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Investigating the Essential Extracellular Invadolysin Metalloprotease

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A thesis submission for the Degree of Doctor of Philosophy

The University of Edinburgh

2023

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Abstract

The invadolysin gene was first identified in the Heck laboratory from the study of *Drosophila melanogaster* mutations that affected chromosome structure and mitotic progression. Invadolysin mutations result in abnormal chromosome condensation and are lethal during the late larval stage. As a novel conserved zinc-metalloprotease, invadolysin is the first protease demonstrated to localise to the surface of lipid droplets in mammalian cultured cells. Mutations of invadolysin influence the development of *Drosophila* larvae with a decreased triglyceride:protein ratio, which suggests a role for invadolysin in lipid storage and metabolism. In addition, invadolysin has been shown to play an important role in insulin signalling and adipogenesis.

As invadolysin is essential for life, it is crucial to explore the biological function of invadolysin in maintaining normal physiology. *In silico* sequence analysis revealed a potential N-terminal signal sequence in invadolysin, which suggests a secreted form(s) of invadolysin. Recent research published by the Heck laboratory characterized an extracellular form of invadolysin in the soluble fractions of vertebrate blood. Furthermore, analysis of a secreted form of invadolysin in invertebrate haemolymph suggested that invadolysin is sexual dimorphic in adult *Drosophila*. To optimize the proteomic research technology, I have developed strategies to enrich invadolysin from human blood fractions for further biochemical characterisation of extracellular invadolysin. My results illustrated that the protein expression form of invadolysin differs between human serum and plasma. I have found no evidence suggesting sexually dimorphic forms of invadolysin existing in vertebrates. During early developmental stages of zebrafish (*Danio rerio*), invadolysin shows dynamic structural changes in yolk and yolk-depleted embryo samples, which suggests a potential role of invadolysin in vertebrate early development. To further explore the potential physiological role(s) of invadolysin, the NAFLD (Non-Alcoholic Fatty Liver Disease) model has been utilized for investigation. Preliminary results

showed that the structural forms of invadolysin in plasma are different between non-NAFLD controls and patients with NAFLD. The *in vitro* hepatic cellular steatosis model and other metabolic associated conditions were used to investigate these differences. The expression form of invadolysin in patients with lung inflammatory diseases, such as idiopathic pulmonary fibrosis (IPF) and coronavirus (COVID-19), have been determined to identify a potential physiological and pathological role of extracellular invadolysin. In the long-term, this project aims to explain whether the extracellular form of invadolysin plays a role in normal physiology and/or serves as a potential biomarker for human diseases.

Lay Summary

Drosophila melanogaster, also known as fruit fly, has been widely used as an animal model to investigate the function of human genes. This organism, which is a popular research model, shares more than 60% of genes with humans, and 75% of genes associated with human genetic diseases can be found in *Drosophila*. Genetic mutations introduced in fruit flies can be recognized by morphology, such as eye colour and body size, which renders mutations visible in adult and larval flies. The Heck laboratory discovered an invadolysin mutation in larval brain tissue, which influences the growth of fruit flies during the early developmental stage. Without functional invadolysin, the fruit fly larva accumulates less body fat, and the development will stop at larval stage. Thus, invadolysin is essential for life. Invadolysin is a conserved protease, which has also been identified in zebrafish, mouse, pig and human. However, the protease function of invadolysin is still not well known. What is the key to switch on and off the action of this protease? Does invadolysin also influence the early development of humans? Does invadolysin have an effect on fat accumulation in humans? Would it be a potential treatment target for metabolic associated diseases, such as obesity and diabetes? Considering these questions, it is important to understand the function of invadolysin protease in humans.

Recently, the Heck laboratory discovered a novel form of invadolysin in human blood. Currently, blood testing is an important diagnostic tool to reveal human health conditions. Studying the function of human blood invadolysin will provide an opportunity to determine whether invadolysin can serve as a diagnostic indicator. For this purpose, I first validated the existence of invadolysin in human blood by separating invadolysin from other proteins in blood using a commercial kit. In addition, I utilized the zebrafish animal model to evaluate invadolysin in zebrafish blood and explore whether invadolysin has an effect on the development of zebrafish embryos. I have found that invadolysin structure is the same in male and female zebrafish blood. Interestingly, it has been shown that invadolysin has different

expression forms in zebrafish yolk and embryo, which suggests a potential role of invadolysin during early developmental stages. Following this, I have developed a biochemical method to improve the methodology of invadolysin extraction and characterization. Compared to the kit protocol, this optimized approach is more flexible and informative, which could assist with the isolation and detection of invadolysin in different biological materials. Moreover, I have discovered that invadolysin structure is different between human serum and plasma. I did not find any evidence to show invadolysin differs between male and female human blood. Lastly, I aimed to decide whether invadolysin is involved in any metabolic related human diseases. Therefore, I tested invadolysin in patients who have Non-Alcoholic Fatty Liver Disease (NAFLD). People with this disease accumulate more fat in liver, and often have other health issues, such as diabetes and heart disease. I have observed that invadolysin protease in NAFLD patients' blood is different from non-NAFLD volunteers' blood. To explain this difference, treating a human liver cell line with a high fat and high sugar diet to mimic the main cause of NAFLD in humans was used as a strategy. In addition, diverse lung inflammatory diseases, such as idiopathic pulmonary fibrosis (IPF) and coronavirus (COVID-19), were selected as clinical models to further explore whether invadolysin in blood is a sign of any inflammatory symptoms. Taken together, my research work established a biochemical approach to investigate a novel form of invadolysin in human blood. This circulating form of invadolysin provides an opportunity to understand its role(s) in metabolism, and as a potential diagnostic tool in the future.

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List of Abbreviations

aa	amino acid
Ab	Antibody
ADAM	Disintegrin-Like Metalloprotease
ADMA	Asymmetric Dimethylarginine
ADRP	Adipose Differentiation-Related Protein
Ag	Antigen
Alt	Alternative
ALT	Alanine Aminotransferase
AMPK	Adenosine Monophosphate (AMP)-activated protein Kinase
ARDS	Adult Respiratory Distress Syndrome
ARF	Acute Renal Failure
ATP	Adenosine triphosphate
BALF	Bronchoalveolar Lavage Fluid
BMI	Body Mass Index
BAT	Brown Adipose Tissue
CatK	Cathepsin K
CL	Cross Linker
COVID-19	Coronavirus Disease
CRISP-Cas9	Clustered Regularly Interspaced Short Palindromic Repeats and CRISP-associated protein 9
C-terminus	Carboxyl terminus
CF	Cystic Fibrosis
C3A	clonal derivative of hepatoma cells Hep G2
D	Aspartic acid
Dm	<i>Drosophila melanogaster</i>
DMP	Dimethyl Pimelimidate
DNL	<i>de novo</i> lipogenesis
DPBS	Dulbecco's Phosphate Buffered Saline
Dr	<i>Danio rerio</i>

DTT	Dithiothreitol
E	Glutamic acid
ECL	Enhanced Chemiluminescent
ECM	Extracellular Matrix
EDTA	Ethylenediamine-N,N,N',N'-tetraacetic acid
ER	Endoplasmic Reticulum
EV	Extracellular Vesicle
FBS	Fetal Bovine Serum
FEV1	Forced Expiratory Volume
FN	Fibronectin
FVC	Forced Vital Capacity
G	Glycine
GGT	Gamma-glutamyl transferase
GPI	Glycosylphosphatidylinositol
GPI-AP	Glycosylphosphatidylinositol-anchor protein
GP63	Leishmanolysin
G6P	Glucose-6-phosphate
H	Histidine
HBSS	Hank's Balanced Salt Solution
HeLa	derivate from cervical cancer cells from Henrietta Lacks
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HDL	High Density Lipoprotein
HK	Hexokinase
hpf	hours post-fertilization
HRP	Horseradish Peroxidase
Hs	Homo sapiens
HSC	Hepatic Stellate Cell
H2B	Histone protein
indels	insertion-deletions
IgG	Immunoglobulin

IIP	Idiopathic Interstitial Pneumonia
IL	Interleukin
ILV	Internal intraluminal vesicles
ILD	Interstitial Lung Disease
INV	Invadolysin
IP	Immunoprecipitation
IPF	Idiopathic Pulmonary Fibrosis
IX-14	Invadolysin gene name
<i>I(3)IX-14¹</i>	Late larval mutation of (first allele) IX-14 on the 3 rd chromosome
kDa	kilodalton
LAM	Lymphangioleiomyomatosis
LCMS	Liquid Chromatography Mass Spectrometry
LD	Light Dark
LD	Lipid Droplet
LMLN	Leishmanolysin Like Peptidase
LPC	Lysophosphatidylcholine
LPON	Lactate; Pyruvate; Octanoate; Ammonia
miR	MicroRNA
mRNA	messenger RNA
MAPK	Mitogen Activated Protein Kinase
MB	Methylthioninium Blue
MEME	Minimum Essential Medium Eagle
MEP	Metalloprotease
MM	Multiple Myeloma
MMP	Matrix Metalloprotease
MOPS	3-(N-morpholino) propansulfonic acid
MSP	Major Surface Protease
MS-222	Tricaine Methanesulfonate
MV	Microvesicle
MVB	Multivesicular bodies

NAFLD	Non-Alcoholic Fatty Liver Disease
NASH	Non-Alcoholic steatohepatitis
NE	Neutrophil Elastase
NEWS	National Early Warning Score
NHS	National Health Service
NMD	Nonsense-mediated mRNA
N-glycosylation	Amide nitrogen of an asparagine residue-glycosylation
N-terminus	Amino terminus
NK	Natural Killer
O-glycosylation	Oxygen atom of serine or threonine residue-glycosylation
PBS	Phosphate-Buffered Saline
PEB	Protein Extraction Buffer
PEG	Polyethylene Glycol Precipitation
RNA	Ribonucleic Acid
RIPA	Radioimmunoprecipitation Assay
ROS	Reactive Oxygen Species
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
S	Serine
SAGA	Spt-Ada-Gcn5-acetyltransferase
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2
SDMA	Symmetric Dimethylarginine
SDS-PAGE	Sodium Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis
SGLT-2i	Sodium Glucose Cotransporter-2 inhibitor
SRP	Signal Recognition Particle
PC	Proprotein Convertase
PCO	Posterior Capsular Opacification
T	Threonine
TEA	Triethanolamine
TIP47	Tail-interacting protein of 47 kDa

TPM	Transcript per Million
TRAIL	Tumor necrosis factor-Related Apoptosis-Inducing Ligand
Tris	Tris[hydroxymethyl]aminomethane
TNF- α	Tumor Necrosis Factor- α
Triton X-100	Octylphenol ethoxylane
Tween-20	Polyoxyethylene-sorbitan monolaurate
T2B	Type 2 Diabetes
T25	25 cm ² Tissue culture flask
T75	75 cm ² Tissue culture flask
U/L	Units per Litre
VDAC	Voltage-Dependent Anion Channel
X	Any amino acid
YD	Yolk-depleted
YSL	Yolk Syncytial Layer
ZP	Zona Pellucida

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Chapter 1 Introduction

1.1 Overview of protease

Proteases interact and co-function with each other to communicate biological information, which forms a dynamic 'protease web' (Overall & Dean, 2006). They are involved in upregulation of cellular growth and angiogenesis in cancers, and thus may have potential as drug targets in cancer treatment. Proteases are essential for living organisms and have been found in bacteria, prokaryotes, viruses, plants and animals (Rawlings & Barrett, 1993). It has been identified, through analysis of the human genome sequence, that approximately 2% of human genes encode proteases (Craik et al., 2011). Proteases are distinguished into different groups based on the amino acid residue(s) responsible for the catalytic event, such as serine proteases, cysteine proteases, aspartic proteases, threonine proteases, and metalloproteases (Gomis-Rüth, 2003; Mótyán et al., 2013; Rawlings & Barrett, 1993) (Figure 1.1). Proteolytic enzymes play important roles in a number of physiological functions, including the blood-clotting cascade and signal transduction (Mótyán et al., 2013; Neurath & Walsh, K. A., 1976). They are also involved in upregulation of cellular growth and angiogenesis in cancers; hence, it has been proposed that proteases may be ideal drug targets in cancer treatment (Overall & Dean, 2006).

Proteases are a wide range of hydrolytic enzymes, from single-domain catalytic molecules to complex multi-functional macromolecules (Boon et al., 2020). Hydrolytic enzymes or hydrolyses, catalyse various chemical bonds within different groups of biomolecules, such as peptides, esters and glycosides (Prabha et al., 2013). Hydrolytic enzymes 'digest' proteins, lipid and fat molecules into simple fragments by speeding up the hydrolysis of corresponding chemical bonds (Prabha et al., 2013; Boon et al., 2020). Before converting to physiological functional forms, proteases are synthesized as inactive precursors (zymogens) (Neurath & Walsh, K. A., 1976). Regulation of proteases are subject to several mechanisms, such as gene expression, transcription, translation and cellular localization (Turk et al.

2012). Besides above functional controls, numerous proteases are produced as 'zymogens' initially, which require further activation to be able to cleave substrate proteins (Boon et al., 2020). Through the cleavage of selective peptide bonds, zymogens become irreversibly physiologically active. Activation of the zymogen triggers the proteolysis pathway, and the presence of interacting proteins, such as cleavage products and inhibitors, increases the complexity of the proteolytic system (Brix et al., 2013; Overall & Dean, 2006). Non-catalytic domains and catalytic domains of proteases are both important for the modulation of enzymatic function, such as providing a guide for enzymatic cellular localization and facilitating interactions with other proteins (López-Bascón et al., 2016). The complexity of the protease web is further amplified by post-translational modifications, such as glycosylation and phosphorylation.

Proteolytic enzymes are classified into three different hierarchical groups, which are 'clan', 'family' and 'peptidase' (Merops n.d.; Rawlings & Barrett, 1993; Rawlings et al., 2018). Clan is a group of families in which all the peptidases evolved from a common ancestor. Family represents peptidases that have homologous amino acid sequences. Peptidase indicates an individual peptidase that is different from others based on genetic, structural, and activity features. Depending on the catalytic properties of different amino acid sequences, proteases are catalogued into nine families, which are serine protease, cysteine protease, aspartic protease, threonine protease, glutamic protease, mixed protease, asparagine protease, and metalloprotease (eg. invadolysin) (Merops n.d.; Hartley, 1960; Rawlings & Barrett, 1993). Proteases that do not belong to the above families are characterized as unknown proteases (eg. aminopeptidase).

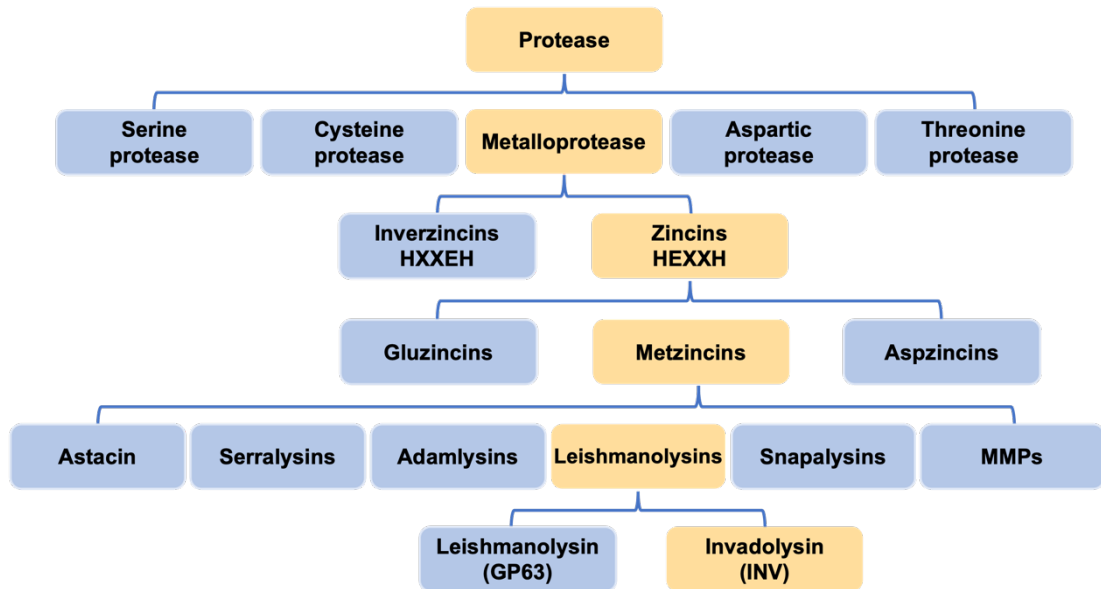


Figure 1.1 Invadolysin in the protease family.

Schematic representing the categories of protease and members from each subgroup. Homologous to leishmanolysin, invadolysin is a metalloprotease, which belongs to the metzincin family with the HEXXH conserved motif. H: histidine; E: glutamic acid; X: any amino acids.

1.2 Introduction to Metalloprotease

Metalloproteases (MEPs) are hydrolytic enzymes, which contribute to diverse physiological responses and activities, including modulation of signal transduction pathways and control of cellular communication (Gomis-Rüth, 2003; Thomas, N. V. & Kim, S.-K., 2010). As described, they are categorized into inverzincins and zincins, with the zincins are subdivided into gluzincins, aspzincins and metzincins. Proteolytic enzymes in the zincins group possess a conserved sequence, HEXXH, which is characterized as the zinc ion binding motif. The two histidine residues are responsible for the zinc binding ligand and the glutamate residue is crucial for enzymatic activity. Metzincins are a subclass of zincins and have a special methionine-containing 1,4- β -turn (the Met-turn). Metzincins are further classified into six sub-families based on the individual structure features (Gomis-Rüth, 2003).

Metalloprotease proteins are classified into five groups depending on respective cellular localization and respective substrates: collagenases (MMP-1, MMP-8, MMP-13 and MMP-18); gelatinases (MMP-2 and MMP-9); stromelysins (e.g., MMP-3, MMP-10 and MMP-11); matrilysins (e.g., MMP-7); membrane-type (MT-MMPs) and other MMPs (Malara et al., 2015; Malara et al., 2018). MMPs are zinc-binding endopeptidases, which process a crucial role in ECM degradation (remodelling mediator) that induces cellular migration via matrix, which can also regulate cell differentiation, angiogenesis, inflammation, and wound healing (Hazra et al., 2012; Malara et al., 2018). The metallic core of enzyme plays an essential role in proteolytic reactions (LibreTexts n.d.). For metalloproteases, a zinc ion is commonly used as the metal ion, which will bind to the active site required for the cleavage of peptide bonds within proteins. As metal ions are important for the physiological functions of metalloprotease, analysis of the interaction between protein and metal ion will help with understanding conformational changes in binding sites and regions of target proteins (Lin & Gross, 2022). Ethylenediaminetetraacetic acid (EDTA) is a strong chelating reagent, which could inhibit activities of MMPs (Hazra et al., 2012). Utilization of EDTA as an inhibitor of metalloprotease has been discussed by Hazra *et al.* (2012),

who observed that EDTA treatment decreased MMP expression and reduced the cellular migration and proliferation in the EDTA-treated eyes in rabbits with posterior capsular opacification (PCO). This further suppressed activities of MMPs and the severity degree of PCO was reduced.

Regulation of the enzymatic activity of metalloproteases is processed at the inactive zymogen form by post-translational modification (Gomis-Rüth, 2003). Metalloproteases are classified into inverzincin and zincin groups (Figure 1.1). Proteases in the inverzincin group possess an inverted zinc-binding HXXEH sequence. *Escherichia coli* pitrilysin and yeast mitochondrial processing peptidase present the structural models of inverzincins. The zincin group is subdivided into gluzincins, aspzincins and metzincins. The conserved sequence, HEXXHXXGXXH/D, which is characterised as the zinc ion binding motif, is present in the metzincin family. Three histidines and /or aspartic acid are zinc ion binding ligands. A special methionine-containing 1,4- β -turn, named as the Met-turn, in addition to the catalytic motif give the special features of metzincins (Gomis-Rüth, 2003). Regulation of proteolytic homeostasis is depending on compartmentalization of proteolysis, where hydrophobic membranes separate proteolytic enzymes' function is distinguished from subcellular localisations (Brix et al., 2013).

The cysteine-switch mechanism has been suggested to be the central component for regulation of the enzymatic activity of matrix metalloprotease (MMP) (Wart & Birkedal-Hansen, 1990; Yao, C. et al., 2003). Members from the MMP gene family are synthesized in a latent and inactive form where Cys⁷³ from the propeptide domain is bound to the essential zinc atom localizing in the catalytic domain (Van Wart 1990; Springman 1990). The zinc atom within the Cys⁷³-zinc complex can be characterized as a 'latent catalytic zinc atom', which is inaccessible to substrates blocked by Cys⁷³. Replacement of Cys⁷³ residue from the zinc atom 'switches' zinc atom from a noncatalytic into a catalytic form, which is called 'cysteine-switch' mechanism. As sequences surrounding the single cysteine residue in propeptide domain and the partial zinc binding site in the catalytic domain are conserved for all MMPs, it has been suggested that the 'cysteine-switch'

mechanism is applicable to the entire MMP family (Van Wart 1990; Springman 1990). This system may explain the regulation of zinc ion binding to the active site of leishmanolysin, which potentially protects the parasite's cells from self-autolysis (because the cleavage sites are not exposed and are thus immobilized in latent protease) (Macdonald et al., 1995; Santos et al., 2006; Wart & Birkedal-Hansen, 1990).

1.3 Leishmanolysin protease: the closest homologue of invadolysin

Leishmanolysin, a member of the metzincins sub-class, is a highly abundant surface protein found in *Leishmania* sp. (Gomis-Rüth, 2003; Schlagenhauf et al., 1998; Yao, C. et al., 2003). There are two distinct morphological forms of *leishmania*, which are the amastigote and the promastigote (Melby 2019). Sand fly is the intermediate host, which transmits the promastigotes from the mid-gut to reservoir animals' mononuclear phagocytes (neutrophils and macrophages). The promastigotes transform into non-motile amastigotes rapidly, and the amastigotes replicate within the acid and hostile environment of the phagolysosome, which eventually lyse the macrophage. To continue the cycle, free amastigotes or amastigote-infected macrophages are ingested by a sandfly (Melby 2019). This pathogenic agent in promastigotes of *Leishmania* is transmitted by sandflies and targets the macrophages of a mammalian host causing cutaneous to visceral leishmaniasis (Gomis-Rüth, 2003; Santos et al., 2006; Schlagenhauf et al., 1998).

Leishmanolysin is the structural model of the membrane-associated metalloproteases, whose conserved catalytic sequence is HEXXHXXGXXH (Gomis-Rüth, 2003; Yao, C. et al., 2003). Unlike other members of the metzincins, 62 amino acids are inserted between glycine and the last histidine (Yao, C. et al., 2003). Leishmanolysin is synthesized in the endoplasmic reticulum (ER), where the N-linked carbohydrate is attached, the C-terminus is replaced by a GPI anchor, and the N-terminal signal sequence is removed co-translationally (Santos et al., 2006).

Leishmanolysin is also known as GP63 since it is derived from a 63 kDa

glycoprotein (Gomis-Rüth, 2003; Santos et al., 2006; Schlagenhauf et al., 1998; Yao, C. et al., 2003)

The role of leishmanolysin in the pathogenesis of *Leishmania sp.* is related to its reaction with immune cells in hosts (Yao, C. et al., 2003). Both intracellular and cell surface bound forms of leishmanolysin have been identified, which have a range of substrate specificity that is pH dependent (Santos et al., 2006). Various substrates (such as casein, gelatin, albumin and other immunoglobulins and hemoglobins) have been shown to be catalysed by leishmanolysin (Yao, C. et al., 2003). *In vitro*, it has the capability to hydrolyse several serum proteins, such as albumin, fibrinogen, hemoglobin and immunoglobulins (Bouvier et al., 1990; Santos et al., 2006). Type IV collagen and fibronectin are also examples of substrates of leishmanolysin, which are localised in the extracellular matrix (McGwire et al., 2003; Santos et al., 2006). It has been reported that GP63 prevents parasite cells from antimicrobial peptide-regulated apoptotic death (Kulkarni et al., 2006; Yao, C. et al., 2003).

Recent research has demonstrated that GP63 is a virulence factor transferred in *Leishmania spp.*-derived exosomes/extracellular vesicles and has an important role in modulation of distinctive macrophage inflammatory responses (Filho et al., 2022). GP63 was discovered in exosomes released from infected macrophages that harbour *Leishmania* promastigotes (Hassani & Olivier, 2013). In infected macrophages, down-regulated GP63 displays dynamic distribution when parasites transform from the promastigote form to the amastigote form (Hsiao et al., 2008; Yao, C. et al., 2003). Parasite-infected macrophages can be deactivated by the degraded forms of fibronectin (FN), which will expand the intracellular activity of parasites. Both surface-derived and secreted forms of leishmanolysin produced by promastigotes can facilitate FN. In this way, invasion can be enhanced by *Leishmania* through degradation of extracellular matrix (Kulkarni et al., 2008). Additionally, it has been elaborated that GP63 is a primordial element in EVs on initial enhanced infection, and this was also confirmed in cutaneous pathology in an *in vivo* animal model (Filho et al., 2022). Taken together, it

has been recommended that extracellular GP63 can be a novel pharmacological treatment target and vaccination candidate for Leishmaniasis (Filho et al., 2022; Gioseffi et al., 2020).

1.4 Background to invadolysin

Drosophila is a classic model used for the characterisation of novel and conserved genes important in cell cycle and cellular physiology. *IX-14¹* is a late larval mutation in *Drosophila melanogaster* with imaginal defects (Shearn et al., 1971). This mutation was discovered during a chemical mutagenesis screen induced by ICR-170 (an acridine compound causing frameshift mutations). A P element (transposon) insertion allele of *I(3)IX-14¹* (first allele identified for *IX-14* on the third chromosome of *Drosophila*), termed as *I(3)IX-14^{4Y7}* (4Y7 refers to the P element), was generated in the Heck lab to further clone and identify the *IX-14* gene (McHugh et al., 2004). Monopolar spindle, hypercondensed lengthwise neuroblast chromosomes and abnormal accumulation of high levels of nuclear envelope proteins have been observed in *IX-14* mutant mitotic cells (Gatti & Baker, 1989; McHugh et al., 2004; Shearn & Garen, 1974; Shearn et al., 1971) (Figure 1.2). The *IX-14* gene encoding invadolysin was then characterized as a novel metalloprotease and it is conserved in eukaryotes, such as fruit flies (*Drosophila melanogaster*), zebrafish (*Danio rerio*) and humans (McHugh et al., 2004; Cobbe et al., 2009; Vass & Heck, 2013). In zebrafish embryos, three different forms of invadolysin protein have been observed by immunoblotting at different stages of development. This has also been observed by immunofluorescence (Vass & Heck, 2013). In human cells, four splice variants of the invadolysin gene have been identified by RT-PCR (Cobbe et al., 2009) (Figure 1.3). Localization and level of invadolysin change dramatically during adipogenesis (Chang et al. 2016). It has been shown that invadolysin was localized to lipid droplets (LDs) in the early stages of differentiation (day 2 to day 4) in murine 3T3-L1 cells (fibroblasts isolated from murine embryos), and dissociated from LDs to the cytosol dispersedly. The mRNA level of invadolysin increased significantly at the 8th day of adipocyte differentiation (Change et al. 2016).

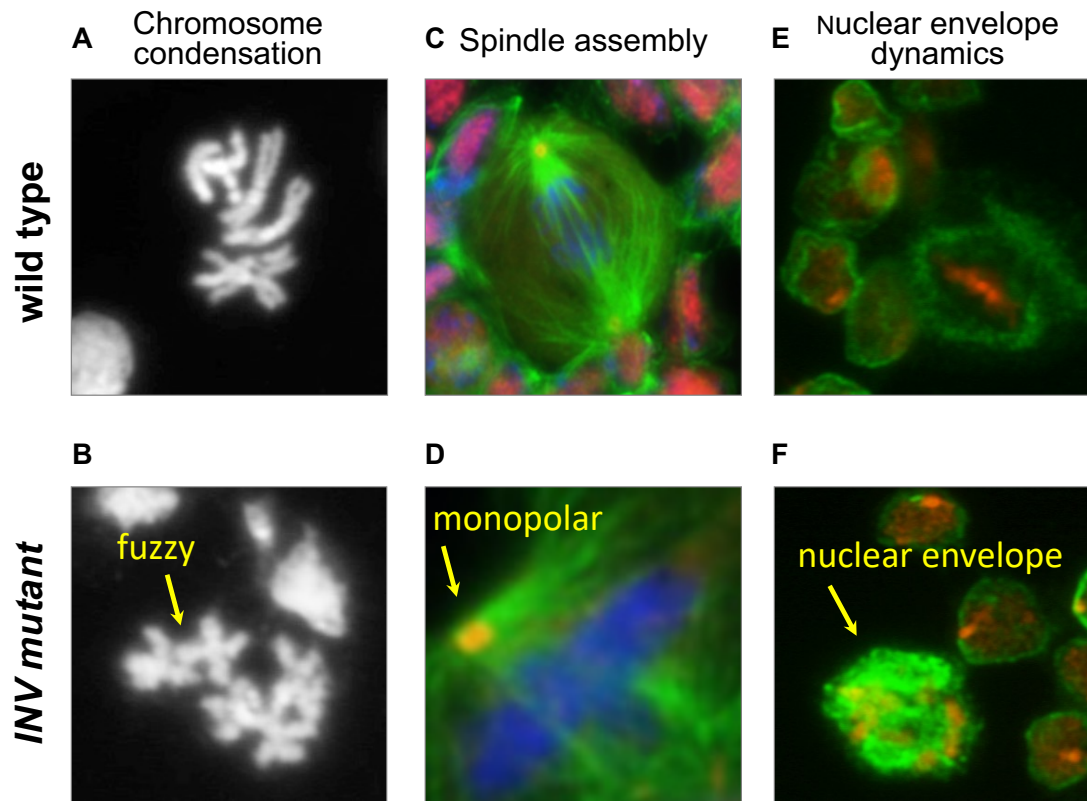


Figure 1.2 Chromosome defects in *I(3)IX-14* (Adapted from McHugh et al., 2004).

(A, B) Mitotic chromosomes of wild-type and homozygous *I(3)IX-14* mutant third instar larval brains After colchicine treatment, the *IX-14* chromosomes are unable to condense as tightly as the wild-type chromosomes. (C, D) Larval neuroblasts are stained with α -tubulin (green), CP190 (red), and DAPI (blue). Bipolar spindles with two centrosomes are in wild-type metaphase figure. Monopolar spindle phenotype is enlarged in *IX-14* mutant neuroblast. (E, F) Larval brains from wild-type and *IX-14* homozygous mutant cells were stained for lamin (E, green) and otefin (F, green) visualization. Over-accumulated otefin is shown in nuclear envelope of mutant cells during mitosis.

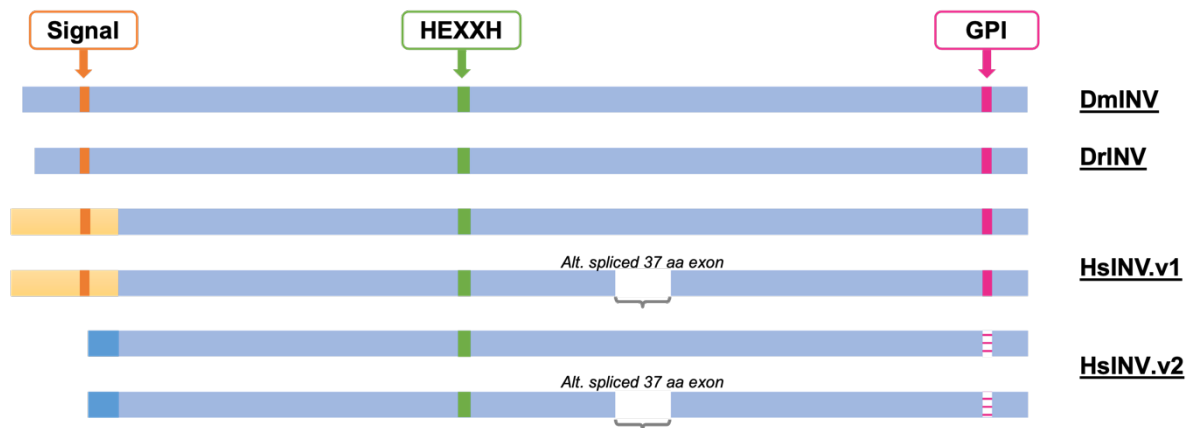


Figure 1.3 Invadolysin is a conserved metalloprotease in invertebrates and vertebrates.

Schematic sequences representing *Drosophila melanogaster* invadolysin (DmINV), zebrafish invadolysin (DrINV) and four human invadolysin variants (HsINV.v1+37, Hsinv.v1 Δ 37, HsINV.v2+37 and HsINV.v2 Δ 37) with N-terminal signal sequence (orange), metalloprotease motif (HEXXH)(green), alternative spliced 37 amino acid exons (grey bracket) and predicted GPI (deep pink) attachment site. H: histidine; E: glutamic acid; X: any amino acids; GPI: glycoposphatidylinositol.

Mechanisms of how transcription of invadolysin is regulated and modified are currently under investigation. It has been shown that invadolysin mutations have lethal effects on late larva stages of *Drosophila* (McHugh et al., 2004). Given this observation, it has been suggested that invadolysin is essential for living organisms. This is based on the demonstration that invadolysin has various physiological activities in cells. For example, invadolysin mutations induce mitotic arrest in *Drosophila* cells, which suggests that invadolysin has an important role(s) in cellular division (McHugh et al., 2004). It also regulates chromosome architecture by interacting with the SAGA complex and subsequently modulates monoubiquitination of H2B (Rao et al., 2015). Abnormal distribution of melanophores and neuromasts has been identified in invadolysin-depleted (induced by morpholino interference) zebrafish embryos, providing evidence that invadolysin is involved in cellular migration (Vass & Heck, 2013). Due to the observation of invadolysin on the surface of lipid droplets, it has been suggested that it is involved in regulation of metabolism (Cobbe et al., 2009). For instance, invadolysin mutations in *Drosophila* larvae cause a significant decrease in triglyceride:protein ratio (Cobbe et al., 2009). Similarly, whilst wild type *Drosophila* larvae accumulate triglyceride when exposed to a high sucrose diet (Musselman et al., 2011), this is impaired in invadolysin mutant larvae. This suggests, therefore, that invadolysin plays positive roles in homeostasis and in the regulation of insulin signaling (Chang, C.-W. et al., 2017). Furthermore, it has been suggested that invadolysin contributes to the modulation of mitochondrial activity (Di Cara et al., 2013). For example, oxidative stress reaction regulated by invadolysin (through cooperation with subunits of ATP synthase) was observed in mitochondria in *Drosophila* (Di Cara et al., 2013).

As a metzincin metalloprotease, invadolysin belongs to a subgroup of the M8 family with a conserved 'HEXXHXXG[X]_NH' motif which is responsible for catalytic activity (McHugh et al., 2004). Invadolysin shares homology with the *Leishmania* sp. protease leishmanolysin (GP63) (McHugh et al., 2004; Yao, C. et al., 2003) (Figure 1.4). Notably, however, the proteolytic activity of invadolysin has only been partly investigated. The catalytic activity of invadolysin has been verified and there is evidence that invadolysin in lysates

of wild type fly larvae brain has the ability to degrade lamin *in vitro* (McHugh et al., 2004). There are diverse modes available for multilevel regulation of zymogen in different environments, such as intracellular, cell surface and extracellular environment, which could be involved in invadolysin regulation (Gaffney et al., 2023; De Almeida et al., 2022; Hadler-Olsen et al., 2010). For instance, at the cell surface, a two-step activation of proMMP-2 by MT1-MMP (Metallothionein-1 matrix metalloproteinase) is introduced where a complex including proMMP-2-MT1-MMP and tissue inhibitor of metalloproteinases 2 (TIMP-2) initially and MMP-2 is activated through the cleavage of pro-MMP-2 by MT1-MMP. At the extracellular environment, cysteine-switch zinc system has been proposed as one mechanism for activation of a functional matrix metalloprotease zymogen as described in Chapter 1.3 (Wart & Birkedal-Hansen, 1990). This mechanism destabilizes the conformation of the propeptide domain and disconnects the interaction between the Cys⁷³ and the catalytic zinc atom. The most well-known mechanism for MMP zymogen activation is proteolysis of the pro-domain of zymogen including trypsin or other MMPs, which shields the catalytic machinery and prevents MMPs from catalytic activation (Wart & Birkedal-Hansen, 1990; Hadler-Olsen et al., 2010). Further research on invadolysin-mediated hydrolysis is ongoing and the mechanisms that regulate activation of invadolysin under different physiological conditions still need to be understood.

As invadolysin is a novel and highly conserved metalloprotease, discovering its physiological function(s) is important. Analysed using *in silico* techniques, multiple potential post-modification sites (such as phosphorylation, glycosylation, GPI-anchor addition) of the invadolysin sequence have been identified (Abhinav et al., 2019). The soluble fractions of both invertebrate hemolymph and vertebrate blood have been examined to verify the secreted form of invadolysin. It has been found that a secreted form of invadolysin is present in *Drosophila* hemolymph, and in vertebrate blood, serum, and plasma. As invadolysin is essential for life, it has been suggested that the secreted form of invadolysin may play an important role in maintaining normal physiology. Therefore, much attention has been given to study the



Figure 1.4 Invadolysin structure based on Leishmanolysin (Adapted from McHugh et al., 2004)

This is the three-dimensional structure of leishmanolysin. The black numbered spheres represent the 'insertions' of high eukaryotic, which localize on the surface of the crystal structure of GP63 and predicts the interfere with substrates, regulators, or other binding partners. The zinc ion is represented by the internal magenta sphere required for catalysis.

cellular forms of invadolysin. Recently, it has been shown that invadolysin is present in extracellular vesicles purified from human plasma. However, whether invadolysin is enriched in a specific soluble fraction of vertebrate serum and plasma has not been investigated. Therefore, it is important to determine what extracellular forms of invadolysin are present in different soluble fractions, and how the expression of these secreted forms may be regulated. As a novel metalloprotease, whether enzymatic activity of invadolysin can be detected in human blood is not yet known. In addition, what roles might the extracellular form of invadolysin play in normal physiological conditions, such as glucose metabolism and immune response, has still to be determined. It will be important to explore any role invadolysin may have in disease pathogenesis; especially in diseases associated with lipid metabolism, such as obesity and diabetes.

1.5 Zebrafish as a research model organism

In spite of the rodent models, zebrafish (*Danio rerio*) has emerged as a powerful model among the scientific community to understand the molecular mechanisms and genetics of human diseases since the 1980s (Bailone et al., 2020; Adhish & Manjubala, 2023). The initial contribution of this teleost model was the developmental biology (Adhish & Manjubala, 2023). Since then, highly conserved genes associated with different diseases have been identified in thousands of zebrafish mutant models. Until now, the zebrafish model has been widely used in various fields, such as genetic and embryonic research on vertebrate development, neurobiology, cardiology and oncology (Lachowicz et al., 2021; Adhish & Manjubala, 2023).

Zebrafish genome has 25 chromosomes and shares genetic similarities with human (Lachowicz et al., 2021). One of the important fascinating facts is that 71.4% of human genes have at least one zebrafish orthologue, which was conducted from a direct comparison of zebrafish and human protein-coding genes feature characterized by Ensembl Compara (Vilella et al., 2009; Howe et al., 2013; Goessling & Sadler, 2015). Reasons of zebrafish model becoming a popular animal model choice are that zebrafish has a high

embryo yield (around 200 eggs laid at one time) and rapid reproduction rate (Lachowicz et al., 2021; Adhish & Manjubala, 2023). Zebrafish embryos are transparent and developing outside of the uterus, which renders the post-fertilization of embryos readily accessible for diverse studies. Once a larva hatched from an egg, the zebrafish embryo transits into a ready morphological development, which will be accomplished on the third or the fourth day after fertilization, and the embryonic development lasts around 6 weeks (Lachowicz et al., 2021). Within the 48 h post fertilization (hpf), head, chest, and cartilage of zebrafish embryos are developing, and most internal organs are formed completely until 72 hpf. Normally, it takes about three months for an embryonic form to grow and develop into a matured adult form. Therefore, zebrafish embryonic modification and morphological development observation can be facilitated steadily (Kaveh et al., 2020). Other advantages of using zebrafish model are its small body size, external fertilization, economical husbandry and breeding, fewer required space for maintenance, and lesser ethical issues comparing to rodent models (Clark 2005; Keller & Keller, 2018; Lachowicz et al., 2021; Adhish & Manjubala, 2023).

From 2000 to 2023, increased utility of zebrafish disease models has been widely seen in diverse research area, such as tissue regeneration, depression research, cardiac regeneration, renal diseases, environmental toxicology and vaccination research (Bailone et al., 2020; Bruton et al., 2022; Choi et al., 2021; Lachowicz et al., 2021; Adhish & Manjubala, 2023). Zebrafish embryos live the aquatic environment which makes it easy to examine the effects of substances, such as water-soluble drugs and chemical, on embryos and young larvae by administration directly in the E3 medium (zebrafish embryonic control medium) (Williams & Renquist, 2016; Keller & Keller, 2018). Recently, a novel zebrafish model with acute renal failure (ARF), where renal tubular epithelial cells were absent, has been established to screen new therapeutic drugs for complications of ARF, especially cardiorenal syndrome type 3 (CRS-3) (Liao et al., 2021). The effectiveness of zebrafish model was tested by the administration of digitoxin and enalapril, which are two commonly used drugs for clinical treatment of

CRS-3. The symptoms of zebrafish CRS-3, such as slow heart rate and heart enlargement, have been alleviated, and the survival rate of ARF zebrafish was increased. The use of zebrafish model has also been expanding to the platform of human vaccination research (Bailone et al., 2020). There are numbers of evidence showing that a well-established zebrafish model, such as the *Mycobacterium tuberculosis* infection model in adult zebrafish, could be a promising approach for pre-clinical screening of tuberculosis immune reaction and identification of vaccine antigens (Oksanen et al., 2013; Myllymäki et al., 2018; Ji et al., 2019; Torracca et al., 2019; Cen et al., 2020; Cheng et al., 2020; Harijula et al., 2020). Furthermore, proteome analysis of zebrafish peripheral blood provides a possibility to access the physiological status of an organism, such as health condition and reproductive cycles, by analysis of changes in serum biochemistry and cellular composition (Rey Vazquez & Guerrero 2007; Nordlie 2009; Babaei et al. 2013). Early in 1996, Roche and Bogé demonstrated that the zebrafish model is a representative avenue for plasma biomarker discovery of chemical (metallic or organic compounds) and physical (temperature) stresses though addressing the plasma levels of stress indicators, such as glucose, cortisol and peroxidase activity (Roche & Bogé, 1996).

1.6 The novel extracellular form of invadolysin in invertebrate hemolymph and vertebrate blood

In silico analysis has revealed potential sites of post-translational modification in the invadolysin sequence, which suggests that invadolysin possesses an extracellular form (Abhinav et al., 2019). The same investigation demonstrated that invadolysin is present in the soluble fraction of serum and plasma from vertebrates, especially in the extracellular vesicle of human plasma. In addition, it showed a potential sexual-dimorphic form of invadolysin in male and female *Drosophila* hemolymph. These results indicate that an extracellular form of invadolysin is a conserved component in vertebrate and invertebrate blood. Although a secreted form of invadolysin has been identified in higher vertebrate serum and plasma, this novel form in human blood has not been well characterised (Abhinav et al., 2019).

1.6.1 An extracellular form of invadolysin has been predicted by *in silico* sequence analysis

In silico analysis demonstrated four splice variants of human invadolysin (Abhinav et al., 2019; Cobbe et al., 2009). The N-terminus of invadolysin variant 1 has been predicted to encode a signal peptide by the first longer exon and the C-terminus of variant 1 is a potential GPI modification site (Cobbe et al., 2009) (Figure 1.2). The signal peptide sequence is absent in variant 2 as the N-terminus of variant 2 is encoded by an alternative open reading frame. It has also been suggested that variant 2 has a different intracellular localisation due to the lack of the predicted signal peptide cleavage site. Each human *invadolysin* has two alternative forms depending on the presence or absence of a 37 amino acid region encoded by exon 12 (v1+37, v1 Δ 37, v2+37 and v2 Δ 37). As a result, the structure of four different splicing variant proteins is expected to be different remarkably (Schlagenhauf et al., 1998). It has been estimated that among all currently known human *invadolysin* variants, the relative abundance of v1+37 is the most prevalent, whereas v2 Δ 37 is the least abundant variant (Cobbe et al., 2009).

Proteolytic processing is an irreversible post-translational modification, which includes cleavage of proteins into small polypeptides by a protease (Rogers & Overall, 2013). These events make a contribution to a series of activities, including but not limited to activation and inactivation to alter protein function, which regulates numerous biological processes, such as cell cycle progression, cell proliferation and pathological processes (inflammation, cancer and cardiovascular disease). The proprotein convertase (PC) family facilitates post-translational modification of precursor proteins in the Golgi, at the cell surface and in endosomes within immature secretory granules, which plays a significant role in regulation of biological pathways, disease progression and pathogenic infections (Seidah & Prat, 2012). Furin is one of the seven PCs members, which recognize target amino acid sequence Arg-X-(Arg/Lys)-Arg (Thomas, 2002). The Uniprot predicts the internal cleavage site of human invadolysin as Arg-Gln-Lys-Arg, when the 37 amino acids exon is present (Uniprot n.d.).

In a conventional secretory pathway, the signal peptide motif at the N-terminal of a newly formed protein is recognised by a signal recognition particle (SRP) on the ER membrane (Akopian et al., 2013; Bendtsen et al., 2004; Kellogg et al., 2021; Lippincott-Schwartz et al., 2000; Lührink & Sinning, 2004). Synthesis and post-translation of the nascent protein are mediated in the ER and the secretory cargo is transported to the Golgi apparatus via membrane-bound vesicles for further modification and maturation. After modification, the nascent protein leaves the Golgi apparatus and may be translocated to the cell surface or secreted into extracellular space (Bendtsen et al., 2004; Klee & Sosa, 2007; Lippincott-Schwartz et al., 2000).

Recently, potential N-glycosylation sites in invadolysin have been predicted across different species (Abhinav et al., 2019). The glycosylation sites in invadolysin sequence were characterised by the N-X-S/T sequence, where X is represented by any amino acid excluding proline (Nita-Lazar et al., 2005). Proteins are modified by N-glycosylation in the ER and the degree of N-glycosylation is selectively increased for secretory proteins (Aebi, 2013; Cherepanova et al., 2016). The NetNGlyc 1.0 Server predicted O-glycosylation sites in invadolysin sequences, where the hydroxyl group in serine or threonine is the potential attachment site for glycans (Abhinav et al., 2019; Spiro, 2002). Like N-glycosylation, O-glycosylation is involved in translational modification, which frequently occurs in the Golgi apparatus (Spiro, 2002). Glycosylation is a crucial stage in protein post-translational modification, which plays an important role in protein folding, cellular distribution and secretion processes required for eukaryotic protein enzymes (Goettig, 2016). Carbohydrates can be characterised by lectin-staining cytochemistry to understand glycans releasing from glycoprotein enzymatically and chemically (Miwa 2015). For instance, a dicalcin-dependent change has been observed by Miwa (2015) in the lectin-staining pattern in the zona pellucida (ZP), a glycoprotein layer outside the plasma membrane of oocytes. This study suggested that dicalcin has the capacity to modulate the affinity between lectin and glycoproteins' oligosaccharides attachments. In addition, mass spectrometry reveals more conserved

carbohydrate components of unfertilized ZP by comprising terminal sequences on N- and O- glycans between different species.

Potential GPI-anchor sites in invadolysin sequences have been predicted by the Big-PI Predictor. These are located at the C-terminus of both variant 1 and variant 2 (Abhinav et al., 2019; Eisenhaber, B. et al., 1999). GPI anchor is synthesized in ER's phosphatidylinositol lipid via a number of enzymatic reactions (Kinoshita 2016). In the ER lumen, the GPI anchor sequence (the carboxyl terminus) within the nascent protein functions as a hydrophobic signal sequence, which initiates the addition of the GPI anchor (Fujita & Kinoshita, 2012; Kinoshita 2016; Muñoz & Riezman, 2016). During this process, the C-terminus of hydrophobic amino acids is cleaved off and replaced by the preassembled GPI anchor through a transamidation reaction (Kinoshita 2016). The GPI-anchor is further modified by remodelling enzymes, which direct GPI-anchored proteins (GPI-APs) to be transported into the Golgi apparatus (Fujita & Kinoshita, 2012; Kinoshita 2016; Muñoz & Riezman, 2016). This modification process will remodel both glycans and lipids, such as mannose-linked phosphoethanolamine and inositol-linked acyl chain (Kinoshita 2016). After several maturation stages, GPI-anchored proteins are transported to the cell surface. Ectodomain shedding is the process known as proteolytic release from the cell surface (Hundhausen et al., 2003). Regulation of shedding is important for cellular signaling, which has been detected for growth factors, cytokines, and respective receptors. For example, ADAM17 is a member of the protease family disintegrin-like metalloproteinases (ADAMs), which can facilitate shedding of tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6) receptor, and this is important in cell adhesion modulation. Lipids and proteins could diffuse in biomembrane freely, which is known as lateral diffusion (Jacobson et al., 1987). It has been found that GPI-anchored protein has a higher mobility with transient incorporation into nanodomains in live-cell plasma membrane, which could be explained by an interaction between GPI-anchored proteins with lipid that is needed for domain formation (Schneider et al., 2017).

Predicted N-terminal signal sequences, N- and O-glycosylation sites, and the GPI-anchor sequences in the invadolysin sequence suggest that invadolysin may be translocated to the cell surface or secreted into the extracellular environment (Abhinav et al., 2019; Muñiz & Riezman, 2016). Human post-translational modification of extracellular invadolysin has been analyzed by Abhinav *et al.* (2019). Enriched invadolysin from human plasma was treated with calf intestinal phosphatase (CIP; phosphorylation), PI-PLC (GPI-anchor), PNGase F (N-linked glycosylation) and O-glycosylase (O-linked glycosylation). The results demonstrated that extracellular invadolysin from human plasma is N- and O- glycosylated.

The N-terminal signal is a classical sequence used during protein secretion through diverse conventional pathways (Lippincott-Schwartz et al., 2000). This signal sequence targets to the endoplasmic reticulum and the Golgi apparatus subsequently. Recently, the presence of N-terminal signal sequence in human *invadolysin* variant 1 has been predicted by SignalIP 4.1c (a signal sequence prediction server) (Abhinav et al. 2019). This analysis suggests that human invadolysin has an extracellular form and this novel form may target translation of invadolysin to the secretory pathway.

1.6.2 A novel extracellular form of invadolysin is present in human plasma

As described, *in silico* analysis has predicted a potential extracellular form of invadolysin, on the basis of sequence features, such as the N-terminal sequence, N- and O- glycosylation sites, and GPI-anchor addition sites (Abhinav et al., 2019). To further validate the potential extracellular form of invadolysin and investigate its physiological functions, human blood products have been utilised for proteomic analysis. This has similarities with the use of human serum and plasma for the identification of novel biomarkers of human diseases (Bellei et al., 2011; Liu, B. et al., 2011; Polaskova et al., 2010). The dominant challenge in examination of the secreted form of invadolysin is blood sample preparation. Depletion of highly abundant proteins from serum and plasma is important for reducing interference in

proteomic analysis. Albumin and immunoglobulin heavy chain contribute to 70-80% of the total protein content of serum and plasma, which could mask the detection of low abundance proteins using proteomics (Abhinav et al., 2019; Liu, B. et al., 2011; Steel et al., 2003). Recent studies have confirmed that invadolysin is present in human plasma soluble fractions (Abhinav et al., 2019). Removal of the top 12 highly abundant proteins from human plasma using a commercial kit improves the detection of invadolysin in immunoblotting with increased resolution and higher intensity. In addition, immunoblotting of extracellular invadolysin displays different migration patterns after depletion of highly abundant proteins from human plasma.

Extracellular vesicles play important roles in cellular communication and in regulation of physiological processes, such as blood coagulation, tissue regeneration and the immune response (Andaloussi et al., 2013).

Furthermore, extracellular vesicles are associated with the spread of cancer as they can stimulate tumour cell proliferation and angiogenesis. Given these physiological and pathophysiological effects, it has been proposed that extracellular vesicles can be used both as disease biomarkers and as useful targets for drug therapy (Andaloussi et al., 2013; Raposo & Stoorvogel, 2013). Three major classes of extracellular vesicle have been described: exosomes, microvesicles, and apoptotic bodies, which can be isolated from blood and other body fluids. Internal intraluminal vesicles (ILV) of multivesicular bodies (MVBs) are the origin of exosomes, which is formed when the endosomal membrane of the MVB is budding inward (Beer and Wehman, 2017). Exosomes are secreted outside the cell when an MVB is fused with the plasma membrane. Microvesicles can be released directly by plasma membrane budding to the extracellular space. There is evidence that invadolysin is present in all plasma fractions (Abhinav et al., 2019).

Additionally, invadolysin has been identified in the plasma fractions enriched for extracellular vesicles (both microvesicles and exosomes), which may have a potential role in cell biology of extracellular vesicles.

Recently, *LmIn* knockout mice were generated to characterize the physiological function of invadolysin, using different strategies, by the Takahashi laboratory (unpublished). Results have shown that deletion of a

single exon from invadolysin gene can generate mice homozygous for invadolysin deletion. However, *LmIn*^{-/-} mice (invadolysin deletion) exhibited no significant phenotype, and extracellular invadolysin could still be detected in their blood. Subsequently, an alternative strategy was used to generate invadolysin null mice by deletion of multiple exons. Through homozygous mice were still not available, it is worth understanding the structure of extracellular invadolysin in genetically modified mice serum and plasma.

1.7 The role of metalloproteases in metabolic inflammatory diseases

1.7.1 Invadolysin is an essential metalloprotease for normal physiology

Since Invadolysin was first described, as a novel conserved metalloprotease, during characterization of mutations related to cellular mitosis in *Drosophila melanogaster* (McHugh et al., 2004), it has been shown on the leading edge of human migrating macrophages and with an invadopodia-like cellular localization in both *Drosophila* and humans. Protease activity of invadolysin has been detected in *Drosophila* larval brain *in vitro*. This work demonstrates that the invadolysin gene is essential for *Drosophila*, as a result of its role in the cell cycle, cellular migration, chromosome structure maintenance, and potentially in disease pathogenesis (McHugh et al., 2004; Rao et al., 2015; Vass & Heck, 2013).

Analysis of invadolysin mutants in *Drosophila* has revealed that they result in a significant decrease in the ratio of triglyceride:protein and diminished fat body thickness (Cobbe et al., 2009). Invadolysin is localized on the surface of lipid droplets and plays a role in lipid accumulation and localised in the cytoplasmic structures resembling invadopodia, which makes invadolysin as a unique metalloprotease (McHugh et al., 2004). Thus, it has been suggested that invadolysin is important for the regulation of the dynamic lipid organelle and relevant metabolism. Subsequent research has demonstrated that defective function of invadolysin influences the normal function of mitochondria (Di Cara et al., 2013). For example, reduced production of ATP

and excessive storage of ROS have been detected in third-instar larvae of flies with invadolysin deletion. Recent research has established that there are different structure forms of invadolysin observed in lipid droplets during adipogenesis and, in *Drosophila*, invadolysin decreases glycogen accumulation (Abhinav et al., 2019; Di Cara et al., 2013; Chang, C.-W. et al., 2017; Vass & Heck, 2013).

Insulin is synthesised from its precursor, proinsulin, in the pancreatic islets of Langerhans β cells (Gisela Wilcox 2005). There are 51 amino acids in the insulin molecule, which consists of two chains (A chain: 21 amino acids; B chain: 30 amino acids) linked by two disulfide bonds (Donner & Sarkar, 2023). After removing of the signal peptide, proinsulin (86 amino acids in a single-chain), is synthesised from pre-proinsulin, which is assembled in the ribosomes of the rough endoplasmic reticulum (RER). Via secretory vesicles, proinsulin is transferred from RER to the Golgi apparatus (Gisela Wilcox 2005; Donner & Sarkar, 2023). After processing, proinsulin is packed into immature granules and cleaved into insulin and C-peptide (a connecting peptide) by enzymes outside the Golgi. Glucose and other insulin secretagogues, including both nutrient and non-nutrient secretagogues, stimulate insulin secretion in a biphasic approach (Gisela Wilcox 2005). In response to hyperglycaemia, insulin, stored in secretory granules, is initiated to release within 1 minute and lasts for 10 minutes, which is characterised as the 'first phase' or 'rapid phase'. In the second phase, insulin is secreted after glucose bolus administration shortly, which originates from both newly synthesised and stored insulin. The principal function of insulin is to balance the micronutrient levels and regulate the energy supply during food intake (Burks & White, 2001). Insulin is responsible for the transportation of intracellular glucose to insulin-dependent cells or tissues. For example, in liver organ, insulin is important for transferring excessive glucose into fatty acid and precursor triglyceride (Rahman et al., 2021). Other hormones, such as glycagon, adrenaline and cortisol, are secreted during hypoglycaemia, which play an insulin-antagonistic role in the liver and peripheral tissues (Lager 1991; Jia et al., 2022).

Insulin signaling is crucial for adipogenesis as essential mediators, such as PI₃K (Phosphoinositide 3-kinase) and Akt (alpha serine/threonine-protein kinase, downstream mediator of insulin signaling; downstream of PI₃K), have positive roles in adipogenesis (Imamura et al., 2002). Chang *et al.* (2016) has shown that invadolysin has a role in insulin signaling, where pAkt levels were decreased in *invadolysin* mutant larvae and insulin signaling is impaired. Chang *et al.* (2016) further demonstrated that a form of invadolysin at 66 kDa increased during adipogenesis of SGBS *in vitro*. Blocking of adipogenesis by PI₃K inhibition not only prevents from the increase of the 66 kDa form of invadolysin, but also reduce the pAkt level, which illustrated the impairment of insulin signaling pathway. As a result, it has been speculated that invadolysin is responsible for adipocyte differentiation through the involvement of the insulin signaling pathway (Chang et al., 2016). Site-directed mutagenesis of transgenic *Drosophila* performed by Abhinav *et al.* (2019), demonstrated that the proteolytic activity of invadolysin has a role in lipid accumulation as overexpression of protease-dead forms of invadolysin has decreased ability to accumulate a high level of triglyceride. In recent research, a potential tumor suppressor of Leishmanolysin Like Peptidase (LMLN) has been elucidated by Xiang *et al.* (2021) who demonstrated that overexpression of LMLN inhibits formation of multiple myeloma (MM) cell colonies and exerts an effect on MM cell migration and invasion (Xiang et al., 2021).

1.7.2 Potential role(s) of extracellular invadolysin in liver disease diagnosis

Changes of lipid and protein catabolism and hyperglycemia, caused by a relative insulin deficiency, have been reviewed in articles for type 2 diabetes with metabolic insulin resistance (Williams et al., 2013). The liver regulating glucose flux and metabolism displays an insulin-dependent processes. The relationship between type 2 diabetes and nonalcoholic fatty liver disease (NAFLD) is clear as diabetes predicts the development of NAFLD, and vice versa, and serves as a progression factor for each other (Williams et al., 2013). As described, it has been hypothesized that invadolysin has a role in

glycolysis and lipid metabolism, thus, it is important to validate whether invadolysin plays a role in liver physiology (Abhinav et al., 2019; Cobbe et al., 2009).

1.7.2.1 Metabolic functions in liver physiology

Metabolic pathways in hepatocytes have been found to be regulated by glycolysis or gluconeogenesis, which controls carbon flux and will further influence urea production, lipid metabolism and glutamine metabolism (Iyer et al., 2010). Increased glycolytic activity is a recognised signature of tumor cells: for example, it has been verified that rat hepatomas exhibit a higher glycolytic flux than normal hepatocytes (Marín-Hernández et al., 2006; Moreno-Sánchez et al., 2007; Zu & Guppy, 2004). An explanation was proposed by Gatenby and Gillies (2004), who hypothesized that the enhanced glycolytic capacity of tumour cells has developed as a metabolic strategy to adapt to dynamic environments with low levels of oxygen (Gatenby & Gillies, 2004). Regulation of glycolytic pathways could be influenced by mitochondrially-bound hexokinase (HK) II, which needs ATP to transform glucose into glucose-6-phosphate (G6P) (Mathupala et al., 2006; Takanaga et al., 2008).

Hepatic lipid metabolism, such as lipid uptake and lipid accumulation, is important for the maintenance of normal liver function (Listenberger & Brown, 2008; Pei et al., 2020). Disruption in the balance of lipid metabolism, also known as dyslipidemia, such as increased levels of plasma free fatty acids and triglycerides, is verified as a major cause of inflammation and liver damage (Chu et al., 2014; Fabbrini et al., 2010; Li, Y. et al., 2017). Aberrant liver function accompanied by accumulation of lipid droplets is an early sign of insulin resistance and steatosis (Fabbrini et al., 2010; Xu, H. et al., 2016). Triglyceride, stored in lipid droplets, is accumulated in intrahepatic cells, which has been defined as a main characteristic of NAFLD (Morgan et al., 2022). Furthermore, abnormalities in lipid metabolism have been found directly or indirectly relating to NAFLD (Pei et al., 2020).

NAFLD has been characterized by hepatic steatosis where triglyceride accumulated in hepatocytes exceeds 5% of liver weight (Kneeman et al., 2012). Type II diabetes, obesity, insulin resistance and the metabolic syndrome, such as dyslipidemia and hypertension, are the primary risk factors of NAFLD (Angulo, 2007; Pouwels et al., 2022). Other causes, other than metabolic syndrome, could also be associated with NAFLD (Kneeman et al., 2012). For example, disorders of lipid metabolism, including glycogen storage disease and familial combined hyperlipidemia, are related to the development of NAFLD. Usually, there are no symptoms for patients with NAFLD, however, sometimes they might suffer from fatigue, pain in the upper right quadrant, hepatomegaly and lipomatosis (Bacon et al., 1994). Even though, there is no specific medicine treatment for NAFLD, a combination of treatment targets, such as smoking/alcohol cessation, increasing physical activity and lifestyle adjustments (a Mediterranean diet and increased intake of monosaturated and omega-3 fatty liver), have been proved to be positive for liver fat management (Nseir et al., 2014; Rmero-Gómez, et al. 2017; Pintó et al., 2019). In addition, weight loss by both conservative and surgical treatment can be beneficial for liver histology and biochemistry and improve the quality of patients' life (Petersen et al., 2005; Promrat et al., 2010; Keating et al., 2012; Knobler et al., 1999; Ueno et al., 1997). Promising hepatic outcomes by utilising pioglitazone and glucagon-like peptide-1 receptor agonists have been observed in patients with NAFLD in most recent years (Colca & Scherer 2021; Takahashi et al., 2022; Ghosal et al., 2021; Rezaei et al., 2021).

In 2013, Di Cara *et al.* discovered that invadolysin is an essential element for mitochondrial function. It has been described that, invadolysin interacts with three mitochondrial ATP synthase subunits α , β and d physically (Di Cara et al., 2013). In *invadolysin* mutants, levels of ATP are decreased in third-instar larvae and reactive oxygen species (ROS) are over accumulated. These observations recommend a role of *invadolysin* in mitochondria structure or physiological function. Previously, Cobbe *et al.* (2009) suggested that invadolysin has been found to be localised on the surface of lipid droplets in human and mouse cells. Thus, it has been suggested that the interaction

between invadolysin and ATP-synthase could be found on lipid droplets in *Drosophila* and mammalian cells (Di Cara et al., 2013). Cobbe *et al.* (2009) described that there is a significant decrease in the ratio of triglyceride:protein in *invadolysin* mutant *Drosophila*. Together with the fact that fat body thickness of fat body cells and cross-sectional area of cells comprising lipid droplets reduce in third instar larvae, it has been suggested that invadolysin has a role in the development of fat body and lipid accumulation. This role of invadolysin was further supported by Abhinav *et al.* (2019), who discovered that the proteolytic activity of invadolysin is crucial for lipid accumulation. However, declined level of glycogen detected in *invadolysin* mutant flies could not be explained by invadolysin transgenes (Abhinav et al., 2019). It is still not well known that whether impaired insulin signalling or abnormal glycogen reservation metabolism in *invadolysin* mutants is responsible for these observations. Thus, it would be interesting to determine whether invadolysin plays a role in metabolic functions of liver, especially in glycolysis or gluconeogenesis.

1.7.2.2 Roles of extracellular vesicle enriched MMPs in NAFLD

As described, Abhinav *et al.* (2019) demonstrated that invadolysin is enriched in the extracellular vesicle (EV) fraction (microvesicles and exosomes) of human plasma and other blood fractions in different vertebrates. However, the role of extracellular form of invadolysin has not been investigated in depth. In the past decade, research on EV biogenesis pathways have revealed that EVs are membrane-enclosed structures with the capacity to shuttle molecules from one cell to another (Verweij et al., 2021). It has been demonstrated that EVs are catalysed into diverse subtypes, such as endosome-derived vesicles, exosomes and other microvesicles budding from the plasma membrane (Verweij et al., 2021). EVs play significant roles in inflammation, cancer progression, pathogenic infections and metabolic homeostasis. The use of EVs, especially exosomes, in clinical research as carriers of biomarkers have been well characterised for diagnosis purposes (Doyle & Wang 2019). Circulating cell-specific EVs have been used as a powerful tool for the diagnosis of various liver diseases (Newman, Muller et

al., 2022). For instance, increased levels of Natural Killer (NK) T-cell-derived EVs have been found in serum from patients with NAFLD (Kornek et al., 2011). Increased levels of circulating EVs in humans with non-alcoholic steatohepatitis (NASH) have been reported by Srinivas *et al.* (2020), who proposed that hepatocyte derived EVs play an important role in pathogenesis and progression of non-alcoholic fatty liver disease (Srinivas et al., 2021; Wu, D. et al., 2021).

An elevated number of hepatocyte-derived EVs was detected in plasma obtained from patients with this condition (Newman, Useckaite et al., 2022). Furthermore, cargos such as miR-122, miR-192, and miR-128-3p identified in liver-specific EVs are associated with disease severity. Variations in levels of EVs released by hepatocytes were also described by Newman *et al.* (2020), who suggested that lipotoxic hepatocyte-derived EVs are key to liver inflammation and fibrosis (Newman et al., 2020). For example, a death receptor ligand, TRAIL, within lipotoxic hepatocyte-derived EVs can trigger hepatocyte death and macrophage activation (with release of the pro-inflammatory cytokines IL-1 β and IL-6) (Hirsova et al., 2016). In addition, changes in expression of MMP-7 in hepatocytes and MMP-9 in hepatic stellate cells (HSCs), induced by EVs from visceral adipose tissue, are involved in the dysregulation of TGF β signalling pathway, which may result in liver fibrosis in NASH (Koeck et al., 2014). This link between elevated levels of EVs and a number of liver diseases has suggested that global, and/or liver-specific EVs, can be used as a means for clarifying the relationship between extracellular invadolysin and the development of NAFLD (Newman, Muller et al., 2022).

1.7.3 Latent function(s) of extracellular invadolysin in lung inflammatory diseases

The surface GP63 shares structural and biochemical similarities with matrix metalloproteases, which are significant for promoting the migration of parasite through extracellular matrix, such as collagen IV and fibronectin, to enhance invasion (McGwire et al. 2003). It has been suggested that

combination of secreted GP63 and surface GP63 has an additive effect on migration. Bandyopadhyay *et al.* (2002) and Ghosh *et al.* (1999) pointed out parasites migration through extracellular matrix (ECM) and basement membrane (BM) proteins could facilitate the access to the blood or lymph circulation for dissemination to diverse sites. Furthermore, attachment to native and degraded ECM is beneficial to parasite migration or host macrophage activation (Yu & Stamenkovic, 2000).

The importance of the extracellular matrix (ECM), which has a key role in injury repair responses and regulation of tissue homeostasis, has been recognized in COVID-19 (Huang, J.-J. *et al.*, 2023; Rozario & DeSimone, 2010; Sarker *et al.*, 2021; Zhou, Y. *et al.*, 2018). Pathological remodelling of ECM through assembly, synthesis and degradation is essential for tissue homeostasis. These processes can be facilitated by secretion of a number of MMPs at sites of inflammation (Huang, J.-J. *et al.*, 2023). MMP-9 is known to influence inflammatory processes, such as the reparative phase, and is associated with pulmonary fibrosis (Gelzo *et al.*, 2022; Moin *et al.*, 2020). Gelzo *et al.* (2022) also pointed out a pathological role for MMP-3 in initiation of lung inflammation; as serum levels of MMP-3 increased in COVID-19 patients and decreased after one week of hospitalization (Gelzo *et al.*, 2022). Therefore, MMP-3 and MMP-9 proteins have potential as biomarkers of the severity of disease in COVID-19 patients.

The MMP protease family is associated with the development and progression of various acute and chronic lung diseases. For example, compared to patients with mild COVID-19, MMP-2 was downregulated in plasma of patients with severe disease (D'Avila-Mesquita *et al.*, 2021). This phenomenon suggests an association between MMP-2 and mortality rates in COVID-19. Similarly, elevated levels of MMP-9 in serum and plasma of patients with progressive COVID-19 were observed by Avila-Mesquita *et al.* (2021) and Gelzo *et al.* (2022). MMPs are also involved in other conditions characterised by lung inflammation. For example, upregulated MMP-28 in bronchoalveolar lavage fluid (BALF) of patients with adult respiratory distress syndrome (ARDS) is found to be correlated with elevated alveolar neutrophils

(Morrell et al., 2020). Similarly, compared with normal lung tissue, expression of MMP-7 is increased in lung tissue from patients with idiopathic pulmonary fibrosis (IPF) (Yu, G. et al., 2018).

Idiopathic pulmonary fibrosis (IPF) is a chronic and progressive lung disease, which usually has a poor prognosis and occurs in middle-aged and elderly adults (Mei et al., 2021). IPF is the most common type of pulmonary fibrosis, and the etiology is unknown as 'idiopathic' means this disease has no known cause. Excessive deposition of extracellular matrix, caused by persistent myofibroblast phenotype, in IPF has been identified as the major histopathological characteristics, which leads to aberrant lung repair and tissue scar formation (Glass et al., 2020). Furthermore, lung architecture is distorted, and lung function is lost irreversibly in patients with IPF. An assumption for the pathophysiological mechanism underlying IPF is that this interstitial lung disease is induced by abnormal epithelial-fibroblast communication, which is triggered by repetitive or sustained micro-injuries to alveolar epithelial cells (Heukels et al., 2019; Martinez et al., 2017; Richeldi et al., 2017). Subsequently, ECM is over accumulated and pathological lung is remodelled abnormally.

As invadolysin is homologous to leishmanolysin (GP63), it is also possible that extracellular invadolysin has a potential role in tissue homeostasis and/or inflammatory responses (through activation of macrophage and ECM remodelling). As mentioned, various MMPs, especially circulated forms, have association with lung inflammation, such as IPF and COVID-19. Therefore, it is worth investigating whether invadolysin, especially the extracellular form, play a role in pulmonary diseases. In the future, whether invadolysin can be served as an inflammatory marker, especially for pulmonary diseases, needs to be confirmed.

1.8 Research Questions

Much attention has been given to the cellular forms of invadolysin. However, whilst invadolysin has been identified in the soluble fraction of vertebrate blood, in invertebrate hemolymph, and in extracellular vesicles isolated from human plasma soluble fractions (Abhinav et al., 2019), the role of a secreted form of invadolysin has not been investigated in depth. Whereas, it remains to be shown whether secreted forms of invadolysin are present in vertebrate serum, how expression of extracellular invadolysin is regulated, and what physiological roles these secreted forms of invadolysin possess. Furthermore, it has not been determined whether invadolysin is enriched in specific fractions of plasma or serum?

As a novel metalloprotease, the proteolytic activities invadolysin possesses in the serum have not been determined. In addition, it remains to be shown whether it is possible to detect catalytic invadolysin in vertebrate serum and whether the proteolytic activity of extracellular invadolysin can be measured and analysed quantitatively and qualitatively. It also remains to be shown whether there are sexually dimorphic structural forms of extracellular invadolysin. Answering these questions will help to determine the roles of invadolysin in physiological pathways, such as homeostasis, metabolism, regeneration and immune response? There is also the unexplored potential of invadolysin serving as a biomarker for pathophysiological disorders.

1.9 Hypothesis and aims of the PhD project

Although various functional roles of invadolysin have been identified in vertebrates and invertebrates, the role(s) of extracellular invadolysin in normal physiology is (are) not well understood. As described in the original paper published by Abhinav *et al.* (2019) in the Heck Lab, it was clear that an extracellular form of invadolysin is present in the soluble fraction of vertebrate blood (including extracellular vesicles), and invertebrate hemolymph (sexually dimorphic forms observed). Invadolysin protein has been found attaching to distinct structures, and the expression form of invadolysin is dynamic during early zebrafish embryonic development (Vass & Heck, 2013). Thus, it has been questioned that whether the extracellular form of invadolysin has a sexually dimorphic form in vertebrates, and whether extracellular form of invadolysin is also involved in dynamic functions, like leishmanolysin/GP63, which has diverse localisations (Yao *et al.*, 2007; Abhinav *et al.*, 2019). Furthermore, this thesis would expand the knowledge of invadolysin's role(s) in early embryonic development.

To explore the understanding of the function of extracellular invadolysin, hypothesis and aims of this thesis have been established as followings:

Hypothesis: Invadolysin influences embryonic development and a novel extracellular form of invadolysin has potential as a marker of disease.

Aims:

1. To develop a biochemical strategy to identify extracellular form of invadolysin.

The first step was to validate the extracellular form of invadolysin is present in blood of vertebrates (adult human and zebrafish plasma). Extracellular form of invadolysin was isolated from human plasma using Pierce™ Top 12 Abundant Protein Depletion Spin Columns and Pierce™ Albumin/IgG Removal Kit, and the retro-orbital blood acquisition approach was utilised to extract blood proteins from adult zebrafish. Expression

forms of extracellular invadolysin was evaluated by immunoblotting with different anti-human and anti-zebrafish invadolysin antibodies. The second step was to develop a biochemical strategy to isolate and enrich extracellular form of invadolysin, which would remove high abundant proteins in blood and could be compatible with immunoblotting. Immunoprecipitation and polyethylene glycol precipitation were two major methodologies modified for this purpose. The results of these experiments are described in Chapter 3 and 4.

2. To determine whether structures of extracellular invadolysin are different in males and females.

For the second aim, extracellular invadolysin from different vertebrate models (human, zebrafish and mouse) was isolated, and the expression form(s) of invadolysin was compared between male and female to determine whether the extracellular form of invadolysin is sexually dimorphic in vertebrate blood. This research was discussed in Chapter 3, 4 and 5.

3. To explore whether invadolysin has a developmental role.

The third aim was to understand how invadolysin from dynamic localisations influences development of zebrafish embryos during early stages (48 and 72 hpf). Zebrafish (*Danio rerio*) was used as a research model to investigate the potential role(s) of invadolysin localizing in yolk and yolk-depleted embryo. Embryo dechoriation and embryo deyolking protocols have been modified to separate yolk from the whole embryo. Results are presented in the Chapter 3.

4. To determine whether extracellular forms of invadolysin can be used as markers of disease.

The fourth aim of this PhD research was to investigate whether extracellular invadolysin is important for normal physiology and the

potential of being a biomarker of any inflammatory disease. The structural forms of extracellular invadolysin were characterized in *LmIn* knockout mice (generated by the Takahashi laboratory). Understanding different structural forms of extracellular invadolysin in patients with inflammatory diseases, such as NAFLD, IPF and COVID-19, should present clues to the role(s) of extracellular invadolysin on physiology and pathophysiology. An *in vitro* model of hepatic cellular steatosis using C3A cell lines would be used to explore the role(s) of invadolysin on glycolysis and lipid metabolism in liver. This analysis was described in Chapter 5.

Chapter 2 Materials and Methods

2.1 Zebrafish lines and husbandry

Ethics and experiments on animals

When utilising animals in research, it is important to notice that animals have a moral status and treatment to animals should be subject to ethical considerations (National Research Ethics Committees, n.d.).

Key positions are described as followings:

- (i) Animals have an intrinsic value which must be respected.
- (ii) Animals are sentient creatures with the capacity to feel pain, and the interests of animals must therefore be taken into consideration.
- (iii) Our treatment of animals, including the use of animals in research, is an expression of our attitudes and influences us as moral actors.

The three Rs are also an established principles that facilitate limitations when using animals for experimental research, which is shown as followings:

Reduction- methods which minimise the number of animals involved.

Refinement- methods which minimise any harm and discomfort and can improve welfare.

Replacement- the used of methods to help avoid or replace animal involvement.

Ethical approval for this PhD research project and each collaborating study has been authorised for the use of animals and biological samples from human (Lockman et al., 2012; Gaughan et al., 2021; Takahashi Labooratory).

Ethical statement

All experiments using *Danio rerio* (zebrafish) were carried out in accordance with the accepted standards of humane animal care under the regulation of the Animal (Scientific Procedures) Act UK 1986 and EU Directive 2010/63/EU; animals were held in a UK Home Office approved and designated facility and subject to ethical review. All animals used in these

experimental studies were under the age of 5 days post fertilization (dpf), which does not require a specific license.

Husbandry and lines

Zebrafish (*Danio rerio*) were used as the animal models for protein extraction and analysis. Zebrafish husbandry, embryo collection, and maintenance were performed according to the accepted standard operating procedures (Nüsslein-Volhard & Dahm, 2002). Wild type line zebrafish were established under constant living systems: temperature 28.5°C, 14:10 hr light-dark (LD) cycle, fed on phytoplankton and *Artemia*. Embryos were collected from random mating and staged correctly (Kimmel, C. B. et al., 1995).

Zebrafish breeding and embryo collection

Fertilized eggs were obtained through in-tank breeding. In-tank breeder was dropped into the fish tank slowly after the onset of light. In-tank breeder was left in the tank around 15 minutes to 1.5 hours (depending on the required number of embryos and mating times) to allow the breeding of zebrafish before removing. Fertilized eggs were collected and raised in 35 mm petri dish (25 embryos per petri dish) and incubated at 28.5°C. Zebrafish embryos less than 120 hours post fertilization (hpf) were raised with Methylene Blue, also named as Methylthioninium Blue (MB) (6.3 μ M), without food. In aquaculture, MB is a known photosensitisers and has been used as a biocide to protect fertilized zebrafish embryos from infection (Costa et al. 2016; Robertson et al., 2009). MB is inherently bactericidal, and detection of bacteria could be observed due to uptake of blue dye (Robertson et al., 2009).

2.2 Zebrafish protein extraction

Early staged embryos dechoriation

For the purpose to extract protein from zebrafish embryos at early developmental stages, protocols for dechoriation and deyolking processes were optimised (Mascha Friedrich, personal communication).

Dechoriation is the first step before yolk separation, especially for embryos

at 3 dpf (Link et al., 2006). Zebrafish embryos were first placed in the warmed Pronase (37°C) (1 mg/mL in Ringer's solution (116 mM NaCl, 5 mM HEPES, 2.9 mM KCl, pH 7.2) and incubated for 10 to 15 minutes at 28.5°C. After incubation, dechoriation was facilitated by gently pipetting with a 1000 µl pipette tip. The embryos without chorions were washed in the 1X PBS (**P**hosphate-**B**uffered **S**aline) (137 mM NaCl, 10 mM Na₂HPO₄, 2.7 mM KCl, 1.8 mM KH₂PO₄, pH 7.4). Zebrafish embryos were then immersed in the overdosed MS-222 (Tricaine **M**ethanesulfonate) (20 mg/ml) (Sigma) for at least 10 minutes and washed in 1X PBS again.

Early staged embryos deyolking

Dechorionated embryos were transferred into a 1.5 ml Eppendorf tube first before the addition of Deyolking buffer to avoid dilution. A pipette with a 200 µl tip was used to remove 1X PBS from the tube as much as possible. After adding the Deyolking buffer (55 mM NaCl, 1.8 mM KCl, 1.25 mM NaHCO₃, freshly added 1X EDTA-free protease inhibitor (Roche)), yolk sacs were disrupted by gentle pipetting. The tube was shaken at 600 rpm for 5 minutes and centrifuged at 300 x **g** for 1 minute at 4°C. The supernatant was collected into another tube as the 'Yolk' sample. The embryonic pellet was washed twice in the embryo washing buffer (110 mM NaCl, 3.5 mM KCl, 10 mM Tris-HCl, pH 8.5) by shaking the Eppendorf tube at 600 rpm for 2 minutes and then centrifuging the Eppendorf tube at 300 x **g** for 1 minute at 4°C. After discarding the fraction of the supernatant, the pellet fraction was re-suspended in the designed buffer (depending on the experimental purposes), which was referred as the 'Embryo' sample. For the analysis of embryonic protein, zebrafish extracts were lysed in the PEB (**P**rotein **E**xtraction **B**uffer, 10 mM Tris-HCl, pH 7.4, 2% Triton X-100 (Sigma), 1X EDTA free protease inhibitor) and mashed by a hand homogenizer.

Adult zebrafish blood extraction

The adult zebrafish blood was extracted from male and female fish respectively. The adult zebrafish, after being humanely culled according to the Home Office Schedule 1 protocol, were exsanguinated by the retro-orbital blood acquisition approach (Vliegenthart et al., 2014). Briefly, one of the

eyes was removed gently by a pair of forceps after euthanasia with MS-222. The heparin solution (25 mg/ml), used to prevent blood clotting, was added into the eye socket. When the orbital space is filled with blood, the blood was transferred into an Eppendorf tube. This step was repeated as many times as possible to collect the maximum volume of the blood. Usually, ~ 5 μ L of blood can be collected from each adult zebrafish.

2.3 Human serum and plasma preparation

Human blood resource

Collection and utilization of healthy human donor blood samples was under the permission granted to the University of Edinburgh Centre for Inflammation Research Blood Resource, under ethical approval 'The role of inflammation in human immunity: AMREC Reference number 15/HV/013.

Serum preparation

The commercial tubes from SARSTEDT (S-Monovette) were used to collect human blood and process the serum preparation. The S-Monovette serum-gel tube (brown capped) contains a polyacrylic ester gel, which forms a stable layer and separates the serum from the blood clot during centrifugation. After filling in blood samples, the S-Monovette serum-gel tube was displaced in an upright position for 30 minutes clotting phase at room temperature without disturbing. The S-Monovette tube was then handled in an upright position and centrifuged at 2,500 x *g* for 10 minutes at 20°C. The supernatant (serum) from the S-Monovette tube was transferred into Eppendorf tubes and stored at -20°C. Serum samples were kept on ice during handling. For long-time storage purpose, serum samples were aliquoted and stored at -80°C. In addition, the Falcon Conical Tubes (15 ml) without anticoagulants were used to prepare serum, which follows the same protocol as the S-Monovette tube.

Plasma preparation

The commercial tubes from SARSTEDT were also used to prepare human plasma samples, which are S-Monovette Potassium EDTA and S-Monovette

Tri-Sodium Citrate tubes. The S-Monovette EDTA K₃ is pre-dosed in the tube and contributes to an average concentration of 1.6 mg EDTA/ml blood during plasma preparation. To process blood samples completely, the filling volume should be more than 80% of 2.7 ml. The S-Monovette Citrate is pre-dosed in the tube with an average concentration of 3.2% trisodium citrate and stringent to the citrate-to-blood ratio 1:9. To ensure an entire blood samples preparation, the filling volume should be as exact as 3 ml. After filling in blood samples, the tube with anticoagulation was inverted at least three times immediately to prevent blood from clotting. Blood sample was centrifuged at 2,300 x *g* for 10 minutes at 20°C. The supernatant (plasma) from the S-Monovette tube was transferred into Eppendorf tubes and stored at -20°C. Plasma samples were kept on ice during handling. For long-time storage purpose, plasma samples were aliquoted and stored at -80°C.

2.4 Human blood sample clean-up

When analysing the target proteins by immunoblotting, the high-abundance proteins interfere the detection of the target proteins, especially when the high-abundant proteins have the similar molecular weights to the target proteins. It has been shown that removing the high-abundance proteins from human plasma improves the detection of the low-abundance proteins (Mrozinski et al. n.d.; (Polaskova et al., 2010). To improve the protein examination technique, the depletion column was used to eliminate the multiple high-abundance proteins in serum and plasma samples, such as albumin and IgG (Anderson, N. L. & Anderson, N. G., 2002; Polaskova et al., 2010). The Pierce™ Albumin/IgG Removal Kit (Thermo Fisher Scientific, Cat. No.:89875) was utilised to remove human serum albumin (HSA) and gamma globulins (IgG) from human blood samples. The top twelve high-abundance proteins in human serum and plasma were removed by the Pierce™ Top 12 Abundant Protein Depletion Spin Columns (Thermo Fisher Scientific, Cat. No.:85165). The list of removed proteins are: Transferrin (80 kDa), IgM (65, 23 kDa), IgG (50, 23 kDa), IgA (55, 23 kDa), Haptoglobin (18, 45 kDa), Fibrinogen (340 kDa), Apolipoprotein A-I (28.3 kDa), Apolipoprotein

A-II (17.4 kDa), Albumin (66.5 kDa), α 2-Macroglobulin (85 kDa), α 1-Antitrypsin (54 kDa), α 1-Acid Glycoprotein (42 kDa) (Abhinav et al., 2019).

2.5 Immunoprecipitation

To isolate and purify invadolysin from protein samples, immunoprecipitation protocols were optimised for protein precipitation using protein samples from different organisms, such as zebrafish embryo (under 1 dpf) and human plasma. Both Dynabeads Protein A (Thermo Fisher Scientific, Cat No.:10002D) and Dynabeads Protein G (Thermo Fisher Scientific, Cat No.:10003D) were utilized in the research. Protein A has been found to be present in the bacterial cell walls of *staphylococcus aureus*, and Protein G originates from the bacterial cell walls of group G *streptococci* (Layer et al., 2000). Immobilized Protein A and Protein G are used in affinity chromatography since they have relative high binding affinity to the Fc region of IgG.

The precipitation protocols were designed on the basis of the provided instructions. Two different protocols were modified and tested to immunoprecipitate invadolysin from protein samples (Figure 2.1). For human plasma and zebrafish embryo, 1X RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 0.1% SDS, 0.5% Sodium Deoxycholate, 1% Triton X-100, freshly added 1X EDTA-free protease inhibitor) was used for protein lysis. For yolk samples lysis, 2X RIPA buffer (containing 245 mM NaCl) was added to the samples since the deyolking buffer contains 55mM of NaCl. Variant invadolysin antibodies utilized to capture the antigen from protein samples, such as R2192, R2357, S172 and S173 are illustrated in Table 2.1. The antigen epitopes for each antibody are shown in Figure 2.2. For each immunoprecipitation, protein samples were prepared freshly and kept on ice.

Protocol A

Antibody Capture

In the first protocol (Figure 2.1A), Dynabeads were prepared by washing 3 times in PBS-0.02% Tw20 (Scientific laboratory supplies). The magnet rack was used to separate the beads from the buffer solution. The capture antibody was prepared in 1XRIPA buffer and added to the beads after discarding the supernatant. Dynabeads were incubated with the capture antibody with rotation at 4°C overnight. After incubation, the supernatant was removed and the Dynabeads-antibody complex was resuspended in PBS-0.02% Tw20.

Crosslinking

To avoid co-elution of the antigen capture antibody, Dynabeads were crosslinked to the antibody before immunoprecipitation. The Dynabeads-Ab complex was washed 2 times in 0.2 M TEA (Triethanolamine, pH 8.2). The supernatant was discarded and the Dynabeads-Ab complex was resuspended in 20 mM DMP (Dimethyl pimelimidate dihydrochloride, in 0.2 M TEA, pH 8.2) (Sigma, Cat No.:D8388) and incubated for 30 minutes at room temperature with rotation. To quench the cross-linking reaction, the Dynabeads-Ab complex was incubated in 50 mM Tris (pH 7.5) for 15min at room temperature with rotation after discarding the cross-linking buffer. The cross-linked Dynabeads were washed 3 times in PBS-0.02% Tw20.

Immunoprecipitation

After discarding the supernatant, the protein sample prepared in 1XRIPA buffer was added to the Dynabeads-Ab complex by gently pipetting. The protein extract containing the antigen was incubated with the Dynabeads-Ab complex at 4°C overnight with rotation. The Dynabeads-Ab-Ag complex was washed 3 times in PBS-0.02% Tw20 for 10 minutes with gentle pipetting and the supernatant was discarded between each wash. After the final wash, the Dynabeads-Ab-Ag complex was resuspended in PBS-0.02% Tw20 and the bead suspension was transferred to a clean Eppendorf to avoid co-elution of proteins bound to the tube wall.

Elution of target antigen

The supernatant was discarded and the Dynabeads-Ab-Ag complex was resuspended in pre-heated (65°C) elution buffer (3X sample buffer with freshly added 20 mM DTT) by gently pipetting. The elution buffer containing the Dynabeads-Ab-Ag complex was heated at 100°C for 10 minutes. After elution, the supernatant was preserved for further analysis.

Protocol B

Antigen Capture

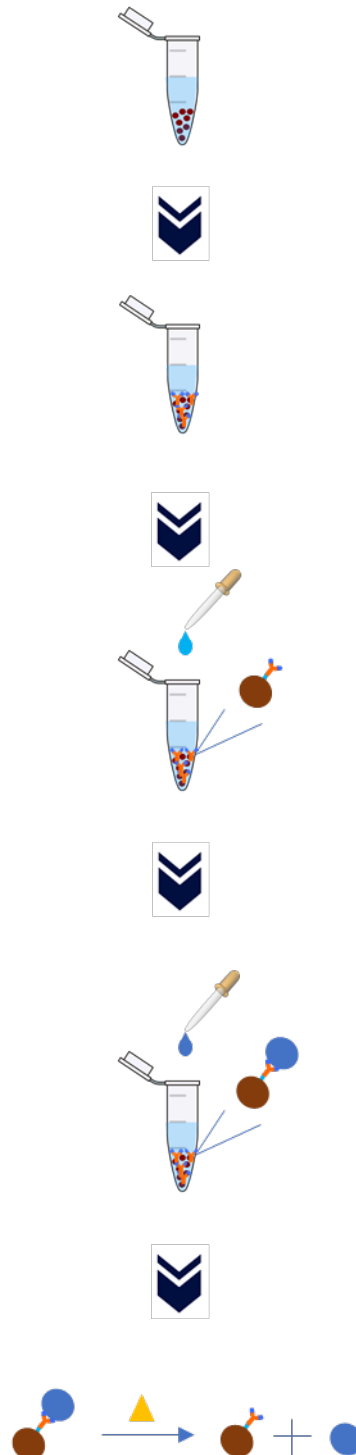
In the second protocol (Figure 2.1B), the protein sample (prepared in 1XRIPA buffer) was incubated with antibody (diluted in 1XRIPA buffer) at 4°C overnight with rotation.

Immunoprecipitation

After incubation, the antibody-antigen complex in solution was captured by prepared Dynabeads (as protocol A) for 4 hours at room temperature with rotation. The Dynabeads-Ab-Ag complex was washed 2 times in PBS-0.02% Tw20 for 10 minutes with gentle pipetting. The Dynabeads-Ab-Ag complex was further washed once in a different washing buffer (20 mM HEPES, 5 mM MgCl₂, 300 mM NaCl, 0.1% Triton X-100) before elution. The supernatant was discarded between each wash. No cross-linker was used in protocol B. Elution of target antigen followed protocol A.

A.

- Antibody
- Cross linker
- Dynabead
- Antigen
- Boiling



B.

- Antibody
- Dynabead
- Antigen
- Boiling



Figure 2.1 Schematic flowchart of immunoprecipitation.

Protocol (A) and protocol (B) were established for protein isolation from human plasma samples. A. Cross-linker DMP was used to strengthen the bonding force between Dynabeads and antibody before antigen capture. The target antigen was eluted from the Cross-linked Dynabeads-Ab-Ag complex by heating at 100°C. B. Dynabeads were used to capture the antibody-antigen complex without cross-linker. Target antigen was eluted from the Dynabeads-Ab-Ag complex by heating at 100°C.

Table 2.1 Invadolysin primary antibodies

Antibody	Species	Host Animals	Dilutions	Manufacturer	Antigen Sequences	Region of Invadolysin
R4100	<i>Dm</i>	<i>Rabbit</i>	1:500	Genosphere Biotech.	CNATGQNCRIDSNTQ	Lower middle of N-terminal sequence before HEXXH
A6971	<i>Dr</i>	<i>Rabbit</i>	1:250	Genosphere Biotech.	YCDSVRSAPLQLTC	Middle of C-terminal sequence after HEXXH
R4624	<i>Hs</i>	<i>Rabbit</i>	1:1000	Genosphere Biotech.	CSLGSSPPCRHHVPS	Variant 1 signal sequence
R4888	<i>Hs</i>	<i>Rabbit</i>	1:1000	Genosphere Biotech.	SGLLGRLPGPEPVAC	Variant 2 N-terminal sequence
G3646	<i>Hs</i>	<i>Rabbit</i>	1:1000	Genosphere Biotech.	YRGGKWPHGAVGVP	Lower middle of N-terminal sequence before HEXXH
R4598	<i>Hs</i>	<i>Rabbit</i>	1:1000	Genosphere Biotech.	CDFVRKSCKFWIDQQ	Spliced exon
R2192 R2357 S172/S173	<i>Hs</i>	<i>Rabbit</i> <i>Rabbit</i> <i>Sheep</i>	1:1000 1:1000 1:1000	PTU/BS (Diagn Scot); Diagnostics Scotland	Fusion protein C-term amino acids 327-629 of $\Delta 37$ variant	His tagged fusion protein expressed in bacteria
G6456	<i>Hs</i>	<i>Rabbit</i>	1:1000	Genosphere Biotech.	DPPATNLTRALPLD	Glycosylation site

Investigating the Essential Extracellular Invadolysin Metalloprotease

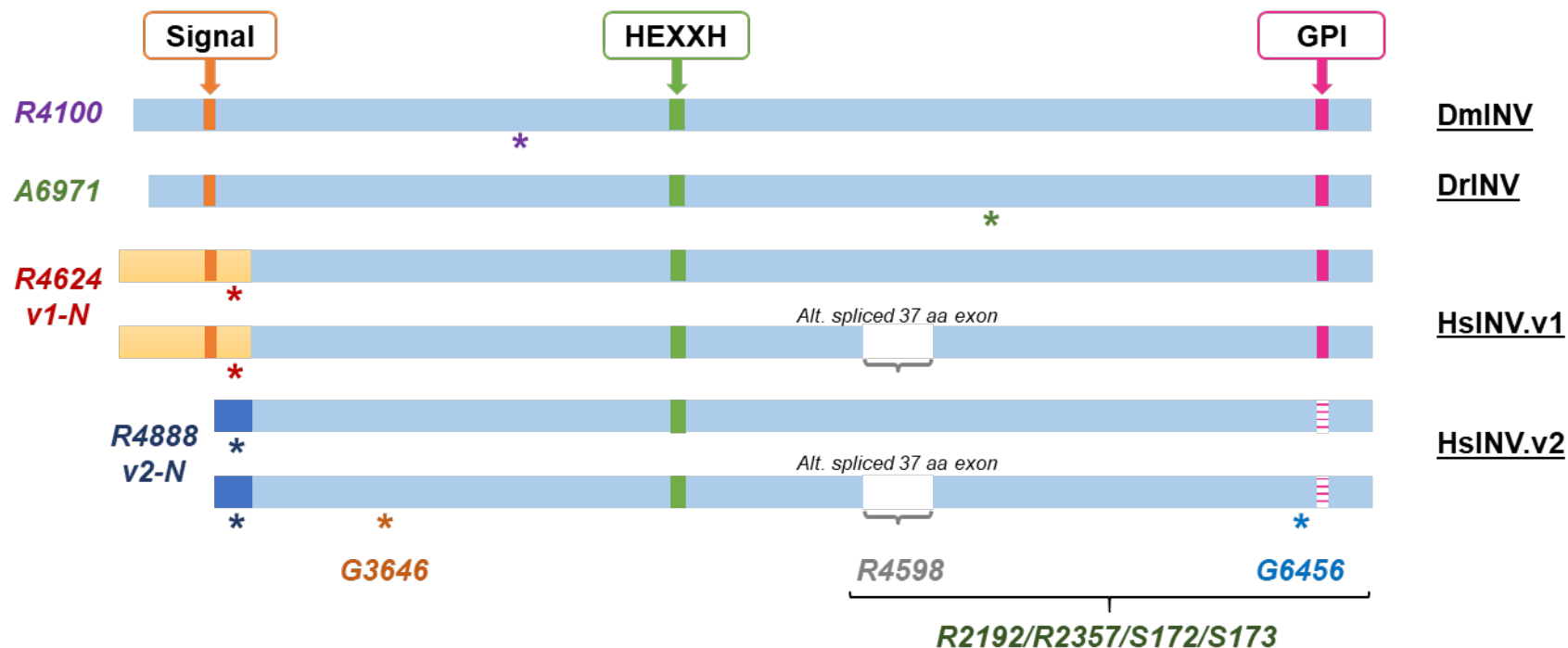


Figure 2.2 Schematic presenting invadolysin antibody epitopes in invertebrate and vertebrate sequences.

The schematic showing invadolysin sequences in invertebrate and vertebrate. Sequences displaced as follows: Dm, *Drosophila melanogaster*; Dr, *Danio rerio*; Hs, *Homo sapiens*. Invadolysin antibody epitopes and antigenic regions are highlighted as asterisks or labelled by brackets.

2.6 Polyethylene glycol precipitation

Polyethylene glycol 6000 (Millipore, Merck Life Science, UK, Cat No.:528877) was used to precipitate proteins from human blood and other protein samples (Briggs et al., 1981; Haskó et al., 1982; Hönig & Kula, 1976; Wang, X. et al., 2011). PEG precipitation protocol has been developed to fractionate blood serum and plasma samples (Abhinav et al., 2019). Equal volumes of protein samples and PEG 6000 solution (usually 8% and 16% in DPBS, **D**ulbecco's **P**hosphate **B**uffered **S**aline (1X), Gibco, Cat No.:14190-094) were mixed thoroughly by pipetting gently. The mixture was incubated on a rotator at 4°C for 30 minutes to produce selective precipitation of proteins. The precipitated proteins were separated by centrifugation at 1000 x **g** and 4°C for 20 minutes. The supernatants were collected in Eppendorf tubes and kept on ice. The precipitated proteins were dissolved in DPBS (an equal volume of the starting protein samples) and incubated at 37°C for at least 30 minutes. During the incubation, the protein sample solution was pipetted gently to ensure the precipitated proteins were dissolved sufficiently. The protein supernatant and pellet samples were aliquoted and stored at -20°C for further study (Figure 2.3).

Investigating the Essential Extracellular Invadolysin Metalloprotease

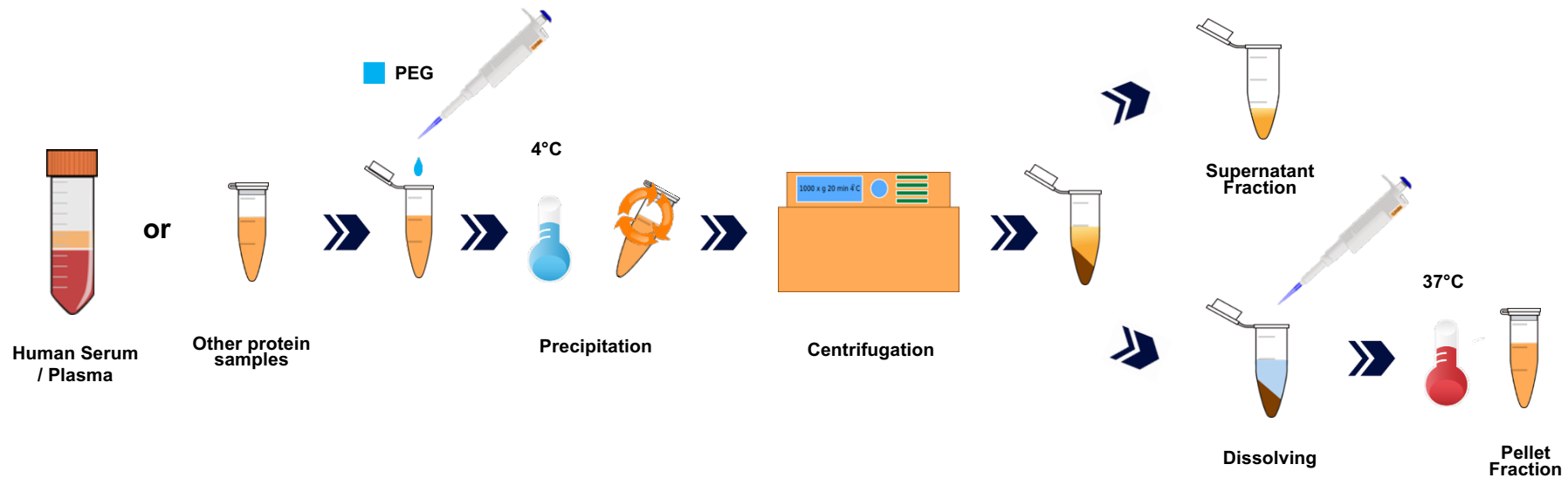


Figure 2.3 Schematic flowchart of the process of polyethylene glycol precipitation.

This protocol was modified to enrich selective proteins from human blood and other protein samples. After collection of freshly prepared human serum and plasma samples, PEG solutions at different concentrations were used to precipitate proteins from blood samples. Protein samples from both supernatant and pellet fractions were used for further analysis.

2.7 Cell culture

To further understand the physiological function of invadolysin in non-alcoholic fatty liver disease, *in vitro* models of hepatic cellular steatosis were established by using human hepatoblastoma C3A cells (Lockman et al., 2012). The C3A cells were kindly gifted by the Plevris Laboratory (University of Edinburgh). The cell culture and passage procedures were following the instructions as in the Hepatology Laboratory. In addition, human HeLa cells derived from cervical cancer cells were examined as a control group in complement with C3A cell line.

Cell culture and passage

C3A cells from the liquid nitrogen were thrown and seeded in 25 cm² tissue culture flasks (T25) and cultured in MEME⁺ (**M**inimum **E**ssential **M**edium **E**agle, Sigma, Merck Life Sciences, UK, Cat No.:M0268; 10% **F**etal **B**ovine **S**erum, Sigma, Merck Life Sciences, UK, Cat No.:12103C; 1% Penicillin-Streptomycin, 100 units/mL penicillin, 100 µg/mL streptomycin, Sigma, Merck Life Sciences, UK, Cat No.:P4333) at 37°C and 5% CO₂ until 70-80% confluent. The cell culture medium was changed every 48 hours and further cell passage was processed every 7-10 days. C3A cells in T25 flasks were transferred into 75 cm² tissue culture flasks (T75) for further experiments. HeLa cells were cultured with MEME⁺ and incubated under the same conditions as C3A cells. HeLa cells were passaged every 5-7 days and grown in T75 flasks.

During cell passaging, HBSS (**H**ank's **B**alanced **S**alt **S**olution, Sigma, Merck Life Sciences, UK, Cat No.:H9394) was used to wash cells by gently moving the flasks and cells were incubated with Trypsin-EDTA solution (Merck Life Sciences, UK, Cat No.:T3924) at 37°C and 5% CO₂ for 3-5 minutes. Once cell dislocation was observed under the microscopy, MEME⁺ was added to each flask to neutralise the digestion reaction by shaking the flasks. For cell collection, cell mixture was collected in the Falcon tube and centrifuged at 600 x **g** for 5 minutes at RT. The supernatant was discard and cells were re-suspended in MEME⁺. After counting the cells, C3A cells were seeded to

T75 flasks with 3×10^5 cells/ cm² cell density and HeLa cells were seeded with a subcultivation ratio of 1:20.

Cell treatment

MEME⁺ supplemented with octanoate (2 mM) (Sigma, Poole, UK, Cat No.:C2875), oleic acid (0.25 mM) (Sigma) and glucose (20 mM) (D-(+)-Glucose, Sigma-Aldrich, Cat No.:G8270) were prepared for cell treatment. Cells were seeded on the 8-well cell culture dish and cultured until 70% confluent. Before treatment experiments, cells were starved for 24 hours to reduce the influence of invadolysin from FBS within MEME⁺. C3A and HeLa cells were cultured in the supplemented media for 24 hr, 48 hr, 72 hr and 96 hr. MEME⁺ (glucose: 5.5 mM) supplemented with glucose at concentrations of 5.5 mM, 27.5 mM and 55 mM were prepared to examine the relationship between invadolysin and glucose metabolism. HeLa cells were seeded on the 8 well cell culture dish and expanded until 70% confluent. Different glucose supplemented media were utilised to treat HeLa cells for 24 hr, 48 hr, 72 hr, and 96 hr after 24 hr starvation. Cell media were refreshed every 48 hr. After each incubation time point, cell culture media and cells were collected for further examination.

Cell culture lysate preparation

Cell culture media from each treatment group was aspirated and kept frozen at -80°C until further analysis. Cells were washed twice with pre-cooled 2 ml of DPBS. Ice-cold RIPA lysis buffer (150 mM NaCl₂, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) was added to cell culture dish to harvest cells gently after discarding DPBS. A cold plastic cell scraper was used to scrape cells off the dish and the cell mixture was transferred to a pre-cooled Eppendorf tube and incubated at 4°C for 30 minutes on a rotator. Cell samples were centrifuged at 12,000 rpm and 4°C for 20 minutes. The tubes were placed on ice and the supernatant was removed to a new tube. The supernatant was kept at -80°C and the pellet was discarded.

2.8 SDS-PAGE and immunoblotting

SDS-polyacrylamide gel electrophoresis

For SDS-PAGE and immunoblotting analysis, the SDS-PAGE sample buffer with freshly added DTT (20 mM) was pre-heated at 65°C and mixed with protein samples. SDS-PAGE sample buffer contains 2% SDS, 50 mM Tris pH 6.8, 10% (w/v) Glycerol, 0.01% (w/v) Bromophenol blue, 2 mM EDTA. Proteins can be denatured by SDS and become negatively charged conferred by SDS (NOVUSBIO n.d.). In this way, proteins can be separated during electrophoresis depending on the respective molecular weight instead of intrinsic electrical charge. Proteins' density is elevated by glycerol, which renders proteins sink down to the well bottom, and promotes even protein loading and minimizes sample overflow. EDTA is a chelating agent that inhibit activities of enzymes in samples by binding to divalent metal ions, such as calcium and magnesium. When migrating during electrophoresis, proteins can be visualized caused by the addition of bromophenol blue, which is an anionic dye. Because the size of bromophenol blue is relative small, migration of bromophenol blue is faster than protein samples, and the front migration benefits to monitoring the electrophoresis progress to prevent running off of samples from the gel.

Protein samples in sample buffer were sonicated three times at 5-10 amplitude for 5 second and boiled at 100°C for 10 minutes. The protocol for SDS-PAGE was following Laemmli's protocol (Laemmli, 1970). Protein extracts in sample buffer were loaded on polyacrylamide gels (NuPAGE 4-12% Bis-Tris Gel-Thermo Fisher Scientific, Cat No.:NP0322; RunBlue 8% Bis-Tris Gel-Expedeon, Cat No.:NXG00812; TruPAGETM Precast Gels 4-8%, Sigma-Aldrich, Cat No.:PCG2017-10EA). The gel was electrophoresed in 1XMOPS buffer (0.8 mM EDTA, 50 mM MOPS 3-(N-morpholino) propansulfonic acid), 50 mM Tris Base, 0.1% SDS) for 45 to 60 minutes at 150V under room temperature.

Immunoblotting

Proteins in polyacrylamide gels were transferred to 10 cm X 10 cm nitrocellulose blotting membranes (GE Healthcare by life sciences) in Towbin buffer (192 mM glycine, 25 mM Tris Base, 20% methanol, 0.1% SDS). The gel and nitrocellulose blotting membranes were prepared in a glass tray containing Towbin buffer (~1.5 L without SDS) in the following order: (negative electrode)-sponge-blotting filter paper-gel-nitrocellulose blotting membrane-blotting filter paper-sponge- (positive electrode). This combination was secured in a cassette, which was then ensembled in the transfer apparatus (BioRad Trans-Blot). The transfer was performed at 300 mA for 3 hours at 4°C. The following incubation and rinsing steps were processed with gentle agitation at room temperature except for a specific statement. After protein transfer, nitrocellulose membranes were stained with 25 ml of Ponceau S solution (5% w/v, 1% acetic acid) for 1min. Membranes were then rinsed in 50 ml of dH₂O for 3 minutes to remove excess stain. The situation of transferred proteins was examined in order to detect any complications, such as ineffective transfer and air bubbles. Nitrocellulose membranes were rinsed with PBS-Tw20 (2.7 mM KCl, 1.5 mM KH₂PO₄, 136.9 mM NaCl, 8.1 mM Na₂HPO₄, 0.1% Tween 20 (Fisher Scientific), pH 7.4) for 5 minutes. Background protein blocking was performed using either 5% milk powder solution (for fruit fly and zebrafish samples; prepared with PBS-Tw20) or 3% Bovine Serum Albumin solution (for cell culture and human blood samples; prepared with PBS-Tw20; Sigma, Cat. No.:SLBX6184) for 1 hr and membranes were washed 3 times with PBS-Tw20 for 15 minutes. This procedure prevents antibodies, both primary and secondary antibodies, from binding to non-specific background proteins on immunoblots. As shown in Table 2.1, a list of primary antibodies was used to probe nitrocellulose membranes overnight at 4°C. Primary antibodies were prepared differently depending on the experimental purposes, such as 1:10,000 in 0.5% milk or 0.3% BSA. Followed by primary antibody incubation, nitrocellulose membranes were washed 3 times with PBS-Tw20 for 15 minutes at room temperature.

Both ECL (Enhanced Chemiluminescent) detection system and LI-COR detection system were utilized for primary antibody detection. For the ECL detection system, the HRP-conjugated secondary antibody (GE Healthcare) (Anti-Rabbit-HRP: 1:10,000 in PBS-Tw20; Anti-Sheep-HRP: 1:20,000 in PBS-Tw20) was prepared and utilized to incubate membranes for 1 hr. Nitrocellulose membranes were rinsed 3 times with PBS-Tw20 for 15min and incubated with 1.5 ml of Detection Reagents (GE Healthcare) for one minute. Nitrocellulose membranes were sandwiched between plastic membranes and excess ECL solution was drained by tissue tower. The chemiluminescence signal was captured by the Lumi-Film Chemiluminescent detection film (Roche) and the film was developed by the Compact X 4 automatic X-ray film processor. For the LI-COR detection system, IRDye 680 RD secondary antibody (LI-COR, Cat No.:C80116-07) and IRDye 800 CW secondary antibody (LI-COR, Cat No.: C30411-01) were used to bound membranes for 30 to 60 minutes with gentle agitation. Nitrocellulose membranes were rinsed 3 times with PBS-Tw20 for 5 minutes. During and after secondary antibodies incubation, nitrocellulose membranes were protected from light using aluminum-foil paper or lightproof coverage. The fluorophore signal was detected by the Odyssey CLx Infrared Imaging System (LI-COR).

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2.9 Analysis software and application

The immunoblotting images were captured by Odyssey DLx LI-COR Imaging System and the protein band signals were analysed by Odyssey CLx under the Image Studio Acquisition Software. The molecular weight from the immunoblotting images was estimated by the algorithm, which was designed by Dr. Neville Cobbe in the Heck Laboratory. Invadolysin signal strength from the immunoblotting images were collected by Image J and graphed by GraphPad Prism 8.0 software. The relationship between cellular treatment and treatment duration was assessed by two-way ANOVA. A value of $P < 0.05$ was considered statistically significant.

Chapter 3 Investigation of the secreted form of invadolysin in vertebrate

3.1 Introduction

As introduced, invadolysin has two variants (each contain two spliced forms) in human, two splice variants in zebrafish and one variant in *Drosophila* (Vass & Heck, 2013) (Table 3.1). *In silico* sequence analysis has revealed that invadolysin has a potential N-terminal signal sequence, suggesting it has a secreted protein form (Abhinav et al., 2019). This was supported by identification of an extracellular form in the soluble fraction of *Drosophila* hemolymph, which is functionally equivalent to vertebrate blood (Abhinav et al., 2019; Wyatt, G. R. et al., 1956). This work also demonstrated that extracellular invadolysin in the hemolymph fraction from adult *Drosophila* exhibits sexually-distinct, structurally-different expression forms (Abhinav et al., 2019).

Hemolymph of *Drosophila* functions as vertebrate blood, which includes soluble plasma and a cellular component, such as crystal cells, plasmatocytes, lamellocytes and precursor cells (Kurucz et al., 2007; Wyatt et al., 1956). Invadolysin is present in *Drosophila* plasma and this extracellular form differs from male (111 kDa) and female (46 kDa). In addition, invadolysin in *Drosophila* gonads demonstrate similarity in molecular weight observed in male (testes) and female (ovaries) respectively (Abhinav et al., 2019). Inhibition of protein secretion by drugs (a cocktail of 10.6 μ M Brefeldin A/ 2 μ M Monensin or 60 μ M Tunicamycin) in *Drosophila* decreased the level of invadolysin in male plasma but not in female plasma. It has been hypothesized that biosynthesis pathways regulating deposition of extracellular invadolysin in male is different from female hemolymph (Abhinav et al., 2019).

Invadolysin is localised on the surface of lipid droplets, and it has been postulated that invadolysin play a role in metabolism regulation (Cobbe et al., 2009). The composition of lipid droplet is a lipid ester core and a surface

phospholipid monolayer (Fujimoto and Parton 2011). Triglycerides and cholesterol esters are the major components of lipid ester. The 'hatching' model is a classic hypothesis that explains the biogenesis of lipid droplets (Ploegh 2007). The phospholipid monolayer covers a homogeneous lipid ester core filled with proteins (Fujimoto and Parton 2011). The lipid ester globule is derived from the cytoplasmic leaflet of the ER membrane via budding. Localisation of invadolysin on the surface of lipid droplets is similar to protein components of lipid droplets, such as PAT family proteins (ADPR and TIP47), which appear as a ring-like structure surrounding the surface of lipid droplets (Cobbe et al., 2009). TIP47 is the first documented exchangeable lipid droplet protein, which is stable in the cytosol and is responsible for lipid loading by (Díaz & Pfeffer 1998). Transportation of TIP-47 is mediated from the endosomal compartment to the trans-Golgi network. Other functions of TIP-47 can also make a contribution to lipid-loading through recruitment of TIP-47 to lipid droplets. Thus, it has been suggested that invadolysin is anchored to the surface of lipid droplets, potentially by GPI-anchored, it regulates adipocyte differentiation progress, and potentially play a role in lipid loading (Cobbe et al., 2009; Bickel et al., 2009).

Further exploration using proteomics proved that the extracellular form of invadolysin could be detected in extracellular vesicles present in human plasma. As invadolysin is a highly conserved metalloprotease among vertebrates and invertebrates, it is important to determine whether the secreted form is present in other eukaryotes (Abhinav et al., 2019; Cobbe et al., 2009; McHugh et al., 2004). A 53 kDa form of invadolysin has been identified in mouse serum and plasma fractions, and is also present in the mouse blood cell fraction (Abhinav et al., 2019). Furthermore, a similar extracellular form of invadolysin has been detected in both rat and pig plasma.

Proteomic analysis is commonly used to establish blood proteome profiles, not only for blood proteins, but also for extracellular proteins secreted from various tissues (Babaei et al., 2013; Li, C. et al., 2016). Zebrafish blood is

Table 3.1 Predicted molecular weight of invadolysin in different species.

Invadolysin Splice Variant	Amino Acids	Predicted Molecular Weight (kDa)	Cleaved Region
HsINV (v1+37)	692	78.12	N/A
HsINV (v1 Δ 37)	655	73.58	(Δ 37) 4.54
HsINV (v2+37)	640	72.79	N/A
HsINV (v2 Δ 37)	603	68.25	(Δ 37) 4.54
DrINV (v1+37)	664	74.66	N/A
DrINV (v2 Δ 37)	627	70.1	(Δ 37) 4.56
DmINV	683	76.98	N/A

widely used as a model to improve understanding of the plasma proteome for the following reasons: zebrafish plasma provides a good model of proteins in human plasma; albumin is absent from zebrafish plasma, which makes proteomic analysis more straight-forward; similar gender-specific expression differences in other plasma proteins have been identified both in zebrafish and in humans (Shaik et al., 2014). For example, several male-dominant plasma proteins have been reported in zebrafish, which are related to cell growth, coagulation and immunological responses (Babaei et al., 2013).

The invadolysin mutation, when it was first characterised in *Drosophila melanogaster*, was shown to give rise to abnormal condensed chromosomes and have a lethal effect on the late larval stages (McHugh et al., 2004). *Drosophila* invadolysin mutants have an impaired energy status, with reduced triglyceride, suggesting that invadolysin might play important roles in cell division and cell migration during development (Cobbe et al., 2009; McHugh et al., 2004). However, it is not clearly understood how the expression of invadolysin is controlled during early developmental stages. It has been hypothesised that regulatory expression of invadolysin is dynamic, since variant forms of invadolysin occur within zebrafish embryos (0-120 hpf) (Vass & Heck, 2013). In this study, analysis of the Morpholino induced knockdown in the zebrafish embryo demonstrated that invadolysin might play crucial roles in cell migration, neuromast deposition, and angiogenesis. The yolk contributes a high proportion of the proteins present in zebrafish embryos, which could complicate proteomic analysis performed using early embryos (Link et al., 2006; Shaik et al., 2014). Removal of yolk proteins improves the signal intensity of embryo proteins when they are analysed by immunoblotting (Link et al., 2006). Proteomic analysis of zebrafish embryos revealed that proteins with a role in energy production and cell cycle control show a higher abundance at 3 dpf compared to 5 dpf (Lucitt et al., 2008). As a consequence, deyolking the zebrafish embryo would provide an opportunity to investigate whether invadolysin plays an important role in cellular progression or the growth of organisms.

3.2 Hypothesis and aims of Chapter 3

This chapter aims to validate the extracellular form of invadolysin in different vertebrate models (thesis aim one). Then, whether extracellular form of invadolysin has sexually dimorphic forms in vertebrate blood would be investigated (thesis aim two). Following, this chapter will discuss whether dynamic localised invadolysin has a role in early embryonic development in zebrafish (thesis aim three).

Hypothesis: Extracellular invadolysin is present in blood in sexually dimorphic forms and contributes to developmental changes in vertebrates.

Aims:

1. To confirm that extracellular invadolysin is present in human and zebrafish plasma.
2. To determine whether invadolysin is present in different structural forms in male compared with female zebrafish.
3. To determine whether extracellular invadolysin has a role in zebrafish development.

3.3 Results

3.3.1 Detection of the secreted form of invadolysin in human plasma

Previous research suggests that invadolysin is a component of the soluble fraction of human plasma (Abhinav et al., 2019). To determine whether invadolysin is enriched in a specific fraction of human blood, blood sample preparation methodologies need to be developed and modified. It has been proved that depletion of the highly abundant proteins from human blood samples improves the detection of invadolysin with qualified resolution (Abhinav et al., 2019). Therefore, elimination of high abundance proteins in human plasma was involved for blood sample preparation. Table 3.4 displays the highly enriched proteins in human plasma, such as albumin and gamma globulins. After ‘clearing up’ the abundant proteins, human plasma samples would be used for analysis of invadolysin.

Whole human plasma and ‘cleared’ plasma samples were used to compare the influence of highly enriched proteins on detecting invadolysin in blood. After removal of albumin and IgG, ‘cleared’ and whole human plasma were loaded equivalently on polyacrylamide gels. The Coomassie blue stained gel documents that abundant proteins ranging around 50 kDa to 70 kDa were absent in ‘cleared’ plasma (Figure 3.1B). To further examine invadolysin in blood fractions, various antibodies recognizing distinct epitopes of human invadolysin were utilized by immunoblotting. A secreted form of invadolysin at 55 kDa was detected in ‘cleared’ plasma by G3646, R4597 and G6456 antibodies (Figure 3.1C-E). This form of invadolysin migrates to ~51 kDa instead of 55 kDa in whole plasma sample without depletion. This protein band was difficult to distinguish from whole plasma fraction, which might be owing to the proximity of albumin and immunoglobulin heavy chain in plasma (Abhinav et al., 2019). The 51 and 64 kDa extracellular forms of invadolysin were detected in whole plasma sample by S173 antibody, which targets to the C-terminal fusion amino acids of $\Delta 37$ variant (Figure 3.1A and F). In the meanwhile, the 64 kDa form is present by R4597 antibody, which also recognizes epitopes on $\Delta 37$ variant (Figure 3.1D). For both R4597 and S173

antibodies, the 51 kDa form of invadolysin was not shown in the 'cleared' plasma sample. However, after depleting abundant proteins from human serum and plasma, R4597 recognized the 51 kDa form of invadolysin in both serum and plasma (Figure 3.1G). The antibody R4597 probed another form of invadolysin at 58 kDa in plasma sample other than 64 kDa (Figure 3.1 D). The same anti-invadolysin antibody (R4597) recognized invadolysin with different molecular forms (Figure D and G). Protein bands migrating above 64 kDa (Figure 3.1 C-F) were more enriched in the whole plasma or not shown in the cleared sample, which may imply that these bands are abundant proteins, such as albumin (66.5 kDa), transferrin (80 kDa) and α -macroglobulin (85 kDa) (Table 3.4). Other proteins bands migrating between 39 and 51 kDa were demonstrating the similar patterns, which could be abundant protein α 1-acid glycoprotein (42 kDa). These results verify that abundant proteins in human plasma have an effect on protein migration on immunoblots. Removal of abundant proteins from blood could improve the detection of invadolysin by immunoblotting to some extent.

3.3.2 Sexually dimorphic form is not detected in zebrafish plasma

As introduced previously, an extracellular form of invadolysin is present in invertebrate and displays sexual-specific differences in adult hemolymph (Abhinav et al., 2019). Thus, it is intriguing to determine whether sexually dimorphic forms of invadolysin could also be detected in vertebrate blood. To further research the extracellular form of invadolysin in higher eukaryotes, adult zebrafish blood from both male and female was utilized for proteomic study.

The A6971 antibody against zebrafish invadolysin recognizes a form of invadolysin at 55 kDa in both male and female zebrafish plasma (Figure 3.2 B). This extracellular form of zebrafish was also probed by two other different anti-human invadolysin antibodies R2357 and G6456 (Figure 3.2 C and D). In addition, these two antibodies recognized a 51 kDa form of invadolysin for both genders. Immunoblotting of adult zebrafish plasma by

G6456 further detected two forms of invadolysin migrating at 65 and 76 kDa in both male and female (Figure 3.2 D). These results support that extracellular form of invadolysin is detectable in adult zebrafish plasma, which was proved by antibodies generated for both zebrafish and human invadolysin. The secreted form of invadolysin in male and female exhibits substantial similarity in molecular weight, which was labelled by both anti-zebrafish and anti-human invadolysin antibodies. These observations indicate the presence of a secreted form of invadolysin in adult zebrafish plasma. Furthermore, these results imply that sexually dimorphic form of invadolysin is not detectable in zebrafish plasma.

3.3.3 Invadolysin exhibits dynamic characteristic in zebrafish yolk depleted embryo and yolk sac

Studies have demonstrated that invadolysin plays important roles in *Drosophila* development, which associates with cellular mitosis and cell migration (Cobbe et al., 2009; McHugh et al., 2004). Compared to wild-type larvae, *invadolysin* mutant larvae display reduced ATP levels and the developmental progress is arrested due to activation of AMP kinase (6 et al., 2013). Therefore, an essential role of invadolysin has been deduced in invertebrate developmental stages. Research of invadolysin in zebrafish embryos at 0- 120 hpf discovered variant expression forms of invadolysin as embryo are developing, which sheds lights on the function of invadolysin during vertebrate development (Vass & Heck, 2013). In order to further characterize the physiological feature of invadolysin in developmental stages in vertebrate, zebrafish embryo model was used for analysis.

After dechoriation, deyolking procedure was requested before examining invadolysin in zebrafish embryos at 48 and 72 hpf. When examining zebrafish embryo proteins, depletion of yolk improves the detection quality by immunoblotting (Link et al., 2006). Invadolysin in yolk and yolk depleted embryo were compared to control embryo at both developmental stages. The Commisive blue stained gel have shown that the amount of proteins around 60 to 80 kDa and 20 to 25 kDa in yolk were removed from the embryo

at 48 hpf and 72 hpf (Figure 3.3 A and B). Immunoblotting of invadolysin in zebrafish embryo was detected by antibody A6971 and R2357 (Figure 3.3 C and D). At 48 and 72 hpf, a form of 55 kDa was observed in zebrafish embryo. When compared to yolk sample, the 55 kDa form of invadolysin is accumulated in yolk depleted embryo at 48 hpf. At 72 hpf, this form of invadolysin is presented in yolk sample instead of yolk depleted embryo. In addition, a band of 22 kDa was detected in zebrafish embryo, which is enriched in zebrafish yolk as developing. When examining the same zebrafish embryo samples with anti-human invadolysin R2357 antibody, similar expression pattern was observed (Figure 3.3 D). These results reveal that there are two abundant forms of invadolysin in zebrafish embryos (48 and 72 hpf) with molecular weight of 22 and 55 kDa. Table 3.3 summaries structural forms of invadolysin with corresponding localisations in zebrafish. The discovery of different expression forms of invadolysin in yolk and yolk depleted embryo of zebrafish suggested that localisation of invadolysin is dynamic in zebrafish embryos and adult plasma.

Investigating the Essential Extracellular Invadolysin Metalloprotease

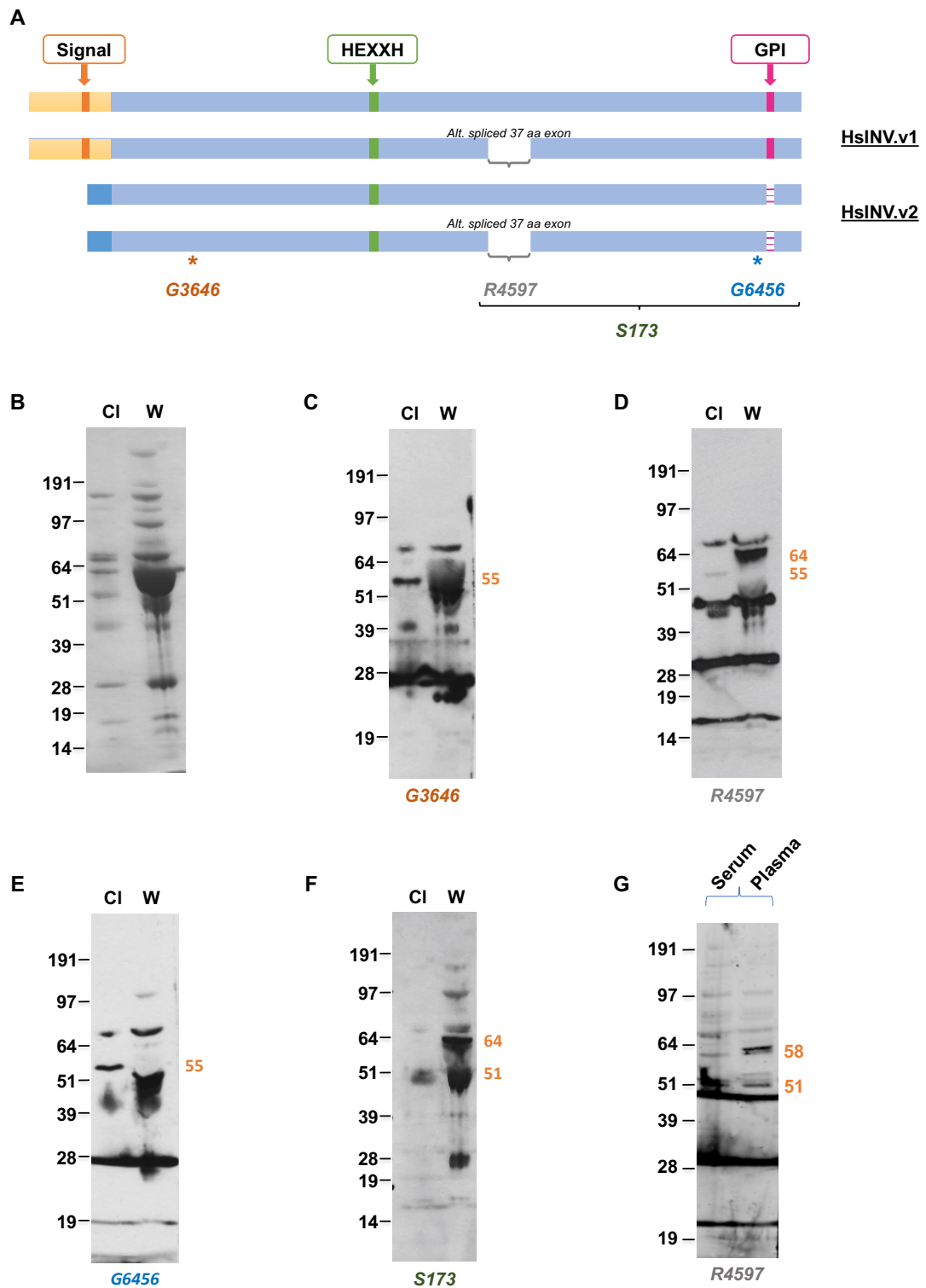


Figure 3.1 Removal of abundant proteins from human plasma improves detection of invadolysin by immunoblotting.

(A) Schematic representing four human invadolysin variants (HsINV.v1+37, HsINV.v1Δ37, HsINV.v2+37 and HsINV.v2Δ37) with highlighted predicted signal sequence (orange), conserved metalloprotease motif (HEXXH)(green) and GPI-anchor addition site (deep pink). Asterisks indicating the antigenic regions recognised by anti-invadolysin antibodies G3646 and G6456. Two other anti-human invadolysin antibodies R4597 and S173 target regions are labelled as brackets. **(B)** Coomassie blue-stained gel compares human plasma protein levels before (W:whole) and after (Cl:cleared) removal of albumin and IgG by the commercial kit. Equivalent volume for each sample was loaded onto the gel (0.3 µl per lane). Depletion of a dominant percentage proteins between 50 kDa to 70 kDa was observed. **(C-F)** Immunoblotting of human cleared and whole plasma samples were probed by anti-invadolysin antibodies G3646, R4597, S173 and G6456. A 55 kDa form of invadolysin was detected by G3656, R4597 and G6456. The secreted form of invadolysin at 64 kDa was enriched in the whole plasma, which was probed by both R4597 and S173. A 51 kDa form of invadolysin was also detected in the whole plasma probed by S173. **(G)** Human serum and plasma probed with R2357 anti-invadolysin antibody after 12 abundant proteins have been removed by Pierce™ Top 12 Abundant Protein Depletion Spin Columns. Biological replications n=3.

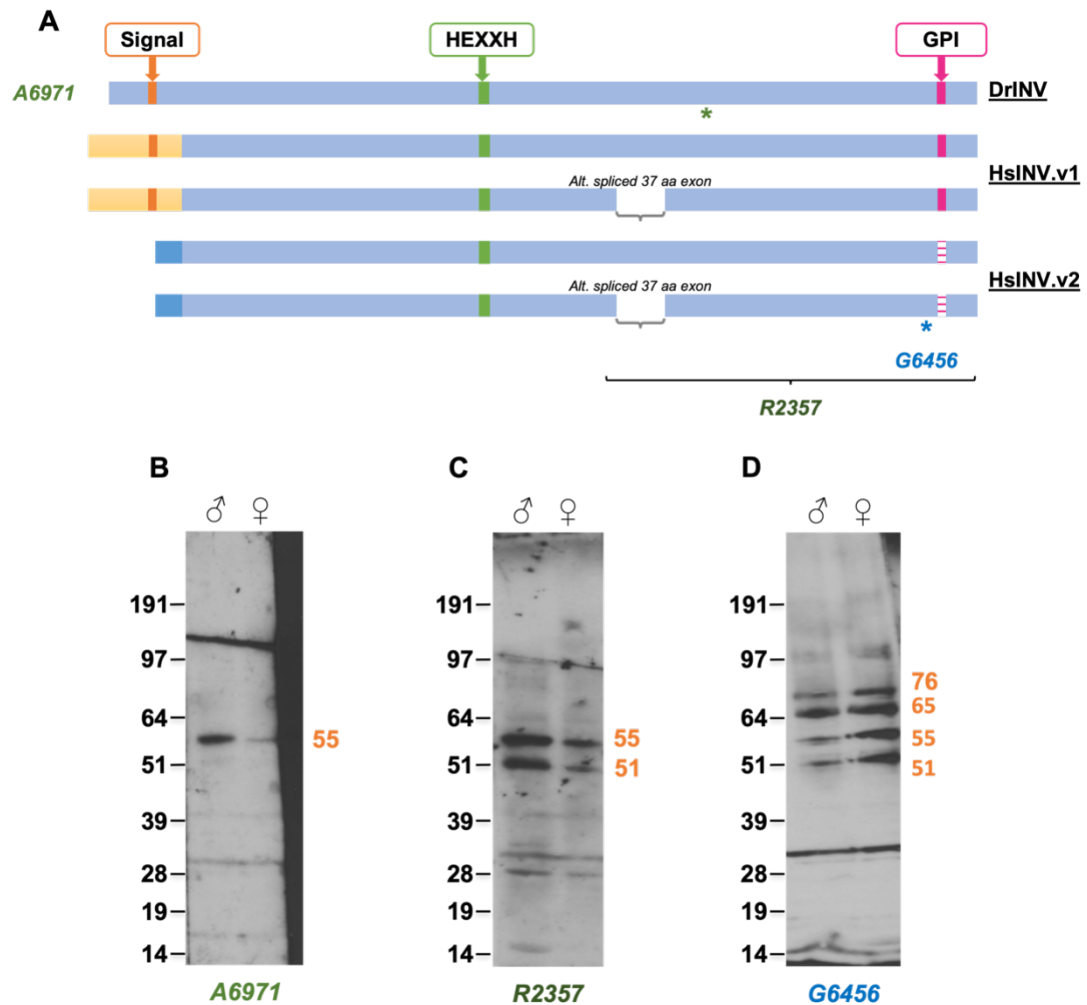


Figure 3.2 The extracellular form of invadolysin is present in zebrafish plasma.

(A) Schematic sequences representing zebrafish invadolysin (DrINV) and two human invadolysin (HsINV.v1 and HsINV.v2) with N-terminal signal sequence (orange), metalloprotease motif (HEXXH)(green), alternative spliced 37 amino acid exons (grey bracket) and predicted GPI (deep pink) attachment site. Asterisks and brackets labelling the epitopes recognized by anti-invadolysin antibodies: A6971(dark green), R2357(forest green) and G6456(sky blue). **(B)** Immunoblotting of adult zebrafish (18 months) plasma was performed to detect and compare the secreted form of invadolysin in male and female. A prominent 55 kDa form of invadolysin was recognized by anti-zebrafish invadolysin antibody (A6971) in both genders. **(C,D)** Immunoblotting of adult zebrafish plasma fraction probed by anti-human invadolysin antibodies R2357 and G6456. Results evidence that the 55 kDa form of invadolysin is enriched in zebrafish plasma fraction. The 51 kDa form was observed by both R2357 and G6456. Other two secreted forms of invadolysin 65 and 76 kDa are present in zebrafish plasma as shown by G6456 antibody. Biological replications n=3.

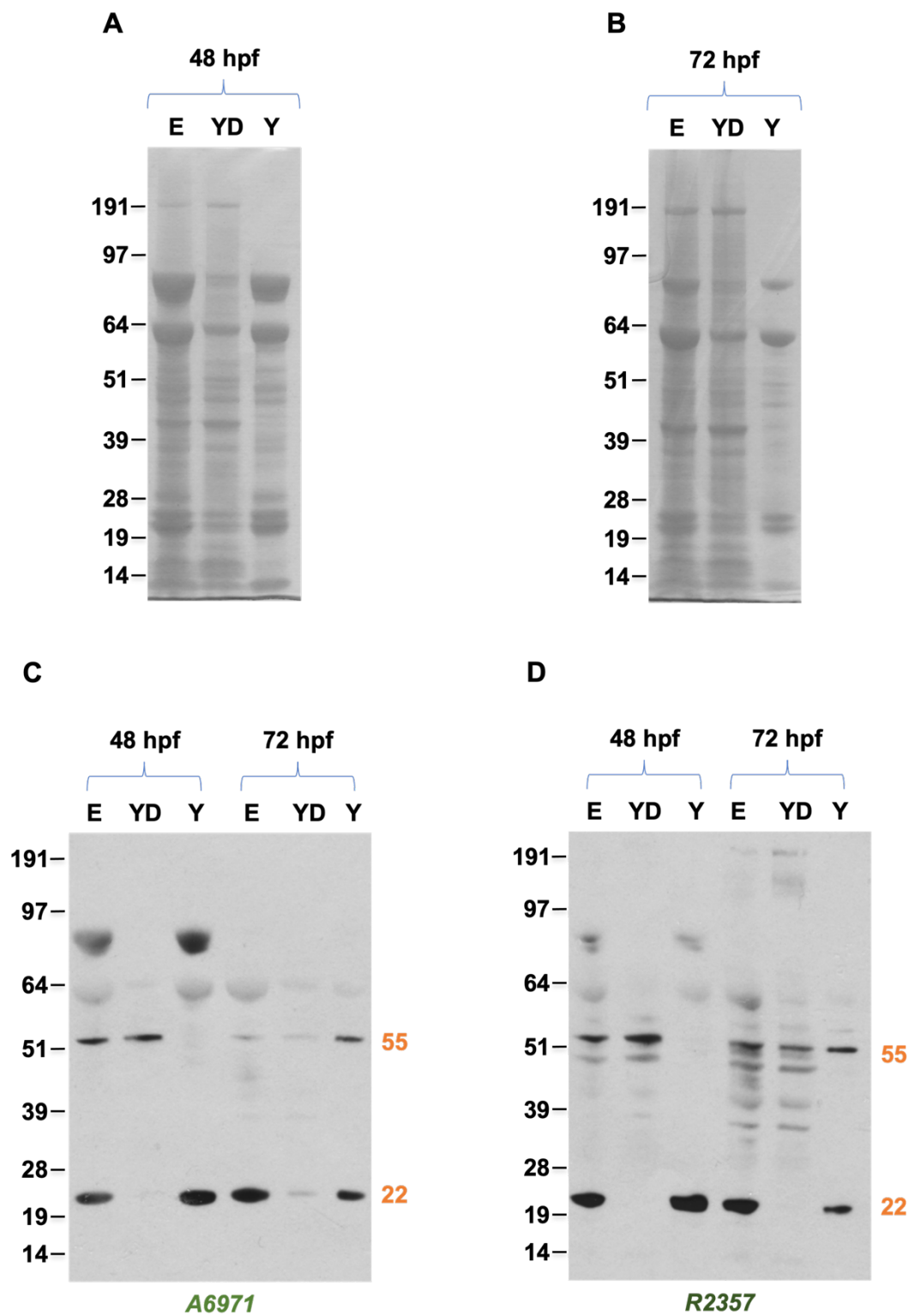


Figure 3.3 Analysis of invadolysin in zebrafish embryo by immunoblotting.

(A,B) Coomassie blue-stained gel represents protein levels in zebrafish embryo (E), yolk depleted embryo (YD) and yolk (Y) at 48 hpf and 72 hpf. Protein amount corresponding to single zebrafish embryo was loaded into each lane. Removal of abundant proteins around 60-80 kDa and 20-25 kDa is observed when comparing yolk depleted embryo and yolk proteins to whole embryo proteins. **(C,D)** Immunoblotting of zebrafish embryo (E), yolk depleted embryo (YD) and yolk (Y) at 48 hpf and 72 hpf by anti-zebrafish invadolysin antibody A6971 and anti-human invadolysin antibody R2357. Protein bands indicating two invadolysin expression forms, 22 kDa and 55 kDa were detected from zebrafish embryo at both developmental stages. The form at 55 kDa is enriched in yolk depleted embryo at 48 hpf and yolk sample from embryo at 72 hpf. The other invadolysin form at 22 kDa is enriched in yolk samples from zebrafish embryos at both 48 and 72 hpf, which is detected by A6971. A similar enrichment pattern of the diversity of invadolysin forms is observed by antibody R2357. Biological replications n=3.

Observed Molecular Weight (kDa)	Antibodies
51	S173
55	G3646, R4597 & G6456
64	R4597 & S173
51	R4597
58	R4597

Table 3.2 Structural forms of extracellular invadolysin in human plasma.

Extracellular invadolysin recognized by anti-human invadolysin antibodies (G3646, S173, R4597 & G6456) in immunoblotting are summarised above with respective molecular weight. Invadolysin was isolated with two commercial kits: Pierce™ Albumin/IgG Removal Kit (light orange) and Pierce™ Top 12 Abundant Protein Depletion Spin Columns (light blue).

Observed Molecular Weight (kDa)	Localisations	Antibodies
22	Yolk	A6971 & R2357
51	Plasma	R2357 & G6456
55	Whole embryo and plasma	A6971, R2357 & G6456
65	Plasma	G6456
76	Plasma	G6456

Table 3.3 Structural forms and localisations of invadolysin in zebrafish.

Dynamic invadolysin forms detected at different localisations in zebrafish recognized by anti-zebrafish invadolysin antibody (A6971) and anti-human invadolysin antibody (R2357 & G6456) in immunoblotting are concluded above with respective molecular weight. The 22 kDa form of invadolysin is enriched in yolk (48 and 72 hpf) and the prominent 55 kDa form of invadolysin is present in both embryo and adult plasma. Anti-human antibodies also detected 51, 65, and 76 kDa forms of invadolysin.

Abundant Plasma Proteins	Molecular Weight (kDa)
Fibrinogen	340
α 2-Macroglobulin	85
Transferrin	80
Albumin	66.5
IgM	65, 23
IgA	55, 23
α 1-Antitrypsin	54
IgG	50, 23
α 1-Acid Glycoprotein	42
Apolipoprotein A-I	28.3
Haptoglobin	18
Apolipoprotein A-II	17.4

Table 3.4 List of the top 12 abundant proteins in human plasma.

Top 12 abundant proteins in human plasma are shown above with respective molecular weight. Albumin and gamma globulins are top two enriched proteins, which make up more than half of plasma proteins. PierceTM Albumin/IgG Removal Kit with Cibacron blue/Protein A gel was used to deplete human serum albumin and IgG from plasma.

3.4 Discussion

3.4.1 A sustainable strategy for invadolysin enrichment from human blood needs to be established

This chapter has been focusing on the examination of invadolysin in vertebrate blood and preliminary exploration of invadolysin's role in early developmental stages. As a highly conserved metalloprotease, it has been shown that an extracellular form of invadolysin is present in vertebrate blood and invertebrate hemolymph (Abhinav et al., 2019). Leishmanolysin, the closest homolog of invadolysin, also possesses an extracellular promastigote form, which has been found in the sand fly vector (McGwire et al., 2002; Yao, C. et al., 2007). When compared to plasma samples without abundant protein removal, protein bands from 'cleared' samples on immunoblots have shown prominent signals with better resolution. Multiple expression forms of invadolysin in human plasma have been discovered, which were supported by various anti-invadolysin antibodies targeting to distinct regions of invadolysin variants. In 2011, Bellei *et al.* suggested that simplification of the complexity of the serum sample is a vital step during proteomics studies by reduction of high-abundance proteins (Bellei et al., 2011). These results support the idea that removal of abundant proteins improves the probability of investigation using blood sample and the requirement for high-abundance protein depletion is essential for detection of invadolysin by immunoblotting (Abhinav et al., 2019; Bellei et al., 2011).

However, the results obtained in this chapter revealed that not only albumin and IgG are removed by the PierceTM Albumin/IgG Removal Kit, invadolysin could also be reduced by non-specific affinity. In addition, distinguishing invadolysin from abundant protein bands in plasma on immunoblots is still difficult after removal of albumin and IgG. It has been discussed that when preparing protein samples using the depletion method, it is possible to remove low-abundance and non-targeted molecules along with removal of abundant proteins (Adkins et al., 2002; Bellei et al., 2011; Granger et al., 2005). Chromatography technology has been introduced for the

improvement of qualified and detectable signals by removal of the top 12 abundant proteins from plasma samples (Table 3.4) (Abhinav et al., 2019). Even though this strategy, which was tested in this study, improves the detection accessibility of invadolysin from human blood sample, high cost and non-recyclable usage features are worth taking into consideration (Figure 3.1). Recent studies have assumed that invadolysin has a functional role in diverse physiological activities as different localizations have been observed in the extracellular vesicle fractions (Abhinav et al., 2019). Whether this form of invadolysin is enriched in a specific soluble fraction of human serum and plasma is still unclear. For all the concerns, a suitable methodology needs to be developed to enrich invadolysin from fractionated serum and plasma samples with repeatable and low-cost benefits (Chapter 4). Indeed, this strategy would improve the efficiency for detection of invadolysin in the soluble fraction of blood.

It has been pointed out that low-abundance proteins are candidates for biomarker discovery, and the establishment of an accurate baseline is a necessary element for proteomics (Bellei et al., 2011; Palmblad et al., 2009; Tirumalai et al., 2003; Zhou, M. et al., 2005). Once a reliable protein preparation strategy has been developed, it will uncover and enrich low-abundance proteins from abundant molecules, which would shed light on the identification of biomarkers in blood samples. In the long-term, it is crucial to determine whether secreted form(s) of invadolysin serve as potential biomarkers for any human disease states.

3.4.2 The sexual dimorphic forms of invadolysin in vertebrate remains to be determined

A recent publication has demonstrated that the extracellular form of invadolysin in adult fruit fly hemolymph displays sexually-dimorphic features (Abhinav et al., 2019). This observation triggers the hypothesis that sexually dimorphic expression of invadolysin might be associated with differential regulation or gender dimorphism development. Furthermore, it would be interesting to investigate whether sexually dimorphic forms of invadolysin

could be identified in vertebrates. In adult zebrafish, an extracellular form of invadolysin was identified by immunoblotting, which was detected by both anti-zebrafish and anti-human antibodies. It has been observed that the absence of albumin in adult zebrafish plasma reduces the complexity of proteomic research. Though it has been proved that the extracellular form of invadolysin is conserved in zebrafish plasma, there is still not enough evidence to demonstrate the existence of sexually dimorphic forms in vertebrate plasma. These observations are not unexpected as only a few gender-biased plasma proteins have been discovered (Li, C. et al., 2016).

It would be of interest to compare invadolysin in male and female zebrafish liver for sexual dimorphism research since liver is the dominant organ in the production of plasma proteins (Li, C. et al., 2016). Similar to the study for gender-difference in adult *Drosophila*, it would be worth examining gene expression of invadolysin in zebrafish ovary and testis. Comparison of the expression forms and protein levels of invadolysin in the liver, plasma and sexual tissues between male and female could help determine whether invadolysin possesses sexual dimorphism in vertebrates and understand the regulation mechanisms for the differences in invadolysin expression between genders.

3.4.3 The dynamic localised invadolysin may have a role during early embryonic development in zebrafish

In the analysis of invadolysin in zebrafish embryo during early developmental stages, two abundant forms of invadolysin were detected in embryos at 48 and 72 hpf. The form at 22 kDa was more predominant in the yolk. The other form of invadolysin (55 kDa) was enriched in the yolk-depleted embryo, which is more prevalent in zebrafish at 48 hpf. Consistent with previous studies, these results further illustrate that the forms of invadolysin are changed temporally during development and the cellular localisation of invadolysin is dynamic (Vass & Heck, 2013). The zebrafish embryo is usually not a suitable model for proteomic studies on the grounds that yolk proteins occupy a higher proportion of embryonic proteins and the zebrafish

embryo at early developmental stages is highly dynamic, which is accompanied by biological events, such as cell differentiation and morphogenesis (Kimmel, C. B. et al., 1995; Shaik et al., 2014; Thomas, R. J., 1968). The introduced yolk depletion strategy assists in reducing the complexity of protein sample preparation when examining invadolysin by immunoblotting. Additionally, this methodology and the dynamic nature of zebrafish embryos establishes an opportunity to explore various expression forms of invadolysin during early developmental stages in vertebrates. Research on invadolysin-depleted zebrafish embryos using morpholino injection has demonstrated an unexpected role of invadolysin in angiogenesis and cellular migration involved in morphological activities (Vass & Heck, 2013).

The established yolk-depletion protein preparation strategy in the zebrafish larval model serves as an ideal system to elucidate the dynamic localisations of invadolysin during developmental stages in vertebrates. However, the role of invadolysin during early embryonic stages is still unknown on the account that the predicted full-length form of invadolysin accumulated in both morphant and control zebrafish embryo extracts from 24 hpf to 72 hpf. Analysis of yolk and yolk-depleted zebrafish embryos from 24 hpf to 120 hpf would further elucidate the role of invadolysin in early developmental stages. Preliminary examination of invadolysin in zebrafish yolk-depleted embryo and yolk by immunoblotting have shown that the predicted full-length form of invadolysin at 74 kDa is abundant in the yolk extracts from 24 to 72 hpf (M. Friedrich, personal communication). From 24 to 96 hpf, the 22 kDa invadolysin form of was observed in the yolk extracts. The third form of invadolysin at 55 kDa was present in both yolk-depleted embryo and yolk extracts for almost all time points though it was more enriched in the yolk depleted embryo samples at 48 hpf. Vass and Heck (2013) examined changes in invadolysin forms during early developmental stages of zebrafish embryos (0-120 hpf). It has been illustrated that invadolysin with molecular weight at 22 (18-96 hpf), 55 (48-120 hpf) and 74 (0-72) kDa are three most abundant forms (Vass & Heck, 2013). Distinct invadolysin forms of zebrafish

detected in this chapter are summarised in Table 3.3. The 22 kDa form of invadolysin is present in the yolk, which is absent from yolk-depleted embryo and adult blood samples (Figure 3.2 and 3.3). Abhinav *et al.* (2019) suggested that post-translational modifications could contribute to the modulation of invadolysin's localisation or activity. For example, the cellular endoprotease furin, which has an important role in embryogenesis, catalyses proprotein substrates for maturation (Thomas, 2007). Thus, it has been postulated that the 22 kDa form of invadolysin has a potential role in early embryonic development in zebrafish, which could be modulated by post-translational modifications. Similar to Vass & Heck's (2013) research, the 55 kDa form of invadolysin was not only detected in zebrafish embryos with potentially dynamic localisations (48 and 72 hpf), but also it was shown to be present in the adult zebrafish plasma. Therefore, these results imply that the 55 kDa form of invadolysin has diverse localisations and the mechanism regulating this form of invadolysin's activity needs to be future examined. Studies on each independent form of invadolysin in yolk and yolk-depleted embryo samples by mass spectrometry would further contribute to predicting the structures and physiological function(s) of invadolysin and understanding how the dynamic expression of invadolysin is regulated at early developmental stages in vertebrates and at the extracellular level.

3.4.4 The potential relationship between invadolysin and embryonic metabolism needs to be investigated

It has been hypothesized that invadolysin plays an important role in metabolism; it was discovered as a novel metalloprotease localized to the surface of lipid droplets in mammalian cell lines (Chang, C.-W. *et al.*, 2017; Cobbe *et al.*, 2009). *Drosophila* invadolysin mutants display a decreased triglyceride:protein ratio and a role in insulin signalling and adipogenesis has been identified (Di Cara *et al.*, 2013; Chang, C.-W. *et al.*, 2017). However, the mechanisms behind how invadolysin is involved in lipid metabolism during embryonic development are not clearly acknowledged. Zebrafish yolk is maternally deposited and functions as nutrient source for zebrafish embryos until 5 days post-fertilization (dpf) (Quinlivan & Farber, 2017).

When the yolk has been exhausted after the first 5 days, zebrafish progress into free-feeding larvae and develop a functional digestive system, including a liver and intestine. Amino acids and lipids in the yolk are exported by the yolk syncytial layer (YSL) into the embryo body (Quinlivan & Farber, 2017; Sant & Timme-Laragy, 2018). The principle yolk lipids are cholesterol, phosphatidylcholine and triglycerides with the supplement of less abundant molecular species, such as cholesteroesters, ceramides and lysophospholipids (Quinlivan & Farber, 2017). It has been reported that lipid synthesis occurs at the earliest developmental stages. For example, phosphatidylcholine is synthesized in embryonic yolk from 18 to 72 hpf at a constant rate. In addition, cholesteroesters are identified in the yolk of zebrafish larvae at 72 hpf.

In conclusion, this chapter validated the extracellular form of invadolysin in human and zebrafish plasma. By examining adult zebrafish plasma, there was no evidence to indicate the existence of sexually dimorphism of invadolysin in vertebrates. It would be worth studying the expression profile of invadolysin, such as protein levels and structural forms of invadolysin in liver and/or ovary and testis of zebrafish, to determine whether invadolysin has sexual-dependent forms in vertebrates. In addition, establishment of dechoriation and deyolking of zebrafish embryo methodology contributes to the study of invadolysin's role in early developmental stages of zebrafish. The structural form of invadolysin is dynamic in yolk and yolk-depleted embryo of zebrafish. Importantly, it has been suggested that the form of invadolysin at 22 kDa may play a role in early developmental stages (48 to 72 hpf) of zebrafish embryo. Whether various forms of invadolysin have a relationship with lipid synthesis and/or remodelling in yolk and embryonic body need to be investigated in the future. Fluorescently-labelled lipid and invadolysin in zebrafish embryonic model would be beneficial for visualisation of the association between invadolysin and lipid metabolism during development.

Chapter 4 Development of approaches to examine the extracellular form of invadolysin

4.1 Introduction

4.1.1 Extracellular forms of invadolysin in blood fractions

The detection of extracellular forms of invadolysin in various higher eukaryotes was followed by the demonstration that the 53 kDa form is enriched in serum, plasma and cells fractionated from mouse blood (Abhinav et al., 2019). Different extracellular forms of invadolysin in higher eukaryotes are summarised in Table 4.1. This work also identified a 66 kDa form of invadolysin in the mouse blood cell fraction which was not present in serum or plasma. Furthermore, it identified other (51 and 53 kDa) extracellular forms of invadolysin in rat and pig plasma. In *Drosophila* hemolymph, a secreted form of invadolysin with diverse molecular weight has been found in plasma and hemocyte fractions. Analysis of invadolysin in plasma, testis and ovary of flies has demonstrated that the extracellular form is expressed in a sexual-dimorphic pattern in adult males and females. In male *Drosophila*, the 111 kDa form of extracellular invadolysin is present in hemolymph, which was also observed in testes. The extracellular form invadolysin in female hemolymph is 46 kDa, which is different from male, and present in ovaries. Abhinav *et al.* (2019) has not discussed whether or not human extracellular invadolysin has a sexually dimorphic form. Therefore, it is still unclear whether sexual organs in invertebrates are the primary source of extracellular invadolysin, and whether such sexually dimorphic forms can be detected in vertebrates. Results reported in Chapter 3 indicate that zebrafish do not exhibit sexually dimorphic forms of invadolysin. Analysis of the extracellular form of invadolysin in different blood fractions from humans provides the opportunity to increase understanding of the physiological role(s) of invadolysin.

Table 4.1 Extracellular form of invadolysin is conserved in higher eukaryotes

Eukaryotes	Observed Molecular Weight (kDa)	Localisations
Human (<i>Homo sapiens</i>)	51 & 58	Plasma
Pig (<i>Sus domesticus</i>)	51	Plasma
Mouse (<i>Mus musculus</i>)	27	Serum, plasma and blood cells
	31	Blood cells
	53	Serum, plasma and blood cells
	66	Blood cells
Rat (<i>Rattus rattus</i>)	53	Plasma

Blood fluid-based extracellular organelles, such as exosomes and apoptotic bodies, are released into the extracellular microenvironment (Mathivanan et al., 2010). Exosomes are important in disease pathogenesis and act as a means of communication necessary for cellular and organelle interaction. Research on extracellular vesicles derived from tumours has suggested that exosomes provide an ideal resource for the identification of biomarkers in the soluble fractions of blood (Mathivanan et al., 2010; Robbins & Morelli, 2014). Recent research illustrated that invadolysin is a negatively-regulated downstream target gene of miR-17-3p, which functions as a tumour suppressor through p21 in multiple myeloma (MM) (Xiang et al., 2021). It has been discovered that overexpression of invadolysin suppresses cellular proliferation in MM, which decreases cellular invasion and migration, reduces tumour volume and tumour weight, and inhibits colony formation. It has been proved that invadolysin is enriched in the exosomes, which indicates it has a potential role in extracellular vesicle biology (Abhinav et al., 2019).

Analysis of the extracellular form of invadolysin in vertebrate blood has the potential to overcome the limitations encountered when using hemolymph from invertebrates, and therefore, to advance research into the physiological role of invadolysin (Abhinav et al., 2019). In particular, investigation of invadolysin in human blood will help further understand its role in regulating biochemical and physiological processes. Consistent with previous publications, the work described in Chapter 3 has shown that the extracellular form of invadolysin possesses various molecular weights in human plasma. However, the dynamic regulation of invadolysin expression in vertebrate blood has not been determined.

4.1.2 Development of a biochemical methodology for invadolysin enrichment

The demonstration by Abhinav *et al.* (2019) that an extracellular form of invadolysin is present in human plasma was achieved by removing the 12 most abundant proteins from plasma using commercial spin columns. The results demonstrated that highly enriched proteins in plasma interfere with

protein migration during immunoblotting, and the molecular weight estimated from protein bands on immunoblots are varied. For instance, the molecular weight of human invadolysin in whole plasma was observed at 51 kDa, whereas it migrated to 58 kDa in cleared plasma sample, where abundant proteins were removed (Abhinav et al., 2019). This was also reinforced by the demonstration in Chapter 3 that removal of proteins during blood sample preparation improved the detection of invadolysin by immunoblotting.

Though, detection of extracellular invadolysin has been improved by Abhinav *et al.* (2019) through removing abundant proteins with chromatography, where Pierce Abundant Protein Depletion Spin Columns was used to remove the top 12 abundant proteins from human plasma. This protocol, using a commercial kit, is difficult to adjust accordingly to meet different experimental purposes. In addition, this protocol is not financial friendly as each column in the kit can only be used for one sample, which is not sustainable and recyclable. A large number of typical plasma fractionation and blood protein purification methods have been summarized by Burnouf (2007), such as affinity chromatography, ion exchange chromatography, immunoaffinity, ethanol precipitation, size-exclusion chromatography and ultrafiltration depending on target protein purification. Therefore, in order to determine whether invadolysin is enriched in a specific soluble fraction of blood, 'customised' and 'novel' biochemical approaches allowing efficient and manageable experiments are required for the investigation of novel invadolysin forms in human blood. Two methodologies were selected for this purpose depending on accessibility, experimental goals and operability.

Immunoprecipitation combined with other techniques is a proteomic research approach, which has been used for target protein capture and biomarker candidate identification from human plasma (Fredolini et al., 2019; Lin, D. et al., 2013). This fractionation assay can enrich antigen from limited available blood samples using only one antibody (Lin, D. et al., 2013). The key to a successful fractionation is the selection of an antibody that recognizes target antigen. Once the target protein has been enriched, proteomic analysis

tools, such as mass spectrometry, can be used for protein validation and molecular quantitation. Binding region of antibody probing target antigen is not modified, folded and denatured, which represents the antibody remains intact (Proteintech n.d.). However, there are still many limitations needed to be considered. The light and heavy chains of antibody used for antigen capture could be detected by the secondary antibody during immunoblotting, which can be visible on immunoblots and renders the difficulties to identify target antigen. Furthermore, there is a higher chance in occurring unwanted interactions between cellular proteome and other contaminants when using Protein A/G and the large antibody molecule. Considering its regulatory and low-cost advantages immunoprecipitation would also contribute to the development of an informative assay for invadolysin enrichment from human plasma.

It has been reported that polyethylene glycol (PEG) can be utilized for blood protein precipitation (Briggs et al., 1981; Haskó et al., 1982). During PEG precipitation, solubility of plasma proteins in a desired fraction can be modified by optimization of a PEG concentration which will not trigger protein denaturation. Previous research has shown that precipitation of albumin can be achieved using PEG 4000 at pH 6.5-7 (Haskó et al., 1982). In addition, Briggs *et al.* (1981) reported that pure low-density lipoproteins could be fractionated using PEG 6000 at 10% (Briggs et al., 1981). On the basis of these experiments, PEG precipitation has potential for development as a systematic methodology for fractionation of soluble components from plasma, by modulating specific biological factors. This promises the possibility that invadolysin could be enriched from specific fractions of human blood.

4.2 Hypothesis and aims of Chapter 4

Chapter 4 focuses on addressing whether novel extracellular form of invadolysin is enriched in a specific soluble fraction in human blood. To answer this question, the initial stage is to develop a suitable protocol to isolate and enrich invadolysin abundant proteins in blood (thesis aim one). Then, the structural expression forms of invadolysin in human serum and plasma would be determined (thesis aim one). In addition, whether extracellular invadolysin has sexually dimorphic form would be investigated in human blood (thesis aim two).

Hypothesis: The extracellular form of invadolysin is enriched in a specific fraction of human blood.

Aims:

1. To develop a biochemical strategy to enrich invadolysin from a soluble fraction of human blood.
2. To determine the molecular weight of the extracellular forms of invadolysin in human serum and plasma.
3. To determine whether the extracellular form of invadolysin is structurally different in blood from male and female humans.

4.3 Results

4.3.1 Immunoprecipitation is adverse to enrich invadolysin for immunoblotting identification

To investigate the extracellular form of invadolysin, immunoprecipitation (IP) methodology was introduced for invadolysin purification. IP protocol was optimised in various aspects as shown in Figure 4.1 to 4.4. IP assay is designed to capture antigen from protein complex, which usually depends on binding affinity between antibody and target antigen. Binding affinity indicates the binding strength between Protein A/G and antibody, and antibody and antigen. Numbers of factors affecting the assembly of the beads-antibody-antigen complex should be taken into consideration during IP protocol optimisation.

The first factor is immobilization of antibody. Anti-human invadolysin antibody S172 was tested as the capture antibody because sheep IgG1/2 has a strong binding affinity to both Protein A and Protein G in general (Thermo Scientific). For the purpose of optimization, only pre-immune sheep serum for S172 and Dynabeads with Protein G were utilized during examination. Since the immunoglobulin concentration of sheep serum and the affinity ability of Protein G Dynabeads to antibody are unknown, it is important to determine a suitable ratio of serum to beads (v:v). A series of serum to beads ratios were examined ranging from 5:1 to 400:1. The supernatant and elution fractions were collected after incubation of serum and Dynabeads. Protein samples in the same volume were loaded from supernatant and elution under each ratio (except 400:1: relative half volume).

The amount of proteins bound to Dynabeads were compared with unbound proteins (Figure 4.1). Coomassie blue-stained gel showed that the amount of captured immunoglobulin heavy chain (~51 kDa) and light chain (~28 kDa) is increased as the ratio amplified (excluding 400:1). The molecular weight of albumin is 66.4 kDa, which might explain the high level of protein observed around 64 kDa in control serum. The band around ~64 kDa was enriched in the supernatant fraction under all conditions, which confirms that Dynabeads

Protein G has no affinity to albumin. The protein level of Ig in elution fraction under 200:1 condition is similar to the amount of Ig in whole serum. By comparing to 200:1, even double amount of serum was used for Ig capture and relative half amount of protein samples were loaded for 400:1, protein level in elution fraction of 400:1 is less than that of 200:1. These observations suggested that serum to bead ratio at 200:1 would be a reasonable ratio for invadolysin antibody immobilization.

Utilisation of crosslinker is a direct method to immobilize the interaction between antibody to Protein A/G. This approach would support the affinity between antibody and Protein A/G by formation of covalent bonds between antibody and Protein A/G. For this reason, to test the affinity effect of crosslinker, DMP was utilized to test its ability in providing antibody immobilization. Pre-immune S172 and S173 are unimmune sera collected from sheep before the second and the third times antigen stimulation for antibody generation. Dynabeads Protein G was first incubated with pre-immune S172 and S173 for 'antibody' capture. Dynabeads-antibody complex was precipitated by magnet and the supernatant fraction was reserved for further examination. An aliquot of the Dynabeads-antibody complex was then incubated with crosslinker, which is known as cross-linking, where Dynabeads Proteins are joined with antigen capture antibody covalently by a crosslinker. Protein fractions eluted from Dynabeads-antibody complex (+/- crosslinking) were inspected to determine the effect of crosslinker on antibody immobilization. No protein sample was used for antigen capture during protocol optimisation at this stage.

For both pre-immune serum S172 and S173 groups, protein levels around ~51 and 28 kDa marker are higher in -CL group other than +CL group (Figure 4.2A and B). In Figure 4.2A, the band around 68 kDa marker was present in the +CL elution group, which was absent in the -CL group. This band was also detected in Figure 4.2B under the +CL group, even though migration of this protein band was smeared. These results demonstrated that crosslinker improves the binding affinity between Dynabeads Protein G and 'antibody'. In addition, it might prevent non-specific binding of non-antibody proteins to

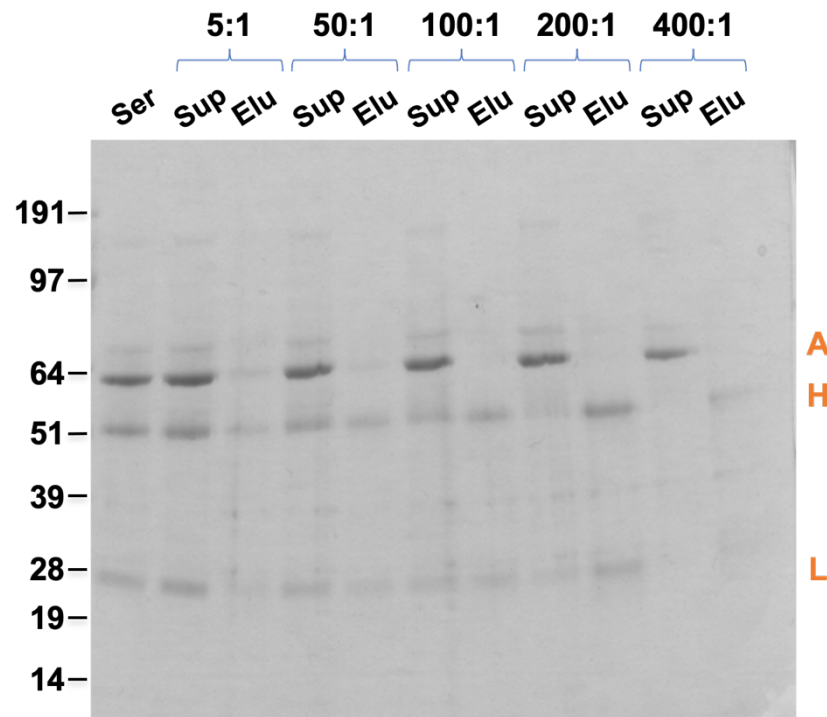


Figure 4.1 Ig binding-capability of Dynabeads is examined for IP protocol optimization.

Pre-immune sheep serum S172 to Dynabeads ratios at 5:1, 50:1, 100:1, 200:1 and 400:1 (v:v) were tested for immunoprecipitation protocol optimization. No human blood was used. Coomassie blue-stained gel showing the amount of protein in the same volume from supernatant (Sup) and elution (Elu) fractions except 400:1 (relative half volume). Pre-immune sheep serum S172 alone was loaded in the first lane as a control. Orange letters denote protein bands of interest, i.e., albumin (A) and immunoglobulin heavy chain (H) and light chain (L).

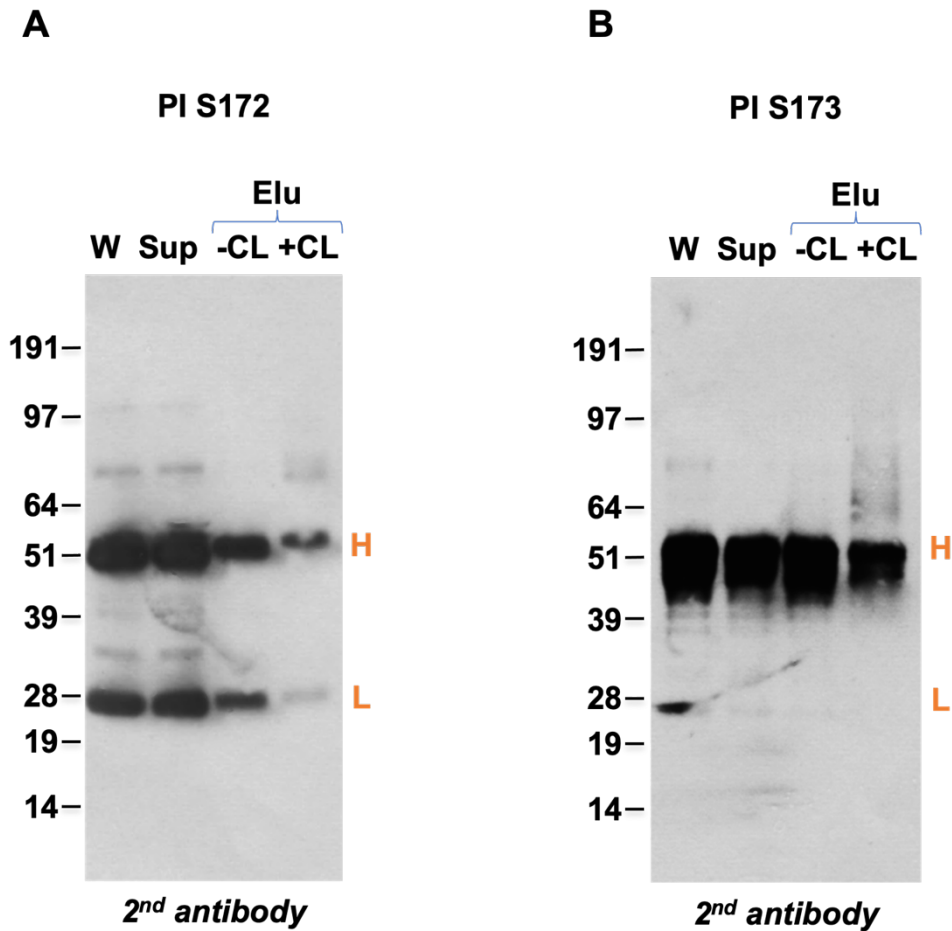


Figure 4.2 Crosslinking improves Ig-binding affinity of Dynabeads.

Protein G Dynabeads (-CL) and Protein G Dynabeads-cross linker (+CL) complex were used to capture Ig from pre-immune S172 (**A**) and S173 serum (**B**). Immunoblotting of proteins in supernatant (Sup) and elution (Elu) fractions were probed by the secondary antibody only (Donkey anti-Rabbit IRDye® 680RD). Whole pre-immune serum (Ser) for each antibody was loaded in the first lane as a control. Equal volume of each sample was loaded for examination. Orange letters representing proteins of interest, i.e., heavy chain (H) and light chain (L) of immunoglobulin.

Dynabeads Protein G. Migrations of proteins from pre-immune S173 serum were smeared around 51 kDa marker, which could be caused by aggregated proteins PI S173 (Figure 4.2B). During immunoprecipitation protocol modification, pre-immune serum was used for testing purpose as well as a negative control in actual antigen capture experiments bond by antibody from immune serum. Taking these factors into account, crosslinker was a suggestive immobilizer for antibody. Furthermore, immune S172 may be a candidate antibody for capture of invadolysin during immunoprecipitation, which would be examined in the following experiments.

The second factor is immobilization of antigen. To capture a prospective antigen from a protein mixture, it is important to optimize a suitable procedure for immobilized antibody-antigen complex assembly. In this research, two approaches were tested by adjusting the addition order of each supportive element. The first protocol was to assemble the Dynabeads-antibody complex (+/- cross linker) and use the complex to precipitate invadolysin from human plasma. The second protocol was to capture invadolysin by antibody, followed by addition of Dynabeads. Antigen was then eluted twice from the Dynabeads-antibody (Ab)-antigen (Ag) complex fraction for both protocols. Antigen from the Dynabeads-Ab-Ag complex solution was eluted by heating. Both supernatant and Dynabeads-Ab-Ag complex were collected, and the supernatant was named as the first elution (1st elution). Then, the Dynabeads-Ab-Ag complex collected from above was resuspended in elution buffer for another elution. Supernatant from this elution process was called the second elution (2nd elution).

Immunoblotting of two elution fractions were probed with anti-human invadolysin antibody R4597 (Figure 4.3A). For the first elution group, a high level of protein bands was observed at 51 kDa under +CL lanes by using pre-immune and immune sera in protocol 1 (Figure 4.3B). Another protein band at 73 kDa was enriched in these fractions, though it could also be observed in the -CL groups. A higher amount of protein was eluted with protocol 2 approach and with -CL methods in protocol 1. Similar results were shown in

the second elution group as high yield of protein were observed between 39 to 51 kDa marker (Figure 4.3C). This could be explained by co-elution of antibody and antigen.

Comparing with whole plasma control, it has been proved that the band at 51 kDa is an extracellular form of invadolysin, which was detected by immunoblotting with immunoprecipitation protocol 1 and 2 approaches (Figure 4.3B and C). An extracellular form of invadolysin migrating at 73 kDa was observed in the lane of immune serum with crosslinker under protocol 1. This form has been predicted as the full length of invadolysin, which was shown in Table 3.1 and detected in zebrafish plasma (Figure 3.2D). The smear background on the top of the lane under 'protocol 1-l-+' is a non-specific binding (Figure 4.3C). The other form of invadolysin at 58 kDa was not shown in any of the elution fractions. Furthermore, the band at 51 kDa was present in the second elution fraction captured by pre-immune serum under the +CL lane from protocol 1, which was absent from the immune serum +CL lane. Therefore, it is difficult to determine whether this 51 kDa form of invadolysin originates from human plasma or from pre-immune serum. These results confirm that crosslinking procedure increases binding affinity between Dynabeads Protein G and antibody, which prevents from co-elution of antibody and antigen. With assistance of cross linker, protocol 1 could be a suitable immunoprecipitation approach for invadolysin precipitation.

Optimization of immunoprecipitation protocol 1 was further processed by modification of essential support factors. Different combinations of Dynabeads and anti-invadolysin antibodies, such as Dynabeads A-(+/- crosslinker)-R2192/S172 and Dynabeads G-(+/- crosslinker)-R2192/S172, were utilized to purify invadolysin from human plasma (Figure 4.4B). Immunoblotting of elution fractions from each experimental group was probed with anti-human antibody R4597. Protein A and Protein G are two immunoglobulin binding proteins which were tested for antibody immobilization. It has been suggested that both Protein A and Protein G have a high binding affinity to rabbit IgG and sheep IgG 1/2. In this case,

antigen capture antibodies R2192 and S172 were utilized for invadolysin precipitation. As shown in the immunoblotting result, a higher amount of proteins were eluted from Dynabeads Protein A-antibody (+/- CL) complexes with protein bands migrating around 35 to 51 kDa. In general, less yield of protein was observed in elution fractions under Dynabeads Protein G-antibody (+/- CL) group, which reduces non-specific protein binding. Within the Dynabeads Protein G-antibody (+/- CL) group, there were higher yield of proteins with molecular weight around 28 and 51 kDa in the elution fraction without the usage of crosslinker for capture antibody R2192.

For capture antibody S172, there were less co-elution of antibody fragments in elution solutions with or without the crosslink method. As shown in the whole plasma sample, two forms extracellular invadolysin were detected in the elution fraction precipitated by capture antibody S172 (-CL). Overall, comparing to Dynabeads A, utilization of Dynabeads G decreases non-specific protein binding during immunoprecipitation, which improves the detection of extracellular invadolysin by immunoblotting. Furthermore, Dynabeads Protein G-S172 could support the precipitation of invadolysin from human plasma in immunoprecipitation method by eliminating co-elution of capture antibody fragments though further modification of immunoprecipitation protocol is still needed.

This immunoprecipitation system was further utilized to purify invadolysin from zebrafish embryo at 72 hpf (Figure 4.4C). Immunoblotting of invadolysin in zebrafish embryos enriched by crosslinking immobilized antibody system (Dynabeads Protein G-S172 (+CL)) was probed with anti-zebrafish invadolysin A6971. The forms at 32 and 45 kDa were predominant in yolk depleted embryo samples. The form at 56 kDa was enriched in both yolk depleted embryo and yolk samples. The protein bands migrating around 51 and 28 kDa could be the heavy chain and light chain of IgG of PI S172 and S172, which were absent in the No Ab control group. These results indicate that immunoprecipitation system can assist the enrichment of invadolysin from zebrafish embryos. However, this protocol may not be necessary for invadolysin precipitation from zebrafish embryos as capture

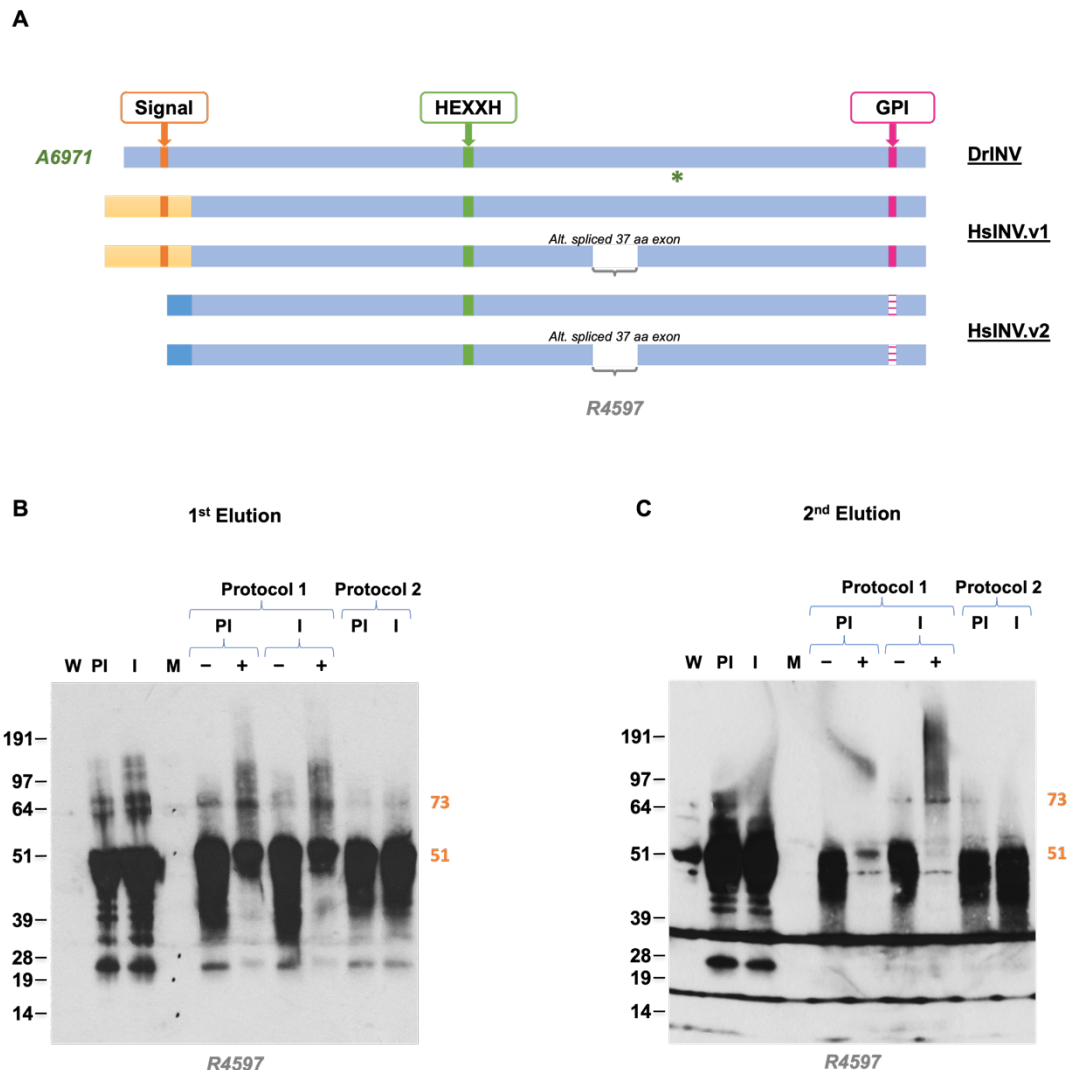


Figure 4.3 Dynabeads-antibody with crosslinker is a potential antigen capture complex for invadolysin enriched by immunoprecipitation.

(A) Schematic representing four human invadolysin variants (HsINV.v1+37, HsINV.v1Δ37, HsINV.v2+37 and HsINV.v2Δ37). Epitopes against to which antibodies are indicated by brackets. **(B,C)** Immunoblotting of invadolysin from human plasma in the first and second elution fractions enriched from immunoprecipitation were probed with R4597 antibody. Different combinations of antigen capture components by using Protein A Dynabeads, antibody R2192 (PI: pre-immune; I: immune) and crosslinker (-: without; + with) were tested for both immunoprecipitation protocols. Whole human plasma (W) (top 12 abundant proteins removed) and R2192 serum (PI/I) were loaded in the first three lanes as controls. Marker was loaded between control and experimental groups to improve a reliable protein detection in immunoblotting. No input protein samples were used. Protein bands of interests were indicated in molecular weight.

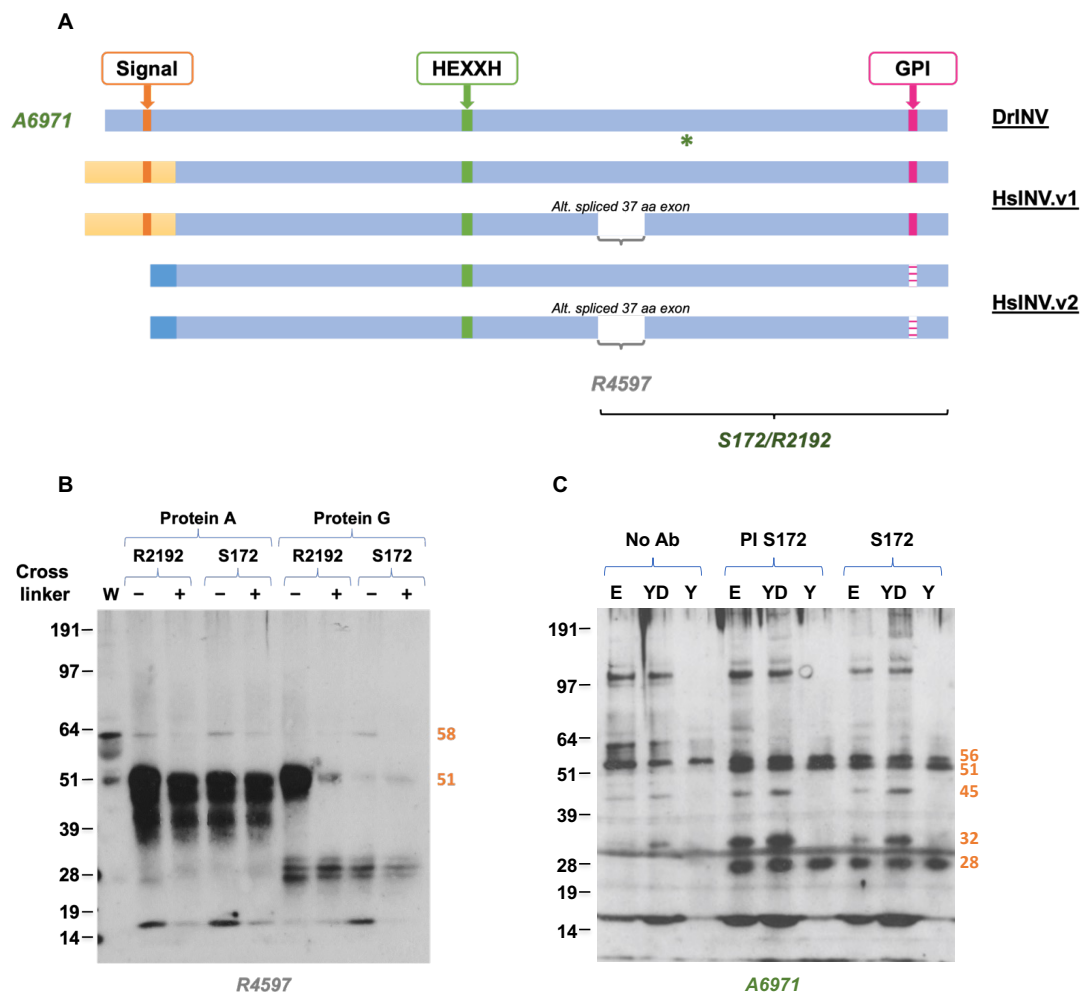


Figure 4.4 Immunoprecipitation of human plasma and zebrafish embryos and identification of invadolysin by immunoblotting.

(A) Schematic of zebrafish invadolysin (DrINV) and alternative variants of human invadolysin (HsINV.v1+37, HsINV.v1Δ37, HsINV.v2+37 and HsINV.v2Δ37). Epitope regions to which the antibody against are labelled with asterisks and brackets (A6971: anti-zebrafish invadolysin antibody; R4597: anti-human invadolysin antibody). R2192 and S172 antibodies were used to capture invadolysin by immunoprecipitation. **(B)** Detection of the extracellular form of invadolysin enriched by IP using Protein A and Protein G Dynabeads (+/-: with or without crosslinker). Immunoblotting of the extracellular form of invadolysin probed with R4597 detected two protein bands at 51 kDa and 58 kDa. Whole human plasma (W) (top 12 abundant proteins depleted) was loaded in the first lane as a control. **(C)** Analysis of invadolysin in immunoprecipitation enriched fractions in zebrafish embryos at 72 hpf. Immunoblotting of zebrafish embryo (E), yolk depleted embryo (YD) and yolk (Y) probed with A6971 discovered various forms of invadolysin. The form at 56 kDa is detected both yolk depleted embryo and yolk samples. Protein bands at 32 and 45 kDa are enriched in yolk depleted embryo lanes. No antibody and the pre-immune S172 serum were used as capture antibody controls during immunoprecipitation.

antibody fragments were still co-eluted with antigen with the utilization of crosslinker. in yolk depleted embryo samples. The form at 56 kDa was enriched in both yolk depleted embryo and yolk samples. The protein bands migrating around 51 and 28 kDa could be the heavy chain and light chain of IgG of PI S172 and S172, which were absent in the No Ab control group. These results indicate that immunoprecipitation system can assist the enrichment of invadolysin from zebrafish embryos. However, this protocol may not be necessary for invadolysin precipitation from zebrafish embryos as capture antibody fragments were still co-eluted with antigen with the utilization of crosslinker.

4.3.2 Polyethylene glycol precipitation can enrich invadolysin from human blood fractions

To fractionate human blood, polyethylene glycol precipitation was introduced as a second strategy for invadolysin enrichment. PEG has been used as a biochemical method to precipitate a special protein fraction from serum and plasma (Haskó et al., 1982). The PEG precipitation protocol has been developed to investigate the extracellular invadolysin in different human plasma fractions (Figure 4.5). Human plasma was fractionated using PEG at a low concentration at 5%. Both supernatant and pellet fractions were analyzed compared with the whole plasma sample. The coomassie blue-stained gel shows that a particular fraction was precipitated in the pellet fraction and abundant proteins migrating around 51 to 64 kDa was left behind in the supernatant fraction (Figure 4.5 B). To determine whether invadolysin can be enriched in a particular plasma fraction, different concentrations of PEG ranging from 4-10% were used to precipitate invadolysin. After fractionated by PEG at each concentration, associated soluble fractions of plasma samples were analyzed by immunoblotting. It has been observed that antibody G3646 recognized a form of invadolysin around 55 kDa in pellet fractions, which was not shown in each supernatant fraction (Figure 4.5C). As the concentration of PEG arises, the level of proteins migrating around 28 and 51 kDa in the precipitated fractions increases. In previous experiments,

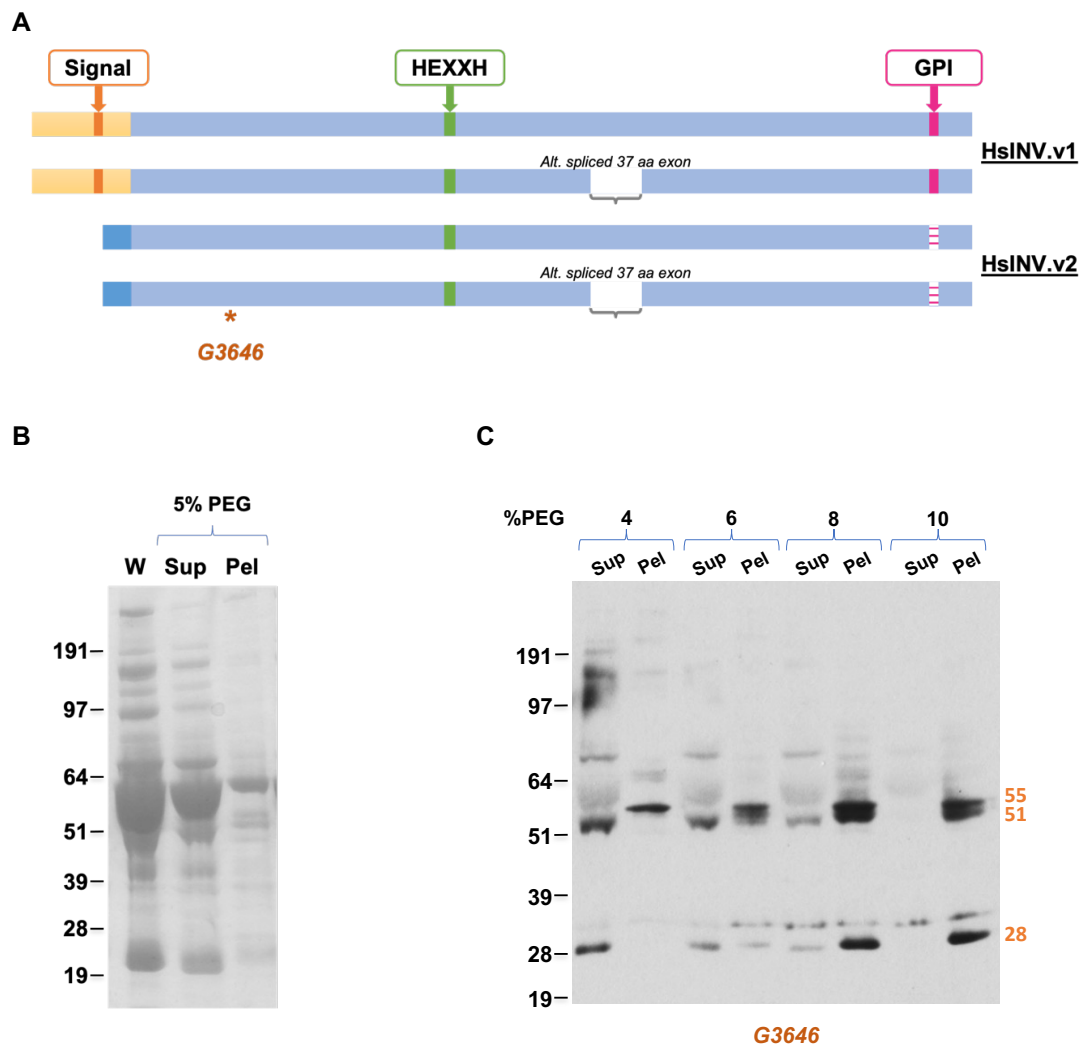


Figure 4.5 The extracellular form of invadolysin can be detected in different fractions enriched by polyethylene glycol precipitation.

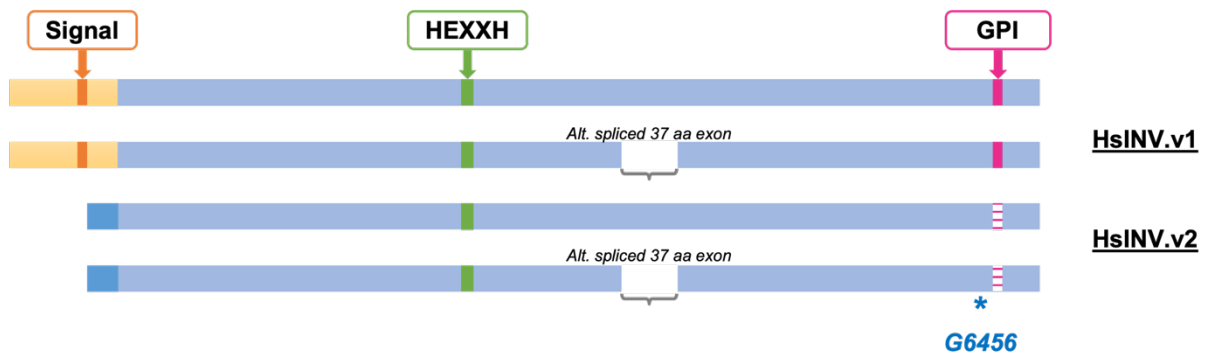
(A) Schematic demonstrating four human invadolysin variants (HsINV.v1+37, HsINV.v1Δ37, HsINV.v2+37 and HsINV.v2Δ37) with the G3646 antibody epitope highlighted with an asterisk. **(B)** Coomassie blue-stained gel showing the amount of protein in whole human plasma, 5% PEG supernatant and 5% PEG pellet (equivalent to the amount of protein in 0.3 ul of human plasma). **(C)** Enrichment of invadolysin in human plasma by PEG 6000 using different PEG concentrations ranging from 4-10%. Immunoblotting of invadolysin in aliquots of supernatant and pellet fractions probed G3646. The prominent bands at ~55 kDa in the pellet fractions represent the secreted form of invadolysin. The levels of protein bands at ~28 kDa and ~51 kDa are higher as the concentration of PEG increased, which are abundant proteins in human plasma, such as light chain (28 kDa) and heavy chain of IgG (51 kDa).

51 kDa and 28 kDa were suggested as the heavy chain and light chain of immunoglobulin, which could be recognized by the secondary antibody (non-specific binding) (Figure 4.2). Thus, these two bands (28 and 51 kDa) observed from Figure 4.5C are representing non-specific binding recognized by the secondary antibody. These results indicate that enrichment of invadolysin can be processed by PEG precipitation method, even with a relatively low PEG concentration, where the extracellular form of invadolysin can be detected in the PEG-pellet fraction by immunoblotting.

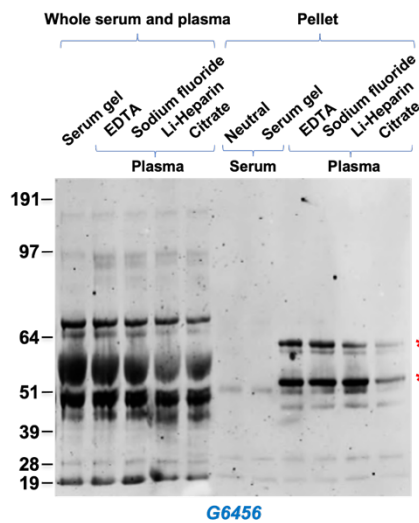
After development of a suitable approach for invadolysin enrichment, it has been questioned whether the extracellular form of invadolysin differs between serum and plasma. In clinical application, a variety of blood fractions preparation methods have been developed and choices of human serum and plasma preparation strategies are depending on the field of application, such as clinical chemistry and haematology (SARSTEDT n.d.). To answer the previous question, the first step is to clarify whether different serum and plasma preparation methods have an influence on the detection of invadolysin in the PEG-pellet fraction by immunoblotting. Before fractionation of blood samples by PEG, human serum and plasma were prepared by various S-Monovette system (SARSTEDT) as demonstrated in Figure 4.6. Comparison of invadolysin in the soluble fractions of serum and plasma after PEG precipitation was examined by immunoblotting probed by G6456 (Figure 4.6B). For PEG-pellet fractions, there was no significant difference in the expression form of invadolysin within different serum preparation approaches, where the similar finding was also observed in plasma pellet fractions. In order to further characterize the extracellular form of invadolysin in PEG-precipitated fractions, immunoblotting results of serum and plasma were compared by using both G6456 and PI G6456 antibodies (Figure 4.6B and C). Protein band signals recognized by G6456 and PI G6456 antibodies were labelled by red and green. Overlay of signals detected by G6456 and PI G6456 demonstrated that a 53 kDa (red bands under 'pellet-serum') form of invadolysin was enriched in the serum fraction whereas the 55 and 58 kDa (red bands under 'pellet-plasma') forms were enriched in the plasma fraction

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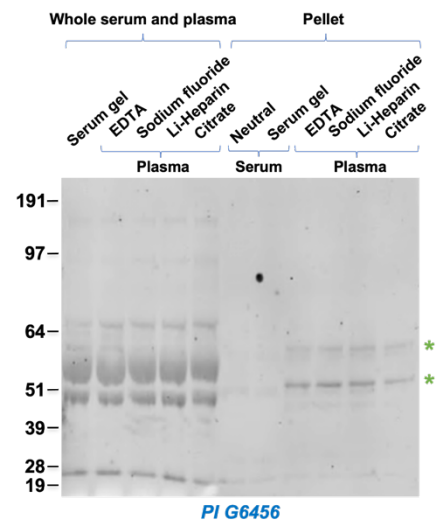
A



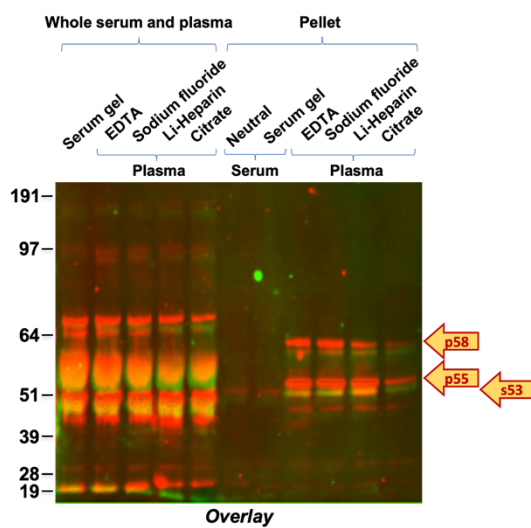
B



C



D



E

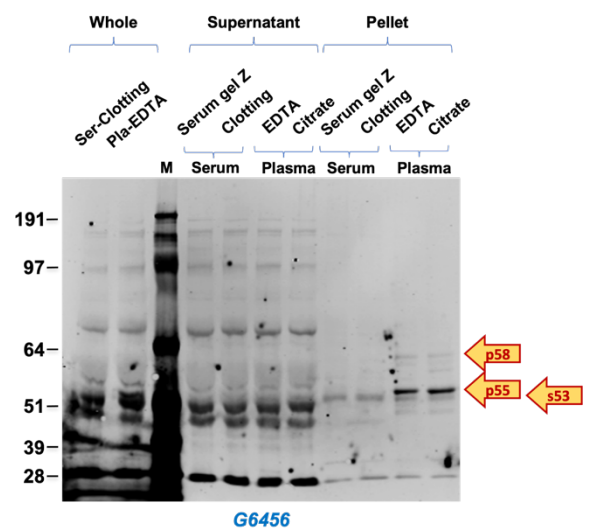


Figure 4.6 Identification of extracellular invadolysin by immunoblotting is not affected by serum and plasma preparation protocols.

(A) Schematic representing four human invadolysin variants (HsINV.v1+37, HsINV.v1 Δ 37, HsINV.v2+37 and HsINV.v2 Δ 37) with epitope (highlighted with blue asterisk) recognized by G6456 antibody. **(B,C)** Analysis of invadolysin in PEG-precipitated human serum and plasma fractions by immunoblotting. Human serum and plasma were collected using the commercial tubes from SARSTEDT (Serum: neutral and serum gel tubes; Plasma: EDTA, Sodium fluoride, Li-Heparin and citrate tubes). PEG 6000 at 4% concentration was used to fractionate serum and plasma samples. Immunoblotting of invadolysin from pellet fractions in serum and plasma was probed with anti-human invadolysin antibody G6456 and pre-immune G6456. Whole serum and plasma samples were loaded as controls. Protein bands of interests are shown as red asterisks for G6456 and green asterisks for PI G6456. **(D)** Identification of extracellular forms of invadolysin from non-specific signals. Overlaying of signals from G6456 (red) and PI G6456 (green) detected a dynamic form of invadolysin in human serum and plasma. A secreted form of invadolysin at 53 kDa (red bands under 'pellet-serum') is enriched in human serum (s53). Two other extracellular forms of invadolysin at 55 and 58 kDa (p55 and p58) (red bands under 'pellet-plasma') are present in the plasma fractions. **(E)** Characteristics of invadolysin in human serum and plasma. Immunoblotting of invadolysin in PEG-precipitated supernatant and pellet fractions probed with G6456 antibody. Human serum and plasma samples were prepared with the commercial tubes (Serum: serum gel Z; Plasma: EDTA and Citrate) or serum-clot. Whole serum (serum-clot) and plasma (EDTA) were loaded in the first two lanes as controls. Marker was loaded between control and experimental groups to encourage a reliable proteomic identification. Molecular weight of prominent bands in serum and plasma pellet fractions are labelled on arrows.

(Figure 4.6D). The 'clotting' method was introduced for serum preparation, which also has no effect on investigation of invadolysin by immunoblotting (Figure 4.6E). In this experiment, the 55 kDa form of invadolysin can be detected in whole plasma and plasma-pellet fractions, which was not shown in whole serum and serum-pellet fractions. The 53 kDa form was enriched in both serum-pellet fractions whereas the 58 kDa was observed in plasma-pellet fractions. The difference of extracellular form of invadolysin was observed in whole serum and plasma, where the 55 kDa form can be detected in whole plasma sample which was not shown in whole serum plasma. These results indicated that identification of extracellular invadolysin in the soluble fraction of human blood by immunoblotting is not affected by different serum and plasma preparation systems. Furthermore, one extracellular form of invadolysin can be enriched in the soluble fraction of serum and plasma.

4.3.3 Diverse expression forms of invadolysin present in human serum and plasma

As invadolysin exhibited different forms in previous experiments, further analysis was facilitated to examine the extracellular form of invadolysin in serum and plasma. Protein enrichment from human serum and plasma was precipitated by PEG ranging from 4% to 8%. Invadolysin from each soluble blood fraction was probed by three anti-human invadolysin antibodies. Immunoblotting with G3646 and R4597 demonstrated that the 51 kDa form was present in serum-pellet fractions (Figure 4.7 B and C). The 53 and 58 kDa forms were abundant in plasma-pellet fractions. Antibody R2192 also identified the extracellular form of invadolysin in serum and plasma respectively (Figure 4.7 D). There were higher amount of proteins precipitated by PEG at 8% compared to 4% and 6%, which were observed by antibody R4597 and R2192. In addition, protein bands migrating around 51 to 64 kDa had more informative signals from 4% PEG sample group than 8% PEG sample group, though the protein yield was similar upon 4%-8% of PEG detected by antibody G3646. These results illustrate that protein enrichment by PEG precipitation with a relative low concentration of PEG provided a

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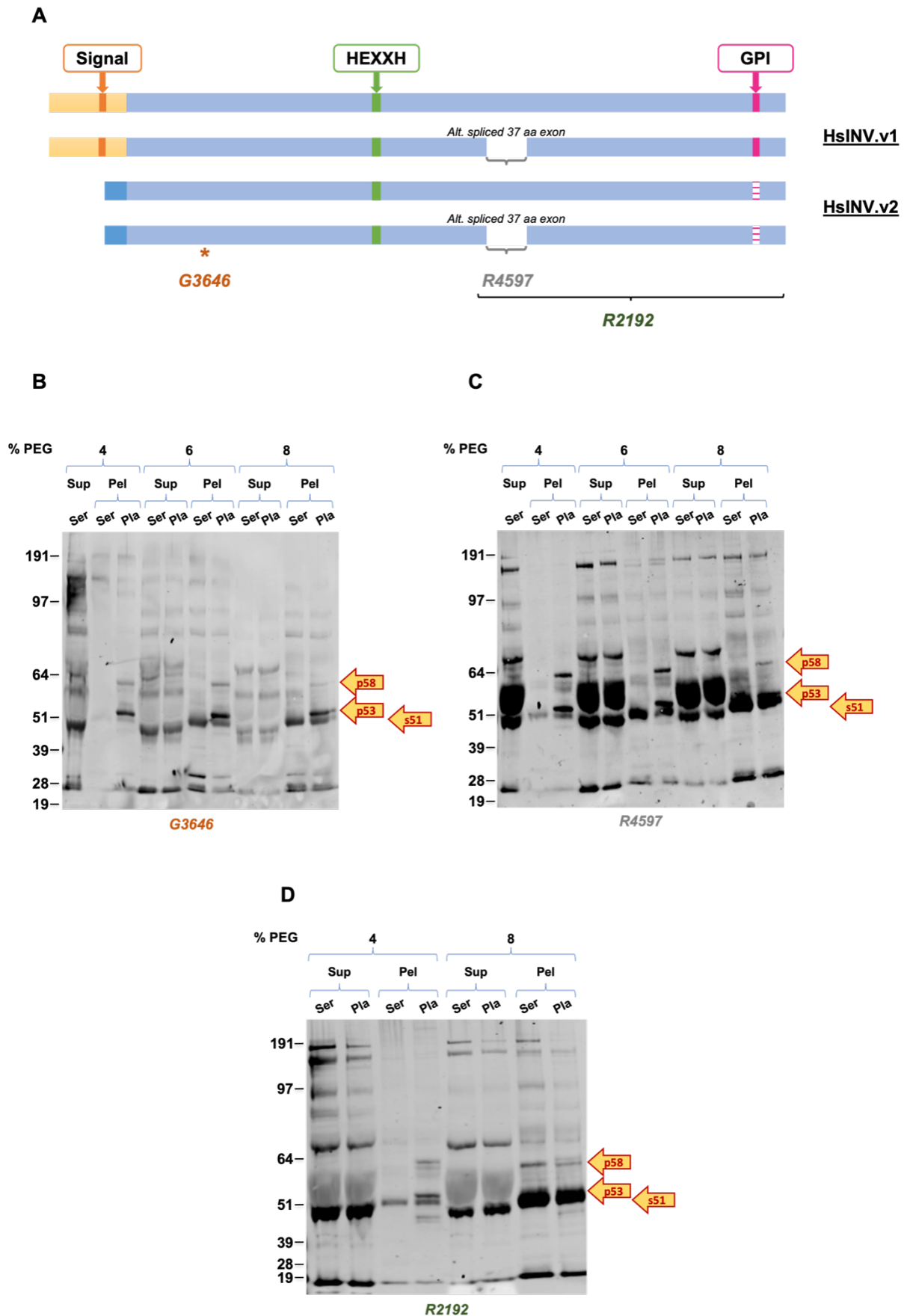


Figure 4.7 The extracellular form of invadolysin is dynamic in human serum and plasma.

(A) Schematic representing four human invadolysin variants (HsINV.v1+37, HsINV.v1Δ37, HsINV.v2+37 and HsINV.v2Δ37). The epitope regions where invadolysin antibodies against are highlighted with the asterisk and brackets. **(B,C)** Analysis of invadolysin in protein enriched serum and plasma fractions. Protein samples were fractionated by PEG 6000 at 4, 6 and 8% and aliquots of supernatant and pellet fractions were probed with G3646 and R4597 antibodies. The form at 51 kDa (s51) is abundant in the serum fraction. The bands at 53 and 58 kDa (p53 and p58) are dominant in plasma fraction. **(D)** Immunoblotting of invadolysin in the serum and plasma protein enriched fractions probed with the R2192 antibody. PEG-precipitated (at 4 and 8% concentrations) supernatant and pellet aliquots were collected for invadolysin examination after protein enrichment. The bands at 55 and 58 kDa (p55 and p58) are detected in the plasma fractions. The form at 53 kDa (s53) is concentrated in the serum fraction. The molecular weight of prominent bands were labelled on arrows.

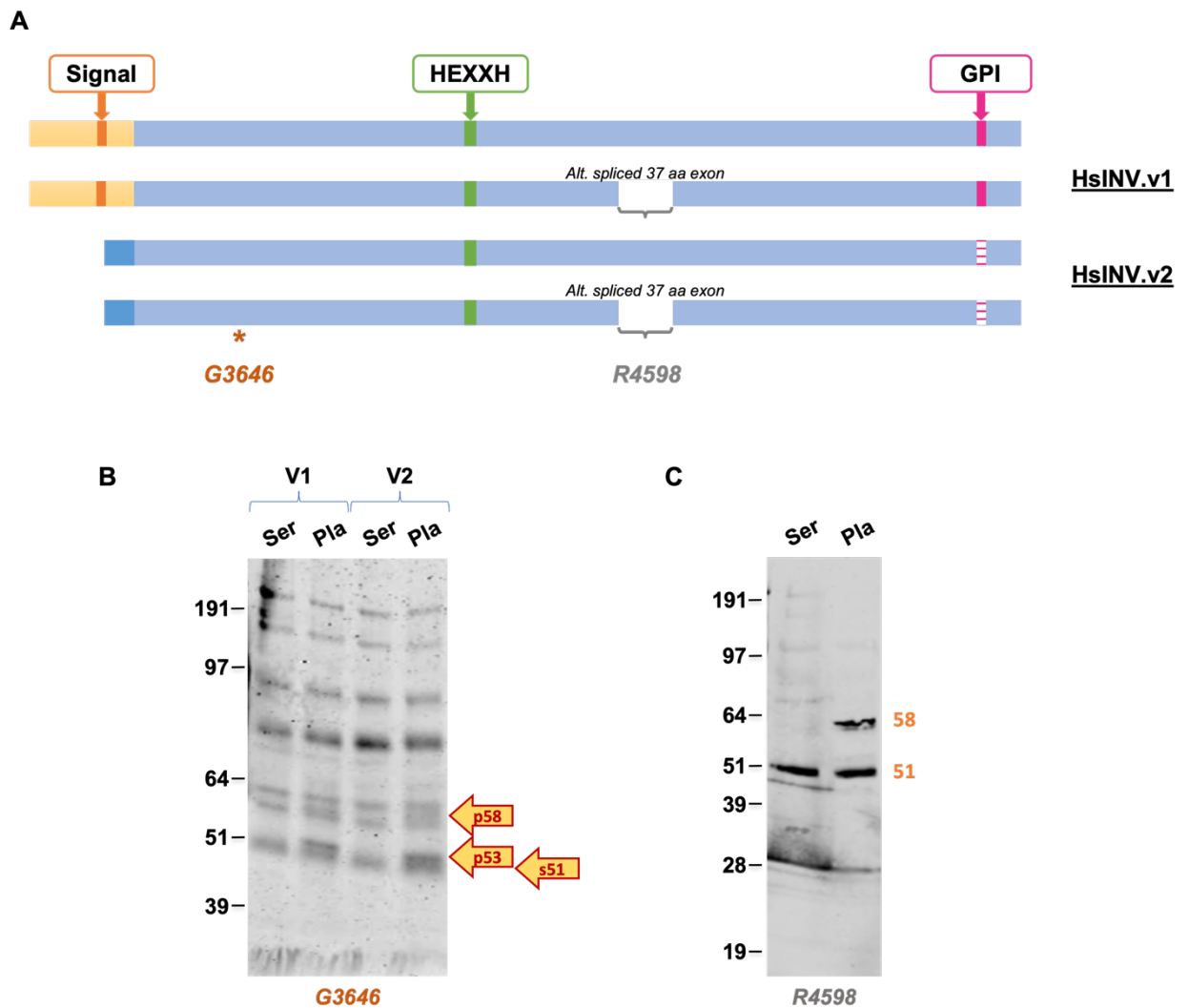


Figure 4.8 The dynamic secreted form of invadolysin in human serum and plasma processed with other proteomic research protocol.

(A) Schematic showing alternative species of human invadolysin. Anti-human invadolysin antibodies recognized regions on invadolysin variants (HsINV.v1+37, HsINV.v1Δ37, HsINV.v2+37 and HsINV.v2Δ37) are highlighted with an orange asterisk (G3646) and brackets (R4598). **(B,C)** Immunoblotting of human serum and plasma (V: volunteer) from after depletion of the top 12 abundant proteins, which occupy up to 90% of the total proteins. The G3646 antibody identified two forms of invadolysin in plasma at 53 and 58 kDa. The 51 kDa form of invadolysin is dominant in human serum. The anti-human antibody R4598 detected the 51 and 58 kDa forms of invadolysin in plasma and the 51 kDa form of invadolysin in serum. Molecular weight of prominent bands are indicated with arrows and numbers.

chance for mapping the difference of extracellular form of invadolysin within serum and plasma.

Alternative protein isolation protocol was further used to validate the extracellular form of invadolysin. As demonstrated before, top 12 abundant proteins in serum and plasma were removed by the Pierce™ commercial kit. The soluble blood fractions were examined by immunoblotting probed with antibodies G3646 and R4598 designed against distinct regions of invadolysin (Figure 4.8 B and C). The G3646 antibody showed that the 51 kDa form is enriched in serum, and the 53 and 58 kDa forms are present in plasma. The R4598 antibody discovered that the 51 kDa form of invadolysin is enriched in both serum and plasma, and the 58 kDa form is also enriched in plasma. These data further confirm that the extracellular form of invadolysin is dynamic within human serum and plasma.

4.3.4 Exploration of sexual dimorphic forms of invadolysin in human serum and plasma

Recent publication has identified that invadolysin is a component of *Drosophila* hemolymph and this form of invadolysin expresses in a sexual-dimorphism pattern (Abhinav et al., 2019). It has been questioned whether extracellular invadolysin could also show differences in males and females of vertebrates. Chapter 3 has shown that the sexually dimorphic form of invadolysin was not observed in adult zebrafish plasma. However, it is still worth investigating how invadolysin's activity is regulated in humans.

To be consistent, human plasma from both male and female were examined for this purpose. Human blood samples were fractionated by PEG precipitation method at 4% and 8% PEG concentrations. Extracellular form of invadolysin from precipitated pellet and supernatant fractions was examined by immunoblotting recognized by various anti-human invadolysin antibodies (Figure 4.9 B-E). As demonstrated before, extracellular forms of invadolysin at 55 and 58 kDa were enriched in the plasma-pellet fraction

detected by antibodies R4624, R4888, R4598 and G6456. The molecular weight of invadolysin in male and female plasma didn't show a difference, which was tested by various anti-human invadolysin antibodies. These results illustrated that there is no indication for the existence of sexually dimorphic forms of invadolysin in adult human plasma

Investigating the Essential Extracellular Invadolysin Metalloprotease

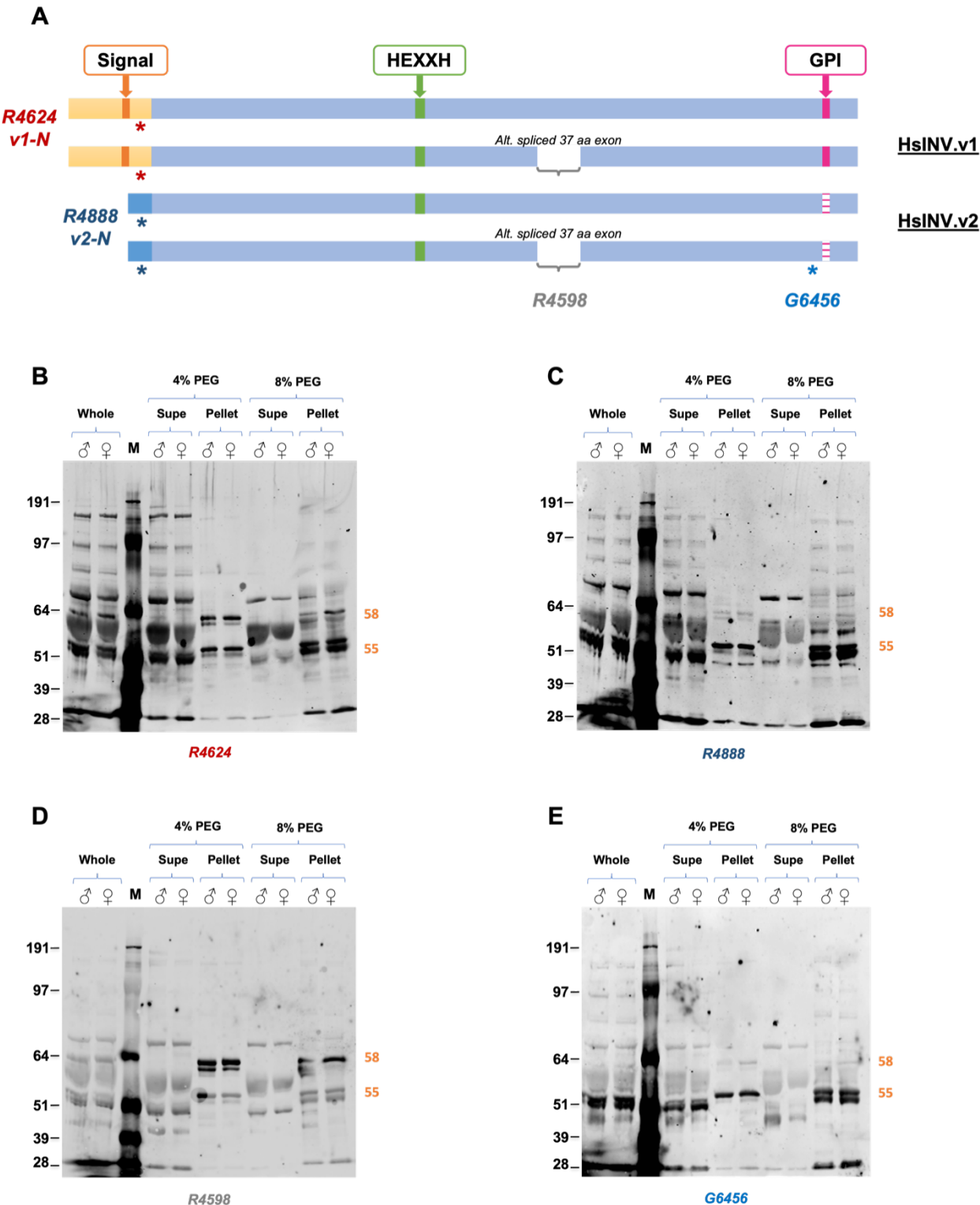


Figure 4.9 Analysis of the extracellular form of invadolysin in human male and female plasma.

(A) Schematic representing alternative species of human invadolysin with asterisks and brackets displaying the epitopes to which the anti-human invadolysin against. (B-D) Immunoblotting of human plasma to examine the secreted form of invadolysin in male and female. PEG 6000 at 4 and 8% concentrations were used for sample fractionation. Aliquots of PEG-precipitated supernatant and pellet sections were probed with anti-human antibodies R4624, R4888, R4598 and G6456. A prominent 55 and 58 kDa form of invadolysin was observed in plasma in both male and female by four antibodies.

4.4 Discussion

This chapter focused on the characterization of the extracellular form of invadolysin in human soluble blood fractions. Previous research has revealed that invadolysin is present in a plasma fraction enriched for extracellular vesicles containing exosomes and microvesicles (Abhinav et al., 2019). The extracellular form of invadolysin has been observed in different soluble fractions of human plasma, such as the extracellular vesicle fraction. Therefore, it has been suggested that extracellular invadolysin has diverse cellular localizations, which implies a potential role for invadolysin in extracellular vesicle biology. Leishmanolysin/GP63 is the closest homology protease to invadolysin, whose enzymatic activity is important for maintaining essential parasite life cycle and exerting pathogenetic roles in the mammalian host and in the insect vector (McGwire et al., 2002; Melo et al., 2006). Three types of extracellular form of this protease have been identified by biochemical analysis: membrane bound, surface-derived, and secreted forms (McGwire et al., 2002; Melo et al., 2006; Yao, C. et al., 2007). It has been questioned whether extracellular invadolysin is expressed differently in each human soluble blood fraction. As a consequence, evaluation of extracellular invadolysin in the soluble fraction of blood is a significant step in understanding the role(s) of invadolysin in normal physiology. As discussed in chapter 3, protein enrichment from blood samples can be prepared using commercial kits by depletion of abundant plasma proteins. However, there are several limitations in this methodology so that an alternative proteomic research protocol needs to be developed to investigate extracellular invadolysin in depth. Several experimental strategies attempting to enrich extracellular invadolysin have been facilitated in this chapter.

4.4.1 The extracellular form of invadolysin can be enriched in a specific soluble blood fraction

With the aim to enrich extracellular invadolysin in a soluble fraction of human blood, modifications on immunoprecipitation and PEG precipitation protocols were optimised for blood fractionation. Immunoprecipitation is a classical

fractionation assay for protein isolation and purification based on binding affinity between each support in a sequential procedure. For different experimental conditions, the IP protocol is usually required to be optimized to ensure a successful antigen elution to precipitate adequate amounts of purified protein. Thus, two main factors affecting assembly of the antigen capture complex in IP have been optimized, which are immobilization of antibody and antigen. Results reported in this chapter have proved that utilization of a crosslinker supports the immobilization of antibody, as it reduced the co-elution of antibody components. However, the dosage of crosslinker is still an important element for IP protocol adjustment as it could influence the number of exposed antibody binding sites for the target antigen.

For immobilization of antigen optimization, an associated variable was taken into consideration, which was the addition order for antibody-antigen complex assembly. The preferred approach for application of IP in this chapter was to immobilize antibody to Dynabeads first using the cross-linking effect, and the complex was further used to purify proteins from plasma samples. This IP procedure improved the detection of extracellular invadolysin in immunoblotting by elimination of nonspecific protein binding as less background signals were observed. Nonetheless, the yield of extracellular invadolysin isolated from plasma samples was not sufficient for general proteomic research (Figure 4.4 B). In the meantime, it is difficult to distinguish whether a detected signal on immunoblot represents an extracellular form of invadolysin originating from plasma samples or from antigen capture antibody. Invadolysin is a highly conserved metalloprotease and the extracellular form of invadolysin has been characterized in vertebrate plasma (Abhinav et al., 2019). Anti-invadolysin antibodies S172 and R2192 used for extracellular invadolysin precipitation were available as poly-clonal antibody, which were not pre-purified and may contain other serum proteins (Cobbe et al., 2009). During modification of the immunoprecipitation protocol, the antibody sera used for antigen capture were not pure antibodies, which not only contained anti-invadolysin antibody, but also involve invadolysin proteins. Thus, the detected invadolysin in the eluted

precipitated protein solutions may come from testing samples or originate from antibody sera used during antigen capture. Immunoprecipitation of invadolysin from zebrafish embryos also evidenced that this protocol is not suitable for extracellular invadolysin isolation and enrichment. PI S172 and antibody S172 captured antigen presented similar protein migration patterns on immunoblots (Figure 4.4C). Non-specific binding was observed in this experiment. Protein bands with 51 kDa and 28 kDa could be heavy chain and light chain of immunoglobulin probed by the secondary antibody during immunoblotting, which was demonstrated similarly in Figure 4.2. These results state that the immunological protocol was not effective and informative for extracellular invadolysin isolation since there are constraints on the available IP antibodies and immunoblotting antibodies.

Subsequently, an alternative attempt in answering whether invadolysin is enriched in a particular soluble fraction of human blood was facilitated using polyethylene glycol precipitation method. Haskó *et al.* have demonstrated that the solubility of plasma proteins in plasma decreases corresponding to the increase of PEG concentration (Haskó *et al.*, 1982). This was based on the analysis of solubility curves of abundant proteins in plasma, which also illustrated that a portion of proteins could be separated from other parts by optimizing pH and PEG concentration. Enrichment of invadolysin in human blood soluble fractions was evaluated using the precipitation method at various PEG 6000 concentrations. Invadolysin was detected in the plasma-pellet precipitated fraction at relatively low concentrations of PEG 6000. The solubility of invadolysin in the soluble fraction of plasma decreased as the concentration of PEG increased from 4% to 10%. This observation confirms the practical conclusion drawn by Haskó *et al.* (1982).

Precipitants and supernatant fractions were readily obtained by centrifugation and each re-dissolved fraction was examined by immunoblotting presenting informative signals without noisy background. The extracellular forms of invadolysin separated using the PEG precipitation method or the commercial spin column illustrated similar results, which shows that extracellular

invadolysin can be enriched by the precipitation approach at a relatively low concentration of PEG. By adjusting PEG concentration, PEG precipitation method could enrich proteins in target fractions with low cost compared to using commercial kits. These experiments may further provide evidence for Juckles' displacement theory that plasma proteins are displaced by PEG from the solutions and PEG would not form insoluble complex with precipitated proteins (Haskó et al., 1982). In addition, isolated proteins remain intact, which means PEG cannot invade into the crystal lattice of precipitated proteins (Juckles, 1971).

Whether or not extracellular invadolysin has been processed by post-translational modifications were investigated by Abhinav *et al.* (2019). Human plasma invadolysin enriched by PEG precipitation (8%) was treated with PNGase F (removing N-linked glycosylation) and O-Glycosidase (removing O-linked glycosylation). From immunoblotting results, protein bands representing invadolysin had a change in migration shifting from ~55 to 50 kDa for both enzymatic treatment, which demonstrated that human plasma invadolysin is N- and O- glycosylated (Abhinav et al. 2019). The relevance of the PEG fractionation method was also demonstrated by Briggs *et al.*, who investigated the stability of precipitated proteins after storage at 4°C (Briggs et al., 1981). The cholesterol value of high-density lipoprotein (HDL) isolated from serum was steady after cold temperature storage, which suggests that PEG precipitation is an acceptable methodology compared to other alternative established strategies as there are less limitations on utilization of enriched proteins, such as enzymatic treatment.

Development of PEG precipitation technique sheds light on the research of extracellular invadolysin in distinct soluble blood fractions. In previous research, a potential role of GP63 in parasites has been discovered by Melo *et al.*, who indicated that GP63 provides nutrients during development of the promastigote stage by digestion of blood proteins from insects (Melo et al., 2006). With respect to the physiological function of invadolysin, investigation of the potential catalytic activity of invadolysin in human blood would be the

next direction of proteomic research. 'In gel' and/or 'in solution' zymography combined with immunoblotting could be further utilized to explore the enzymatic reactions in soluble fractions of human blood and to determine whether an extracellular form of invadolysin is responsible for any observed protease activities. Following the identification of an enzymatically-activated form of invadolysin, peptide sequence and even the molecular structure of such extracellular invadolysin forms will be characterized by mass spectrometry. Along these lines, analysis of molecular synthesis will further contribute to understanding of a potential role of the extracellular form of invadolysin.

4.4.2 Invadolysin forms are variable in human serum and plasma

In clinical and epidemiological studies, human serum and plasma are collected and analysed for proteomic research and diseases diagnosis (Sotelo-Orozco et al., 2021; Yu, Z. et al., 2011). Human blood is a rich and accessible biological matrix, which makes a contribution to the investigation of genetic variation and health conditions, and is commonly stored as serum and plasma for further biological studies. However, a variety of data on programming of proteomic differences between serum and plasma have demonstrated that there are distinct variations in metabolite composition between serum and plasma (Yu, Z. et al., 2011). In addition, blood collection tubes used for serum and plasma preparation have a significant effect on metabolic profile, which would influence the results of downstream epidemiological research. In 2008, Mannello *et al.* presented data showing that inappropriate utilization of serum and plasma samples for clinical diagnosis could result in a wrong decision (Mannello, 2003). As a consequence, it is important to examine whether different blood collection tubes have an influence on evaluation of extracellular form(s) of invadolysin and whether there is a difference in expression of invadolysin between human serum and plasma.

Extracellular invadolysin profiles in human serum and plasma prepared by either the conventional blood preparation approach or using commercial blood collection tubes were compared and analysed by proteomic research. Precipitates in PEG-supernatant and PEG-pellet fractions were examined to determine whether characterization of extracellular invadolysin could be influenced by diverse blood preparation procedures. It has been shown that there was no change in the detection of extracellular form(s) of invadolysin regarding the type of tubes used for serum and plasma collection. During serum preparation, the influence of the conventional method of 'clotting' and utilization of gel separator tubes for 'Neutral' and 'Serum gel' were considered as another factor. It has been proved that there is no alteration in detection of extracellular form(s) of invadolysin when using the conventional protocol or commercial gel separator tubes for serum preparation. A similar hypothesis has been discussed by Sotelo *et al.*, who explained that there should be no difference in metabolite profile between utilization of traditional blood collection tubes and gel separator tubes for proteomic analysis (Sotelo-Orozco et al., 2021). However, several studies have indicated that metabolite identifications in blood, especially amino acids, exhibit variations when using polymeric gel tubes in comparison with conventional tubes (López-Bascón et al., 2016; Sotelo-Orozco et al., 2021; Yu, Z. et al., 2011). It has been introduced in Chapter one that leishmanolysin catalyzes serum proteins including fibrinogen in *in vitro* experiments (Bouvier et al., 1990; Santos et al., 2006). When fibrinogen is circulating in blood, thrombin cleaves fibrinopeptides to produce fibrin, which is a crucial step during coagulation (Kattula et al., 2017). Additionally, it has been reported that higher plasma level of fibrinogen (exceed 7 mg/ml) is observed during acute inflammation. Because invadolysin is homologous to leishmanolysin, it has been suggested that fibrinogen can be a substrate of human invadolysin. In this chapter, protein expression form of invadolysin is different between serum and plasma, thus, it is worth examining whether different isoforms of invadolysin, especially the form exclusive presenting in plasma, play a role in coagulation.

For various *Leishmania* species, the enzymatically active leishmanolysin (GP63) exerts important pathogenetic roles in the mammalian host and the insect vector to maintain the essential parasite life cycle (McGwire et al., 2002; Melo et al., 2006; Yao, C. et al., 2007). Three types of leishmanolysin/GP63 proteases have been discovered by biochemical analysis (McGwire et al., 2002; Melo et al., 2006; Yao, C. et al., 2007). Membrane bound or surface-derived GP63 is pre-located on the cell surface of the parasite, which is released by autoproteolytic cleavage and is important for parasite virulence. Another extracellular form is secreted GP63, which can be freed from the parasite by direct secretion. The predominant forms of the extracellular GP63 are ~63 and 59 kDa, which were discovered by examining the protein lysates from *Leishmania major* promastigotes through immunoblotting (Melo et al., 2006). A third form of leishmanolysin has intracellular expression and may be responsible for degradation of extracellular matrix (Yao, C. et al., 2007). This perspective was studied by McGwire, who identified that internal major surface protease (MSP) could digest collagen type IV and fibronectin on an *L. amazonensis*. (McGwire et al., 2003). In this chapter, it is interesting to find that extracellular invadolysin also displays diverse expression forms in human serum and plasma, which was evidenced by different proteomic research strategies. Antibodies G3646, R4597 and R2192 recognized a 51 kDa polypeptide in serum. These antibodies also detected two polypeptides, of 53 and 58 kDa, which were enriched in the plasma soluble fraction. Moreover, antibody R4598 reacted with a form of invadolysin at 51 kDa in both serum and plasma, as well as with one polypeptide of 58 kDa observed in plasma exclusively. Therefore, it could be postulated that, like leishmanolysin, different extracellular forms of invadolysin found in human serum and plasma are playing diverse roles, such as lipid and glucose transformation and anti-inflammation, in maintaining normal physiology.

Previous studies have described different metabolite profiles between serum and plasma (Aoki et al., 2002; Yu, Z. et al., 2011). For example, a higher concentration of lysophosphatidylcholine (LPC) was found in serum. This

observation could be explained by coagulation, during which thrombin activates platelets to release phospholipases (Aoki et al., 2002). When compared with plasma, Teerlink *et al.* demonstrated that the concentration of arginine was close to 60% higher in serum (Teerlink et al., 2002). This observation was explained by coagulation as increased levels of arginine could be released by platelets. Based on these data, it has also been hypothesized that examination of extracellular form(s) of invadolysin between human serum and plasma can be influenced by the coagulation process.

4.4.3 The physiological role(s) of essential extracellular invadolysin remain(s) to be explored

Abhinav *et al.* have illustrated that sexually dimorphic forms of invadolysin discovered in adult flies provide evidence for a potential pathway of extracellular invadolysin biosynthesis in invertebrates. However, the mechanism regulating invadolysin secretion in vertebrates and invertebrates is still unclear. Work reported in Chapter 3 examined extracellular invadolysin in adult zebrafish plasma, which demonstrated that there is no difference in expression of invadolysin in males and females. In the current chapter, immunoblotting of the soluble fraction of human plasma showed no significance difference in extracellular invadolysin between males and females. Yu *et al.* have reported that serum has a higher sensitivity than plasma when comparing metabolites among subjects with different phenotypes (Yu, Z. et al., 2011). For instance, 40 metabolites were significantly different in mean concentration when type 2 diabetes (T2D) patient and non-T2D volunteer sera were compared, whereas there were 25 metabolite differences were found in plasma. Similarly, such results were confirmed by examining metabolites for smokers vs. non-smokers. Teerlink *et al.* observed that concentrations of symmetric dimethylarginine (SDMA) were 2% higher in serum than in plasma (Teerlink et al., 2002). In healthy volunteer plasma, higher levels of SDMA were discovered in males than in females, though there was no sexually related difference for arginine and asymmetric dimethylarginine (ADMA). It has been argued that serum is suitable for analysis of small molecules due to low protein content, and it is a

candidate matrix for biomarker identification (Denery et al., 2011; Yu, Z. et al., 2011). Interestingly, proteomic profiling of metabolites in serum and plasma for both males and females was performed by Yu *et al.*, who revealed metabolites with significant differences presenting in serum compared with plasma; however, these differences were close to significance in plasma (Yu, Z. et al., 2011). For instance, 69 metabolites with significant differences in mean concentration were found in serum whereas 62 metabolite differences were found in plasma when differences between male and female blood samples were analyzed. As a consequence, the choice of serum or plasma as a research matrix for the study of extracellular invadolysin still needs to be considered. In this case, when exploring extracellular invadolysin expression in humans, it is crucial to consider diverse variations and the potential influence of factors, such as the choice of biological matrices, gender, age, and physiological condition of volunteers.

A previous study has postulated that invadolysin has a potential role in extracellular vesicle (EV) biology based on the diverse cellular localizations of extracellular invadolysin (Abhinav et al., 2019). However, there is limited understanding of the extracellular vesicle-associated invadolysin metalloprotease and the fraction of extracellular vesicles enriched invadolysin was not examined in this chapter. GP63 associated immune modulation in macrophages and exosomes of *Leishmania* parasites was analyzed by Hassani *et al.*, who pointed out that GP63 metalloprotease plays important roles in selection for exosomal protein contents (Hassani et al., 2014). With increasing evidence, Shimoda and Khokha highlighted three potential functions of EV-related metalloproteases: extracellular matrix degradation to remodel tissue microenvironment, regulation of EV contents by ectodomain shedding, and EV-associated metalloprotease transference to target cells (Shimoda & Khokha, 2017). This review also pointed out that the platelet-derived microvesicle (MV) is a resource for metalloproteases, which plays a role during blood coagulation. Thus, it has been asked whether the extracellular form(s) of invadolysin identified exclusively in serum could be released by platelet-derived MVs. Can the proteolytic activation of

invadolysin be detected on EVs and/or platelet-derived MVs? If so, how is invadolysin's activity regulated and what is its role(s) in the network of cellular communication?

Chapter 5 Exploration of invadolysin's role in inflammation

5.1 Introduction

It has been proposed that identification of an extracellular form provides a new opportunity for investigating the role of invadolysin in human physiology. For this purpose, analysis of the relationship of invadolysin to human disease, using samples from clinical trials, has been suggested as means of exploring physiological function. The recent genetic deletion of invadolysin in mice has potential to provide a model that increases understanding of the role of invadolysin in normal physiology. It also provides an opportunity to clarify the association between extracellular forms of invadolysin and functional abnormalities.

The role of matrix metalloproteases in disease has been emphasised by the development of matrix metalloprotease (MMP) inhibitors as a key strategy for treatment of inflammatory and vascular disease (Hu, J. et al., 2007). It has been illustrated that regulation of MMP is relevant to hepatitis. For instance, the generation of mice with selective deletion of *Mmp2*, *Mmp3* or *Mmp9* demonstrated that deficiency of some MMPs increased survival rate and decreased levels of hepatocyte necrosis and apoptosis in mice with hepatitis (Wielockx et al., 2001). This is supported by clinical studies associating MMPs with liver disease. For example, an elevated level of MMP2 in serum was shown in patients with non-alcoholic steatohepatitis (NASH) (Toyoda et al., 2013). In 2016, a novel serum biomarker for acute liver disease was discovered by Irvine, who showed that increased serum levels of MMP7 are associated with significant or advanced liver fibrosis (Irvine et al., 2016). In an extension of this work, Irvine *et al.* (2021) demonstrated that serum MMP7 can serve as a non-invasive independent indicator of advanced liver fibrosis in NAFLD, especially for patients aged over 60 (Irvine et al., 2021). These investigations supported the proposal that NAFLD is an important clinic condition that would be appropriate for exploration of the role of invadolysin in inflammatory disease. The extracellular form of invadolysin will be

analysed in serum and plasma samples from patients with NAFLD to determine whether their expression forms are altered in this disease. In addition, a well-established *in vitro* model of hepatic cellular steatosis will be utilized to address gain further insights into altered invadolysin secretion in fatty liver disease (Lockman et al., 2012).

Another area of interest for clinical research is pulmonary inflammation. There is evidence that many MMPs are important in lung fibrosis (Hu, J. et al., 2007; Rosas et al., 2008; Todd et al., 2019). For example, Mmp7-knockout mice had reduced sensitivity to fibrosis induced in the lung (Zuo et al., 2002). In clinical research, Todd *et al.* reported a higher degree of expression of some circulating proteins in patients with IPF, demonstrating that circulating proteins can be a potential indicator of disease severity (Todd et al., 2019). To determine the potential of extracellular invadolysin as a biomarker of pulmonary inflammation, samples were obtained from two cohorts of patients with lung disease (IPF or coronavirus (COVID-19)).

In a previous clinical study, it was shown that, together with MMP1, gene expression level and protein concentration of MMP7 were higher in lung BALF of patients with IPF (Rosas et al., 2008). By analysis of the circulating proteome, Jenkins *et al.* explained that a patient's MMPs have a potential role in diseases progression as increased levels of protein fragments generated by proteolytic activity of MMPs were discovered in patients with IPF versus controls (Jenkins et al., 2015). In addition, protein levels of MMP7 and MMP1 were increased in serum and plasma suggesting a role for MMP7 and MMP1 in the pathogenesis of IPF and raising the possibility that levels of these enzymes in the blood may serve as a peripheral biomarker for IPF (Rosas et al., 2008; Todd et al., 2019).

Pulmonary fibrosis has been characterized as one of the physiologic sequelae of post-COVID19 (McGroder et al., 2021). In a cohort study of severe COVID-19, 72% of survivors with mechanical ventilation had fibrotic-like abnormal radiographic tests. Furthermore, it has been suggested that a fibrotic-like pattern observed using radiography could be associated with

degradation of pulmonary function, such as cough and frailty. In clinical trials, antifibrotic medicines (previously approved for treatment of IPF) have been used for treatment of post-COVID 19 lung fibrosis (Bazdyrev et al., 2021). For instance, nintedanib prevents activity of pro-fibrotic mediators, such as platelet-derived growth factor, and can inhibit the secretion of matrix metalloprotease. Since it has been estimated that the incidence of post-COVID pulmonary fibrosis can increase to 6% with moderate disease, a treatment strategy for post-COVID 19 lung fibrosis is desired by the global medical community. Therefore, it would be interesting to assess whether levels of invadolysin in the blood are associated with lung-specific inflammatory disease.

In addition to measuring extracellular levels of invadolysin in the blood, similar measurements in fluid secreted from the lungs may help clarify the association of invadolysin with pulmonary inflammation. This hypothesis will be addressed using BALF/blood samples taken from patients with IPF/COVID-19 at different stages of disease severity. This provides an opportunity to validate whether extracellular invadolysin is present in the BALF of patients with IPF/ COVID-19, and can be used as a marker of disease severity.

5.2 Hypothesis and aims of Chapter 5

This chapter aims to discover the extracellular role(s) of invadolysin in normal physiology. The *invadolysin* knockout mouse model would assist with understanding invadolysin deficiency in both male and female adult vertebrate (thesis aims two and four). The *in vitro* model of hepatic steatosis and NAFLD patients' blood samples would help with the exploration of whether invadolysin has a role on glycolysis and lipid metabolism in liver (thesis aim four). Furthermore, whether invadolysin has the potential as a biomarker of inflammatory diseases would be investigated under different pneumonia disorders, such as IPF and COVID-19 (thesis aim four).

Hypothesis: Extracellular Invadolysin plays an important role (s) in normal physiology and has potential as a biomarker of inflammatory disease.

Aims:

1. To determine the impact of invadolysin gene knockdown in *inv*^{-/-} mice, on the expression of extracellular invadolysin in serum and plasma.
2. To determine whether different structural forms of extracellular invadolysin are expressed in patients with non-alcoholic fatty liver disease (NAFLD) and in *in vitro* models of hepatic cellular steatosis.
3. To determine whether different structural forms of extracellular invadolysin are expressed in patients with lung inflammatory diseases, such as idiopathic pulmonary fibrosis (IPF) or coronavirus (COVID-19).

5.3 Results

5.3.1 Extracellular invadolysin is not absent from an *inv*^{-/-} mouse model

A physiological role of invadolysin's conserved catalytic motif has been revealed recently by generation of *invadolysin* mutant *Drosophila* recently (Abhinav et al., 2019). It has been suggested that proteolytic activity other than lipase activity is crucial for lipid storage. Though decreased levels of glycogen were observed in *invadolysin* mutants, neither protease or lipase motif has a significant effect on glycogen accumulation. Utilisation of transgenic invadolysin in invertebrate is an effective biological methodology to investigate the physiological role (s) of invadolysin *in vivo*. Based on these research, it has been recommended to generate knockout mice to address the physiological function of invadolysin in vertebrate. The Takahashi laboratory has attempted to generate *invadolysin* knockout mice with multiple genome editing systems to exploit invadolysin function *in vivo*. Deletion of exons 6-12 of *invadolysin* by CRISPR-Cas9 genome mediation system was processed in the mouse model. The results show that homozygous null mice could survive, however, homozygous mice did not show obvious phenotype. Therefore, it is still of interesting to investigate whether invadolysin in mice sera and plasma is deficient. Therefore, serum and plasma samples from both wild type and *invadolysin*^{-/-} mice, provided by the Takahashi lab, were examined for analysis. Around 200 μ l of blood sample were provided from each animal.

Immunoblotting of nine mice serum samples were probed with two anti-human invadolysin antibodies G3646 and R2192. Wild type and *inv*^{-/-} serum samples were loaded randomly during electrophoresis to avoid bias. Genotype was not labelled on each serum sample when loading for electrophoresis, and it would be revealed until immunoblotting analysis. Both antibodies recognized an extracellular form of invadolysin in mice serum migrating around 51 and 66 kDa (Figure 5.1 B and C). The band at 51 kDa seemed more enriched in *inv*^{wt} (S5) than other serum samples. In the

meanwhile, the form at 66 kDa was more enriched in *inv*^{-/-} (S1) among the serum fraction from different mice models. Overall, there is no significant differences in the expression form or enrichment of extracellular invadolysin in serum between wild type and heterozygous mouse models.

During inspection of secreted invadolysin in *Drosophila* hemolymph, Abhinav *et al.* discovered that extracellular invadolysin in soluble fraction of plasma is expressed in a sexual dimorphic form (Abhinav *et al.*, 2019). Whether such observations could be detected in vertebrate have been investigated in multiple attempts, such as using zebrafish and human plasma (Chapter 3 and 4). To further address this question, male and female serum fractions, collecting from *inv*^{wt} and *inv*^{-/-} mice models, were examined for expression forms of secreted invadolysin. Immunoblotting of mouse serum samples probed with antibody R2192 demonstrated an extracellular form of invadolysin (Figure 5.2). Besides two forms of invadolysin presenting in mouse plasma (51 and 66 kDa), a form of 27 was detected in mouse serum from both *inv*^{wt} and *inv*^{-/-} mice (Figure 5.2 B). In addition, a strong signal of 27 kDa form was enriched in mice plasma from both wild type and heterozygous mice (P4, P6 and P7). Interestingly, a weaker band migrating at 86 kDa was observed in all male serum samples, no matter the genotypes of invadolysin in each mouse model, which needs to be further determined as the molecular weight is higher than the precited full length of invadolysin. This band was absent in all female plasma samples. Protein bands migrating around 93-105 kDa molecular weight range were less readily detected in male. These results demonstrated that investigation of invadolysin in mouse soluble plasma fraction suggests a potential sexual difference in extracellular proteins exists in mammals.

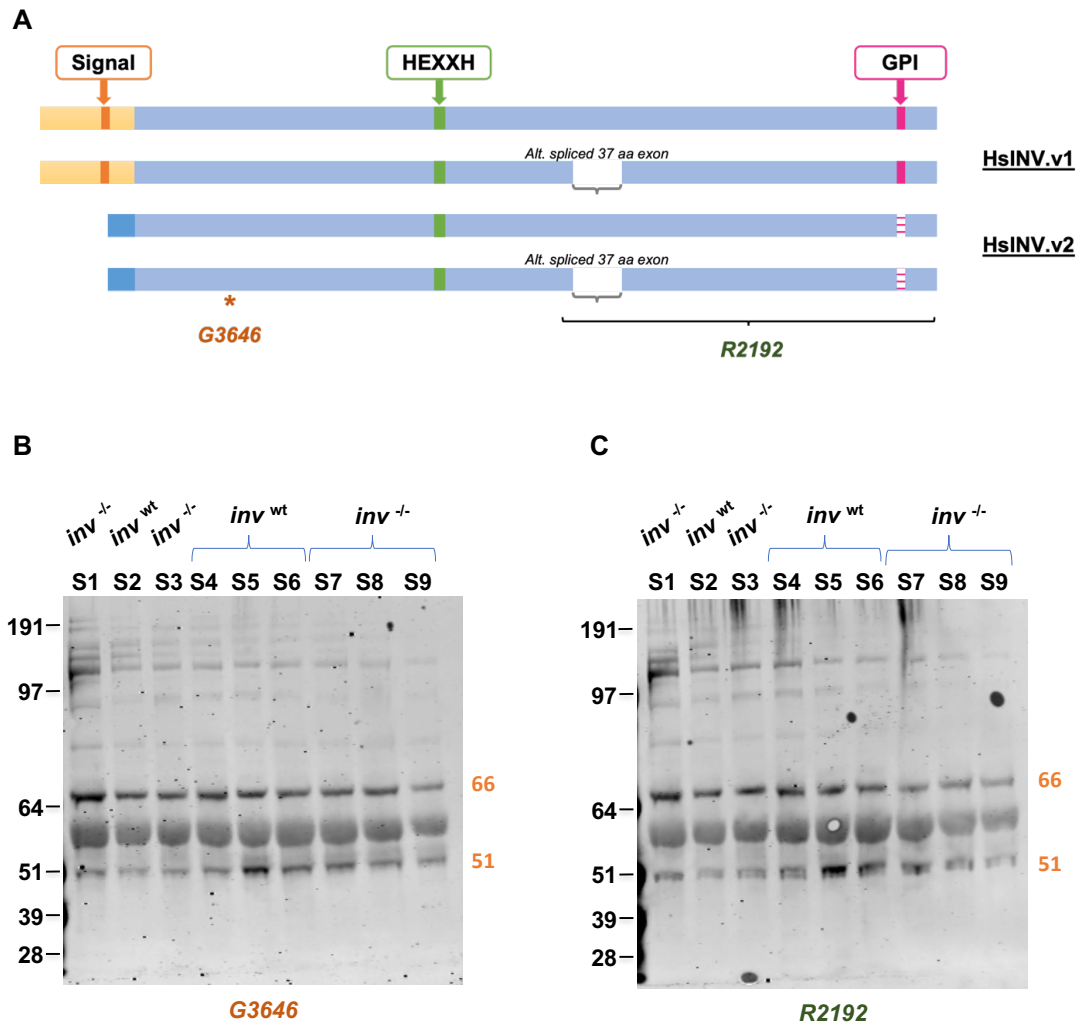


Figure 5.1 Extracellular forms of invadolysin are present in *inv*^{wt} and *inv*^{-/-} mouse serum.

(A) Schematic of four human invadolysin variants (HsINV.v1+37, HsINV.v1Δ37, HsINV.v2+37 and HsINV.v2Δ37). The epitope region recognized by antibody G3646 is highlighted by an asterisk. A bracket represents the region recognized by the antibody R2192. **(B,C)** Immunoblotting of serum (S: serum) from wild type (*inv*^{wt}) and invadolysin heterozygous (*inv*^{-/-}) mouse models. Serum samples were loaded in a single-blind strategy during electrophoresis. Antibodies G3646 and R2192 identified extracellular forms of invadolysin in both *inv*^{wt} and *inv*^{-/-} mice. Two forms of secreted invadolysin were observed, which are 51 and 66 kDa.

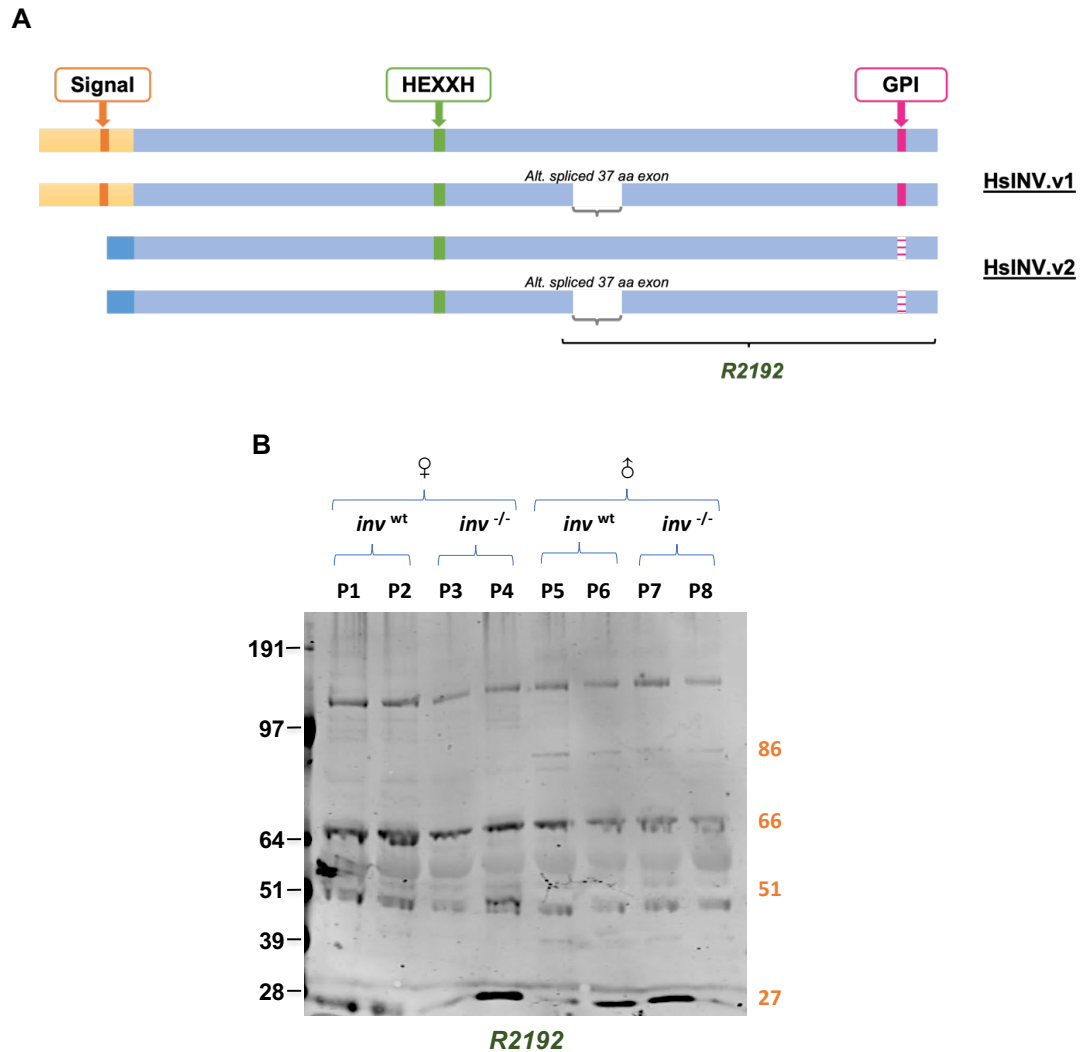


Figure 5.2 An extracellular form of invadolysin is present in plasma from male and female *inv*^{wt} and *inv*^{-/-} mice.

(A) Schematic of four human invadolysin variants (HsINV.v1+37, HsINV.v1Δ37, HsINV.v2+37 and HsINV.v2Δ37) highlighting the epitope region recognised by antibody R2192. **(B)** Immunoblotting of mice plasma (P: plasma) from wild type (*inv*^{wt}) and heterozygous (*inv*^{-/-}) mouse models. Plasma fractions from both female (P1-P4) and male (P5-P8) mice were tested for determination of sexual dimorphism of invadolysin. Secreted forms of invadolysin were detected in mouse plasma, which are 27, 51 and 66 kDa. The band around 86 kDa presenting in male plasma has not been determined. Protein bands in the 93-105 kDa molecular weight range were detected in female plasma.

5.3.2 Expression of serum invadolysin in NAFLD differs from non-NAFLD

In 2017, Chang *et al.* has proposed that invadolysin has a crucial role in the insulin pathway to participate in the regulation of adipocyte differentiation (Chang, C.-W. et al., 2017). During exploration of invadolysin's potential catalytic role in lipid accumulation, Abhinav *et al.* also suggested that invadolysin is important in lipid accumulation and glycogen storage by insulin signalling pathway (Abhinav et al., 2019). As a result, it has been hypothesized that extracellular invadolysin is important in lipid and/or glycogen metabolism. To assess this question, analysis of extracellular invadolysin in metabolic associated disorders would provide an opportunity to examine the physiological role(s) of invadolysin in inflammation. Blood samples from a cohort with NAFLD were collected and collaboratively provided by the Plevris laboratory, where these NAFLD patients were also diagnosed with type 2 Diabetes. Invadolysin in patients' serum and plasma samples were analysed by immunoblotting while comparing with control blood samples received from non-NAFLD volunteers (Figure 5.3). Both serum and plasma blood samples were prepared by SARSTEDT tubes (Plasma: S-Monovette Potassium EDTA/Serum: S-Monovette serum-gel tube) and top 12 abundant proteins were removed before proteomic analysis.

In patients' blood samples, invadolysin serum-specific form 51 kDa and plasma-specific form 53 kDa were detected by antibodies G3646 and G6456 (Figure 5.3B-E). A band around 87 kDa, shown in both control serum and plasma, was absent in all patients' serum samples. Furthermore, a band migrating at 108 kDa was observed in P1 and P2 serum samples, which were not shown in control blood samples (Figure 5.3B). In Figure 5.3D and E, the band 45 kDa was present in control serum, which was absent in P4, P5 and P6 serum samples. The 35 kDa, however, was observed in the patients' serum (P4-P6). NAFLD patients' demographic data was summarized in Table 5.1. Gamma-glutamyl transferase (GGT), alanine aminotransferase (ALT) and albumin are three of the typical liver function

Investigating the Essential Extracellular Invadolysin Metalloprotease

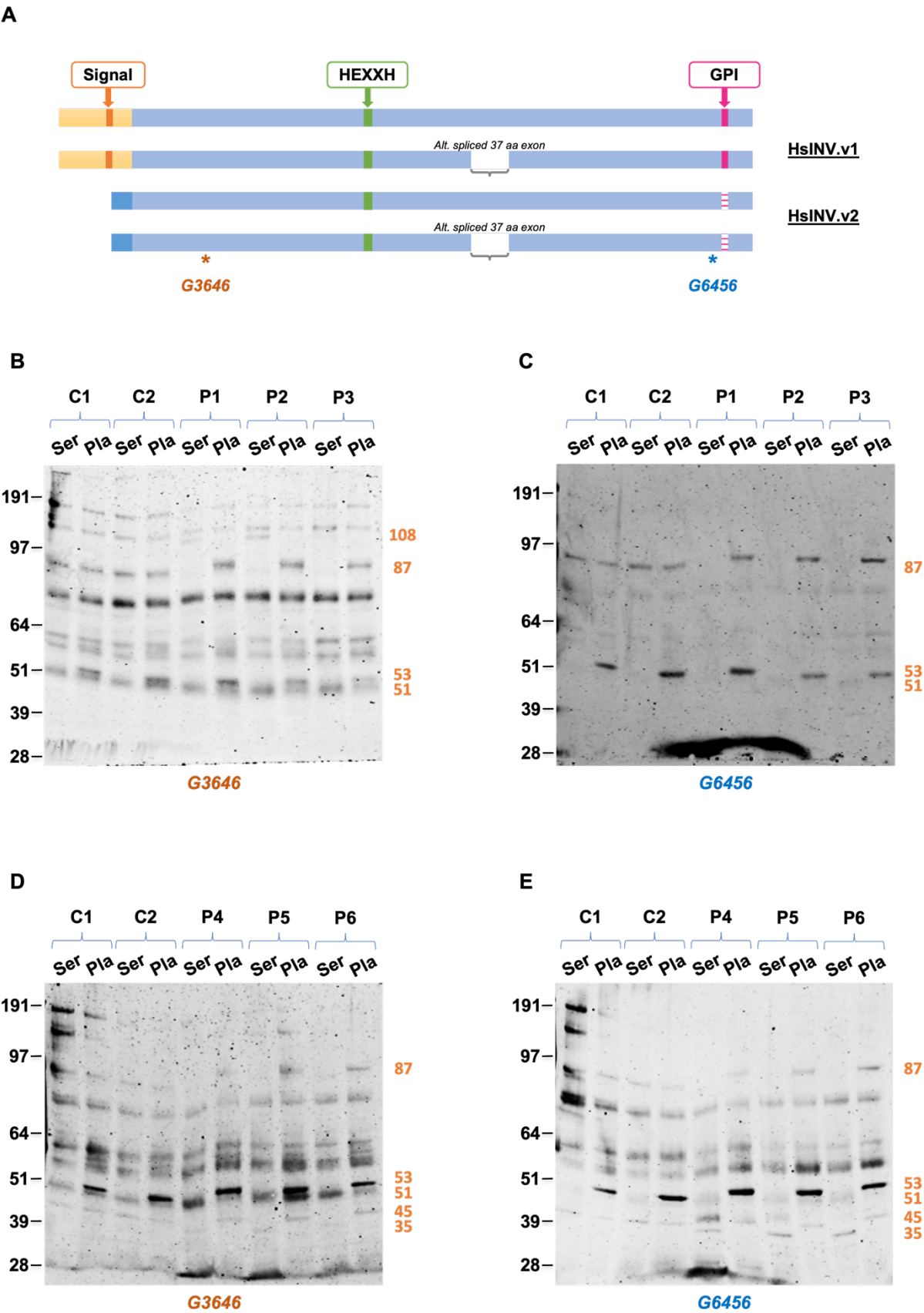


Figure 5.3 Extracellular form of invadolysin is dynamic in NAFLD patients' sera.

(A) Schematic of four variants of human invadolysin (HsINV.v1+37, HsINV.v1 Δ 37, HsINV.v2+37 and HsINV.v2 Δ 37). Epitopes regions against which antibodies G3646 and G6456 were generated are highlighted by asterisks. **(B-E)** Immunoblotting of human serum and plasma from NAFLD patients (P1-P6) and non-NAFLD controls (C1(male) and C2 (male)). Both control and patient samples were collected from different individuals. The top 12 abundant proteins in blood samples were depleted before proteomic analysis by chromatography. The secreted form of invadolysin in serum was dynamic in patients with NAFLD.

Table 5.1 NAFLD patients' demographic and clinical characteristics.

ID	Age	Gender	BMI (kg/m ²)	GGT (U/L)	ALT (U/L)	Albumin (g/L)
1	64	M	26	44	44	46
2	52	M	29	36	24	42
3	62	M	30	66	51	44
4	71	M	35	64	35	43
5	72	F	32	34	20	43
6	61	F	27	16	28	42

tests (Lala et al., 2022). The suggestive interval ranges for GGT, ALT and albumin are 6-50 U/L, 4-36 U/L and 35-50 U/L. The albumin values for all included patients situated between upper and lower limits. The ALT level of P1 and P3 is higher than the upper limit, which may indicate a higher enzymatic activity caused by hepatocellular injury. For P3 and P4, the GGT level is higher than 50 U/L, who also have higher BMI values (>30) compared with other NAFLD patients. These results suggest that serum extracellular invadolysin displays dynamic expressions between NAFLD and non-NAFLD.

5.3.3 *In vitro* models open a gate for research of extracellular invadolysin's role(s) in lipid and glucose metabolism

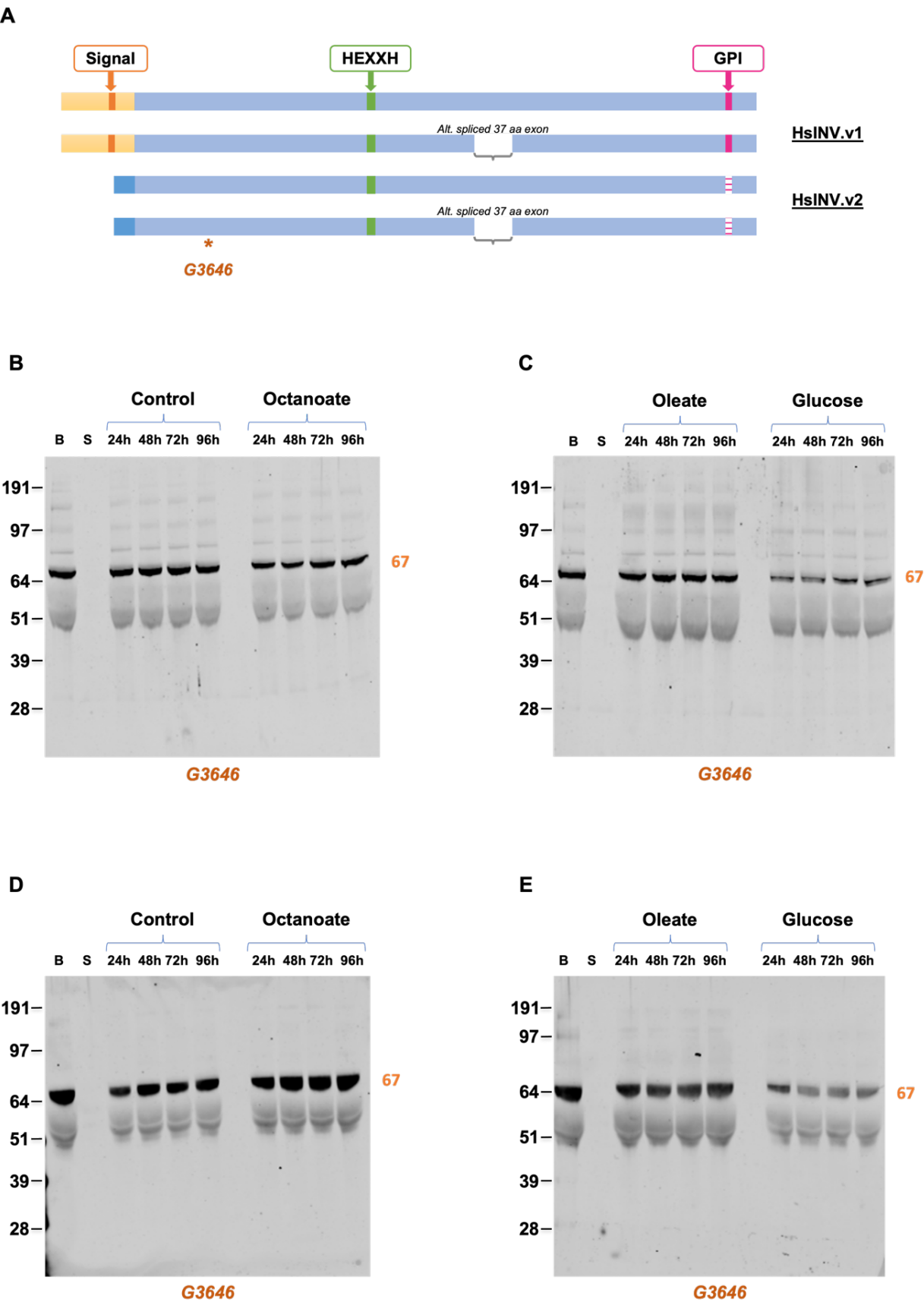
Previous immunoblotting results have shown a significant change in the extracellular form of invadolysin in NAFLD/T2D patients' sera, which implies that extracellular invadolysin has association with lipid deposition and/or glucose metabolism. However, the regulation of extracellular invadolysin during lipid accumulation and initiation of inflammation in liver have not been investigated and fully understood. To better address the regulatory mechanism of invadolysin's expression, a hepatic cellular steatosis model was established using human hepatoblastoma C3A cell line to mimic the NAFL disease model *in vitro* (Lockman et al., 2012). The HeLa cell was also used as a cell culture model to investigate the role(s) of invadolysin under circumstances of triglyceride and glucose accumulation.

Before high fat and high sugar treatment, C3A cells and HeLa cells were starved for 24 hr to set the baseline level of invadolysin in cell culture media and decrease the influence of invadolysin from foetal calf serum. Cells were treated with octanoate (2 mM), oleic acid (0.25 mM) and glucose (20 mM) for 24 hr, 48 hr, 72 hr and 96 hr, and cell culture media were refreshed every 48 hr. Extracted from cell culture media, invadolysin was detected by immunoblotting and analysed by ImageStudio and GraphPad. In both C3A and HeLa cell culture media, a form of 67 kDa was present in before starvation (B) and treatment groups (octanoate, oleic acid and glucose), and was absent in the starvation group (S) (Figure 5.4B-E). Signal strength of

protein bands representing invadolysin from cell culture media were normalised and summarized in Figure 5.4 F and G. For the control group of C3A cell culture model, the level of invadolysin in cell culture medium was not fluctuating dramatically over 96 hr (Figure 5.4F). Unlike C3A cell culture model, signal trend of invadolysin in HeLa cell culture model has a slight fluctuation after 48 hr's incubation (Figure 5.4G). The overall level of invadolysin in oleate-treated culture was close to the control group of both C3A and HeLa cell culture models. In C3A cell culture model, the level of invadolysin in octanoate-treated group decreased after 24 hr's incubation and increased to the highest level gradually starting at 48 hr. Following, the level of invadolysin in cell culture media approached to the highest after 96 hr's incubation. In HeLa cell culture model, when treated with octanoate, invadolysin's level reached to the highest after 48 hr's incubation and dropped gradually. These observations recommend that extracellular invadolysin's expression may be more sensitive to octanoate treatment since changes in the level of invadolysin were observed between 24 to 48 hr. In addition, these results provided evidence that extracellular invadolysin has a potential association with lipid accumulation induced by octanoate treatment at 2 mM.

When C3A cells were treated with glucose at 20 mM, though the level of invadolysin increased after 48 hr's incubation, the overall level of invadolysin in cell culture medium was lower than control group and two high fat diet treated groups. In HeLa cell culture model, levels of invadolysin at each time point was less than respective value for untreated and lipid treatment groups. These results suggest that extracellular invadolysin may be important for cellular glucose metabolism. Therefore, the association between invadolysin and glucose metabolism would be examined further by using HeLa cells.

The concentration of glucose in HeLa cell culture medium was 5.5 mM. HeLa cells were starved for 24 hr and incubated with cell culture medium containing glucose at concentrations of 5.5 mM, 27.5 mM and 55 mM for 24 hr, 48 hr, 72 hr and 96 hr. Cell culture media were refreshed every 48 hr and



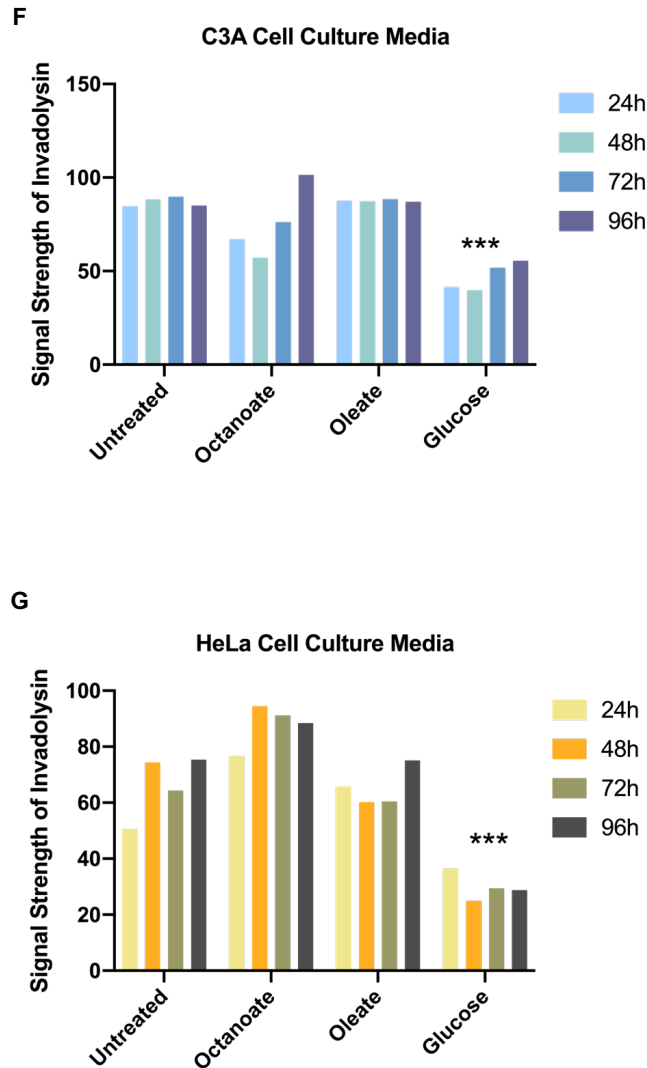


Figure 5.4 Preliminary exploration of expression of invadolysin in cell culture medium during lipid and glucose accumulation in C3A and HeLa cells.

(A) Schematic of human invadolysin with the G3646 antibody highlighted with an asterisk. (B-E) Analysis of the effects of lipid and glucose storage on secretion of invadolysin from C3A (B and C) and HeLa cells (D and E) respectively. Immunoblotting of protein extracts from C3A and HeLa cell culture media gathered at 24, 48, 72 and 96 hrs' incubation was probed with antibody G3646, which recognized a band of 67 kDa. (F,G) Signal strength of invadolysin after 24, 48, 72 and 96 h of treatment on immunoblotting was collected by Image J and analysed by GraphPad. The baseline of invadolysin has been decided depending on the signal strength from 'before starvation'. Untreated: cell culture with standard medium; Octanoate: cell culture with 2 mM of octanoate; Oleate: cell culture with 0.25 mM of oleate; Glucose: cell culture with glucose at 20 mM. Two-way ANOVA test was used for statistical analysis for (F) and (G). *** $P < 0.001$, ** $P < 0.01$ and * $P < 0.05$ compared with untreated cells of the same treatment duration.

invadolysin in cell culture media was analysed by immunoblotting. An extracellular form of invadolysin migrating at 67 kDa was detected by primary antibody G3646 (Figure 5.5B and C). Signal strength of protein bands representing invadolysin from cell culture media were normalised and summarized in Figure 5.5 D. In control group (glucose: 5.5 mM), the level of invadolysin in cell culture medium was increasing gradually after nutrient boost. In 27.5 mM glucose-treated group, the level of invadolysin in cell culture medium reached to the highest at 48 hr and dropped to the bottom at 72 hr. In the highest concentration of glucose-treated group, overall levels of invadolysin are higher than control and 27.5 mM group at each time point. And the level of invadolysin was fluctuated slightly within the group. These results demonstrate that extracellular invadolysin can be released into cell culture medium in response to high glucose treatment in 24 hr, which implies an increased expression of extracellular invadolysin triggered by high glucose diet. In addition, regulation of extracellular invadolysin has a relative higher sensitivity to glucose metabolism.

5.3.4 BALF extracellular invadolysin exhibits variations in expression forms during lung inflammation and lung fibrosis

To further understand the physiological role(s) of extracellular invadolysin in inflammatory, various patient cohorts with interstitial lung diseases (ILD) have been utilised for research. Patient blood and BALF samples were kindly provided by the Hirani laboratory, which has been committed to the development of novel prognosis tools and treatment targets for ILD. Patients' blood and BALF samples were originating from the Edinburgh Lung Fibrosis Biobank (Clinical Trials.gov; Identifier: NCT04016181). Serum samples were prepared by serum-gel tubes and plasma samples were prepared by tubes with EDTA. BAL samples were collected from patients with 40 mL of saline for 6 times. During BALF sample preparation, BAL cells were precipitated by centrifugation at 300xg for 10 min. Supernatant was retrieved and stored as BALF samples for further analysis. In order to examine extracellular

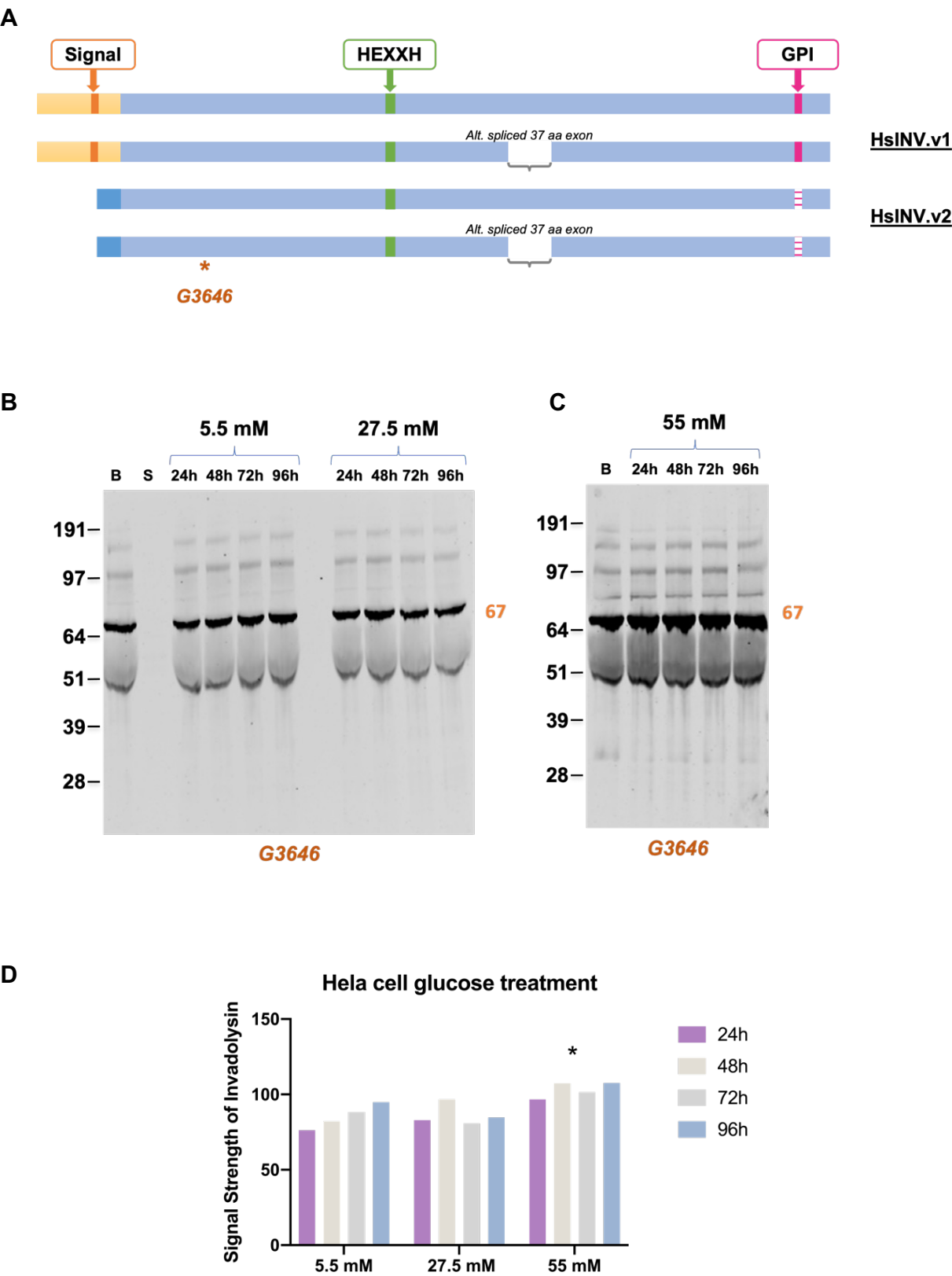


Figure 5.5 Expression of invadolysin in cell culture medium during glucose accumulation in HeLa cells.

(A) Schematic of four human invadolysin variants (HsINV.v1+37, HsINV.v1 Δ 37, HsINV.v2+37 and HsINV.v2 Δ 37) with epitopes recognised by antibody G3646. **(B,C)** Exploration of effects of glucose treatment on secretion of invadolysin from HeLa cells at glucose concentrations of 5.5, 27.5 and 55 mM. Protein extracts isolated from cell culture medium collected at 24, 48, 72 and 96 hrs' incubation probed with G3646 identified a form of invadolysin at 67 kDa. **(D)** Signals of invadolysin on immunoblots were generated by ImageStudio and analysed by GraphPad. Data normalisation was based on the signal of invadolysin under 'before starvation'. 5.5 mM: glucose concentration of standard cell culture medium; 27.5 and 55 mM: final concentrations of glucose in cell culture media. Two-way ANOVA test was used for statistical analysis for **(D)**. *** $P < 0.001$, ** $P < 0.01$ and * $P < 0.05$ compared with untreated cells of the same treatment duration.

invadolysin in patients with ILD, both blood and BAL samples were utilized for proteomic analysis by immunoblotting (Figure 5.6). Blood and BALF samples from patients with ILD, such as IPF and idiopathic interstitial pneumonia (IIP) with and without lung fibrosis, were analysed and compared with non-lung inflammation control samples (Table 5.2).

The 53 kDa extracellular form of invadolysin in serum was detected in three patients (Figure 5.6B). The form of 55 kDa was observed in patient 1 and patient 2 plasma samples, which was validated with antibodies G3646 and G6456 (Figure 5.6B and C). When compared with control serum and plasma, expression of invadolysin in patient blood was not showing a significant difference. Though plasma sample of patient 3 was not available for analysis, these results suggest that blood extracellular invadolysin in patients with ILD is not influenced by lung inflammation.

Even BAL control sample was not accessible in this cohort, it is still questioned whether invadolysin is present in human BAL and whether this form of extracellular invadolysin is associated with lung fibrosis. A band with 53 kDa in BALF from patients with different lung inflammation states was discovered, which was confirmed with both antibodies G3646 and G6456 (Figure 5.6B and C). Furthermore, this form of invadolysin in patients with lung fibrosis (P1 and P2) is not displaying a significant difference when comparing to patient without lung fibrosis (P3). The signal strength of this band in each patient sample was standardized based on total protein concentrations by Image J (Table 5.2). This signal strength is higher in ILD patient without fibrosis (P1: IIP without fibrosis) than patient with fibrosis (P2: IIP with fibrosis; P3: IPF). Moreover, patient with IPF has the least signal strength compared to patients with IIP. IPF is a subset of IIP, which is normally characterized as abnormal lung fibroblast proliferation, reduction in lung function and progressive respiratory failure (Barratt et al., 2018; Sagardia et al., 2021). Compared with other IIPs, IPF has a relative high mortality rate and poor prognosis, which is one of the most aggressive type of IIP. Therefore, analysis of this result revealed that secretion of

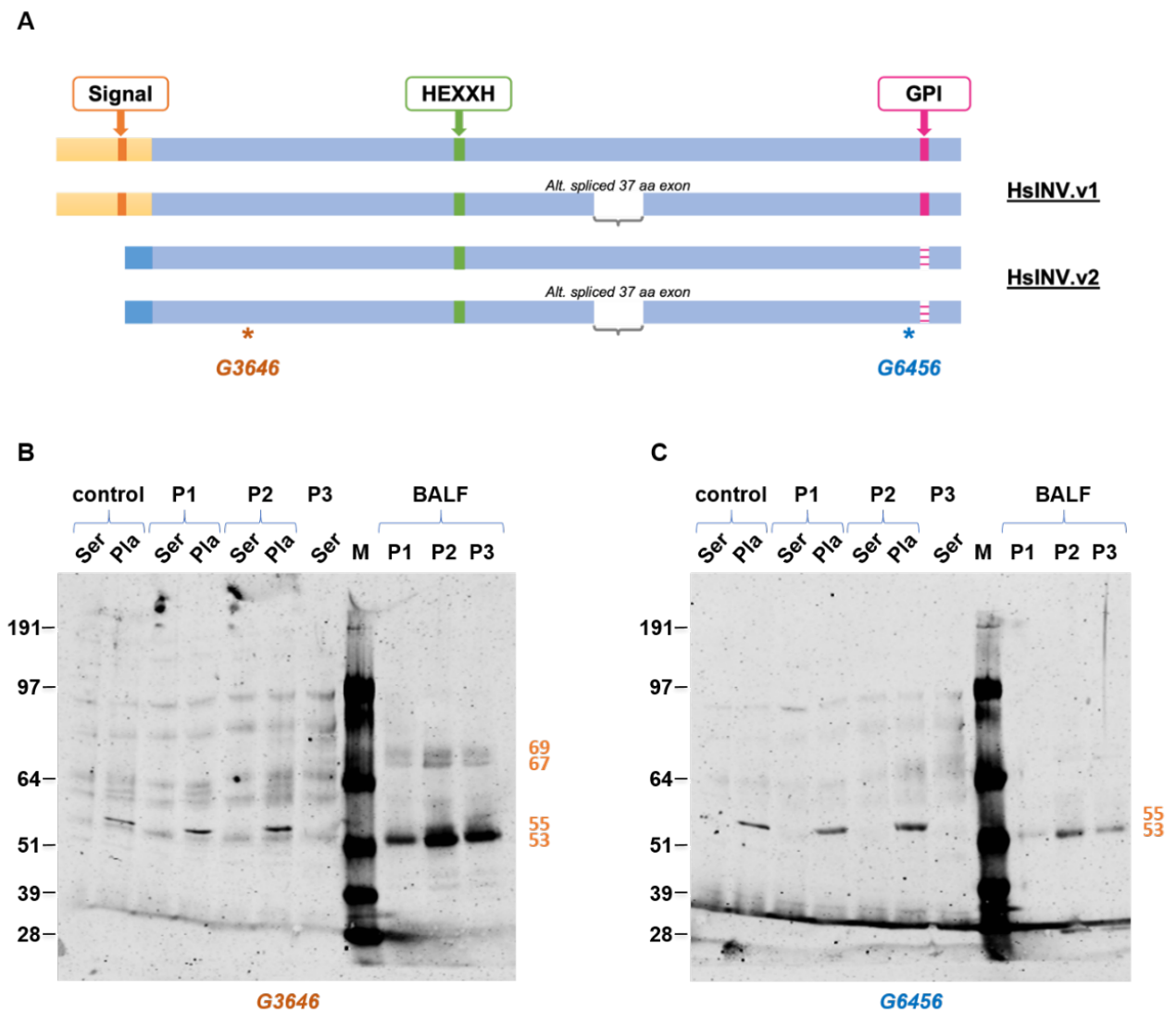


Figure 5.6 Extracellular forms of invadolysin were detected in BALF samples from patients with ILD.

(A) Schematic of four human invadolysin variants (HsINV.v1+37, HsINV.v1Δ37, HsINV.v2+37 and HsINV.v2Δ37). Epitopes for respective antibody were labelled and highlighted individually. **(B,C)** BALF samples from ILD patients (P1, P2 and P3) characterized a secreted form of invadolysin. A form of 53 kDa was identified by antibody G3646, which was also probed by antibody G6456. Two bands migrating at 67 and 69 kDa were further recognized in BALF samples from patient 1, 2 and 3. Blood samples probed by antibodies G3646 and G6456 proved the secreted form of invadolysin. The extracellular form of invadolysin in serum and plasma

Table 5.2 ILD patients' demographic and clinical characteristics.

ID	Age	Gender	Smoking	BMI	Diagnosis	Standardised Signal Strength in BALF	Progression
1	35	F	Current	35	IIP without fibrosis	36.3	NA
2	30	M	Ex-smoker	30	IIP with fibrosis	33.6	NA
3	36	M	Never	36	IPF	27.3	NA

extracellular has no significant difference between control (non-ILD volunteers) and patients with ILD. Invadolysin in BALF declines as the severity of lung diseases increases. Two bands around 67 and 69 kDa were shown in patients' BALF samples, which was only detected by antibody G3646. Overall, these results suggest that an extracellular form of invadolysin is present in human BAL. The expression form of invadolysin in BAL is similar among IPF and IIP. In addition, regulation of the extracellular presence of invadolysin in BAL can be related to severity of IIP and the formation of lung fibrosis.

As invadolysin has been found in BAL from patients with IPF, it has been assumed that extracellular form of invadolysin in BAL is associated with IPF progression. In this cohort, IPF progression or lung function decline was defined as a reduction of forced vital capacity (FVC) $\geq 10\%$ in a follow up period of 12 months (Wuyts et al., 2020). BALF samples from patients at stable and progressed disease status were collected and examined by immunoblotting. To avoid analysing bias, BALF samples were loaded in a double-blinded strategy. Diagnostic and demographic features of IPF patients were concluded in Table 5.3. The bands of 27, 53 and 67 kDa were detected in both stable and progressed disease conditions, which was validated by antibodies G3646 and G6456 (Figure 5.7B and C). A form at 69 kDa has been observed in patients 6 to 8. Furthermore, it is worth pointing out that a band migrating at 55 kDa is present in P8. As shown in Table 5.3, patient 8 has no smoking history, while patient 6 and 7 are current smoker or ex-smoker. This result implies that smoking could be served a risk factor in lung damage, which would influence expression forms of extracellular invadolysin in BAL. There is no obvious evidence to demonstrate a significant difference in regulation of BAL invadolysin between stable and progressed IPF, though a variety of extracellular invadolysin forms displayed in three out of five patients at stable status. Thus, whether regulation of extracellular invadolysin in BAL is associated with IPF progression is still inconclusive.

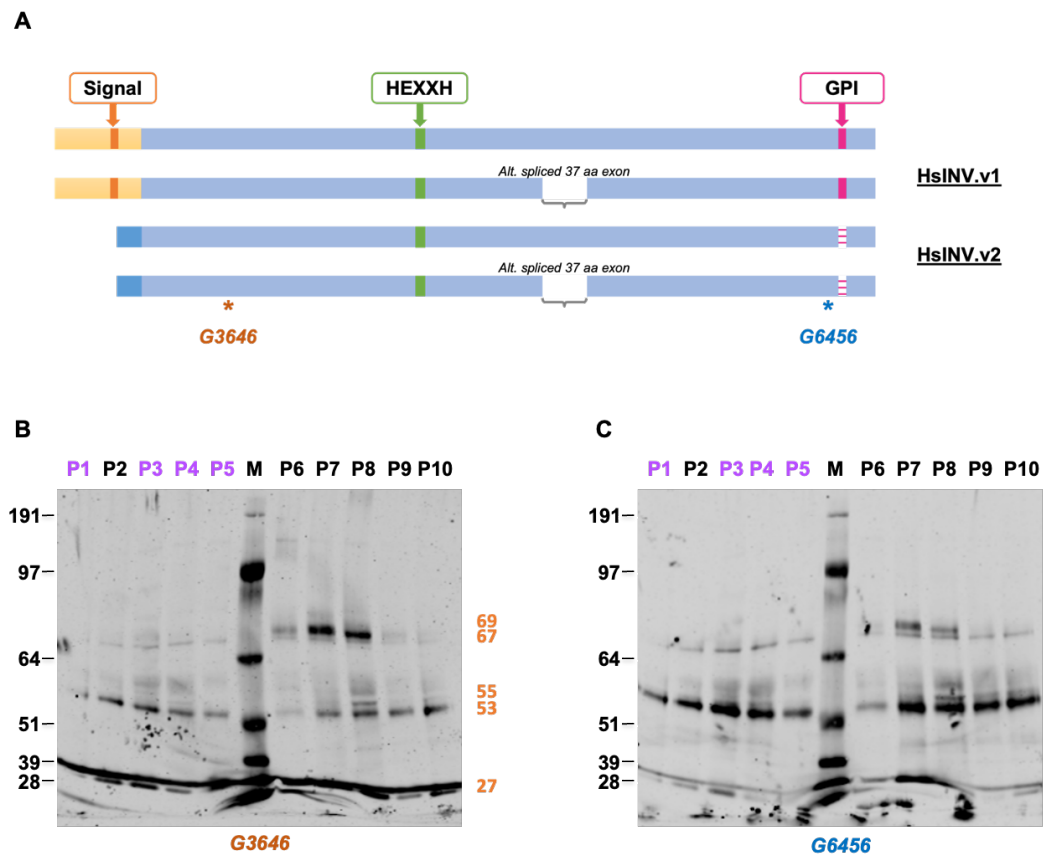


Figure 5.7 Expression of extracellular forms of invadolysin in BALF from IPF patients during stable and progressed stages.

(A) Schematic of four variants of human invadolysin (HsINV.v1+37, HsINV.v1 Δ 37, HsINV.v2+37 and HsINV.v2 Δ 37). Epitopes of antibodies G3646 and G6456 were labelled and highlighted by asterisks. **(B,C)** Patients' BALF samples probed by anti-invadolysin antibodies demonstrated a dynamic expression pattern of secreted form of invadolysin. Three forms of invadolysin were identified in patients' BALF migrating at 27, 53 and 67 kDa. A band at 69 kDa were detected in BALF samples from patient 6, 7 and 8. In patient 8's BALF, a band with molecular weight 55 kDa was also observed. Patients 1, 3, 4 and 5 were diagnosed with IPF in a progressed status (Violet: progressed). Patients 2, 6, 7, 8, 9 and 10 were diagnosed with stable IPF (Black: stable).

Several cohort studies have shown evidence that lung function in a normal population decreases as aging in asymptomatic adults by spirometry measurements, such as forced expiratory volume (FEV1) and forced vital capacity (FVC) (Thomas, E. T. et al., 2019). To further explore how invadolysin is regulated in BAL during IPF progression as lung function decreasing gradually, invadolysin in BALF samples from each patient in following one or two continuous years were analysed by proteomic research. BALF samples probed by anti-invadolysin antibodies G3646 and G6456 recognized two forms of invadolysin, which were migrating at 27 and 53 kDa. (Figure 5.8B and C).

The level of 27 kDa form is dynamic after one- or two-years' illness progression. Similar to previous results, the band at 67 kDa was detected in BAL from P2 and P3, and the expression form of this band was relative stable as lung function reducing in one or two years. In previous result, the 69 kDa form was present in patients 7 and 8 (Figure 5.7B and C). As lung function decreased, this form of invadolysin was absent after 1 year in BALF samples collected from patients 7 and patients 8. Furthermore, this form was present in patient 9's BAL after two years. It is worth noting that the form at 55 kDa, enriched in patient 8's BAL, was absent during one year's lung function decline. It has been observed that expression of BAL invadolysin is dynamic in three out of four IPF patients with stable status diagnosis. These results suggested that regulation of extracellular invadolysin may be associated with lung function decline.

5.3.5 Plasma invadolysin displays a dynamic expression between coronavirus and non-coronavirus

It has been shown that extracellular invadolysin in BALF is dynamic during under lung inflammation conditions of ILD patients. This finding warranted a further investigation of whether regulation of extracellular invadolysin is related to severe respiratory diseases, such as COVID-19 caused by SARS-CoV-2, and demonstrates modifications in expression during

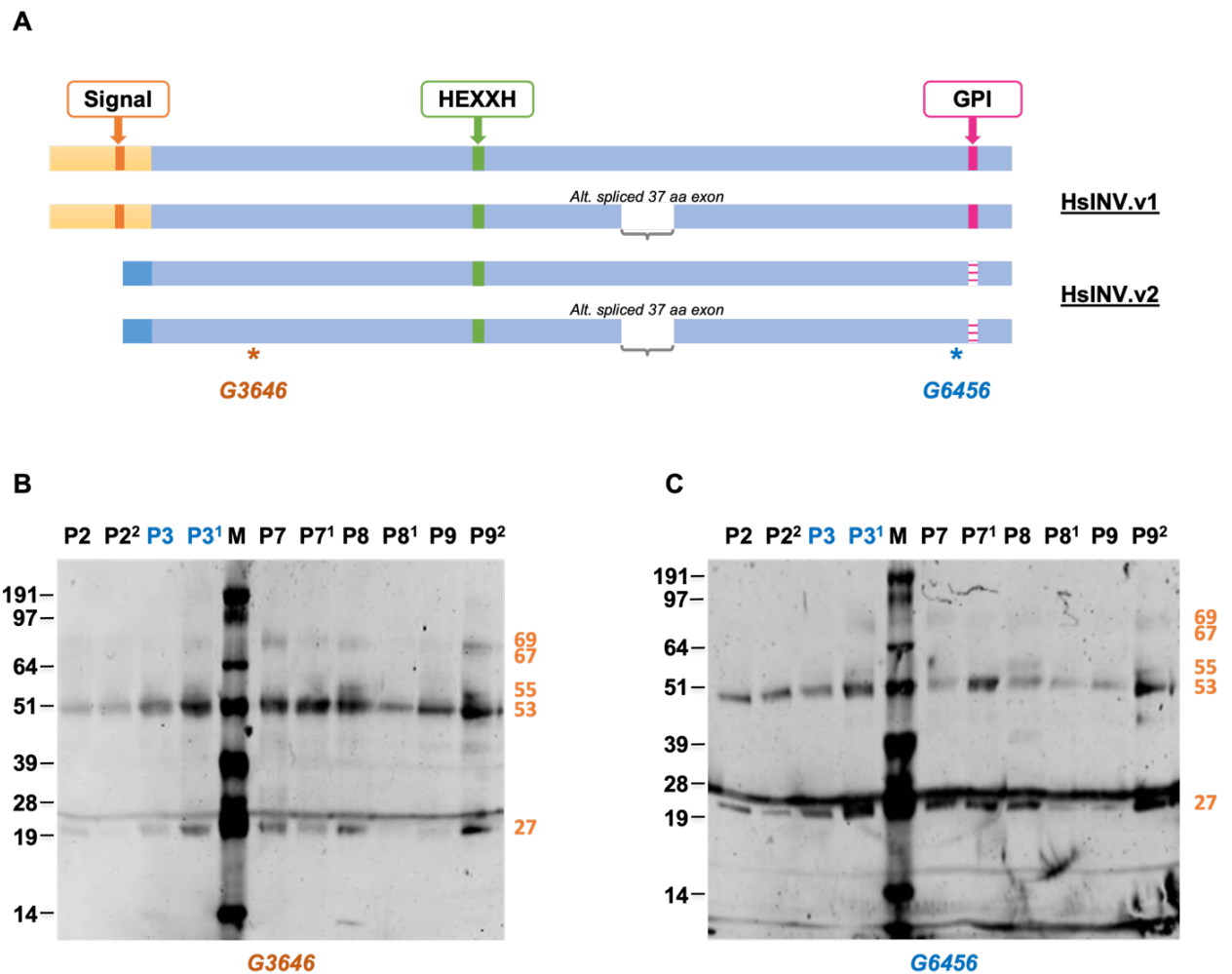


Figure 5.8 Regulation of extracellular forms of invadolysin is associated with lung function decline.

(A) Schematic of four human invadolysin variants (HsINV.v1+37, HsINV.v1 Δ 37, HsINV.v2+37 and HsINV.v2 Δ 37) with epitope regions recognised by antibodies G3646 and G6456. **(B,C)** Effects of IPF progression on expression of secreted invadolysin in BALF samples from patients. BALF samples collected from the same patient with IPF within one¹ or two² consecutive years were analysed by immunoblotting. Expression level of 27 kDa form is fluctuating in patients' BALF during progression of IPF. A 55 kDa form was absent in P8¹ and present in P9². The band at 69 kDa was enriched in P9² and reduced in P7¹ and P8¹. Patients 3 was diagnosed with IPF in a progressed status (Blue: progressed). Patients 2, 7, 8 and 9 were diagnosed with stable IPF (Black: stable).

Table 5.3 IPF patients' demographic and clinical characteristics.

ID	Age	Gender	Smoking	BMI	Diagnosis	Progression
1	67	F	Current	32	IPF	Yes
2	68	F	Ex-smoker	38	IPF	No
3	79	M	Ex-smoker	29	IPF	Yes
4	77	M	Ex-smoker	29	IPF	Yes
5	67	M	Ex-smoker	34	IPF	Yes
6	71	F	Current	32	IPF	No
7	69	M	Ex-smoker	36	IPF	No
8	74	M	Never	24	IPF	No
9	57	M	Ex-smoker	29	IPF	No
10	65	F	Ex-smoker	32	IPF	No

immunopathology. For this purpose, COVID-19 patients' plasma samples were examined to address a potential role of extracellular invadolysin in lung inflammation. Cohort samples were kindly provided by the Hirani laboratory, who has been focusing on the study of a clinical trial known as DEFINE (University of Edinburgh COVID-19 Define trial, 2020) (Gaughan et al., 2023). DEFINE is an exploratory medicine platform aiming at the evaluation of the safety, and pharmacokinetics and pharmacodynamics of GB0139 for COVID-19 treatment (Gaughan et al., 2021; Gaughan et al., 2023). GB0139 is an inhaled galectin-3 inhibitor for treating patients with COVID-19, which was first reported by DEFINE (Gaughan et al., 2023). In this clinical trial, participant patients were confirmed with COVID-19 pneumonitis and had an add-on treatment with inhaled GB0139 as a complement to the standard of care (SoC) (Gaughan et al., 2022). Patients were administered with GB0139 by inhalation at 10mg twice daily for 48 hours. And the dose of GB0139 was reduced to 10mg once daily in following up to 14 days. Plasma samples from five patients with COVID-19 were collected at day 0 (D0: without medical treatment) and day 3 (D3: patients had received therapy for 3 days). Protein samples were extracted and enriched by PEG precipitation at concentration of 4%. Both supernatant and pellet fractions were analysed through immunoblotting in comparison with plasma and/or serum controls from non-COVID volunteers.

Antibodies G3646 and G6456 recognized 55 and 65 kDa forms of invadolysin in control and patients' plasma pellet fractions at D0 and D3 (Figure 5.9B and C). A band at 53 kDa was detected in control serum pellet fraction. In P1 plasma, extracellular form of invadolysin with molecular weight at 65 kDa decreased after 3 days. In addition, two bands migrating around 70 and 35 kDa were observed in P1, which were not present in control and P2 plasma. To validate the experimental results, three more groups of plasma samples from patients with COVID-19 (P3-P5) were included for examination along with plasma samples from P1. Control plasma from three non-COVID-19 volunteers (C1 and 2: male; C3: female) were utilized in this experiment. Both antibodies 4888 and G6456 detected the extracellular form of invadolysin at 55 kDa and 65 kDa (Figure 5.10B and C). In addition,

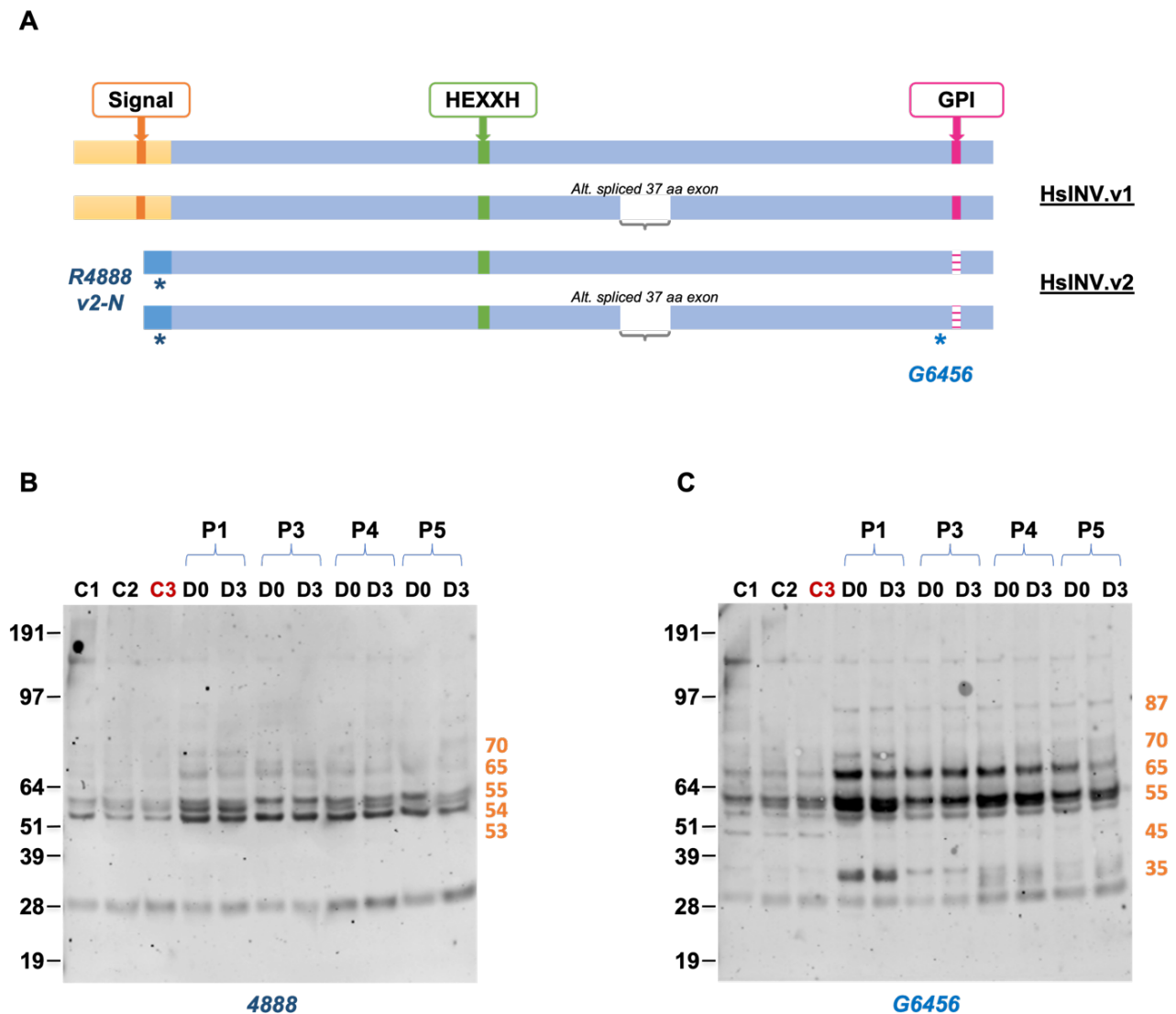


Figure 5.10 The effect of severity of lung infection on extracellular forms of invadolysin.

(A) Schematic of four variants of human invadolysin (HsINV.v1+37, HsINV.v1Δ37, HsINV.v2+37 and HsINV.v2Δ37). Epitopes recognised by antibodies were labelled and highlighted by an asterisks. **(B,C)** Expression of extracellular invadolysin in plasma from patients with COVID-19 at pre-treatment (D0) and administration of GB0139 plus SoC for three days (D3). Evaluation of secreted invadolysin in PEG-precipitated pellet, after 4% PEG 6000 precipitation of controls (C1 and 2: male in black colour; C3: female in red colour) and patients (P1-P5) plasma. The prominent bands at 53, 55 and 65 kDa in human plasma were detected by antibodies 4888 and G6456. The forms of 35 and 70 kDa were enriched in P1. The bands at 45 kDa in controls was probed with G6456. The band at 87 kDa in examined patients' samples were detected by G6456, exclusively. For patient 5, the level of 55 and 65 kDa forms decreased at D3.

Table 5.4 COVID-19 patients' demographic and clinical characteristics.

ID	Age	Gender	Smoking	BMI	NEWS2 D0	NEWS2 D1	NEWS2 D2	NEWS2 D3
1	80	M	Never	26	8	7	5	5
2	82	F	Ex-smoker	42	8	5	7	5
3	66	F	Never	34	4	5	2	4
4	73	M	Never	34	6	5	2	2
5	53	F	Ex-smoker	31	2	1	3	3

antibody 4888 probed a form of invadolysin at 53 kDa in both control and patients' plasma. Another form migrating around 54 kDa was detected in control C2 and C3, and in patients P1, P4 and P5 at D3. This form could also be validated by G6456 in C2 and C3, though the band migrating at 55 kDa, with strong strength of signal, may influence the detection of band at 54 kDa in patients' plasma. It has shown that the 55 kDa form of invadolysin reduced at D3 of P5 (Figure 5.10B). Furthermore, the 65 kDa form also decreased for the same patient at D3 (Figure 5.10C). As discovered before, the band with molecular weight 35 and 70 kDa having a stronger signal were observed in P1 plasma. This 35 kDa form was also detected in P3, P4 and P5 at different days. Moreover, antibody G6456 probed a band migrating at 87 kDa from all patients' plasma at D0 and D3, which was not present in control plasma samples.

Demographic features of COVID-19 patients were summarised in Table 5.4. This table demonstrates smoking history, BMI value and NEWS (National Early Warning Score) 2 of each patient. NEWS2 is an updated standardised system used widely by NHS to assess the severity of acute-illness since 2017 (Royal College of Physicians 2017). A NEW score of 5 or more is defined as a vital threshold, which indicates a potential deterioration. A prompt response or urgent clinical therapy would be determined on the basis of NEWS. In general, the NEW scores in 4 consecutive days for patient 5 were below the threshold value and relative lower than scores of other four patients. Overall, the NEW scores of P1 and P2 were above the threshold in first 4 days and relatively higher than those of other three patients. The BMI values for P1 and P2 were in polarization, which were 26 and 42. The NEW scores of Patient 3 and Patient 4 lied in the range of medium to low intervals. The BMI values for P3, P4 and P5 were in the middle comparing with those values of P1 and P2. All together, these results recommended that structural forms of extracellular invadolysin in COVID-19 patients' plasma can be related to lung infection and the severity of lung inflammation, which implies a role of extracellular form of invadolysin in lung diseases.

5.4 Discussion

The main purpose of the work described in chapter 5 was to identify a potential physiological function of invadolysin. In a previous review, it was suggested that the zinc-metalloprotease GP63 is one of the virulence factors secreted by *Leishmania* species (Isnard et al., 2012; Lambertz et al., 2012; Olivier et al., 2012). *Leishmania* has developed several defensive systems to protect itself from the innate immune response of host macrophage. GP63 has been found to be involved in the activation of protein tyrosine phosphatases, inactivation of transcription factors and modulation of kinase pathways, such as MAPK (Mitogen Activated Protein Kinase) signalling, which together inhibits signalling activities and vital antimicrobial effect of macrophages. GP63 mediates diverse mechanisms important for *leishmania* pathogenesis, thus it has been proposed that GP63 could be used as a novel target for treatment of Leishmaniasis and vaccine development (Filho et al., 2022; Gioseffi et al., 2020; Lambertz et al., 2012). As GP63 is the prototype of invadolysin in the M8 family of metalloproteases, it has been hypothesised that invadolysin possess an important role in normal physiology, such as in the innate immune response. To further explore the physiological function of invadolysin, both *in vivo* and *in vitro* models have been established to investigate invadolysin's expression. In addition, expression of extracellular invadolysin in a cohort of patients with various inflammatory conditions was examined and analysed in combination with patients' demographic and clinical characteristics.

5.4.1 Preliminary research of invadolysin's function in *in vivo* and *in vitro* models

5.4.1.1 Conventional methods have limitations to establish *invadolysin* knockout mice model

McHugh *et al.* have demonstrated that invadolysin makes an important contribution to the maintenance of normal mitotic cell activities (McHugh et al., 2004). In *IX-14* mutant *Drosophila*, abnormalities in mitotic cells were observed, such as defects in chromosome condensation, mutant phenotype of spindles-monopolar, disorganized and asymmetric structures, and

overexpression of the nuclear envelope. Moreover, invadolysin has a lethal effect at the late larval developmental stages of *Drosophila* and displays a significant role in cell migration and angiogenesis in zebrafish (McHugh et al., 2004; Vass & Heck, 2013). These studies emphasize that *invadolysin* is a life essential gene, and thus support further research of extracellular invadolysin in an *in vivo invadolysin* knockout model.

CRISPR-Cas9 genome editing was used to introduce deletions of exon 6 to exon 12 in sequences to generate a null mutation of *invadolysin* in mice. The assumption of this experiment was that the stop codon would be present at exon 13 after genetic modification, which would trigger nonsense-mediated mRNA (NMD) machinery degradation. Under this experimental design, loss-of-function (LoF) alleles or 'null' alleles of invadolysin could be created for functional analysis. Examination of extracellular invadolysin in wild type and mutant mouse serum and plasma shows that the secreted form of invadolysin is present in mouse blood, which agrees with previous research that the secreted form of invadolysin is a component of vertebrate blood (Abhinav et al., 2019). Interestingly, neither the expressed form of this secreted invadolysin nor the degree of enrichment in wildtype and mutant blood showed any significant differences. However, it is important to note that there was no homozygous mouse available in this experiment and *invadolysin* mutant mice healthy. The Takahashi lab has suggested that embryonic lethality occurred in homozygous mice and investigation of embryonic lethality would be further investigated.

A similar phenomenon was interpreted by Lalonde *et al.*, who stated that the splicing pattern was influenced by CRISPR-Cas9 introduced frameshift insertion-deletions (indels), which cause shorter and in-frame transcripts (Lalonde et al., 2017). For example, exon skipping caused by genome editing engineer could result in encoding of partially functional proteins, such as truncated proteins, and even fully functional proteins. This discovery was further discussed by Bello *et al.*, who indicated that differentially joining, skipping or overlapping of exons in a pre-mRNA transcript leads to splice variants in gene expression (Bello et al., 2020). Genetically-modified

homozygous mice could still survive with a mutant protein since a shorter version of the expressed protein has sufficient functional properties to support a normal physiology.

5.4.1.2 The transcriptome reveals a potential sexually-dimorphic form of invadolysin in mice

It has been noted that when evaluating expression forms of secreted invadolysin in mouse plasma, there is a tendency towards sex-dependent differences. In recent research, sexually-dimorphic gene expression profiles in brown adipose tissue (BAT) were screened by Kaikaew *et al.*, who identified an important role of progesterone in regulation of the transcription of sexually-differentially expressed genes (Kaikaew *et al.*, 2021).

Assessment of the transcriptome of BAT revealed expression levels of invadolysin in male (3TPM; **T**ranscript **p**er **M**illion) and female (2TPM) C57BL/6J mice. Correspondingly, transcriptional profiles of human skeletal muscle were explored by Lindholm *et al.*, who aimed to document a baseline reference for genes in female and male skeletal muscle (Lindholm *et al.*, 2014). RNA sequencing of skeletal muscle genes showed that invadolysin was slightly higher in male (1TPM) than female (0.8TPM). Furthermore, Lindholm *et al.* (2014) demonstrated that the majority of enriched genes in females were localized to mitochondria, and were involved in oxidative biological activities, such as fatty acid metabolism and cellular respiration. In these experiments, even though differences in transcriptional levels of invadolysin between genders were not significant (< 2-fold), additional factors could be considered during interpretation of sex-related variations in gene expression, such as proteinase activity in combination with protein level and/or mRNA levels (Abhinav *et al.*, 2019; Kaikaew *et al.*, 2021; Lindholm *et al.*, 2014). In 2001, Bucci and Murphy investigated responses of four phosphatase enzymes, alkaline phosphatase, 5'-nucleotidase, thiamine pyrophosphatase and adenosine triphosphatase, to hormonal treatments in uterine epithelial cells of female rats. The results have shown that expression of these enzymes is under control of ovarian hormones oestrogen and progesterone (Bucci & Murphy, 2001). These enzymes displayed

different reactions to progesterone, while they are all upregulated by oestrogen in the epithelial cells.

As introduced in Chapter one, invadolysin has been found as the first metalloprotease localising on the surface of lipid droplets, which implies a role of invadolysin in lipid storage or metabolism (Cobbe et al., 2009). Besides energy storage, adipose tissue displays an endocrine function and produces multiple factors (Luo & Liu, 2016). Among these factors, adipokines are characterised as cytokines, such as leptin, resistin and adiponectin, that regulate insulin sensitivity, inflammation, cell growth and reproduction. The physiological function of adipose tissues, in turn, is also modulated by production and secretion of adipokines, where dysfunctional adipose tissues have an impact on obesity-induced insulin resistance and inflammation. In addition to the roles in diabetes and metabolic disorders, adiponectin is important for embryonic development and female reproductive function (Čikoš 2012; Santos et al., 2012). Secreted by maternal adipose tissue or organs of the female reproductive tract, adiponectin's action on lipid and glucose metabolism promotes the supply of energy to embryo (Čikoš 2012). There are mainly three isoforms of adiponectin, which are low molecular weight trimers (67 kDa), medium-molecular weight hexamers (~120 kDa) and high-molecular weight oligomers (>300 kDa) (Luo & Liu, 2016; Richard et al., 2020). An important feature of adiponectin is that circulating levels of adiponectin in plasma are higher than other adipokines, such as adipisin, resistin, leptin and insulin (in a decreasing order) (Čikoš 2012; Richard et al., 2020). Furthermore, levels of adiponectin in female are higher than male, especially for the higher molecular isoforms (Combs et al., 2003; Nishizawa et al., 2002). Diverse functions of adiponectin have been discovered in different cell types, which is isoform and cell-type dependent (Čikoš 2012). For instance, Čikoš *et al.* (2010) has demonstrated that recombinant full-length adiponectin exerts stimulatory effect on preimplantation development, whereas the globular isoform (truncated trimer) has no effect on preimplantation of embryos in *in vitro* mouse models. Other signalling molecules, such as PI₃K and AMPK are also involved in the adiponectin regulated metabolic signalling processes, which play a significant

role in preimplantation and implantation of embryos (Čikoš 2012). Therefore, it is worth investigating whether intra- and/or extracellular invadolysin's expression and/or activity is under hormonal control, such as adiponectin and sex hormones, in energy expenditure, embryonic development and inflammation.

5.4.1.3 The metabolic role of invadolysin in NAFLD remains to be determined

Research on understanding protease and lipase activities of invadolysin have been explored by using *invadolysin* transgenic *Drosophila* (Abhinav *et al.*, 2019). Mutant flies with *protease*-dead forms of invadolysin accumulated lower levels of triglyceride compared with the wild-type form of invadolysin, whereas the *lipase*-form of invadolysin had no significant effect on triglyceride storage. Though decreased levels of glycogen were observed in *invadolysin* mutant flies, there was no direct evidence to prove that any of the motifs was responsible for impaired glycogen accumulation. Such an observation might be explained by a failure in the response to insulin signalling or impaired glycogen metabolism (Chang, C.-W. *et al.*, 2017).

Extracellular invadolysin in patients with NAFLD was examined in this chapter to determine whether regulation of invadolysin expression is associated with impaired liver metabolism. It clearly demonstrated that the expression of serum extracellular invadolysin is different in patients with NAFLD by comparing serum invadolysin in volunteers under non-NAFLD condition. Accordingly, *in vitro* cellular models of hepatic cellular steatosis were established to further investigate the role of invadolysin in lipid and glycogen accumulation during inflammation. Compared with control cells, higher expression levels of invadolysin in the cell culture medium was induced by octanoate in the first 96 hr. For the oleate-treated group, the expression level of invadolysin started to increase from 24 hr and reached to a plateau phase over next 72 hr. The overall invadolysin level of oleate-treated cells was similar to that of the control cells.

In a study of the relationship between cellular steatosis and triglyceride concentrations, Lockman *et al.* found that there was a significant difference in the pattern of triglyceride accumulation in hepatic cells triggered by oleate or octanoate (Lockman et al., 2012). Triglyceride levels in octanoate-treated cells reached a significantly higher level after 72 hr compared with untreated controls. When examining oleate-treated cells, higher concentrations of triglyceride were initiated significantly in 24 hr and the level was maintained at 72 hr. Similar observations were shown in LPON-treated (L: lactate; P: pyruvate; O: octanoate; N: ammonia) C3A cells compared with oleate-treated cells. These data could possibly explain the relatively higher expression level of invadolysin in octanoate-treated cell culture medium, which further provides evidence that extracellular invadolysin has a role in triglyceride accumulation.

Regarding the importance of glycolysis in tumour cells, this chapter explored the effects of hyperglycemia on the metabolism of tumour cells for invadolysin secretion. In the 'normoglycemia' group (5.5 mM), expression levels of invadolysin were increasing from day 1 to day 4 in HeLa cell culture media. In the hyperglycemic (55 mM) environment, expression levels of invadolysin increased gradually from day 1 to day 2, and subsequently reached to a plateau at day 4. The overall levels of invadolysin triggered by high glucose were higher than seen in the normoglycemia group. Previously, the effects of glucose treatment on cellular metabolism in HepG2-C3A cells have been examined by Iyer *et al.* (Iyer et al., 2010). Within five days of glucose treatment, cell growth was sustained without the influence of glucose concentrations (High glucose: 17 mM; Low glucose: 5.6 mM; Glucose-free: 0 mM). It is worth noting that, in the low glucose cell culture medium, glycolytic flux increased from day 2 to day 3 and dropped to the lowest at day 5. For high glucose-induced glycolytic flux, the value decreased from day 2, and reached to a plateau at day 5. The overall glycolytic flux of C3A cells at day 2, 3 and 5 were lower in high glucose medium than low glucose medium. Similar fluctuations in glucose uptake were also detected in high glucose- and low glucose-treated cells, regardless of abundant availability of glucose. This study points toward a hypothesis

that invadolysin may play a role in cellular glycolysis. Whether intracellular invadolysin and/or extracellular invadolysin is related to changes in glycolytic flux induced by variant glucose concentrations needs to be examined.

It has been discussed that regulation of glycolytic pathways could be affected by multiple factors, such as mitochondrially-bound hexokinase (HK) II, which converts glucose into glucose-6-phosphate (G6P) with the assistance of ATP (Mathupala et al., 2006; Takanaga et al., 2008). The voltage-dependent anion channel (VDAC), localised on the outer mitochondrial membrane, renders access of ATP (generated by ATP synthase) to HK II, which phosphorylates glucose for G6P formation (Mathupala et al., 2006). It has been identified that *invadolysin* mutant fruit fly larvae have an impaired energy status, where lower cellular concentrations of ATP and accumulated reactive oxygen species (ROS) were observed (Di Cara et al., 2013). In this research, Di Cara *et al.* (2013) postulated that invadolysin is required for biosynthesis of the ATP synthase complex and that it has a role in the formation of a functional electron transport chain. Taken together, these studies further imply that invadolysin may participate in glycolysis through the regulation of the ATP synthase complex in mitochondria.

In 2006, Marin-Herandez *et al.* (2006) illustrated that feedback from elevated concentrations of G6P could depress glycolysis facilitated by HK enzymes in tumour cells (Marín-Hernández et al., 2006). Furthermore, the availability of ATP and phosphorylating activity have been suggested as other factors that contribute to a low glucose uptake in higher glucose medium (Iyer et al., 2010). Thus, these studies could explain the higher expression levels of invadolysin in 'hyperglycemic' medium, even though whether invadolysin is involved in glucose uptake and glycolysis in tumour cells still needs to be determined.

5.4.2 The extracellular form of invadolysin is a novel candidate for physiological research

5.4.2.1 The potential link between extracellular invadolysin and NAFLD

The extracellular expression form of invadolysin in serum of patients with NAFLD was variable in comparison with controls. Within the established *in vitro* cellular NAFLD models, it was postulated that secreted levels of invadolysin were related to lipotoxicity and glycolysis. Thus, it has been questioned whether extracellular invadolysin is relevant to the development of NAFLD. In previous research, Abhinav *et al.* (2019) demonstrated that invadolysin is enriched in the extracellular vesicle fraction of human plasma. Increased levels of circulating EVs in humans with NASH have been observed by Srinivas *et al.*, who proposed that hepatocyte-derived EVs play an important role in pathogenesis and progression of NAFLD (Srinivas *et al.*, 2021; Wu, D. *et al.*, 2021). Variations in levels of EVs released by hepatocytes were also described by Newman *et al.*, who suggested that lipotoxic hepatocyte-derived EVs indicate liver function and pathological development of NAFLD because molecular cargos of small EVs, such as miRNA and liver-abundant proteins, reflect the physiological conditions of liver (Newman *et al.*, 2020). For instance, increased levels of EV were observed by Hirsova *et al.* (2016) in induced lipotoxicity by either palmitate or lysophosphatidylcholine. In addition, a death receptor ligand, TRAIL, within lipotoxic hepatocyte-derived EVs can trigger hepatocyte death and macrophage activation (with release of proinflammatory cytokines IL-1 β and IL-6) (Hirsova *et al.*, 2016). Changes in expression of MMP-7 in hepatocytes and MMP-9 in hepatic stellate cells (HSCs), induced by EVs from visceral adipose tissue, are involved in the dysregulation of TGF β signalling pathway, which may result in liver fibrosis in NASH (Koeck *et al.*, 2014).

Circulating cell-specific EVs have been used as a powerful target for the diagnosis of various liver diseases (Newman, Muller *et al.*, 2022). For instance, increased levels of NK T-cell-derived EVs have been found in NAFLD patient serum (Kornek *et al.*, 2011). An elevated number of hepatocyte-derived EVs was detected in plasma from patients with NAFLD

(Newman, Useckaite et al., 2022). Furthermore, cargos such as miR-122, -192, and -128-3p identified in liver-specific EVs are associated with disease severity. As invadolysin is enriched in circulating EVs, it is important to determine the source of EVs that carry invadolysin as a molecular cargo, and whether invadolysin in this type of EV is associated with any physiological status of liver and pathological process of liver disorders. In the long-term, whether or not invadolysin enriched EVs, such as global EVs and/or liver-specific EVs, can serve as a diagnostic approach for liver diseases would be determined.

5.4.2.2 The role of extracellular invadolysin in lung inflammation remains to be determined

The extracellular form of invadolysin was identified in BALF of patients with IPF and IIP. It seems that regulation of this form of extracellular invadolysin is associated with the severity of lung diseases, such as pulmonary fibrosis and decline in lung function. Moreover, it has been suggested that smoking history could be a risk factor affecting lung damage, which has an influence on extracellular invadolysin regulation in BALF. There is no evidence to demonstrate whether extracellular invadolysin in blood fluid has a relationship with the development of lung inflammation since the expression form of invadolysin in serum and plasma of patients with ILD was similar to control samples. However, the expression form of invadolysin in the plasma fraction of COVID-19 patients displays a dynamic feature during medical treatment (Figure 5.10 and Table 5.4). This suggests a connection between circulating extracellular invadolysin and lung inflammation, and also lung disease progression.

The importance of the extracellular matrix (ECM) has been recognized in COVID-19, which is responsible for injury repair responses and regulation of tissue homeostasis (Huang, J.-J. et al., 2023; Rozario & DeSimone, 2010; Sarker et al., 2021; Zhou, Y. et al., 2018). Pathological remodulation of ECM through assembly, synthesis and degradation is essential for tissue homeostasis, which can be facilitated by secretion of many MMPs at sites of

inflammation (Huang, J.-J. et al., 2023). The MMP protease family is associated with the development and progression of various acute and chronic lung diseases (Huang, J.-J. et al., 2023). For example, compared to patients with mild COVID-19, MMP-2 was downregulated in plasma of patients with severe COVID-19 (D'Avila-Mesquita et al., 2021). This phenomenon suggests an association between MMP-2 and mortality rate of COVID-19. Elevated levels of MMP-9 in serum and plasma of patients with progressive COVID-19 was observed by D'Avila-Mesquita *et al* and Gelzo *et al* (D'Avila-Mesquita et al., 2021; Gelzo et al., 2022). MMP-9 is further characterised to be involved in the inflammatory process, such as in the repairing phase, and associated with pulmonary fibrosis (Gelzo et al., 2022; Moin et al., 2020). Gelzo *et al.* (2022) also pointed out a pathological role of MMP-3 in initiation of lung inflammation as serum levels of MMP-3 increased in COVID-19 patients and decreased after one week hospitalization. Therefore, MMP-3 and MMP-9 proteins can serve as a biomarker for defining the severity of COVID-19 patients. Physiological roles of MMPs have been identified in other inflammatory lung conditions. For example, compared with normal lung tissue, expression of MMP-7 is increased in lung tissue of IPF patients (Yu, G. et al., 2018). Upregulated MMP-28 in BALF of patients with adult respiratory distress syndrome (ARDS) is found to be correlated with elevated alveolar neutrophils (Morrell et al., 2020).

In summary, the results first reported the extracellular form of invadolysin (67 kDa) presenting in BALF samples of patients with IPF. In addition, this form of invadolysin was detected extracellularly in lipotoxicity induced cellular inflammation models. Therefore, it is worth examining whether changes in this extracellular form of invadolysin reflects the physiological status of lung. The significant role of proteases in pathobiology of the cystic fibrosis (CF) has been established that proteases not only function as a catalytic enzyme for protein degradation, but also participate in disease progression as a signaling molecule no matter whether proteases are secreted by lung or derived from immune cells (Birrer et al., 1994; Voynow et al., 2008; Turk et al., 2012; McKelvey et al., 2019). Additionally, increased proteolytic activity of extracellular neutrophil elastase (NE) has been found in BALF of children

(3 months of age) with persistent bronchiectasis, which was believed to be related to CF pathogenesis (Sly et al., 2013). Human cathepsins K (CatK) is a monomeric endopeptidase, which has been hypothesized as a potential biomarker and a novel therapeutic target in lymphangioleiomyomatosis (LAM) (Marchand-Adam et al., 2023). LAM is a multisystemic disease with cystic pulmonary destruction and lymphangiomas, which has a majority effect on women. This disease usually progresses to chronic decline in respiratory function and patients with this disease have a higher risk of osteoporosis-related bone fractures. Because expression level of CatK associates with the severity of LAM and CatK is responsible for lung dysfunction, it has been suggested that inhibition of CatK is a differential therapeutic target. Dysregulation of CatK expression also contributes to bone resorption disorders since CatK promotes tumor progression through degradation of ECM proteins and inactivation/activation of cytokines.

To investigate the correlation of extracellular invadolysin with lung inflammation, it is worth examining the expression forms and the secretion levels of extracellular invadolysin from BALF, serum and plasma from patients with lung diseases. In addition, comparing these data with markers of inflammation promises to provide clarify whether extracellular invadolysin is an indicator of inflammation. Moreover, the physiological role of invadolysin in extracellular vesicles remains unclear. Analysis of invadolysin in global-, tissue- and cell-specific EVs would help with assessing the significance of alterations in the protein level, expression form and functional activity of invadolysin in disease. Combining vesicle isolation with other analytical methods (such as LCMS and Flow cytometry) promises to help determine whether extracellular invadolysin can act as a biomarker for inflammatory diseases, and, potentially, as an indicator of disease severity.

Chapter 6 Discussion

6.1 Main findings and implications

In 2009, Cobbe *et al.* discovered that invadolysin is localised on the surface of lipid droplets, which suggested a role for invadolysin in lipid storage by examining the morphology of the fat body and triglyceride levels in *Drosophila* larvae (Cobbe *et al.*, 2009). Following this study, a crucial function of invadolysin in metabolism was discovered by Chang *et al.* (2016) and Di Cara *et al.* (2013), who identified that invadolysin is necessary for normal mitochondrial activity and insulin signalling. Further proteomic approaches have been utilised to address the potential enzymatic activity of invadolysin. *In silico* analysis revealed potential sites of post-translational modification in invadolysin sequence, which suggested that invadolysin possesses an extracellular form (Abhinav *et al.*, 2019). This research also demonstrated that invadolysin is present in the soluble fraction of vertebrate (human and mouse) serum and plasma, especially in the extracellular vesicle in human plasma. In addition, a potential sexually dimorphic form of invadolysin was observed in male and female *Drosophila* hemolymph. In fractionated plasma of adult fruit flies, extracellular form of invadolysin was present as a 111 kDa form in male and a 46 kDa form in female (Abhinav *et al.*, 2019). Following, Abhinav *et al.* (2019) discovered that this secreted form of invadolysin was shown in testes and ovaries respectively. Thus, Abhinav *et al.* (2019) suggested that an extracellular form of invadolysin is conserved in vertebrates and invertebrates. Although this work showed an extracellular form of invadolysin in serum and plasma from higher vertebrates, the novel form in human blood had not been well characterised. This prompted a number of questions. Is invadolysin enriched in a specific fraction of human blood? Are there structurally-different forms of extracellular invadolysin in males and females? Does extracellular invadolysin influence embryonic development? Can extracellular invadolysin serve as a biomarker for disease? The research described in this thesis was designed to focus on enrichment and isolation of the novel extracellular form of invadolysin from vertebrate serum and plasma, and on exploration of its

potential role(s) in maintaining normal physiological function. An overview of the work presented in this thesis, comprising the research questions, experimental strategy and main findings, is summarised in the following schematic (Figure 6.1).

Investigating the Essential Extracellular Invadolysin Metalloprotease

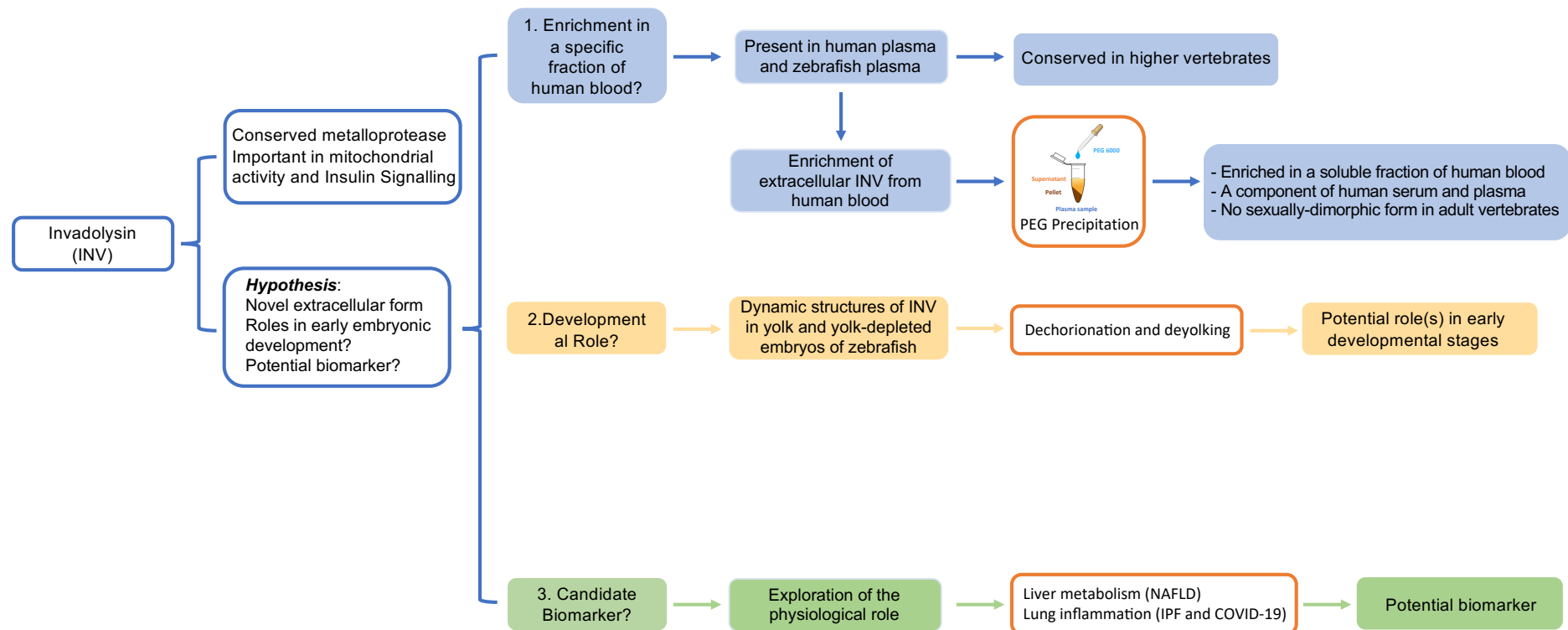


Figure 6.1 The schematic overview of PhD research.

This PhD research project aims to investigate the physiological function(s) of novel extracellular form of invadolysin. This study has discussed whether invadolysin has a role in early embryonic development and whether extracellular form of invadolysin has a potential in biomarker (**Hypothesis**). Three research aims and respective research processes are demonstrated in blue, yellow and green boxes, where orange boxes indicate methods and disease models, and conclusions are explicated at the end of each aim. INV: invadolysin; PEG: Polyethylene Glycol Precipitation; NAFLD: Non-Alcoholic Fatty Liver Disease; IPF: Idiopathic Pulmonary Fibrosis; COVID-19: Coronavirus Disease.

6.1.1 The extracellular form of invadolysin is present in human serum and plasma

6.1.1.1 The extracellular form of invadolysin is conserved in higher vertebrates

To investigate whether invadolysin is enriched in a specific fraction of human blood, the first stage was to evaluate whether the extracellular form of invadolysin can be detected in human blood. The findings in Chapter 3 demonstrated that an extracellular form of invadolysin is present in human plasma. Furthermore, it was shown that an extracellular form of invadolysin was present in zebrafish plasma. These findings confirmed the work of Abhinav *et al.* which demonstrated that an extracellular form of invadolysin is conserved in higher vertebrates (Abhinav et al., 2019). Different structural forms of invadolysin in vertebrates have been discovered throughout the thesis, and these forms are summarised in Table 6.1.

6.1.1.2 The extracellular form of invadolysin can be enriched in a soluble fraction of human blood by polyethylene glycol precipitation

The second stage of determining whether the extracellular form of invadolysin is enriched in a specific fraction of blood involved development of a biochemical strategy for invadolysin enrichment and precipitation. When examining the expressed form of invadolysin by immunoblotting, enriched proteins in human plasma interfere with the migration of protein bands (Abhinav et al., 2019). To overcome this interference, highly abundant proteins, such as albumin and immunoglobulins, were depleted during plasma sample preparation. It was confirmed that this strategy improved detection of the expressed form of invadolysin by immunoblotting. However, these results suggested that the protein level of invadolysin could be diminished during plasma sample preparation, as a consequence of non-specific binding, when using commercially-available plasma albumin and IgG protein removal kits. In addition, the commercially-available kits for removal of abundant proteins (as applied in Abhinav *et al.* (2019)'s research or utilised in Chapter 3) are not readily optimized for specific experimental

Localisations	Molecular Weight (kDa)
C3A and HeLa cell medium	67
Zebrafish Yolk (48 and 72 hpf)	22
Zebrafish Embryo (48 and 72 hpf)	55
Zebrafish Plasma	51, 55, 65, 76
Mouse Serum	51, 66
Mouse Male Plasma	27, 51, 66
Mouse Female Plasma	27, 51, 66
Human Serum	51, 53
Human Serum with Inflammation	51, 53
Human Plasma	35, 51, 53, 55, 58, 64, 67, 69
Human Plasma with Inflammation	35, 51, 53, 54, 55, 65, 70
BALF of ILD	27, 53, 55, 67, 69

Table 6.1 Different structural forms of invadolysin in vertebrates under various physiological conditions.

The form of Invadolysin is dynamic at different localisations. Different extracellular forms of invadolysin have been detected in C3A and HeLa cell culture medium, zebrafish blood, mouse (serum and plasma), human (serum and plasma), and in BALF (Bronchoalveolar Lavage Fluid) of patients with ILD (Interstitial lung Disease). Zebrafish embryos from 48 and 72 hpf were used for observation. Blood samples from human with different inflammation, such as non-alcoholic fatty liver disease and lung diseases (idiopathic pulmonary fibrosis and COVID-19). Blood samples were all collected from adult vertebrates.

purposes (such as isolation of invadolysin from a specific fraction of blood or enrichment of invadolysin protein from small volumes of blood). As a result, it was necessary to develop a biochemical approach to enrich and isolate the extracellular form of invadolysin efficiently and sustainably.

In Chapter 4, two strategies for enrichment and isolation of the extracellular form of invadolysin were considered: immunoprecipitation (IP) and polyethylene glycol precipitation (PEG). The results demonstrated that invadolysin isolated from human plasma and zebrafish embryo using IP can be detected by immunoblotting by reducing background noise. However, it was still difficult to design a complete protocol of IP that was compatible with immunoblotting. Numerous factors in the IP workflow (including antibody and antigen immobilization) may affect examination of the extracellular form of invadolysin by immunoblotting. As an alternative approach, the PEG protocol was optimized to precipitate proteins from human soluble fractions with relatively low concentrations of PEG (4-10%). The results clearly illustrated that the extracellular form of invadolysin in the PEG-pellet can be identified by immunoblotting. Precipitated proteins remain intact and unaffected by PEG invasion (Juckles, 1971). Isolated proteins in the PEG-pellet fraction can be concentrated for desired experimental purposes, especially when the amount of available samples is limited. This strategy is compatible with immunoblotting for the detection of invadolysin from human blood. Overall, the PEG protocol developed in Chapter 4 can enrich and precipitate the extracellular form of invadolysin present in a soluble fraction of human blood. This protocol avoids the incompatibilities between the protein isolation strategy and the protein examination approach, which improves the ability to achieve the desired experimental goal. Subsequently, the polyethylene glycol precipitation procedure will contribute to the characterization of invadolysin structure and physiological function in vertebrates (Abhinav et al., 2019).

6.1.1.3 The extracellular form of invadolysin is a component of human serum and plasma

The extracellular form of invadolysin was found to be present in both serum and plasma fractions of human blood. Intriguingly, it was discovered that the expression form of secreted invadolysin is different in human serum and plasma, which has been verified by multiple invadolysin enrichment and isolation experiments. Therefore, these findings not only display consistency with the research of Abhinav *et al.*, who stated that invadolysin is present in vertebrate blood, but also provide supplementary evidence to support that invadolysin is a component of human serum and plasma (Abhinav *et al.*, 2019).

In the study of the metabolite profile of human blood, several researchers have proved that the levels of particular metabolites are different between serum and plasma (Aoki *et al.*, 2002; Teerlink *et al.*, 2002; Yu, Z. *et al.*, 2011). The observed differences have been considered a result of coagulation. Furthermore, a similar theory has been suggested by Shimoda and Khokha, who stated that metalloproteases located on platelet-derived microvesicles play a significant role in blood coagulation (Shimoda & Khokha, 2017). On the other hand, several publications have revealed that matrix metalloproteases in human serum or plasma are involved in the progression of pathological diseases, such as tumour growth and metastasis (Huang, H., 2018; Huang, J.-J. *et al.*, 2023; Roy, R. *et al.*, 2009; Wang, H. *et al.*, 2021; Wong *et al.*, 2015). Thus, it has been questioned whether the extracellular forms of invadolysin in serum and plasma have a physiological role in blood. In addition, whether the serum and/or plasma enriched invadolysin is associated with maintaining normal physiology in human needs to be investigated.

6.1.1.4 The sexually-dimorphic form of extracellular invadolysin is not present in adult vertebrates

Abhinav *et al.* validated the presence of invadolysin in *Drosophila* hemolymph, where the extracellular form has various molecular weights in adult male and female hemolymph (Abhinav et al., 2019). An extracellular form at 111 kDa is exclusively expressed in male soluble hemolymph and testes. The 46 and 39 kDa form of invadolysin is present in female hemolymph and ovaries. Though these two forms of invadolysin are also observed in male hemolymph, they are not enriched in testes. Therefore, it was interesting to determine whether the sexually-dimorphic form of invadolysin was also detectable in vertebrates and the role(s) of sexually dimorphic forms of invadolysin. The first attempt was facilitated using adult zebrafish plasma (Chapter 3). This illustrated that the sexually-dimorphic form of extracellular invadolysin is not present in adult zebrafish plasma. The second attempt was to address whether the sexually-dimorphic form of the extracellular invadolysin could be detected in human plasma (Chapter 4). This showed that the expression form of extracellular invadolysin is the same in human adult male and female plasma samples. The third attempt was processed during the analysis of the physiological function of invadolysin using invadolysin knockout mice model designed by the Takahashi lab (Chapter 5). There were no homozygous mice available and the extracellular form of invadolysin was detected in both *inv*^{+/-} and wild type mice serum and plasma. The expression form of invadolysin in *inv*^{+/-} was the same in the wild type mice; however, there is a potentially sexually-dimorphic form of invadolysin was observed in mouse plasma during early development.

Moreira *et al.* have demonstrated that the expression level of invadolysin differs in testis, ovary and brain at different early developmental stages in humans (Cardoso-Moreira et al., 2019). Based on the above research, it has been proposed that invadolysin is an active participant in physiological activities during differentiation at early stages in vertebrates. Whether the sexually-dimorphic form of invadolysin can be detected in humans during

early developmental stages, and whether invadolysin plays a crucial role in early physiological development, need to be further explored.

6.1.2 Preliminary exploration of the physiological role of invadolysin

6.1.2.1 A potential role of invadolysin during the early development period

Developmental progress is a favourable period for the research of genetic phenotypes (DeFalco & Capel, 2009; Pantalacci & Sémon, 2015; Silbereis et al., 2016). This developmental phase is implemented with evolutionarily-related phenotypic features. To understand the gene expression profile across mammals during early developmental stages, Moreira *et al.* (2019) examined transcriptomes of seven organs in different species: brain, cerebellum, heart, kidney, ovary and testis. During human organ development, the expression level of invadolysin increases in testis during the toddler stage, and in the ovary during the 13 to 18 week post conception stages. In addition, invadolysin displayed a higher expression in brain tissues, such as forebrain and hindbrain, during weeks 4 and 5 post conception (Cardoso-Moreira et al., 2019). In the same year, Kraiczy *et al.* observed a low transcription level of invadolysin in human intestinal epithelial organoids derived from foetal and paediatric intestines (Kraiczy et al., 2019). Moreira *et al.* explained that changes in gene transcription usually occur in two developmental periods (Cardoso-Moreira et al., 2019). The first period is the embryonic developmental stage, during which genes related to organ-specific functions have increased expression and genes contributing to cellular division and morphogenesis display a reduced expression. The second period is associated with postnatal stages or birth, which varies in different species and is dependent on the maturity of the newborns. Therefore, it has been questioned whether invadolysin has an important role in early development stages.

A potential role of invadolysin during *Drosophila* developmental stages was described in Di Cara *et al.* where reduced level of ATP in *invadolysin* mutation larvae triggered the activation of the energy sensor AMPK, which

prevented larvae from further developing (Di Cara et al., 2013). Similarly, the essential function of invadolysin during early developmental stages of zebrafish was proposed by Vass and Heck (Vass & Heck, 2013). They observed that the expression form of invadolysin from whole zebrafish embryo extracts was dynamic during 0-120 hpf. Thus, it has been proposed that invadolysin plays a role in embryonic development of zebrafish. However, yolk proteins in zebrafish embryo may affect the detection of embryonic proteins by immunoblotting (Link et al., 2006). Work described in Chapter 3 demonstrated an alternative methodology to examine the expression form of invadolysin in both yolk and yolk-depleted (YD) embryo of zebrafish at 48 and 72 hpf. It was shown that the expression form of invadolysin in yolk and YD embryo is dynamic, which suggests a potential role of invadolysin in early developmental stages. The establishment of the yolk-depleted model sheds light on the investigation of the physiological role of invadolysin in embryonic development in vertebrates. Additionally, this model would help determine whether invadolysin is a developmentally dynamic gene (Cardoso-Moreira et al., 2019).

6.1.2.2 The extracellular form of invadolysin is a candidate biomarker

In recent years, novel biomarkers have been characterized from the metalloprotease family, which have been demonstrated playing significant roles in early diagnosis, risk assessment and prognosis (D'Avila-Mesquita et al., 2021; Gelzo et al., 2022; Moin et al., 2020; Morrell et al., 2020; Yu, G. et al., 2018). Changes in protein levels of MMPs in serum or plasma have been noticed during initiation and progression of inflammatory diseases, such as idiopathic pulmonary fibrosis and coronavirus. The diagnostic value of metalloproteases has been recognized, which would contribute to the improvement of clinical treatment outcome. It has been questioned whether the extracellular form of invadolysin can be used as a biomarker for the detection of any pathophysiological states. Therefore, to explore the physiological function of invadolysin, comparison of the expression form of extracellular invadolysin between control and inflammatory diseases conditions was conducted in Chapter 5.

The first aim was to examine whether the regulation of extracellular invadolysin expression is associated with liver metabolism. It was shown that the expression form of serum invadolysin in patients with NAFLD was different from control sera. Correspondingly, the expression form and the protein level of secreted invadolysin was examined in the *in vitro* cellular model of hepatic cellular steatosis. The results showed that there were no significant changes in the expression form of invadolysin in cellular culture media during lipid and glucose accumulation. However, protein levels of extracellular invadolysin were enhanced in the octanoate-treated cell culture medium. In addition, the overall reduced protein levels of invadolysin induced by hyperglycemia were observed in comparison with normoglycemic groups. This could be explained by the participation of invadolysin in glycolysis through the regulation of the ATP synthase complex in mitochondria. These results suggested that the extracellular form of invadolysin plays a role in cellular triglyceride accumulation and glycolysis. The *in vitro* model of hepatic cellular steatosis opens a gate for understanding the role(s) of invadolysin in lipid and glucose metabolism.

The second aim was to investigate the physiological role of extracellular invadolysin during lung inflammation. The expression forms of invadolysin in serum, plasma and BALF of patients with ILD were analysed. The results demonstrated that there was no direct evidence that regulation of serum or plasma invadolysin is associated with lung inflammation. The extracellular form of invadolysin was identified in human BALF, and its expression was shown to be dynamic in ILD patients with different lung inflammation states. These results suggest that the regulation of BALF invadolysin expression is correlated with lung fibrosis formation, lung function decline, and the severity of idiopathic interstitial pneumonia (IIP). Moreover, it has been postulated that as a risk factor, smoking is important for the regulation of the extracellular invadolysin in BALF. However, it is still not clear whether there is a connection between the regulation of BALF invadolysin expression and IPF progression.

The potential role of plasma invadolysin in severe respiratory disease was also investigated in COVID-19. The expression form of secreted invadolysin in patient plasma is dynamic during lung infection compared with non-COVID-19 controls. It has been hypothesized that expression of circulating invadolysin plays a role in lung infection and progression. Overall, the discovery of dynamic expression forms of extracellular invadolysin in blood and BALF encourages the idea that the extracellular form of invadolysin is a potential candidate for a biomarker of inflammatory diseases. To benefit from the treatment outcome, whether extracellular invadolysin can be used as an initiator of inflammation and/or an effector of inflammation progression is worth further investigation.

6.2 Limitations and future work

During the exploration of a physiological role of an extracellular form of invadolysin, various limitations have been recognized, which restrict the understanding of the regulatory mechanisms of invadolysin in NAFLD. The first limitation occurred during the investigation of invadolysin's role in hepatic lipid metabolism and glycolysis. There are several limitations in the establishment of an *in vitro* hepatic cellular steatosis model, which was utilized to recapitulate the effects that occur in NAFLD. Firstly, given the results described in Chapter 5, additional data is highly required. It has been recommended that invadolysin may have a role in triglyceride accumulation. To further support this hypothesis, it is important to identify the extracellular and/or intracellular protein levels of invadolysin, the expression forms of invadolysin, and the concentrations of cellular triglyceride.

Secondly, the established hepatic cellular steatosis model could not satisfy the research aims in depth. Only simple steatosis was developed in cellular models by treating human hepatoblastoma C3A cells with either octanoate, oleate or glucose. Even though the research data has provided low to moderate evidence to suggest that invadolysin could be a participant in glycolysis, through modulation of mitochondrial activity, a mitochondrial dysfunction model is still highly required. The determinant of mitochondrial dysfunction in the hepatic cellular steatosis was studied by Lockman *et al.* (2012). These researchers found that a failure in mitochondrial respiration can be triggered by LPON treatment after 72 h. In addition, the level of triglyceride accumulation in LPON-treated cells was similar to that seen in the simple steatosis model, which was induced by oleate treatment accompanied by unperturbed mitochondrial activity. Therefore, to further understand the importance of invadolysin in hepatic metabolic functions, such as glycolysis, *de novo* lipogenesis (DNL) and gluconeogenesis, it is worth utilizing the LPON hepatic cell steatosis model.

Thirdly, this study is lacking inhibitors of hepatic steatosis to explore the association of invadolysin with NAFLD. In recent years, numerous research

publications have indicated that the sodium glucose cotransporter-2 inhibitor (SGLT-2i) is performing as a promising therapeutic option for NAFLD (Raj, H. et al., 2019; Zhang, E. et al., 2021). SGLT-2i is an antidiabetic drug that has been demonstrated to promote an improved glycaemic control by mediating renal glucose reabsorption and excreting glucose into urine (Chao, 2014; Raj, H. et al., 2019; Vallon & Thomson, 2017; Zhang, E. et al., 2021). In clinical liver disease cases, patients with NAFLD and NASH can benefit from the treatment with some SGLT-2is in terms of accumulation of hepatic fat, insulin resistance, liver fibrosis indices, and abnormal inflammatory responses (Raj, H. et al., 2019; Zhang, E. et al., 2021). In addition, this protective effect has been validated in several *in vitro* studies (Androutsakos et al., 2022; Hawley et al., 2016; Soret et al., 2020; Xiao et al., 2019; Zhou, J. et al., 2020). In these studies, it has been suggested that activation of AMPK signalling is responsible for the improvement of NAFLD induced by SGLT-2i. For example, dapagliflozin, one type of SGLT-2i, reduced intracellular lipid accumulation in palmitic acid-stimulated L02 and C3A cell lines, which contributed to regulation of AMPK signalling pathway (Androutsakos et al., 2022; Soret et al., 2020; Xiao et al., 2019). Interestingly, Di Cara *et al.* (2013) suggested that AMPK could be antagonistic to invadolysin since hyper-activation of AMPK was observed in *invadolysin* fly larvae. Hence, SGLT-2i could be used as an inhibitor in cellular hepatic steatosis to characterise a role of invadolysin in NAFLD.

Abhinav *et al.* (2019) suggested that identification of protein partners interacting with invadolysin could help with understanding the regulatory mechanism of invadolysin. Furthermore, as a zinc-metalloprotease, the latent proteolytic activity of invadolysin is still not well known. Predicted information of protein-protein interaction has been considered as a novel strategy to achieve a comprehensive knowledge of the cellular machinery (Szklarczyk et al., 2018; Szklarczyk et al., 2020; Szklarczyk et al., 2022). Combining the biological data and protein-complex knowledge predicted by STRING, Yeung *et al.* (2015) suggested that NAFLD is an independent risk factor for cardiovascular disease through the upregulation of fibrinogen

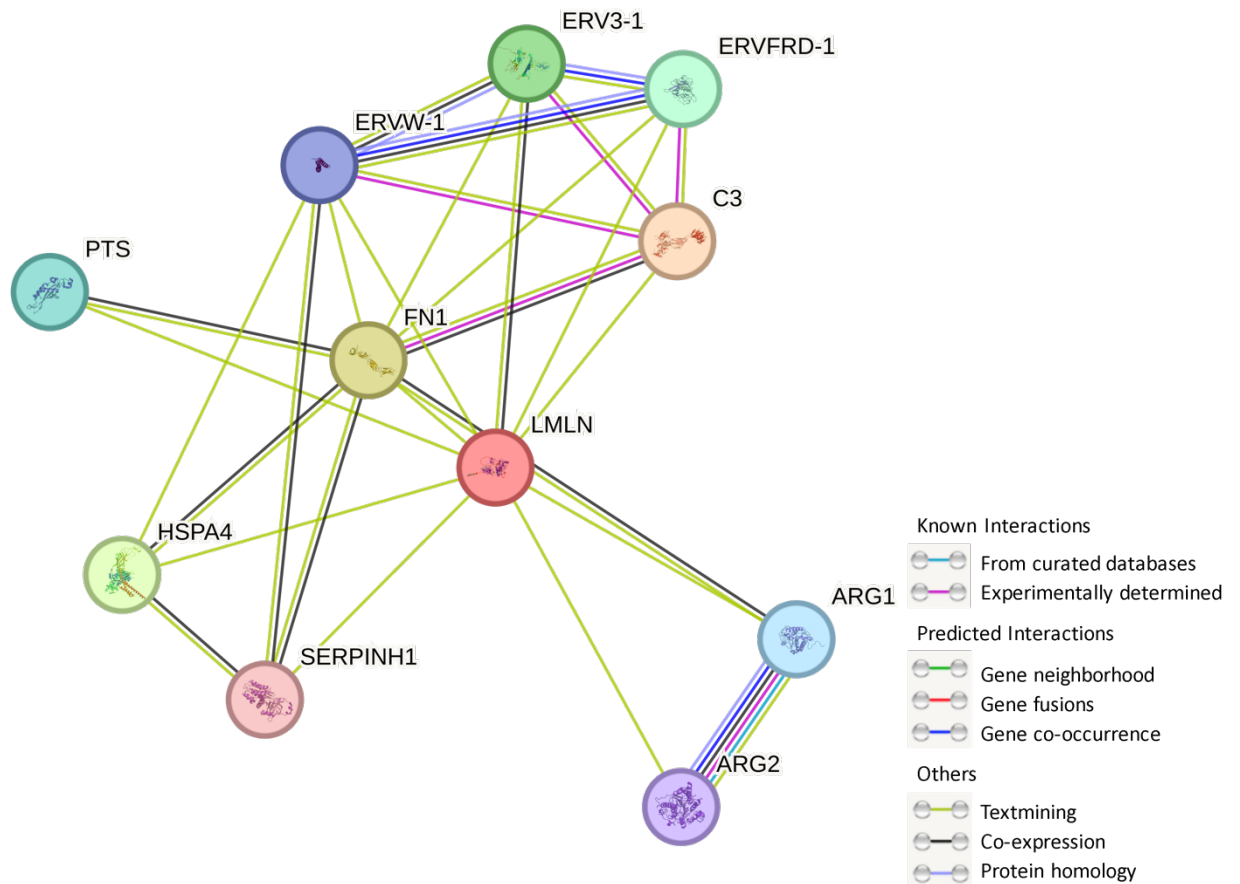


Figure 6.2 Associated partners of invadolysin predicted by STRING.

Proteins with potential functional relationship with invadolysin (LMLN: leishmanolysin-like peptidase) are shown as the coloured nodes in this network web. Proteins having predicted or known 3D structure are filled within the nodes, and the empty nodes represent proteins having unknown 3D structure. The specific protein-protein interactions are shown as edges with different colours (STRING12.0 2023).

coagulation factor (Yeung, E. N. W. et al., 2015). Thus, the application of bioinformatics research tools might shed light on the investigation of invadolysin's putative actions. Various evidence can be provided by STRING. For instance, functional associations of invadolysin predicted by STRING are shown in Figure 6.2, which demonstrates the potential interactions of partners and other genetic context information (Szkarczyk et al., 2018). This figure predicts a relationship between C3, fibronectin (FN1) and human invadolysin (LMLN). As introduced in chapter 1, invadolysin is homologous to leishmanolysin (McHugh et al., 2004; Yao, C. et al., 2003). Leishmanolysin has the capacity to degrade fibronectin (FN1), which would deactivate host macrophages and expand parasites' activity intracellularly (Hsiao et al., 2008; Kulkarni et al., 2008; Yao, C. et al., 2003). Thus, it has been suggested that fibronectin could be a substrate of human invadolysin. Schistosomiasis is induced by infection of *Schistosoma mansoni*, which utilised a number of immunomodulating factors, including proteases, to overcome the immune response from human hosts (Hambrook & Hanington, 2023). Recently, Hambrook and Hanington (2023) discovered a cercarial form of invadolysin (SmCI-1), secreted from the *S. mansoni*'s acetabular glands, is responsible for parasite's survival against the immune response from host. SmCI-1 functions as a metalloprotease and catalyzes collagen type IV, gelatin, fibrinogen, and especially, complement component C3b (a fragment of the central complement protein C3, crucial element in innate immune system), which causes inhibition of both the classical and alternative complement pathways and commits an immunomodulatory role (Hambrook & Hanington, 2023). In human peripheral blood mononuclear cells, SmCI-1 initiates an anti-inflammatory strategy via promoting levels of IL-10 and downregulating the production of inflammatory cytokines, such as IL1B and Eotaxin-1. As fibronectin has been found as a substrate of different proteases belonging to invadolysin family, it would be worth investigating whether human invadolysin has the capacity to catalyze fibronectin and other ECM proteins and determining whether this cleavage activity of human invadolysin is involved in physiological conditions, such as lipid and glucose metabolism, and immune response.

In humans, the circulating form of invadolysin has been identified in the soluble fractions of serum and plasma, human plasma EVs, and BALF from patients with ILD. In addition, different structural forms of extracellular invadolysin may be associated with inflammation and the severity of disease states. Nevertheless, questions remained to be answered about the physiological role (s) of specific forms of extracellular invadolysin observed under different physiological conditions. Recently, Filho *et al.* (2022) indicated that leishmanolysin derived from exosomes secreted by *Leishmania spp.* stimulates innate inflammatory responses in the host. Researchers have discovered that different expression levels of leishmanolysin in EVs elicit distinctive inflammatory responses in macrophages. Newman *et al.* (2022) concluded that global EVs and/or tissue-specific EVs provide a powerful tool to understand the pathological processes of diseases, such as chronic liver diseases. Thus, it is important to determine whether circulating invadolysin has a pathophysiological role. Mass spectrometry could contribute to understanding the extracellular role of invadolysin by analysis of molecular structure of invadolysin from human samples (global EVs and/or tissue-specific EVs, serum and plasma) under control and disease states. In the long-term, mass spectrometry will further contribute to determining whether invadolysin can be used as a biomarker for any human metabolic associated diseases.

This thesis has discussed the potential roles of extracellular invadolysin in physiological metabolism and inflammation. Further investigation regarding understanding invadolysin's characteristics in mediating energy restoration and innate response in different cells and tissues can be considered. The first aim is to continue explore invadolysin's role in early embryonic development. As shown in chapter 3, different isoforms of invadolysin are processed in diverse localisations of zebrafish embryos, thus, there is a need to address the regulation mechanism of invadolysin isoforms. Is the process modulated by maternal hormones with the participation of yolk lipids and other energy molecular species, such as cholesterol, triglycerides and ceramides, leading to transformation during embryonic development; is this a physiological function of invadolysin isoforms or cell type-dependent? Thus,

the embryonic zebrafish models and/or mouse models would be utilised to address whether invadolysin is responsible for energy expenditure, female reproduction, and embryonic development through metabolic signaling pathways (Brinks et al., 2011; Lachowicz et al., 2021; Adhish and Manjubala 2023).

The second aim is to understand extracellular invadolysin's role in inflammatory responses in different organs, such as liver, kidney, and lung. Recent studies demonstrate different proteinases from the invadolysin family act as an immunomodulator in innate immune system through ECM proteins' cleavage and subsequently regulate enhancement and suppression of cytokines. In addition, several studies have discovered that EVs are key to diagnosis and treatment of diverse disorders as the level of EVs' molecular cargos reflects the physiological progression and disease severity. Since the discovery that invadolysin is a component of EVs (microvesicles and exosomes) of human plasma, it is important to examine the source of invadolysin enriched EVs. Does it derive from a specific cell type or tissue? Do changes in the level of invadolysin enriched EVs indicate any physiological status? Additionally, does extracellular invadolysin, whether or not localising on EVs, catalyse ECM proteins, such as laminin, collagen and fibronectin? This will answer whether these degradation activities promote and/or suppress inflammatory responses.

The third aim is to decide whether extracellular invadolysin can serve as a diagnosis indicator, treatment target and a biomarker for any metabolic disorders and inflammatory diseases. Given that invadolysin is a dynamic protease, which processes different protein expression forms and dynamic cellular localisations, either intra- or extra-cellularly localised, it is worth understanding the regulation mechanisms of each isoform, and in the long term, research of invadolysin in the extracellular environment will provide more opportunities to gain insight in this conserved metalloprotease (Abhinav et al., 2019).

6.3 Challenges of research arising from COVID-19 pandemic

The COVID-19 pandemic had an impact on the research progress and the completion of thesis writing due to numbers of complicated circumstances and unforeseen difficulties. During the COVID-19 lockdown, the research buildings (QMRI) had been signed off for nearly three months. Additional actions for the support of PhD study during quarantine had been taken, such as literature research, thesis planning, attending virtual seminars, presenting at conferences (Society of Endocrinology BES Conference; Biochemical Society 'The Adipocyte across biological scales' Conference; Chinese Life Scientists Society Annual Conference in the UK), and joining the IAD online academic workshops for thesis writing and viva defending. However, the lab-based research work was still necessary and crucial for thesis submission. When the research buildings opened for repopulation, it was possible to continue experimental research, however, the accessibility to the laboratories was still restricted. At that moment, the PhD progress was at the crucial stage where the study was focusing on the analysis of invadolysin's expression form(s) in fractionated human blood samples from the general population, and developing the experimental strategies to examine the expression form(s) of extracellular invadolysin in control and disease conditions. As a consequence, 'fresh' human blood from healthy donors is the vital experimental materials. During the pandemic period, it was difficult to access to the sufficient human blood samples, and the availability for blood was under severe regulations. In addition, some experimental materials were not manufactured during the pandemic period, and it took extra some time to figure out the alternative solutions. Furthermore, other inevitable factors were affecting the progress of PhD study, such as experiencing the difficulties from the unconventional and remote supervision modes.

References

Abhinav, K., Feng, L., Morrison, E., Jung, Y., Dear, J., Takahashi, S. & Heck, M. M. S. (2019) The Conserved Metalloprotease Invadolysin Is Present in Invertebrate Haemolymph and Vertebrate Blood. *Biology Open*, 8 (11), p. bio044073.

Adhish, M. and Manjubala, I. 2023. Effectiveness of zebrafish models in understanding human diseases-A review of models. *Heliyon* 9(3), p. e14557.

Adkins, J. N., Varnum, S. M., Auberry, K. J., Moore, R. J., Angell, N. H., Smith, R. D., Springer, D. L. & Pounds, J. G. (2002) Toward a Human Blood Serum Proteome Analysis By Multidimensional Separation Coupled With Mass Spectrometry*. *Molecular & Cellular Proteomics*, 1 (12), pp. 947–955.

Aebi, M. (2013) N-Linked Protein Glycosylation in the ER. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, 1833 (11), pp. 2430–2437.

Akopian, D., Shen, K., Zhang, X. & Shan, S. (2013) Signal Recognition Particle: An Essential Protein-Targeting Machine. *Annual Review of Biochemistry*, 82 (1), pp. 693–721.

Andaloussi, S. E., Mäger, I., Breakefield, X. O. & Wood, M. J. A. (2013) Extracellular Vesicles: Biology and Emerging Therapeutic Opportunities. *Nature Reviews Drug Discovery*, 12 (5), pp. 347–357.

Anderson, N. L. & Anderson, N. G. (2002) The Human Plasma Proteome History, Character, and Diagnostic Prospects*. *Molecular & Cellular Proteomics*, 1 (11), pp. 845–867.

Androutsakos, T., Nasiri-Ansari, N., Bakasis, A.-D., Kyrou, I., Efstathopoulos, E., Randeva, H. S. & Kassi, E. (2022) SGLT-2 Inhibitors in NAFLD: Expanding Their Role beyond *Diabetes* and Cardioprotection. *International Journal of Molecular Sciences*, 23 (6), p. 3107.

Angulo, P. 2007. GI Epidemiology: nonalcoholic fatty liver disease. *Alimentary Pharmacology & Therapeutics* 25(8), pp. 883–889.

Aoki, J., Taira, A., Takanezawa, Y., Kishi, Y., Hama, K., Kishimoto, T., Mizuno, K., Saku, K., Taguchi, R. & Arai, H. (2002) Serum Lysophosphatidic Acid Is Produced through Diverse Phospholipase Pathways*. *Journal of Biological Chemistry*, 277 (50), pp. 48737–48744.

Babaei, F., Ramalingam, R., Tavendale, A., Liang, Y., Yan, L. S. K., Ajuh, P., Cheng, S. H. & Lam, Y. W. (2013) Novel Blood Collection Method Allows Plasma Proteome Analysis from Single *Zebrafish*. *Journal of Proteome Research*, 12 (4), pp. 1580–1590.

Bacon, B.R., Farahvash, M.J., Janney, C.G. and Neuschwander-Tetri, B.A. 1994. Nonalcoholic steatohepatitis: An expanded clinical entity. *Gastroenterology* 107(4), pp. 1103–1109.

Bailone, R.L. et al. 2020. Zebrafish as an alternative animal model in human and animal vaccination research. *Laboratory Animal Research* 36(1), p. 13.

Bandyopadhyay, K., Karmakar, S., Ghosh, A. and Das, P.K. 2002. High affinity binding between laminin and laminin binding protein of *Leishmania* is stimulated by zinc and may involve laminin zinc - finger like sequences. *European Journal of Biochemistry* 269(6), pp. 1622–1629.

Barratt, S. L., Creamer, A., Hayton, C. & Chaudhuri, N. (2018) Idiopathic Pulmonary Fibrosis (IPF): An Overview. *Journal of Clinical Medicine*, 7 (8), p. 201.

Bazdyrev, E., Rusina, P., Panova, M., Novikov, F., Grishagin, I. & Nebolsin, V. (2021) Lung Fibrosis after COVID-19: Treatment Prospects. *Pharmaceuticals*, 14 (8), p. 807.

Beer, K.B. and Wehman, A.M. 2017. Mechanisms and functions of extracellular vesicle release in vivo-What we can learn from flies and worms. *Cell Adhesion & Migration* 11(2), pp. 135–150.

Bellei, E., Bergamini, S., Monari, E., Fantoni, L. I., Cuoghi, A., Ozben, T. & Tomasi, A. (2011) High-Abundance Proteins Depletion for Serum Proteomic

Analysis: Concomitant Removal of Non-Targeted Proteins. *Amino Acids*, 40 (1), pp. 145–156.

Bello, S. M., Perry, M. N. & Smith, C. L. (2020) Know Your Model: A Knockout Does Not Always Make a Null. *Lab Animal*, 49 (3), pp. 59–60.

Bendtsen, J. D., Jensen, L. J., Blom, N., Heijne, G. von & Brunak, S. (2004) Feature-Based Prediction of Non-Classical and Leaderless Protein Secretion. *Protein Engineering, Design and Selection*, 17 (4), pp. 349–356.

Bickel, P.E., Tansey, J.T. and Welte, M.A. 2009. PAT proteins, an ancient family of lipid droplet proteins that regulate cellular lipid stores. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* 1791(6), pp. 419–440.

Birrer, P. et al. 1994. Protease-antiprotease imbalance in the lungs of children with cystic fibrosis. *American Journal of Respiratory and Critical Care Medicine* 150(1), pp. 207–213.

Boon, L., Ugarte-Berzal, E., Vandooren, J. and Opdenakker, G. 2020. Protease propeptide structures, mechanisms of activation, and functions. *Critical Reviews in Biochemistry and Molecular Biology* 55(2), pp. 111–165.

Bouvier, J., Schneider, P., Etges, R. & Bordier, C. (1990) Peptide Substrate Specificity of the Membrane-Bound Metalloprotease of *Leishmania*. *Biochemistry*, 29 (43), pp. 10113–10119.

Briggs, C. J., Anderson, D., Johnson, P. & Deegan, T. (1981) Evaluation of the Polyethylene Glycol Precipitation Method for the Estimation of High-Density Lipoprotein Cholesterol. *Annals of Clinical Biochemistry*, 18 (3), pp. 177–181.

Brinks, V., Jiskoot, W. and Schellekens, H. 2011. Immunogenicity of therapeutic proteins: the use of animal models. *Pharmaceutical Research* 28(10), pp. 2379–2385.

Brix, K., Scott, C. J. & Heck, M. M. S. (2013) *Proteases: Structure and Function*. pp. 85–125.

Bruton, F.A. et al. 2022. Macrophages trigger cardiomyocyte proliferation by increasing epicardial vegfaa expression during larval zebrafish heart regeneration. *Developmental Cell* 57(12), pp. 1512-1528.e5.

Bucci, M. and Murphy, C.R. 2001. Hormonal control of enzyme activity during the plasma membrane transformation of uterine epithelial cells. *Cell Biology International* 25(9), pp. 859–871.

Burks, D.J. and White, M.F. 2001. IRS proteins and beta-cell function. *Diabetes* 50 Suppl 1, pp. S140-145.

Burnouf, T. 2007. Modern Plasma Fractionation. *Transfusion Medicine Reviews* 21(2), pp. 101–117.

Di Cara, F., Duca, E., Dunbar, D. R., Cagney, G. & Heck, M. M. S. (2013) Invadolysin, a Conserved Lipid-Droplet-Associated Metalloproteinase, Is Required for Mitochondrial Function in *Drosophila*. *Journal of Cell Science*, 126 (20), pp. 4769–4781.

Cardoso-Moreira, M., Halbert, J., Valloton, D., Velten, B., Chen, C., Shao, Y., Liechti, A., Ascensão, K., Rummel, C., Ovchinnikova, S., Mazin, P. V., Xenarios, I., Harshman, K., Mort, M., Cooper, D. N., Sandi, C., Soares, M. J., Ferreira, P. G., Afonso, S., Carneiro, M., Turner, J. M. A., VandeBerg, J. L., Fallahshahroudi, A., Jensen, P., Behr, R., Lisgo, S., Lindsay, S., Khaitovich, P., Huber, W., Baker, J., Anders, S., Zhang, Y. E. & Kaessmann, H. (2019) Gene Expression across Mammalian Organ Development. *Nature*, 571 (7766), pp. 505–509.

Cen, J. et al. 2020. Particulate matter (PM10) induces cardiovascular developmental toxicity in zebrafish embryos and larvae via the ERS, Nrf2 and Wnt pathways. *Chemosphere* 250, p. 126288.

Cheng, T., Kam, J.Y., Johansen, M.D. and Oehlers, S.H. 2020. High content analysis of granuloma histology and neutrophilic inflammation in adult zebrafish infected with *Mycobacterium marinum*. *Micron* 129, p. 102782.

Chang, C.-W., Abhinav, K., Di Cara, F., Panagakou, I., Vass, S. & Heck, M. M. S. (2017) A Role for the Metalloprotease Invadolysin in Insulin Signaling and Adipogenesis. *Biological Chemistry*, 398 (3), pp. 373–393.

Chao, E. C. (2014) SGLT-2 Inhibitors: A New Mechanism for Glycemic Control. *Clinical Diabetes*, 32 (1), pp. 4–11.

Cherepanova, N., Shrimal, S. & Gilmore, R. (2016) N-Linked Glycosylation and Homeostasis of the Endoplasmic Reticulum. *Current Opinion in Cell Biology*, 41, pp. 57–65.

Choi, T.-Y., Choi, T.-I., Lee, Y.-R., Choe, S.-K. and Kim, C.-H. 2021. Zebrafish as an animal model for biomedical research. *Experimental & Molecular Medicine* 53(3), pp. 310–317.

Chu, Y., Rosso, L. G., Huang, P., Wang, Z., Xu, Y., Yao, X., Bao, M., Yan, J., Song, H. & Wang, G. (2014) Liver Med23 Ablation Improves Glucose and Lipid *Metabolism* through Modulating FOXO1 Activity. *Cell Research*, 24 (10), pp. 1250–1265.

Choi, T.-Y., Choi, T.-I., Lee, Y.-R., Choe, S.-K. and Kim, C.-H. 2021. Zebrafish as an animal model for biomedical research. *Experimental & Molecular Medicine* 53(3), pp. 310–317.

Cikoš, S. 2012. Adiponectin and its receptors in preimplantation embryo development. *Vitamins and Hormones* 90, pp. 211–238.

Čikoš, Š., Burkuš, J., Bukovská, A., Fabian, D., Reháč, P. and Koppel, J. 2010. Expression of adiponectin receptors and effects of adiponectin isoforms in mouse preimplantation embryos. *Human Reproduction* (Oxford, England) 25(9), pp. 2247–2255.

Clark, D.P. 2005. Molecular biology: understanding the genetic revolution. Amsterdam: Elsevier Academic Press.

Cobbe, N., Marshall, K. M., Rao, S. G., Chang, C.-W., Di Cara, F. D., Duca, E., Vass, S., Kassan, A. & Heck, M. M. S. (2009) The Conserved Metalloprotease Invadolysin Localizes to the Surface of Lipid Droplets. *Journal of Cell Science*, 122 (18), pp. 3414–3423.

Combs, T.P. et al. 2003. Sexual differentiation, pregnancy, calorie restriction, and aging affect the adipocyte-specific secretory protein adiponectin. *Diabetes* 52(2), pp. 268–276.

Costa, S.R.D., Monteiro, M.D.C., Da Silva Júnior, F.M.R. and Sandrini, J.Z. 2016. Methylene blue toxicity in zebrafish cell line is dependent on light exposure. *Cell Biology International* 40(8), pp. 895–905.

Craik, C. S., Page, M. J. & Madison, E. L. (2011) Proteases as Therapeutics. *Biochemical Journal*, 435 (1), pp. 1–16.

D`Avila-Mesquita, C., Couto, A. E. S., Campos, L. C. B., Vasconcelos, T. F., Michelon-Barbosa, J., Corsi, C. A. C., Mestriner, F., Petroski-Moraes, B. C., Garbellini-Diab, M. J., Couto, D. M. S., Jordani, M. C., Ferro, D., Sbragia, L., Joviliano, E. E., Evora, P. R., Santana, R. de C., Martins-Filho, O. A., Polonis, K., Menegueti, M. G., Ribeiro, M. S., Auxiliadora-Martins, M. & Becari, C. (2021) MMP-2 and MMP-9 Levels in Plasma Are Altered and Associated with Mortality in COVID-19 Patients. *Biomedicine & Pharmacotherapy*, 142, p. 112067.

De Almeida, L.G.N. et al. 2022. Matrix Metalloproteinases: From Molecular Mechanisms to Physiology, Pathophysiology, and Pharmacology. Schulte, G. ed. *Pharmacological Reviews* 74(3), pp. 714–770.

DeFalco, T. & Capel, B. (2009) Gonad Morphogenesis in Vertebrates: Divergent Means to a Convergent End. *Annual Review of Cell and Developmental Biology*, 25 (1), pp. 457–482.

Denery, J. R., Nunes, A. A. K. & Dickerson, T. J. (2011) Characterization of Differences between Blood Sample Matrices in Untargeted Metabolomics. *Analytical Chemistry*, 83 (3), pp. 1040–1047.

Díaz, E. and Pfeffer, S.R. 1998. TIP47: A Cargo Selection Device for Mannose 6-Phosphate Receptor Trafficking. *Cell* 93(3), pp. 433–443.

Díaz, E. and Pfeffer, S.R. 1998. TIP47: A Cargo Selection Device for Mannose 6-Phosphate Receptor Trafficking. *Cell* 93(3), pp. 433–443.

Donnor, T. and Sarkar, S. 2000. Insulin- Pharmacology, Therapeutic Regimens and Principles of Intensive Insulin Therapy. In: Feingold, K. R. et al. eds. *Endotext*. South Dartmouth (MA): MDText.com, Inc.

Dos Santos, E., Pecquery, R., de Mazancourt, P. and Dieudonné, M.-N. 2012. Adiponectin and reproduction. *Vitamins and Hormones* 90, pp. 187–209.

Doyle, L. and Wang, M. 2019. Overview of Extracellular Vesicles, Their Origin, Composition, Purpose, and Methods for Exosome Isolation and Analysis. *Cells* 8(7), p. 727.

Eisenhaber, B., Bork, P. & Eisenhaber, F. (1999) Prediction of Potential GPI-Modification Sites in Proprotein Sequences. *Journal of Molecular Biology*, 292 (3), pp. 741–758.

Fabbrini, E., Sullivan, S. & Klein, S. (2010) Obesity and Nonalcoholic Fatty Liver Disease: Biochemical, Metabolic, and Clinical Implications. *Hepatology*, 51 (2), pp. 679–689.

Filho, A. da S. L., Fajardo, E. F., Chang, K. P., Clément, P. & Olivier, M. (2022) Leishmania Exosomes/Extracellular Vesicles Containing GP63 Are Essential for Enhance Cutaneous Leishmaniasis Development Upon Co-Inoculation of Leishmania Amazonensis and Its Exosomes. *Frontiers in Cellular and Infection Microbiology*, 11, p. 709258.

Fredolini, C., Byström, S., Sanchez-Rivera, L., Ioannou, M., Tamburro, D., Pontén, F., Branca, R. M., Nilsson, P., Lehtiö, J. & Schwenk, J. M. (2019) Systematic Assessment of Antibody Selectivity in Plasma Based on a Resource of Enrichment Profiles. *Scientific Reports*, 9 (1), p. 8324.

Freishtat, R. J. & Nadler, E. P. (2014) Adipocyte Exosomes Induce Transforming Growth Factor Beta Pathway Dysregulation in Hepatocytes: A Novel Paradigm for Obesity-Related Liver Disease. *Journal of Surgical Research*, 192 (2), pp. 268–275.

Fujimoto, T. and Parton, R.G. 2011. Not just fat: the structure and function of the lipid droplet. *Cold Spring Harbor Perspectives in Biology* 3(3), p. a004838.

Fujita, M. & Kinoshita, T. (2012) GPI-Anchor Remodeling: Potential Functions of GPI-Anchors in Intracellular Trafficking and Membrane Dynamics. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*, 1821 (8), pp. 1050–1058.

Gaffney, J., Solomonov, I., Zehorai, E. and Sagi, I. 2015. Multilevel regulation of matrix metalloproteinases in tissue homeostasis indicates their molecular specificity in vivo. *Matrix Biology* 44–46, pp. 191–199.

Gatenby, R. A. & Gillies, R. J. (2004) Why Do Cancers Have High Aerobic Glycolysis? *Nature Reviews Cancer*, 4 (11), pp. 891–899.

Gaughan, E. E., Quinn, T. M., Mills, A., Bruce, A. M., Antonelli, J., MacKinnon, A., Aslanis, V., Li, F., O'Connor, R., Boz, C., Mills, R., Emanuel, P., Burgess, M., Rinaldi, G., Valanciute, A., Mills, B., Scholefield, E., Hardisty, G., Findlay, E. G., Parker, R. A., Norrie, J., Dear, J. W., Akram, A. R., Koch, O., Templeton, K., Dockrell, D. H., Walsh, T. S., Partridge, S., Humphries, D., Wang-Jairaj, J., Slack, R. J., Schambye, H., Phung, D., Gravelle, L., Lindmark, B., Shankar-Hari, M., Hirani, N., Sethi, T. & Dhaliwal, K. (2022) An Inhaled Galectin-3 Inhibitor in COVID-19 Pneumonitis (DEFINE): A Phase Ib/IIa Randomised Controlled Trial. *American Journal of Respiratory and Critical Care Medicine*.

Gaughan, E. et al. 2021. Evaluation of new or repurposed treatments for COVID-19: protocol for the phase Ib/IIa DEFINE trial platform. *BMJ open* 11(12), p. e054442.

Gatti, M. & Baker, B. S. (1989) Genes Controlling Essential Cell-Cycle Functions in *Drosophila Melanogaster*. *Genes & Development*, 3 (4), pp. 438–453.

Gelzo, M., Cacciapuoti, S., Pinchera, B., Rosa, A. D., Cernera, G., Scialò, F., Comegna, M., Mormile, M., Fabbrocini, G., Parrella, R., Corso, G., Gentile, I. & Castaldo, G. (2022) Matrix Metalloproteinases (MMP) 3 and 9 as *Biomarkers* of Severity in COVID-19 Patients. *Scientific Reports*, 12 (1), p. 1212.

Ghosh, A., Bandyopadhyay, K., Kole, L. and Das, P.K. 1999. Isolation of a laminin-binding protein from the protozoan parasite *Leishmania donovani* that may mediate cell adhesion. *The Biochemical Journal* 337 (Pt 3)(Pt 3), pp. 551–558.

Gioseffi, A., Hamerly, T., Van, K., Zhang, N., Dinglasan, R. R., Yates, P. A. & Kima, P. E. (2020) *Leishmania*-Infected Macrophages Release Extracellular Vesicles That Can Promote Lesion Development. *Life Science Alliance*, 3 (12), p. e202000742.

Glass, D.S. et al. 2020. Idiopathic pulmonary fibrosis: Molecular mechanisms and potential treatment approaches. *Respiratory Investigation* 58(5), pp. 320–335.

Goessling, W. and Sadler, K.C. 2015. Zebrafish: an important tool for liver disease research. *Gastroenterology* 149(6), pp. 1361–1377.

Goettig, P. (2016) Effects of Glycosylation on the Enzymatic Activity and Mechanisms of Proteases. *International Journal of Molecular Sciences*, 17 (12), p. 1969.

Gomis-Rüth, F. X. (2003) Structural Aspects of the Metzincin Clan of Metalloendopeptidases. *Molecular Biotechnology*, 24 (2), pp. 157–202.

Granger, J., Siddiqui, J., Copeland, S. & Remick, D. (2005) Albumin Depletion of Human Plasma Also Removes Low Abundance Proteins Including the Cytokines. *PROTEOMICS*, 5 (18), pp. 4713–4718.

Hadler - Olsen, E., Fadnes, B., Sylte, I., Uhlin - Hansen, L. and Winberg, J. 2011. Regulation of matrix metalloproteinase activity in health and disease. *The FEBS Journal* 278(1), pp. 28 – 45.

Hambrook, J.R. and Hanington, P.C. 2023. A cercarial invadolysin interferes with the host immune response and facilitates infection establishment of *Schistosoma mansoni*. *PLoS pathogens* 19(2), p. e1010884.

Harjula, S.-K.E. et al. 2020. Characterization of immune response against *Mycobacterium marinum* infection in the main hematopoietic organ of adult zebrafish (*Danio rerio*). *Developmental & Comparative Immunology* 103, p. 103523.

Hartley, B.S. 1960. PROTEOLYTIC ENZYMES. *Annual Review of Biochemistry* 29(1), pp. 45–72.

Haskó, F., Vaszileva, R. & Halász, L. (1982) Solubility of Plasma Proteins in the Presence of Polyethylene Glycol. *Biotechnology and Bioengineering*, 24 (9), pp. 1931–1939.

Hassani, K. & Olivier, M. (2013) Immunomodulatory Impact of Leishmania-Induced Macrophage Exosomes: A Comparative Proteomic and Functional Analysis. *PLoS Neglected Tropical Diseases*, 7 (5), p. e2185.

Hassani, K., Shio, M. T., Martel, C., Faubert, D. & Olivier, M. (2014) Absence of Metalloprotease GP63 Alters the Protein Content of Leishmania Exosomes. *PLoS ONE*, 9 (4), p. e95007.

Hawley, S. A., Ford, R. J., Smith, B. K., Gowans, G. J., Mancini, S. J., Pitt, R. D., Day, E. A., Salt, I. P., Steinberg, G. R. & Hardie, D. G. (2016) The

Na⁺/Glucose Cotransporter Inhibitor Canagliflozin Activates AMPK by Inhibiting Mitochondrial Function and Increasing Cellular AMP Levels. *Diabetes*, 65 (9), pp. 2784–2794.

Hazra, S. et al. 2012. Modulation of matrix metalloproteinase activity by EDTA prevents posterior capsular opacification. *Molecular Vision* 18, pp. 1701–1711.

Heukels, P., Moor, C.C., von der Thüsen, J.H., Wijsenbeek, M.S. and Kool, M. 2019. Inflammation and immunity in IPF pathogenesis and treatment. *Respiratory Medicine* 147, pp. 79–91.

Hirsova, P., Ibrahim, S. H., Verma, V. K., Morton, L. A., Shah, V. H., LaRusso, N. F., Gores, G. J. & Malhi, H. (2016) Extracellular Vesicles in Liver Pathobiology: Small Particles with Big Impact. *Hepatology*, 64 (6), pp. 2219–2233.

Hönig, W. & Kula, M.-R. (1976) Selectivity of Protein Precipitation with Polyethylene Glycol Fractions of Various Molecular Weights. *Analytical Biochemistry*, 72 (1–2), pp. 502–512.

Howe, K. et al. 2013. The zebrafish reference genome sequence and its relationship to the human genome. *Nature* 496(7446), pp. 498–503.

Hsiao, C.-H. C., Yao, C., Storlie, P., Donelson, J. E. & Wilson, M. E. (2008) The Major Surface Protease (MSP or GP63) in the Intracellular Amastigote Stage of *Leishmania Chagasi*. *Molecular and Biochemical Parasitology*, 157 (2), pp. 148–159.

Hu, J., Steen, P. E. V. den, Sang, Q.-X. A. & Opdenakker, G. (2007) Matrix Metalloproteinase Inhibitors as Therapy for Inflammatory and Vascular Diseases. *Nature Reviews Drug Discovery*, 6 (6), pp. 480–498.

Huang, H. (2018) Matrix Metalloproteinase-9 (MMP-9) as a Cancer Biomarker and MMP-9 Biosensors: Recent Advances. *Sensors*, 18 (10), p. 3249.

Huang, J.-J., Wang, C.-W., Liu, Y., Zhang, Y.-Y., Yang, N.-B., Yu, Y.-C., Jiang, Q., Song, Q.-F. & Qian, G.-Q. (2023) Role of the Extracellular Matrix in COVID-19. *World Journal of Clinical Cases*, 11 (1), pp. 73–83.

Hundhausen, C. et al. 2003. The disintegrin-like metalloproteinase ADAM10 is involved in constitutive cleavage of CX3CL1 (fractalkine) and regulates CX3CL1-mediated cell-cell adhesion. *Blood* 102(4), pp. 1186–1195.

Imamura, M., Inoguchi, T., Ikuyama, S., Taniguchi, S., Kobayashi, K., Nakashima, N. and Nawata, H. 2002. ADRP stimulates lipid accumulation and lipid droplet formation in murine fibroblasts. *American Journal of Physiology-Endocrinology and Metabolism* 283(4), pp. E775–E783.

Irvine, K. M., Okano, S., Patel, P. J., Horsfall, L. U., Williams, S., Russell, A. & Powell, E. E. (2021) Serum Matrix Metalloproteinase 7 (MMP7) Is a Biomarker of Fibrosis in Patients with Non-Alcoholic Fatty Liver Disease. *Scientific Reports*, 11 (1), p. 2858.

Irvine, K. M., Wockner, L. F., Hoffmann, I., Horsfall, L. U., Fagan, K. J., Bijin, V., Lee, B., Clouston, A. D., Lampe, G., Connolly, J. E. & Powell, E. E. (2016) Multiplex Serum Protein Analysis Identifies Novel Biomarkers of Advanced Fibrosis in Patients with Chronic Liver Disease with the Potential to Improve Diagnostic Accuracy of Established Biomarkers. *PLoS ONE*, 11 (11), p. e0167001.

Isnard, A., Shio, M. T. & Olivier, M. (2012) Impact of Leishmania Metalloprotease GP63 on Macrophage Signaling. *Frontiers in Cellular and Infection Microbiology*, 2, p. 72.

Iyer, V. V., Yang, H., Ierapetritou, M. G. & Roth, C. M. (2010) Effects of Glucose and Insulin on HepG2-C3A Cell Metabolism. *Biotechnology and Bioengineering*, 107 (2), pp. 347–356.

Jacobson, K., Ishihara, A. and Inman, R. 1987. Lateral Diffusion of Proteins in Membranes. *Annual Review of Physiology* 49(1), pp. 163–175.

Jenkins, R. G., Simpson, J. K., Saini, G., Bentley, J. H., Russell, A.-M., Braybrooke, R., Molyneaux, P. L., McKeever, T. M., Wells, A. U., Flynn, A.,

Hubbard, R. B., Leeming, D. J., Marshall, R. P., Karsdal, M. A., Lukey, P. T. & Maher, T. M. (2015) Longitudinal Change in Collagen Degradation Biomarkers in Idiopathic Pulmonary Fibrosis: An Analysis from the Prospective, Multicentre PROFILE Study. *The Lancet Respiratory Medicine*, 3 (6), pp. 462–472.

Ji, J., Torrealba, D., Thwaite, R., Gomez, A.-C., Parra, D. and Roher, N. 2019. Nanostructured TNF α protein targets the zebrafish (*Danio rerio*) immune system through mucosal surfaces and improves the survival after *Mycobacterium marinum* lethal infection. *Aquaculture* 510, pp. 138–149.

Jia, Y., Liu, Y., Feng, L., Sun, S. and Sun, G. 2022. Role of Glucagon and Its Receptor in the Pathogenesis of Diabetes. *Frontiers in Endocrinology* 13, p. 928016.

Juckles, I. R. M. (1971) Fractionation of Proteins and Viruses with Polyethylene Glycol. *Biochimica et Biophysica Acta (BBA) - Protein Structure*, 229 (3), pp. 535–546.

Kaikaew, K., Grefhorst, A., Steenbergen, J., Swagemakers, S. M. A., McLuskey, A. & Visser, J. A. (2021) Sex Difference in the Mouse BAT Transcriptome Reveals a Role of Progesterone. *Journal of Molecular Endocrinology*, 66 (2), pp. 97–113.

Kattula, S., Byrnes, J.R. and Wolberg, A.S. 2017. Fibrinogen and Fibrin in Hemostasis and Thrombosis. *Arteriosclerosis, Thrombosis, and Vascular Biology* 37(3), pp. e13–e21.

Kaveh, A. et al. 2020. Live Imaging of Heart Injury in Larval Zebrafish Reveals a Multi-Stage Model of Neutrophil and Macrophage Migration. *Frontiers in Cell and Developmental Biology* 8, p. 579943.

Keating, S.E., Hackett, D.A., George, J. and Johnson, N.A. 2012. Exercise and non-alcoholic fatty liver disease: a systematic review and meta-analysis. *Journal of Hepatology* 57(1), pp. 157–166.

Keller, J.M. and Keller, E.T. 2018. The Use of Mature Zebrafish (*Danio rerio*) as a Model for Human Aging and Disease. In: Conn's Handbook of Models for Human Aging. Elsevier, pp. 351–359.

Kellogg, M. K., Miller, S. C., Tikhonova, E. B. & Karamyshev, A. L. (2021) SRPassing Co-Translational Targeting: The Role of the Signal Recognition Particle in Protein Targeting and mRNA Protection. *International Journal of Molecular Sciences*, 22 (12), p. 6284.

Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B. & Schilling, T. F. (1995) Stages of Embryonic Development of the Zebrafish. *Developmental Dynamics*, 203 (3), pp. 253–310.

Kinoshita, T. 2016. Glycosylphosphatidylinositol (GPI) Anchors: Biochemistry and Cell Biology: Introduction to a Thematic Review Series. *Journal of Lipid Research* 57(1), pp. 4–5.

Klee, E. W. & Sosa, C. P. (2007) Computational Classification of Classically Secreted Proteins. *Drug Discovery Today*, 12 (5–6), pp. 234–240.

Koeck, E. S., Iordanskaia, T., Sevilla, S., Ferrante, S. C., Hubal, M. J.,

Kneeman, J.M., Misdraji, J. and Corey, K.E. 2012. Secondary causes of nonalcoholic fatty liver disease. *Therapeutic Advances in Gastroenterology* 5(3), pp. 199–207.

Knobler, H. et al. 1999. Fatty liver--an additional and treatable feature of the insulin resistance syndrome. *QJM: monthly journal of the Association of Physicians* 92(2), pp. 73–79.

Kornek, M., Popov, Y., Libermann, T. A., Afdhal, N. H. & Schuppan, D. (2011) Human T Cell Microparticles Circulate in Blood of Hepatitis Patients and Induce Fibrolytic Activation of Hepatic Stellate Cells. *Hepatology*, 53 (1), pp. 230–242.

Kraiczy, J., Nayak, K. M., Howell, K. J., Ross, A., Forbester, J., Salvestrini, C., Mustata, R., Perkins, S., Andersson-Rolf, A., Leenen, E., Liebert, A.,

Vallier, L., Rosenstiel, P. C., Stegle, O., Dougan, G., Heuschkel, R., Koo, B.-K. & Zilbauer, M. (2019) DNA Methylation Defines Regional Identity of Human Intestinal Epithelial Organoids and Undergoes Dynamic Changes during Development. *Gut*, 68 (1), p. 49.

Kulkarni, M. M., Jones, E. A., McMaster, W. R. & McGwire, B. S. (2008) Fibronectin Binding and Proteolytic Degradation by *Leishmania* and Effects on Macrophage Activation. *Infection and Immunity*, 76 (4), pp. 1738–1747.

Kulkarni, M. M., McMaster, W. R., Kamysz, E., Kamysz, W., Engman, D. M. & McGwire, B. S. (2006) The Major Surface-metalloprotease of the Parasitic Protozoan, *Leishmania*, Protects against Antimicrobial Peptide-induced Apoptotic Killing. *Molecular Microbiology*, 62 (5), pp. 1484–1497.

Kurucz, É. et al. 2007. Definition of *Drosophila* hemocyte subsets by cell-type specific antigens. *Acta Biologica Hungarica* 58(Supplement 1), pp. 95–111.

Lachowicz, J., Niedziałek, K., Rostkowska, E., Szopa, A., Świąder, K., Szponar, J. and Serefko, A. 2021. Zebrafish as an Animal Model for Testing Agents with Antidepressant Potential. *Life (Basel, Switzerland)* 11(8), p. 792.

Lager, I. 1991. The insulin-antagonistic effect of the counterregulatory hormones. *Journal of Internal Medicine*. Supplement 735, pp. 41–47.

Laemmli, U. K. (1970) Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature*, 227 (5259), pp. 680–685.

Lala V, Zubair M, Minter DA. Liver Function Tests. 2022 Oct 5. StatPearls Publishing; 2023 Jan.

Lalonde, S., Stone, O. A., Lessard, S., Lavertu, A., Desjardins, J., Beaudoin, M., Rivas, M., Stainier, D. Y. R. & Lettre, G. (2017) Frameshift Indels Introduced by Genome Editing Can Lead to In-Frame Exon Skipping. *PLoS ONE*, 12 (6), p. e0178700.

Lambertz, U., Silverman, J. M., Nandan, D., McMaster, W. R., Clos, J., Foster, L. J. & Reiner, N. E. (2012) Secreted Virulence Factors and Immune Evasion in Visceral Leishmaniasis. *Journal of Leukocyte Biology*, 91 (6), pp. 887–899.

Layer, A., Schneider, P., Tissot, J.-D. & Duchosal, M. A. (2000) Encyclopedia of Separation Science. Appendices, (Journal of Biological Chemistry 261 1986), pp. 4553–4559.

Li, C., Tan, X. F., Lim, T. K., Lin, Q. & Gong, Z. (2016) Comprehensive and Quantitative Proteomic Analyses of Zebrafish Plasma Reveals Conserved Protein Profiles between Genders and between Zebrafish and Human. *Scientific Reports*, 6 (1), p. 24329.

Li, Y., Ma, Z., Jiang, S., Hu, W., Li, T., Di, S., Wang, D. & Yang, Y. (2017) A Global Perspective on FOXO1 in Lipid Metabolism and Lipid-Related Diseases. *Progress in Lipid Research*, 66, pp. 42–49.

Liao, S. et al. 2021. Establishment of a Drug Screening Model for Cardiac Complications of Acute Renal Failure. *Biomolecules* 11(9), p. 1370.

Lin, Y. and Gross, M.L. 2022. Mass Spectrometry-Based Structural Proteomics for Metal Ion/Protein Binding Studies. *Biomolecules* 12(1), p. 135.

LibreTexts Chemistry, Metalloprotease, accessed 12 December 2023, < [https://chem.libretexts.org/Bookshelves/Biological_Chemistry/Supplemental_Modules_\(Biological_Chemistry\)/Enzymes/Metalloproteases](https://chem.libretexts.org/Bookshelves/Biological_Chemistry/Supplemental_Modules_(Biological_Chemistry)/Enzymes/Metalloproteases) >.

Lin, D., Alborn, W. E., Slebos, R. J. C. & Liebler, D. C. (2013) Comparison of Protein Immunoprecipitation-Multiple Reaction Monitoring with ELISA for Assay of Biomarker Candidates in Plasma. *Journal of Proteome Research*, 12 (12), pp. 5996–6003.

Lindholm, M. E., Huss, M., Solnestam, B. W., Kjellqvist, S., Lundeberg, J. & Sundberg, C. J. (2014) The Human Skeletal Muscle Transcriptome: Sex Differences, Alternative Splicing, and Tissue Homogeneity Assessed with

RNA Sequencing. *The FASEB Journal*, 28 (10), pp. 4571–4581.

Link, V., Shevchenko, A. & Heisenberg, C.-P. (2006) Proteomics of Early Zebrafish Embryos. *BMC Developmental Biology*, 6 (1), p. 1.

Lippincott-Schwartz, J., Roberts, T. H. & Hirschberg, K. (2000) SECRETORY PROTEIN TRAFFICKING AND ORGANELLE DYNAMICS IN LIVING CELLS1. *Annual Review of Cell and Developmental Biology*, 16 (1), pp. 557–589.

Listenberger, L. L. & Brown, D. A. (2008) Lipid Droplets. *Current Biology*, 18 (6), pp. R237–R238.

Liu, B., Qiu, F., Voss, C., Xu, Y., Zhao, M., Wu, Y., Nie, J. & Wang, Z. (2011) Evaluation of Three High Abundance Protein Depletion Kits for Umbilical Cord Serum Proteomics. *Proteome Science*, 9 (1), pp. 24–24.

Lockman, K. A., Baren, J. P., Pemberton, C. J., Baghdadi, H., Burgess, K. E., Plevris-Papaioannou, N., Lee, P., Howie, F., Beckett, G., Pryde, A., Jaap, A. J., Hayes, P. C., Filippi, C. & Plevris, J. N. (2012) Oxidative Stress Rather than Triglyceride Accumulation Is a Determinant of Mitochondrial Dysfunction in in Vitro Models of Hepatic Cellular Steatosis. *Liver International*, 32 (7), pp. 1079–1092.

López-Bascón, M. A., Priego-Capote, F., Peralbo-Molina, A., Calderón-Santiago, M. & Castro, M. D. L. de (2016) Influence of the Collection Tube on Metabolomic Changes in Serum and Plasma. *Talanta*, 150, pp. 681–689.

López-Otín, C. and Bond, J.S. 2008. Proteases: multifunctional enzymes in life and disease. *The Journal of Biological Chemistry* 283(45), pp. 30433–30437.

Lubet, R.A., Turner-Lubet, M. and Whitson, G.L. 1975. The relationship of cellular metabolism of benzo(a)pyrene to the induction of cytotoxicity and cell transformation in fetal hamster cells in vitro. *European Journal of Cancer* 11(3), pp. 139–144.

Lucitt, M. B., Price, T. S., Pizarro, A., Wu, W., Yocum, A. K., Seiler, C., Pack, M. A., Blair, I. A., FitzGerald, G. A. & Grosser, T. (2008) Analysis of the Zebrafish Proteome during Embryonic Development*. *Molecular & Cellular Proteomics*, 7 (5), pp. 981–994.

Luirink, J. & Sinning, I. (2004) SRP-Mediated Protein Targeting: Structure and Function Revisited. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, 1694 (1–3), pp. 17–35.

Luo, L. and Liu, M. 2016. Adipose tissue in control of metabolism. *Journal of Endocrinology* 231(3), pp. R77–R99.

Macdonald, M. H., Morrison, C. J. & McMaster, W. R. (1995) Analysis of the Active Site and Activation Mechanism of the Leishmania Surface Metalloproteinase GP63. *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology*, 1253 (2), pp. 199–207.

Malara, A., Abbonante, V., Di Buduo, C.A., Tozzi, L., Currao, M. and Balduini, A. 2015. The secret life of a megakaryocyte: emerging roles in bone marrow homeostasis control. *Cellular and Molecular Life Sciences* 72(8), pp. 1517–1536.

Malara, A., Ligi, D., Di Buduo, C.A., Mannello, F. and Balduini, A. 2018. Sub-Cellular Localization of Metalloproteinases in Megakaryocytes. *Cells* 7(7), p. 80.

Marchand-Adam, S., Pronost, M., Saidi, A., Lecaille, F. and Lalmanach, G. 2023. Cathepsin K: both a likely biomarker and a new therapeutic target in lymphangioleiomyomatosis? *Rare Disease and Orphan Drugs Journal* 2(1), p. 3.

Martinez, F.J. et al. 2017. Idiopathic pulmonary fibrosis. *Nature Reviews. Disease Primers* 3, p. 17074.

Mannello, F. (2003) Effects of Blood Collection Methods on Gelatin Zymography of Matrix Metalloproteinases. *Clinical Chemistry*, 49 (2), pp.

339–340.

Marín-Hernández, A., Rodríguez-Enríquez, S., Vital-González, P. A., Flores-Rodríguez, F. L., Macías-Silva, M., Sosa-Garrocho, M. & Moreno-Sánchez, R. (2006) Determining and Understanding the Control of Glycolysis in Fast-growth Tumor Cells. *The FEBS Journal*, 273 (9), pp. 1975–1988.

Mathivanan, S., Ji, H. & Simpson, R. J. (2010) Exosomes: Extracellular Organelles Important in Intercellular Communication. *Journal of Proteomics*, 73 (10), pp. 1907–1920.

Mathupala, S. P., Ko, Y. H. & Pedersen, P. L. (2006) Hexokinase II: Cancer's Double-Edged Sword Acting as Both Facilitator and Gatekeeper of Malignancy When Bound to Mitochondria. *Oncogene*, 25 (34), pp. 4777–4786.

McGroder, C. F., Zhang, D., Choudhury, M. A., Salvatore, M. M., D'Souza, B. M., Hoffman, E. A., Wei, Y., Baldwin, M. R. & Garcia, C. K. (2021) Pulmonary Fibrosis 4 Months after COVID-19 Is Associated with Severity of Illness and Blood Leucocyte Telomere Length. *Thorax*, 76 (12), pp. 1242–1245.

McGwire, B. S., Chang, K.-P. & Engman, D. M. (2003) Migration through the Extracellular Matrix by the Parasitic Protozoan *Leishmania* Is Enhanced by Surface Metalloprotease Gp63. *Infection and Immunity*, 71 (2), pp. 1008–1010.

McGwire, B. S., O'Connell, W. A., Chang, K.-P. & Engman, D. M. (2002) Extracellular Release of the Glycosylphosphatidylinositol (GPI)-Linked *Leishmania* Surface Metalloprotease, Gp63, Is Independent of GPI Phospholipolysis IMPLICATIONS FOR PARASITE VIRULENCE*. *Journal of Biological Chemistry*, 277 (11), pp. 8802–8809.

McGwire, B.S., Chang, K.-P. and Engman, D.M. 2003. Migration through the Extracellular Matrix by the Parasitic Protozoan *Leishmania* Is Enhanced by Surface Metalloprotease gp63. *Infection and Immunity* 71(2), pp. 1008–1010.

McHugh, B., Krause, S. A., Yu, B., Deans, A.-M., Heasman, S., McLaughlin, P. & Heck, M. M. S. (2004) Invadolysin. *The Journal of Cell Biology*, 167 (4), pp. 673–686.

McKelvey, M.C., Weldon, S., McAuley, D.F., Mall, M.A. and Taggart, C.C. 2020. Targeting Proteases in Cystic Fibrosis Lung Disease. Paradigms, Progress, and Potential. *American Journal of Respiratory and Critical Care Medicine* 201(2), pp. 141–147.

Mei, Q., Liu, Z., Zuo, H., Yang, Z. and Qu, J. 2022. Idiopathic Pulmonary Fibrosis: An Update on Pathogenesis. *Frontiers in Pharmacology* 12, p. 797292.

Melby, P.C., Travi, B.L. and Osorio, E.Y. 2019. *Leishmania*☆. In: Schmidt, T. M. ed. *Encyclopedia of Microbiology* (Fourth Edition). Fourth Edition. Oxford: Academic Press, pp. 769 – 779.

Melo, A. C. N. de, Levy, C. M. d'Avila-, Dias, F. A., Armada, J. L. A., Silva, H. D., Lopes, A. H. C. S., Santos, A. L. S., Branquinha, M. H. & Vermelho, A. B. (2006) Peptidases and Gp63-like Proteins in *Herpetomonas Megasteliae*: Possible Involvement in the Adhesion to the Invertebrate Host. *International Journal for Parasitology*, 36 (4), pp. 415–422.

MEROPS, The Peptidase Database, accessed 12 December 2023, <<http://www.ebi.ac.uk/merops/>>.

Miwa, N. 2015. Protein-Carbohydrate Interaction between Sperm and the Egg-Coating Envelope and Its Regulation by Dicalcin, a *Xenopus laevis* Zona Pellucida Protein-Associated Protein. *Molecules* 20(5), pp. 9468–9486.

Moin, A. S. M., Sathyapalan, T., Atkin, S. L. & Butler, A. E. (2020) Pro-Fibrotic M2 Macrophage Markers May Increase the Risk for COVID19 in Type 2 Diabetes with Obesity. *Metabolism*, 112, p. 154374.

Moreno-Sánchez, R., Rodríguez-Enríquez, S., Marín-Hernández, A. & Saavedra, E. (2007) Energy Metabolism in Tumor Cells. *The FEBS Journal*, 274 (6), pp. 1393–1418.

Morgan, K., Suppiah, S. L., Masterton, G., Samuel, K., Johnston, S., Hayes,

P. C., Lockman, K. A., Koutsos, V. & Plevris, J. N. (2022) The Effect of Eicosapentaenoic Acid on Hepatic Metabolism and Intracellular Lipid Droplet Dynamics in An in Vitro Model of Hepatic Steatosis.

Morrell, E. D., Mikacenic, C., Gong, K.-Q., Kosamo, S., Wurfel, M. M. & Manicone, A. M. (2020) Alveolar MMP28 Is Associated with Clinical Outcomes and Measures of Lung Injury in Acute Respiratory Distress Syndrome. *Critical Care*, 24 (1), p. 141.

Mótyán, J. A., Tóth, F. & Tózsér, J. (2013) Research Applications of Proteolytic Enzymes in Molecular Biology. *Biomolecules*, 3 (4), pp. 923–942.

Muñiz, M. & Riezman, H. (2016) Trafficking of Glycosylphosphatidylinositol Anchored Proteins from the Endoplasmic Reticulum to the Cell Surface. *Journal of Lipid Research*, 57 (3), pp. 352–360.

Musselman, L. P., Fink, J. L., Narzinski, K., Ramachandran, P. V., Hathiramani, S. S., Cagan, R. L. & Baranski, T. J. (2011) A High-Sugar Diet Produces Obesity and Insulin Resistance in Wild-Type *Drosophila*. *Disease Models & Mechanisms*, 4 (6), pp. 842–849.

Myllymäki, H., Niskanen, M., Luukinen, H., Parikka, M. and Rämetsä, M. 2018. Identification of protective postexposure mycobacterial vaccine antigens using an immunosuppression-based reactivation model in the zebrafish. *Disease Models & Mechanisms* 11(3), p. dmm033175.

National Research Ethics Committees n.d., Guidelines, <
<https://www.forskningsetikk.no/en/guidelines/science-and-technology/ethical-guidelines-for-the-use-of-animals-in-research/>>.

Neurath, H. & Walsh, K. A. (1976) Role of Proteolytic Enzymes in Biological Regulation (a Review). *Proceedings of the National Academy of Sciences*, 73 (11), pp. 3825–3832.

Newman, L. A., Muller, K. & Rowland, A. (2022) Circulating Cell-Specific Extracellular Vesicles as Biomarkers for the Diagnosis and Monitoring of Chronic Liver Diseases. *Cellular and Molecular Life Sciences*, 79 (5), p. 232.

Newman, L. A., Sorich, M. J. & Rowland, A. (2020) Role of Extracellular Vesicles in the Pathophysiology, Diagnosis and Tracking of Non-Alcoholic Fatty Liver Disease. *Journal of Clinical Medicine*, 9 (7), p. 2032.

Newman, L. A., Useckaite, Z., Johnson, J., Sorich, M. J., Hopkins, A. M. & Rowland, A. (2022) Selective Isolation of Liver-Derived Extracellular Vesicles Redefines Performance of MiRNA Biomarkers for Non-Alcoholic Fatty Liver Disease. *Biomedicines*, 10 (1), p. 195.

Nishizawa, H. et al. 2002. Androgens decrease plasma adiponectin, an insulin-sensitizing adipocyte-derived protein. *Diabetes* 51(9), pp. 2734–2741.

Nita-Lazar, M., Wacker, M., Schegg, B., Amber, S. & Aepli, M. (2005) The N-X-S/T Consensus Sequence Is Required but Not Sufficient for Bacterial N-Linked Protein Glycosylation. *Glycobiology*, 15 (4), pp. 361–367.

Nordlie, F.G. 2009. Environmental influences on regulation of blood plasma/serum components in teleost fishes: a review. *Reviews in Fish Biology and Fisheries* 19(4), pp. 481–564.

Novus Biologicals n.d., Western Blot Sample Preparation, <<https://www.novusbio.com/support/support-by-application/western-blot-sample-preparation>>.

Nseir, W., Hellou, E. and Assy, N. 2014. Role of diet and lifestyle changes in nonalcoholic fatty liver disease. *World Journal of Gastroenterology* 20(28), pp. 9338–9344.

Nüsslein-Volhard, C. and Dahm, R. eds. 2005. Zebrafish: a practical approach. Reprinted. Oxford: Oxford Univ. Press.

Oksanen, K.E. et al. 2013. An adult zebrafish model for preclinical tuberculosis vaccine development. *Vaccine* 31(45), pp. 5202–5209.

Olivier, M., Atayde, V. D., Isnard, A., Hassani, K. & Shio, M. T. (2012) Leishmania Virulence Factors: Focus on the Metalloprotease GP63. *Microbes and Infection*, 14 (15), pp. 1377–1389.

Overall, C. M. & Dean, R. A. (2006) Degradomics: Systems Biology of the Protease Web. Pleiotropic Roles of MMPs in Cancer. *Cancer and Metastasis Reviews*, 25 (1), pp. 69–75.

Palmblad, M., Tiss, A. & Cramer, R. (2009) Mass Spectrometry in Clinical Proteomics – from the Present to the Future. *PROTEOMICS - Clinical Applications*, 3 (1), pp. 6–17.

Pantalacci, S. & Sémon, M. (2015) Transcriptomics of Developing Embryos and Organs: A Raising Tool for Evo–Devo. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution*, 324 (4), pp. 363–371.

Pei, K., Gui, T., Kan, D., Feng, H., Jin, Y., Yang, Y., Zhang, Q., Du, Z., Gai, Z., Wu, J. & Li, Y. (2020) An Overview of Lipid Metabolism and Nonalcoholic Fatty Liver Disease. *BioMed Research International*, 2020, p. 4020249.

Petersen, K.F., Dufour, S., Befroy, D., Lehrke, M., Hendler, R.E. and Shulman, G.I. 2005. Reversal of nonalcoholic hepatic steatosis, hepatic insulin resistance, and hyperglycemia by moderate weight reduction in patients with type 2 diabetes. *Diabetes* 54(3), pp. 603–608.

Pintó, X. et al. 2019. A Mediterranean Diet Rich in Extra-Virgin Olive Oil Is Associated with a Reduced Prevalence of Nonalcoholic Fatty Liver Disease in Older Individuals at High Cardiovascular Risk. *The Journal of Nutrition* 149(11), pp. 1920–1929.

Ploegh, H.L. 2007. A lipid-based model for the creation of an escape hatch from the endoplasmic reticulum. *Nature* 448(7152), pp. 435–438.

Polaskova, V., Kapur, A., Khan, A., Molloy, M. P. & Baker, M. S. (2010) High-abundance Protein Depletion: Comparison of Methods for Human Plasma Biomarker Discovery. *ELECTROPHORESIS*, 31 (3), pp. 471–482.

Pouwels, S. et al. 2022. Non-alcoholic fatty liver disease (NAFLD): a review of pathophysiology, clinical management and effects of weight loss. *BMC Endocrine Disorders* 22(1), p. 63.

Prabha, M., Ravi, V. and Ramachandra Swamy, N. 2013. Activity of Hydrolytic Enzymes in Various Regions of Normal Human Brain Tissue. *Indian Journal of Clinical Biochemistry* 28(3), pp. 283–291.

Promrat, K. et al. 2010. Randomized controlled trial testing the effects of weight loss on nonalcoholic steatohepatitis. *Hepatology* (Baltimore, Md.) 51(1), pp. 121–129.

Proteintech n.d., Blog, accessed 11 December 2023, <
<https://www.ptglab.com/news/blog/advantages-and-limitations-of-different-antibody-formats-in-immunoprecipitation/>>.

Quinlivan, V. H. & Farber, S. A. (2017) Lipid Uptake, Metabolism, and Transport in the Larval Zebrafish. *Frontiers in Endocrinology*, 8, p. 319.

Rahman, M.S. et al. 2021. Role of Insulin in Health and Disease: An Update. *International Journal of Molecular Sciences* 22(12), p. 6403.

Raj, H., Durgia, H., Palui, R., Kamalanathan, S., Selvarajan, S., Kar, S. S. & Sahoo, J. (2019) SGLT-2 Inhibitors in Non-Alcoholic Fatty Liver Disease Patients with Type 2 Diabetes Mellitus: A Systematic Review. *World Journal of Diabetes*, 10 (2), pp. 114–132.

Rao, S. G., Janiszewski, M. M., Duca, E., Nelson, B., Abhinav, K., Panagakou, I., Vass, S. & Heck, M. M. S. (2015) Invadolysin Acts Genetically via the SAGA Complex to Modulate Chromosome Structure. *Nucleic Acids Research*, 43 (7), pp. 3546–3562.

Raposo, G. & Stoorvogel, W. (2013) Extracellular Vesicles: Exosomes, Microvesicles, and Friends. *The Journal of Cell Biology*, 200 (4), pp. 373–383.

Rawlings, N. D. & Barrett, A. J. (1993) Evolutionary Families of Peptidases. *Biochemical Journal*, 290 (1), pp. 205–218.

Rawlings, N.D., Barrett, A.J., Thomas, P.D., Huang, X., Bateman, A. and Finn, R.D. 2018. The MEROPS database of proteolytic enzymes, their substrates and inhibitors in 2017 and a comparison with peptidases in the PANTHER database. *Nucleic Acids Research* 46(D1), pp. D624–D632.

Rey Vázquez, G. and Guerrero, G.A. 2007. Characterization of blood cells and hematological parameters in *Cichlasoma dimerus* (Teleostei, Perciformes). *Tissue and Cell* 39(3), pp. 151–160.

Richard, A.J., White, U., Elks, C.M. and Stephens, J.M. 2000. Adipose Tissue: Physiology to Metabolic Dysfunction. In: Feingold, K. R. et al. eds. *Endotext*. South Dartmouth (MA): MDTText.com, Inc.

Richeldi, L., Collard, H.R. and Jones, M.G. 2017. Idiopathic pulmonary fibrosis. *Lancet* (London, England) 389(10082), pp. 1941–1952.

Robertson, P.K.J. et al. 2009. A new generation of biocides for control of crustacea in fish farms. *Journal of Photochemistry and Photobiology B: Biology* 95(1), pp. 58–63.

Robbins, P. D. & Morelli, A. E. (2014) Regulation of Immune Responses by Extracellular Vesicles. *Nature Reviews Immunology*, 14 (3), pp. 195–208.

Roche, H. and Bogé, G. 1996. Fish blood parameters as a potential tool for identification of stress caused by environmental factors and chemical intoxication. *Marine Environmental Research* 41(1), pp. 27–43.

Rogers, L.D. and Overall, C.M. 2013. Proteolytic post-translational modification of proteins: proteomic tools and methodology. *Molecular & cellular proteomics: MCP* 12(12), pp. 3532–3542.

Romero-Gómez, M., Zelber-Sagi, S. and Trenell, M. 2017. Treatment of NAFLD with diet, physical activity and exercise. *Journal of Hepatology* 67(4), pp. 829–846.

Rosas, I. O., Richards, T. J., Konishi, K., Zhang, Y., Gibson, K., Lokshin, A. E., Lindell, K. O., Cisneros, J., MacDonald, S. D., Pardo, A., Sciurba, F., Dauber, J., Selman, M., Gochuico, B. R. & Kaminski, N. (2008) MMP1 and MMP7 as Potential Peripheral Blood Biomarkers in Idiopathic Pulmonary Fibrosis. *PLoS Medicine*, 5 (4), p. e93.

Roy, R., Yang, J. & Moses, M. A. (2009) Matrix Metalloproteinases As Novel Biomarkers and Potential Therapeutic Targets in Human Cancer. *Journal of Clinical Oncology*, 27 (31), pp. 5287–5297.

Rozario, T. & DeSimone, D. W. (2010) The Extracellular Matrix in Development and Morphogenesis: A Dynamic View. *Developmental Biology*, 341 (1), pp. 126–140.

Sagardia, L. M., Taylor, B. E. & Mehta, I. (2021) Idiopathic Interstitial Pneumonias: A Review of the Past and Emerging Therapies. *Journal of Pharmacy Practice*, p. 089719002110532.

Sant, K. E. & Timme-Laragy, A. R. (2018) Zebrafish as a Model for Toxicological Perturbation of Yolk and Nutrition in the Early Embryo. *Current Environmental Health Reports*, 5 (1), pp. 125–133.

Santos, A. L. S., Branquinha, M. H. & D'Avila-Levy, C. M. (2006) The Ubiquitous Gp63-like Metalloprotease from Lower Trypanosomatids: In the Search for a Function. *Anais da Academia Brasileira de Ciências*, 78 (4), pp. 687–714.

Sarker, H., Haimour, A., Toor, R. & Fernandez-Patron, C. (2021) The Emerging Role of Epigenetic Mechanisms in the Causation of Aberrant MMP Activity during Human Pathologies and the Use of Medicinal Drugs. *Biomolecules*, 11 (4), p. 578.

SARSTEDT n.d., Manuals, accessed 27 October 2022, <
<https://www.sarstedt.com/en/download/manuals/>>.

Schlagenhauf, E., Etges, R. & Metcalf, P. (1998) The Crystal Structure of the Leishmania Major Surface Proteinase Leishmanolysin (Gp63). *Structure*, 6 (8), pp. 1035–1046.

Schneider, F. et al. 2017. Diffusion of lipids and GPI-anchored proteins in actin-free plasma membrane vesicles measured by STED-FCS. *Molecular Biology of the Cell* 28(11), pp. 1507–1518.

Seidah, N.G. and Prat, A. 2012. The biology and therapeutic targeting of the proprotein convertases. *Nature Reviews Drug Discovery* 11(5), pp. 367–383.

Shaik, A. A., Wee, S., Li, R. H. X., Li, Z., Carney, T. J., Mathavan, S. & Gunaratne, J. (2014) Functional Mapping of the Zebrafish Early Embryo Proteome and Transcriptome. *Journal of Proteome Research*, 13 (12), pp. 5536–5550.

Shearn, A. & Garen, A. (1974) Genetic Control of Imaginal Disc Development in *Drosophila*. *Proceedings of the National Academy of Sciences*, 71 (4), pp. 1393–1397.

Shearn, A., Rice, T., Garen, A. & Gehring, W. (1971) Imaginal Disc Abnormalities in Lethal Mutants of *Drosophila*. *Proceedings of the National Academy of Sciences*, 68 (10), pp. 2594–2598.

Shimoda, M. & Khokha, R. (2017) Metalloproteinases in Extracellular Vesicles. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, 1864 (11), pp. 1989–2000.

Silbereis, J. C., Pochareddy, S., Zhu, Y., Li, M. & Sestan, N. (2016) The Cellular and Molecular Landscapes of the Developing Human Central Nervous System. *Neuron*, 89 (2), pp. 248–268.

Sly, P.D. et al. 2013. Risk Factors for Bronchiectasis in Children with Cystic Fibrosis. *New England Journal of Medicine* 368(21), pp. 1963–1970.

Soret, P.-A., Magusto, J., Housset, C. & Gautheron, J. (2020) In Vitro and In Vivo Models of Non-Alcoholic Fatty Liver Disease: A Critical Appraisal. *Journal of Clinical Medicine*, 10 (1), p. 36.

Sotelo-Orozco, J., Chen, S.-Y., Hertz-Picciotto, I. & Slupsky, C. M. (2021) A Comparison of Serum and Plasma Blood Collection Tubes for the Integration of Epidemiological and Metabolomics Data. *Frontiers in Molecular Biosciences*, 8, p. 682134.

Spiro, R. G. (2002) Protein Glycosylation: Nature, Distribution, Enzymatic Formation, and Disease Implications of Glycopeptide Bonds. *Glycobiology*, 12 (4), pp. 43R-56R.

Springman, E.B., Angleton, E.L., Birkedal-Hansen, H. and Van Wart, H.E. 1990. Multiple modes of activation of latent human fibroblast collagenase: evidence for the role of a Cys73 active-site zinc complex in latency and a 'cysteine switch' mechanism for activation. *Proceedings of the National Academy of Sciences of the United States of America* 87(1), pp. 364–368.

Srinivas, A. N., Suresh, D., Santhekadur, P. K., Suvarna, D. & Kumar, D. P. (2021) Extracellular Vesicles as Inflammatory Drivers in NAFLD. *Frontiers in Immunology*, 11, p. 627424.

Steel, L. F., Trotter, M. G., Nakajima, P. B., Mattu, T. S., Gonye, G. & Block, T. (2003) Efficient and Specific Removal of Albumin from Human Serum Samples*. *Molecular & Cellular Proteomics*, 2 (4), pp. 262–270.

STRING 12.0, STRING website, Swiss Institute of Bioinformatics, Novo Nordisk Foundation Center Protein Research and European Molecular Biology Laboratory, accessed 2 December 2023, < <https://string-db.org/> >.

Szklarczyk, D., Gable, A. L., Lyon, D., Junge, A., Wyder, S., Huerta-Cepas, J., Simonovic, M., Doncheva, N. T., Morris, J. H., Bork, P., Jensen, L. J. & von Mering, C. (2018) STRING V11: Protein–Protein Association Networks with Increased Coverage, Supporting Functional Discovery in Genome-Wide Experimental Datasets. *Nucleic Acids Research*, 47 (Database issue), p. gky1131.

Szklarczyk, D., Gable, A. L., Nastou, K. C., Lyon, D., Kirsch, R., Pyysalo, S., Doncheva, N. T., Legeay, M., Fang, T., Bork, P., Jensen, L. J. & von Mering, C. (2020) The STRING Database in 2021: Customizable Protein–Protein Networks, and Functional Characterization of User-Uploaded Gene/Measurement Sets. *Nucleic Acids Research*, 49 (D1), pp. gkaa1074-.

Szklarczyk, D., Kirsch, R., Koutrouli, M., Nastou, K., Mehryary, F., Hachilif, R., Gable, A. L., Fang, T., Doncheva, N. T., Pyysalo, S., Bork, P., Jensen, L. J. & Mering, C. von (2022) The STRING Database in 2023: Protein–Protein Association Networks and Functional Enrichment Analyses for Any Sequenced Genome of Interest. *Nucleic Acids Research*, 51 (D1), pp. D638–D646.

Takanaga, H., Chaudhuri, B. & Frommer, W. B. (2008) GLUT1 and GLUT9 as Major Contributors to Glucose Influx in HepG2 Cells Identified by a High Sensitivity Intramolecular FRET Glucose Sensor. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 1778 (4), pp. 1091–1099.

Teerlink, T., Nijveldt, R. J., Jong, S. de & Leeuwen, P. A. M. van (2002) Determination of Arginine, Asymmetric Dimethylarginine, and Symmetric Dimethylarginine in Human Plasma and Other Biological Samples by High-Performance Liquid Chromatography. *Analytical Biochemistry*, 303 (2), pp. 131–137.

Thomas, E. T., Guppy, M., Straus, S. E., Bell, K. J. L. & Glasziou, P. (2019) Rate of Normal Lung Function Decline in Ageing Adults: A Systematic Review of Prospective Cohort Studies. *BMJ Open*, 9 (6), p. e028150.

Thomas, G. 2002. Furin at the cutting edge: From protein traffic to embryogenesis and disease. *Nature Reviews Molecular Cell Biology* 3(10), pp. 753–766.

Thomas, N. V. & Kim, S.-K. (2010) Metalloproteinase Inhibitors: Status and Scope from Marine Organisms. *Biochemistry Research International*, 2010, p. 845975.

Thomas, R. J. (1968) Yolk Distribution and Utilization during Early Development of a Teleost Embryo (Brachydanio Rerio). *Journal of embryology and experimental morphology*, 19 (2), pp. 203–215.

Tirumalai, R. S., Chan, K. C., Prieto, D. A., Issaq, H. J., Conrads, T. P. & Veenstra, T. D. (2003) Characterization of the Low Molecular Weight Human Serum Proteome**S. Molecular & Cellular Proteomics*, 2 (10), pp. 1096–1103.

Todd, J. L., Neely, M. L., Overton, R., Durham, K., Gulati, M., Huang, H., Roman, J., Newby, L. K., Flaherty, K. R., Vinisko, R., Liu, Y., Roy, J., Schmid, R., Strobel, B., Hesslinger, C., Leonard, T. B., Noth, I., Belperio, J. A., Palmer, S. M., Asi, W., Baker, A., Beegle, S., Belperio, J. A., Condos, R., Cordova, F., Culver, D. A., Andrade, J. A. M. de, Dilling, D., Flaherty, K. R., Glassberg, M., Gulati, M., Guntupalli, K., Gupta, N., Case, A. H., Hotchkin, D., Huie, T., Kaner, R., Kim, H., Kreider, M., Lancaster, L., Lasky, J., Lederer, D., Lee, D., Liesching, T., Lipchik, R., Lobo, J., Mageto, Y., Menon, P., Morrison, L., Namen, A., Oldham, J., Raj, R., Ramaswamy, M., Russell, T., Sachs, P., Safdar, Z., Sigal, B., Silhan, L., Strek, M., Suliman, S., Tabak, J., Walia, R. & Whelan, T. P. (2019) Peripheral Blood Proteomic Profiling of Idiopathic Pulmonary Fibrosis Biomarkers in the Multicentre IPF-PRO Registry. *Respiratory Research*, 20 (1), p. 227.

Torraca, V., Gomes, M.C., Sarris, M. and Mostowy, S. 2019. Meeting report: Zebrafish Infection and Immunity 2019. *Lab Animal* 48(10), pp. 284–287.

Toyoda, H., Kumada, T., Kiriya, S., Tanikawa, M., Hisanaga, Y., Kanamori, A., Tada, T. & Murakami, Y. (2013) Higher Hepatic Gene Expression and Serum Levels of Matrix Metalloproteinase-2 Are Associated with Steatohepatitis in Non-Alcoholic Fatty Liver Diseases. *Biomarkers*, 18 (1), pp. 82–87.

Turk, B., Turk, D. and Turk, V. 2012. Protease signalling: the cutting edge: Protease signalling. *The EMBO Journal* 31(7), pp. 1630–1643.

Uniprot, UniprotKB, accessed 12 December 2023, <<https://www.uniprot.org/>>.

Vallon, V. & Thomson, S. C. (2017) Targeting Renal Glucose Reabsorption to Treat Hyperglycaemia: The Pleiotropic Effects of SGLT2 Inhibition. *Diabetologia*, 60 (2), pp. 215–225.

Vass, S. & Heck, M. M. S. (2013) Perturbation of Invadolysin Disrupts Cell Migration in Zebrafish (*Danio Rerio*). *Experimental Cell Research*, 319 (8),

pp. 1198–1212.

Van Wart, H.E. and Birkedal-Hansen, H. 1990. The cysteine switch: a principle of regulation of metalloproteinase activity with potential applicability to the entire matrix metalloproteinase gene family. *Proceedings of the National Academy of Sciences* 87(14), pp. 5578–5582.

Verweij, F.J. et al. 2021. The power of imaging to understand extracellular vesicle biology in vivo. *Nature Methods* 18(9), pp. 1013–1026.

Vilella, A.J., Severin, J., Ureta-Vidal, A., Heng, L., Durbin, R. and Birney, E. 2009. EnsemblCompara GeneTrees: Complete, duplication-aware phylogenetic trees in vertebrates. *Genome Research* 19(2), pp. 327–335.

Vliegenthart, A. D. B., Lewis, P. S., Tucker, C. S., Pozo, J. D., Rider, S., Antoine, D. J., Dubost, V., Westphal, M., Moulin, P., Bailey, M. A., Moggs, J. G., Goldring, C. E., Park, B. K. & Dear, J. W. (2014) Retro-Orbital Blood Acquisition Facilitates Circulating MicroRNA Measurement in Zebrafish with Paracetamol Hepatotoxicity. *Zebrafish*, 11 (3), pp. 219–226.

Voynow, J.A., Fischer, B.M. and Zheng, S. 2008. Proteases and cystic fibrosis. *The International Journal of Biochemistry & Cell Biology* 40(6–7), pp. 1238–1245.

Wang, H., Li, H., Yan, Q., Gao, S., Gao, J., Wang, Z. & Sun, Y. (2021) Serum Matrix Metalloproteinase-13 as a Diagnostic Biomarker for Cutaneous Squamous Cell Carcinoma. *BMC Cancer*, 21 (1), p. 816.

Wang, X., Schmidt, D. R., Joyce, E. J. & Kao, W. J. (2011) Application of MS-Based Proteomics to Study Serum Protein Adsorption/Absorption and Complement C3 Activation on Poly(Ethylene Glycol) Hydrogels. *Journal of Biomaterials Science, Polymer Edition*, 22 (10), pp. 1343–1362.

Wart, H. E. V. & Birkedal-Hansen, H. (1990) The Cysteine Switch: A Principle of Regulation of Metalloproteinase Activity with Potential Applicability to the Entire Matrix Metalloproteinase Gene Family. *Proceedings of the National*

Academy of Sciences, 87 (14), pp. 5578–5582.

Wielockx, B., Lannoy, K., Shapiro, S. D., Itoh, T., Itohara, S., Vandekerckhove, J. & Libert, C. (2001) Inhibition of Matrix Metalloproteinases Blocks Lethal Hepatitis and Apoptosis Induced by Tumor Necrosis Factor and Allows Safe Antitumor Therapy. *Nature Medicine*, 7 (11), pp. 1202–1208.

Wilcox, G. 2005. Insulin and insulin resistance. *The Clinical Biochemist. Reviews* 26(2), pp. 19–39.

Williams, K.H., Shackel, N.A., Gorrell, M.D., McLennan, S.V. and Twigg, S.M. 2013. Diabetes and Nonalcoholic Fatty Liver Disease: A Pathogenic Duo. *Endocrine Reviews* 34(1), pp. 84–129.

Williams, S.Y. and Renquist, B.J. 2016. High Throughput Danio Rerio Energy Expenditure Assay. *Journal of Visualized Experiments: JoVE* (107), p. e53297.

Wong, T.-S., Gao, W. & Li, Z.-H. (2015) Biomarkers in Cancer. *Biomarkers in Disease: Methods, Discoveries and Applications*, pp. 771–790.

Wu, D., Zhu, H. & Wang, H. (2021) Extracellular Vesicles in Non-Alcoholic Fatty Liver Disease and Alcoholic Liver Disease. *Frontiers in Physiology*, 12, p. 707429.

Wuyts, W. A., Wijsenbeek, M., Bondue, B., Bouros, D., Bresser, P., Cordeiro, C. R., Hilberg, O., Magnusson, J., Manali, E. D., Morais, A., Papiris, S., Shaker, S., Veltkamp, M. & Bendstrup, E. (2020) Idiopathic Pulmonary Fibrosis: Best Practice in Monitoring and Managing a Relentless Fibrotic Disease. *Respiration*, 99 (1), pp. 73–82.

Wyatt, G. R., Loughheed, T. C. & Wyatt, S. S. (1956) THE CHEMISTRY OF INSECT HEMOLYMPH. *The Journal of General Physiology*, 39 (6), pp. 853–868.

Xiang, P., Yeung, Y. T., Wang, J., Wu, Q., Du, R., Huang, C., Jia, X., Gao, Y., Zhi, Y., Guo, F., Wei, H., Zhang, D., Liu, Y., Liu, L., Liang, L., Wang, J., Song, Y., Liu, K. & Fang, B. (2021) MiR-17-3p Promotes the Proliferation of Multiple Myeloma Cells by Downregulating P21 Expression through LMLN Inhibition. *International Journal of Cancer*, 148 (12), pp. 3071–3085.

Xiao, Q., Zhang, S., Yang, C., Du, R., Zhao, J., Li, J., Xu, Y., Qin, Y., Gao, Y. & Huang, W. (2019) Ginsenoside Rg1 Ameliorates Palmitic Acid-Induced Hepatic Steatosis and Inflammation in HepG2 Cells via the AMPK/NF-KB Pathway. *International Journal of Endocrinology*, 2019, p. 7514802.

Xu, H., Zhou, Y., Liu, Y., Ping, J., Shou, Q., Chen, F. & Ruo, R. (2016) Metformin Improves Hepatic IRS2/PI3K/Akt Signaling in Insulin-Resistant Rats of NASH and Cirrhosis. *Journal of Endocrinology*, 229 (2), pp. 133–144.

Yao, C., Donelson, J. E. & Wilson, M. E. (2003) The Major Surface Protease (MSP or GP63) of *Leishmania* Sp. Biosynthesis, Regulation of Expression, and Function. *Molecular and Biochemical Parasitology*, 132 (1), pp. 1–16.

Yao, C., Donelson, J. E. & Wilson, M. E. (2007) Internal and Surface-Localized Major Surface Proteases of *Leishmania* Spp. and Their Differential Release from Promastigotes. *Eukaryotic Cell*, 6 (10), pp. 1905–1912.

Yeung, E. N. W., Treskes, P., Martin, S. F., Manning, J. R., Dunbar, D. R., Rogers, S. M., Bihan, T. L., Lockman, K. A., Morley, S. D., Hayes, P. C., Nelson, L. J. & Plevris, J. N. (2015) Fibrinogen Production Is Enhanced in an In-Vitro Model of Non-Alcoholic Fatty Liver Disease: An Isolated Risk Factor for Cardiovascular Events? *Lipids in Health and Disease*, 14 (1), p. 86.

Yu, G., Tzouvelekis, A., Wang, R., Herazo-Maya, J. D., Ibarra, G. H., Srivastava, A., Castro, J. P. W. de, Deluliis, G., Ahangari, F., Woolard, T., Aurelien, N., Drigo, R. A. e, Gan, Y., Graham, M., Liu, X., Homer, R. J., Scanlan, T. S., Mannam, P., Lee, P. J., Herzog, E. L., Bianco, A. C. & Kaminski, N. (2018) Thyroid Hormone Inhibits Lung Fibrosis in Mice by Improving Epithelial Mitochondrial Function. *Nature Medicine*, 24 (1), pp. 39–49.

Yu, Q. and Stamenkovic, I. 2000. Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor

invasion and angiogenesis. *Genes & Development* 14(2), pp. 163–176.

Yu, Z., Kastenmüller, G., He, Y., Belcredi, P., Möller, G., Prehn, C., Mendes, J., Wahl, S., Roemisch-Margl, W., Ceglarek, U., Polonikov, A., Dahmen, N., Prokisch, H., Xie, L., Li, Y., Wichmann, H.-E., Peters, A., Kronenberg, F., Suhre, K., Adamski, J., Illig, T. & Wang-Sattler, R. (2011) Differences between Human Plasma and Serum Metabolite Profiles. *PLoS ONE*, 6 (7), p. e21230.

Zhang, E., Zhao, Y. & Hu, H. (2021) Impact of Sodium Glucose Cotransporter 2 Inhibitors on Nonalcoholic Fatty Liver Disease Complicated by Diabetes Mellitus. *Hepatology Communications*, 5 (5), pp. 736–748.

Zhou, J., Zhu, J., Yu, S.-J., Ma, H.-L., Chen, J., Ding, X.-F., Chen, G., Liang, Y. & Zhang, Q. (2020) Sodium-Glucose Co-Transporter-2 (SGLT-2) Inhibition Reduces Glucose Uptake to Induce Breast Cancer Cell Growth Arrest through AMPK/MTOR Pathway. *Biomedicine & Pharmacotherapy*, 132, p. 110821.

Zhou, M., Conrads, T. P. & Veenstra, T. D. (2005) Proteomics Approaches to Biomarker Detection. *Briefings in Functional Genomics*, 4 (1), pp. 69–75.

Zhou, Y., Horowitz, J. C., Naba, A., Ambalavanan, N., Atabai, K., Balestrini, J., Bitterman, P. B., Corley, R. A., Ding, B.-S., Engler, A. J., Hansen, K. C., Hagood, J. S., Kheradmand, F., Lin, Q. S., Neptune, E., Niklason, L., Ortiz, L. A., Parks, W. C., Tschumperlin, D. J., White, E. S., Chapman, H. A. & Thannickal, V. J. (2018) Extracellular Matrix in Lung Development, Homeostasis and Disease. *Matrix Biology*, 73, pp. 77–104.

Zu, X. L. & Guppy, M. (2004) Cancer Metabolism: Facts, Fantasy, and Fiction. *Biochemical and Biophysical Research Communications*, 313 (3), pp. 459–465.

Zuo, F., Kaminski, N., Eugui, E., Allard, J., Yakhini, Z., Ben-Dor, A., Lollini, L., Morris, D., Kim, Y., DeLustro, B., Sheppard, D., Pardo, A., Selman, M. & Heller, R. A. (2002) Gene Expression Analysis Reveals Matrilysin as a Key Regulator of Pulmonary Fibrosis in Mice and Humans. *Proceedings of the National Academy of Sciences*, 99 (9), pp. 6292–6297.