suggested an intracellular role for CD59 in T cells wherein it interacts with Ras and affects Ras/MAPK signalling, thus influencing T cell activation (100).

Onwards, CD59 has been identified as a receptor for intermedilysin, a toxin produced by the pathogenic bacterium *Streptococcus intermedius*. Intermedilysin forms pores in the surface of target cells, leading to cell lysis. It binds to the same domain of human CD59 that is involved in the binding of C8 and C9 (101). Moreover, the calcium-binding protein calreticulin typically resides in the ER, yet it has been observed on the surface of neutrophils. As it does not contain a transmembrane domain of its own, the cell surface localisation is dependent on an adaptor present at said location. In neutrophils, CD59 has been implicated as an adaptor protein facilitating calreticulin's surface localisation (102). Furthermore, CD59 has been found to be upregulated in both solid tumours and in some cancer cell lines. This could be a mechanism employed by the cancerous cells to shield themselves from complement-mediated damage (52). Conversely, an absence of CD59 has been observed for both leukemic cell lines and patient-derived leukaemia cells (103).

CD59 also contains a glycation site within its active site, and it was found that CD59 can be glycated at Lys66 (Lys41 in the mature protein) in the presence of glucose. This is referred to as glycation-inactivation since it abrogates the complement-regulatory role of CD59. Consistent with this, erythrocytes from diabetic individuals were found to be more susceptible to MAC-mediated cytolysis than erythrocytes from healthy individuals. Furthermore, the inactivation of CD59 through glycation has been suggested to play a role in several diabetic complications. For instance, atherosclerosis, which is a well-known diabetic complication, was found to be accelerated in diabetic mice lacking CD59. Additionally, glycated CD59 colocalised with MAC deposits in tissues associated with diabetic complications (104). Soluble glycated CD59 can be found in both urine and blood, and it has been shown that glycated CD59 can serve as a sensitive biomarker for diabetics and their glycaemic control (105). Interestingly, the glycation motif has only been identified in human CD59 and not in other species (106).

Pathology

A disease associated with CD59 is paroxysmal nocturnal haemoglobinuria (PNH), which is a rare acquired haematological disorder. Due to complement-mediated cytolysis of erythrocytes, afflicted individuals suffer from haemolytic anaemia. It develops as a result of a somatic mutation in a haematopoietic stem cell, which may then undergo clonal expansion. The somatic mutation occurs in a gene known as *PIG-A* (phosphatidylinositol glycan class A), the product of which is involved in the initial step of GPI-anchor synthesis. As a result, the biosynthesis of GPI anchors is abrogated. Clonal expansion of the aberrant cell harbouring the *PIG-A* mutation will lead to circulating cells in the blood devoid of GPI-anchored proteins. Most notable is the absence of CD59 and CD55 (107). The lack of these complement inhibitors renders the cells susceptible to complement-mediated lysis. The PNH-like symptoms exhibited by patients with congenital CD59 deficiency suggest that the absence of CD59 is more critical. Although the GPI-anchored complement inhibitors are absent on the surface of leukocytes as well, erythrocytes are particularly susceptible. Unlike the leukocytes, erythrocytes are also devoid of CD46 (107). The most common cause of death in PNH patients is thromboembolism, i.e. blood clots (108). Several factors may contribute to the thrombotic events in PNH patients, including the proinflammatory processes encompassing complement and platelet activation (109). PNH is effectively treated and managed with Eculizumab, a humanised antibody targeting C5, thus inhibiting the terminal complement pathway. Not only is it preventing haemolysis, but it is also markedly lowering the risk of thrombotic events in PNH patients (108).

Additionally, six different mutations giving rise to congenital CD59 deficiency have been identified and described in patients. Five of these are located in exon sequences encoding the mature CD59 mRNA. Two of them, p.Ala41Alafs and p.Asp49Valfs, cause a frameshift, while the remaining three are missense mutations (110). The two frameshift mutations, p.Ala41Alafs and p.Asp49Valfs, result in truncated variants. Albeit containing an N-terminal signal peptide, they lack a GPI anchor and are consequently absent from the cell surface. Instead, they were found intracellularly and are either secreted or targeted for proteasomal degradation. Contrastingly, the missense mutants p.Cys89Tyr and p.Asp49Val were both present at the cell surface, yet known antibodies targeting CD59 could not recognise them, suggesting an abnormal protein structure. Furthermore, both p.Cys89Tyr and p.Asp49Val, as well as the frameshift variants, are incapable of inhibiting MAC-mediated cytolysis (111). The patients in which the p.Cys89Tyr, p.Asp49Val, p.Ala41Alafs and p.Asp49Valfs were identified all suffered from haemolytic anaemia. All but one patient was suffering from peripheral neuropathy, and many of them had recurrent strokes (111).

Another homozygous missense mutation, p.Tyr29Asp, has been identified in a young girl who was first diagnosed with Guillain-Barré syndrome and later with systemic lupus erythematosus. CD59 was found to be absent on both erythrocytes

and leukocytes in the patient, and although she exhibited neuropathy, she did not present with PNH (112).

The sixth mutation described was identified in a young boy who was first diagnosed with Guillain-Barré syndrome and later with atypical haemolytic uremic syndrome. Genome sequencing identified a homozygous mutation in a splice donor site in intron 1 (c.67 + 1G > T) of the gene encoding CD59. Consequently, CD59 could not be detected on erythrocytes, and essentially no mRNA expression could be seen. The patient is being treated with Eculizumab, which is now used as a therapeutic intervention for congenital deficiencies of CD59 (110).

CD59 in mice

Unlike humans, mice possess two *CD59* genes as a result of gene duplication; these are named *CD59A* and *CD59B* (113, 114). Both are located on chromosome 2 and together, they span a region of 45.6 kb, with *CD59A* being located about 11.6 kb downstream of *CD59B*. The length of the *CD59B* and *CD59A* genes is 15- and 19 kb, respectively. At both genomic- and amino acid level, the two genes or proteins are 60 % identical. At the level of mRNA, they are 83 % identical. Both genes are composed of four exons exhibiting different levels of homology (114). While exon 1 of the two genes share no homology, exon 2 is identical. Exons 3 and 4 share 86- and 84 % homology, respectively (113). For both genes, the 5´untranslated region is contained within exon 1 and the beginning of exon 2. The rest of exon 2, along with exons 3 and 4, provide the coding sequence (115). Both CD59A and CD59B contain a N-glycosylation site. However, CD59A also contains a second potential N-glycosylation site at Asn94 (Asn71 in the mature protein), which explains its slightly larger molecular mass despite being eleven amino acids shorter than CD59B (116). Both CD59A and CD59B have been shown to inhibit MAC formation and protect against complement-mediated lysis (113, 117).

A broad tissue expression for CD59A is and has been the general consensus. The tissue expression pattern for CD59B, however, has been debated. Although a broad expression pattern has been suggested (114), most reports argue a tissue-restricted expression pattern and show that CD59B is primarily expressed in testes and spermatozoa (113, 116, 117). Further complicating the gene structure and regulation of expression, an alternative exon 1 located slightly upstream of the “canonical” exon 1 was identified for *CD59B*. This alternative exon 1 is identical to exon 1 of *CD59A*. Furthermore, an alternative exon 1 for *CD59A* has been identified between the “canonical” exons 1 and 2. This alternative exon 1 of *CD59A* shares 97 % identity with the “canonical” exon 1 of *CD59B*. Consequently, two variants of each mouse *CD59* gene can be expressed, differing only in their 5´untranslated region. Each of these two variants has a separate promoter region, conferring a particular expression pattern, one exhibiting a broad tissue expression while the other is seemingly restricted to the testes tissue (115).

Although preliminary studies suggested that CD59B was a more potent complement inhibitor than CD59A (114), a later study disputed this and showed that the contribution of CD59B in erythrocyte protection was irrelevant in the presence of CD59A. This could potentially be explained by the much higher presence of CD59A than CD59B on the surface of these cells. However, in the absence of CD59A, CD59B was shown to confer protection against complement cytolysis (116). Interestingly, though, even if erythrocytes from *CD59A* knockout mice exhibited increased susceptibility to complement-mediated lysis *in vitro* and the mice showed signs of intravascular haemolysis, the *CD59A* knockout mice were not anaemic (118). In contrast, *CD59B* knockout mice developed haemolytic anaemia and a strong PNH-like phenotype. Erythrocytes from *CD59B* knockout mice were also susceptible to complement orchestrated cytolysis (119). However, it was later revealed that the knockout of *CD59B* conferred a concomitant downregulation of CD59A in the mouse model, which may explain the contradictory results. In the *CD59A* knockout mouse model, CD59B was not found to be downregulated (115).

Interestingly, inflammatory conditions such as tumour necrosis factor-α (TNF-α) and lipopolysaccharides have been found to upregulate CD59B as a protective response. This upregulation was mediated by serum response factor and nuclear factor-kappa B (NF-κB). This induced expression could not be seen for CD59A, whose constitutive expression was regulated by the transcription factor Sp1. In humans, Sp1 regulates normal expression of CD59, whereas NF-κB is involved in induced expression (120). Moreover, consistent with the idea that CD59B plays a role in reproduction based on its high expression in the testes and spermatozoa (117), male *CD59B* knockout mice progressively developed infertility (119). This was associated with impaired mobility, abnormal shape and reduced number of sperm cells.

Intracellular complement

What has been described thus far is considered the canonical extracellular function of complement. This includes its role in inflammation, opsonisation, and direct killing of pathogens through MAC formation. However, the view of complement has changed with the discovery of intracellular complement. Its intracellular presence comes as no surprise, as it needs to be produced and secreted by the cells to function extracellularly; the surprising discovery was that it has important functional roles within the cells. Although extracellular non-canonical roles of complement have also been identified, the focus here will be on non-canonical intracellular functions, which constitute an emerging area of research. Although some of the functions that will be described are arguably related to the canonical function, either the means of activation, source of complement, or function can be

considered non-canonical. In reference to intracellular active complement, the term "complosome" has been introduced. Worth noting, however, is that the concept of active intracellular complement is a controversial topic plagued by many questions (121, 122).

Intracellular complement has been shown to affect a wide range of cellular processes, such as autophagy, metabolism, and gene expression. Much of the focus has concerned C3 and C5, including their cleavage products and the cognate anaphylatoxin receptors. Different modes of intracellular C3 and C5 activation have been reported. For example, the presence of intracellular alternative pathway convertases driving the generation of C5a has been reported for monocytes and macrophages during sterile inflammation. By acting on the C5aR1 present in mitochondria, the convertase-generated C5a altered mitochondrial function and promoted IL-1β production. Given the expression of C4 and C2 and the detection of C4b and C2b in monocytes, the authors of the study also suggested the plausibility of intracellular classical pathway convertases (123). Another mechanism for the intracellular generation of C5a has been reported to occur in colorectal cancer cells. According to this study, C5 present in endosomal or lysosomal compartments can be cleaved by cathepsin D. The resultant generation of C5a subsequently promoted β-catenin stabilisation through its activation of C5aR1. As β-catenin then drives the expression of oncogenes, the intracellular generation of C5a was found to promote tumourigenesis (124).

Similar to the case with C5, intracellular C3 stored in lysosomes can be cleaved into C3a and C3b by cathepsin L. In resting $CD4^+$ T cells, for example, the intracellularly generated C3a will act on its cognate receptor on lysosomes, which promotes survival through low-level activation of mTOR. Additionally, upon engagement of the T cell receptor, the intrinsically produced C3b will act on CD46, which causes the cytoplasmic domain of the complement inhibitor to translocate to the nucleus. This results in metabolic changes that are needed for the induction of interferon-γ (IFNγ) producing Th1 cells (125, 126). The cytoplasmic domain of CD46 will induce the expression of both glucose- and amino acid transporters, which enables the T cells to meet the increased metabolic demand encompassing activation. Furthermore, in resting $CD4^+$ T cells, CD46 is associated with Jagged1. Upon C3b engagement of CD46, Jagged1 is freed and can interact with Notch1, which contributes to the induction of Th1 cells. In addition to playing a costimulatory role in the induction of IFNγ-producing Th1 cells, CD46 can also, in the presence of interleukin-2 (IL-2), contribute to a phenotypic switch of Th1 cells, wherein they go from producing IFNγ to instead produce the immunosuppressive IL-10 (126). Similarly, CD46 also plays an important role in the function of $CD8^+$ T cells by affecting their metabolism (125). Moreover, in $CD4^+$ T cells, intracellular C5a engages the C5aR1, leading to the generation of reactive oxygen species (ROS). This, in turn, causes inflammasome activation and IL-1β secretion. In an autocrine

fashion, the secreted IL-1β promotes IFNγ production and induction of Th1 cells. It is worth noting that the alternative C5a receptor, C5L2, present at the cell surface, negatively regulated inflammasome activation (127).

Several studies have identified novel functions for the central complement system component C3. For instance, C3 has been found to interact with ATG16L1, which plays an important role in autophagy. Through this interaction, invasive bacteria opsonised with C3 are targeted for xenophagy, a type of autophagy specific for the degradation of pathogens (128). The process of autophagy and the role of ATG16L1 in it will be described in detail in a later section. Another study found that cell-intrinsic intracellular C3 can opsonise invasive bacteria. This did not affect the survival of the bacteria within the cells, but it did facilitate phagocytosis of the bacteria following their escape from the cells (129). Although this is related to its canonical function, the source of the opsonising C3 was non-canonical. Moreover, intracellular C3 has also been found to confer cytoprotective effects in pancreatic β-cells. These will be described later on in the context of complement in diabetes.

A non-canonical role for factor H has been identified in lung adenocarcinoma cells and in renal carcinoma cells. The intracellular factor H in these cells was localised, together with C3, in lysosomes and its presence was associated with a poor prognosis. Additionally, the intracellular factor H was found to affect viability, proliferation, gene expression and migration (130). Moreover, apoptotic cells have been found to actively internalise factor H, which then acts as a cofactor for cathepsin L-mediated cleavage of endogenous C3. This led to increased opsonisation of the apoptotic cells, thus facilitating phagocytosis (131).

Moreover, an intracellular role for the alpha chain of C4BP has been reported in colorectal cancer cells. The C4BP alpha chain was found to interact with a member of the NF-κB pathway and regulate anti-apoptotic NF-κB signalling. Increased intracellular levels of the C4BP alpha chain sensitised the cancer cells to apoptosis induced by a chemotherapeutic agent (132). Another cancer-related non-canonical function of a complement inhibitor concerns CSMD1, which functions as a breast cancer suppressor. In triple-negative breast cancer cell lines, CSMD1 was found to interact with the epidermal growth factor receptor (EGFR), inhibit its activation and downstream signalling, and alter the intracellular trafficking of the receptor. Furthermore, this sensitised the cancer cells to chemotherapy (133).

Intracellular CD59 has been implicated in T cell-mediated anti-tumour immunity. Through a direct interaction with Ras, CD59 regulates the subcellular localisation of Ras, thus affecting the Ras/MAPK signalling pathway, which plays a role in the proliferation and activation of T cells. Compared to wild type mice, tumour growth was suppressed in CD59 double knockout mice. This tumour suppression was mediated by T cells in the knockout mice (100). Another non-canonical complement function related to T cells concerns C1q. In addition to its presence on the cell surface, the receptor for the globular domain of C1q can also be found in

mitochondria. By acting on its cognate receptor, extrinsically generated C1q that is taken up can affect the metabolism and function of mitochondria in $CD8^+$ T cells, which consequently affects the function of these T cells (121).

As mentioned at the beginning of this section, the discovery of intracellular complement has been met with many questions. One such question is whether complement proteins present within the cells have distinct functions that are unrelated to their extracellular role or if canonical complement functions occur inside the cells (121). A major concern that has been raised is that the intracellular environment is very different from the extracellular one. As such, the proper folding of complement proteins may not be permitted within the cells, thus prohibiting their functions (122).

In the case of C3, its extracellular function is linked to its structure, which depends on disulphide bonds that may not be allowed to form in the reducing environment of the cytosol. However, C3 was found to be resistant towards a reducing agent present in the cytosol, and it has been proposed that the folding of C3 may shield the disulphide bonds (121). Moreover, as a result of retrotranslocation and the use of an alternative translation start site, two distinct forms of cytosolic C3 have been observed, differing in their redox state. By using an alternative translation start site downstream of the signal peptide, this form is translated immediately to the cytosol and is, therefore, both reduced and non-glycosylated. Importantly, though, it still functions and was found to opsonise invading bacteria, which facilitated the subsequent phagocytosis once the bacteria escaped the cells (129). Evidently, forms of C3 differing from hepatic extracellular C3 can both exist and function intracellularly (122). Additionally, the fact that the intracellular environment differs from the extracellular one does not exclude the possibility that complement can function within the cell. Inside the cell, complement factors encounter a whole other set of proteins that are not present in the extracellular environment. As such, non-canonical interactions may occur, and complement proteins may thus have distinct intracellular functions that are unrelated to their canonical extracellular functions. A prime example of this is the interaction between C3 and ATG16L1, which links C3 to the process of autophagy. Furthermore, complement may also function intracellularly by a different mechanism. For instance, the potential absence of convertases may be compensated for by cathepsins that mediate the cleavage activation of C3 and C5 (121).

Another question that has been raised concerning intracellular complement is where it comes from. However, only two possible sources exist: intrinsic and extrinsic, meaning that complement is either expressed by the cells themselves or derived from the extracellular environment. With the exception of membrane-bound complement inhibitors and receptors, complement proteins are typically viewed as being secreted from the cells and present in the circulatory system. Proteins destined to be secreted possess a signal peptide that will guide the translated polypeptide into

the ER lumen, where protein folding will occur. Following subsequent modifications and processing in the ER and Golgi, the protein is trafficked to the plasma membrane in secretory vesicles and then released to the exterior space (121). This process will be exemplified and described in greater detail later on in the context of insulin processing.

To use C3 as an example, four distinct mechanisms whereby it gains access to the intracellular space have been reported, three of which have already been touched upon. For instance, invasive bacteria opsonised by C3 can carry it into the cells (128). Proteins can also be retrotranslocated, meaning that they escape the secretory pathway to access the cytosol. This mode of accessing the cytosolic space has been proposed for both C3 and CD59 (129, 134). As described earlier, C3 can also be translated from an alternative start site downstream of the signal peptide. This form of C3 is thus translated straight into the cytosolic space (129). Lastly, a pathway by which C3(H_2O) is taken up by cells has also been described. The internalised C3(H_2O) constitutes a source for intracellular C3a, but it was also found to be recycled to the exterior environment (135). As such, many plausible mechanisms for complement to gain access to the cell interior exist, and these may differ depending on the complement protein, cell type and circumstances.

Diabetes mellitus

Introduction

Diabetes mellitus refers to a group of metabolic diseases characterised by an inability to regulate blood glucose levels (136). They revolve around insulin, a metabolic hormone acting to lower blood glucose levels (137). Insulin acts in concert with the functionally antagonistic hormone glucagon to regulate and maintain blood glucose homeostasis (137). Persistent hyperglycaemia, a state of elevated blood glucose levels caused by insufficient insulin secretion and/or impaired insulin action and function, is a hallmark of diabetes mellitus (136). Most of the time, diabetes cases can be broadly classified into type 1- and type 2 diabetes mellitus (T1D and T2D), yet additional subtypes have also been characterised (138). Some of the different subtypes of diabetes mellitus are described later on. Note that even though diabetes mellitus is not the only type of diabetes, the term diabetes will refer to diabetes mellitus throughout this thesis.

The history of diabetes, or "the pissing evil", as the 17th-century physician Thomas Willis so elegantly put it, seemingly dates back to ancient times. In Ebers papyrus, presumably dating back to around 1550 B.C., recordings of a disease involving polyuria can be found. This may represent an early description of diabetes symptoms. In a Hindu document from around 400 B.C., the Indian physician Sushruta described another symptom of diabetes, namely glycosuria or "honeyed urine", as he phrased it in reference to its sweetness. Similar to Sushruta, the English physician Thomas Willis described the sweet taste of urine and brought attention to diabetes in Europe during the 17th century. During the 2nd century A.D., the Greek physician Aretaeus provided an impressive description of the disease for his time. He described the excessive urination and the chronic nature of the disease. He further described that it takes a long time for the disease to develop but that once it has been established, the patient is short-lived. Aretaeus is responsible for introducing the term "diabetes", which comes from a Greek word and refers to the excessive urination characteristic of afflicted individuals (136, 139). At the end of the 18th century, in reference to the sweetness of urine, John Rollo introduced the suffix "mellitus", which in Latin means honey (139).

Today, we, of course, know much more about diabetes. However, a major breakthrough in the early 1920s warrants a mention of four pioneers in the field. In 1921 and 1922, Fredrick Banting, John Macleod, Charles Best and James Collip

managed to successfully produce insulin for therapeutic use. Although not without controversy, Banting and Macleod received the Nobel Prize in medicine for their work in 1923 (136).

According to the International Diabetes Federation (140), it was estimated that about 537 million adults worldwide had diabetes in 2021. This number is predicted to reach 783 million by the year 2045. According to their estimates, diabetes-related causes were responsible for about 6.7 million deaths in 2021 (140). That corresponds to almost 13 people per minute. Furthermore, the health expenditure globally in 2021 caused by diabetes in adults was estimated to be 966 billion US dollars, a cost that is predicted to increase (140). Diabetes thus represents a global societal burden, both in terms of health and in terms of economy. Insulin insufficiency, i.e. diabetes, can lead to a range of complications affecting various parts of the body. These complications include cardiovascular diseases, nephropathy, neuropathy, retinopathy (which can cause blindness), and the need for amputation of lower limbs (140).

Islets of Langerhans and pancreatic β-cells

The structure and function of pancreatic islets

The pancreas is a multifunctional organ with key roles influencing metabolism. The pancreas has an important exocrine function mediated by acinar cells. These cells produce and secrete digestive enzymes into the pancreatic duct. The digestive enzymes make up the pancreatic juice that is released into the duodenum and aids in the digestion of macronutrients. In addition to its exocrine function, the pancreas also has an important endocrine function. The endocrine function of the pancreas is mediated by micro-organs known as pancreatic islets (137). These pancreatic islets, also known as islets of Langerhans, were first described by Paul Langerhans in 1869 (141).

The islets of Langerhans are highly vascularised clusters of endocrine cells that are scattered throughout the pancreas (142). The number of islets in a human pancreas has been estimated to range between 3.6 and 14.8 million with a combined volume of 0.5-1.3 cm^3 (143). The islets of Langerhans are thought to constitute about 1-2 % of the pancreas (144, 145). Worth noting is that the size of islets does not assume a normal distribution, and only around 20 % of the islets have a diameter equal to or above 100 μm, yet they constitute around 75 % of the total volume of all islets (146, 147). Furthermore, it has been stated that islets in the 90^{th} percentile in terms of size are accounting for half of the total volume of β-cells (145). It has been estimated that an islet with a diameter of 150 μm contains around 1560 cells (148).

The endocrine cells of the pancreatic islets secrete hormones that dictate whole-body metabolism. The islets are composed of five types of endocrine cells present at different proportions (137). The cell composition of islets can vary slightly, but a human islet contains about 30-40 % α-cells, 50-60 % β-cells and 10 % δ-cells (149, 150). γ-cells (PP-cells) and ε-cells (Ghrelin cells) make up the rest but are present in low numbers in adults (151, 152). Some noteworthy differences exist between human and rodent islets, both in terms of cellular composition and architecture. Mouse islets are composed of a higher proportion of β-cells and a lower proportion of α-cells in comparison to human islets. Additionally, in rodent islets, β-cells are present within the core, while the other islet cells are present at the periphery, forming a mantle around the β-cells. In contrast, the endocrine cells of human islets appear to be more randomly distributed within the islets (149, 150).

Pancreatic α- and β-cells are crucial for the regulation of metabolism and blood glucose levels. β-cells secrete the anabolic hormone insulin and are thus of critical importance in the context of diabetes mellitus. Conversely, α-cells produce and secrete the catabolic hormone glucagon, which antagonises the effect of insulin. Insulin and glucagon have opposing effects and are regulated reciprocally. Pancreatic δ-cells produce and secrete somatostatin, which has a paracrine function and inhibits both insulin- and glucagon secretion. PP-cells produce pancreatic polypeptide which can both stimulate the exocrine function of the pancreas and suppress appetite (153, 154). The fifth endocrine cell of the islets, the ghrelin- or ε-cells, produce ghrelin. Ghrelin seemingly has paracrine functions, affecting the secretion of other islet hormones (155).

Insulin biosynthesis, secretion and function

Insulin biosynthesis

Insulin is secreted by pancreatic β-cells, and it is an anabolic hormone that lowers blood glucose levels by stimulating glucose uptake. Insulin is highly expressed by β-cells; insulin mRNA accounts for about 10-15 % of the total mRNA pool in these cells (156). The insulin mRNA is stabilised by an mRNA-binding protein, and this binding is induced by glucose (157). Furthermore, glucose also affects the expression and activity of three transcription factors (Pdx-1, NeuroD1 and MafA) that are specific to β-cells and play a key role in the regulation of insulin expression (158). In addition to affecting expression and mRNA stability, glucose also induces translation and synthesis of proinsulin (159).

Upon translation of the insulin mRNA, an N-terminal signal peptide in the nascent preproinsulin is recognised by the Signal Recognition Particle, which then facilitates the translocation of the preproinsulin into the ER (160). Once it has entered the ER, the signal peptide of preproinsulin is cleaved off to form proinsulin. The proinsulin

subsequently adopts the proper folding in a chaperone-assisted manner (161). Insulin is composed of an A-chain and a B-chain. These polypeptide chains are held together by two disulphide bridges that are formed during the folding process of proinsulin in the ER. A third internal disulphide bond present within the A-chain is also formed (162).

Folded and processed proinsulin is subsequently transported to the Golgi apparatus and is sorted and packaged into immature secretory granules at the Trans-Golgi Network (163). In this Zn^{2+} and Ca^{2+}-rich environment, the proinsulin is present in hexamers, the structure of which is dependent on the presence of the divalent cations (164). The insulin granule maturation process involves acidification (163). This is orchestrated by the concerted action of an ATP-dependent H^+ pump and a Cl^- channel. While the proton pump acidifies the interior of the granule, the Cl^- channel allows for Cl^- uptake. This prevents the build-up of an electrical gradient, thus permitting further acidification (165). During insulin granule maturation, proinsulin is also processed into insulin (**Figure 2**). This is mediated by two prohormone convertases (PC1/3 and PC2) as well as carboxypeptidase E. PC1/3 and PC2 are calcium-dependent endoproteases with an optimal activity at pH 5.5. The intragranular environment with a Ca^{2+} concentration of up to 10 mM and a pH of about 5.5 is thus well suited for these endoproteases. PC1/3 and PC2 cleave off the C-peptide that links together the A- and B-chain of insulin (166). Basic residues are subsequently trimmed off at the cleavage site by carboxypeptidase E (167). The insulin hexamer has a much lower solubility than proinsulin hexamers. The liberation of the C-peptide thus leads to the crystallisation of insulin within the secretory granules (164). While the crystallised insulin constitutes the core, the soluble C-peptide is localised to the periphery of the secretory granule's interior (168). Insulin and C-peptide are secreted at equimolar concentrations, and it has been reported that C-peptide, upon secretion, aids in the dissociation of insulin hexamers to generate biologically active insulin monomers (169).

In addition to insulin and C-peptide, other proteins and molecules present within the insulin granules are also co-secreted. For instance, islet amyloid polypeptide (IAPP) is also released at a varying ratio (168). IAPP will be described in more detail in the context of type 2 diabetes pathogenesis. Initially, it was estimated that mouse β-cells contain about 13000 insulin granules (170), but a more recent study suggests around 9000 (171).

Insulin secretion

Following a meal, blood glucose levels are elevated, which represents the main stimulus for β-cells to secrete insulin. The mechanism by which plasma glucose promotes insulin secretion is termed "stimulus-secretion coupling" (137), and is illustrated in **Figure 2**. Pancreatic β-cells function as glucose sensors and maintain blood glucose levels within a suitable range by adjusting the amount of insulin released (154). Glucose is taken up by the β-cells through glucose transporters.

GLUT1 is the main glucose transporter in human β-cells, while GLUT2 is the transporter in murine β-cells (172). Upon entry into the β-cells, glucose is phosphorylated by glucokinase, also known as hexokinase IV. This leads to the formation of glucose-6-phosphate and constitutes the rate-limiting step in glucose-stimulated insulin secretion. Several properties of glucokinase make it an important glucose sensor in pancreatic β-cells (145, 154). The phosphorylated glucose will undergo glycolysis to generate pyruvate, which is then transferred into the mitochondria, where it is used as a substrate for the tricarboxylic acid cycle. Glycolysis, the tricarboxylic acid cycle and subsequent oxidative phosphorylation are processes that together result in the formation of ATP (136).

Similar to neurons, β-cells are electrically excitable cells with a resting membrane potential of about -70 mV (173). This membrane potential is maintained by an ATP-sensitive octameric potassium channel composed of four Kir6.2 channel subunits and four regulatory subunits called sulfonylurea receptor 1 (SUR1) (163). Upon increased intracellular ATP levels resulting from the cellular respiration of glucose, the K_{ATP} channel closes (173, 174). ATP binding to Kir6.2 causes the channel to close, and SUR1 is thought to sensitise Kir6.2 to ATP-mediated inhibition (175). The closure of the K_{ATP} channel prohibits K^+ efflux, causing membrane depolarisation. This membrane depolarisation, in turn, leads to the activation of L-type voltage-dependent calcium channels. Consequently, due to the electrochemical gradient, Ca^{2+} starts flooding into the cell, where it will trigger insulin release (176).

Insulin is released from β-cells in a biphasic manner with a rapid first phase lasting up to 10 minutes and a sustained second phase where the rate of insulin release is lower than during the first phase, but it lasts for a prolonged period of time (145). This biphasic pattern of insulin release reflects the existence of two separate insulin granule pools within the β-cells. About 1-5 % of the insulin granules constitute the readily releasable pool (RRP) and are primed, docked and release-competent. The insulin granules of the RRP do not need further modification or processing and are released during the first phase. The remaining >95 % of the secretory granules belong to the second pool, the reserve pool (163, 177). Following the depletion of the RRP during the first phase of insulin secretion, insulin granules from the reserve pool are recruited and released during the second phase (163). The requirement for mobilisation and priming of these reserve granules reflects the slower nature of the second phase (178). Furthermore, the recruitment and processing of the reserve pool granules to make them release-competent is both calcium- and ATP-dependent (163). The second phase of insulin release can only be elicited by metabolic fuels, such as glucose (179). Potassium-stimulated insulin secretion, for instance, only triggers the first phase (171). Individuals with type 2 diabetes typically have an impaired first phase of insulin release (180, 181).

Insulin granules can be transported on both microtubules and filamentous actin, and it has been reported that long-range trafficking occurs on microtubules while short-

range trafficking depends on filamentous actin (178, 182-184). In its GTP-bound active form, Rab27A is associated with the granule membrane (185). It can either directly or indirectly interact with filamentous actin motor proteins of the myosin family by being in a complex with its effectors exophilin-8, granuphilin or rabphilin (182, 184, 186, 187). In a myosin-dependent fashion, the insulin granules are translocated to the plasma membrane along the actin filaments (184). Both Rab3 and Rab27A are important for the formation, regulation and replenishment of the RRP and the generation of granules ready for immediate release. They exhibit both distinct and overlapping functions, and their concerted actions generate release-competent insulin granules (185).

At the core of the exocytotic machinery governing insulin release, we have the soluble N-ethylmaleimide-sensitive factor attachment protein receptors, or SNARE proteins for short. The SNARE proteins involved in insulin release are part of a superfamily of proteins with over 35 members that all contain a so-called SNARE domain (163). Target (t)- and vesicular (v)-membrane SNARE proteins together make up the SNARE complex that orchestrates insulin exocytosis (188). In β-cells, the two t-SNARE proteins SNAP-25 and Syntaxin-1 are localised on the plasma membrane, while the v-SNARE protein VAMP2, also known as synaptobrevin, is associated with the vesicular membrane (163). It should be noted, however, that Syntaxin-3, Syntaxin-4 and VAMP-8 have also been implicated as mediators of insulin exocytosis. These SNARE proteins contribute to the biphasic nature of insulin secretion, where Syntaxin-3 and VAMP-8 are involved in the release of so-called newcomer granules that are recruited (188-190). Nevertheless, in addition to the SNARE complex constituents, several other proteins are also needed, including the accessory proteins Munc13 and Munc18, as well as the active zone protein RIM (178).

Stable and successful docking of insulin granules requires an interaction between Rab3 and RIM2α (191). Rab3 can also bind Munc18, which in turn interacts with Syntaxin-1. The recruitment and clustering of Munc18 and Syntaxin-1 at the docking site is required for stable docking (192). The binding of Munc18 to Syntaxin-1 maintains the SNARE protein in a closed conformation unable to interact with VAMP2 and SNAP-25 (193, 194). Based on studies of neuronal SNARE complexes, subsequent priming of insulin granules is thought to require Munc13 which cooperates with Munc18 to facilitate proper assembly of the ternary SNARE complex with a stoichiometry of 1:1:1. This Munc-mediated assembly of Syntaxin-1, VAMP2 and SNAP-25 leads to a so-called “half-zippered” SNARE complex and causes Syntaxin-1 to be released from Munc18 (178, 195, 196). This half-zippered SNARE complex is thought to be stabilised by complexin, which thereby prohibits unstimulated exocytosis (197, 198).

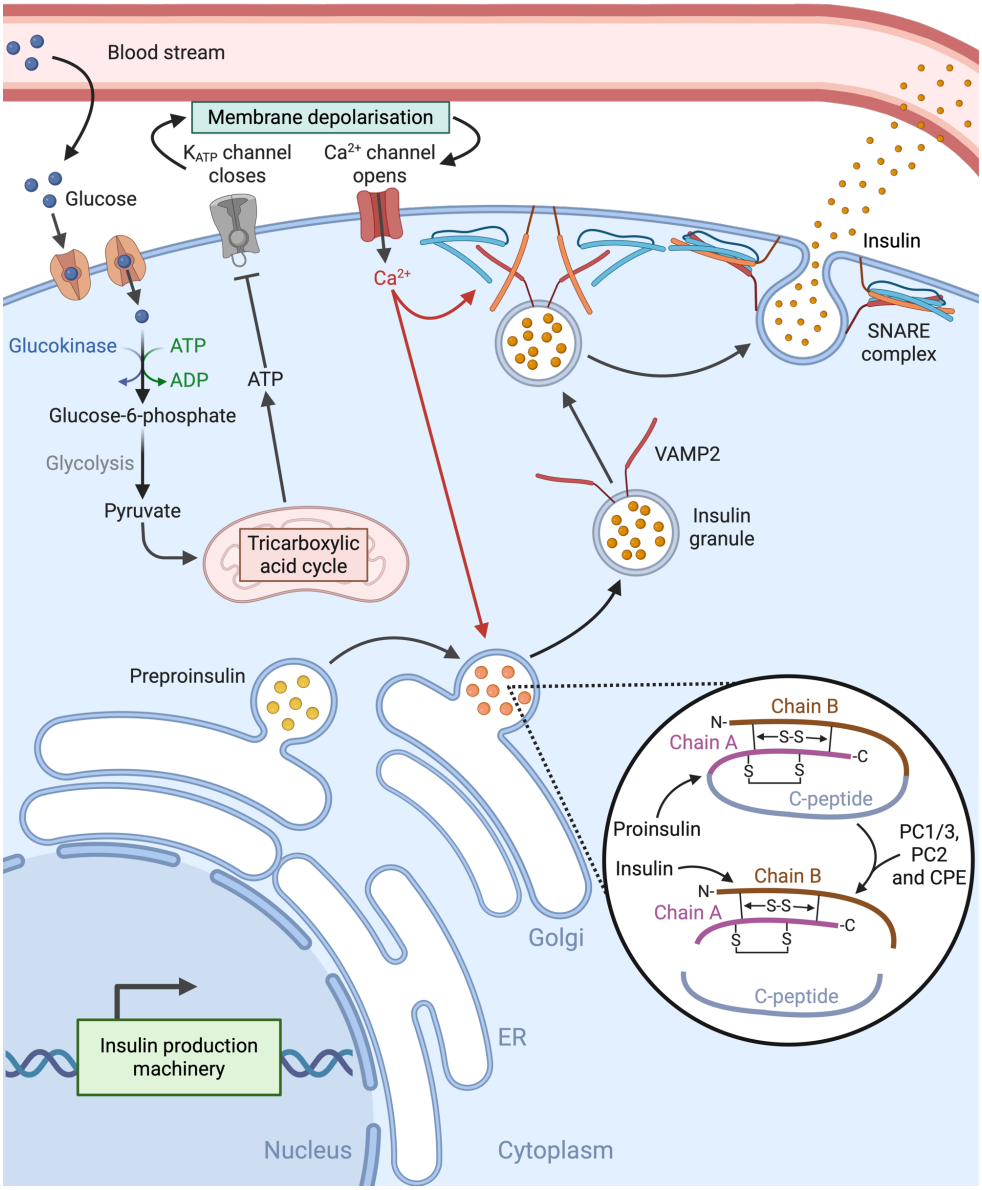


Figure 2. Schematic illustrating the mechanism of glucose-stimulated insulin secretion. Glucose is taken up by the β-cell and converted into glucose-6-phosphate by glucokinase. The glucose is used to generate ATP through glycolysis, the tricarboxylic acid cycle and the electron transport chain. The increased cellular ATP levels lead to the closure of K_{ATP} channels, membrane depolarisation and opening of voltage-gated Ca^{2+} channels. The resultant influx of calcium will trigger insulin release through its effect on the SNARE complex. The figure further illustrates proinsulin processing into insulin during the insulin granule maturation process.

Several members of the exocytotic machinery have been found to interact with the L-type calcium channel, such as RIM, Munc13, Syntaxin-1, SNAP-25 and synaptotagmin. The recruitment of these channels to sites with release-competent insulin granules ensures that the granule and exocytotic machinery are highly exposed to Ca^{2+} upon the opening of the voltage-gated calcium channels. This allows insulin exocytosis to be both rapid and synchronised with depolarising events (163, 199). In Ca^{2+}-dependent exocytosis, synaptotagmin is likely serving the function of Ca^{2+}-sensor (163, 200). Synaptotagmin is a large family of proteins that contain two Ca^{2+}-binding C2 domains, yet not all members function as calcium sensors (201). In addition to calcium, synaptotagmins can also bind phospholipids (200). While the expression of synaptotagmin family members in β-cells appears to vary between species, several of them have been suggested to be involved in Ca^{2+}-dependent exocytosis in β-cells (145, 163, 202-205). However, the exact mechanism by which synaptotagmins trigger exocytosis in response to Ca^{2+} is not entirely clear, and several models have been proposed (197, 206-208). One such model, based on results obtained when studying neurons, suggests a competitive binding between synaptotagmin-1 and complexin for the SNARE complex and that in response to Ca^{2+}, synaptotagmin-1 can dislodge complexin and trigger exocytosis (197). Nevertheless, the influx of Ca^{2+} and subsequent activation of calcium sensors allows for full zippering of the SNARE complex, thus causing the secretory vesicle to fuse with the plasma membrane, which results in insulin secretion (178).

Insulin function

Secreted insulin circulates in the bloodstream and exerts its function by binding to its cognate receptor. The insulin receptor exists in two isoforms as a result of alternative splicing (209). Additionally, the insulin receptor is a tetramer composed of two α- and two β subunits. It is a tyrosine kinase receptor that, in response to ligand-induced activation, will undergo conformational changes, leading to transphosphorylation of the β subunits and activation of the kinase activity. Following its autoactivation, receptor substrates such as Shc and members of a protein family known as insulin receptor substrate (IRS) are recruited and activated. Shc will activate the Ras-MAPK signalling pathway, which causes changes in gene expression and promotes proliferation. Activated IRS proteins, however, will, in turn, activate a phosphatidylinositol 3-kinase (PI3K) that subsequently converts phosphatidylinositol-4,5-biphosphate (PIP_2) into phosphatidylinositol-3,4,5-triphosphate (PIP_3). This will lead to the recruitment and activation of PDK1, which in turn activates protein kinase C (PKC) and Akt. PKC and Akt will then propagate the signal further and activate a variety of pathways. Many of the metabolic effects of insulin, including glycogenesis, lipid synthesis and regulation of glucose transport, stem from the activation of Akt and its downstream effects (210).

The liver, skeletal muscles and adipose tissues are insulin-sensitive tissues that play important roles in the regulation of blood glucose levels (211). One of the effects of insulin on adipose tissue and skeletal muscles is to greatly enhance glucose uptake from the bloodstream. The cells in these tissues express a glucose transporter named GLUT4, which normally resides intracellularly. However, in response to insulin, GLUT4 is translocated to the plasma membrane, which results in glucose uptake and lowering of blood glucose levels (212). The glucose taken up by the adipose tissue is used to generate glycerol-3-phosphate, which is a substrate for triglyceride synthesis. The main effects of insulin on the adipose tissue are to promote glucose uptake, suppress lipolysis and promote triglyceride synthesis. In doing so, insulin promotes adipogenesis. The adipose tissue is the body's main fatty acid reservoir, and upon lipolysis, fatty acids are released into the circulation. The main effects of insulin on skeletal muscle, in addition to increasing glucose uptake, are to promote protein synthesis, induce glycogenesis and inhibit glycogen breakdown. Insulin also induces a shift from fatty acid oxidation to oxidation of glucose. The liver plays an important role in the regulation of both glucose- and insulin levels in the circulation (211). It was found that the liver can extract around 40-70 % of the secreted insulin (213), thus preventing hyperinsulinemia, which can lead to peripheral insulin resistance (211). Insulin acting on the liver inhibits gluconeogenesis, glycogenolysis and lipolysis. It also promotes glucose uptake, glycogenesis and lipogenesis (211, 214).

Classification of diabetes mellitus subtypes

Type 1 diabetes

Type 1 diabetes (T1D) is generally considered a chronic autoimmune disorder, accounting for about 5-10 % of all diabetes cases (215). The prevalence, however, varies substantially between different parts of the world (216). Inulin deficiency and consequent hyperglycaemia due to β-cell loss are characteristics of T1D. It should be noted that around 10 % of T1D patients do not exhibit any signs of autoimmunity, which has led to the subclasses autoimmune-mediated (or type 1a) and idiopathic (type 1b) T1D. The aetiology of idiopathic T1D is not known, but genetic components play a major role (217, 218). The rest of this section will focus on autoimmune T1D.

T1D is an insulin-dependent form of diabetes with an early onset. However, although it usually presents during childhood or early adulthood, it can develop at any age (219). T1D is characterised by the presence of autoantibodies targeting glutamic acid decarboxylase (GAD65 or GADA), insulin (IAA), zinc transporter 8 (ZnT8), and insulinoma-associated protein 2 (IA-2). The first autoantibodies to

appear are usually IAA or GADA. The autoantibodies can be present months or even years before clinical presentation of T1D. Although the autoantibodies serve as good biomarkers for autoimmunity, they are not considered pathogenic (217). The aforementioned four autoantibodies are the ones most frequently observed in T1D patients. The risk for developing T1D is quite small if only one of these is present, but the risk increases dramatically if two or more of these autoantibodies are present (219).

T1D diabetes is known to develop as a result of autoimmune-mediated destruction of pancreatic β-cells, but the exact pathological mechanism, as well as the trigger for autoimmunity, remains unknown. However, both genetic- and environmental factors are likely involved (217, 219). It is thought that the presence- and presentation of β-cell autoantigens lead to activation of T cells and B cells. β-cell stress may cause them to present autoantigens to cytotoxic T cells, leading to immune-mediated cell death. Although not major effectors, the B cell-derived autoantibodies may enhance the autoimmune response driven by T cells. A hallmark of T1D-associated β-cell destruction is islet infiltration by immune cells. The predominant immune cell found in the islets is $CD8^+$ T cells, but $CD4^+$ T cells, as well as B cells and macrophages, are also present (219).

Genetic factors have been estimated to account for about 40-50 % of the susceptibility to developing T1D. More than 50 genomic loci have been found to contribute to the risk for T1D (220). The region containing the human leukocyte antigen (HLA) class II genes confers a major risk for developing T1D. Two loci in particular, namely HLA-DR and HLA-DQ, are of major importance. Both haplotypes conferring susceptibility and haplotypes conferring protection have been identified. For instance, the haplotypes DR3-DQ2 and DR4-DQ8 are associated with an increased risk for T1D, while the haplotype DR15-DQ6 is strongly protective (218, 219). Out of the non-HLA-related genetic risk factors for the development of T1D, the strongest candidates are polymorphisms in the genes encoding insulin (*INS*) or a protein tyrosine phosphatase present in lymphocytes (*PTPN22*) (219).

In terms of environmental factors, viral infections have been implicated in the pathogenesis. Enteroviruses, including Coxsackievirus B, are thought to play a role (221-223). Coxsackievirus B4 has been isolated from islets of T1D patients with a recent onset, wherein β-cells exhibited signs of infection (224).

Type 2 diabetes

Type 2 diabetes (T2D) represents the predominant form of diabetes, thought to account for about 90 % of all diabetes cases (215, 225). While T1D used to be referred to as juvenile-onset diabetes or insulin-dependent diabetes, T2D was previously known as non-insulin-dependent diabetes or adult-onset diabetes (215).

Due to the generally slow progression of the disease, people can be prediabetic and asymptomatic for many years before it is detected. In fact, it has been estimated that almost 45 % of diabetic individuals were unaware of their condition. Despite being asymptomatic, people with prediabetes or undiagnosed T2D may still develop diabetic complications (226).

T2D is a chronic and heterogeneous metabolic disorder. In addition to genetic predisposition, a multitude of environmental factors have been implicated in T2D development. These include, for instance, diet, physical activity and smoking (225, 227). The most prominent risk factor for developing T2D is obesity, which is linked to metabolic aberrations promoting insulin resistance. Insulin resistance, together with β-cell dysfunction and insufficient insulin release are characteristics of T2D, and their conjoint effect leads to hyperglycaemia (225). Additionally, both T2D and obesity are characterised by low-grade, chronic inflammation, and T2D has even been described as an inflammatory disorder (228-231). Not only is inflammation implicated in the pathophysiology of T2D, but proinflammatory markers are also predictive of the disease (228).

Initially, in order to cope with insulin resistance, the endocrine pancreas adopts compensatory mechanisms to counter the imminent threat of overt hyperglycaemia. This adaptation involves both an increased output of insulin from β-cells and an expansion of β-cell mass (232, 233). If or when the adaptive response becomes inadequate, hyperglycaemia develops, and T2D progresses. This leads to β-cell dysfunction and a decrease in β-cell mass (232). Concordant with a decrease in β-cell mass in T2D (234-236), increased β-cell apoptosis has been identified in T2D (235, 237, 238). Additionally, compared to controls, the total islet mass was found to be reduced in individuals with T2D, and their islets tended to be smaller than those from non-diabetic controls (239). However, apoptosis does not alone account for insulin deficiency, β-cell dysfunction also plays a role (232). Additionally, apoptosis might not be the only factor contributing to a loss of β-cell mass (232, 233). Nevertheless, there is a decrease in functional β-cell mass leading to an impaired insulin secretory response and hampered glucose clearance. The persistent hyperglycaemia contributes to further injury to the functioning β-cell mass, generating a vicious cycle proposed to drive the transition from an asymptomatic state of T2D to clinical manifestation (232).

Some of the mechanisms underlying β-cell dysfunction and a reduction in β-cell mass in the context of T2D progression are further elaborated upon later. Still, they include metabolic-, oxidative-, inflammatory-, and ER stress, for instance (225).

Moreover, in addition to negatively impacting the uptake of glucose in adipose tissue, liver and muscle, insulin resistance promotes hepatic gluconeogenesis, which adds to the insult and further aggravates the hyperglycaemic state (225). Impaired glucose tolerance and T2D are associated with a loss of 1st phase insulin release,

which otherwise plays a vital role in suppressing gluconeogenesis. The loss of 1st phase insulin release is a sign of T2D development (240). Additionally, it has been shown that impaired glucose tolerance and the progression into T2D are associated with a decrease in β-cells' sensitivity to glucose. The sensitivity of β-cells to glucose was significantly reduced in individuals with impaired glucose tolerance compared to individuals with normal glucose tolerance and was even further reduced in type 2 diabetics (241).

The insulin resistance associated with T2D and obesity generally stems from interference with the signalling pathway downstream of the insulin receptor. Several triggers, including mitochondrial dysfunction and generation of reactive oxygen species (ROS), ER stress and the unfolded protein response, proinflammatory cytokines, and accumulation of ectopic lipids in the liver and muscles, can promote insulin resistance. These triggers lead to the activation of various kinases, such as PKC and c-Jun N-terminal kinases, that can phosphorylate serine residues in IRS. In addition to prohibiting IRS activation through tyrosine phosphorylation, it can also promote IRS degradation (227). This will impair insulin receptor signalling, which, as described earlier, goes through IRS. Insulin receptor signalling normally increases glucose uptake by promoting GLUT4 translocation to the plasma membrane.

Diagnosis

The clinical tests employed to diagnose diabetes measure blood glucose levels in different ways. The guidelines and criteria for a diagnosis of diabetes according to the American Diabetes Association will be described here. Fasting blood glucose levels, measured following an at least 8-hour fasting period that is equal to, or above, 7 mmol/L is a sign of diabetes. Fasting blood glucose levels in the range of 5.6-6.9 mmol/L is a sign of impaired fasting glucose and prediabetes. A random blood glucose test, irrespective of fasting or time of the day, can be used for diagnosis if accompanied by diabetic symptoms such as unintended weight loss, polyuria or polydipsia. For a random blood glucose test, the cut-off for a diagnosis of diabetes is a blood glucose concentration of at least 11.1 mmol/L. Another test, the oral glucose tolerance test, requires the patient to take an oral glucose load corresponding to 75 grams of glucose dissolved in water. Blood glucose levels measured two hours later that are in the range of 7.8-11.0 mmol/L is a sign of prediabetes and impaired glucose tolerance. Blood glucose levels above 11.0 is a sign of diabetes. A commonly used test relies on the non-enzymatic glycation of haemoglobin upon glucose exposure. Glycated haemoglobin, or HbA1c, reflects the average blood glucose levels during a period of up to about 120 days, corresponding to erythrocytes' lifespan. It provides an average of haemoglobin in erythrocytes that is bound to glucose and is given in percentage. While HbA1c in the range of 5.7-6.4 % is considered a sign of prediabetes, the cut-off for a diabetes diagnosis is HbA1C equal to, or above, 6.5 % (218).

Type 2 diabetes subtypes

The conventional classification of diabetes subtypes is essentially based on the presence of autoantibodies to primarily distinguish between T1D and T2D. Latent autoimmune diabetes in adults (LADA, described later) is a third subtype and is characterised by the presence of GADA autoantibodies. Albeit phenotypically resembling T2D early on, it progressively becomes more like T1D with time. Moreover, the monogenic forms of diabetes are identified and classified based on genetic testing. However, T2D represents a highly heterogeneous disease, and a more precise classification would be clinically advantageous. Not only could treatment regimens be tailored to each patient, but patients at risk for particular diabetic complications could also be identified and treated accordingly.

A novel classification of T2D subgroups has been proposed based on results from a cluster analysis. The study identified five distinct patient clusters, the first one being "severe autoimmune diabetes" (SAID), which had some overlap with both T1D and LADA. Patients in this cluster were characterised by an early onset of diabetes, insulin deficiency and being positive for GADA. The second cluster, termed "severe insulin-deficient diabetes" (SIDD), was also characterised by an early onset and poor insulin secretion. However, these patients were not positive for GADA. The third cluster was characterised by high insulin resistance, hence the name "severe insulin-resistant diabetes" (SIRD). The fourth cluster was named "mild obesity-related diabetes" (MOD), and the patients therein were obese yet not insulin-resistant. Patients in the fifth and last cluster were distinguished by their higher age compared to other clusters. This cluster was called "mild age-related diabetes" (MARD) and exhibited only mild metabolic disorder, similar to MOD.

The two clusters SAID and SIDD were both characterised by a relatively low BMI, while SIRD was characterised by a high BMI. Moreover, while patients in the SIDD cluster were more prone to exhibit early signs of retinopathy, the patients in the SIRD cluster suffered the highest risk of developing both chronic kidney disease and diabetic kidney disease (242).

Pathophysiology

The metabolic perturbation occurring in T2D can lead to chronic hyperglycaemia and chronic hyperlipidaemia, both of which can contribute to β-cell dysfunction. This is referred to as gluco- and lipotoxicity, respectively (243). Palmitate has been found to induce carboxypeptidase E degradation. This leads to improper processing of insulin, and the ensuing ER stress, leading to cell death, is likely a consequence of the accumulation of proinsulin (244). Palmitate has also been found to induce ER Ca^{2+} depletion, thus impeding the functional integrity of the ER. As the protein folding capacity of the ER is compromised, the unfolded protein response is induced (245, 246). Moreover, exposure to free fatty acids was accompanied by a downregulation of the anti-apoptotic protein Bcl-2 in islets and induction of caspase-mediated cell death (247). Similarly, another study found that elevated

glucose levels in combination with palmitate promoted caspase-3 activation and β-cell death (248). Elevated levels of free fatty acids can also contribute to insulin resistance, which increases the demand for insulin output from β-cells. This, in turn, can lead to ER stress. Free fatty acids can also lead to the generation of ROS in β-cells. This can lead to mitochondrial dysfunction and apoptosis (249).

When it comes to glucotoxicity, it has, for example, been shown that it causes alterations in the expression of Bcl family members, leading to an imbalance between pro- versus anti-apoptotic signals that favour apoptosis, thus leading to β-cell death (250). β-cells are inherently at risk for developing ER stress due to the demand for high levels of insulin biosynthesis, especially when coping with insulin resistance. The ER stress imposed on the β-cells may lead to activation of the unfolded protein response. If ER homeostasis cannot be restored by the ensuing stress response, apoptosis is induced (249). The overstimulation conferred by prolonged hyperglycaemia can thus lead to ER stress, activation of the unfolded protein response and, eventually, cell death (251). Moreover, glucotoxicity can also lead to the generation of ROS, which causes oxidative stress, cellular damage and β-cell dysfunction. Owing to the low expression of antioxidant enzymes, β-cells are particularly sensitive to oxidative stress (251, 252).

Inflammation also contributes to β-cell dysfunction. The proinflammatory cytokine interleukin-1β (IL-1β) exerts dual opposing effects on pancreatic β-cells. Limited exposure to IL-1β, both time-wise and concentration-wise, can have beneficial effects. Under such circumstances, IL-1β can augment insulin secretion, promote proliferation and have an anti-apoptotic effect. Conversely, high concentration and/or prolonged exposure to IL-1β causes β-cell dysfunction and promotes apoptosis (253). An interesting side note is that a synergistic effect of insulin and IL-1β in glucose clearance has been described. The mechanism entailed IL-1β release from macrophages in response to glucose, which potentiated insulin secretion. By activating its cognate receptor on macrophages, insulin stimulated glucose uptake and metabolism, which led to the generation of ROS and activation of the NLRP3 inflammasome. This, in turn, led to more IL-1β release, thus forming a loop promoting glucose clearance (254). However, in β-cells, hyperglycaemia can also induce the expression of both IL-1β and FAS, which is a pro-apoptotic receptor (255, 256). The IL-1 receptor is highly expressed by β-cells and the released IL-1β can thus act in an autocrine fashion, promoting autostimulation (257, 258). This leads to activation of the transcription factor NF-κB, which drives further expression of both IL-1β and FAS (255, 257). This forms a loop wherein the glucose-induced expression of IL-1β and FAS promotes β-cell apoptosis (255, 256). Additionally, free fatty acids, such as palmitate, can also promote IL-1β release (228). By acting on Toll-like receptors 2 and 4, free fatty acids can modulate the activity of NF-κB and promote the expression of cytokines and chemokines (258-260). This, in turn, results in the recruitment of proinflammatory

macrophages that further propagate the local inflammation, leading to β-cell dysfunction (260). It has also been proposed that the proinflammatory cytokines IL-1β and IFNγ can downregulate the expression of sarcoendoplasmic reticulum Ca^{2+} ATPase, which loads the ER with Ca^{2+}. Consequently, the ER gets depleted of Ca^{2+}, leading to ER stress and potentially β-cell death (261). Moreover, if the unfolded protein response cannot resolve the ER stress imposed on the β-cells by hyperglycaemia and/or hyperlipidaemia, it cannot only trigger apoptosis but it can also promote inflammation. The unfolded protein response can activate transcription factors, including NF-κB, that regulate the expression of various proinflammatory cytokines and chemokines (262). Additionally, the oxidative stress stemming from nutritional overload can lead to the activation of stress pathways involving, for example, JNK. These pathways can upregulate the expression of proinflammatory cytokines (263).

As described earlier, IAPP is co-secreted with insulin from pancreatic β-cells. IAPP, also known as amylin, is a polypeptide hormone with many functions. For instance, IAPP has been found to play a role in controlling both satiety and gastric emptying. Furthermore, IAPP is also involved in regulating glucose homeostasis. In addition to suppressing the release of glucagon, IAPP has also been suggested to inhibit insulin release (264). IAPP is prone to aggregate and form amyloid deposits that are cytotoxic and contribute to the progression of T2D. The precise trigger for amyloid formation is not entirely clear, and the exact pathological mechanism by which it promotes β-cell death remains to be delineated. However, several toxic effects have been proposed (264-266). For example, IAPP has been suggested to cause β-cell death by disrupting the membrane integrity of the cells (267, 268). Additionally, ER stress can cause an accumulation of pro-IAPP, which can activate the unfolded protein response. Not only can this stress be damaging by itself, but pro-IAPP can also aggregate and form fibrils, which can damage the cell from within (264-266). Moreover, IAPP amyloids can also activate the NLRP3 inflammasome, which leads to the production of IL-1β. This proinflammatory cytokine contributes to the progression of T2D and can induce β-cell apoptosis (269). It has also been suggested that IAPP can impair autophagy in β-cells, which otherwise serves a protective role against the toxic effects of IAPP (270). Worth noting is that rodent IAPP does not aggregate and form amyloids (264, 265).

As described above, T2D is associated with a decrease in β-cell mass. This can, in part, be attributed to β-cell apoptosis, yet the relatively low rate of apoptosis cannot account for the total decrease in β-cell mass (271). A phenomenon termed dedifferentiation has emerged as a potential key contributor to the deterioration of β-cell mass in T2D. Dedifferentiation has been demonstrated in humans and animal models and is now reckoned to play a role in the pathogenesis of T2D (272-275). In the process of dedifferentiation, β-cells lose their identity, become less differentiated, and can even revert to a progenitor-like state. While physiological

glucose levels have been implicated in the regulation of a differentiated β-cell phenotype, chronic hyperglycaemia is considered a driver of dedifferentiation. Several transcription factors, including MAFA, PDX-1, PAX6, FOXO1 and NKX6.1, play an important role in regulating the expression of β-cell-enriched genes and preserving β-cell identity. Diabetes and glucotoxicity are associated with an altered expression and/or nuclear localisation of these transcription factors (271). In addition to reverting to a less differentiated state, there is also evidence of so-called transdifferentiation, wherein β-cells undergo conversion into other endocrine cells. β-cell transdifferentiation into both α- and δ-cells has been reported (274-277). Conversely, transdifferentiation of both α- and δ-cells into insulin-positive β-like cells has also been reported. This plasticity of endocrine cells within the pancreatic islets is intriguing (278, 279). Furthermore, at the periphery of pancreatic islets, the presence of cells termed "virgin β-cells" has been reported. These cells are transcriptionally and functionally immature β-cells that can be described as an intermediate of α- and β-cells (280).

Latent autoimmune diabetes in adults

Latent autoimmune diabetes in adults (LADA) can be described as an intermediate of T1D and T2D with features characteristic of each of these two forms of diabetes. Similar to T1D, LADA is autoimmune-mediated, yet the adult-onset of the disease resembles T2D (281). Additionally, LADA patients exhibit some degree of insulin resistance but not at the level observed for T2D patients (282). Despite being pathologically closer to T1D, LADA patients are often wrongfully diagnosed with T2D (281). Somewhere in the range of 3-12 % of all adult diabetes cases have been estimated to be LADA. LADA is characterised by the presence of autoantibodies, similar to T1D. However, it progresses slower and milder than T1D. Patients with LADA remain insulin-independent longer than patients with T1D but become insulin-dependent faster than individuals with T2D (282). C-peptide levels in LADA patients are usually lower than in people with T2D but higher than in individuals with T1D (281). In a study assessing the presence of autoantibodies in LADA patients, only 24 % of those positive for an autoantibody were positive for at least two. Furthermore, 90 % of the patients exhibiting the presence of autoantibodies were positive for GADA. Autoantibodies against IA-2A and ZnT8A constituted the remaining 10 % (283).

Monogenic diabetes

Monogenic diabetes is estimated to account for about 1-5 % of all diabetes cases. However, monogenic diabetes is estimated to be undiagnosed in ≥80 % of all cases. As implied by the name, they are caused by mutations in a single gene and over 50

causative genetic variants have been identified (284). Despite this heterogeneity in the group, it is characterised by diabetic onset at an early age (285). The mutations typically affect either the function or development of pancreatic islets. The two main variants of monogenic diabetes are neonatal diabetes mellitus and maturity-onset diabetes of the young (286).

Neonatal diabetes mellitus

Neonatal diabetes mellitus is a rare congenital affliction with an estimated occurrence of 1 in about 90,000-160,000 births. It presents very early in life, and the traditional cut-off is within six months of age. However, it has now been shown that it may take up to twelve months for it to present.

Based on the phenotypic properties, neonatal diabetes mellitus can be classified into three groups: syndromic, transient and permanent. The number of genetic causes identified exceeds 20, with the two most common being mutations in *KCNJ11* and *ABCC8*. Both of these mutations affect the K_{ATP} channel in β-cells, and together, they constitute over 50 % of all neonatal diabetes mellitus cases (287).

Maturity-onset diabetes of the young (MODY)

While the cut-off for neonatal diabetes mellitus presentation is within the first six months, MODY typically presents within the first 25 years of life. The most common variants are *HNF1A*-MODY (MODY3) and *GCK*-MODY (MODY2).

HNF1A-MODY involves a mutation in the gene encoding hepatocyte nuclear factor 1 homeobox A (*HNF1A*) (286). HNF1A is a transcription factor involved in the regulation of genes that are important for the function and development of β-cells (288). However, despite the defects in β-cell function, *HNF1A*-MODY usually presents in early adulthood. HNF1A also controls the expression of sodium-glucose cotransporter 2, which plays a role in the reabsorption of glucose in the proximal tubules of the kidney. The lower expression of the transporter leads to an increase in glucosuria, which masks the hyperglycaemic state caused by β-cell defects (286).

GCK-MODY is a mutation affecting the enzyme glucokinase. Glucokinase functions as a glucose sensor in pancreatic β-cells, and impairments raise the threshold for glucose-stimulated insulin secretion. It mediates glucose conversion into glucose-6-phosphate, which is the initial step for both glycogenesis and glycolysis. Consequently, *GCN* mutations result in defective glycolysis, a decrease in intracellular ATP levels and thus impairments in stimulated insulin release (286).

Gestational diabetes

In the absence of a prior diagnosis of diabetes, hyperglycaemia and poor blood glucose regulation that presents during pregnancy is termed gestational diabetes.

Both insulin resistance and impaired β-cell function contribute to the disorder. It is estimated to affect around 14 % of all pregnancies (289). The exact pathogenesis is not entirely clear but appears to involve environmental, genetic and metabolic factors (290). Both maternal age and obesity are among the risk factors for gestational diabetes. Pregnancy comes with metabolic changes, including insulin resistance, which may exacerbate a pre-existing decline in insulin sensitivity. The growing insulin resistance leads to an increased demand for insulin output from β-cells, which in turn leads to β-cell stress and, eventually, β-cell dysfunction. Additionally, genes associated with an increased susceptibility often relate to the function of β-cells and the metabolic stress encompassing pregnancy may expose even minor defects in β-cell function. Although gestational diabetes is, in itself, a pregnancy complication, it may also cause further pregnancy-related complications such as preeclampsia and a need for caesarean section. Following the birth of the child, gestational diabetes is often resolved. However, both the mother and child suffer an increased risk of developing type 2 diabetes and cardiovascular disease later in life (289).

Links between diabetes and the complement system

Several links between diabetes and complement have been identified, both pertaining to diabetes progression and to the pathogenesis of diabetic complications (104, 291). Inflammation plays a well-recognised role in T2D and is involved in driving insulin resistance (228, 292). By virtue of its proinflammatory properties, the complement system can fuel the diabetes-related inflammation (291). Additionally, diabetes can also perturb complement regulation and activation. As a result of diabetes-associated hyperglycaemia, cell surface molecules may undergo modifications in the form of non-enzymatic glycations, which result in advanced glycation end products (AGE) (293). AGE may function as neo-antigens capable of complement activation. For example, AGE may be recognised by MBL, leading to complement activation via the lectin pathway (294). Additionally, AGE may be recognised by autoantibodies, which could then trigger the classical pathway of complement activation (295).

The complement system has also been implicated in the pathology of various diabetic complications, including diabetic nephropathy, retinopathy, neuropathy and diabetic cardiovascular complications. The complement system's involvement was initially indicated by the presence of complement activation fragments as well as MAC deposition in these tissues. In addition to cytolysis, the MAC can also trigger various signalling pathways that play a well-recognised role in mediating tissue damage in tissues afflicted by diabetic complications. Furthermore, as described in the context of CD59, hyperglycaemia can lead to glycation inactivation

of CD59, rendering it unable to inhibit MAC-formation. Glycated CD59 has been found to colocalise with MAC deposits in tissues of diabetic individuals, including nerves and kidneys (104). Glycated CD59 may also serve as a good biomarker for diabetes, especially gestational diabetes (296).

For both obesity and T2D, chronic low-grade inflammation is recognised as a key pathological feature. This inflammation occurs in the peripheral tissues acted upon by insulin, such as the adipose tissue (297). In adipose tissue, this inflammation is considered a driving force for insulin resistance (298), and the alternative pathway is seemingly highly involved in this process. Adipose tissue is a major site of factor D (also known as adipsin) expression (299). However, adipose cells also express both factor B and C3. As such, adipose cells and tissue express all three factors needed for alternative pathway activation. Consequently, adipose cells are able to induce alternative pathway activation on their own, leading to the generation of factor B and C3 cleavage fragments, such as the anaphylatoxin C3a (300). In mice, proinflammatory macrophages have been shown to infiltrate the adipose tissue in a C3aR-dependent manner. Furthermore, the adipose tissue macrophages, as well as the adipocytes themselves, express high levels of C3aR (301). The infiltrating macrophages produce proinflammatory cytokines such as TNFα (298), which is known to promote insulin resistance (302, 303). Additionally, adipocytes themselves also produce TNFα and the expression is upregulated in obese and diabetic rodent models (302), as well as in obese humans (303).

The alternative complement pathway and adipocyte-derived factor D also influence β-cell metabolism and function. Factor D knockout mice on a high-fat diet exhibit impaired glycaemic control and defective insulin release. Interestingly, though, both insulin secretion and glycaemic control could be improved in obese diabetic mice by reintroducing factor D expression. The effect on insulin secretion was found to be mediated by C3a. C3a was found to augment insulin release in the presence of other secretory stimuli by acting on the C3aR. The underlying mechanism involved an increase in cellular respiration and ATP levels, as well as changes in intracellular calcium levels (299). As such, the alternative pathway can be linked to both insulin secretion and insulin resistance.

Moreover, it has been found that uptake of glucose and fatty acids by adipocytes is promoted by C3a and C5a (304). It has also been suggested that C3a-desArg can stimulate triglyceride synthesis in adipocytes by acting on the anaphylatoxin receptor C5L2 (305, 306). However, this has been disputed by others (307).

Interestingly, in a proteomic study of serum samples from T2D individuals and healthy individuals, several complement proteins, such as C4BP and C3, were upregulated in diabetic individuals. In fact, in T2D individuals, the most upregulated pathway was identified as the complement system. Factor D, however, was found to be enriched in the healthy non-diabetic group compared to the T2D group (308).

IAPP has been found to trigger complement activation (309, 310). However, C4BP is also capable of binding IAPP, and it was found to limit IAPP-induced complement activation (309). C4BP was also found to provide a cytoprotective effect by promoting IAPP fibril formation, thus limiting the cells' exposure to the cytotoxic IAPP oligomers capable of lysing cells (311). Additionally, the actions of C4BP preserve the viability and function of β-cells by restraining IAPP-induced inflammation. When phagocytosed by macrophages, IAPP can trigger inflammasome activation by disrupting the integrity of the phagolysosome. However, IAPP-induced inflammasome activation was inhibited in the presence of C4BP, which thereby prevented inflammasome-generated IL-1β that otherwise negatively impacts β-cell viability and function. The fact that C4BP is produced by the islets themselves is indicative of the importance of C4BP in limiting the IAPP-induced inflammation (312).

Moreover, intracellular C3 has been found to serve a protective role in pancreatic β-cells by interacting with ATG16L1 and facilitating autophagy. Autophagy, which is described in greater detail later on, plays an essential homeostatic role in cells and confers protection against cellular stress. β-cells lacking C3 exhibited defective autophagy and were more sensitive to known β-cell stressors, such as IAPP and lipotoxicity. Consequently, the upregulation of C3 in pancreatic islets observed in T2D was suggested to be a protective mechanism employed by the islets (313). Additionally, intracellular C3 also serves a protective role against IL-1β-induced apoptosis in β-cells. The pro-survival function of intracellular C3 in this context is twofold as it both suppresses pro-apoptotic signalling downstream of the IL-1 receptor and it indirectly, through an interaction with Fyn-Related Kinase, promotes increased activation of Akt (314). As will be described later on, Akt signalling promotes survival.

Cancer

Introduction

Cancer predates the human race by many millions of years, as indicated by studies showing that even dinosaurs were afflicted by it (315). Given that, it comes as no surprise that humans have been plagued by cancer since ancient times. The oldest recordings that are thought to describe cancer in humans come from Egyptian papyri writings dating back to around 1500 BC. One of these writings, the Edvin Smith papyrus, contains what is thought to be the first description of breast cancer. Moreover, Peruvian and Egyptian mummies dating back around 3500 years have provided the earliest evidence of human cancer growth. Modern techniques have also evidenced disseminated prostate cancer in a Scythian king thought to have lived around 700 years BC. Descriptions of cancer originating from antiquity have also been discovered (316). Scholars of this era, such as Hippocrates and Galen, used the terms *onkos*, *karkinos* and *karkinomas* in reference to cancerous malignancies. The Greek words *karkinos* and *karkinomas* translate to "crab" and "crab-like", respectively. This naming is thought to stem from a resemblance of cancer with crabs, either that it can be aggressive and persistent just like crabs or that swollen blood vessels surrounding the tumour resemble crab legs. The term *karkinos* was eventually adopted and changed into the Latin word *cancer* (316, 317).

Today, we know much more about cancer than the scholars active during antiquity, both in terms of pathogenesis and treatment. However, cancer constitutes a global health issue. Due to population ageing, risk-associated habits and behaviour, as well as the exposure to carcinogens present in our surroundings, recent decades have seen a rise in the prevalence of cancer (316). It has been estimated that, in 2020, 19.3 million new cases of cancer were diagnosed worldwide. Additionally, it was estimated that about 10 million people died of cancer the same year (318).

What is cancer?

Cancer is not a single disease; instead, it is an umbrella term used for a large number of distinct diseases. In fact, over 100 different cancers have been identified, and a particular organ can also present with distinct tumour subtypes (319). Based on the definitions of cancer provided by multiple different sources (320), it can be described as a group of diseases characterised by unhinged proliferation of abnormal cells. These cancer cells may be capable of forming tumours, invade the surrounding tissue and even colonise distant parts of the body in a process called metastasis.

Four distinct theories for the origin of cancer cells have been proposed. The first theory concerns somatic mutations and postulates that cancer cells arise as a result of accumulating mutations in so-called tumour suppressor genes and oncogenes. Another theory, referred to as "the bad luck theory", suggests that cancer cells develop as a result of random DNA replication-induced mutations in stem cells that, upon self-renewal, will give rise to malignant daughter cells and eventually cancer. A third theory proposes that disruption of tissue homeostasis and aberrant interactions between the tissue parenchyma and tissue stroma leads to dysplasia and, eventually, cancer. The fourth theory, the "ground state" theory, essentially combines elements of the aforementioned three theories and incorporates both intrinsic and extrinsic factors that converge on cells and drive the generation of cancer (321).

Hallmarks of cancer

In 2000, Douglas Hanahan and Robert Weinberg described six characteristic features of cancer, the acquisition of which was attributed to genome instability and susceptibility to mutations (319). Since then, the list of so-called cancer hallmarks has grown, and new candidates have been proposed (322, 323). Each of these hallmarks will be briefly described here. The hallmarks of cancer and the features enabling the acquisition of these hallmarks are summarised in **Figure 3**.

Sustained proliferation

A key characteristic of cancer cells is their propensity for sustained proliferation. The sustained signalling that promotes proliferation can be achieved in various ways. Not only can they produce and release growth factors that, in an autocrine fashion, will stimulate proliferation, but they can also signal to the tumour stromal cells to provide growth factors. Additionally, cancer cells may also upregulate growth factor receptors. Sustained signalling promoting proliferation can also be achieved through gain-of-function mutations in components of the signalling

pathways downstream of these receptors or through loss-of-function mutations in negative regulators of these pathways (319, 322).

Resisting growth suppression

A second cancer hallmark, which is somewhat linked to the one already described, concerns cancer cells' ability to evade growth suppressors. Many tumour suppressors that negatively regulate proliferation have been identified. Two well-characterised examples of such tumour suppressors are retinoblastoma-associated protein and P53. They function as gatekeepers of the cell cycle, and upon unfavourable conditions, they inhibit progression through the cell cycle. P53 can also initiate an apoptotic program. In many human cancers, the pathway involving retinoblastoma-associated protein is defective, which allows for sustained proliferation (322). Similarly, mutations causing dysfunction of the P53 protein have been observed in more than half of human cancers, but the frequency of P53 mutations differs substantially among different types of cancer (319, 324).

Cell death resistance

The third hallmark concerns cancer cells' ability to resist apoptosis. Apoptosis is regarded as a natural defence against cancer development and can be triggered by different stressors that cancer cells are exposed to. For instance, the tumour suppressor P53 can trigger apoptosis in response to DNA damage. However, as mentioned, P53 is often lost or dysfunctional in cancer cells, which represents one way by which these cells evade programmed cell death. Additionally, apoptosis is regulated by Bcl-2 family members. This family comprises both pro-apoptotic and anti-apoptotic members, the balance of which will determine the cell's fate. Tumour cells can resist cell death by increasing and/or decreasing the expression of anti-apoptotic and pro-apoptotic regulators, respectively (319, 322). Autophagy, which will be described later, represents another pro-survival mechanism.

Replicative immortality

Replicative immortality represents another hallmark of cancer cells and refers to their ability to bypass the Hayflick limit. In contrast to the limitless proliferative capacity of cancer cells, normal cells can only progress through the cell cycle a fixed number of times before entering a non-proliferative senescent state. Cells that are able to break this barrier will enter a crisis phase, leading to cell death. Chromosomal ends are protected by telomeres, which are composed of thousands of repeats of a hexanucleotide sequence. These telomeres are shortened with each replication. In the absence of telomeres, chromosomes can fuse, leading to cell death. The Hayflick limit is determined by the length of telomeres, which is maintained in up to 90 % of all malignant cells through the upregulation of an enzyme called telomerase that extends the telomeres. The rest instead employs a recombination-based mechanism called "alternative lengthening of telomeres".

Irrespective of the mechanism used, the cancer cells can progress through an infinite number of cell cycles as the length of the telomeres is maintained (319, 322).

Angiogenesis

Similar to other tissues, tumours are dependent on the supply of oxygen and nutrients from the vasculature. Neovascularisation in tumours accommodates this need and constitutes another hallmark of cancer. Angiogenesis is regulated by a so-called "angiogenic switch" that, based on the balance between proangiogenic and antiangiogenic factors, will be either on or off. In the context of tumour progression, the switch tends to be constitutively turned on, causing new, typically somewhat aberrant blood vessels to be formed continuously. Oncogene signalling can cause an upregulation of vascular endothelial growth factor expression, which is a key proangiogenic factor (319, 322). Additionally, the inflammatory tumour microenvironment can also act to promote angiogenesis (325).

Invasion and metastasis

The invasive and metastatic properties of cancer cells constitute another hallmark. These characteristics are associated with a number of cellular changes, such as alterations in the expression of adhesion molecules. Typically, adhesion molecules facilitating cell-to-cell contacts, such as E-cadherin, and those facilitating anchoring to the extracellular matrix are downregulated in cancer cells. Adhesion molecules that instead are associated with migratory processes, such as N-cadherin, tend to be upregulated (322). The process of invasion and metastasis can be depicted with a couple of sequential steps. Following the initial transformation into a cancerous cell, the formation of a primary tumour and the encompassing angiogenic processes, local tissue invasion can ensue. Eventually, the cancer cells will enter the circulatory system, detach, and then spread by means of the vascular system. Upon circulatory arrest, the cancer cells can, through extravasation and invasion of the new tissue, begin to colonise the new environment. New tumours can then eventually be established through proliferation and vascularisation (326).

The epithelial-mesenchymal transition is a developmental program orchestrated by a set of transcription factors and plays an important role in facilitating invasion and metastasis. In addition to morphological changes, this developmental program is associated with a downregulation of E-cadherin and loss of intercellular adhesions, apoptosis resistance, expression of enzymes capable of degrading the extracellular matrix, and heightened motility. All of these traits contribute to increased invasiveness (322).

Metabolic reprogramming

The aforementioned cancer hallmarks were described by Hanahan and Weinberg in 2000 (319). However, since then, additional hallmarks have emerged and been proposed. This includes metabolic reprogramming and something referred to as the

Warburg effect. Even under aerobic conditions, glucose utilisation in cancer cells leads to the generation of lactate rather than pyruvate. As such, far less ATP is produced per molecule of glucose, which may seem counterintuitive as cancer cells need a lot of energy to support their high growth rate. However, glucose uptake is enhanced in tumours, and although aerobic glycolysis is an inefficient way of generating ATP, it is a fast way of generating ATP. Additionally, it has been proposed that the glycolytic intermediates can be used for the biosynthesis of macromolecules needed for growth (322, 327).

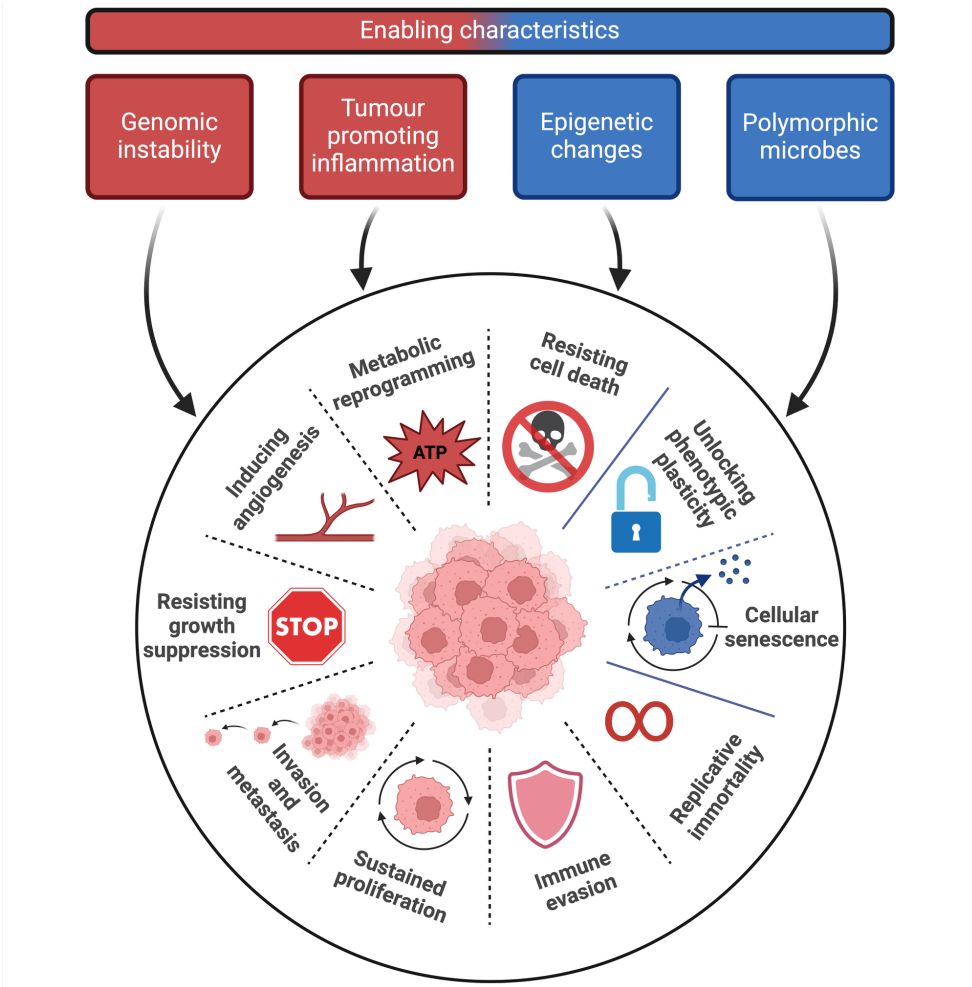


Figure 3. Hallmarks of cancer and the characteristic traits enabling the acquisition of the hallmarks. The most recently proposed hallmarks and enabling characteristics are shown in blue, while the previously established ones are shown in red.

Immune evasion

Another hallmark that has emerged is cancer cells' ability to evade the immune system. The immune system, including $CD4^+$ and $CD8^+$ T cells, normally prohibits the development of cancer. However, chronic inflammation can promote the development of cancer. Cancer cells and tumours can manipulate the immune system in their favour by various means, including the production of immunosuppressive cytokines. They can also recruit immunosuppressive immune cells such as regulatory T cells. The production of transforming growth factor-β, also promotes the conversion of helper T cells into immunosuppressive regulatory T cells. Furthermore, an impaired antigen presentation capability, leading to the absence of tumour antigens, impedes the anti-tumour immune response. The lack of costimulatory molecules on tumour cells also affects the anti-tumour response of T cells and promotes tolerance. Tumour cells can also induce tolerance through the expression of immune inhibitors such as PD-L1 (328).

Newly proposed cancer hallmarks

Recently, phenotypic plasticity and cellular senescence were proposed as potential hallmarks (**Figure 3**). Senescence refers to a state of arrested cellular proliferation and is generally thought to confer protection against cancerous cells. However, these cells can secrete various chemokines, cytokines and other bioactive molecules that, in a paracrine manner, can affect nearby cancer cells. When it comes to phenotypic plasticity, it refers to an ability to either dedifferentiate and revert into a progenitor-like state or transdifferentiate and thereby switch to a different developmental path. In doing so, cancer cells can adopt a phenotype that is distinct from the cell that originally gave rise to the cancer cells (323).

Acquisition of the cancer hallmarks

The originally described trait of cancer cells that enables the acquisition of the various hallmarks was genome instability and a propensity for acquiring mutations that confer a selective advantage. The increased mutational rate exhibited by cancer cells can be achieved through impaired maintenance of genome integrity. Defects in a variety of factors involved in either DNA damage detection, activation of DNA repair mechanisms, or that are part of the DNA repair machinery themselves have been reported. An example of this is P53, which stops the cell cycle upon DNA damage but is lost in many cancers. Inflammation has emerged as another trait enabling the development of cancer hallmarks. For instance, ROS can be released by inflammatory cells and have a mutagenic effect on cancer cells, thereby promoting the accumulation of mutations. Inflammation can also induce cancer progression by supplying the tumour microenvironment with factors promoting growth, survival, angiogenesis, etc, thus contributing to the acquisition of the various hallmarks (319, 322). Recently, both epigenetic changes and the

microbiome were proposed as potential traits enabling the acquisition of cancer hallmarks (323).

Breast cancer

It has been estimated that globally, in 2020, 2.3 million new breast cancer cases in women were diagnosed. This corresponds to 11.7 % of all new cancer cases globally, making breast cancer in women the most frequently diagnosed type of cancer that year. The same report also estimated that, in addition to having the highest incidence among new cancer cases worldwide, breast cancer in women represents the fifth most common cause of cancer-related deaths. Among women in 2020, breast cancer was both the most frequently diagnosed type of cancer and the primary cause of cancer-related deaths (318).

Risk factors for developing breast cancer include an older age at first pregnancy, menarche at a younger age, delayed menopause, alcohol, obesity and diabetes. Mutations in *BRCA1* and *BRCA2*, two tumour suppressor genes, are also associated with a high risk of developing breast cancer. These high-penetrance genes encode DNA repair proteins. Another tumour suppressor, P53, has been found to be mutated in 41 % of breast cancer tumours. In a subtype of breast cancer called basal-like breast cancer (described later on), the prevalence of P53 mutations is 84 % (329). Moreover, the terminal ductal lobular unit is the site from which the majority of breast cancer originates (330). Common metastatic sites in advanced breast cancer include axillary lymph nodes, bone, liver and lungs (329).

Classification

Breast cancer can be categorised both histologically and molecularly, which dictates treatment regimens and provides prognostic information (329). With regard to clinical presentation, morphology and behaviour, invasive breast cancer tumours exhibit a broad heterogeneity. Histologically, at least 18 distinct types of invasive breast cancer can be distinguished, according to the World Health Organization. Based on various features, such as growth- and cytological patterns, around one-quarter of invasive breast cancers can be classified as particular histological subtypes. However, tumours that histologically cannot be classified as a special subtype are diagnosed as invasive ductal carcinoma, also referred to as “no special type”. This subgroup constitutes up to 80 % of invasive breast cancers (331).

Although an updated version takes gene expression and biomarkers into account, a breast cancer staging system based on anatomical features proposed by the American Joint Committee on Cancer has been accepted internationally. Also referred to as TNM staging, it entails an assessment of tumour size (T), the degree

to which the cancer has spread to lymph nodes (N), and the presence or absence of distant metastasis (M). Each factor is given a categoric rank of increasing severity, which is then grouped to give an overall anatomic stage. This ranges from stage 0 to stage IV, with an increasingly worse prognosis (331, 332). In addition to a staging system, a histological grading system can also be used. The Nottingham grading system is a well-established system for histological grading of breast cancer. A score ranging from 1 to 3 with decreasing favourability is assigned to each of three distinct morphological features: the mitotic count, tubular formation, and nuclei morphology in terms of shape and size. The score is then combined to give an overall grade ranging from 1 to 3 (331).

Independent of the histological subgroup, breast cancer can also be classified into molecular subtypes. Based on gene expression data, Perou et al. identified four distinct breast cancer subtypes (333). The identification of these subtypes, referred to as intrinsic subtypes, led to a shift in the clinical approach to managing breast cancer, going from one dictated by tumour burden to one that is more biology-oriented. A surrogate classification system based on the intrinsic subtypes as well as histological characteristics is typically used in the clinic today (329). The intrinsic subtypes are luminal A, luminal B, human epidermal growth factor receptor 2 (HER2)-enriched, and basal-like breast cancer. Luminal A breast cancer is characterised by the expression of oestrogen and progesterone receptors as well as the lack of HER2. This subtype is slow-growing, has a lower clinical grade and typically has a good prognosis. Luminal B tumours also express the oestrogen receptor, but typically at a lower level than luminal A tumours. Additionally, they may lack the progesterone receptor and can either be positive or negative for HER2 expression. The luminal B subtype typically has a higher grade and poorer prognosis than luminal A. The luminal molecular subtypes together constitute the majority of breast cancers. HER2-enriched breast cancer is characterised by an absence of both the oestrogen receptor and the progesterone receptor whilst having a high expression of HER2. HER2-enriched breast cancers exhibit faster growth than luminal breast cancers, have a high grade and are associated with a poor prognosis. Basal-like breast cancer is a triple-negative breast cancer, which refers to the lack of all three receptors characterising the other subtypes, i.e. the oestrogen receptor, progesterone receptor and HER2. Additionally, basal-like breast cancer is associated with a high grade and a poor prognosis. While basal-like breast cancer represents only about 15 % of all breast cancers, it represents up to 80 % of all triple-negative breast cancers. Triple-negative breast cancer constitutes a heterogenic group that can be further classified into six or more distinct subgroups (329, 331, 334, 335). Both triple-negative and HER2-enriched breast cancer have a higher proliferation, death rate and immunogenicity than the luminal subtypes (329).

In addition to the well-established subtypes luminal A, luminal B, HER2-enriched, and basal-like, other proposed subtypes have been described. This includes, for

example, claudin-low breast cancer, which tends to be triple-negative and has a poor prognosis (334).

Treatment and prognosis

Breast cancer detected at an early stage is curable in about 70-80 % of cases, but due to distant recurrence, around 20-30 % of early breast cancer patients ultimately succumb to metastatic disease. The primary cause of death in breast cancer is metastasis. In contrast to early-stage breast cancer, advanced metastatic breast cancer is regarded as incurable with the treatment options available today. As such, patients receive palliative treatment. Metastatic breast cancer can either be present at the time of diagnosis or result from disease recurrence. In the case of the latter, it tends to have increased resistance to treatments and be more aggressive. Although most of the oncogenic drivers of the primary tumour are retained in metastases, subclonal differences may be present. Metastases can develop new drivers as they acquire mutations that distinguish them from the primary tumour. These alterations can develop following the introduction of treatment pressure. Moreover, alterations in growth dependence can also occur as the metastasising cancer cells adapt to the new environment. Despite possible differences between the primary tumour and the metastases, the treatment is usually based on the characteristics of the primary tumour. However, breast cancer metastases may develop resistance to treatment. For instance, as many as 30 % of hormone receptor-positive breast cancers can become resistant to endocrine therapy. Luminal-like primary tumours can spawn metastases that have undergone subtype switching into triple-negative or HER2-enriched breast cancers that are resistant to endocrine therapy. A characteristic of breast cancer is a long interval following surgery, during which a patient may relapse. The time until disease recurrence can range between months and decades. This feature of breast cancer has been suggested to be caused by tumour dormancy. In fact, breast cancer cells are able to remain in a dormant state for decades before emerging and causing metastatic recurrence (329, 336, 337).

Besides the lymph nodes, the most common metastatic sites of breast cancer are bone, brain, liver and lungs, yet the molecular subtypes exhibit distinct propensities for these various tissues. In triple-negative breast cancer, the incidence of metastatic recurrence peaks within the first 2-3 years, and it commonly metastasises to the lungs and brain. Similarly, HER2-enriched breast cancer also metastasises to the brain despite the progress made with HER2-targeting therapies. As for triple-negative breast cancer, an early recurrence within the first few years is also seen for the HER2-enriched subtype. The luminal subtypes, on the other hand, have a better prognosis as they exhibit a low rate of recurrence during the first half-decade. Both luminal A and luminal B breast cancers are prone to metastasising to the bone (329, 336, 337).

For early-stage breast cancer, absent metastases, surgical removal of the primary tumour is the main treatment option. Conversely, surgery is a controversial option for late-stage metastatic breast cancer. However, radiation therapy is considered beneficial for both late-stage metastatic breast cancer and postoperatively in early-stage breast cancer. Moreover, factors such as the intrinsic subtype and tumour burden will dictate the treatment strategy. Chemotherapy is the standard treatment for triple-negative breast cancer and may, depending on the circumstances, also be used for the other subtypes. The standard treatment for HER2-positive breast cancer, both HER2-enriched and luminal-like, is anti-HER2 therapy. Moreover, regardless of the presence or absence of HER2, endocrine therapy is used for luminal breast cancers that are hormone receptor-positive. For patients carrying *BRCA* mutations, poly (ADP-ribose) polymerase inhibitors can be used. Immunotherapy represents another treatment option that can be employed against breast cancer (329).

The epidermal growth factor receptor

The human epidermal growth factor receptor (EGFR) family is composed of four members, called ErbB1-4 or HER1-4 (338). The second member of this family, HER2, was introduced previously in the context of molecular classification of breast cancer. The first member of this family, ErbB1, is generally referred to as EGFR and will henceforth be referred to as such here as well. As the EGFR is of particular relevance in this thesis, much of the focus will be on it rather than the other family members.

The ErbB family of receptors are tyrosine kinase receptors. Ligand-induced EGFR activation entails structural rearrangements and dimerisation. Both homodimers and heterodimers can be formed. The dimerisation of the receptors leads to subsequent transautophosphorylation of the cytoplasmic tyrosine kinase domain (339). This, in turn, enables the activation of downstream signalling pathways, which are described later on. Albeit capable of being phosphorylated and triggering downstream signalling, ErbB3 lacks a kinase domain, and homodimers, therefore, have limited potential for autophosphorylation. Additionally, no ligand-binding domain is present in HER2, and as such, EGFR and ErbB4 are the only fully functional receptors of this family (338, 340). Several ligands have been identified for EGFR and ErbB4. For EGFR, these include the epidermal growth factor, amphiregulin, epigen, betacellulin and transforming growth factor-α. ErbB4 can also be activated by epigen and betacellulin, as well as epiregulin and neuregulin 1-4. Neuregulin 1 and 2 can also activate ErbB3 (338).

Nearly all types of cells express members of the ErbB family, apart from haematopoietic cells. It has been estimated that normal cells express around 40-100 thousand copies of the EGFR. However, it is overexpressed in many cancers,

including breast cancer, and these cells may have over a million molecules of the receptor (340, 341). Although overexpression of the EGFR has been seen in all breast cancer subtypes, it is more frequently observed in triple-negative breast cancer (342). In fact, the EGFR is overexpressed in the majority of cases with triple-negative breast cancer (343).

EGFR signalling

Following ligand-induced receptor activation, dimerisation and transautophosphorylation to activate the cytoplasmic kinase domain, the downstream signalling pathways can be induced. Depending on the activating ligand, the downstream effects of receptor activation differ. This may be due to a preference for a particular dimerisation partner, depending on the ligand. The EGFR activates a complex signalling network of several interlinked pathways known to inhibit apoptosis and promote migration, growth, proliferation and differentiation. In cancer, the downstream signalling pathways promoting these processes are often dysregulated. Combined with the EGFR mutations occurring in cancer, as well as the aforementioned overexpression of the receptor in cancer cells, it has become an appealing therapeutic target. The signalling pathways activated by the EGFR include PI3K-Akt-mTOR, RAS-ERK MAPK, PLC-γ1-PKC, SRC, JAK-STAT and JNK (340). The first two will be briefly described here.

The RAS-ERK MAPK pathway starts with the two adaptors GRB2 (growth factor receptor binding protein 2) and SHC (Src homology and collagen) being recruited to the activated EGFR. SHC becomes phosphorylated and associates with GRB2, which in turn binds SOS (son of sevenless). SOS subsequently activates RAS, which can then interact with RAF-1. RAF-1 is then activating MEK1/2 (mitogen-activated protein kinase (MAPK) kinase 1/2) directly. MEK1/2 are then finally activating the MAPKs ERK1/2. ERK1/2 have over a hundred downstream target substrates that they phosphorylate to induce a wide range of effects. ERK1/2 can, for instance, regulate the synthesis of pyrimidines, ribosome synthesis, and translation of proteins, as well as inhibit apoptosis (340).

The PI3K-Akt-mTOR pathway regulates, for instance, cell size, proliferation, metabolism and survival. Due to mutations in EGFR, PI3K and Akt, this pathway tends to be hyperactivated in cancer (340). Adding to the insult, the negative regulator of PI3K activity PTEN (phosphatase and tensin homolog) is one of the tumour suppressor genes with the highest frequency of mutations in cancer (344). Downstream of the EGFR, a class I PI3K is activated and recruited to the receptor. PI3K will then phosphorylate PIP_2, which results in the formation of PIP_3 (340). The suppressor PTEN functions by dephosphorylating PIP_3 (344). Onwards, PIP_3 causes the serine/threonine kinase Akt, also known as protein kinase B, to translocate to the plasma membrane. Akt then binds PIP_3 and is phospho-activated by PDK1 and mTORC2. Akt has a multitude of downstream target substrates and is known to regulate growth, proliferation, survival, protein synthesis, metabolism and

migration through them. A major outcome of Akt activation is the ensuing activation of mTORC1. Akt can both phosphorylate mTOR directly and phosphorylate the mTORC1 suppressor tuberous sclerosis 2 (TSC2), thereby relieving mTORC1 of the suppression and promoting its activation. When activated, mTORC1 plays an important role in stimulating growth and protein synthesis (340). Worth noting is that ERK1/2 can also promote mTORC1 activation by phosphorylating TSC2 (345).

As described in the context of cancer hallmarks, an altered metabolism is a characteristic feature of cancer cells. The Warburg effect, which refers to cancer cells' propensity for lactate generation rather than oxidative phosphorylation, creates an increased demand for glucose uptake. Akt has been described as a key figure driving the metabolic shift in cancer cells and inducing the Warburg effect (346). This may be facilitated by its ability to upregulate and maintain the number of glucose transporters present on the cell surface, which leads to increased glucose uptake (347).

Worth noting is that an overexpression of EGFR is not associated with a constitutive activation of Akt (348). In HER2-overexpressing breast cancer cells, however, Akt has been found to be constitutively active even when serum and growth factors are absent (349).

Nuclear translocation of EGFR can occur in response to epidermal growth factor stimulation as well as to various stressors such as UV and hydrogen peroxide. An increased presence of nuclear EGFR has been observed in various cancers. When present in the nucleus, the EGFR is involved in the transcriptional regulation of genes encoding factors that mediate cell cycle progression (340).

EGFR regulation

The regulation of EGFR signalling involves internalisation of the receptor. The EGFR can be internalised through both clathrin-mediated endocytosis and non-clathrin-mediated endocytosis. The latter operates in the presence of high epidermal growth factor concentration and plays an important role in long-term repression of receptor signalling through lysosomal degradation of the receptor (339). It has also been suggested that the mode of internalisation depends, to at least some extent, on the ligand that induced activation of the receptor (350). In clathrin-mediated endocytosis, clathrin-coated pits containing the receptors will pinch off to form endocytic vesicles. Following a subsequent fusion between the vesicles and early endosomes, the fate of the receptors will be determined. The receptor can either be directed for lysosomal degradation or recycled back to the plasma membrane (351).

The ubiquitin ligase Cbl (Casitas B-lineage lymphoma) will ubiquitinate the EGFR, a process thought to be important for the translocation of EGFR to clathrin-coated pits. The binding of Cbl to the EGFR can occur either directly or via the adaptor protein GRB2. The ubiquitination of EGFR will eventually target it for lysosomal degradation. The fate of the receptors in the early endosomes depends on the

stability of the ligand-receptor complexes, which in turn depends on the acidic pH in the endosomes (351). Additionally, both the type of receptor dimer and the activating ligand affect the fate of the receptors. For example, the relatively stable EGFR homodimers will remain associated with each other and be sorted for lysosomal degradation. Conversely, the less stable EGFR-HER2 heterodimer will dissociate in the early endosomes, leading to receptor recycling to the plasma membrane (352).

As described earlier, several ligands for the EGFR have been identified. These different ligands will also affect the outcome of EGFR internalisation. Following activation with betacellulin or heparin-binding EGF-like growth factor, all EGFRs will be directed for lysosomal degradation. Contrastingly, a complete recycling of the receptors occurs following activation with epiregulin or transforming growth factor-α. Similarly, amphiregulin also promotes receptor recycling. When it comes to receptor activation by the epidermal growth factor, some of the EGFRs will be recycled, but most will be directed to the lysosomes for degradation (353).

An imbalance between the two fates of the receptor, recycling versus degradation, has been observed in cancers. Abnormal EGFR trafficking may stem from either overexpression or mutations (339).

Non-canonical functions of the EGFR

The aforementioned ligand-induced activation and induction of downstream signalling pathways is regarded as the canonical function of the EGFR. However, the EGFR also has non-canonical functions, some of which are independent of its kinase activity (339). That the EGFR has kinase-independent functions was first suggested when it was found that EGFR knockout mice suffer from developmental defects and are not viable (354-356) but that mice expressing a kinase-dead mutant EGFR variant are viable (357). Using cells expressing kinase-dead mutant EGFR variants, it has been found that independent of its kinase activity, the EGFR can promote cell survival (358), DNA replication (359), and expression of certain genes (360). Additionally, in cancer cells, the EGFR can also stabilise a glucose transporter at the plasma membrane in a manner that does not depend on its kinase activity. This maintains glucose uptake in the cancer cells and promotes their survival (361). Another identified kinase-independent function of EGFR is related to autophagy and will be described later in the context of autophagy in cancer. Additional non-canonical functions of the EGFR include its internalisation in response to stress. Stressors such as tyrosine-kinase inhibitors, UV, cisplatin, hypoxia, oxidative stress, et cetera can trigger EGFR internalisation. Depending on the stressor, this can lead to endosomal arrest, nuclear translocation, degradation or recycling of the EGFR. In most cases, however, it leads to the induction of autophagy (362).

It is possible that there are more non-canonical and kinase-independent functions of the EGFR yet to be discovered. These non-canonical functions may have been underappreciated in the context of EGFR targeting cancer treatments. Given its overexpression in many cancers and the downstream effects of the signalling pathways it activates, the EGFR has been an attractive therapeutic target. Consequently, various EGFR targeting agents have been assessed for their therapeutic effect. However, despite efficiently inhibiting the ligand-induced kinase activity of the receptor, these agents have had limited effect on most tumours. This may very well be due to the non-canonical and kinase-independent functions of the receptor (362).

The complement system and cancer

The role of complement in cancer is complex, dualistic and context-dependent (76). Complement is activated by pattern recognition molecules recognising, for example, aberrant sugar motifs, dying cells and immunoglobulins (76, 363). The genetic and morphological changes associated with carcinogenesis, leading to alterations in glycosylation and the presence of tumour antigens, result in immunogenic cells distinct from normal cells. Such damage-associated molecular patterns may be recognised by the complement system, triggering its activation (363, 364). Originally, complement was thought to confer protection against tumours through the formation of MAC, an idea prompted by the deposition of complement components in tumour tissues (365). Theoretically, if allowed to run its entire course, the complement system could eliminate malignant cells through MAC formation and complement-mediated cytotoxicity. However, most cancers express high levels of complement inhibitors, and the terminal pathway inhibitor CD59 is, in most tumour types, one of the complement genes with the highest expression. This may reflect an adaptation employed by cancer cells to escape complement-mediated cytolysis (76). Of note, though, sublytic MAC abundance can have tumour-promoting downstream effects by activating oncogenic signalling pathways promoting proliferation and inhibiting apoptosis (366, 367).

However, as described in a previous section, complement promotes inflammation by means of the anaphylatoxins C3a and C5a. As also described previously, inflammation is recognised as a characteristic feature of cancer, enabling the acquisition of cancer hallmarks (322). Chronic inflammation not only predisposes an individual to develop cancer, but an inflammatory environment can also promote the progression of the disease (325). Using mouse models, both pro-tumoural (365, 368-378) and anti-tumoural (379-381) effects of complement have been observed for various cancers. Many of the identified pro-tumoural effects were mediated by the C3aR and C5aR1, the receptors for the inflammatory anaphylatoxins (365, 369, 370, 376-378).

As described in the section concerning complement, the complement anaphylatoxins can promote vascularisation, leukocyte recruitment and release of inflammatory mediators. In the context of tumours, locally produced anaphylatoxins similarly influence leukocyte recruitment (76). For instance, myeloid-derived suppressor cells can be recruited to the tumour microenvironment by C5a. These cells will then suppress $CD8^+$ T cells, thus promoting tumour growth. C5a was also found to augment the suppressive capability of the myeloid-derived suppressor cells (365). Moreover, C3aR signalling has been found to restrict the recruitment of neutrophils and $CD4^+$ T cells, thereby having a pro-tumoural function (370). C3aR signalling can also favour tumour progression by affecting the cytokine profile of $CD4^+$ T cells (378). Additionally, C3a and C5a induced signalling through the anaphylatoxin receptors expressed by tumour-infiltrating $CD8^+$ T cells inhibits their IL-10 expression and, thereby, the autocrine IL-10 stimulation of their cytolytic activity. Furthermore, the tumour infiltrating $CD8^+$ T cells express C3 themselves, which can act in an autocrine fashion to inhibit IL-10 production (382). In addition to affecting the recruitment and activity of leukocytes, C3a and C5a also play a role in neovascularisation (76, 377). The complement anaphylatoxins can also mediate effects that are unrelated to inflammation. Some cancer cells express anaphylatoxin receptors themselves, which allows for autocrine stimulation as these cells can also express either C3, C5, or both (76). Cancer cell-derived C3 and C5 can be activated locally and act on the cancer cells in an autocrine manner, activating signalling pathways that promote proliferation (369). By inducing the expression of metalloproteinases, enhancing migration and promoting angiogenesis, C5a can also promote metastasis (76).

When complement gene expression was assessed in various solid human tumours, all cancer types evidently expressed high levels of C3 as well as classical pathway components. Conversely, whilst some exhibited a heterogenous expression level, an overall low expression of genes related to the lectin pathway was observed for most cancers. The alternative pathway genes encoding factor B and factor D were lowly expressed in some cancers but higher in others. Moreover, when comparing the complement gene expression levels in tumourous and matched normal tissues, most complement genes were found to be upregulated in about half of the different types of tumours. In the remaining types of cancers assessed, complement was found to be downregulated. When the expression of complement genes related to the alternative and classical pathways were assessed for their prognostic impact in different types of cancers, four distinct groups were observed. Two of the groups directly contrasted each other and contained tumour types where the expression of these complement genes was associated with either a good or a bad prognosis. A third group contained tumour types where the expression of C3 was associated with a favourable prognosis. In the fourth group of tumours, no discernible prognostic pattern could be identified (76). Taken together, the diverse context-dependent effects of various complement proteins in different types of malignancies warrant further investigation and consideration.

Autophagy

Christian de Duve first coined the term “autophagy” in 1963, which translates to “self-eating” in Greek (383). The initial spark in the research field came from his discovery of the lysosome a few years earlier, in 1955 (384). However, it was not until Miki Tsukada and Yoshinori Ohsumi managed to identify 15 autophagy-related genes in yeast in 1993 that the research field really gained popularity (385). For his work related to autophagy, Yoshinori Ohsumi received the Nobel Prize in Physiology or Medicine in 2016.

Introduction

Autophagy refers to a cellular mechanism whereby cytoplasmic constituents are targeted for lysosomal degradation (386). The process of autophagy is, evolutionarily, highly conserved and occurs in all eukaryotes (387). Autophagy serves a crucial homeostatic function in cells and is constantly operating at low levels but can be further induced in response to cellular stress (388, 389). A wide range of cellular constituents and components can be degraded by autophagy, including protein aggregates, lipid droplets, ribosomes, intracellular pathogens and organelles such as peroxisomes, endosomes, ER and mitochondria (388, 390). The degradation of excess- and/or damaged organelles is crucial for cellular homeostasis and organelle integrity to be maintained, and it protects cells from potential harm. For instance, autophagic degradation of dysfunctional mitochondria prohibits their production of ROS, which can damage the cells (388). The degradation products resulting from autophagy, such as amino acids, are eventually recycled to the cytoplasm, where they can be used for energy production or macromolecule biosynthesis (391, 392).

Autophagy serves as an adaptive response induced by cellular stress. It is triggered by, for example, nutrient or growth factor deprivation, ER stress, oxidative stress, hypoxia, and the presence of protein aggregates, intracellular pathogens or damaged organelles (393). Autophagy generally functions as a protective mechanism promoting cell survival and host health. However, depending on the circumstances, such as a pathological setting, autophagy can have either positive or negative effects (389). Additionally, autophagy is also linked to cell death, and both

autophagy-dependent and autophagy-mediated modes of cell death have been described (394).

Different types of autophagy have been characterised and described, namely chaperone-mediated autophagy, microautophagy and macroautophagy. A major difference between the three is how the autophagic cargo that is to be degraded is delivered to the lysosome (389, 391). Chaperone-mediated autophagy depends on chaperones, such as heat shock cognate 71 kDa protein (HSC70), for directing proteins to the lysosome. Unlike microautophagy and macroautophagy, proteins are the only substrate that can be degraded via chaperone-mediated autophagy. The cytosolic chaperone HSC70 recognises and binds a particular amino acid sequence motif present in about 40 % of all mammalian proteins. HSC70 directs the target protein to the lysosome, where it is translocated across the lysosomal membrane by lysosome-associated membrane protein type 2A (395). In microautophagy, the endosomal or lysosomal membrane invaginates and engulfs the cytoplasmic material that is to be degraded. The membrane invagination will then be pinched off, generating a microautophagic body within the endosome or lysosome (396).

Macroautophagy is of particular interest in this thesis, and its mechanism will be described in detail in the following section. In literature, the term autophagy generally refers to macroautophagy, and similarly, in this thesis, the term autophagy will henceforth refer to macroautophagy.

Macroautophagy

Autophagy is initiated at a phosphatidylinositol 3-phosphate (PI3P)-rich ER subdomain called the omegasome (390). Initiation entails the recruitment of autophagy-related (ATG) proteins and nucleation of a cup-shaped isolation membrane termed phagophore (397). The omegasome serves as a platform for phagophore formation (398). Following the nucleation of the phagophore, the autophagy machinery governs the growth of the phagophore, which will eventually be sealed. This results in the formation of a double-layered membranous compartment known as the autophagosome, in which cytosolic constituents are sequestered (**Figure 4**). Once formed, the autophagosomes will fuse with lysosomes, resulting in the formation of autolysosomes and degradation of the sequestered autophagic cargo (397). Autophagosomes may also fuse with endosomes, forming intermediate structures called amphisomes that will subsequently fuse with lysosomes (399). As described above, the degradation products resulting from the fusion with lysosomes are then recycled to the cytoplasm. Worth noting is that the autophagosome is not formed from pre-existing membranous compartments through membrane budding but is instead synthesised *de novo* (400). Several different membrane sources for the formation of the

autophagosome have been proposed, including the ER, Golgi apparatus and endosomes (401). However, local phospholipid synthesis in close proximity to the autophagosome formation site has also been reported (402, 403).

Autophagy can be both selective and non-selective. Non-selective, or bulk, autophagy can be triggered by starvation and results in non-specific engulfment of cytoplasmic constituents (397). Fatty acids and amino acids resulting from the lysosomal degradation are then recycled and reused by the cell (389). In selective autophagy, specific targets are enwrapped by the elongating phagophore. Different terms are used depending on the autophagic cargo, such as mitophagy for mitochondria, aggrephagy for aggregated proteins, xenophagy for intracellular bacteria, and so on. Selective autophagy depends on so-called selective autophagy receptors (SARs) that can be either membrane-bound or soluble. It also depends on the microtubule-associated protein light chain 3 (LC3) subfamily of proteins, which includes LC3A, LC3B and LC3C (386). As described later, LC3 can be cleaved and lipidated to generate LC3-II, which is incorporated into the growing phagophore (397). LC3-II functions as an adaptor, recruiting proteins harbouring a motif called LC3-interacting region, which is present in SARs. SARs associated with the autophagic cargo can, through their interaction with LC3-II, facilitate the recruitment of the cargo to the phagophore for autophagic degradation. An example of a SAR is p62, also known as sequestosome-1. In addition to being a substrate for autophagy itself, it also facilitates the autophagic degradation of ubiquitinated cargos, for instance (386).

Mechanism

The initiation and nucleation to form the phagophore depend on two key protein complexes, the ULK1 (unc-51-like kinase 1) complex and the PI3K complex. The ULK family of kinases has four members (ULK1-4). However, the most important member in the context of autophagy is ULK1. The ULK1 complex is composed of ULK1, ATG13, ATG101 and FIP200. The PI3K complex, on the other hand, is composed of the class III PI3K VPS34, Beclin-1, p150 and ATG14L (388, 390).

mTOR is a key regulator of autophagy in mammals and is known to be a part of two different protein complexes called mTORC1 and mTORC2, but only mTORC1 is directly involved in autophagy regulation (404). Under nutrient-rich conditions, both ULK1 and ATG13 are present in an inactive phosphorylated state bound to mTORC1. Nutrient deprivation, however, causes ULK1 to be dephosphorylated and dissociated from mTORC1. Subsequent autophosphorylation of ULK1 results in its activation, enabling it to phosphorylate both ATG13 and FIP200 (397). This leads to activation of the complex responsible for autophagy initiation (404). Once activated, the ULK1 complex translocates and initiates autophagy at the phagophore assembly site. The ULK1 complex proceeds to phosphorylate its substrate proteins, thus promoting autophagy progression. ULK1-mediated phosphorylation of Beclin-

1 leads to activation of the PI3K complex that is recruited to the phagophore assembly site in a manner dependent on its subunit ATG14L. The activated PI3K complex then starts to generate PI3P by phosphorylating phosphatidylinositol (390). This constitutes an essential step in the initiation of autophagy. The recruitment and activation of the ULK1 complex and the PI3K complex leads to autophagy initiation and nucleation of the phagophore (392, 405).

The PI3K complex is subject to extensive regulatory input. At least six different kinases are, for example, regulating Beclin-1 through phosphorylation. Beclin-1 can be phosphorylated by AMP-activated kinase (AMPK), which promotes autophagy (390). AMPK is a heterotrimeric complex composed of two regulatory subunits, β and γ, and a catalytic α subunit. Conserved in all eukaryotic cells, AMPK functions as a metabolic switch that, in response to cues concerning energy and nutrient levels, regulates metabolism. Upon a decreased ATP:AMP ratio in the cell, AMPK is activated by AMP binding and phosphorylation by Liver Kinase B1 (LKB1) (406). AMPK is subsequently able to exert many pro-autophagic functions. Activated AMPK suppresses the activity of mTORC1 by phospho-activating TSC2, which has an inhibitory effect on mTORC1. AMPK can also suppress the activity of mTORC1 by phosphorylating its subunit known as Raptor (406). As mTORC1 is a well-known suppressor of autophagy, AMPK induces autophagy by relieving the mTORC1-mediated suppression. AMPK can also bypass mTORC1 and promote autophagy initiation through direct phospho-activation of ULK1. However, under nutrient-rich conditions, AMPK-mediated activation of ULK1 is prohibited by mTORC1 (407). In addition to being phosphorylated by ULK1 and AMPK, both death-associated protein kinase (DAPK) and mitogen-activated protein kinase-activated protein kinase 2 (MAPKAP2) can also phosphorylate Beclin-1. While phosphorylations by these kinases promote autophagy, both Akt and the EGFR can phosphorylate Beclin-1 in a suppressive manner (390). How EGFR affects Beclin-1 will be described in a later subsection concerning autophagy in cancer.

Moreover, Bcl-2, which is an anti-apoptotic protein, can bind Beclin-1 and inhibit autophagy by suppressing the kinase activity of VPS34. The PI3K complex can also be bound by Autophagy and Beclin-1 Regulator 1 (AMBRA1), which promotes autophagy. AMBRA1 can be phosphorylated by ULK1, which contributes to PI3K complex activation. Finally, regarding the regulation of autophagy at this step, mTORC1 can also suppress autophagy through phosphorylation of ATG14L (390).

ATG9-positive vesicles are also recruited to the initiation site. ATG9 is a transmembrane protein essential for autophagosome formation. ATG9 is present in small vesicles in the trans-Golgi network but can also be trafficked through other membranous compartments such as the ER, Golgi and endosomes. Various components of the machinery orchestrating autophagy interact with ATG9-positive vesicles. In addition to the proposed contribution of a seed membrane for the formation of an autophagosome precursor, these vesicles have been suggested to

function as a platform where the autophagy machinery components can assemble (398). The local generation of PI3P by the PI3K complex leads to the recruitment of WD repeat protein interacting with phosphoinositide (WIPI), which is in a complex with ATG2. While WIPI is a PI3K-binding protein, ATG2 is able to associate with the ER. Consequently, this complex tethers the phagophore to the ER and plays an important role in autophagosome biogenesis. This complex can contribute to the elongation of the autophagosome precursor through lipid transfer from the ER (401).

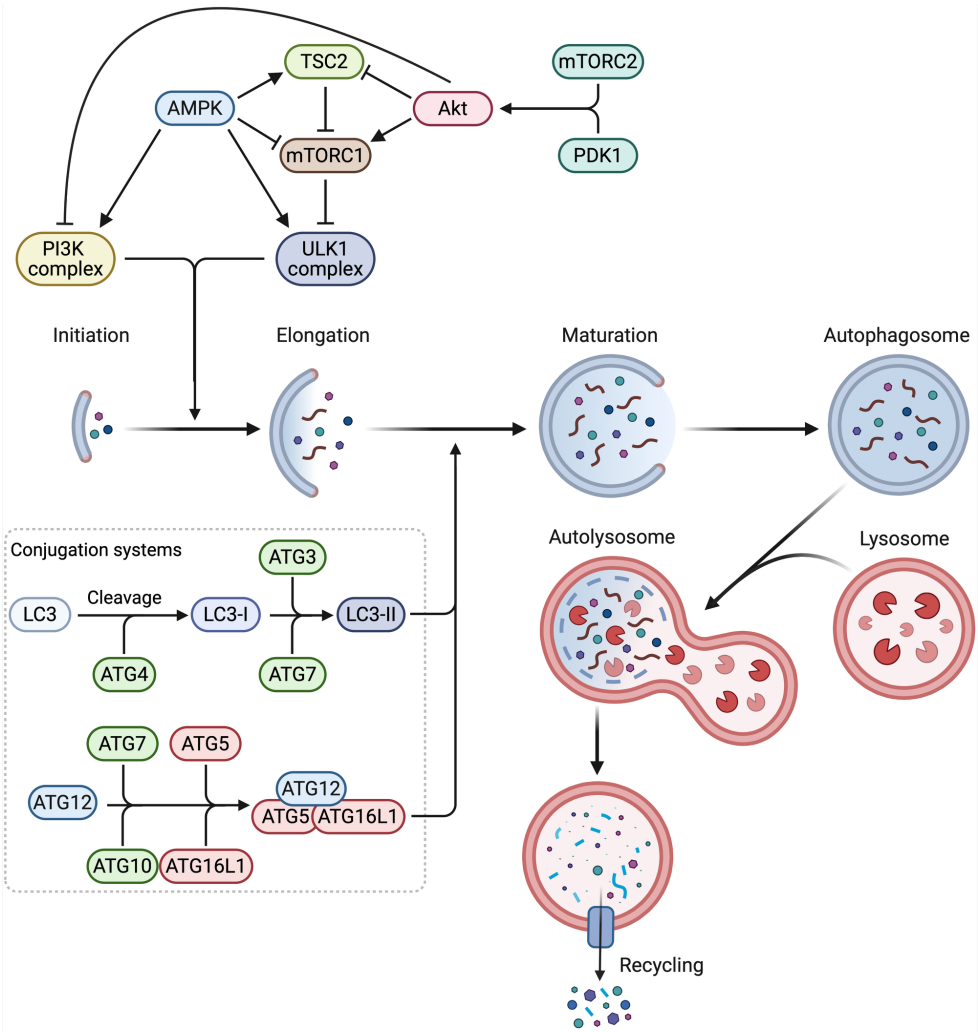


Figure 4. Simplified overview of the mechanism of autophagy. Key complexes influencing the initiation of autophagy and the conjugation systems involved in phagophore elongation are shown. The phagophore engulfs cytosolic constituents in a structure called the autophagosome. The autophagosome will fuse with a lysosome, resulting in the degradation and recycling of the engulfed material.

Two different ubiquitin-like systems are subsequently important for the elongation of the phagophore (**Figure 4**). The first of these facilitates the formation of the ATG12-ATG5-ATG16L1 complex. The ubiquitin-like protein ATG12 is first activated and conjugated to ATG5 through the consecutive actions of the E1-like ubiquitin activating enzyme ATG7 and the E2-like ubiquitin conjugating enzyme ATG10. ATG5 also interacts with ATG16L1, which is present in dimers. As such, a complex with ATG12-ATG5-ATG16L1 in a 2:2:2 stoichiometry is formed (391, 404). The ATG12-ATG5-ATG16L1 complex is recruited to the phagophore assembly site through an interaction between WIPI2 and ATG16L1 (397). LC3 processing is orchestrated by the second ubiquitin-like system and is important for phagophore elongation. The protease ATG4 first cleaves cytosolic LC3 to generate LC3-I, which is then activated by the E1-like enzyme ATG7. The ensuing lipidation of activated LC3-I by conjugating it to phosphatidylethanolamine to generate LC3-II, as well as its incorporation into the elongating phagophore, is governed by the E2- and E3-like activities of ATG3 and the ATG12-ATG5-ATG16L1 complex, respectively. As such, the ATG12-ATG5-ATG16L1 complex promotes the generation of LC3-II and its subsequent incorporation into the growing phagophore, which is important for the elongation (388, 391, 397).

Once the formation of the autophagosome is complete, it is transported on microtubules to a lysosome. Subsequent autophagosome fusion with the lysosome results in an autolysosome, in which the autophagic cargo is degraded. The fusion with the lysosome is orchestrated by a set of SNARE proteins, namely Syntaxin-17, SNAP-29 and VAMP8 (390). When forming the autolysosome, the autophagosome's outer membrane layer is fused with the membrane of the lysosome. A single-membrane layered autophagic structure is thus released into the lysosome, where it is degraded together with its content (397).

Autophagy in cancer

Considering the dually opposing effects, the role of autophagy in cancer is complex. The functional outcome of autophagy in cancer is seemingly dependent on the context, including the tumour stage. Currently, autophagy is thought to suppress carcinogenesis and tumour initiation but then serve a pro-tumoural effect in later stages.

Several observations argue a suppressive effect of autophagy on tumour development. Allelic deletions of the gene encoding Beclin-1 have been seen in many breast cancer cell lines, and it is frequently observed in cases of both ovarian cancer and breast cancer (408, 409). Additionally, while homozygous deletion of the gene encoding Beclin-1 is lethal, hemizygous mice spontaneously develop tumours in the lymphatic tissue, liver and lungs (410, 411). Together, this depicts

Beclin-1 as a tumour suppressor and suggests a protective effect of autophagy against cancer. It should be noted, however, that the view of Beclin-1 as a tumour suppressor in the context of human cancers has been disputed. Given the close chromosomal location of the Beclin-1 gene with the tumour suppressor gene *BRCA1*, the allelic deletion of the gene encoding Beclin-1 may be the result of gene linkage (412). Nevertheless, mice with a mosaic deletion of ATG5 spontaneously develop hepatic tumours. Such tumours in the liver also develop in mice with a liver-specific deficiency of ATG7 (413).

Additionally, as described earlier, the PI3K-Akt-mTOR axis is often dysregulated in cancer. This pathway leads to the activation of mTORC1, which is frequently overactive in many cancers (414). A consequence of this is the inhibition of autophagy through mTORC1-mediated suppression of ULK1. Conversely, many so-called tumour suppressors, such as AMPK, LKB1, PTEN, Beclin-1, TSC, and P53, either directly or indirectly promote autophagy. The function of most of these has already been described, except for P53 (415). P53 can promote autophagy through transcriptional regulation of autophagy-related genes (416). However, both activation and inhibition/deficiency of P53 can promote autophagy. It has been reported that while P53 localised in the nucleus can induce autophagy, cytoplasmic P53 can suppress it (417). Cancer cells' propensity for overactive signalling that suppresses autophagy, together with the autophagy-promoting effect of many tumour suppressors, could serve as an indication of autophagy's anti-tumoural effect.

Autophagy functions as an adaptive response to cellular stress. This applies to both normal cells and cancer cells. As such, the same exact function of autophagy can either suppress tumourigenesis or promote tumour progression. The normal homeostatic role of autophagy is important for maintaining cellular health. Autophagy enables cells to endure metabolic stress, and it mitigates the potentially harmful effects of misfolded proteins, protein aggregates and damaged organelles, the effect of which could render the cell susceptible to malignant transformation. For example, autophagy plays an important role in maintaining the mitochondrial pool by degrading old and damaged mitochondria. By preventing damaged mitochondria from accumulating in the cells, autophagy limits the generation of ROS, which could otherwise promote mutagenesis by inducing DNA damage (418, 419). In line with this, it has been shown that metabolic stress leads to increased DNA damage in cells with defective autophagy, suggesting a role for autophagy in maintaining the integrity of the genome (420).

In tumour cells, autophagy is induced in response to hypoxia, growth factor depletion and starvation. As a result of inadequate vascularisation and, thus, poor blood supply, tumour cells may be subjected to increased metabolic stress caused by the limited availability of growth factors, nutrients and oxygen. Tumour cells can also suffer from increased metabolic stress caused by intrinsic factors. The inefficient generation of ATP as a consequence of the metabolic switch to aerobic

glycolysis, as well as the high rate of proliferation, results in an increased metabolic demand. Therefore, even if autophagy serves a similar function in both normal cells and tumour cells, the latter may have a greater dependence on autophagy as they may experience increased metabolic stress. Moreover, autophagy also functions as a pro-survival mechanism that is able to sustain tumour cells with defects in apoptosis for a long period of time (418). Another pro-tumoural role of autophagy is its repression of the tumour suppressor P53, which otherwise promotes cell cycle arrest or apoptosis in response to cellular stress such as DNA damage (416).

Counterintuitively, the pro-survival role of autophagy in apoptosis-deficient tumour cells may serve an anti-tumoural effect. In this setting, autophagy may prohibit necrosis and the encompassing inflammatory response, which could otherwise be beneficial for the tumour (421). Autophagy has also been shown to play a role in immune evasion. In pancreatic ductal adenocarcinoma cells, the major histocompatibility complex I is selectively degraded by autophagy, resulting in decreased antigen presentation to $CD8^+$ T cells (422).

Autophagy has also been found to play a seemingly context-dependent role in metastasis. In addition to being able to promote migration in some settings, autophagy has also been proposed to either restrict or promote the epithelial-mesenchymal transition of cancer cells. Moreover, cellular detachment from the extracellular matrix leads to an upregulation of autophagy. This occurs in cancer cells during metastasis, where autophagy then serves a protective role against anoikis, a type of cell death induced by the absence of integrin signalling. Autophagy can also play a role in metabolic adaptation and enable cancer cells to persevere under potentially nutrient-scarce conditions in the new environment. Another role for autophagy is related to dormancy. Autophagy is upregulated in dormant cells and is thought to be important for maintaining the dormant state and promoting survival. Inhibition of autophagy can lead to escape from dormancy and metastatic outgrowth (419, 423).

Additionally, various anti-cancer therapies induce autophagy, which may then promote cell survival and confer a mechanism of resistance. In response to treatment, autophagy can also enable cancer cells to enter a dormant state. Treatments that have been found to induce autophagy include various chemotherapeutics and radiotherapy (415). How autophagy can be induced in response to cancer treatment will be exemplified in the following subsection with therapeutics targeting the EGFR.

The EGFR regulates autophagy

As described earlier, the EGFR is overexpressed and/or mutated in many cancers. Additionally, its downstream signalling pathways, including the PI3K-Akt-mTOR

pathway, are often dysregulated. The EGFR and its signalling pathways are directly linked to the process of autophagy. For example, activation of the PI3K-Akt-mTOR pathway suppresses autophagy. mTOR, which is activated by Akt, inhibits autophagy by suppressing ULK1. Akt can also suppress autophagy through direct phosphorylation of Beclin-1 (424). Moreover, activated EGFR can directly bind and phosphorylate Beclin-1. This enhances the interaction between Beclin-1 and its negative regulators, Rubicon and Bcl-2, thus suppressing autophagy (425). The activation of EGFR and the downstream PI3K-Akt-mTOR signalling pathway thus illustrates a mechanism whereby autophagy is suppressed in cancer.

However, an autophagy-promoting mechanism of EGFR has also been identified. While it inhibits autophagy during nutrient-rich conditions, it induces autophagy in response to serum deprivation. During such conditions, inactive EGFR accumulates in endosomes, where it associates with lysosomal-associated transmembrane protein 4B (LAPTM4B) and the Sec5 exocyst complex. Subsequently, the inactive EGFR interacts with Rubicon, which leads to its dissociation from Beclin-1. Once Beclin-1 is released from Rubicon, it can initiate autophagy (426).

EGFR-targeting tyrosine-kinase inhibitors and EGFR-targeting antibodies have been developed and assessed for their therapeutic potential. However, they have been found to induce autophagy in cancer cells, which serves a cytoprotective function. It has, therefore, been suggested that a combinatorial treatment strategy targeting both the EGFR and autophagy may be clinically advantageous (427-432). The use of these therapeutic agents, such as tyrosine-kinase inhibitors, mimics the effect of serum starvation. Concordantly, tyrosine-kinase inhibitors induce autophagy by a similar mechanism. The treatment with tyrosine-kinase inhibitors leads to an endosomal accumulation of EGFR, where it interacts with both the exocyst complex and Rubicon, thus relieving Beclin-1 of the inhibition imposed by Rubicon. In contrast to the situation with serum starvation, autophagy induced by tyrosine-kinase inhibitors was not dependent on LAPTM4B (426).

Methodology

Cell lines

Cell lines were used as models throughout the studies included in this thesis. Cell lines represent a useful tool in research, and they come with many advantages. They offer a pure and uniform population of cells, which promotes reproducibility. As they can be easily expanded, they constitute an unlimited sample resource. They are also easily manipulated and can with ease be genetically engineered to suit one's needs. Additionally, cell lines are cost-efficient models, and from an ethical standpoint, it is easier to motivate using them instead of other models. There are important limitations that must be considered, though. The physiological relevance of studies based on cell lines is lower as the cells are not studied in their natural physiological setting, thus being devoid of environmental cues, for instance. Furthermore, immortalised cell lines are genetically altered, which may lead to differences in behaviour, function and phenotype. Genotypic and phenotypic differences may also arise due to genetic drift as they are cultivated for extended periods of time. As such, results obtained using cell lines may not always be translatable to an *in vivo* setting or even adequately represent primary cells.

INS-1 832/13 cells

INS-1 832/13 is a rat β-cell line derived from a rat insulinoma (433). The parental cell population, INS-1, was originally established through dispersion of transplantable radiation-induced insulinoma from New England Deaconess Hospital rats (434). Inherent restraints to the methodological approach led to the procurement of a heterogenic polyclonal cell population (433). The heterogeneity of the INS-1 cell population was evidenced when stable subclonal cell populations were generated through transfection of INS-1 cells with a plasmid carrying human insulin cDNA under the control of the cytomegalovirus promoter. Following antibiotic selection, insulin secretion was tested in the obtained clones, where the INS-1 832/13 subclone secreted the most insulin in response to glucose stimulation. The glucose responsiveness of the INS-1 832/13 cells was comparable to that of isolated rat pancreatic islets and exhibited an 8-11-fold increase in glucose-stimulated insulin secretion that remained stable for up to 7.5 months. Comparably, the parental INS-1 cells only demonstrate a 2-4-fold increase in glucose-stimulated insulin secretion that diminishes after 2-3 months in culture (433). The INS-

1 832/13 cell line is a monoclonal insulin-secreting β-cell line that serves as a suitable model for studies related to β-cell function and mechanisms of metabolic signalling and stimulated exocytosis. Worth noting is that INS-1 832/13 cells are inherently resistant to G418 as a result of the transfection of the parental cell line (433), which is something that must be considered in the context of subsequent transfections. Additionally, INS-1 cells depend on the presence of β-mercaptoethanol for continuous growth and cell culturing (434).

MIN6 cells

MIN6 is a mouse β-cell line obtained from insulinomas of a transgenic C57BL/6 mouse. A fusion gene composed of the human insulin promoter coupled to the gene encoding the SV40 T antigen was microinjected into fertilised eggs to generate transgenic C57BL/6 mice (435). The SV40 T antigen acts on tumour suppressors and induces cell transformation and tumour formation (436). Consequently, the transgenic mice developed insulinomas from which two cell lines could be established, MIN6 and MIN7. In contrast to MIN7 cells, MIN6 cells exhibited glucose responsiveness and secreted significantly more insulin in response to a higher glucose concentration. Originating from mouse pancreatic β-cells, the MIN6 cell line represents a morphologically homogenous cell population growing in clusters and that functions in stimulus-coupled insulin secretion (435). However, impaired glucose-stimulated insulin secretion has been reported for MIN6 cells at high passage numbers, yet they retain the ability for potassium-stimulated insulin release (437).

EndoC-βH1 cells

EndoC-βH1 is a human β-cell line derived from a foetal pancreas. Human pancreases were obtained from foetuses of terminated pregnancies. The foetal pancreases were transduced with lentiviral vectors carrying the SV40LT gene coupled to the rat insulin promoter. Next, the lentiviral vector-transduced pancreatic tissue was transplanted into SCID (Severe Combined Immunodeficient) mice. β-cell proliferation induced by the expression of SV40LT led to the formation of insulinomas. Following isolation and dissociation of primary insulinomas, resulting cell clusters were transduced with a second lentiviral vector. With lentiviral vectors encoding human telomerase reverse transcriptase, the second transduction aimed to bypass the Hayflick limit and inhibit senescence. Enrichment of β-cells was subsequently achieved by transplantation of transduced cell clusters into SCID mice. Following the expansion of insulin-expressing cells, the resulting tissue, from which the EndoC-βH1 cells could be retrieved, was isolated. EndoC-βH1 was selected for its high insulin content, yet it contains less insulin than human β-cells. EndoC-βH1 cells are glucose-responsive insulin-secreting β-cells. In addition to secreting insulin in a glucose concentration-dependent manner, insulin release could be further augmented by the incretin GLP1 and known secretagogues. Furthermore,

several β-cell identity genes were found to be expressed in the EndoC-βH1 cells. In addition to the low insulin content compared to human β-cells, another disadvantage of the EndoC-βH1 cell line is the low proliferation rate with a 5-day population doubling time. However, insulin content and glucose-stimulated insulin secretion remain consistent at high passages (438).

BT20 cells

BT20 is a human breast cancer cell line obtained from a primary tumour of advanced infiltrating duct cell carcinoma of a 74-year-old Caucasian woman (439, 440). BT20 cells were isolated in 1958 (439), making it the oldest breast cancer cell line (441). According to the molecular classification, BT20 cells belong to the basal-like A subgroup of breast cancer cells (442, 443). BT20 cells carry a missense mutation in the P53 tumour suppressor gene and overexpress the EGFR (444, 445). It also carries an oncogenic mutation in PIK3CA (444), which encodes a catalytic subunit of PI3K (446).

MDA-MB-468 cells

First described in 1978 (447), MDA-MB-468 is a human triple-negative breast cancer cell line obtained from pleural effusion of a black 51-year-old woman with adenocarcinoma of the breast (440, 447). Similar to BT20 cells, MDA-MB-468 belong to the basal-like A subtype of breast cancer cells based on molecular classification (442, 443). Furthermore, like BT20 cells, MDA-MB-468 cells carry an oncogenic missense mutation in the P53 tumour suppressor gene (444) and overexpress the EGFR (448). Additionally, MDA-MB-468 cells also carry an oncogenic mutation in PTEN (444).

4T1 cells

4T1 is a tumour-derived cell line originating from a mouse mammary carcinoma (449). A mammary tumour arose spontaneously in a BALB/cfC3H mouse carrying the mouse mammary tumour virus, from which four cell lines were retrieved (450). Transplantation of cells from the parental tumour for *in vivo* passage eventually led to the generation of a cell line denoted 4.10, which was derived from lung nodule metastasis (451). Four generations of transplantation later, the tumour-derived cell line 410.4 was obtained (452), from which two sublines were ultimately derived – one being the 4T1 cell line (453). 4T1 cells can be cultured *in vitro* but can also be transplanted into mice and effectively model human breast cancer. Several properties make it a suitable model, such as the invasiveness, tumourigenicity and the fact that the primary tumour readily metastasises. Additionally, by transplanting into the correct anatomical site, the primary tumour will form in the appropriate site, which further contributes to its suitability in modelling human mammary carcinoma (449).

Mouse models

Mouse models represent a valuable tool for research of human pathologies. Unlike cell lines, mouse models allow for investigations in whole-body systems, which increases the physiological relevance of the results. It should be noted, however, that results obtained using mouse models cannot be directly extrapolated to a human setting due to limited genetic diversity and inherent species differences. Additionally, genetic manipulation is less feasible than in cell lines, although several well-established models for a variety of pathologies are already available. An important difference between the use of cell lines and mouse models is the ethical aspect of using animals. The use of animals, e.g. mice, in research needs to be properly motivated, and one should always adhere to the three R's of animal research: Replace, Reduce and Refine.

Syngeneic mouse model

Syngeneic mouse tumour models involve transplantation of *in vitro*-cultured murine cancer cells into a mouse strain with the same genetic background as the host from which the tumour cell line is derived (454). As this allows for studies in immunocompetent hosts, syngeneic models are widely used for investigations pertaining to cancer therapy, including cancer immunotherapy (454, 455). As the cells can rapidly be expanded, a major advantage of syngeneic models is the ease with which sufficiently large study groups can be obtained compared to genetically engineered- or patient-derived xenograft models (454). Additionally, prior to transplantation, the tumour cells can be genetically manipulated to facilitate studies of a particular cell-intrinsic property (454). There are, however, limitations to consider. The rapid formation and growth of the tumours may cause too narrow a time window for studies related to the efficacy of immunotherapies or the effect of therapeutic agents at the developmental stages of the tumour. Furthermore, the therapeutic relevance of the results is also hampered by the lack of heterogeneity. The tumour heterogeneity caused by a mutational propensity of cells in the tumour microenvironment, as well as interpatient differences, is absent. Instead, a monoclonal, or at least poorly diversified, population of cells is transplanted into genetically identical inbred hosts. The transplantation process by itself can also induce an inflammatory response, but the tumour cells can, in some cases, be transplanted to the correct anatomical site to better mimic the appropriate tumour microenvironment (454, 455). To investigate the effect of SUSD4 expression on tumour growth, a syngeneic mouse model was used. As described previously, 4T1 is a murine breast cancer cell line derived from BALB/c mice. To evaluate the effect of SUSD4 expression, 4T1 cells expressing or lacking SUSD4 were transplanted into the mammary fat pads of BALB/c mice. Since SUSD4 is a complement inhibitor, its expression could be advantageous for the tumour. As such, we opted

for a syngeneic model, which allowed for an investigation in a system with an uncompromised immune background.

CD59 double knockout mice

To study the role of CD59 in insulin secretion *in vivo*, a CD59 double knockout mouse model (CD59abKO) was employed. As previously described, the CD59 gene in mice has undergone duplication, resulting in the existence of *CD59A* and *CD59B*. With *CD59A* being present about 11.6 kb downstream of *CD59B*, the two CD59 genes together span a region of 45.6 kb. The CD59abKO mouse model was generated by removing a large genomic region that, in addition to the region between the two CD59 genes, contained critical exons of each gene. The 24 kb fragment that was removed included *CD59B* exon 4, which encodes around 60 % of the mature protein. It also contained both exons 1 and 2 of *CD59A*, which encodes the start codon as well as the signal peptide. Consequently, the mice do not have any detectable CD59A or CD59B and present with haemolytic anaemia as a result of complement-mediated lysis of erythrocytes (456). This seemed like a good model for investigating CD59's role in blood glucose homeostasis.

Methodological approaches

General

Every experimental model and method comes with inherent advantages and problems. To improve the reliability of the research, it is therefore good to perform complementary experiments using other approaches and to use different models in parallel. By showing that an observed phenomenon can be detected by various approaches and that it is not specific to a particular cell line, model or species, the research findings become more reliable. How such rigour was achieved in the three papers can be exemplified in many ways. For instance, several distinct approaches were used to validate the identified protein interactions. Both the interaction between SUSD4 and EGFR in paper I and the interactions between IRIS isoforms and SNARE proteins in paper II were demonstrated using ELISA, proximity ligation assay and co-immunoprecipitation. Moreover, an impaired insulin secretion caused by the absence of CD59 was shown in β-cells of three distinct species – human, mouse and rat. Additionally, intracellular splice forms of CD59 capable of rescuing this impaired insulin secretion were identified in both mice and humans. Moreover, multiple breast cancer cell lines were used in paper I, two of which were used in parallel in most of the experiments. Autophagy was also investigated using distinct approaches, both in terms of autophagic marker and the technique used. Additionally, in paper III, blood glucose homeostasis in the CD59abKO mice was looked at in different ways, directly by assessing fasting blood glucose levels and glucose tolerance and indirectly by looking at glucose-stimulated insulin secretion

with isolated pancreatic islets. Several other examples can be mentioned from the different projects, which contribute to the reliability of the results.

However, an important drawback that should be noted is the use of "unnatural" models. In paper I, for example, breast cancer cell lines were transfected with constructs inducing an overexpression of SUSD4. Such overexpression may cause disparities relative to a natural setting. An alternative approach would be to use triple-negative breast cancer cell lines naturally expressing SUSD4, and in fact, two such cell lines were available, namely HCC1187 and HCC1143. But, these were slow-growing and not as easy to work with. Additionally, to study the effect of SUSD4 in these cells, CRISPR/cas9 would have had to be used to knockout SUSD4. Such gene editing using CRISPR/cas9 is more difficult in slow-growing cell lines. Although this would also have entailed an "unnatural" setting, it would constitute an additional approach which would have strengthened the findings. Moreover, both BT20 cells and MDA-MB-468 cells, which were used throughout paper I, carry mutations in genes affecting the downstream signalling from the EGFR, which could have affected the results.

Another example of an unnatural model is the overexpression of human IRIS-1 and IRIS-2 in the rat β-cell line INS-1 832/13. This was because of the feasibility of using INS-1 832/13 cells. This is a well-established β-cell model that grows fast, is easy to work with and secretes insulin well. Conversely, EndoC-βH1 cells are slow-growing and not as easy to work with. Knocking out CD59 and then generating clones that stably express the IRIS isoforms by transfection was more feasible in INS-1 832/13 cells. For the same reason, the CD59ba hybrid identified in the mouse model was expressed and functionally characterised in INS-1 832/13 cells instead of MIN6 cells.

Gene-editing and transfections

Gene-edited cells were used in all three papers. CRISPR/cas9 had previously been used to generate INS-1 832/13 CD59 knockout cells that were used in papers II and III, and it was used to generate BT20 EGFR knockout cells in paper I. CRISPR/cas9 represents a useful tool for genetically engineering cells. It does, however, come with some disadvantages, as it can be time-consuming. One may also have to screen a large number of clones to identify cells in which the desired editing occurred. It can also have off-target effects, causing unwanted genomic alterations and, consequently, phenotypic alterations. As such, there may be clonal disparities that one must account for by using multiple clones. An alternative to CRISPR/cas9-mediated gene knockout is to use siRNA to knock down the expression of a particular gene. siRNA was used in papers II and III.

Similarly, stably transfected cells were also used in all three papers. Transfections represent an easy way to induce the expression of a particular construct. Although, depending on the proliferation rate of the cell line of choice, this might also be time-

consuming. Similar to the case with CRISPR/cas9, transfections can also have background effects, which lead to phenotypic differences between clones. As such, when generating stably transfected clones, several of them will have to be used and compared to ensure that an observed effect is not clone-specific.

To account for potential off-target effects and background effects resulting from the gene editing and/or transfections, several different clones were used and compared in the projects. In paper III, the need for using multiple clones was circumvented by using heterogeneous cell populations stemming from the transfections rather than individual clones.

Antibodies

In addition to the many commercially available antibodies used in the projects, novel non-commercial antibodies were also used. In paper I, a homemade (Agrisera) antibody against SUSD4 was used. As has been described (68), this polyclonal antibody was generated by immunising rabbits with purified Fc-tagged SUSD4. Using affinity chromatography, antibodies specific for the Fc-tag were removed. The removal of such antibodies was verified by ELISA. In the project, several breast cancer cell lines were transfected with either a plasmid encoding human SUSD4 or an empty mock plasmid to generate SUSD4-expressing cells and mock control cells. Consistently, in various applications, the antibody generated a signal for SUSD4-expressing cells but not for mock control cells. For example, while a band signifying SUSD4 could be detected by western blot using lysate of SUSD4-expressing cells, no such band could be detected for mock control cells. The results obtained from the parallel use of cells, either expressing or lacking SUSD4, testified to the validity of the antibody.

Similarly, novel homemade antibodies (Capra Science) were used for the detection of human IRIS-1 and IRIS-2 in paper II. Rabbits were immunised with peptide antigens designed based on the unique C-terminal domains of IRIS-1 and IRIS-2. Affinity-purified polyclonal antibodies targeting the distinct C-terminals were thus generated. These antibodies were extensively validated. Using purified plate-bound IRIS-1 or IRIS-2, the sensitivity of the antibodies was assessed by ELISA. In addition to INS-1 832/13 cells and CD59 knockout INS-1 832/13 cells, INS-1 832/13 cells lacking CD59 but stably expressing flag-tagged IRIS-1 or IRIS-2 were used. By using these cells and by comparing with the flag-tag detection, the specificity of the IRIS antibodies could be validated. Furthermore, the specificity of the antibodies was also assessed by peptide-blocking. Here, the antibodies were pre-incubated with the peptide antigens used for the immunisation of rabbits, which led to a diminished detection of the proteins. This was done for ELISA, western blot and confocal microscopy. This showed that the antibody raised against IRIS-1 did not detect IRIS-2 and vice versa. The parallel use of the antibodies against IRIS-1 or IRIS-2 with an anti-flag tag antibody further contributed to the validity of the results as the findings could be verified using a different antibody.

Present investigations

Paper I

Sushi domain-containing protein 4 binds to epithelial growth factor receptor and initiates autophagy in an EGFR phosphorylation independent manner

Background and aim

SUSD4 had previously been described as a potential breast cancer suppressor, but no mechanistic function of SUSD4 in breast cancer cells had been identified. The expression of SUSD4 in both tumour cells and tumour-infiltrating T cells was found to be associated with a favourable prognosis for breast cancer patients. Additionally, *in vitro* experiments showed that the expression of SUSD4 affected the migration, invasion, growth and clonogenicity of breast cancer cells. Together, these results indicated breast cancer suppressive properties of SUSD4, but the underlying mechanism was not elucidated (70). Therefore, this study aimed to identify the function of SUSD4 in breast cancer cells and elucidate the mechanism underlying the breast cancer suppressive effect.

Main findings

Using a syngeneic mouse model, further support for the previously proposed breast cancer suppressive effect of SUSD4 was obtained. To then identify the functional implications of the expression of SUSD4, a broad screening for differentially expressed proteins was performed using breast cancer cells either expressing or lacking SUSD4. The largest difference could be seen for the EGFR, which was markedly upregulated in SUSD4-expressing cells. However, this was not due to a difference in either mRNA expression or receptor degradation. Moreover, SUSD4 was found to interact with growth factor receptors in triple-negative breast cancer cells, including the EGFR. The EGFR plays a well-established role in autophagy, and a plausible effect of SUSD4 on autophagy was therefore investigated. SUSD4 was found to promote autophagy in a manner strictly dependent on the presence of the EGFR. This effect on autophagy required the complete form of the receptor but was independent of its kinase activity. In line with SUSD4's ability to promote autophagy, an altered phosphorylation status of key components of the autophagic machinery, favouring the induction of autophagy, was observed in SUSD4-

expressing cells (**Figure 5**). This includes ULK1, Beclin-1 and ATG14. Additionally, using direct activators of AMPK, an increased susceptibility to AMPK activation was observed for SUSD4-expressing cells. Moreover, both SUSD4 and EGFR were found to colocalise with various endosomal markers, including a marker for recycling endosomes. This suggests that SUSD4 may play a role in EGFR trafficking and recycling.

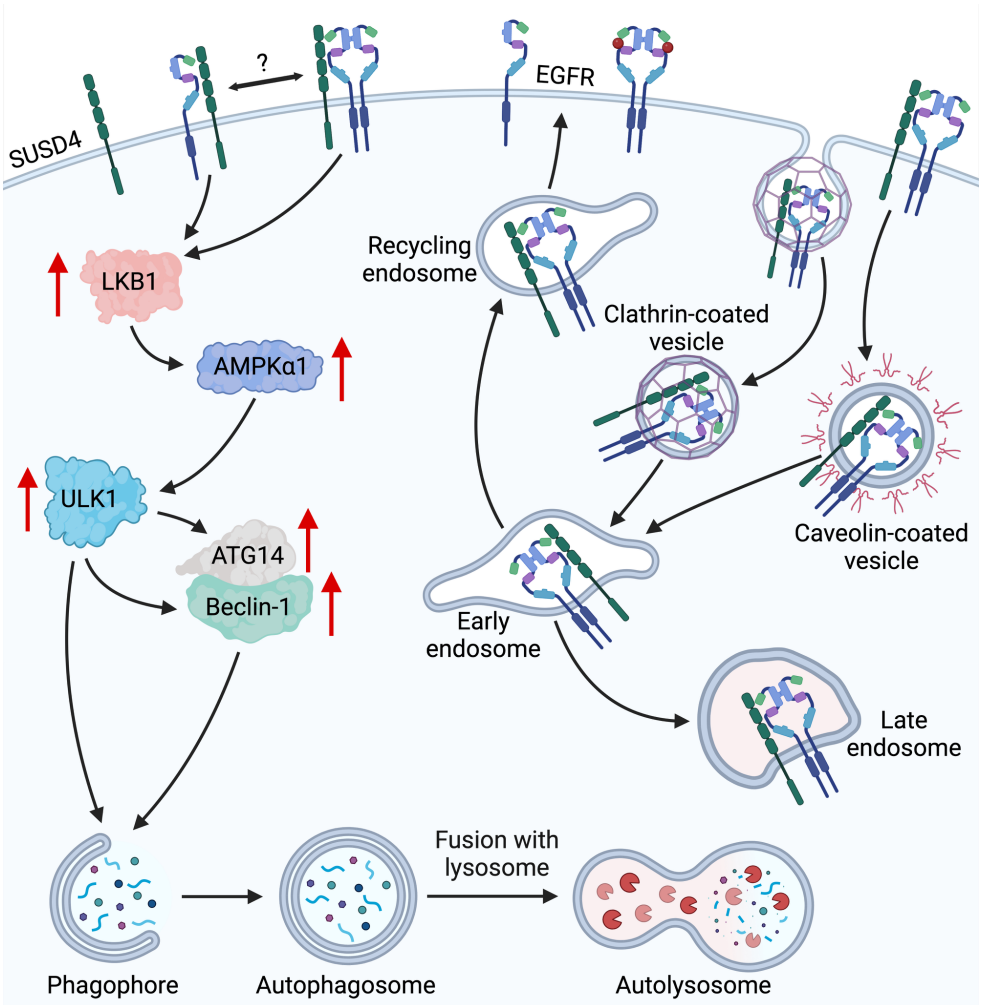


Figure 5. Illustration summarising the main findings of paper I. SUSD4 was found to interact with the EGFR in triple-negative breast cancer cells. In the presence of the EGFR, SUSD4 promoted autophagy. Accordantly, an altered phosphorylation status of various complexes influencing the initiation of autophagy was observed. Increased phospho-activation of LKB1, AMPK, ATG14 and Beclin-1 was observed for SUSD4-expressing cells. A lower level of phospho-inhibited ULK1 was also observed for triple-negative breast cancer cells expressing SUSD4. Both SUSD4 and the EGFR were found to colocalise with various endosomal markers, which suggests a role for SUSD4 in EGFR trafficking.

Paper II

Alternative splicing encodes functional intracellular CD59 isoforms that mediate insulin secretion and are down-regulated in diabetic islets

Background and aim

CD59 was identified as the most highly expressed complement gene in human pancreatic islets, and interestingly, it was found to be downregulated in pancreatic islets of rodent models of diabetes (CD59B in mice). After seeing that CD59 was present intracellularly and colocalised with insulin, it was shown that an intracellular pool of CD59 functions in insulin secretion in the rat β-cell line INS-1 832/13. CD59 silenced cells exhibited impaired stimulated insulin secretion. Subsequently, CD59 was shown to interact with the SNARE proteins VAMP2 and Syntaxin-1A (457). A subsequent study, wherein a set of mutant CD59 variants were assessed for their ability to mediate insulin secretion, demonstrated different structural requirements for the two functions of CD59: MAC inhibition and insulin secretion (134). The aim of this study was to further investigate the role of CD59 in β-cells and to answer the question of how endogenous CD59 enters the cytosol to function in insulin release.

Main findings

An impaired insulin secretion caused by the absence of CD59 had previously only been demonstrated using a rat β-cell line. However, here we showed that the lack of CD59 (CD59B in mice) also leads to impaired insulin secretion in both a human and a mouse β-cell line. The main finding, however, was the discovery of two previously undescribed intracellular splice forms of CD59 (**Figure 6**). In contrast to canonical CD59, these novel isoforms lack the GPI-anchor attachment site and instead harbour unique C-terminal domains. Both were quite broadly expressed, but pancreatic islets and the placenta demonstrated the highest expression. These isoforms, which were named IRIS-1 and IRIS-2 (Isoform rescuing insulin secretion 1 and 2), colocalised with insulin and interacted with the SNARE proteins. Consistently, both IRIS-1 and IRIS-2 were able to rescue the impaired insulin secretion in CD59 knockout INS-1 832/13 cells, thus earning their name. The results also indicate that IRIS-1 is involved in the 1st phase of insulin release while IRIS-2 functions in the 2nd phase. Interestingly, both IRIS-1 and IRIS-2 were downregulated in human pancreatic islets from T2D donors compared to healthy donors. Additionally, IRIS-1 was downregulated in human islets upon exposure to glucotoxic conditions. Together, these results point to a link between the IRIS isoforms and the pathogenesis of T2D.

The aforementioned CD59 isoforms refer to human splice forms of CD59. However, intracellular splice forms of mouse CD59B were also identified, and these were named mouse CD59B-IRIS-1 and 2. These lack the GPI-anchor attachment site and

instead possess unique C-terminal domains, similar to the human IRIS isoforms. While both CD59B-IRIS-1 and CD59B-IRIS-2 were able to rescue the impaired insulin secretion in *CD59B* knockout MIN6 cells, only CD59B-IRIS-2 was found to be downregulated in pancreatic islets of a mouse model of diabetes.

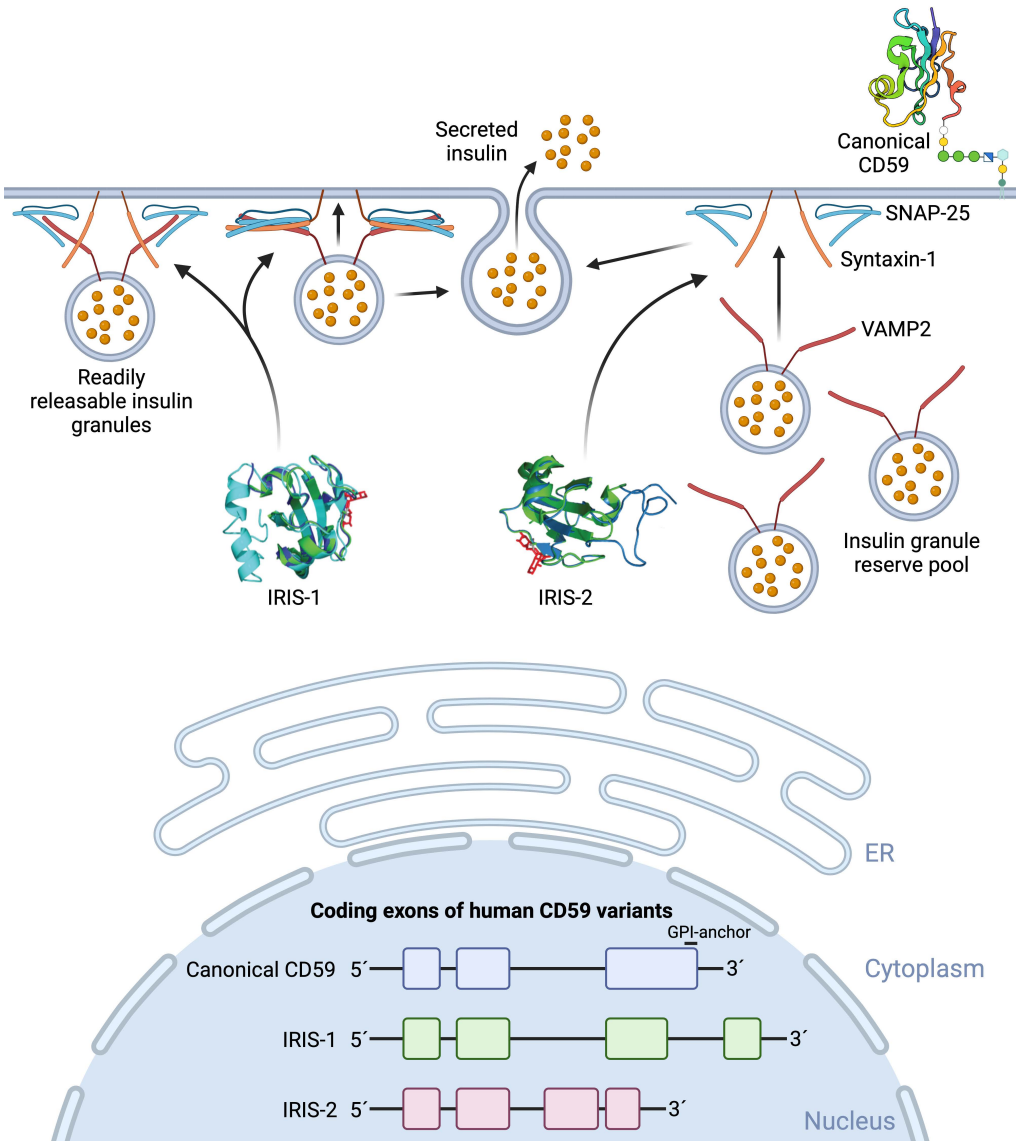


Figure 6. Illustration of the main findings of paper II. Two alternative splice forms of CD59 present intracellularly were identified. These isoforms were named IRIS-1 and IRIS-2 and could rescue the impaired insulin secretion in CD59-deficient β-cells. The results suggest that IRIS-1 is involved in the 1st of insulin secretion while IRIS-2 plays a role in the 2nd phase of insulin release.

Paper III

CD59 double knockout mice express a CD59ba hybrid fusion protein that mediates insulin secretion

Background and aim

The background to this project was essentially the same as that described for paper II. The non-canonical role of CD59 in mediating insulin secretion was first observed in the rat β-cell line INS-1 832/13. In paper II, impaired insulin secretion in the absence of CD59 was further demonstrated in both the human β-cell line EndoC-βH1 and the mouse β-cell line MIN6. As such, the importance of CD59 for insulin release has been observed in multiple cell lines of different species. The next step was, therefore, to study the role of CD59 in insulin secretion *in vivo*. Thus, this project aimed to investigate insulin secretion and blood glucose homeostasis in a CD59 double knockout mouse model (CD59abKO). This model lacks critical exons of both *CD59A* and *CD59B*. Consequently, the CD59abKO mice were hypothesised to have impaired insulin secretion and, thereby, perturbed blood glucose homeostasis.

Main findings

After having verified the mouse model by ensuring the lack of transcripts encoding CD59A, CD59B and the mouse CD59B-IRIS isoforms in different tissues, the investigation pertaining to blood glucose homeostasis was commenced. No difference could be observed when comparing fasting blood glucose levels between the wild type and the CD59abKO mice. Additionally, no difference in glucose tolerance could be detected between the wild type and the CD59abKO mice, suggesting that the knockout mice do not have impaired blood glucose homeostasis. In line with this, no difference in glucose-stimulated insulin secretion using isolated pancreatic islets could be detected. These results directly opposed the initial hypothesis and sparked an investigation as to why no phenotype could be observed for the knockout mice.

This led to the identification of an expressed transcript in the CD59abKO mice encoded by the remaining exons of each CD59 gene spliced together (**Figure 7**). To further characterise this CD59ba hybrid, it was expressed in INS-1 832/13 cells. Similar to canonical CD59, the CD59ba hybrid was found to be present at the cell surface. Furthermore, the CD59ba hybrid was shown to be glycosylated, which is a prerequisite for canonical CD59's ability to function in insulin secretion. The CD59ba hybrid also proved capable of maintaining normal glucose-stimulated insulin secretion following knockdown of canonical CD59. This indicates that the CD59ba hybrid is rescuing the phenotype pertaining to blood glucose homeostasis in the CD59abKO mice.

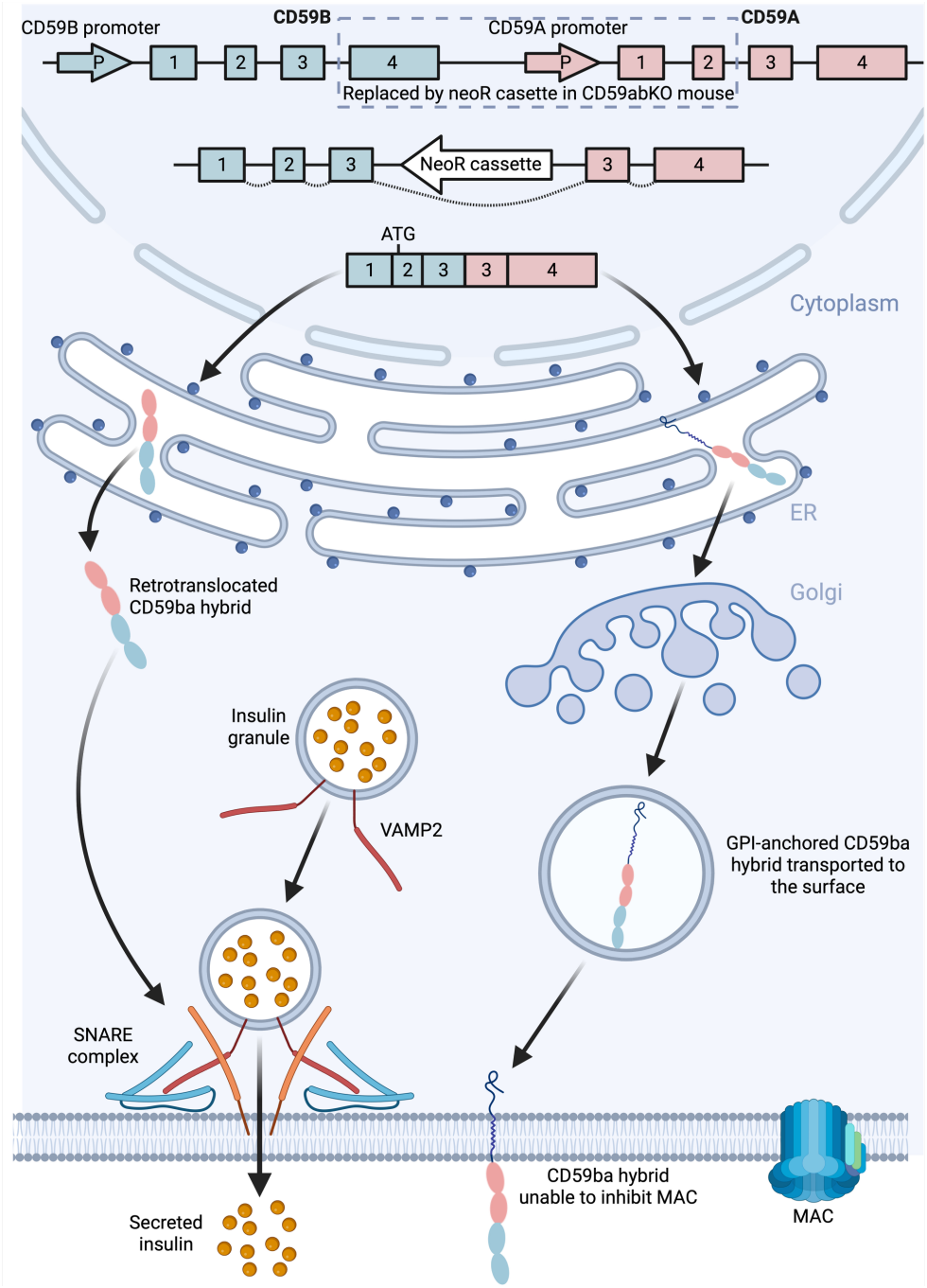


Figure 7. Illustration summarising the findings of paper III. The CD59abKO mice express a CD59ba hybrid gene product resulting from the splicing together of the remaining exons of each CD59 gene. The product of the CD59ba hybrid transcript is both present at the cell surface and can function in insulin secretion.

Author contribution

Paper I

In paper I, I was involved in both the *in vivo* and *in vitro* experiments. For the *in vivo* experiments, I monitored the mice and tracked the tumour growth. When it comes to the *in vitro* experiments, I compared total EGFR levels between SUSD4-expressing cells and mock control cells, compared the activation of AMPK between the cells expressing or lacking SUSD4, and assessed the phosphorylation status of both Akt and ULK1. I was also involved in generating BT20 EGFR knockout clones and in the subsequent generation of SUSD4-expressing EGFR knockout clones. I then assessed the effect of SUSD4 on autophagy in EGFR knockout cells. Additionally, I performed the ELISAs showing that SUSD4 interacts with both the EGFR and the platelet-derived growth factor receptor. In addition to my practical contribution in terms of experiments, I also wrote the manuscript.

Paper II

My main contribution was the assessment of the novel isoform-specific antibodies. I was involved in the purification of IRIS-2 protein and later assessed the purity of the purified IRIS-1 and IRIS-2. I then performed the ELISA with the purified recombinant proteins, wherein the sensitivity of the isoform-specific antibodies was evaluated. Moreover, I also performed the ELISA, where the specificity of the antibodies was assessed through peptide-antigen blocking. Similarly, I also performed the western blot with the lysates of INS-1 832/13 cells either expressing or lacking IRIS-1 or IRIS-2, where the same peptide-antigen blocking strategy was employed to assess the specificity of the isoform-targeting antibodies. Moreover, in addition to writing about the parts related to the experiments I performed, I was critically reading and making corrections in the mature manuscript.

Paper III

The project leading up to paper III was initially intended as a continuation of the project in paper II, as we then wanted to investigate the novel function of CD59 *in vivo* using a mouse model. However, due to the presence of the CD59ba hybrid gene product that appears to rescue the phenotype in the mouse model, the plans for this project were substantially changed along its course. Nevertheless, apart from the predicted structure models, which a collaborator made, I generated all the data presented in the paper. I performed all of the experiments, analysed the data and wrote the manuscript.

Discussion and future perspectives

This thesis focused on novel non-canonical roles of the two complement inhibitors SUSD4 and CD59 in the context of two major human diseases. Only a very limited number of publications have addressed potential functions of SUSD4. As such, it is a poorly understood protein, and there is much left to learn about it. SUSD4 had previously been described as a complement inhibitor and breast cancer suppressor. However, the underlying mechanism of the proposed breast cancer suppressive effect was not identified. Conversely, a non-canonical role for CD59 in mediating insulin secretion had already been discovered. Yet, there is a lot left to learn about the intracellular role of CD59 in β-cells. A major question to be answered was how the plasma membrane-anchored protein CD59 enters the cytosol, where it can function in insulin release. As such, the aim of this thesis was two-fold: First, we wanted to elucidate the role and function of SUSD4 in breast cancer cells. Second, we wanted to explore the role of CD59 in insulin secretion further, both its mechanisms in β-cells and its role in regulating blood glucose homeostasis *in vivo*.

Using a syngeneic mouse model, we obtained additional support for a breast cancer suppressive effect of SUSD4. Further, in triple-negative breast cancer cells, we found that SUSD4 interacts with growth factor receptors, including the EGFR. We also found that SUSD4 promotes autophagy in a manner strictly dependent on the presence of the EGFR. Our results also indicated a plausible role for SUSD4 in EGFR trafficking. As such, a novel role for SUSD4 in breast cancer cells was identified. However, both complement and autophagy have complex relationships with cancer. Whether they are beneficial or detrimental depends on the context. An intriguing question is, therefore, whether the breast cancer suppressive effect of SUSD4 is related to its complement inhibitory function, the autophagy-promoting function, both, or neither. The fact that a role for SUSD4 in inducing autophagy was identified does not exclude the possibility that SUSD4 has other functions in breast cancer cells that are yet to be discovered. Other effects and interaction partners may be awaiting discovery. For example, SUSD4 was also found to interact with the platelet-derived growth factor receptor, the functional implications of which remain to be deciphered. Moreover, we also corroborated the previous findings and found that a high expression of SUSD4 was associated with a favourable prognosis for patients. SUSD4 may, therefore, have some value as a prognostic marker. Additionally, given that autophagy can function as a mechanism to resist cancer

treatment, the fact that SUSD4 promotes autophagy in triple-negative breast cancer cells may have therapeutic implications.

Worth noting is that our results concerning the effect of SUSD4 on the downstream signalling from the EGFR were inconclusive. In BT20 cells, higher levels of phospho-activated Akt could be seen for the mock control cells relative to the SUSD4-expressing cells. However, no difference in mTOR phosphorylation could be seen. Conversely, in MDA-MB-468 cells, a lower level of phosphorylated mTOR was seen for the SUSD4-expressing cells compared to the cells lacking SUSD4, but no difference in the phosphorylation of Akt was detected. In the third cell line used, HS-578T, no difference in either phospho-Akt or phospho-mTOR could be detected. These nondefinitive results may, however, be explained by the fact that all three cell lines carry mutations that, in one way or another, affect the PI3K-Akt-mTOR signalling pathway.

Whether or not SUSD4 interacts with the EGFR and promotes autophagy in breast cancer subtypes other than triple-negative breast cancer cells should also be investigated. The effect of SUSD4 on autophagy was quickly assessed in two luminal A breast cancer cell lines with a comparably low expression of EGFR, but further investigation in other subtypes would be interesting. Furthermore, the role of SUSD4 in other types of cancers should also be investigated. Depending on the cancer type, either a high or low expression of SUSD4 is associated with a poor prognosis in various cancers (75).

Moreover, SUSD4 is highly expressed in the brain, where it seems to have important functions. In addition to playing a role in synaptic plasticity, there are also indications that SUSD4 might affect synaptic pruning. This, together with the observed phenotype for both humans and mice lacking SUSD4, makes the role of SUSD4 in the brain an intriguing area that warrants further research. Regarding its role in synaptic plasticity, SUSD4 was found to interact with an AMPA receptor and affect its turnover. SUSD4 also interacts with a ubiquitin ligase that targets the AMPA receptor for degradation (69). Although we did not detect a difference in EGFR degradation between the SUSD4-expressing cells and the mock control cells, our results did suggest a potential role for SUSD4 in EGFR trafficking. Thus, interacting with receptors and affecting their trafficking and turnover may be a common mechanism of SUSD4. Additionally, SUSD4 is expressed in both $CD4^+$- and $CD8^+$ T cells, and the expression of SUSD4 changes upon T cell stimulation (70). Given that SUSD4 is structurally similar to CD46, which has a costimulatory role in T cells, the role and function of SUSD4 in T cells should be investigated.

To conclude, we identified a novel function of SUSD4 that might be related to its breast cancer suppressive effect. Finally, little is known about the functions of SUSD4 in various settings, and there may be a lot of interesting discoveries left to be made.

In contrast to SUSD4, much more is known about CD59. However, there is still more to learn concerning its non-canonical intracellular role in β-cell exocytosis. CD59 was initially shown to be essential for insulin secretion in a rat β-cell line. However, here, we showed that knockdown of CD59 in a human β-cell line and knockout of CD59B in a mouse β-cell line also leads to impaired insulin secretion. Furthermore, in both humans and mice, we identified two intracellular splice forms of CD59 that we named IRIS-1 and IRIS-2. In humans, these were quite broadly expressed, but the highest expression was seen in pancreatic islets and the placenta. Onwards, both IRIS-1 and IRIS-2 were found to interact with SNARE proteins and to rescue the impaired insulin secretion in CD59-deficient β-cells. Our results also indicate that while IRIS-1 is involved in the first phase of insulin release, IRIS-2 functions in the second phase of insulin secretion. This suggests that the two IRIS isoforms have similar yet distinct and complementary functions in β-cell exocytosis. How this is reflected by potential differences in interaction partners, for instance, requires further investigation. Additionally, given that both IRIS-1 and IRIS-2 were downregulated in pancreatic islets from T2D donors and that IRIS-1 was downregulated in islets upon exposure to glucotoxic conditions, our results also suggest a potential link between the IRIS isoforms and the pathogenesis of T2D. This warrants further attention and will be investigated by assessing how the levels of IRIS-1 and IRIS-2 are regulated and affected by exposure to various diabetogenic factors.

Here, we have identified intracellular splice forms of CD59 functioning in insulin secretion in both humans and mice, but we hypothesise that such CD59 variants are also present in other species. In human IRIS-1, the last exon encoding the mature protein is derived from the highly conserved open reading frame *C11orf91*. The *CD59* gene and *C11orf91* are adjacently located in mammalian genomes, arguing the potential existence of conserved homologs of IRIS-1 in mammals. Moreover, due to a gene truncation, the *CD59* gene in guinea pigs has been described as a pseudogene, in part because it lacks the GPI-anchor signal sequence and could not be detected at the cell surface (458). However, even though it is seemingly absent at the surface and has lost the complement inhibitory function, it is possible that the intracellular function is retained. Whether or not the truncated guinea pig CD59 is expressed in β-cells and if the insulin secretory function, in that case, is preserved should be assessed. If so, that would testify to the importance of the intracellular function of CD59.

Moreover, these newly identified proteins might also have other functions and interaction partners. As they were found to be quite broadly expressed, they likely have functions in other cell types and tissues, which would be interesting to investigate. The high expression in the placenta, for example, is intriguing. Nevertheless, the functions of IRIS-1 and IRIS-2 should be further explored, both in the context of pancreatic β-cells and other cell types. For example, whether or not

these isoforms play a role in regulated secretion in other cell types, such as neuronal cells and $CD8^+$ T cells, should also be investigated.

We recently performed RNA sequencing with INS-1 832/13 cells, INS-1 832/13 CD59 knockout cells and INS-1 832/13 CD59 knockout cells expressing either IRIS-1 or IRIS-2. From these data, several differentially regulated pathways were identified, some of which were particularly intriguing and will be the focus of future investigations. We are also planning to use a proximity labelling technique called APEX to identify the interactome of IRIS-1 and IRIS-2 in pancreatic β-cells. By identifying additional interaction partners, other functions and mechanisms of the IRIS isoforms may be unveiled. Albeit just with canonical CD59, we have previously conducted a protein array wherein potential CD59 interaction partners were sought. Some of the candidates identified from this array will also be the subject of future investigations.

Moreover, given the shared N-terminal region between CD59, IRIS-1, and IRIS-2, the functional properties that facilitate insulin release are likely located in this region. Identifying the necessary structural motifs could spark the development of small peptides or molecules based on the structure of the IRIS isoforms that enhance insulin secretion. If so, such peptides or molecules could be of therapeutic interest in the future. Therefore, the structural requirements for the insulin secretory function of CD59 and its intracellular isoforms should be investigated.

The non-canonical role of CD59 in facilitating insulin secretion had previously only been studied *in vitro*. We, therefore, wanted to take the next step and investigate the role of CD59 in insulin secretion and blood glucose homeostasis *in vivo*. This was done using a CD59 double knockout mouse model, which, on paper, seemed like an appropriate model. Due to the lack of CD59, we hypothesised that these mice would have impaired insulin secretion and, thus, perturbed blood glucose homeostasis. However, that was not the case. We could not detect any difference in either fasting blood glucose levels or glucose tolerance between the CD59-deficient mice and wild type controls. Additionally, isolated pancreatic islets from the CD59 double knockout mice did not exhibit impaired insulin secretion. Together, these results argued against a role for CD59 in insulin exocytosis and, thereby, challenged the previously obtained *in vitro* results. Despite the apparent impairment in glucose-stimulated insulin secretion in human-, rat-, and mouse β-cell lines lacking CD59, no functional defects related to insulin secretion could be observed *in vivo*.

However, as it turns out, this proved to be a good example of how "nature always finds a way". As a mere consequence of how the model was generated, the CD59abKO mice express a product that does not normally exist in nature. Comprising the remaining exons of each CD59 gene still present in the model, the CD59ba hybrid gene product, as we came to call it, was found to be expressed in pancreatic islets, for example. When expressed in a β-cell line, the CD59ba hybrid was found to be present at the cell surface. However, the CD59abKO mice are

reportedly suffering from haemolytic anaemia, suggesting that the CD59ba hybrid has lost the complement inhibitory function of canonical CD59. Importantly, though, the CD59ba hybrid was able to maintain normal glucose-stimulated insulin secretion in a β-cell line following knockdown of canonical CD59. This indicates that the CD59ba hybrid functions in insulin secretion and explains the lack of a phenotype in the CD59abKO mouse model. How the CD59ba hybrid mechanistically functions in insulin secretion was not delineated. However, it presumably functions in the same manner as canonical CD59 and the IRIS isoforms. Canonical CD59 has previously been shown to function in insulin secretion by interacting with SNARE complex components (134, 457). Similarly, in paper II, we showed that the IRIS isoforms can interact with SNARE proteins. It is likely that the CD59ba hybrid also interacts with SNARE proteins, yet this remains to be elucidated.

It is worth noting, however, that the CD59ba hybrid was detected *in vitro* because a flag-tagged construct was expressed in the β-cell line. Available antibodies against both CD59A and CD59B fail to detect it, and, as such, it is yet to be detected at protein level in the CD59abKO mice. Nevertheless, further support for the ability of the CD59ba hybrid to mediate insulin secretion and rescue the phenotype in the CD59abKO mice comes from its N-terminal region. Mouse CD59B-IRIS-1, which we identified and described in paper II, can rescue the impaired insulin secretion in *CD59B* knockout MIN6 cells. CD59B-IRIS-1 is merely 32 amino acids in size, and the same amino acid sequence can be found in its entirety in the N-terminal region of the CD59ba hybrid. This not only provides further support for the possibility that the CD59ba hybrid can rescue the phenotype in the mouse model, but it also further suggests that the N-terminal region of CD59/IRIS harbours the necessary functional properties for the insulin secretory function. In addition to providing information regarding the structural requirements that are important for the insulin secretory function of CD59, it also serves as a prime example of how genetic editing can have unforeseen consequences. One should, therefore, always be careful when employing genetically edited research models.

Despite the fact that no impairment in insulin secretion or blood glucose homeostasis was detected in the CD59abKO mice, the results do not discredit the functional importance of CD59 in β-cells as the identified gene product proved capable of mediating insulin release. However, the investigation concerning the role of CD59 and its intracellular splice forms in mediating insulin secretion *in vivo* will have to be continued in a different model. Two potential options are CD59B single knockout mice and CD59 knockout rats. Concerning the former, one would first have to ensure that these mice do not express any of the intracellular IRIS isoforms, which in mice were identified within the *CD59B* gene. It should also be ruled out that no compensatory mechanism is offered by CD59A in these mice, even though that was not the case in a mouse β-cell line. When it comes to the CD59 knockout rat model, that is not without risks either. While intracellular splice forms of CD59

functioning in insulin secretion have been identified in humans and mice, no such isoforms have been discovered in rats yet. Therefore, one would have to closely examine how the model was generated and ensure that no gene products are being expressed.

In conclusion, we identified novel intracellular splice forms of CD59 that play an essential role in insulin secretion, and that may be linked to the pathogenesis of T2D. T2D is a disease that affects hundreds of millions of people worldwide and is characterised by impaired insulin secretion. By fully elucidating the mechanisms at play in β-cells, novel and more efficient treatments for T2D can be developed. Here, we identified two previously unknown proteins involved in the process of insulin secretion, thus contributing to an improved understanding of key β-cell mechanisms. The functions of these novel proteins should be further investigated in both β-cells and other secretory cells. Regarding the role of CD59 in insulin secretion *in vivo*, the investigation will have to proceed in a new model. However, the gene product identified in the mouse model did provide additional support for the importance of the N-terminal region of CD59/IRIS for the insulin secretory function.

Popular science summary

Our immune system plays an essential role in maintaining health as it protects us against potential threats in our body, such as invading bacteria. To ensure that the immune system is protective and not causing self-harm, it must accurately distinguish between healthy host cells and foreign or damaged cells. To avoid self-harm, our cells express a set of inhibitors that stop the immune system from targeting them. Two such inhibitors are SUSD4 and CD59, which are typically present on the surface of our cells. However, both SUSD4 and CD59 have functions that are unrelated to their role in the immune system. This thesis has focused on novel roles of these proteins in the context of two major human diseases, breast cancer and type 2 diabetes.

Breast cancer is the most common type of cancer in women. In fact, in 2020, breast cancer in women was the most frequently diagnosed type of cancer worldwide, with 2.3 million new cases. Thus, it represents a global health issue. In a previous study, SUSD4 was portrayed as a potential breast cancer suppressor, but how it functions in these cells was not elucidated. We, therefore, aimed to identify the mechanism underlying this effect of SUSD4. Not only did we obtain further support for a breast cancer suppressive effect of SUSD4, but we also identified a new function by which it operates in breast cancer cells. We found that SUSD4 can promote a process called autophagy, which can be described as a form of self-cannibalism where the cell eats parts of itself. Autophagy is an essential cellular mechanism contributing to normal maintenance in the cell, but it also serves a protective role in enabling cells to persevere in response to stress. The relationship between autophagy and cancer is complex as it can both prevent the development of cancer, but it can also benefit already established cancer cells. It is worth noting that autophagy can also protect cancer cells against various cancer treatments. The fact that SUSD4 can promote this process may, therefore, be of clinical importance. Additionally, the presence of SUSD4 is associated with a better prognosis for breast cancer patients. SUSD4 may, therefore, also be of clinical relevance as it could be used as an indicator of patient prognosis.

Regarding CD59, the work has centred on its new role, which is related to type 2 diabetes. Hundreds of millions of people worldwide have type 2 diabetes, and it constitutes a global health burden in our society. Type 2 diabetes revolves around the hormone insulin, which stimulates tissues to take up glucose from the blood. Insulin is produced and secreted by so-called β-cells in the pancreas. The β-cells

reside in cell clusters called islets of Langerhans that are present in the pancreas. Type 2 diabetes is characterised by an inability to regulate blood glucose levels, partly due to insufficient insulin secretion. CD59 was recently found to be essential for insulin secretion, but since it is typically present on the cell surface, a question that remained was how it gains access to the insulin secretory machinery. Here, we identified intracellular variants of CD59 in both humans and mice. These variants are present in β-cells and were found to play an essential role in insulin secretion. Moreover, lower levels of these variants were found in islets of individuals with type 2 diabetes than in islets from healthy individuals. The level of one of the CD59 variants also decreased in response to prolonged exposure to a high glucose concentration. These results indicate a potential link between the CD59 variants and the development of type 2 diabetes.

Although many treatments are available for type 2 diabetes patients, new and better alternatives are needed. To develop such treatments, an increased understanding of cellular processes in β-cells is required. Here, we identified new proteins involved in the process of insulin secretion, which led to an increased understanding of a key mechanism in β-cells. Increased knowledge of the function of CD59 and its variants in β-cells may pave the way for new approaches in the treatment of type 2 diabetes.

Popular science summary (Swedish)

Vårt immunförsvar spelar en viktig roll i upprätthållandet av vår hälsa då det skyddar oss från potentiella hot i kroppen, såsom inkräktande bakterier. För att säkerställa att immunförsvaret spelar en skyddande roll och inte orsakar skada så måste det kunna skilja på friska kroppsegna celler och främmande eller skadade celler. För att undvika att bli attackerade av immunförsvaret så uttrycker våra celler olika typer av hämmare som stoppar immunförsvaret från att angripa dem. Två sådana hämmare är SUSD4 och CD59 som båda normalt sett sitter förankrade på ytan av våra celler. Både SUSD4 och CD59 har däremot andra funktioner också som inte är relaterade till deras roll i immunförsvaret. Den här avhandlingen har kretsat kring nyupptäckta funktioner hos SUSD4 och CD59 samt dessa funktionernas betydelse för sjukdomstillstånd såsom bröstcancer och typ 2 diabetes.

Bröstcancer är den vanligaste sortens cancer bland kvinnor. Faktum är att år 2020 så var bröstcancer den mest diagnostiserade sortens cancer världen över med 2.3 miljoner nya fall. Det utgör därmed ett globalt hälsoproblem. In en tidigare studie beskrevs SUSD4 som en potentiell bröstcancerhämmare, men vad det har för funktion i bröstcancerceller identifierades inte. Vi syftade därför att identifiera mekanismen som ligger till grund för dess hämmande effekt i bröstcancer. Våra resultat gav dels vidare stöd för en bröstcancerhämmande effekt hos SUSD4, men vi lyckades också identifiera en funktion som proteinet har i bröstcancerceller. Vi fann att SUSD4 främjar en process som kallas autofagi, vilket kan ses som en typ av självkannibalism där cellen äter delar av sig själv. Autofagi är en viktig cellfunktion då det dels utför en typ av underhållningsarbete, men det kan också spela en skyddande roll och göra det möjligt för celler att uthärda stressfyllda förhållanden. Autofagi har ett komplext förhållande till cancer eftersom det både kan motverka bildandet av cancerceller och gynna redan etablerade cancerceller. Autofagi kan även fungera som ett skydd mot olika cancerbehandlingar. Det faktum att SUSD4 främjar autofagi kan därför ha klinisk betydelse vid behandlingen av bröstcancer. Vidare så är närvaron av SUSD4 i bröstcancer kopplat till en mer positiv prognos för patienter. Därmed kan det eventuellt finnas ett värde i att använda SUSD4 som en indikation på patienters prognos.

Vad gäller CD59 så har arbetet fokuserat på dess nya roll som har betydelse för typ 2 diabetes. Hundratals miljoner människor världen över har typ 2 diabetes och det utgör ett globalt samhälleligt problem. Typ 2 diabetes kretsar kring hormonet insulin vilket stimulerar kroppens vävnader att ta upp socker från blodet. Insulin produceras

och utsöndras i blodet av så kallade β-celler i bukspottskörteln. β-cellerna sitter i cellkluster som kallas för Langerhanska öar och som finns utspridda i bukspottskörteln. Typ 2 diabetes kännetecknas av en oförmåga att reglera blodsockernivån i kroppen, vilket delvis beror på defekt insulinutsöndring. Nyligen fann man att CD59 spelar en viktig roll i utsöndringen av insulin, men eftersom CD59 normalt sett sitter på cellernas yta så var det oklart hur det får tillgång till det maskineri som styr insulinutsöndringen. Här så lyckades vi identifiera varianter av CD59 i både människor och möss som finns inuti cellerna. Dessa varianter finns närvarande i β-celler och visade sig spela en viktig roll i utsöndringen av insulin. Vidare så fann vi att Langerhanska öar från individer med typ 2 diabetes har lägre nivåer av dessa varianterna än Langerhanska öar från friska individer. Nivån av en av dessa varianterna minskade även i Langerhanska öar som utsatts för en förhöjd sockerkoncentration. Våra resultat indikerar därmed på en potentiell koppling mellan CD59 varianterna och sjukdomsförloppet i typ 2 diabetes.

Även om flertalet läkemedelsbehandlingar finns tillgängliga för typ 2 diabetiker så finns det ett behov för nya och mer effektiva alternativ. För att utveckla nya och bättre läkemedel behövs en ökad förståelse för olika processer i β-celler. Här har vi identifierat nya proteiner som är involverade i insulinutsöndringsprocessen vilket har lett till en ökad förståelse för en väsentlig mekanism i β-celler. En ökad förståelse kring den funktion som CD59 och dess varianter har i β-celler kan eventuellt öppna upp för nya behandlingsmöjligheter för typ 2 diabetes.

Acknowledgements

First of all, I would like to thank **Anna** for the opportunity to pursue my PhD in her group. This has been a rollercoaster of an experience, and I am very grateful for the guidance and supervision through it all. I have learned a lot from you, both in terms of pure science, critical thinking, and planning and structuring a project. I also want to thank my co-supervisor, **Ben,** who also guided and supervised me throughout my PhD. I learned a lot from you, both practically in the lab and from all our discussions. You always have a lot of creative ideas for the projects, and your enthusiasm is inspiring. Thank you also for always taking the time to answer my questions when I come barging into your office. I would also like to thank **Erik** for agreeing to be my co-supervisor and for the collaboration that led to two of the publications included in this thesis. Thank you also to **Kostas** for the past supervision and our work together on the SUSD4 project. I also learned a lot from you during our time together.

Ewelina, thank you for all the help during my PhD, answering all my questions related to the project or other things that were part of the PhD experience. Thank you also for all the entertaining conversations over the years. Thank you, **Karolina**, for all the support and it was great to work together and discuss the projects during the later part. **Frida**, thank you for all the help, for always answering my many questions and for everything you are doing to keep things working. Even if our conversations sometimes took a dark turn, I always enjoyed them. Thank you, **Elena** and **Kasia,** for making the days at work a bit better. Thank you for "the happy corner", all the after-work beers, and all the very random but entertaining discussions. **Mario**, you were also part of "the happy corner", and we had a lot of fun during the late evenings in the cell lab. **Michał**, thanks for all the great conversations in the office and for being an outlet for my "need" to discuss sports. It has been great to share the office with you. **Damien** and **Mateusz**, you were also always up for discussing sports, and thanks for all the fun but sometimes frustrating evening sessions. **Rebecca**, it has been great to share the office with you as well; we have had many enjoyable conversations covering all kinds of topics. **Emre**, thank you for keeping me company at work during the weekends and for all the jokes and fun conversations. **Saleh** and **Robin,** I appreciate the company during the weekends and long evenings in the office and the well-needed breaks for some good chats while writing this thesis. **Aleksandra**, I always enjoyed the interesting trivia

sessions in the cell lab, and the dad jokes in the office on Fridays. **Lucie**, you were a great neighbour in the lab. Thank you for all the good times in the lab, the hockey game and the after-work beers. I also want to thank all the rest of the past and present members of the research group who have been a part of my everyday life during the past few years, including **Serena**, **Tomi**, **Gilar**, **Myriam**, **Vaishnavi**, **Thu-ha**, **Marina, Niko**, **Klaudia** and **Karolina**. Thank you for all the good times, both in and outside the lab.

Thank you also to all the collaborators on the different projects and for your contributions to the publications.

Last but not least, I want to thank my friends and family for the immense support that made this possible. Your encouragement, help and distraction from all the stress have been invaluable. Thank you!

Bibliography

1. Volanakis JE, Frank MM. The human complement system in health and disease. New York: Marcel Dekker; 1998. 656 p.
2. Nesargikar PN, Spiller B, Chavez R. The complement system: history, pathways, cascade and inhibitors. Eur J Microbiol Immunol (Bp). 2012;2(2):103-11.
3. Cavaillon J-M, Sansonetti P, Goldman M. 100th Anniversary of Jules Bordet's Nobel Prize: Tribute to a Founding Father of Immunology. Frontiers in Immunology. 2019;10.
4. Bohlson SS, Garred P, Kemper C, Tenner AJ. Complement Nomenclature-Deconvoluted. Front Immunol. 2019;10:1308.
5. Dunkelberger JR, Song W-C. Complement and its role in innate and adaptive immune responses. Cell Research. 2010;20(1):34-50.
6. Qu H, Ricklin D, Lambris JD. Recent developments in low molecular weight complement inhibitors. Mol Immunol. 2009;47(2-3):185-95.
7. Noris M, Remuzzi G. Overview of complement activation and regulation. Semin Nephrol. 2013;33(6):479-92.
8. Merle NS, Church SE, Fremeaux-Bacchi V, Roumenina LT. Complement System Part I - Molecular Mechanisms of Activation and Regulation. Front Immunol. 2015;6:262.
9. Almitairi JOM, Venkatraman Girija U, Furze CM, Simpson-Gray X, Badakshi F, Marshall JE, et al. Structure of the C1r–C1s interaction of the C1 complex of complement activation. Proceedings of the National Academy of Sciences. 2018;115(4):768-73.
10. Gaboriaud C, Thielens NM, Gregory LA, Rossi V, Fontecilla-Camps JC, Arlaud GJ. Structure and activation of the C1 complex of complement: unraveling the puzzle. Trends in Immunology. 2004;25(7):368-73.
11. Ghai R, Waters P, Roumenina LT, Gadjeva M, Kojouharova MS, Reid KBM, et al. C1q and its growing family. Immunobiology. 2007;212(4):253-66.
12. Diebolder CA, Beurskens FJ, de Jong RN, Koning RI, Strumane K, Lindorfer MA, et al. Complement is activated by IgG hexamers assembled at the cell surface. Science. 2014;343(6176):1260-3.

13. Roumenina LT, Popov KT, Bureeva SV, Kojouharova M, Gadjeva M, Rabheru S, et al. Interaction of the globular domain of human C1q with Salmonella typhimurium lipopolysaccharide. Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics. 2008;1784(9):1271-6.
14. Jiang H, Cooper B, Robey FA, Gewurz H. DNA binds and activates complement via residues 14-26 of the human C1q A chain. Journal of Biological Chemistry. 1992;267(35):25597-601.
15. Païdassi H, Tacnet-Delorme P, Lunardi T, Arlaud GJ, Thielens NM, Frachet P. The lectin-like activity of human C1q and its implication in DNA and apoptotic cell recognition. FEBS Letters. 2008;582(20):3111-6.
16. Martin M, Leffler J, Blom AM. Annexin A2 and A5 serve as new ligands for C1q on apoptotic cells. J Biol Chem. 2012;287(40):33733-44.
17. Païdassi H, Tacnet-Delorme P, Garlatti V, Darnault C, Ghebrehiwet B, Gaboriaud C, et al. C1q binds phosphatidylserine and likely acts as a multiligand-bridging molecule in apoptotic cell recognition. J Immunol. 2008;180(4):2329-38.
18. Ogden CA, deCathelineau A, Hoffmann PR, Bratton D, Ghebrehiwet B, Fadok VA, et al. C1q and mannose binding lectin engagement of cell surface calreticulin and CD91 initiates macropinocytosis and uptake of apoptotic cells. J Exp Med. 2001;194(6):781-95.
19. Steinø A, Jørgensen CS, Laursen I, Houen G. Interaction of C1q with the Receptor Calreticulin Requires a Conformational Change in C1q. Scandinavian Journal of Immunology. 2004;59(5):485-95.
20. Terrasse R, Tacnet-Delorme P, Moriscot C, Pérard J, Schoehn G, Vernet T, et al. Human and pneumococcal cell surface glyceraldehyde-3-phosphate dehydrogenase (GAPDH) proteins are both ligands of human C1q protein. J Biol Chem. 2012;287(51):42620-33.
21. Gaboriaud C, Ling WL, Thielens NM, Bally I, Rossi V. Deciphering the fine details of c1 assembly and activation mechanisms: "mission impossible"? Front Immunol. 2014;5:565.
22. Garred P, Genster N, Pilely K, Bayarri-Olmos R, Rosbjerg A, Ma YJ, et al. A journey through the lectin pathway of complement—MBL and beyond. Immunological Reviews. 2016;274(1):74-97.
23. Beltrame MH, Catarino SJ, Goeldner I, Boldt AB, de Messias-Reason IJ. The lectin pathway of complement and rheumatic heart disease. Front Pediatr. 2014;2:148.
24. Degn SE, Kjaer TR, Kidmose RT, Jensen L, Hansen AG, Tekin M, et al. Complement activation by ligand-driven juxtaposition of discrete pattern recognition complexes. Proc Natl Acad Sci U S A. 2014;111(37):13445-50.
25. Gál P, Harmat V, Kocsis A, Bián T, Barna L, Ambrus G, et al. A True Autoactivating Enzyme: STRUCTURAL INSIGHT INTO MANNOSE-BINDING LECTIN-ASSOCIATED SERINE PROTEASE-2 ACTIVATIONS*. Journal of Biological Chemistry. 2005;280(39):33435-44.

26. Héja D, Kocsis A, Dobó J, Szilágyi K, Szász R, Závodszky P, et al. Revised mechanism of complement lectin-pathway activation revealing the role of serine protease MASP-1 as the exclusive activator of MASP-2. Proceedings of the National Academy of Sciences. 2012;109(26):10498-503.
27. Kjaer TR, Thiel S, Andersen GR. Toward a structure-based comprehension of the lectin pathway of complement. Molecular Immunology. 2013;56(3):222-31.
28. Oroszlán G, Kortvely E, Szakács D, Kocsis A, Dammeier S, Zeck A, et al. MASP-1 and MASP-2 Do Not Activate Pro–Factor D in Resting Human Blood, whereas MASP-3 Is a Potential Activator: Kinetic Analysis Involving Specific MASP-1 and MASP-2 Inhibitors. The Journal of Immunology. 2016;196(2):857-65.
29. Ma YJ, Hein E, Munthe-Fog L, Skjoedt M-O, Bayarri-Olmos R, Romani L, et al. Soluble Collectin-12 (CL-12) Is a Pattern Recognition Molecule Initiating Complement Activation via the Alternative Pathway. The Journal of Immunology. 2015;195(7):3365-73.
30. Stahl GL, Xu Y, Hao L, Miller M, Buras JA, Fung M, et al. Role for the alternative complement pathway in ischemia/reperfusion injury. Am J Pathol. 2003;162(2):449-55.
31. Pangburn MK, Müller-Eberhard HJ. The C3 convertase of the alternative pathway of human complement. Enzymic properties of the bimolecular proteinase. Biochem J. 1986;235(3):723-30.
32. Schwaeble W, Dippold WG, Schäfer MK, Pohla H, Jonas D, Luttig B, et al. Properdin, a positive regulator of complement activation, is expressed in human T cell lines and peripheral blood T cells. J Immunol. 1993;151(5):2521-8.
33. SCHWAEBLE W, HUEMER HP, MÖST J, DIERICH MP, STRÖBEL M, CLAUS C, et al. Expression of properdin in human monocytes. European Journal of Biochemistry. 1994;219(3):759-64.
34. Kimura Y, Miwa T, Zhou L, Song WC. Activator-specific requirement of properdin in the initiation and amplification of the alternative pathway complement. Blood. 2008;111(2):732-40.
35. Fearon DT, Austen KF. Properdin: binding to C3b and stabilization of the C3b-dependent C3 convertase. J Exp Med. 1975;142(4):856-63.
36. Xu W, Berger SP, Trouw LA, de Boer HC, Schlagwein N, Mutsaers C, et al. Properdin Binds to Late Apoptotic and Necrotic Cells Independently of C3b and Regulates Alternative Pathway Complement Activation12. The Journal of Immunology. 2008;180(11):7613-21.
37. Spitzer D, Mitchell LM, Atkinson JP, Hourcade DE. Properdin Can Initiate Complement Activation by Binding Specific Target Surfaces and Providing a Platform for De Novo Convertase Assembly1. The Journal of Immunology. 2007;179(4):2600-8.
38. Bubeck D. The Making of a Macromolecular Machine: Assembly of the Membrane Attack Complex. Biochemistry-Us. 2014;53(12):1908-15.
39. Aleshin AE, DiScipio RG, Stec B, Liddington RC. Crystal structure of C5b-6 suggests structural basis for priming assembly of the membrane attack complex. J Biol Chem. 2012;287(23):19642-52.

40. Immunobiology, 5th edition: The Immune System in Health and Disease: Garland Publishing; 2001.
41. Brannen CL, Sodetz JM. Incorporation of human complement C8 into the membrane attack complex is mediated by a binding site located within the C8β MACPF domain. Molecular Immunology. 2007;44(5):960-5.
42. Bubeck D, Roversi P, Donev R, Morgan BP, Llorca O, Lea SM. Structure of human complement C8, a precursor to membrane attack. J Mol Biol. 2011;405(2):325-30.
43. Morgan BP, Boyd C, Bubeck D. Molecular cell biology of complement membrane attack. Seminars in Cell & Developmental Biology. 2017;72:124-32.
44. Xie CB, Jane-Wit D, Pober JS. Complement Membrane Attack Complex: New Roles, Mechanisms of Action, and Therapeutic Targets. Am J Pathol. 2020;190(6):1138-50.
45. Serna M, Giles JL, Morgan BP, Bubeck D. Structural basis of complement membrane attack complex formation. Nature Communications. 2016;7(1):10587.
46. Tschopp J, Engel A, Podack ER. Molecular weight of poly(C9). 12 to 18 C9 molecules form the transmembrane channel of complement. Journal of Biological Chemistry. 1984;259(3):1922-8.
47. Dudkina NV, Spicer BA, Reboul CF, Conroy PJ, Lukoyanova N, Elmlund H, et al. Structure of the poly-C9 component of the complement membrane attack complex. Nature Communications. 2016;7(1):10588.
48. Barnum SR. C4a: An Anaphylatoxin in Name Only. J Innate Immun. 2015;7(4):333-9.
49. Klos A, Tenner AJ, Johswich KO, Ager RR, Reis ES, Köhl J. The role of the anaphylatoxins in health and disease. Mol Immunol. 2009;46(14):2753-66.
50. Krych-Goldberg M, Atkinson JP. Structure–function relationships of complement receptor type 1. Immunological Reviews. 2001;180(1):112-22.
51. Helmy KY, Katschke KJ, Gorgani NN, Kljavin NM, Elliott JM, Diehl L, et al. CRIg: A Macrophage Complement Receptor Required for Phagocytosis of Circulating Pathogens. Cell. 2006;124(5):915-27.
52. Morgan BP, Harris CL. Complement regulatory proteins. San Diego: Academic Press; 1999. 382 p.
53. Ojha H, Ghosh P, Singh Panwar H, Shende R, Gondane A, Mande SC, et al. Spatially conserved motifs in complement control protein domains determine functionality in regulators of complement activation-family proteins. Communications Biology. 2019;2(1):290.
54. Kopp A, Hebecker M, Svobodová E, Józsi M. Factor h: a complement regulator in health and disease, and a mediator of cellular interactions. Biomolecules. 2012;2(1):46-75.
55. Blom AM, Villoutreix BO, Dahlbäck B. Complement inhibitor C4b-binding protein—friend or foe in the innate immune system? Molecular Immunology. 2004;40(18):1333-46.
56. Kim DD, Song W-C. Membrane complement regulatory proteins. Clinical Immunology. 2006;118(2):127-36.

57. Birmingham DJ, Hebert LA. CR1 and CR1-like: the primate immune adherence receptors. Immunological Reviews. 2001;180(1):100-11.
58. Khera R, Das N. Complement Receptor 1: disease associations and therapeutic implications. Mol Immunol. 2009;46(5):761-72.
59. Kovács KG, Mácsik-Valent B, Matkó J, Bajtay Z, Erdei A. Revisiting the Coreceptor Function of Complement Receptor Type 2 (CR2, CD21); Coengagement With the B-Cell Receptor Inhibits the Activation, Proliferation, and Antibody Production of Human B Cells. Frontiers in Immunology. 2021;12.
60. Nilsson SC, Sim RB, Lea SM, Fremeaux-Bacchi V, Blom AM. Complement factor I in health and disease. Molecular Immunology. 2011;48(14):1611-20.
61. Naponelli V, Bettuzzi S. Chapter 32 - Clusterin. In: Barnum S, Schein T, editors. The Complement FactsBook (Second Edition): Academic Press; 2018. p. 341-9.
62. Su Y-C, Riesbeck K. Chapter 33 - Vitronectin. In: Barnum S, Schein T, editors. The Complement FactsBook (Second Edition): Academic Press; 2018. p. 351-60.
63. Davis AE, 3rd, Lu F, Mejia P. C1 inhibitor, a multi-functional serine protease inhibitor. Thromb Haemost. 2010;104(5):886-93.
64. Matsushita M, Thiel S, Jensenius JC, Terai I, Fujita T. Proteolytic Activities of Two Types of Mannose-Binding Lectin-Associated Serine Protease. The Journal of Immunology. 2000;165(5):2637-42.
65. Escudero-Esparza A, Kalchishkova N, Kurbasic E, Jiang WG, Blom AM. The novel complement inhibitor human CUB and Sushi multiple domains 1 (CSMD1) protein promotes factor I-mediated degradation of C4b and C3b and inhibits the membrane attack complex assembly. The FASEB Journal. 2013;27(12):5083-93.
66. Matthews KW, Mueller-Ortiz SL, Wetsel RA. Carboxypeptidase N: a pleiotropic regulator of inflammation. Molecular Immunology. 2004;40(11):785-93.
67. Gialeli C, Gungor B, Blom AM. Novel potential inhibitors of complement system and their roles in complement regulation and beyond. Molecular Immunology. 2018;102:73-83.
68. Holmquist E, Okroj M, Nodin B, Jirström K, Blom AM. Sushi domain-containing protein 4 (SUSD4) inhibits complement by disrupting the formation of the classical C3 convertase. The FASEB Journal. 2013;27(6):2355-66.
69. González-Calvo I, Iyer K, Carquin M, Khayachi A, Giuliani FA, Sigoillot SM, et al. Sushi domain-containing protein 4 controls synaptic plasticity and motor learning. Elife. 2021;10.
70. Englund E, Reitsma B, King BC, Escudero-Esparza A, Owen S, Orimo A, et al. The human complement inhibitor Sushi Domain-Containing Protein 4 (SUSD4) expression in tumor cells and infiltrating T cells is associated with better prognosis of breast cancer patients. BMC Cancer. 2015;15:737.
71. Tu Z, Cohen M, Bu H, Lin F. Tissue distribution and functional analysis of Sushi domain-containing protein 4. Am J Pathol. 2010;176(5):2378-84.
72. Rosenfeld JA, Lacassie Y, El-Khechen D, Escobar LF, Reggin J, Heuer C, et al. New cases and refinement of the critical region in the 1q41q42 microdeletion syndrome. European Journal of Medical Genetics. 2011;54(1):42-9.

73. Shaffer LG, Theisen A, Bejjani BA, Ballif BC, Aylsworth AS, Lim C, et al. The discovery of microdeletion syndromes in the post-genomic era: review of the methodology and characterization of a new 1q41q42 microdeletion syndrome. Genetics in Medicine. 2007;9(9):607-16.

74. Zhu H, Meissner LE, Byrnes C, Tuymetova G, Tifft CJ, Proia RL. The Complement Regulator Susd4 Influences Nervous-System Function and Neuronal Morphology in Mice. iScience. 2020;23(3):100957.

75. Zhong Y, Zheng C, Zhang W, Wu H, Zhang Q, Li D, et al. Pan-cancer analysis of Sushi domain-containing protein 4 (SUSD4) and validated in colorectal cancer. Aging (Albany NY). 2024;16(7):6417-44.

76. Roumenina LT, Daugan MV, Petitprez F, Sautès-Fridman C, Fridman WH. Context-dependent roles of complement in cancer. Nature Reviews Cancer. 2019;19(12):698-715.

77. Granata S, Santoro G, Signorini L, Malerba G, Patuzzo C, Gambaro G, et al. Comparative transcriptome analysis of peripheral blood mononuclear cells in renal transplant recipients in everolimus- and tacrolimus-based immunosuppressive therapy. Eur J Pharmacol. 2019;859:172494.

78. Galat A. The three-fingered protein domain of the human genome. Cell Mol Life Sci. 2008;65(21):3481-93.

79. Galat A, Gross G, Drevet P, Sato A, Ménez A. Conserved structural determinants in three-fingered protein domains. The FEBS Journal. 2008;275(12):3207-25.

80. Leath KJ, Johnson S, Roversi P, Hughes TR, Smith RA, Mackenzie L, et al. High-resolution structures of bacterially expressed soluble human CD59. Acta Crystallogr Sect F Struct Biol Cryst Commun. 2007;63(Pt 8):648-52.

81. Sugita Y, Nakano Y, Oda E, Noda K, Tobe T, Miura N-H, et al. Determination of Carboxyl-Terminal Residue and Bisulfide Bonds of MACIF (CD59), a Glycosyl-Phosphatidylinositol-Anchored Membrane Protein1. The Journal of Biochemistry. 1993;114(4):473-7.

82. Fletcher CM, Harrison RA, Lachmann PJ, Neuhaus D. Structure of a soluble, glycosylated form of the human complement regulatory protein CD59. Structure. 1994;2(3):185-99.

83. Tone M, Walsh LA, Waldmann H. Gene structure of human CD59 and demonstration that discrete mRNAs are generated by alternative polyadenylation. Journal of Molecular Biology. 1992;227(3):971-6.

84. Petranka JG, Fleenor DE, Sykes K, Kaufman RE, Rosse WF. Structure of the CD59-encoding gene: further evidence of a relationship to murine lymphocyte antigen Ly-6 protein. Proc Natl Acad Sci U S A. 1992;89(17):7876-9.

85. Morgan P. Chapter 34 - CD59. In: Barnum S, Schein T, editors. The Complement FactsBook (Second Edition): Academic Press; 2018. p. 361-7.

86. Kinoshita T. Biosynthesis and biology of mammalian GPI-anchored proteins. Open Biology. 2020;10(3):190290.

87. Uszewski MK, Farries TC, Lublin DM, Rooney IA, Atkinson JP. Control of the Complement System. In: Dixon FJ, editor. Advances in Immunology. 61: Academic Press; 1996. p. 201-83.

88. Rudd PM, Morgan BP, Wormald MR, Harvey DJ, van den Berg CW, Davis SJ, et al. The Glycosylation of the Complement Regulatory Protein, Human Erythrocyte CD59*. Journal of Biological Chemistry. 1997;272(11):7229-44.
89. Couves EC, Gardner S, Voisin TB, Bickel JK, Stansfeld PJ, Tate EW, et al. Structural basis for membrane attack complex inhibition by CD59. Nature Communications. 2023;14(1):890.
90. Ninomiya H, Sims PJ. The human complement regulatory protein CD59 binds to the alpha-chain of C8 and to the "b"domain of C9. Journal of Biological Chemistry. 1992;267(19):13675-80.
91. Meri S, Lehto T, Sutton CW, Tyynelä J, Baumann M. Structural composition and functional characterization of soluble CD59: heterogeneity of the oligosaccharide and glycophosphoinositol (GPI) anchor revealed by laser-desorption mass spectrometric analysis. Biochem J. 1996;316 (Pt 3)(Pt 3):923-35.
92. Bodian DL, Davis SJ, Morgan BP, Rushmere NK. Mutational analysis of the active site and antibody epitopes of the complement-inhibitory glycoprotein, CD59. J Exp Med. 1997;185(3):507-16.
93. Hahn WC, Menu E, Bothwell ALM, Sims PJ, Bierer BE. Overlapping but Nonidentical Binding Sites on CD2 for CD58 and a Second Ligand CD59. Science. 1992;256(5065):1805-7.
94. Deckert M, Kubar J, Zoccola D, Bernard-Pomier G, Angelisova P, Horejsi V, et al. CD59 molecule: A second ligand for CD2 in T cell adhesion. European Journal of Immunology. 1992;22(11):2943-7.
95. Binder C, Cvetkovski F, Sellberg F, Berg S, Paternina Visbal H, Sachs DH, et al. CD2 Immunobiology. Front Immunol. 2020;11:1090.
96. van der Merwe PA, Barclay AN, Mason DW, Davies EA, Morgan BP, Tone M, et al. Human cell-adhesion molecule CD2 binds CD58 (LFA-3) with a very low affinity and an extremely fast dissociation rate but does not bind CD48 or CD59. Biochemistry-Us. 1994;33(33):10149-60.
97. Arulanandam AR, Moingeon P, Concino MF, Recny MA, Kato K, Yagita H, et al. A soluble multimeric recombinant CD2 protein identifies CD48 as a low affinity ligand for human CD2: divergence of CD2 ligands during the evolution of humans and mice. J Exp Med. 1993;177(5):1439-50.
98. Naderi S, Hofmann P, Seiter S, Tilgen W, Abken H, Reinhold U. CD2-mediated CD59 stimulation in keratinocytes results in secretion of IL-1alpha, IL-6, and GM-CSF: implications for the interaction of keratinocytes with intraepidermal T lymphocytes. Int J Mol Med. 1999;3(6):609-23.
99. Menu E, Tsai BC, Bothwell AL, Sims PJ, Bierer BE. CD59 costimulation of T cell activation. CD58 dependence and requirement for glycosylation. J Immunol. 1994;153(6):2444-56.
100. Li L, Ding P, Lv X, Xie S, Li L, Chen J, et al. CD59-Regulated Ras Compartmentalization Orchestrates Antitumor T-cell Immunity. Cancer Immunol Res. 2022;10(12):1475-89.

101. Giddings KS, Zhao J, Sims PJ, Tweten RK. Human CD59 is a receptor for the cholesterol-dependent cytolysin intermedilysin. Nature Structural & Molecular Biology. 2004;11(12):1173-8.
102. Ghiran I, Klickstein LB, Nicholson-Weller A. Calreticulin Is at the Surface of Circulating Neutrophils and Uses CD59 as an Adaptor Molecule*. Journal of Biological Chemistry. 2003;278(23):21024-31.
103. Hatanaka M, Seya T, Matsumoto M, Hara T, Nonaka M, Inoue N, et al. Mechanisms by which the surface expression of the glycosyl-phosphatidylinositol-anchored complement regulatory proteins decay-accelerating factor (CD55) and CD59 is lost in human leukaemia cell lines. Biochem J. 1996;314 (Pt 3)(Pt 3):969-76.
104. Ghosh P, Sahoo R, Vaidya A, Chorev M, Halperin JA. Role of complement and complement regulatory proteins in the complications of diabetes. Endocr Rev. 2015;36(3):272-88.
105. Ghosh P, Sahoo R, Vaidya A, Cantel S, Kavishwar A, Goldfine A, et al. A specific and sensitive assay for blood levels of glycated CD59: a novel biomarker for diabetes. Am J Hematol. 2013;88(8):670-6.
106. Acosta J, Hettinga J, Flückiger R, Krumrei N, Goldfine A, Angarita L, et al. Molecular basis for a link between complement and the vascular complications of diabetes. Proc Natl Acad Sci U S A. 2000;97(10):5450-5.
107. Risitano AM, Rotoli B. Paroxysmal nocturnal hemoglobinuria: pathophysiology, natural history and treatment options in the era of biological agents. Biologics. 2008;2(2):205-22.
108. Hillmen P, Muus P, Dührsen U, Risitano AM, Schubert J, Luzzatto L, et al. Effect of the complement inhibitor eculizumab on thromboembolism in patients with paroxysmal nocturnal hemoglobinuria. Blood. 2007;110(12):4123-8.
109. Brodsky RA. Paroxysmal nocturnal hemoglobinuria. Blood. 2014;124(18):2804-11.
110. Chai JN, Azad AK, Kuan K, Guo X, Wang Y. A Splice Site Mutation Associated with Congenital CD59 Deficiency. Hematol Rep. 2022;14(2):172-8.
111. Karbian N, Eshed-Eisenbach Y, Tabib A, Hoizman H, Morgan BP, Schueler-Furman O, et al. Molecular pathogenesis of human CD59 deficiency. Neurol Genet. 2018;4(6):e280.
112. Javadi Parvaneh V, Ghasemi L, Rahmani K, Shiari R, Mesdaghi M, Chavoshzadeh Z, et al. Recurrent angioedema, Guillain-Barré, and myelitis in a girl with systemic lupus erythematosus and CD59 deficiency syndrome. Autoimmunity Highlights. 2020;11(1):9.
113. Qian Y-M, Qin X, Miwa T, Sun X, Halperin JA, Song W-C. Identification and Functional Characterization of a New Gene Encoding the Mouse Terminal Complement Inhibitor CD59. The Journal of Immunology. 2000;165(5):2528-34.
114. Qin X, Miwa T, Aktas H, Gao M, Lee C, Qian Y-M, et al. Genomic structure, functional comparison, and tissue distribution of mouse Cd59a and Cd59b. Mammalian Genome. 2001;12(8):582-9.
115. Qin X, Ferris S, Hu W, Guo F, Ziegeler G, Halperin JA. Analysis of the promoters and 5′-UTR of mouse Cd59 genes, and of their functional activity in erythrocytes. Genes & Immunity. 2006;7(4):287-97.

116. Baalasubramanian S, Harris CL, Donev RM, Mizuno M, Omidvar N, Song W-C, et al. CD59a Is the Primary Regulator of Membrane Attack Complex Assembly in the Mouse1. The Journal of Immunology. 2004;173(6):3684-92.
117. Harris CL, Hanna SM, Mizuno M, Holt DS, Marchbank KJ, Morgan BP. Characterization of the mouse analogues of CD59 using novel monoclonal antibodies: tissue distribution and functional comparison. Immunology. 2003;109(1):117-26.
118. Holt DS, Botto M, Bygrave AE, Hanna SM, Walport MJ, Morgan BP. Targeted deletion of the CD59 gene causes spontaneous intravascular hemolysis and hemoglobinuria. Blood. 2001;98(2):442-9.
119. Qin X, Krumrei N, Grubissich L, Dobarro M, Aktas H, Perez G, et al. Deficiency of the Mouse Complement Regulatory Protein mCd59b Results in Spontaneous Hemolytic Anemia with Platelet Activation and Progressive Male Infertility. Immunity. 2003;18(2):217-27.
120. Chen J, Du Y, Ding P, Zhang X, Zhang L, Wang N, et al. Mouse Cd59b but not Cd59a is upregulated to protect cells from complement attack in response to inflammatory stimulation. Genes & Immunity. 2015;16(7):437-45.
121. King BC, Blom AM. Intracellular complement: Evidence, definitions, controversies, and solutions. Immunol Rev. 2023;313(1):104-19.
122. Singh P, Kemper C. Complement, complosome, and complotype: A perspective. Eur J Immunol. 2023;53(12):e2250042.
123. Niyonzima N, Rahman J, Kunz N, West EE, Freiwald T, Desai JV, et al. Mitochondrial C5aR1 activity in macrophages controls IL-1β production underlying sterile inflammation. Sci Immunol. 2021;6(66):eabf2489.
124. Ding P, Xu Y, Li L, Lv X, Li L, Chen J, et al. Intracellular complement C5a/C5aR1 stabilizes β-catenin to promote colorectal tumorigenesis. Cell Reports. 2022;39(9):110851.
125. West EE, Kemper C. Complosome - the intracellular complement system. Nat Rev Nephrol. 2023;19(7):426-39.
126. Freeley S, Kemper C, Le Friec G. The "ins and outs" of complement-driven immune responses. Immunol Rev. 2016;274(1):16-32.
127. Arbore G, West EE, Spolski R, Robertson AAB, Klos A, Rheinheimer C, et al. T helper 1 immunity requires complement-driven NLRP3 inflammasome activity in $CD4^+$ T cells. Science. 2016;352(6292):aad1210.
128. Sorbara MT, Foerster EG, Tsalikis J, Abdel-Nour M, Mangiapane J, Sirluck-Schroeder I, et al. Complement C3 Drives Autophagy-Dependent Restriction of Cyto-invasive Bacteria. Cell Host & Microbe. 2018;23(5):644-52.e5.
129. Kremlitzka M, Colineau L, Nowacka AA, Mohlin FC, Wozniak K, Blom AM, et al. Alternative translation and retrotranslocation of cytosolic C3 that detects cytoinvasive bacteria. Cell Mol Life Sci. 2022;79(6):291.
130. Daugan MV, Revel M, Thouenon R, Dragon-Durey M-A, Robe-Rybkine T, Torset C, et al. Intracellular Factor H Drives Tumor Progression Independently of the Complement Cascade. Cancer Immunology Research. 2021;9(8):909-25.

131. Martin M, Leffler J, Smoląg KI, Mytych J, Björk A, Chaves LD, et al. Factor H uptake regulates intracellular C3 activation during apoptosis and decreases the inflammatory potential of nucleosomes. Cell Death & Differentiation. 2016;23(5):903-11.
132. Olcina MM, Kim RK, Balanis NG, Li CG, von Eyben R, Graeber TG, et al. Intracellular C4BPA Levels Regulate NF-κB-Dependent Apoptosis. iScience. 2020;23(10):101594.
133. Gialeli C, Tuysuz EC, Staaf J, Guleed S, Paciorek V, Mörgelin M, et al. Complement inhibitor CSMD1 modulates epidermal growth factor receptor oncogenic signaling and sensitizes breast cancer cells to chemotherapy. Journal of Experimental & Clinical Cancer Research. 2021;40(1):258.
134. Golec E, Rosberg R, Zhang E, Renström E, Blom AM, King BC. A cryptic non-GPI-anchored cytosolic isoform of CD59 controls insulin exocytosis in pancreatic β-cells by interaction with SNARE proteins. Faseb j. 2019;33(11):12425-34.
135. Elvington M, Liszewski MK, Bertram P, Kulkarni HS, Atkinson JP. A C3(H20) recycling pathway is a component of the intracellular complement system. J Clin Invest. 2017;127(3):970-81.
136. Joslin EP, Kahn CR. Joslin's diabetes mellitus. Philadelphia, Pa.: Lippincott Williams & Willkins; 2005.
137. Röder PV, Wu B, Liu Y, Han W. Pancreatic regulation of glucose homeostasis. Experimental & Molecular Medicine. 2016;48(3):e219-e.
138. Diagnosis and classification of diabetes mellitus. Diabetes Care. 2010;33 Suppl 1(Suppl 1):S62-9.
139. Tattersall RB. The History of Diabetes Mellitus. Textbook of Diabetes2010. p. 1-23.
140. Magliano DJ, Boyko EJ. IDF DIABETES ATLAS. IDF Diabetes Atlas. 10th ed. Brussels2021.
141. Sakula A. Paul Langerhans (1847-1888): a centenary tribute. J R Soc Med. 1988;81(7):414-5.
142. Brissova M, Powers AC. Architecture of Pancreatic Islets. In: Seino S, Bell GI, editors. Pancreatic Beta Cell in Health and Disease. Tokyo: Springer Japan; 2008. p. 3-11.
143. Saito K, Iwama N, Takahashi T. Morphometrical analysis on topographical difference in size distribution, number and volume of islets in the human pancreas. Tohoku J Exp Med. 1978;124(2):177-86.
144. Ichise M, Harris PE. Imaging of beta-cell mass and function. J Nucl Med. 2010;51(7):1001-4.
145. Rorsman P, Ashcroft FM. Pancreatic β-Cell Electrical Activity and Insulin Secretion: Of Mice and Men. Physiol Rev. 2018;98(1):117-214.
146. Korsgren O, Nilsson B, Berne C, Felldin M, Foss A, Kallen R, et al. Current Status of Clinical Islet Transplantation. Transplantation. 2005;79(10):1289-93.
147. Kim A, Miller K, Jo J, Kilimnik G, Wojcik P, Hara M. Islet architecture: A comparative study. Islets. 2009;1(2):129-36.
148. Pisania A, Weir GC, O'Neil JJ, Omer A, Tchipashvili V, Lei J, et al. Quantitative analysis of cell composition and purity of human pancreatic islet preparations. Lab Invest. 2010;90(11):1661-75.

149. Brissova M, Fowler MJ, Nicholson WE, Chu A, Hirshberg B, Harlan DM, et al. Assessment of Human Pancreatic Islet Architecture and Composition by Laser Scanning Confocal Microscopy. Journal of Histochemistry & Cytochemistry. 2005;53(9):1087-97.
150. Cabrera O, Berman DM, Kenyon NS, Ricordi C, Berggren P-O, Caicedo A. The unique cytoarchitecture of human pancreatic islets has implications for islet cell function. Proceedings of the National Academy of Sciences. 2006;103(7):2334-9.
151. Wierup N, Svensson H, Mulder H, Sundler F. The ghrelin cell: a novel developmentally regulated islet cell in the human pancreas. Regulatory Peptides. 2002;107(1):63-9.
152. Larsson LI, Sundler F, Håkanson R. Immunohistochemical localization of human pancreatic polypeptide (HPP) to a population of islet cells. Cell Tissue Res. 1975;156(2):167-71.
153. Da Silva Xavier G. The Cells of the Islets of Langerhans. J Clin Med. 2018;7(3).
154. Campbell JE, Newgard CB. Mechanisms controlling pancreatic islet cell function in insulin secretion. Nature Reviews Molecular Cell Biology. 2021;22(2):142-58.
155. Wierup N, Sundler F, Heller RS. The islet ghrelin cell. Journal of Molecular Endocrinology. 2014;52(1):R35-R49.
156. Goodge KA, Hutton JC. Translational regulation of proinsulin biosynthesis and proinsulin conversion in the pancreaticβ-cell. Seminars in Cell & Developmental Biology. 2000;11(4):235-42.
157. Tillmar L, Carlsson C, Welsh N. Control of Insulin mRNA Stability in Rat Pancreatic Islets: REGULATORY ROLE OF A 3′-UNTRANSLATED REGION PYRIMIDINE-RICH SEQUENCE*. Journal of Biological Chemistry. 2002;277(2):1099-106.
158. Andrali Sreenath S, Sampley Megan L, Vanderford Nathan L, Özcan S. Glucose regulation of insulin gene expression in pancreatic β-cells. Biochemical Journal. 2008;415(1):1-10.
159. Greenman IC, Gomez E, Moore CE, Herbert TP. The selective recruitment of mRNA to the ER and an increase in initiation are important for glucose-stimulated proinsulin synthesis in pancreatic beta-cells. Biochem J. 2005;391(Pt 2):291-300.
160. Magro MG, Solimena M. Regulation of β-cell function by RNA-binding proteins. Mol Metab. 2013;2(4):348-55.
161. Ghiasi SM, Dahlby T, Hede Andersen C, Haataja L, Petersen S, Omar-Hmeadi M, et al. Endoplasmic Reticulum Chaperone Glucose-Regulated Protein 94 Is Essential for Proinsulin Handling. Diabetes. 2019;68(4):747-60.
162. Chang S-G, Choi K-D, Jang S-H, Shin H-C. Role of Disulfide Bonds in the Structure and Activity of Human Insulin. Molecules and Cells. 2003;16(3):323-30.
163. Hou JC, Min L, Pessin JE. Insulin granule biogenesis, trafficking and exocytosis. Vitam Horm. 2009;80:473-506.
164. Dunn MF. Zinc–Ligand Interactions Modulate Assembly and Stability of the Insulin Hexamer – A Review. Biometals. 2005;18(4):295-303.

165. Barg S, Huang P, Eliasson L, Nelson DJ, Obermüller S, Rorsman P, et al. Priming of insulin granules for exocytosis by granular Cl− uptake and acidification. Journal of Cell Science. 2001;114(11):2145-54.
166. Davidson HW, Rhodes CJ, Hutton JC. Intraorganellar calcium and pH control proinsulin cleavage in the pancreatic β cell via two distinct site-specific endopeptidases. Nature. 1988;333(6168):93-6.
167. Davidson HW, Hutton JC. The insulin-secretory-granule carboxypeptidase H. Purification and demonstration of involvement in proinsulin processing. Biochem J. 1987;245(2):575-82.
168. Suckale J, Solimena M. The insulin secretory granule as a signaling hub. Trends in Endocrinology & Metabolism. 2010;21(10):599-609.
169. Shafqat J, Melles E, Sigmundsson K, Johansson BL, Ekberg K, Alvelius G, et al. Proinsulin C-peptide elicits disaggregation of insulin resulting in enhanced physiological insulin effects. Cell Mol Life Sci. 2006;63(15):1805-11.
170. Dean PM. Ultrastructural morphometry of the pancreatic β-cell. Diabetologia. 1973;9(2):115-9.
171. Olofsson CS, Göpel SO, Barg S, Galvanovskis J, Ma X, Salehi A, et al. Fast insulin secretion reflects exocytosis of docked granules in mouse pancreatic B-cells. Pflügers Archiv. 2002;444(1):43-51.
172. Caspi I, Tremmel DM, Pulecio J, Yang D, Liu D, Yan J, et al. Glucose Transporters Are Key Components of the Human Glucostat. Diabetes. 2024;73(8):1336-51.
173. Ashcroft FM, Harrison DE, Ashcroft SJH. Glucose induces closure of single potassium channels in isolated rat pancreatic β-cells. Nature. 1984;312(5993):446-8.
174. Cook DL, Hales N. Intracellular ATP directly blocks K+ channels in pancreatic B-cells. Nature. 1984;311(5983):271-3.
175. Tucker SJ, Gribble FM, Zhao C, Trapp S, Ashcroft FM. Truncation of Kir6.2 produces ATP-sensitive K+ channels in the absence of the sulphonylurea receptor. Nature. 1997;387(6629):179-83.
176. Jacobson DA, Philipson LH. Action potentials and insulin secretion: new insights into the role of Kv channels. Diabetes Obes Metab. 2007;9 Suppl 2(Suppl 2):89-98.
177. Rorsman P, Renström E. Insulin granule dynamics in pancreatic beta cells. Diabetologia. 2003;46(8):1029-45.
178. Omar-Hmeadi M, Idevall-Hagren O. Insulin granule biogenesis and exocytosis. Cell Mol Life Sci. 2021;78(5):1957-70.
179. Rorsman P, Eliasson L, Renström E, Gromada J, Barg S, Göpel S. The Cell Physiology of Biphasic Insulin Secretion. Physiology. 2000;15(2):72-7.
180. Davis SN, Piatti PM, Monti L, Brown MD, Branch W, Hales CN, et al. Proinsulin and insulin concentrations following intravenous glucose challenges in normal, obese, and non-insulin-dependent diabetic subjects. Metabolism. 1993;42(1):30-5.
181. Hosker JP, Rudenski AS, Burnett MA, Matthews DR, Turner RC. Similar reduction of first- and second-phase B-cell responses at three different glucose levels in type II diabetes and the effect of gliclazide therapy. Metabolism. 1989;38(8):767-72.

182. Varadi A, Tsuboi T, Rutter GA. Myosin Va transports dense core secretory vesicles in pancreatic MIN6 beta-cells. Mol Biol Cell. 2005;16(6):2670-80.
183. Meng YX, Wilson GW, Avery MC, Varden CH, Balczon R. Suppression of the expression of a pancreatic beta-cell form of the kinesin heavy chain by antisense oligonucleotides inhibits insulin secretion from primary cultures of mouse beta-cells. Endocrinology. 1997;138(5):1979-87.
184. Ivarsson R, Jing X, Waselle L, Regazzi R, Renström E. Myosin 5a Controls Insulin Granule Recruitment During Late-Phase Secretion. Traffic. 2005;6(11):1027-35.
185. Cazares VA, Subramani A, Saldate JJ, Hoerauf W, Stuenkel EL. Distinct actions of Rab3 and Rab27 GTPases on late stages of exocytosis of insulin. Traffic. 2014;15(9):997-1015.
186. Brozzi F, Diraison F, Lajus S, Rajatileka S, Philips T, Regazzi R, et al. Molecular Mechanism of Myosin Va Recruitment to Dense Core Secretory Granules. Traffic. 2012;13(1):54-69.
187. Fan F, Matsunaga K, Wang H, Ishizaki R, Kobayashi E, Kiyonari H, et al. Exophilin-8 assembles secretory granules for exocytosis in the actin cortex via interaction with RIM-BP2 and myosin-VIIa. Elife. 2017;6.
188. Zhu D, Koo E, Kwan E, Kang Y, Park S, Xie H, et al. Syntaxin-3 regulates newcomer insulin granule exocytosis and compound fusion in pancreatic beta cells. Diabetologia. 2013;56(2):359-69.
189. Spurlin BA, Thurmond DC. Syntaxin 4 Facilitates Biphasic Glucose-Stimulated Insulin Secretion from Pancreatic β-Cells. Molecular Endocrinology. 2006;20(1):183-93.
190. Zhu D, Zhang Y, Lam Patrick PL, Dolai S, Liu Y, Cai Erica P, et al. Dual Role of VAMP8 in Regulating Insulin Exocytosis and Islet β Cell Growth. Cell Metabolism. 2012;16(2):238-49.
191. Yasuda T, Shibasaki T, Minami K, Takahashi H, Mizoguchi A, Uriu Y, et al. Rim2α Determines Docking and Priming States in Insulin Granule Exocytosis. Cell Metabolism. 2010;12(2):117-29.
192. Gandasi NR, Barg S. Contact-induced clustering of syntaxin and munc18 docks secretory granules at the exocytosis site. Nature Communications. 2014;5(1):3914.
193. Dulubova I, Sugita S, Hill S, Hosaka M, Fernandez I, Südhof TC, et al. A conformational switch in syntaxin during exocytosis: role of munc18. Embo j. 1999;18(16):4372-82.
194. Yang B, Steegmaier M, Gonzalez LC, Jr., Scheller RH. nSec1 binds a closed conformation of syntaxin1A. J Cell Biol. 2000;148(2):247-52.
195. Lai Y, Choi UB, Leitz J, Rhee HJ, Lee C, Altas B, et al. Molecular Mechanisms of Synaptic Vesicle Priming by Munc13 and Munc18. Neuron. 2017;95(3):591-607.e10.
196. Wang S, Li Y, Gong J, Ye S, Yang X, Zhang R, et al. Munc18 and Munc13 serve as a functional template to orchestrate neuronal SNARE complex assembly. Nature Communications. 2019;10(1):69.

197. Tang J, Maximov A, Shin O-H, Dai H, Rizo J, Südhof TC. A Complexin/Synaptotagmin 1 Switch Controls Fast Synaptic Vesicle Exocytosis. Cell. 2006;126(6):1175-87.

198. Abderrahmani A, Niederhauser G, Plaisance Vr, Roehrich M-E, Lenain V, Coppola T, et al. Complexin I regulates glucose-induced secretion in pancreatic β-cells. Journal of Cell Science. 2004;117(11):2239-47.

199. Gandasi NR, Yin P, Riz M, Chibalina MV, Cortese G, Lund PE, et al. Ca2+ channel clustering with insulin-containing granules is disturbed in type 2 diabetes. J Clin Invest. 2017;127(6):2353-64.

200. Barg S. Mechanisms of Exocytosis in Insulin-Secreting B-Cells and Glucagon-Secreting A-Cells. Pharmacology & Toxicology. 2003;92(1):3-13.

201. Wolfes AC, Dean C. The diversity of synaptotagmin isoforms. Current Opinion in Neurobiology. 2020;63:198-209.

202. Lang J, Fukuda M, Zhang H, Mikoshiba K, Wollheim CB. The first C₂ domain of synaptotagmin is required for exocytosis of insulin from pancreatic β‐cells: action of synaptotagmin at low micromolar calcium. The EMBO Journal. 1997;16(19):5837-46.

203. Iezzi M, Kouri G, Fukuda M, Wollheim CB. Synaptotagmin V and IX isoforms control Ca2+-dependent insulin exocytosis. Journal of Cell Science. 2004;117(15):3119-27.

204. Gut A, Kiraly CE, Fukuda M, Mikoshiba K, Wollheim CB, Lang J. Expression and localisation of synaptotagmin isoforms in endocrine β-cells: their function in insulin exocytosis. Journal of Cell Science. 2001;114(9):1709-16.

205. Gao Z, Reavey-Cantwell J, Young RA, Jegier P, Wolf BA. Synaptotagmin III/VII Isoforms Mediate Ca2+-induced Insulin Secretion in Pancreatic Islet β-Cells*. Journal of Biological Chemistry. 2000;275(46):36079-85.

206. Honigmann A, van den Bogaart G, Iraheta E, Risselada HJ, Milovanovic D, Mueller V, et al. Phosphatidylinositol 4,5-bisphosphate clusters act as molecular beacons for vesicle recruitment. Nature Structural & Molecular Biology. 2013;20(6):679-86.

207. Martens S, Kozlov MM, McMahon HT. How Synaptotagmin Promotes Membrane Fusion. Science. 2007;316(5828):1205-8.

208. Wang J, Bello O, Auclair SM, Wang J, Coleman J, Pincet F, et al. Calcium sensitive ring-like oligomers formed by synaptotagmin. Proceedings of the National Academy of Sciences. 2014;111(38):13966-71.

209. Mosthaf L, Grako K, Dull TJ, Coussens L, Ullrich A, McClain DA. Functionally distinct insulin receptors generated by tissue-specific alternative splicing. Embo j. 1990;9(8):2409-13.

210. Boucher J, Kleinridders A, Kahn CR. Insulin receptor signaling in normal and insulin-resistant states. Cold Spring Harb Perspect Biol. 2014;6(1).

211. Norton L, Shannon C, Gastaldelli A, DeFronzo RA. Insulin: The master regulator of glucose metabolism. Metabolism. 2022;129:155142.

212. Bryant NJ, Govers R, James DE. Regulated transport of the glucose transporter GLUT4. Nature Reviews Molecular Cell Biology. 2002;3(4):267-77.

213. Eaton RP, Allen RC, Schade DS. Hepatic Removal of Insulin in Normal Man: Dose Response to Endogenous Insulin Secretion*. The Journal of Clinical Endocrinology & Metabolism. 1983;56(6):1294-300.
214. Rui L. Energy metabolism in the liver. Compr Physiol. 2014;4(1):177-97.
215. Association AD. Diagnosis and Classification of Diabetes Mellitus. Diabetes Care. 2013;37(Supplement_1):S81-S90.
216. Atkinson MA, Eisenbarth GS, Michels AW. Type 1 diabetes. The Lancet. 2014;383(9911):69-82.
217. Katsarou A, Gudbjörnsdottir S, Rawshani A, Dabelea D, Bonifacio E, Anderson BJ, et al. Type 1 diabetes mellitus. Nature Reviews Disease Primers. 2017;3(1):17016.
218. Committee ADAPP. 2. Diagnosis and Classification of Diabetes: Standards of Care in Diabetes—2024. Diabetes Care. 2023;47(Supplement_1):S20-S42.
219. Ilonen J, Lempainen J, Veijola R. The heterogeneous pathogenesis of type 1 diabetes mellitus. Nature Reviews Endocrinology. 2019;15(11):635-50.
220. Robertson CC, Rich SS. Genetics of type 1 diabetes. Current Opinion in Genetics & Development. 2018;50:7-16.
221. Vehik K, Lynch KF, Wong MC, Tian X, Ross MC, Gibbs RA, et al. Prospective virome analyses in young children at increased genetic risk for type 1 diabetes. Nature Medicine. 2019;25(12):1865-72.
222. Hyöty H. Viruses in type 1 diabetes. Pediatric Diabetes. 2016;17(S22):56-64.
223. Ashton MP, Eugster A, Walther D, Daehling N, Riethausen S, Kuehn D, et al. Incomplete immune response to coxsackie B viruses associates with early autoimmunity against insulin. Sci Rep. 2016;6:32899.
224. Dotta F, Censini S, van Halteren AG, Marselli L, Masini M, Dionisi S, et al. Coxsackie B4 virus infection of beta cells and natural killer cell insulitis in recent-onset type 1 diabetic patients. Proc Natl Acad Sci U S A. 2007;104(12):5115-20.
225. Galicia-Garcia U, Benito-Vicente A, Jebari S, Larrea-Sebal A, Siddiqi H, Uribe KB, et al. Pathophysiology of Type 2 Diabetes Mellitus. Int J Mol Sci. 2020;21(17).
226. Ogurtsova K, Guariguata L, Barengo NC, Ruiz PL-D, Sacre JW, Karuranga S, et al. IDF diabetes Atlas: Global estimates of undiagnosed diabetes in adults for 2021. Diabetes Research and Clinical Practice. 2022;183:109118.
227. DeFronzo RA, Ferrannini E, Groop L, Henry RR, Herman WH, Holst JJ, et al. Type 2 diabetes mellitus. Nature Reviews Disease Primers. 2015;1(1):15019.
228. Donath MY, Shoelson SE. Type 2 diabetes as an inflammatory disease. Nature Reviews Immunology. 2011;11(2):98-107.
229. Pickup JC, Mattock MB, Chusney GD, Burt D. NIDDM as a disease of the innate immune system: association of acute-phase reactants and interleukin-6 with metabolic syndrome X. Diabetologia. 1997;40(11):1286-92.
230. Spranger J, Kroke A, Möhlig M, Hoffmann K, Bergmann MM, Ristow M, et al. Inflammatory Cytokines and the Risk to Develop Type 2 Diabetes: Results of the Prospective Population-Based European Prospective Investigation into Cancer and Nutrition (EPIC)-Potsdam Study. Diabetes. 2003;52(3):812-7.

231. Herder C, Illig T, Rathmann W, Martin S, Haastert B, Müller-Scholze S, et al. Inflammation and Type 2 Diabetes: Results from KORA Augsburg. Gesundheitswesen. 2005;67(S 01):115-21.
232. Chen C, Cohrs CM, Stertmann J, Bozsak R, Speier S. Human beta cell mass and function in diabetes: Recent advances in knowledge and technologies to understand disease pathogenesis. Mol Metab. 2017;6(9):943-57.
233. Christensen AA, Gannon M. The Beta Cell in Type 2 Diabetes. Current Diabetes Reports. 2019;19(9):81.
234. Rahier J, Guiot Y, Goebbels RM, Sempoux C, Henquin JC. Pancreatic beta-cell mass in European subjects with type 2 diabetes. Diabetes Obes Metab. 2008;10 Suppl 4:32-42.
235. Butler AE, Janson J, Bonner-Weir S, Ritzel R, Rizza RA, Butler PC. β-Cell Deficit and Increased β-Cell Apoptosis in Humans With Type 2 Diabetes. Diabetes. 2003;52(1):102-10.
236. Butler AE, Dhawan S, Hoang J, Cory M, Zeng K, Fritsch H, et al. β-Cell Deficit in Obese Type 2 Diabetes, a Minor Role of β-Cell Dedifferentiation and Degranulation. J Clin Endocrinol Metab. 2016;101(2):523-32.
237. Marchetti P, Del Guerra S, Marselli L, Lupi R, Masini M, Pollera M, et al. Pancreatic Islets from Type 2 Diabetic Patients Have Functional Defects and Increased Apoptosis That Are Ameliorated by Metformin. The Journal of Clinical Endocrinology & Metabolism. 2004;89(11):5535-41.
238. Marchetti P, Bugliani M, Lupi R, Marselli L, Masini M, Boggi U, et al. The endoplasmic reticulum in pancreatic beta cells of type 2 diabetes patients. Diabetologia. 2007;50(12):2486-94.
239. Deng S, Vatamaniuk M, Huang X, Doliba N, Lian M-M, Frank A, et al. Structural and Functional Abnormalities in the Islets Isolated From Type 2 Diabetic Subjects. Diabetes. 2004;53(3):624-32.
240. Del Prato S. Loss of early insulin secretion leads to postprandial hyperglycaemia. Diabetologia. 2003;46(1):M2-M8.
241. Ferrannini E, Gastaldelli A, Miyazaki Y, Matsuda M, Mari A, DeFronzo RA. β-Cell Function in Subjects Spanning the Range from Normal Glucose Tolerance to Overt Diabetes: A New Analysis. The Journal of Clinical Endocrinology & Metabolism. 2005;90(1):493-500.
242. Ahlqvist E, Storm P, Käräjämäki A, Martinell M, Dorkhan M, Carlsson A, et al. Novel subgroups of adult-onset diabetes and their association with outcomes: a data-driven cluster analysis of six variables. The Lancet Diabetes & Endocrinology. 2018;6(5):361-9.
243. Poitout V, Robertson RP. Minireview: Secondary β-Cell Failure in Type 2 Diabetes—A Convergence of Glucotoxicity and Lipotoxicity. Endocrinology. 2002;143(2):339-42.
244. Jeffrey KD, Alejandro EU, Luciani DS, Kalynyak TB, Hu X, Li H, et al. Carboxypeptidase E mediates palmitate-induced beta-cell ER stress and apoptosis. Proc Natl Acad Sci U S A. 2008;105(24):8452-7.

245. Gwiazda KS, Yang T-LB, Lin Y, Johnson JD. Effects of palmitate on ER and cytosolic Ca2+ homeostasis in β-cells. American Journal of Physiology-Endocrinology and Metabolism. 2009;296(4):E690-E701.
246. Cunha DA, Hekerman P, Ladrière L, Bazarra-Castro A, Ortis F, Wakeham MC, et al. Initiation and execution of lipotoxic ER stress in pancreatic beta-cells. J Cell Sci. 2008;121(Pt 14):2308-18.
247. Lupi R, Dotta F, Marselli L, Del Guerra S, Masini M, Santangelo C, et al. Prolonged Exposure to Free Fatty Acids Has Cytostatic and Pro-Apoptotic Effects on Human Pancreatic Islets: Evidence that β-Cell Death Is Caspase Mediated, Partially Dependent on Ceramide Pathway, and Bcl-2 Regulated. Diabetes. 2002;51(5):1437-42.
248. El-Assaad W, Buteau J, Peyot M-L, Nolan C, Roduit R, Hardy S, et al. Saturated Fatty Acids Synergize with Elevated Glucose to Cause Pancreatic β-Cell Death. Endocrinology. 2003;144(9):4154-63.
249. Lytrivi M, Castell AL, Poitout V, Cnop M. Recent Insights Into Mechanisms of β-Cell Lipo- and Glucolipotoxicity in Type 2 Diabetes. J Mol Biol. 2020;432(5):1514-34.
250. Federici M, Hribal M, Perego L, Ranalli M, Caradonna Z, Perego C, et al. High Glucose Causes Apoptosis in Cultured Human Pancreatic Islets of Langerhans: A Potential Role for Regulation of Specific Bcl Family Genes Toward an Apoptotic Cell Death Program. Diabetes. 2001;50(6):1290-301.
251. Bensellam M, Laybutt DR, Jonas J-C. The molecular mechanisms of pancreatic β-cell glucotoxicity: Recent findings and future research directions. Molecular and Cellular Endocrinology. 2012;364(1):1-27.
252. Poitout V, Robertson RP. Glucolipotoxicity: Fuel Excess and β-Cell Dysfunction. Endocrine Reviews. 2008;29(3):351-66.
253. Donath MY, Böni-Schnetzler M, Ellingsgaard H, Halban PA, Ehses JA. Cytokine production by islets in health and diabetes: cellular origin, regulation and function. Trends in Endocrinology & Metabolism. 2010;21(5):261-7.
254. Dror E, Dalmas E, Meier DT, Wueest S, Thévenet J, Thienel C, et al. Postprandial macrophage-derived IL-1β stimulates insulin, and both synergistically promote glucose disposal and inflammation. Nature Immunology. 2017;18(3):283-92.
255. Maedler K, Sergeev P, Ris F, Oberholzer J, Joller-Jemelka HI, Spinas GA, et al. Glucose-induced beta cell production of IL-1beta contributes to glucotoxicity in human pancreatic islets. J Clin Invest. 2002;110(6):851-60.
256. Maedler K, Spinas GA, Lehmann R, Sergeev P, Weber M, Fontana A, et al. Glucose Induces β-Cell Apoptosis Via Upregulation of the Fas Receptor in Human Islets. Diabetes. 2001;50(8):1683-90.
257. Böni-Schnetzler M, Thorne J, Parnaud G, Marselli L, Ehses JA, Kerr-Conte J, et al. Increased interleukin (IL)-1beta messenger ribonucleic acid expression in beta -cells of individuals with type 2 diabetes and regulation of IL-1beta in human islets by glucose and autostimulation. J Clin Endocrinol Metab. 2008;93(10):4065-74.
258. Böni-Schnetzler M, Boller S, Debray S, Bouzakri K, Meier DT, Prazak R, et al. Free Fatty Acids Induce a Proinflammatory Response in Islets via the Abundantly Expressed Interleukin-1 Receptor I. Endocrinology. 2009;150(12):5218-29.

259. Igoillo-Esteve M, Marselli L, Cunha DA, Ladrière L, Ortis F, Grieco FA, et al. Palmitate induces a pro-inflammatory response in human pancreatic islets that mimics CCL2 expression by beta cells in type 2 diabetes. Diabetologia. 2010;53(7):1395-405.

260. Eguchi K, Manabe I, Oishi-Tanaka Y, Ohsugi M, Kono N, Ogata F, et al. Saturated Fatty Acid and TLR Signaling Link β Cell Dysfunction and Islet Inflammation. Cell Metabolism. 2012;15(4):518-33.

261. Cardozo AK, Ortis F, Storling J, Feng Y-M, Rasschaert J, Tonnesen M, et al. Cytokines Downregulate the Sarcoendoplasmic Reticulum Pump Ca2+ ATPase 2b and Deplete Endoplasmic Reticulum Ca2+, Leading to Induction of Endoplasmic Reticulum Stress in Pancreatic β-Cells. Diabetes. 2005;54(2):452-61.

262. Meyerovich K, Ortis F, Allagnat F, Cardozo AK. Endoplasmic reticulum stress and the unfolded protein response in pancreatic islet inflammation. Journal of Molecular Endocrinology. 2016;57(1):R1-R17.

263. Lytrivi M, Igoillo-Esteve M, Cnop M. Inflammatory stress in islet β-cells: therapeutic implications for type 2 diabetes? Current Opinion in Pharmacology. 2018;43:40-5.

264. Akter R, Cao P, Noor H, Ridgway Z, Tu LH, Wang H, et al. Islet Amyloid Polypeptide: Structure, Function, and Pathophysiology. J Diabetes Res. 2016;2016:2798269.

265. Raleigh D, Zhang X, Hastoy B, Clark A. The β-cell assassin: IAPP cytotoxicity. Journal of Molecular Endocrinology. 2017;59(3):R121-R40.

266. Asthana S, Mallick B, Alexandrescu AT, Jha S. IAPP in type II diabetes: Basic research on structure, molecular interactions, and disease mechanisms suggests potential intervention strategies. Biochimica et Biophysica Acta (BBA) - Biomembranes. 2018;1860(9):1765-82.

267. Mirzabekov TA, Lin M-c, Kagan BL. Pore Formation by the Cytotoxic Islet Amyloid Peptide Amylin (∗). Journal of Biological Chemistry. 1996;271(4):1988-92.

268. Janson J, Ashley RH, Harrison D, McIntyre S, Butler PC. The mechanism of islet amyloid polypeptide toxicity is membrane disruption by intermediate-sized toxic amyloid particles. Diabetes. 1999;48(3):491-8.

269. Masters SL, Dunne A, Subramanian SL, Hull RL, Tannahill GM, Sharp FA, et al. Activation of the NLRP3 inflammasome by islet amyloid polypeptide provides a mechanism for enhanced IL-1β in type 2 diabetes. Nature Immunology. 2010;11(10):897-904.

270. Rivera JF, Gurlo T, Daval M, Huang CJ, Matveyenko AV, Butler PC, et al. Human-IAPP disrupts the autophagy/lysosomal pathway in pancreatic β-cells: protective role of p62-positive cytoplasmic inclusions. Cell Death & Differentiation. 2011;18(3):415-26.

271. Bensellam M, Jonas J-C, Laybutt DR. Mechanisms of β-cell dedifferentiation in diabetes: recent findings and future research directions. Journal of Endocrinology. 2018;236(2):R109-R43.

272. Jonas J-C, Sharma A, Hasenkamp W, Ilkova H, Patanè G, Laybutt R, et al. Chronic Hyperglycemia Triggers Loss of Pancreatic β Cell Differentiation in an Animal Model of Diabetes*. Journal of Biological Chemistry. 1999;274(20):14112-21.

273. Kjørholt C, Åkerfeldt MC, Biden TJ, Laybutt DR. Chronic Hyperglycemia, Independent of Plasma Lipid Levels, Is Sufficient for the Loss of β-Cell Differentiation and Secretory Function in the db/db Mouse Model of Diabetes. Diabetes. 2005;54(9):2755-63.
274. Talchai C, Xuan S, Lin Hua V, Sussel L, Accili D. Pancreatic β Cell Dedifferentiation as a Mechanism of Diabetic β Cell Failure. Cell. 2012;150(6):1223-34.
275. Cinti F, Bouchi R, Kim-Muller JY, Ohmura Y, Sandoval PR, Masini M, et al. Evidence of β-Cell Dedifferentiation in Human Type 2 Diabetes. The Journal of Clinical Endocrinology & Metabolism. 2016;101(3):1044-54.
276. Spijker HS, Ravelli RB, Mommaas-Kienhuis AM, van Apeldoorn AA, Engelse MA, Zaldumbide A, et al. Conversion of mature human β-cells into glucagon-producing α-cells. Diabetes. 2013;62(7):2471-80.
277. Brereton MF, Iberl M, Shimomura K, Zhang Q, Adriaenssens AE, Proks P, et al. Reversible changes in pancreatic islet structure and function produced by elevated blood glucose. Nature Communications. 2014;5(1):4639.
278. Chera S, Baronnier D, Ghila L, Cigliola V, Jensen JN, Gu G, et al. Diabetes recovery by age-dependent conversion of pancreatic δ-cells into insulin producers. Nature. 2014;514(7523):503-7.
279. Thorel F, Népote V, Avril I, Kohno K, Desgraz R, Chera S, et al. Conversion of adult pancreatic alpha-cells to beta-cells after extreme beta-cell loss. Nature. 2010;464(7292):1149-54.
280. van der Meulen T, Mawla AM, DiGruccio MR, Adams MW, Nies V, Dólleman S, et al. Virgin Beta Cells Persist throughout Life at a Neogenic Niche within Pancreatic Islets. Cell Metab. 2017;25(4):911-26.e6.
281. O'Neal KS, Johnson JL, Panak RL. Recognizing and Appropriately Treating Latent Autoimmune Diabetes in Adults. Diabetes Spectr. 2016;29(4):249-52.
282. Carlsson S. Etiology and Pathogenesis of Latent Autoimmune Diabetes in Adults (LADA) Compared to Type 2 Diabetes. Frontiers in Physiology. 2019;10.
283. Hawa MI, Kolb H, Schloot N, Beyan H, Paschou SA, Buzzetti R, et al. Adult-onset autoimmune diabetes in Europe is prevalent with a broad clinical phenotype: Action LADA 7. Diabetes Care. 2013;36(4):908-13.
284. Riddle MC, Philipson LH, Rich SS, Carlsson A, Franks PW, Greeley SAW, et al. Monogenic Diabetes: From Genetic Insights to Population-Based Precision in Care. Reflections From a Diabetes Care Editors' Expert Forum. Diabetes Care. 2020;43(12):3117-28.
285. Bonnefond A, Unnikrishnan R, Doria A, Vaxillaire M, Kulkarni RN, Mohan V, et al. Monogenic diabetes. Nature Reviews Disease Primers. 2023;9(1):12.
286. Yang Y, Chan L. Monogenic Diabetes: What It Teaches Us on the Common Forms of Type 1 and Type 2 Diabetes. Endocrine Reviews. 2016;37(3):190-222.
287. Lemelman MB, Letourneau L, Greeley SAW. Neonatal Diabetes Mellitus: An Update on Diagnosis and Management. Clin Perinatol. 2018;45(1):41-59.

288. Murphy R, Ellard S, Hattersley AT. Clinical implications of a molecular genetic classification of monogenic β-cell diabetes. Nature Clinical Practice Endocrinology & Metabolism. 2008;4(4):200-13.

289. Plows JF, Stanley JL, Baker PN, Reynolds CM, Vickers MH. The Pathophysiology of Gestational Diabetes Mellitus. Int J Mol Sci. 2018;19(11).

290. Modzelewski R, Stefanowicz-Rutkowska MM, Matuszewski W, Bandurska-Stankiewicz EM. Gestational Diabetes Mellitus-Recent Literature Review. J Clin Med. 2022;11(19).

291. King BC, Blom AM. Non-traditional roles of complement in type 2 diabetes: Metabolism, insulin secretion and homeostasis. Mol Immunol. 2017;84:34-42.

292. Mraz M, Haluzik M. The role of adipose tissue immune cells in obesity and low-grade inflammation. Journal of Endocrinology. 2014;222(3):R113-R27.

293. Hevey R, Pouw RB, Harris C, Ricklin D. Sweet turning bitter: Carbohydrate sensing of complement in host defence and disease. British Journal of Pharmacology. 2021;178(14):2802-22.

294. Østergaard JA, Ruseva MM, Malik TH, Hoffmann-Petersen IT, Pickering MC, Thiel S, et al. Increased Autoreactivity of the Complement-Activating Molecule Mannan-Binding Lectin in a Type 1 Diabetes Model. J Diabetes Res. 2016;2016:1825738.

295. Uesugi N, Sakata N, Nangaku M, Abe M, Horiuchi S, Hisano S, et al. Possible mechanism for medial smooth muscle cell injury in diabetic nephropathy: Glycoxidation-mediated local complement activation. American Journal of Kidney Diseases. 2004;44(2):224-38.

296. Ma D, Luque-Fernandez MA, Bogdanet D, Desoye G, Dunne F, Halperin JA. Plasma Glycated CD59 Predicts Early Gestational Diabetes and Large for Gestational Age Newborns. J Clin Endocrinol Metab. 2020;105(4):e1033-40.

297. Rohm TV, Meier DT, Olefsky JM, Donath MY. Inflammation in obesity, diabetes, and related disorders. Immunity. 2022;55(1):31-55.

298. Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, et al. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. J Clin Invest. 2003;112(12):1821-30.

299. Lo JC, Ljubicic S, Leibiger B, Kern M, Leibiger IB, Moede T, et al. Adipsin is an adipokine that improves β cell function in diabetes. Cell. 2014;158(1):41-53.

300. Choy LN, Rosen BS, Spiegelman BM. Adipsin and an endogenous pathway of complement from adipose cells. Journal of Biological Chemistry. 1992;267(18):12736-41.

301. Mamane Y, Chung Chan C, Lavallee G, Morin N, Xu LJ, Huang J, et al. The C3a anaphylatoxin receptor is a key mediator of insulin resistance and functions by modulating adipose tissue macrophage infiltration and activation. Diabetes. 2009;58(9):2006-17.

302. Hotamisligil GS, Shargill NS, Spiegelman BM. Adipose Expression of Tumor Necrosis Factor-α: Direct Role in Obesity-Linked Insulin Resistance. Science. 1993;259(5091):87-91.

303. Hotamisligil GS, Arner P, Caro JF, Atkinson RL, Spiegelman BM. Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance. J Clin Invest. 1995;95(5):2409-15.
304. Lim J, Iyer A, Suen JY, Seow V, Reid RC, Brown L, et al. C5aR and C3aR antagonists each inhibit diet-induced obesity, metabolic dysfunction, and adipocyte and macrophage signaling. The FASEB Journal. 2013;27(2):822-31.
305. Kalant D, Cain SA, Maslowska M, Sniderman AD, Cianflone K, Monk PN. The Chemoattractant Receptor-like Protein C5L2 Binds the C3a des-Arg77/Acylation-stimulating Protein*. Journal of Biological Chemistry. 2003;278(13):11123-9.
306. Baldo A, Sniderman AD, St-Luce S, Avramoglu RK, Maslowska M, Hoang B, et al. The adipsin-acylation stimulating protein system and regulation of intracellular triglyceride synthesis. J Clin Invest. 1993;92(3):1543-7.
307. Johswich K, Martin M, Thalmann J, Rheinheimer C, Monk PN, Klos A. Ligand Specificity of the Anaphylatoxin C5L2 Receptor and Its Regulation on Myeloid and Epithelial Cell Lines*. Journal of Biological Chemistry. 2006;281(51):39088-95.
308. Li R-X, Chen H-B, Tu K, Zhao S-L, Zhou H, Li S-J, et al. Localized-Statistical Quantification of Human Serum Proteome Associated with Type 2 Diabetes. PLOS ONE. 2008;3(9):e3224.
309. Sjölander J, Westermark GT, Renström E, Blom AM. Islet amyloid polypeptide triggers limited complement activation and binds complement inhibitor C4b-binding protein, which enhances fibril formation. J Biol Chem. 2012;287(14):10824-33.
310. Klegeris A, McGeer PL. Complement activation by islet amyloid polypeptide (IAPP) and α-synuclein 112. Biochemical and Biophysical Research Communications. 2007;357(4):1096-9.
311. Sjölander J, Byman E, Kulak K, Nilsson SC, Zhang E, Krus U, et al. C4b-binding Protein Protects β-Cells from Islet Amyloid Polypeptide-induced Cytotoxicity. J Biol Chem. 2016;291(41):21644-55.
312. Kulak K, Westermark GT, Papac-Milicevic N, Renström E, Blom AM, King BC. The human serum protein C4b-binding protein inhibits pancreatic IAPP-induced inflammasome activation. Diabetologia. 2017;60(8):1522-33.
313. King BC, Kulak K, Krus U, Rosberg R, Golec E, Wozniak K, et al. Complement Component C3 Is Highly Expressed in Human Pancreatic Islets and Prevents β Cell Death via ATG16L1 Interaction and Autophagy Regulation. Cell Metabolism. 2019;29(1):202-10.e6.
314. Kulak K, Kuska K, Colineau L, McKay M, Maziarz K, Slaby J, et al. Intracellular C3 protects β-cells from IL-1β-driven cytotoxicity via interaction with Fyn-related kinase. Proc Natl Acad Sci U S A. 2024;121(8):e2312621121.
315. Rothschild BM, Tanke DH, Helbling M, Martin LD. Epidemiologic study of tumors in dinosaurs. Naturwissenschaften. 2003;90(11):495-500.
316. Faguet GB. A brief history of cancer: Age-old milestones underlying our current knowledge database. International Journal of Cancer. 2015;136(9):2022-36.
317. Panegyres K. The story of how cancer got its name. Cancer. 2024;130(20):3401-3.

318. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA: A Cancer Journal for Clinicians. 2021;71(3):209-49.
319. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell. 2000;100(1):57-70.
320. Brown JS, Amend SR, Austin RH, Gatenby RA, Hammarlund EU, Pienta KJ. Updating the Definition of Cancer. Mol Cancer Res. 2023;21(11):1142-7.
321. Jassim A, Rahrmann EP, Simons BD, Gilbertson RJ. Cancers make their own luck: theories of cancer origins. Nature Reviews Cancer. 2023;23(10):710-24.
322. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144(5):646-74.
323. Hanahan D. Hallmarks of Cancer: New Dimensions. Cancer Discov. 2022;12(1):31-46.
324. Leroy B, Anderson M, Soussi T. TP53 Mutations in Human Cancer: Database Reassessment and Prospects for the Next Decade. Human Mutation. 2014;35(6):672-88.
325. Colotta F, Allavena P, Sica A, Garlanda C, Mantovani A. Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability. Carcinogenesis. 2009;30(7):1073-81.
326. Talmadge JE, Fidler IJ. AACR centennial series: the biology of cancer metastasis: historical perspective. Cancer Res. 2010;70(14):5649-69.
327. Liberti MV, Locasale JW. The Warburg Effect: How Does it Benefit Cancer Cells? Trends Biochem Sci. 2016;41(3):211-8.
328. Vinay DS, Ryan EP, Pawelec G, Talib WH, Stagg J, Elkord E, et al. Immune evasion in cancer: Mechanistic basis and therapeutic strategies. Seminars in Cancer Biology. 2015;35:S185-S98.
329. Harbeck N, Penault-Llorca F, Cortes J, Gnant M, Houssami N, Poortmans P, et al. Breast cancer. Nature Reviews Disease Primers. 2019;5(1):66.
330. Tabár L, Dean PB, Tucker FL, Yen AM-F, Fann JC-Y, Lin AT-Y, et al. Breast cancers originating from the terminal ductal lobular units: In situ and invasive acinar adenocarcinoma of the breast, AAB. European Journal of Radiology. 2022;152:110323.
331. Łukasiewicz S, Czeczelewski M, Forma A, Baj J, Sitarz R, Stanisławek A. Breast Cancer—Epidemiology, Risk Factors, Classification, Prognostic Markers, and Current Treatment Strategies—An Updated Review. Cancers. 2021;13(17):4287.
332. Kalli S, Semine A, Cohen S, Naber SP, Makim SS, Bahl M. American Joint Committee on Cancer's Staging System for Breast Cancer, Eighth Edition: What the Radiologist Needs to Know. RadioGraphics. 2018;38(7):1921-33.
333. Perou CM, Sørlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, et al. Molecular portraits of human breast tumours. Nature. 2000;406(6797):747-52.
334. Johnson KS, Conant EF, Soo MS. Molecular Subtypes of Breast Cancer: A Review for Breast Radiologists. Journal of Breast Imaging. 2020;3(1):12-24.

335. Dai X, Li T, Bai Z, Yang Y, Liu X, Zhan J, et al. Breast cancer intrinsic subtype classification, clinical use and future trends. Am J Cancer Res. 2015;5(10):2929-43.
336. Riggio AI, Varley KE, Welm AL. The lingering mysteries of metastatic recurrence in breast cancer. British Journal of Cancer. 2021;124(1):13-26.
337. Jin X, Mu P. Targeting Breast Cancer Metastasis. Breast Cancer (Auckl). 2015;9(Suppl 1):23-34.
338. Wieduwilt MJ, Moasser MM. The epidermal growth factor receptor family: Biology driving targeted therapeutics. Cellular and Molecular Life Sciences. 2008;65(10):1566-84.
339. Sigismund S, Avanzato D, Lanzetti L. Emerging functions of the EGFR in cancer. Mol Oncol. 2018;12(1):3-20.
340. Wee P, Wang Z. Epidermal Growth Factor Receptor Cell Proliferation Signaling Pathways. Cancers (Basel). 2017;9(5).
341. Herbst RS. Review of epidermal growth factor receptor biology. International Journal of Radiation Oncology*Biology*Physics. 2004;59(2, Supplement):S21-S6.
342. Masuda H, Zhang D, Bartholomeusz C, Doihara H, Hortobagyi GN, Ueno NT. Role of epidermal growth factor receptor in breast cancer. Breast Cancer Res Treat. 2012;136(2):331-45.
343. Dent R, Trudeau M, Pritchard KI, Hanna WM, Kahn HK, Sawka CA, et al. Triple-Negative Breast Cancer: Clinical Features and Patterns of Recurrence. Clinical Cancer Research. 2007;13(15):4429-34.
344. Salmena L, Carracedo A, Pandolfi PP. Tenets of PTEN Tumor Suppression. Cell. 2008;133(3):403-14.
345. Ma L, Chen Z, Erdjument-Bromage H, Tempst P, Pandolfi PP. Phosphorylation and Functional Inactivation of TSC2 by Erk: Implications for Tuberous Sclerosisand Cancer Pathogenesis. Cell. 2005;121(2):179-93.
346. Elstrom RL, Bauer DE, Buzzai M, Karnauskas R, Harris MH, Plas DR, et al. Akt Stimulates Aerobic Glycolysis in Cancer Cells. Cancer Research. 2004;64(11):3892-9.
347. Wieman HL, Wofford JA, Rathmell JC. Cytokine stimulation promotes glucose uptake via phosphatidylinositol-3 kinase/Akt regulation of Glut1 activity and trafficking. Mol Biol Cell. 2007;18(4):1437-46.
348. Okano J-i, Gaslightwala I, Birnbaum MJ, Rustgi AK, Nakagawa H. Akt/Protein Kinase B Isoforms Are Differentially Regulated by Epidermal Growth Factor Stimulation*. Journal of Biological Chemistry. 2000;275(40):30934-42.
349. Zhou BP, Hu MCT, Miller SA, Yu Z, Xia W, Lin S-Y, et al. HER-2/neu Blocks Tumor Necrosis Factor-induced Apoptosis via the Akt/NF-κB Pathway*. Journal of Biological Chemistry. 2000;275(11):8027-31.
350. Henriksen L, Grandal MV, Knudsen SL, van Deurs B, Grøvdal LM. Internalization mechanisms of the epidermal growth factor receptor after activation with different ligands. PLoS One. 2013;8(3):e58148.

351. Seshacharyulu P, Ponnusamy MP, Haridas D, Jain M, Ganti AK, Batra SK. Targeting the EGFR signaling pathway in cancer therapy. Expert Opin Ther Targets. 2012;16(1):15-31.
352. Lenferink AE, Pinkas-Kramarski R, van de Poll ML, van Vugt MJ, Klapper LN, Tzahar E, et al. Differential endocytic routing of homo- and hetero-dimeric ErbB tyrosine kinases confers signaling superiority to receptor heterodimers. Embo j. 1998;17(12):3385-97.
353. Roepstorff K, Grandal MV, Henriksen L, Knudsen SL, Lerdrup M, Grøvdal L, et al. Differential effects of EGFR ligands on endocytic sorting of the receptor. Traffic. 2009;10(8):1115-27.
354. Threadgill DW, Dlugosz AA, Hansen LA, Tennenbaum T, Lichti U, Yee D, et al. Targeted disruption of mouse EGF receptor: effect of genetic background on mutant phenotype. Science. 1995;269(5221):230-4.
355. Sibilia M, Wagner EF. Strain-dependent epithelial defects in mice lacking the EGF receptor. Science. 1995;269(5221):234-8.
356. Miettinen PJ, Berger JE, Meneses J, Phung Y, Pedersen RA, Werb Z, et al. Epithelial immaturity and multiorgan failure in mice lacking epidermal growth factor receptor. Nature. 1995;376(6538):337-41.
357. Luetteke NC, Phillips HK, Qiu TH, Copeland NG, Earp HS, Jenkins NA, et al. The mouse waved-2 phenotype results from a point mutation in the EGF receptor tyrosine kinase. Genes Dev. 1994;8(4):399-413.
358. Ewald JA, Wilkinson JC, Guyer CA, Staros JV. Ligand- and kinase activity-independent cell survival mediated by the epidermal growth factor receptor expressed in 32D cells. Experimental Cell Research. 2003;282(2):121-31.
359. Coker KJ, Staros JV, Guyer CA. A kinase-negative epidermal growth factor receptor that retains the capacity to stimulate DNA synthesis. Proc Natl Acad Sci U S A. 1994;91(15):6967-71.
360. Eldredge ER, Korf GM, Christensen TA, Connolly DC, Getz MJ, Maihle NJ. Activation of c-fos gene expression by a kinase-deficient epidermal growth factor receptor. Mol Cell Biol. 1994;14(11):7527-34.
361. Weihua Z, Tsan R, Huang WC, Wu Q, Chiu CH, Fidler IJ, et al. Survival of cancer cells is maintained by EGFR independent of its kinase activity. Cancer Cell. 2008;13(5):385-93.
362. Tan X, Lambert PF, Rapraeger AC, Anderson RA. Stress-Induced EGFR Trafficking: Mechanisms, Functions, and Therapeutic Implications. Trends Cell Biol. 2016;26(5):352-66.
363. Pio R, Corrales L, Lambris JD. The role of complement in tumor growth. Adv Exp Med Biol. 2014;772:229-62.
364. Berraondo P, Minute L, Ajona D, Corrales L, Melero I, Pio R. Innate immune mediators in cancer: between defense and resistance. Immunological Reviews. 2016;274(1):290-306.
365. Markiewski MM, DeAngelis RA, Benencia F, Ricklin-Lichtsteiner SK, Koutoulaki A, Gerard C, et al. Modulation of the antitumor immune response by complement. Nat Immunol. 2008;9(11):1225-35.

366. Vlaicu SI, Tegla CA, Cudrici CD, Danoff J, Madani H, Sugarman A, et al. Role of C5b-9 complement complex and response gene to complement-32 (RGC-32) in cancer. Immunologic Research. 2013;56(1):109-21.

367. Tegla CA, Cudrici C, Patel S, Trippe R, 3rd, Rus V, Niculescu F, et al. Membrane attack by complement: the assembly and biology of terminal complement complexes. Immunol Res. 2011;51(1):45-60.

368. Roumenina LT, Daugan MV, Noé R, Petitprez F, Vano YA, Sanchez-Salas R, et al. Tumor Cells Hijack Macrophage-Produced Complement C1q to Promote Tumor Growth. Cancer Immunology Research. 2019;7(7):1091-105.

369. Cho MS, Vasquez HG, Rupaimoole R, Pradeep S, Wu S, Zand B, et al. Autocrine effects of tumor-derived complement. Cell Rep. 2014;6(6):1085-95.

370. Nabizadeh JA, Manthey HD, Steyn FJ, Chen W, Widiapradja A, Md Akhir FN, et al. The Complement C3a Receptor Contributes to Melanoma Tumorigenesis by Inhibiting Neutrophil and CD4+ T Cell Responses. The Journal of Immunology. 2016;196(11):4783-92.

371. Lalli PN, Strainic MG, Yang M, Lin F, Medof ME, Heeger PS. Locally produced C5a binds to T cell-expressed C5aR to enhance effector T-cell expansion by limiting antigen-induced apoptosis. Blood. 2008;112(5):1759-66.

372. Okroj M, Hsu Y-F, Ajona D, Pio R, Blom AM. Non-small cell lung cancer cells produce a functional set of complement factor I and its soluble cofactors. Molecular Immunology. 2008;45(1):169-79.

373. Riihilä P, Nissinen L, Farshchian M, Kivisaari A, Ala-aho R, Kallajoki M, et al. Complement Factor I Promotes Progression of Cutaneous Squamous Cell Carcinoma. Journal of Investigative Dermatology. 2015;135(2):579-88.

374. Riihilä P, Nissinen L, Farshchian M, Kallajoki M, Kivisaari A, Meri S, et al. Complement Component C3 and Complement Factor B Promote Growth of Cutaneous Squamous Cell Carcinoma. The American Journal of Pathology. 2017;187(5):1186-97.

375. Bulla R, Tripodo C, Rami D, Ling GS, Agostinis C, Guarnotta C, et al. C1q acts in the tumour microenvironment as a cancer-promoting factor independently of complement activation. Nature Communications. 2016;7(1):10346.

376. Piao C, Zhang W-M, Li T-T, Zhang C-c, Qiu S, Liu Y, et al. Complement 5a stimulates macrophage polarization and contributes to tumor metastases of colon cancer. Experimental Cell Research. 2018;366(2):127-38.

377. Corrales L, Ajona D, Rafail S, Lasarte JJ, Riezu-Boj JI, Lambris JD, et al. Anaphylatoxin C5a creates a favorable microenvironment for lung cancer progression. J Immunol. 2012;189(9):4674-83.

378. Kwak JW, Laskowski J, Li HY, McSharry MV, Sippel TR, Bullock BL, et al. Complement Activation via a C3a Receptor Pathway Alters CD4(+) T Lymphocytes and Mediates Lung Cancer Progression. Cancer Res. 2018;78(1):143-56.

379. Bandini S, Macagno M, Hysi A, Lanzardo S, Conti L, Bello A, et al. The non-inflammatory role of C1q during Her2/neu-driven mammary carcinogenesis. Oncoimmunology. 2016;5(12):e1253653.

380. Bandini S, Curcio C, Macagno M, Quaglino E, Arigoni M, Lanzardo S, et al. Early onset and enhanced growth of autochthonous mammary carcinomas in C3-deficient Her2/neu transgenic mice. Oncoimmunology. 2013;2(9):e26137.
381. Block I, Müller C, Sdogati D, Pedersen H, List M, Jaskot AM, et al. CFP suppresses breast cancer cell growth by TES-mediated upregulation of the transcription factor DDIT3. Oncogene. 2019;38(23):4560-73.
382. Wang Y, Sun SN, Liu Q, Yu YY, Guo J, Wang K, et al. Autocrine Complement Inhibits IL10-Dependent T-cell-Mediated Antitumor Immunity to Promote Tumor Progression. Cancer Discov. 2016;6(9):1022-35.
383. Klionsky DJ. Autophagy revisited: A conversation with Christian de Duve. Autophagy. 2008;4(6):740-3.
384. De Duve C, Pressman BC, Gianetto R, Wattiaux R, Appelmans F. Tissue fractionation studies. 6. Intracellular distribution patterns of enzymes in rat-liver tissue. Biochem J. 1955;60(4):604-17.
385. Tsukada M, Ohsumi Y. Isolation and characterization of autophagy-defective mutants of Saccharomyces cerevisiae. FEBS Letters. 1993;333(1-2):169-74.
386. Lamark T, Johansen T. Mechanisms of Selective Autophagy. Annual Review of Cell and Developmental Biology. 2021;37(Volume 37, 2021):143-69.
387. Yorimitsu T, Klionsky DJ. Autophagy: molecular machinery for self-eating. Cell Death & Differentiation. 2005;12(2):1542-52.
388. Anding AL, Baehrecke EH. Cleaning House: Selective Autophagy of Organelles. Dev Cell. 2017;41(1):10-22.
389. Levine B, Kroemer G. Autophagy in the Pathogenesis of Disease. Cell. 2008;132(1):27-42.
390. Hurley JH, Young LN. Mechanisms of Autophagy Initiation. Annu Rev Biochem. 2017;86:225-44.
391. Glick D, Barth S, Macleod KF. Autophagy: cellular and molecular mechanisms. J Pathol. 2010;221(1):3-12.
392. Lamb CA, Yoshimori T, Tooze SA. The autophagosome: origins unknown, biogenesis complex. Nature Reviews Molecular Cell Biology. 2013;14(12):759-74.
393. Kroemer G, Mariño G, Levine B. Autophagy and the integrated stress response. Mol Cell. 2010;40(2):280-93.
394. Liu S, Yao S, Yang H, Liu S, Wang Y. Autophagy: Regulator of cell death. Cell Death & Disease. 2023;14(10):648.
395. Kaushik S, Cuervo AM. The coming of age of chaperone-mediated autophagy. Nature Reviews Molecular Cell Biology. 2018;19(6):365-81.
396. Schuck S. Microautophagy – distinct molecular mechanisms handle cargoes of many sizes. Journal of Cell Science. 2020;133(17).
397. Dikic I, Elazar Z. Mechanism and medical implications of mammalian autophagy. Nature Reviews Molecular Cell Biology. 2018;19(6):349-64.
398. Holzer E, Martens S, Tulli S. The Role of ATG9 Vesicles in Autophagosome Biogenesis. Journal of Molecular Biology. 2024;436(15):168489.

399. Fader CM, Colombo MI. Autophagy and multivesicular bodies: two closely related partners. Cell Death & Differentiation. 2009;16(1):70-8.
400. Yang Z, Klionsky DJ. An overview of the molecular mechanism of autophagy. Curr Top Microbiol Immunol. 2009;335:1-32.
401. Nakatogawa H. Mechanisms governing autophagosome biogenesis. Nature Reviews Molecular Cell Biology. 2020;21(8):439-58.
402. Schütter M, Giavalisco P, Brodesser S, Graef M. Local Fatty Acid Channeling into Phospholipid Synthesis Drives Phagophore Expansion during Autophagy. Cell. 2020;180(1):135-49.e14.
403. Nishimura T, Tamura N, Kono N, Shimanaka Y, Arai H, Yamamoto H, et al. Autophagosome formation is initiated at phosphatidylinositol synthase-enriched ER subdomains. Embo j. 2017;36(12):1719-35.
404. Abada A, Elazar Z. Getting ready for building: signaling and autophagosome biogenesis. EMBO Rep. 2014;15(8):839-52.
405. Karanasios E, Stapleton E, Manifava M, Kaizuka T, Mizushima N, Walker SA, et al. Dynamic association of the ULK1 complex with omegasomes during autophagy induction. Journal of Cell Science. 2013;126(22):5224-38.
406. Shackelford DB, Shaw RJ. The LKB1–AMPK pathway: metabolism and growth control in tumour suppression. Nature Reviews Cancer. 2009;9(8):563-75.
407. Kim J, Kundu M, Viollet B, Guan K-L. AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. Nature Cell Biology. 2011;13(2):132-41.
408. Aita VM, Liang XH, Murty VVVS, Pincus DL, Yu W, Cayanis E, et al. Cloning and Genomic Organization of Beclin 1, a Candidate Tumor Suppressor Gene on Chromosome 17q21. Genomics. 1999;59(1):59-65.
409. Liang XH, Jackson S, Seaman M, Brown K, Kempkes B, Hibshoosh H, et al. Induction of autophagy and inhibition of tumorigenesis by beclin 1. Nature. 1999;402(6762):672-6.
410. Yue Z, Jin S, Yang C, Levine AJ, Heintz N. Beclin 1, an autophagy gene essential for early embryonic development, is a haploinsufficient tumor suppressor. Proc Natl Acad Sci U S A. 2003;100(25):15077-82.
411. Qu X, Yu J, Bhagat G, Furuya N, Hibshoosh H, Troxel A, et al. Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. J Clin Invest. 2003;112(12):1809-20.
412. Laddha SV, Ganesan S, Chan CS, White E. Mutational landscape of the essential autophagy gene BECN1 in human cancers. Mol Cancer Res. 2014;12(4):485-90.
413. Takamura A, Komatsu M, Hara T, Sakamoto A, Kishi C, Waguri S, et al. Autophagy-deficient mice develop multiple liver tumors. Genes Dev. 2011;25(8):795-800.
414. Menon S, Manning BD. Common corruption of the mTOR signaling network in human tumors. Oncogene. 2008;27 Suppl 2(0 2):S43-51.
415. Verma AK, Bharti PS, Rafat S, Bhatt D, Goyal Y, Pandey KK, et al. Autophagy Paradox of Cancer: Role, Regulation, and Duality. Oxidative Medicine and Cellular Longevity. 2021;2021(1):8832541.
416. White E. Autophagy and p53. Cold Spring Harb Perspect Med. 2016;6(4):a026120.

417. Tasdemir E, Maiuri MC, Galluzzi L, Vitale I, Djavaheri-Mergny M, D'Amelio M, et al. Regulation of autophagy by cytoplasmic p53. Nature Cell Biology. 2008;10(6):676-87.
418. White E, DiPaola RS. The double-edged sword of autophagy modulation in cancer. Clin Cancer Res. 2009;15(17):5308-16.
419. Debnath J, Gammoh N, Ryan KM. Autophagy and autophagy-related pathways in cancer. Nature Reviews Molecular Cell Biology. 2023;24(8):560-75.
420. Mathew R, Kongara S, Beaudoin B, Karp CM, Bray K, Degenhardt K, et al. Autophagy suppresses tumor progression by limiting chromosomal instability. Genes Dev. 2007;21(11):1367-81.
421. Degenhardt K, Mathew R, Beaudoin B, Bray K, Anderson D, Chen G, et al. Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis. Cancer Cell. 2006;10(1):51-64.
422. Yamamoto K, Venida A, Yano J, Biancur DE, Kakiuchi M, Gupta S, et al. Autophagy promotes immune evasion of pancreatic cancer by degrading MHC-I. Nature. 2020;581(7806):100-5.
423. Marsh T, Tolani B, Debnath J. The pleiotropic functions of autophagy in metastasis. J Cell Sci. 2021;134(2).
424. Wang RC, Wei Y, An Z, Zou Z, Xiao G, Bhagat G, et al. Akt-mediated regulation of autophagy and tumorigenesis through Beclin 1 phosphorylation. Science. 2012;338(6109):956-9.
425. Wei Y, Zou Z, Becker N, Anderson M, Sumpter R, Xiao G, et al. EGFR-mediated Beclin 1 phosphorylation in autophagy suppression, tumor progression, and tumor chemoresistance. Cell. 2013;154(6):1269-84.
426. Tan X, Thapa N, Sun Y, Anderson RA. A kinase-independent role for EGF receptor in autophagy initiation. Cell. 2015;160(1-2):145-60.
427. Li X, Fan Z. The epidermal growth factor receptor antibody cetuximab induces autophagy in cancer cells by downregulating HIF-1alpha and Bcl-2 and activating the beclin 1/hVps34 complex. Cancer Res. 2010;70(14):5942-52.
428. Zou Y, Ling YH, Sironi J, Schwartz EL, Perez-Soler R, Piperdi B. The autophagy inhibitor chloroquine overcomes the innate resistance of wild-type EGFR non-small-cell lung cancer cells to erlotinib. J Thorac Oncol. 2013;8(6):693-702.
429. Sobhakumari A, Schickling BM, Love-Homan L, Raeburn A, Fletcher EV, Case AJ, et al. NOX4 mediates cytoprotective autophagy induced by the EGFR inhibitor erlotinib in head and neck cancer cells. Toxicol Appl Pharmacol. 2013;272(3):736-45.
430. Li Y-y, Lam S-k, Mak JC-w, Zheng C-y, Ho JC-m. Erlotinib-induced autophagy in epidermal growth factor receptor mutated non-small cell lung cancer. Lung Cancer. 2013;81(3):354-61.
431. Han W, Pan H, Chen Y, Sun J, Wang Y, Li J, et al. EGFR tyrosine kinase inhibitors activate autophagy as a cytoprotective response in human lung cancer cells. PLoS One. 2011;6(6):e18691.

432. Dragowska WH, Weppler SA, Wang JC, Wong LY, Kapanen AI, Rawji JS, et al. Induction of autophagy is an early response to gefitinib and a potential therapeutic target in breast cancer. PLoS One. 2013;8(10):e76503.
433. Hohmeier HE, Mulder H, Chen G, Henkel-Rieger R, Prentki M, Newgard CB. Isolation of INS-1-derived cell lines with robust ATP-sensitive K+ channel-dependent and -independent glucose-stimulated insulin secretion. Diabetes. 2000;49(3):424-30.
434. Asfari M, Janjic D, Meda P, Li G, Halban PA, Wollheim CB. Establishment of 2-mercaptoethanol-dependent differentiated insulin-secreting cell lines. Endocrinology. 1992;130(1):167-78.
435. Miyazaki JI, Araki K, Yamato E, Ikegami H, Asano T, Shibasaki Y, et al. Establishment of a Pancreatic Beta-Cell Line That Retains Glucose-Inducible Insulin-Secretion - Special Reference to Expression of Glucose Transporter Isoforms. Endocrinology. 1990;127(1):126-32.
436. Ahuja D, Sáenz-Robles MT, Pipas JM. SV40 large T antigen targets multiple cellular pathways to elicit cellular transformation. Oncogene. 2005;24(52):7729-45.
437. Cheng K, Delghingaro-Augusto V, Nolan CJ, Turner N, Hallahan N, Andrikopoulos S, et al. High Passage MIN6 Cells Have Impaired Insulin Secretion with Impaired Glucose and Lipid Oxidation. PLOS ONE. 2012;7(7):e40868.
438. Ravassard P, Hazhouz Y, Pechberty S, Bricout-Neveu E, Armanet M, Czernichow P, et al. A genetically engineered human pancreatic β cell line exhibiting glucose-inducible insulin secretion. J Clin Invest. 2011;121(9):3589-97.
439. Lasfargues EY, Ozzello L. Cultivation of Human Breast Carcinomas2. JNCI: Journal of the National Cancer Institute. 1958;21(6):1131-47.
440. Chavez KJ, Garimella SV, Lipkowitz S. Triple negative breast cancer cell lines: one tool in the search for better treatment of triple negative breast cancer. Breast Dis. 2010;32(1-2):35-48.
441. Holliday DL, Speirs V. Choosing the right cell line for breast cancer research. Breast Cancer Research. 2011;13(4):215.
442. Kao J, Salari K, Bocanegra M, Choi Y-L, Girard L, Gandhi J, et al. Molecular Profiling of Breast Cancer Cell Lines Defines Relevant Tumor Models and Provides a Resource for Cancer Gene Discovery. PLOS ONE. 2009;4(7):e6146.
443. Neve RM, Chin K, Fridlyand J, Yeh J, Baehner FL, Fevr T, et al. A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. Cancer Cell. 2006;10(6):515-27.
444. Hollestelle A, Nagel JHA, Smid M, Lam S, Elstrodt F, Wasielewski M, et al. Distinct gene mutation profiles among luminal-type and basal-type breast cancer cell lines. Breast Cancer Research and Treatment. 2010;121(1):53-64.
445. Lebeau J, Goubin G. Amplification of the epidermal growth factor receptor gene in the BT20 breast carcinoma cell line. International Journal of Cancer. 1987;40(2):189-91.
446. Reinhardt K, Stückrath K, Hartung C, Kaufhold S, Uleer C, Hanf V, et al. PIK3CA-mutations in breast cancer. Breast Cancer Res Treat. 2022;196(3):483-93.
447. Cailleau R, Olivé M, Cruciger QVJ. Long-term human breast carcinoma cell lines of metastatic origin: Preliminary characterization. In Vitro. 1978;14(11):911-5.

448. Filmus J, Pollak MN, Cailleau R, Buick RN. MDA-468, a human breast cancer cell line with a high number of epidermal growth factor (EGF) receptors, has an amplified EGF receptor gene and is growth inhibited by EGF. Biochemical and Biophysical Research Communications. 1985;128(2):898-905.
449. Pulaski BA, Ostrand-Rosenberg S. Mouse 4T1 Breast Tumor Model. Current Protocols in Immunology. 2000;39(1):20.2.1-.2.16.
450. Dexter DL, Kowalski HM, Blazar BA, Fligiel Z, Vogel R, Heppner GH. Heterogeneity of tumor cells from a single mouse mammary tumor. Cancer Res. 1978;38(10):3174-81.
451. Hager JC, Russo J, Ceriani RL, Peterson JA, Fligiel S, Jolly G, et al. Epithelial characteristics of five subpopulations of a heterogeneous strain BALB/cfC3H mouse mammary tumor. Cancer Res. 1981;41(5):1720-30.
452. Miller BE, Miller FR, Heppner GH. Interactions between tumor subpopulations affecting their sensitivity to the antineoplastic agents cyclophosphamide and methotrexate. Cancer Res. 1981;41(11 Pt 1):4378-81.
453. Heppner GH, Miller FR, Shekhar PM. Nontransgenic models of breast cancer. Breast Cancer Res. 2000;2(5):331-4.
454. Olson B, Li Y, Lin Y, Liu ET, Patnaik A. Mouse Models for Cancer Immunotherapy Research. Cancer Discov. 2018;8(11):1358-65.
455. Chulpanova DS, Kitaeva KV, Rutland CS, Rizvanov AA, Solovyeva VV. Mouse Tumor Models for Advanced Cancer Immunotherapy. Int J Mol Sci. 2020;21(11).
456. Qin X, Hu W, Song W, Grubissich L, Hu X, Wu G, et al. Generation and phenotyping of mCd59a and mCd59b double-knockout mice. Am J Hematol. 2009;84(2):65-70.
457. Krus U, King Ben C, Nagaraj V, Gandasi Nikhil R, Sjölander J, Buda P, et al. The Complement Inhibitor CD59 Regulates Insulin Secretion by Modulating Exocytotic Events. Cell Metabolism. 2014;19(5):883-90.
458. Boshra H, Zelek WM, Hughes TR, Rodriguez de Cordoba S, Morgan BP. Absence of CD59 in Guinea Pigs: Analysis of the Cavia porcellus Genome Suggests the Evolution of a CD59 Pseudogene. J Immunol. 2018;200(1):327-35.

All the figures in this thesis were created using BioRender.com

Printed by Media-Tryck, Lund 2025 NORDIC SWAN ECOLABEL 3041 0903

LUND UNIVERSITY

FACULTY OF MEDICINE

Division of Medical Protein Chemistry
Department of Translational Medicine

Lund University, Faculty of Medicine
Doctoral Dissertation Series 2025:19
ISBN 978-91-8021-672-2
ISSN 1652-8220