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The Role of Coagulation Factor V in Breast Cancer

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Biotechnology

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Sammendrag

I tillegg til den velkjente sammenhengen mellom kreft og trombotiske sykdommer, er økt koagulasjonsaktivitet kjent for å være forbundet med tumorprogresjon. Å studere de underliggende molekylære mekanismene kan føre til en bedre forståelse av sammenhengen mellom kreft og hemostase, og muligens føre til en mer individualisert behandling for pasienter som lider av kreft eller kreft-relatert trombose. Rollen til koagulasjonsfaktor V (FV) i kreft er ikke nøye studert, men FV er kjent for å uttrykke både prokoagulante og antikoagulante egenskaper som bidrar til en balanse i koagulasjonssystemet. Dessuten er det rapportert en assosiasjon mellom flere *F5* SNPs og risiko for brystkreft. Målet med denne avhandlingen var å få en bedre forståelse av hvordan FV er relatert til brystkreft, studert ved hjelp av både et klinisk brystkreft materiale og *in vitro* brystkreft celler.

Den kliniske betydningen av FV ble undersøkt i et sammenslått brystkreft datasett (n = 1881, fra GOBO) og i pasienter fra OsloII studien (FV plasma protein nivåer fra n = 366 og FV tumor mRNA nivåer fra n = 152). En FV overekspresjonscellemodell ble benyttet for å studere FV villtype og FV genvariantene rs6025 (FV Leiden), rs6028 og en ikke tidligere rapportert mutasjon (A2184T) i brystkreftcellelinjen MDA-MB-231, og deres effekt på genekspresjon, proteinsekresjon, samt funksjonelle virkninger på cellevekst, celledød og inflammatoriske markører.

Økt FV mRNA ekspresjon ble observert hos pasienter med tumorer med aggressive karakteristika; ER-negative tumorer, HER2-enriched tumorer, basal-lignende tumorer, og tumorer av høy grad. Blant pasienter med disse karakteristika ble høyt FV mRNA uttrykk forbundet med økt overlevelse. *F5* ble funnet assosiert direkte eller indirekte, til celleproliferasjon, celledifferensiering og immunrespons. Gjennom *in vitro* studiene fant vi at FV wt viste høyt FV overutrykk, redusert cellevekst, økt apoptose men redusert nekrose, og forhøyede nivåer av proinflammatoriske cytokiner. FV variantene viste varierende forskjeller for FV wt, med økning i cellevekst, uforandret celledød og økning i inflammatoriske markører. FV rs6028 avvikte med økt cellevekst i forhold til de andre

FV variantene, og FV A2184T avvikte med økte nivåer av celledød og pro-inflammatoriske markører i forhold til de andre FV variantene.

For å konkludere tyder resultatene i denne avhandlingen på at *F5* har tumor-suppressor-gen-egenskaper sett av økt overlevelse hos pasienter med økte FV nivåer. *F5* kan være et godt kandidat gen for nye behandlingsmetoder for pasienter som lider av brystkreft eller kreft-relatert trombose. Genetisk variasjon i *F5* påvirker suppressor-gen-effekten, hovedsakelig på grunn av reduserte FV nivåer. I tillegg ble det antatt en link til endret biologisk funksjon forårsaket av A2184T.

Abstract

In addition to the well-known link between cancer and risk of thrombotic diseases, it is known that increased coagulation activity is associated with tumor progression. Studying the underlying molecular mechanisms could lead to a better understanding of the association between cancer and hemostasis, and possibly lead to a more individualized treatment for patients suffering cancer or cancer-related thrombosis. The role of coagulation factor V (FV) in cancer has not been studied extensively, but FV is known to express both procoagulant and anticoagulant properties contributing to a balance in the coagulation system. Also, several *F5* SNPs are reported to be associated with risk of breast cancer. The aim of this thesis was to gain a better understanding of how coagulation FV relates to breast cancer, using both a clinical breast cancer material and *in vitro* breast cancer cells.

The clinical significance of FV was studied in a merged breast cancer dataset (n=1881, from GOBO) and in patients from the OsloII study (FV plasma protein levels from n=366 and FV tumor mRNA levels from n=152). A FV overexpression cell model was used to study FV wild type and the FV gene variants rs6025 (FV Leiden), rs6028 and a novel mutation (A2184T) in the MDA-MB-231 breast cancer cell line, and their effects on gene expression, protein secretion as well as functional effects on cell growth, cell death and inflammatory markers.

Increased FV mRNA expression was observed in patients with more aggressive tumor subtypes; ER-negative, HER2-enriched, basal-like, and high-grade tumors. Interestingly, within these patient subgroups, high FV mRNA expression was associated with increased survival rates. *F5* was found associated, directly or indirectly, to cell proliferation, differentiation and immune response. Through the *in vitro* studies we found that FV wt was highly overexpressed, showed reduced cell growth, increased apoptosis but decreased necrosis and elevated levels of pro-inflammatory cytokines. The FV variants showed varying differences to FV wt, with increase in cell growth, unchanged cell death and increase in inflammatory markers. Deviating, FV rs6028

showed a more elevated increase in cell growth than the other FV variants, and FV A2184T highly increased levels of cell death and pro-inflammatory markers.

In conclusion, the results of this thesis indicate that *F5* acts as a suppressor gene, showing increased survival in patients with increased levels. *F5* may be a good candidate gene for new treatment procedures for patients suffering breast cancer or cancer-related thrombosis. Genetic variation in *F5* does affect the suppressor effect, mostly due to reduced levels of FV. Nevertheless, a link to altered biological function caused by the A2184T was assumed.

Abbreviations

aPC: activated protein C
aPCR: activated protein C resistance
AT: antithrombin
bp: base pair
cDNA: complementary DNA
DAVID: the Database for Annotation, Visualization and Integrated Discovery
ddNTP: dideoxyribonucleotide
DMFS: Distant metastasis-free survival
DNA: deoxyribonucleic acid
dNTP: deoxyribonucleotide
E.coli: Escherichia coli
ELISA: Enzyme linked immunosorbent assay
EPCR: endothelial protein C receptor
ER: Estrogen receptor
F: Factor
FV: factor V
FVa: activated factor V
GO: gene ontology
GOBO: Gene Expression-Based Outcome for Breast Cancer Online
GSA: Gene Set Analysis
HER2: Human epidermal growth factor receptor 2
HR: hormone receptor
IHC: immunohistochemistry
IL: interleukin
ISH: in situ hybridization
KEGG: Kyoto Encyclopedia of Genes and Genomes
LD: linkage disequilibrium
LN: lymph node
MAC: macrophage

MAF: minor allele frequency

MAPK: mitogen-activated protein kinase

mRNA: messenger ribonucleic acid

NCBI: National Center for Biotechnology Information

NTC: non-template control

OS: Overall survival

PAM50: Prediction analysis of microarray 50

PI3K: phosphoinositide 3-kinase

PR: Progesterone receptor

pRb: retinoblastoma protein

qRT-PCR: quantitative reverse transcriptase polymerase chain reaction

RefSeq: The Reference Sequence

RFS: Relapse-free survival

RNA: ribonucleic acid

SD: standard deviation

SEM: standard error of the mean

SNP: single-nucleotide polymorphism

SPSS: statistic package for the social sciences

TAM: tumor-associated macrophages

TF: tissue factor

TFPI: tissue factor pathway inhibitor

TLR: toll-like receptor

TM: thrombomodulin

TMB: tetramethylbenzidine

TNF: tumor necrosis factor

VEGF: vascular endothelial growth factor

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1. Introduction

1.1 Cancer

Cancers is a heterogeneous disease caused by mutations in the genome. Cancer cells derive from an apparent normal cell, which is exposed to mutations not corrected by the many control systems of the cell. If the cell does not self-destruct, a multistep process causing mutations in several genes may result in uncontrolled growth, and the formation of a cancer cell. The genes causing formation of cancerous cells are commonly divided into two classes: The proto-oncogenes, a result of gain-of-function mutations, and the tumor suppressor genes a result of loss-of-function mutations. From a cancer cell a malignant tumor may be formed. A malignant tumor may use the blood vessels and lymph system to invade new tissues, thus initiating metastasis (Alberts et al. 2015; Hanahan & Weinberg 2000; Parham 2015). Studies show that 5-10% of the cancer risk is caused by genetic factors, while the remaining 90-95% are caused by environmental- and life style factors (Alberts et al. 2015; Anand et al. 2008).

1.1.1 The hallmarks of cancer

According to Hanahan and Weinberg (2011) there are eight biological alterations and two enabling characteristics that are essential for tumor growth and progression (Figure 1). The biological alterations are:

- Sustaining proliferate signaling
- Evading growth suppressors
- Activating invasion and metastasis
- Enabling replicative immortality
- Inducing angiogenesis
- Resisting cell death
- Reprogramming of energy metabolism
- Evading immune destruction

The enabling characteristics are:

- Genome instability and mutation
- Tumor promoting inflammation

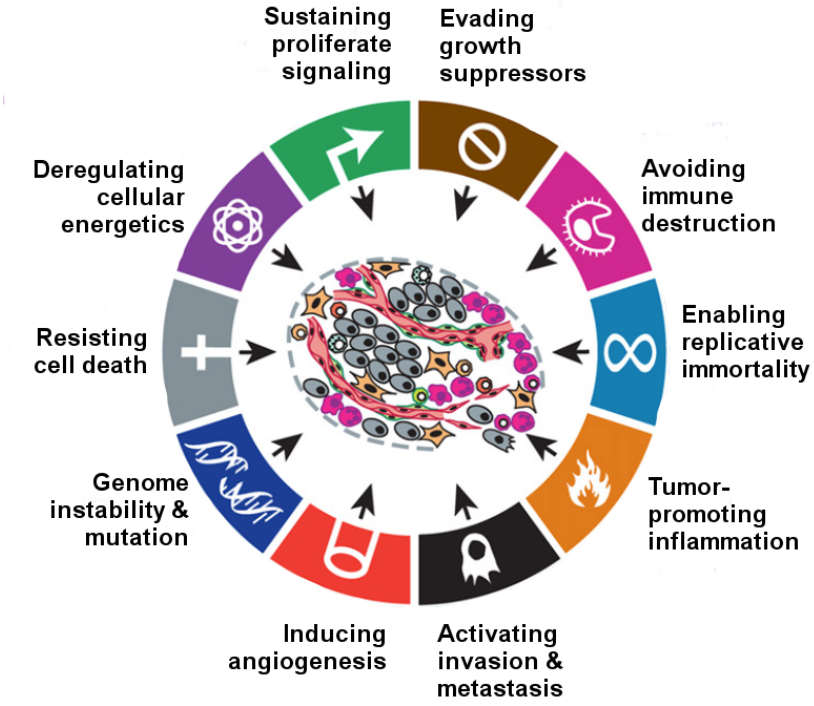

Figure 1: The biological alterations and enabling characteristics essential for tumor growth and progression (Hanahan & Weinberg 2011).

Sustaining proliferative signaling and evading growth suppressors

In a well-balanced homeostatic tissue environment, cells receive external signals controlling their pathways, leading to promotion or suppression of cell growth and division. However, cancer cells are found to have mechanisms controlling their pathways without external signals (Alberts et al. 2015). Commonly, cancer cells have the ability to produce their own growth signals, such as platelet-derived growth factor (PDGF) (Lokker et al. 2002), which can initiate proliferation signals through pathways such as mitogen-activated protein kinase (MAPK) and Akt/mTOR (Holland et al. 2000).

Enhanced proliferation can also be due to the evasion of growth suppressors. In many tumor cells the tumor suppressor gene *TP53* carries missense mutations. The task of p53, which is to detect DNA damage and arrest the cell in the cell cycle to prevent growth and division, may then be impaired (Levine 1997). Moreover, a loss-of-function mutation in the tumor suppressor gene retinoblastoma protein (pRb) may cause cells to enter the cell cycle unintentionally (Alberts et al. 2015).

Resisting cell death

Programmed cell death (apoptosis) is a normal and crucial event in all tissues eliminating any malfunctioning cells. A cell can receive apoptotic signals through both the intrinsic and extrinsic pathway (Elmore 2007; Fadeel & Orrenius 2005). During apoptosis a cell experience shrinkage with blebbing of the plasma membrane, and degradation of cytoskeletal and nuclear proteins. This causes fragmentation of the cell, producing smaller membrane-enclosed particles, known as apoptotic bodies. These particles will be recognized and engulfed by phagocytic cells, such as macrophages (Elmore 2007; Fadeel & Orrenius 2005; Kerr et al. 1972). Cancer cells may evolve the ability to evade stress stimuli that normally leads to apoptosis (Kerr et al. 1972; Levine 1997). Moreover, the p53 protein has an important role in the intrinsic pathway of apoptosis, but by inactivation of *TP53* the cell may evade apoptosis (Fadeel & Orrenius 2005).

Enabling replicative immortality

The ends of all chromosomes have protecting replicative ends, called telomeres. Due to the nature of DNA polymerase, the telomeres become shorter after each cell division. When the shortening extends into the coding area, cell death is induced (Cong et al. 2002; Lin & Elledge 2003; Zhang et al. 1999). By overexpression of the telomerase enzyme, that reverse transcribes the telomeres, the chromosomes of cancer cells will continuously be elongated. Thus the cell is not hindered to enter the cell cycle, and a phenomenon of replicative immortality has occurred (Lin & Elledge 2003).

Inducing angiogenesis

All cells need to be in close proximity of a blood vessel in order to get sufficient amounts of oxygen and nutrition, and to get rid of carbon dioxide and metabolic waste. As tumors grow the original vessels become insufficient for these exchanges, hence new blood vessels are needed (Hanahan & Folkman 1996). Through a process called angiogenesis, the endothelial cells of a blood vessel will migrate and proliferate into a protruding new vessel (Hanahan & Weinberg 2011). Angiogenesis is controlled by the so called angiogenic switch relying on expression of activators and inhibitors (Hanahan & Folkman 1996). Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) are two examples of activators of angiogenesis (Anan et al. 1996).

Activating invasion and metastasis

Metastasis is a multistep process where cancer cells from a primary tumor spread to a new site and form a secondary tumor. First, some cancer cells from the primary tumor enter the circulation system. The evading cancer cells may stick to the inside of a blood vessel and evade into new tissues where they can give rise to a secondary tumor (Alberts et al. 2015; Talmadge & Fidler 2010). When circulating in the lymphatic system an aggregate of cancer cells may attach in a lymph node and with the right conditions cause lymph node metastasis. Though several cancer cells may evade from the primary tumor and enter the circulation system only a few, if any, will lead to metastasis (Alberts et al. 2015). The endothelial cell-expressed protein E-cadherin is important in the suppression of invasion and metastasis, through its cell-to-cell interactions (Hanahan & Weinberg 2000). In fact, loss of function of E-cadherin proteins are found in the majority of cancers (Christofori & Semb 1999). Also integrins play an important role in invasion and metastasis. Changes in integrin expression may help the cancer cells adapt to the new environment and interact with substrates on extracellular matrix (Hanahan & Weinberg 2000; Lukashev & Werb 1998). Moreover, cancer cells may cause change in expression of proteases that assist in the breakdown and remodeling of extracellular matrix (Friedl & Wolf 2008).

Reprogramming of energy metabolism

Tumor cells proliferate with a higher rate than normal cells, hence they need higher levels of metabolites (and energy). Cancer cells have the ability to reprogram their metabolism, by for instance up-regulating the transport of glucose into the cytoplasm. This will favor glycolysis even under anaerobic conditions. This is a contradiction to normal cells that need aerobic conditions in order to limit oxidative phosphorylation after glycolysis (Jones & Thompson 2009).

Evasion of immune destruction

The task of the immune system is to protect against disease, thus it is evident that cancer cells somehow evade this system (Hanahan & Weinberg 2011). Though the mechanisms in which the cancer cells evade immune destruction is not fully understood, studies show that patients with colon and ovarian tumors that also have high levels of killer lymphocytes have better prognosis than those with lower levels (Pages et al. 2010). Moreover, studies by Sica and colleagues (2000) indicated that the reduced

ability of tumor-associated macrophages (TAMs) to generate interleukin (IL) 12 (IL-12), increase the ability of tumors to evade immune destruction.

The enabling characteristics: genome instability and mutations and tumor promoting inflammation

Genome instability is characterized by high frequency of alterations in the genome that by errors in the DNA repair systems leads to somatic mutations. Such genomic alterations may be changes in the nucleotide sequence such as deletion, insertion, substitution or inversion of a few bases, but can also be large rearrangements or deletions of chromosome segments or aneuploidy (abnormal number of chromosomes) (Langie et al. 2015; Shen 2011). During tumor transformation and progression these genetic changes can happen in different cell populations at different points in time, causing a heterogeneous background in cancer (Shen 2011).

Many types of cancer have been associated with inflammatory processes: For example there is a documented link between Crohn's disease and colorectal cancer (Eiró & Vizoso 2012). In fact, 15-25% of all cancer cases are associated with underlying infections and inflammatory reactions. When an inflammation is not terminated by the immune system, it can become chronic, change the cellular microenvironment and facilitate tumor transformation (Balkwill & Mantovani 2001; Eiró & Vizoso 2012). In addition to cancer cells themselves having the ability to produce cytokines and chemokines, inflammatory cells may secrete cytokines, growth factors, chemokines and proteases that induce proliferation and invasiveness of the cancer cell (Eiró & Vizoso 2012). In a balanced cellular microenvironment the toll-like receptors (TLRs) are a link between the innate and adaptive immunity that assist the immune system in defeating pathogens. Nevertheless, increased expression levels of TLRs have been found in human tumors (Eiró & Vizoso 2012).

1.2 Breast cancer

Breast cancer is one of the most common cancers in the world (Borg et al. 2011). In fact, it was the second most frequent diagnosed cancer in 2012 with 12% of all new cancer diagnoses, including incidences in both men and women (Ferlay et al. 2015). Considering women only, breast cancer was the most common cancer worldwide in 2012, with 25% of all incidences. Despite that, breast cancer is ranked 5th worldwide when it comes to mortality rate, highly due to good screening programs and good treatment procedures. That is probably the reason higher survival rates are reported in more developed regions (Ferlay et al. 2015).

1.2.1 Characterization of breast cancer

Breast cancer is a heterogeneous disease, with extensive variation in both molecular and clinical characteristics. Based on the physical characteristics of the tumors, they are commonly classified into subgroups according to lymph node (LN) status, tumor size and histological grade (low, medium and high). The molecular markers estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) are also important in the subgrouping of breast cancer (Parker et al. 2009). Immunohistochemistry (IHC) is commonly used to determine the status of these receptors, and is reported as either positive or negative (present or not present). HER2 status can also be detected by in situ hybridization (ISH) (Kittaneh et al. 2013). Positive status for ER and PR is found in about 80% of breast tumors. ER and PR are hormonal receptors, and tumors positive for both markers respond well to hormonal therapy. Nevertheless, about 40% of ER positive tumors are PR negative, and these tumors tend to have a poorer response to tamoxifen (hormone therapy) than tumors positive for both ER and PR, due to being more aggressive. Tumors that are negative for ER, PR, and HER2, called triple negative, constitute 10-15% of breast tumors, and tend to be very aggressive thus associated with a poor prognosis (Vuong et al. 2014).

Tumors may also be classified according to their gene expression patterns. The so-called molecular subtypes include basal-like, luminal A, luminal B, HER2 enriched and normal-like tumors (Parker et al. 2009). Tumors within a subtype tend to correspond to clinicopathological characteristics (Table 1). Tumors in the luminal A and luminal B

subtypes are most often both ER positive. Luminal A tumors are PR positive and HER2 negative, while luminal B can be PR negative or positive, as well as HER2 negative or positive (Sotiriou & Pusztai 2009; Vuong et al. 2014). Patients with luminal A tumors have better survival rates than the other subtypes (Vuong et al. 2014). Basal-like tumors tend to be triple negative (Sotiriou & Pusztai 2009). The basal-like tumors show high diversity, and are associated with aggressive clinical behavior. The HER2 enriched tumors are, as the name suggest, HER2 positive. Beyond that, there is an overlap between clinicopathological characterizations. Many HER2 enriched tumors are ER positive and fall into the luminal B subtype, while some HER2 enriched tumors are ER negative and fall into the basal-like subtype (Vuong et al. 2014). Moreover, HER2 enriched tumors tend to be PR negative (Kennecke et al. 2010). Tumors in the normal-like subtype are often associated with genes related to stromal cells, like adipose tissue, but by some normal-like tumors are not considered an intrinsic molecular subtype (Vuong et al. 2014).

Table 1: Immunohistochemical phenotype of molecularly defined breast cancer subtypes. Breast cancer subtypes (luminal A, luminal B, HER2 and basal-like) and presence/absence of breast cancer tumor characteristics.

Intrinsic molecular subtypes	**Histological grade**	**ER status**	**PR status**	**HER2 status**
Luminal A	G1	+	+	-
Luminal B	G3	+	+/-	+/-
HER2-enriched	G3	*	*	+
Basal-like	G3	-	-	-

1.3 Blood coagulation

1.3.1 Cell based model of blood coagulation

Blood coagulation has been described in multiple ways in literature (Davie & Ratnoff 1964; Macfarlane 1964). The cell based model of blood can be described as a cascade of reactions initiated by a rupture of the endothelial cell layer of a blood vessel and terminated by the formation of a fibrin mesh and a clot, thus preventing further blood loss. Many coagulation factors contribute to this process (Sjaastad et al. 2010; Versteeg et al. 2013).

After breakage upon a vessel, coagulation factor (F) VII, or activated FVII (FVIIa), found circulating in blood, binds to TF expressed on extravascular cells. TF supports activation of FVII to FVIIa, leading to further formation of TF-FVIIa complexes. The TF-FVIIa complexes also activate FIX and FX to FIXa and FXa, respectively. FXa form prothrombinase complexes with its cofactor FVa, resulting in the cleavage of prothrombin (FII) to thrombin (FIIa). FXa is inactivated when dissociated from TF, resulting in limited production of thrombin. The small amounts of thrombin are nevertheless able to activate FV to FVa, as well as FVIII and FXI to FVIIIa and FXIa, respectively. After generation of additional prothrombinase and intrinsic tenase (FIXa-FVIIIa) complexes additional thrombin is generated through a positive feedback-loop. Moreover, thrombin also activates platelets, resulting in a procoagulant surface of negatively charged phospholipids that creates an activation-spot for the coagulation factors. Binding of coagulation factors to a membrane surface enhances the enzymatic reactions of the coagulation cascade. Fibrinogen is cleaved into insoluble fibrin monomers that polymerize and form a mesh, where platelets and red blood cells get stuck to form a clot (Figure 2). FXIII, activated to FXIIIa by thrombin, assists in the stabilization of the mesh (not shown in figure) (Smith 2009; Versteeg et al. 2013). After the formation of a blood clot either of two processes may occur: Migration of connective tissue cells into the clot for gradually replacement of connective tissue, or dissolving of the clot by fibrinolysis (Sjaastad et al. 2010).

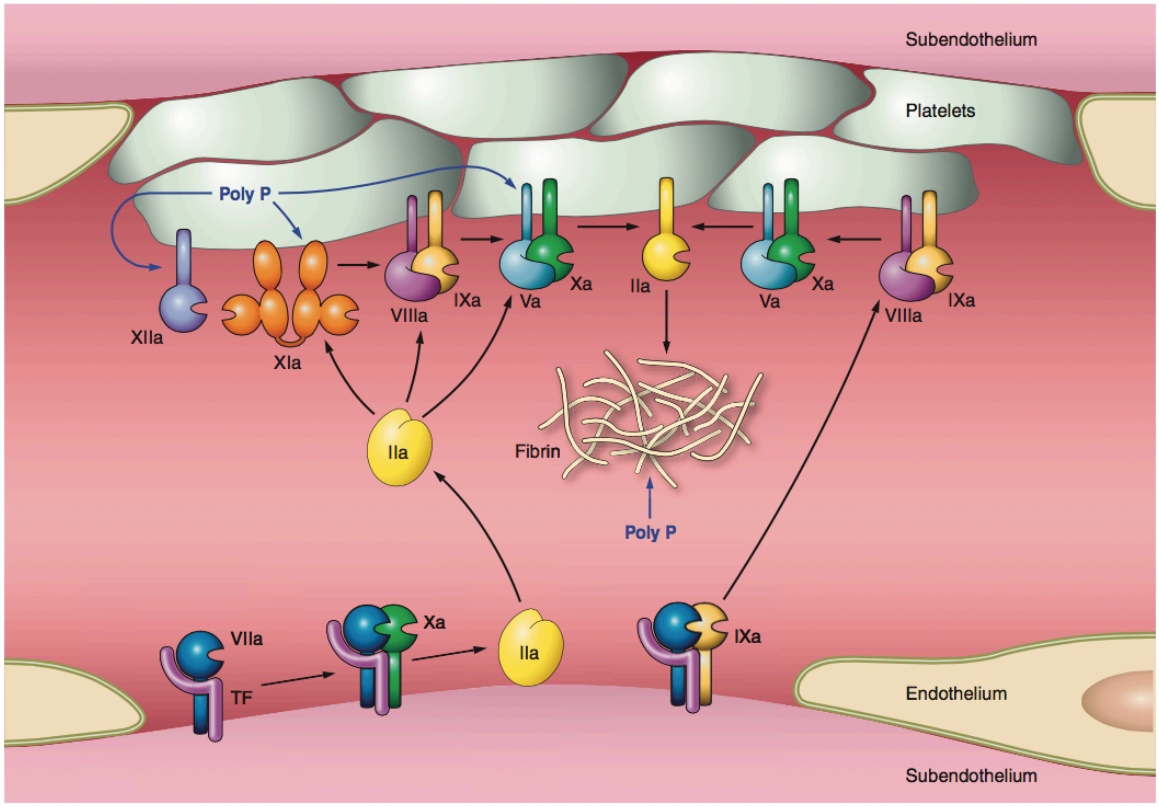

Figure 2: Cell based model of blood coagulation. After a rupture in the endothelial cell layer, FVIIa binds to TF expressed on extravascular cells forming the TF-FVIIa complex that activate FX and FIX to FIXa and FXa, respectively. The prothrombinase complex cleaves prothrombin to thrombin initiating the formation of a fibrin mesh preventing blood loss (Versteeg et al. 2013)

1.3.2 Inhibitors of blood coagulation

Anticoagulant pathways serve to control blood coagulation. Different protease inhibitors targeted towards specific coagulation factors are important to inhibit excessive and potentially harmful coagulation. Tissue factor pathway inhibitor (TFPI), protein C and antithrombin (AT) are examples of inhibitors contributing to normal hemostatic conditions. TFPI inhibits the coagulation cascade by binding to FXa or the TF-FVIIa-FXa complex, resulting in less generated thrombin. Binding of protein S to TFPI increases its inhibitory activity (Smith 2009). Procoagulant thrombin changes to anticoagulant thrombin when bound to thrombomodulin (TM) due to the immediate generation of aPC. This result in inactivation of FVa and FVIIIa hence reduced thrombin generation (aPC inactivation of FVa, see section 1.5.2). AT is a serine protease inhibitor that inhibits several key coagulation factors, including thrombin, FXa and FIXa (Smith 2009; Versteeg et al. 2013).

1.4 Blood coagulation, cancer and inflammation

Studies suggest a link between cancer biology and blood coagulation, where blood coagulation regulates malignant transformation, tumor angiogenesis and metastasis. Moreover, there is also a link between cancer biology and inflammation, and coagulation and inflammation.

1.4.1 Blood coagulation, cancer and thrombosis

Patients with thrombosis have been reported to have higher risk of cancers than the general population, where a study found that 10% of patients diagnosed with venous thromboembolism (VTE) was also diagnosed with cancer within 12 months (Carrier et al. 2008). Moreover, patients diagnosed with cancer had a general 7-fold increased risk of venous thrombosis (VT) compared to the general population (Blom et al. 2005), and after the cancer itself, VT was the second most common cause of mortality (Khorana, A. et al. 2007). Although, the risk of VT was highest within the first 3 months of cancer diagnosis, it was still elevated risk was seen even 2 years after diagnosis. However, the risk depended on time since onset, type of cancer and treatment (Blom et al. 2005). The cancer types with the highest rate of VT were pancreas (8,1%), kidney (5,6%), ovary (5,6%), lung (5,1%) and stomach (4,9%). Breast cancer patients had a lower risk of VT, affecting ~2% of the patients (Khorana, A. A. et al. 2007). Breast tumor cells have been found to possess the ability to induce procoagulant properties and inhibit anticoagulant properties in several cell types, increasing the risk of thrombosis (Caine et al. 2003).

1.4.2 Inflammation and cancer progression

As one of the enabling characteristics of cancer (see section 1.1.1), inflammation is evidently linked to cancer, and an inflammatory microenvironment supports cancer progression. Studies suggest that inflammation supports tumor initiation, progression and metastasis by enhancing processes like induction of genomic changes, inhibition of apoptosis, stimulation of angiogenesis, stimulation of cell proliferation, invasion and metastasis (Eiró & Vizoso 2012; Grivennikov et al. 2010; Kraus & Arber 2009). Moreover, a connection between inflammation and coagulation is indicated by common triggers (Davalos & Akassoglou 2012).

Tumor cells use the same mechanisms as leukocytes to spread during an inflammation, aided by for example adhesion molecules or cytokines. Moreover, tumor cells as well as tumor-associated leucocytes and platelets have the ability to produce inflammatory cytokines, chemokines and growth factors, all important in the progression and migration of cancer (Balkwill & Mantovani 2001; Eiró & Vizoso 2012). Cytokines involved in specific and sustained immune responses, are normally absent in tumors (Balkwill & Mantovani 2001). By altering the genomic sequence and inhibiting DNA repair systems, as well as inactivating the *TP53* gene the inflammatory cytokines lead to proliferation of tumor cells. Additionally, the cytokines interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF), as well as inflammatory macrophages such as tumor-associated macrophages (TAMs), are important in the production of angiogenic factors, such as vascular endothelial growth factor (VEGF). In case of hypoxia angiogenesis is also stimulated by TAMs and VEGF (Balkwill & Mantovani 2001; Leek et al. 1999). As a result of TAMs production of growth and angiogenic factors, TAMs contribute substantially to the control of cell proliferation, invasion, metastasis, and angiogenesis (Balkwill & Mantovani 2001; Mocellin et al. 2005; Sica et al. 2000). Due to TAMs found evenly spread around a tumor, the task of supporting cancer growth becomes easier (Balkwill & Mantovani 2001). Moreover, a link between high levels of macrophages in focal areas and reduced relapse-free survival (RFS) and overall survival (OS) in breast cancer was found ((Leek et al. 1997) as stated by Leek et al. (1999)). Also, the cytokine TNF may both suppress and promote tumor progression and adaptive immune response. As the name implies TNF induces necrosis, but can also stimulate growth of fibroblasts. Moreover, TNF both suppresses and promotes angiogenesis (Kollias et al. 1999; Mocellin et al. 2005). Elevated levels of toll-like receptors (TLRs) have been found in tumors of patients with different types of cancers, and are also associated with inflammation and tumor progression. In fact, studies indicate a relation between specific TLRs and higher probability of metastasis in breast cancer patients (Eiró & Vizoso 2012).

Common mediators trigger both inflammatory responses and the coagulation cascade, which indicates a link between them. Several mediators of the coagulation cascade such as TF, thrombin and fibrin have been associated with inflammatory responses in several diseases from stroke and Alzheimer's to cancers. TF and thrombin are associated with

changes in levels of inflammatory cytokines like IL6 and IL8, affecting the inflammatory processes (Cimmino et al. 2011; Davalos & Akassoglou 2012; Sower et al. 1995; Szaba & Smiley 2002). Both TF and thrombin can activate protease-activated receptors (PARs) which initiates a vast variety of signal transductions important in inflammation and coagulation, as well as cancer biology (Dugina et al. 2002; Ossovskaya & Bunnett 2004). For example, PAR activation can lead to anti-inflammatory and cytoprotective effects of aPC in several cells, like innate immune cells and vascular endothelium (Liang et al. 2015). In addition to preventing blood loss by formation of a fibrin mesh, fibrin is associated with inflammatory processes through initiating macrophage secretion of inflammatory markers (Davalos & Akassoglou 2012; Smiley et al. 2001).

1.5 Coagulation factor V

Coagulation factor V (FV) is a cofactor that has a significant role in the regulation of the coagulation process. As for other cofactors in the coagulation cascade, it is crucial that the cofactors are activated only when needed to fulfill a reaction. FV expresses both procoagulant and anticoagulant cofactor properties.

1.5.1 Structure and biology of coagulation factor V

Coagulation factor V (FV) is a single-chain protein (Camire 2011) mainly synthesized by the hepatocytes in the liver (Dashty et al. 2012). FV circulates in plasma with an average concentration of 20 nM (7 μg/mL) in humans. About 20% of FV in whole blood is stored in platelet α-granules (Asselta et al. 2006; Camire et al. 1998). Circulating FV and platelet derived FV have divergent physical and functional properties. Platelet derived FV show a significantly lower cofactor activity after thrombin activation than plasma FV (Gould et al. 2004), and platelet FV probably lack anticoagulant cofactor properties for aPC (Cramer & Gale 2012). Recently, Dashty *et al.* (2012) demonstrated that FV is also expressed by monocytes as well as hepatocytes (Figure 3), while the same study reported low FV expression in monocyte-derived macrophages (Figure 3) as well as other tested cell types (data not shown).

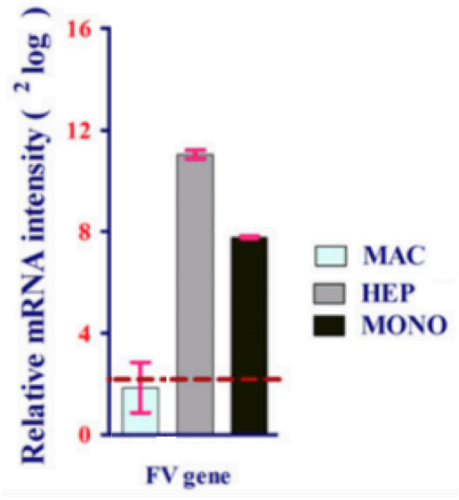

Figure 3: Expression of FV across macrophages (MAC), hepatocytes (HEP) and monocytes (MONO). Significance value marked as dotted line (Dashty et al. 2012).

The F5 protein is encoded by the *F5* gene located on chromosome 1. *F5* spans about 80kb, and comprises 25 exons (Figure 4A). Its mRNA length is 6,8kb, while the coding protein consists of 2224 amino acids where 28 comprise the signal peptide. FV circulates as an inactive pro-cofactor, with a six-domain organization; A1-A2-B-A3-C1-C2 (Figure 4B). In the activated FV (FVa) the domains A1 and A2 comprise the heavy chain, while the domains A3, C1 and C2 comprise the light chain of the protein. FV undergoes post-translational modifications such as glycosylation, phosphorylation and sulfation, all playing important functional roles. The cleavage of the heavily glycosylated B domain is essential for the function of FV, and thus the important contribution FV provides to the coagulation process (Asselta et al. 2006; Mann & Kalafatis 2003; Steen et al. 2008; Wiencek et al. 2013).

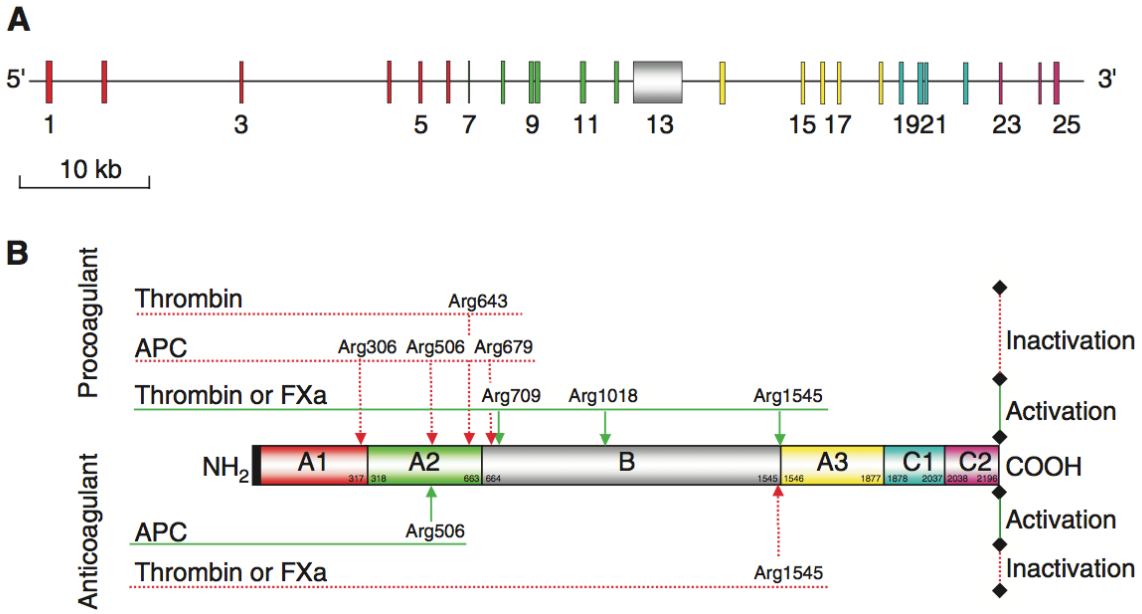

Figure 4: Schematic structure of the *F5* gene and the F5 protein. (A) Exon-intron structure of the *F5* gene. Exons and introns are presented by colored boxes. (B) Domain organization of the F5 protein. Proteolytic cleavage sites categorized into FV activation and inactivation of the procoagulant and anticoagulant forms are indicated by arrows. Domains A1-A2-B-A3-C1-C2 are colored to match with the exons in figure A. The black box at the N-terminal represent the signal peptide. The numbers within each box represent the amino acid number in the FV protein. (Asselta et al. 2006)

1.5.2 Procoagulant FV

Activation of FV to procoagulant FVa

The single-chain, pro-cofactor FV binds to membrane surfaces due to electrostatic and hydrophobic properties of the C1-C2 domain in the light chain (Figure 5A). This binding is crucial for the cleavage and removal of the B domain and thereby the activation of the FV pro-cofactor to procoagulant FV. Thrombin, FXa and plasmin activate the pro-cofactor into the active cofactor FVa, also called procoagulant FV (Figure 4) (Esmon 1979; Huang & Koerper 2008; Mann & Kalafatis 2003; Wiencek et al. 2013). Procoagulant FV acts as a cofactor for FXa in the activation of prothrombin to thrombin (Figure 6A).

Thrombin activates FV to FVa through limited proteolysis at the sites Arg709, Arg1018 and Arg1545 (Figure 4B & Figure 5A-B) (Jenny et al. 1987). First the light chain is separated by cleavage at Arg709. Then cleavage at Arg1018 and Arg1545 splits the B domain in two, resulting in removal of the heavy chain (Monkovic & Tracy 1990; Suzuki

et al. 1982). The cleavage at Arg1545 is the most important for FV procoagulant function (Jenny et al. 1987; Keller et al. 1995). After the extensive part of the B domain is cleaved off, a non-covalently association between the heavy chain and the light chain occur, which is stabilized by the presence of divalent metal ions (Cramer & Gale 2012; Krishnaswamy et al. 1989; Singh et al. 2003). This cleavage process occurs early in the coagulation cascade, and is considered of higher biological importance than activation by FXa (Mann & Kalafatis 2003). Single-chain FV bound to a membrane surface does not have the ability to bind FXa, hence the presence of the small amounts of thrombin activated FVa is crucial for the generation of the prothrombinase complex, which activates prothrombin to thrombin (Figure 5C) (Esmon et al. 1973; Guinto & Esmon 1984; Toso & Camire 2004). Consequently, the rate of thrombin activated FV significantly increases (Mann & Kalafatis 2003; Nesheim et al. 1979).

FXa activates FV to FVa by cleavage at the same sites as thrombin (Thorelli et al. 1997), though in the order: Arg1018, Arg709 and Arg1545 (Monkovic & Tracy 1990; Suzuki et al. 1982). Maroney and Mast (2015) suggest that a part within the C-terminal region of tissue factor pathway inhibitor α (TFPIα) has sequence homology with a region in the B domain of FV composed of basic amino acids. This indicates that TFPIα can impede the generation of prothrombinase due to the basic region of TFPIα homologous to the region of the FV B domain binding to the acidic region of the FV B domain, which assure that FV stays in its pro-cofactor conformation. The same function is not seen in thrombin activated FV, due to the differences in the order FV is cleaved (Bos & Camire 2012; Maroney & Mast 2015).

The serine protease plasmin briefly activates the FV into FVa, nevertheless, plasmin also inactivates procoagulant FV when FVa is bound to a membrane surface. In fact, when FV/FVa is bound to a membrane surface plasmin inactivation of FVa is favored over plasmin activation of FV (Lee & Mann 1989).

Inactivation of procoagulant factor Va

Inactivation of FVa is important to control the coagulation process and may contribute to reduce the risk of thrombosis and acute inflammation triggered by coagulation (Nogami et al. 2014; van der Neut Kolfschoten et al. 2004). The procoagulant FVa can be

inactivated (FVai) by aPC, thrombin and plasmin (Cramer & Gale 2012; Krishnaswamy et al. 1986; Lee & Mann 1989).

aPC binds to and proteolytically cleaves FVa at the sites Arg306, Arg506 and Arg679 on the FVa heavy chain (Figure 4B). This results in loss of FVa procoagulant activity. However, cleavage at only position Arg506 results in decreased FVa activity due to reduced affinity for FXa (Krishnaswamy et al. 1986; Nogami et al. 2014; Steen et al. 2008). The presence of protein S enhances the aPC-driven inactivation of FVa (Solymoss et al. 1988), due to protein S resulting in a conformational change of aPC (Yegneswaran et al. 1997).

Thrombin inactivates FVa to FVai by proteolytic cleavage at Arg643 in the presence of endothelial cells. This cleavage results in reduced affinity between the heavy and the light chain of FV (Asselta et al. 2006). Plasmin proteolytically cleaves FVa at Arg348, Lys1656 and Arg1765 resulting in inactivation, and thus loss of cofactor activity (Lee & Mann 1989; Zeibdawi & Pryzdial 2001).

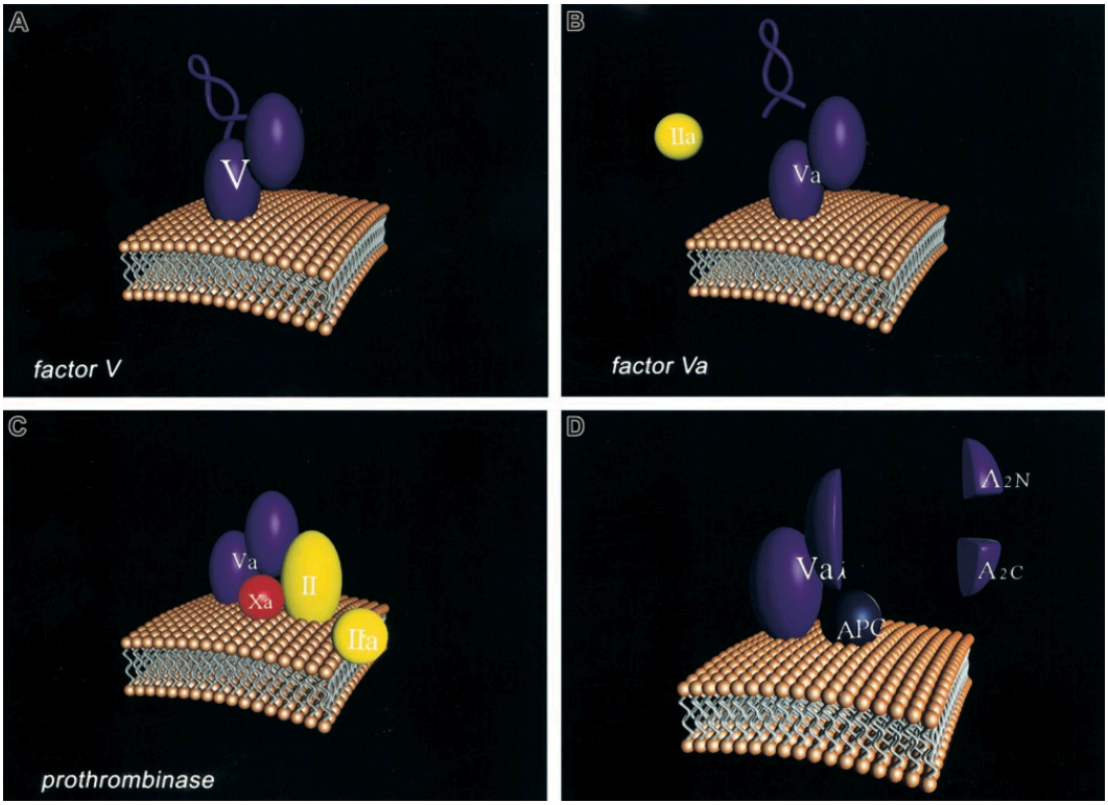

Figure 5: Contribution of factor V in the coagulation cascade. (A) FV bound to a membrane surface; light chain bound to the membrane connected with the heavy chain

(both illustrated as bulbs) through the B domain (illustrated as a loop). (B) Activation of FV to FVa by thrombin cleavage. The light and heavy chain are non-covalently connected. (C) Activation of prothrombin to thrombin by the FVa-FXa complex. (D) Cleavage and inactivation of FVa (FVai) by aPC (Mann & Kalafatis 2003).

1.5.3 Anticoagulant properties of FV

Activation of anticoagulant FV

Some refers to FV as the chameleon co-factor, since it can express anticoagulant properties in addition to procoagulant properties (Figure 6) (Cramer & Gale 2012).

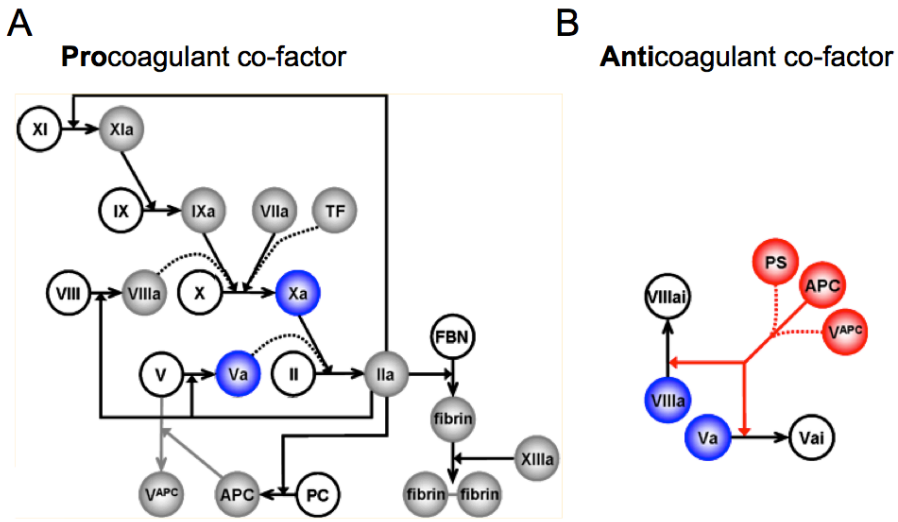

Figure 6: The chameleon co-factor, FV. (A) FV procoagulant function as co-factor for FXa in the activation of prothrombin and (B) FV anticoagulant function as co-factor for aPC in the inactivation of FVIIIa and FVa. Modified from Cramer and Gale (2012).

The anticoagulant FV is generated when aPC cleaves single-chain FV proteolytically at the site Arg506 (Figure 4B), before thrombin or FXa has cleaved the single chain into procoagulant FV (described in section 1.5.2)(Cramer & Gale 2012; Mann & Kalafatis 2003; Nogami et al. 2014). The anticoagulant FV functions as a cofactor for aPC in the inactivation of FVa and FVIIIa. For FV to express anticoagulant cofactor activity two requirements are essential: The presence of protein S and the presence of the C-terminal end of the B domain (Cramer & Gale 2012; Nogami et al. 2014). Protein S is a cofactor for aPC like FV, and presence of protein S is essential for the cofactor function of anticoagulant FV towards aPC (Lu et al. 1996; Shen & Dahlbäck 1994; Thorelli et al. 1999; Varadi et al. 1996; Yegneswaran et al. 1997). The C-terminal part of the B domain

contains an acidic region as well as potential sites for N-linked glycosylation (Jenny et al. 1987; Schuijt et al. 2013) which may be of importance in the event where anticoagulant FV binds to aPC and protein S (Cramer & Gale 2012). Also, the linkage between the C-terminal end of the B domain and the A3 domain of the light chain is essential for the cofactor activity of anticoagulant FV (Thorelli et al. 1998).

Inactivation of anticoagulant FV

Thrombin and FXa proteolytically cleaves anticoagulant FV at Arg1545, leading to de-attachment between the B and A3 domain, and loss of FV anticoagulant properties (Figure 4B) (Lu et al. 1996; Thorelli et al. 1999)

1.5.4 Anti-inflammatory properties of FV

A link between anticoagulant FV and anti-inflammatory response has been indicated (Liang et al. 2015). In the presence of PS, FV acts as a cofactor for aPC, which can destabilize the TF-FVIIa-FXa complex. This results in inhibition of the EPCR-dependent activation of the inflammatory PAR2 signaling, hence no inflammatory response (Sun 2015).

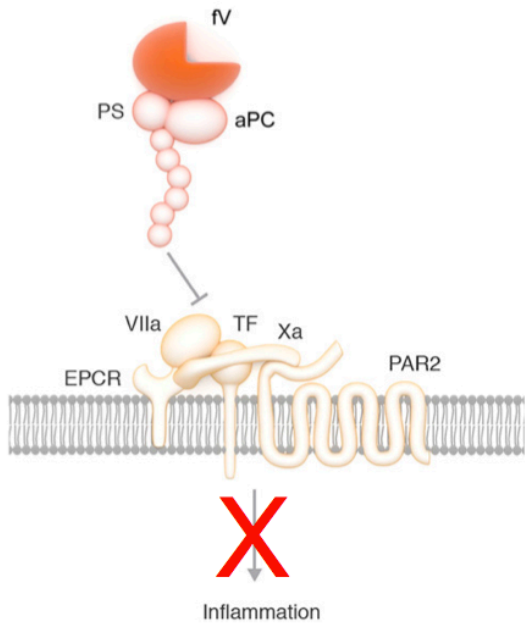

Figure 7: Anti-inflammatory FV, as a cofactor for aPC in the presence of PS, FV induces the activation of EPCR-dependent PAR2 signaling by the TF-FVIIa-FXa complex (Sun 2015)

1.6 Human genetic variation

Genetic variation is an important basis of evolution that makes every species different and every individual unique. With the whole genome sequencing technology came the possibility to study the genetic differences across species and between individuals (Lesk 2012). Genetic variation at the nucleotide level incudes insertions, deletions, duplications, and inversions of one or several bases. Alterations at the chromosomal level also contribute to genetic variation, such as rearrangements or deletions of chromosome segments or aneuploidy (abnormal number of chromosomes). The most common type of genetic variation is the change of a single base. When the allele frequency of a single base substitution is higher than 1%, it is commonly referred to as a single nucleotide polymorphism (SNP), whereas a frequency less than 1% is referred to as a mutation (Frazer et al. 2009). SNP with minor allele frequency ≥5% in a population is referred to as common SNPs (Consortium 2012).

The genetic code is unambiguous. Several three-base codons codes for the same amino acid, meaning that a change in one of the bases of a codon does not necessarily lead to an altered amino acid (Lesk 2012). When a base substitution does lead to change of amino acid the mutation is said to be non-synonymous. An altered amino acid sequence in the coding region may lead to altered protein function. Interestingly, 88% of SNPs associated with disease are found in the intron or intergenic regions (Hindorff et al. 2009). These SNPs may contribute to changes in the regulation of gene expression.

Genetic association studies and linkage disequilibrium (LD)

Possible associations between complex diseases and genetic variations can be studied through genetic associations studies, and candidate genes or genome regions that contribute to a specific disease can be identified. SNPs are the most commonly used marker in such studies (Lewis & Knight 2012). Most common diseases are complex and caused by the effect of the interplay between several different genetic factors (Lesk 2012).

A SNP may be directly linked to a disease phenotype, but can also be indirectly linked through LD with directly linked SNPs (Figure 8) (Lewis & Knight 2012). LD is about the distribution of allelic patterns, and two alleles are said to be in LD when they co-occur at

a higher frequency than expected by chance (Lesk 2012; Wall & Pritchard 2003). The strength of the LD between loci are dependent on the crossover rate and the number of generations since the allele variant occurred (Lunetta 2008). When SNPs are inherited together as a block they are said to express a haplotype (Lesk 2012; Wall & Pritchard 2003). There are multiple ways to measure the LD between SNP alleles, including the traditional pairwise measures of D' and r2, where r2 is the more conservative method (Mueller 2004).

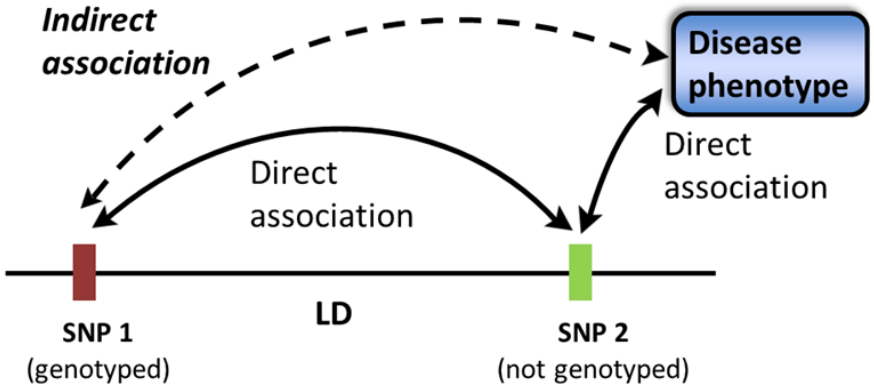

Figure 8: SNPs in LD and association to disease. A SNP may have indirect association to a disease if in strong LD with a SNP known to have direct association to the disease (Tinholt et al. 2016).

1.6.1 Genetic variants and association to cancer and coagulation

Several SNPs have been associated with complex diseases, such as cancers (Frazer et al. 2009) and thrombosis (Blom et al. 2005), and with the interplay between the two it is of interest to study possible genetic variations of common association.

Many genetic variants in genes associated with the coagulation cascade have been associated with risk of cancers, thrombosis and also bleeding disorders. Six SNPs in the clotting factor genes *F5, F10* and *EPCR* were found associated with risk of breast cancer (Tinholt et al. 2014). In the *FV* gene alone several exon SNPs have been associated with increased risk of thrombosis, mostly due to reduced inactivation of FVa and reduced function of anticoagulant FV leading to loss of aPC co-factor activity (Nogami et al. 2014; Norstrøm et al. 2002; Steen et al. 2004). As the genetic risk factor with highest

associations to thrombosis (Pabinger et al. 2015), the FV Leiden has been widely studied.

According to Rosendaal and Reitsma (2009) and Nogami *et al.* (2014) 20% of patients with Caucasian descent suffering from thrombosis are carriers of FV Leiden (homo-, and heterozygous). The FV Leiden variant also known as SNP rs6025, is located in exon 10, and expresses glutamine at amino acid 506 instead of arginine (Koster et al. 1994). Patients homozygous for FV Leiden showed a 2-8 fold increased risk of VT, while the increase was 10-80 fold higher in heterozygous carriers (Kujovich 2011). Homozygous carriers of FV Leiden were found, in average, to suffer thrombosis at a much younger age (31 years of age) than heterozygous carriers (44 years of age) and individuals without the variant (46 years of age) (Rosendaal et al. 1995). Moreover, FV Leiden is associated with aPCR, which was found to be the underlying cause of more than 80% of aPCR cases (Lucotte & Mercier 2001). In presence of FV Leiden aPC is not able to cleave FV at Arg506, which leads to no activation of anticoagulant FV (Castoldi et al. 2004), and reduced inactivation of procoagulant FV (Castoldi et al. 2004; van der Neut Kolfschoten et al. 2004). Both forms are assumed to contribute equally to the FV Leiden-caused aPCR (Castoldi et al. 2004), and lead to increased risk of thrombosis (Rosendaal et al. 1995).

The four *FV* intron SNPs rs12120605, rs6427202, rs9332542 and rs6427199 were reported to be associated with risk of breast cancer and independent of the FV Leiden variant (Tinholt et al. 2014), but the three later were found to express a haplotype effect (Tinholt et al. 2016). Interestingly, the intron SNP rs9332542 was found to be in LD with the *FV* exon SNP rs6028, which makes it a good candidate SNP to study the functional effects of this haplotype.

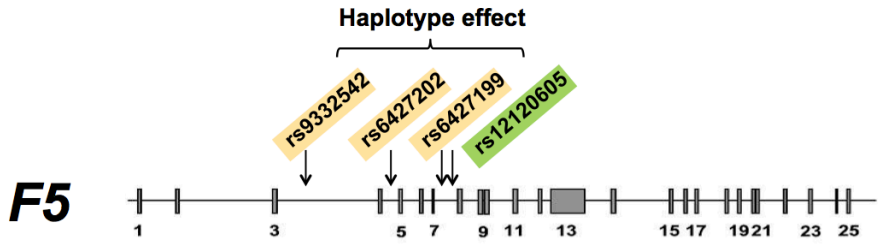

Figure 9: Location of four intron SNPs of *FV* associated with risk of breast cancer. Rs9332542, rs6427202 and rs6427199 express a haplotype effect.

Several studies have investigated the association between FV Leiden and risk of solid cancers. Vossen and colleagues (2011) found that homozygous carriers of FV Leiden had a 5,8-fold increased risk of colorectal cancer. However, several studies have failed to show an association between FV Leiden and risk of solid cancers (Battistelli et al. 2006; Ghasemi et al. 2014; Paspatis et al. 2002; Pihusch et al. 2002; Sciacca et al. 2004; Tinholt et al. 2014; Tormene et al. 2008; Vairaktaris et al. 2005; Vylliotis et al. 2013).

Genetic variations in *F5* are not only associated with thrombosis, but also to increased tendency of bleeding. Coagulation factor V was in fact discovered through a patient with bleeding incidences, found to lack procoagulant FV (Huang & Koerper 2008; Owen & Cooper 1955). Today over 60 polymorphisms are reported to be associated with FV deficiency (Huang & Koerper 2008).

2. Aims of the study

The aim of this thesis was to characterize the role of coagulation factor V in breast cancer. The link between cancer and risk of thrombotic diseases is well known, and increased coagulation activity is associated with tumor progression. With a better understanding of the underlying molecular mechanisms of these links, a more individualized therapy for patients not only suffering cancer, but cancer-related thrombosis may be possible. Breast cancer patients have a lower risk of thrombotic diseases than patients suffering from other types of cancers, thus breast cancer serves a useful model to study cancer progression. In this thesis the clinical significance of FV was studied in a clinical breast cancer material, and expression- and functional effects of selected *FV* gene variants were studied *in vitro*. The following specific objectives were addressed in this thesis:

I. The clinical significance of FV:
   - Study FV mRNA expression and FV plasma protein levels in breast cancer patients in relation to breast tumor characteristics and breast cancer subtypes.
   - Study relation of FV mRNA expression in breast tumor characteristics and breast cancer subtypes and possible association to survival.
   - Study possible biological functions associated with FV and co-expressed genes.

II. *In vitro* cell studies:
   - Construct a FV overexpression model for FV overexpression studies.
   - Study FV overexpression in FV wt:
     - And effects on gene expression and protein levels.
     - And functional effects of FV overexpression on cell growth, cell death and inflammatory markers
   - Study FV overexpression of FV variants
     - And effects on gene expression and protein levels, compared to FV wt.
     - And possible altered functional effects on cell growth, cell death and inflammatory markers, compared to FV wt.

3. Materials

3.1 Clinical studies of FV gene expression in breast cancer

3.1.1 Clinical breast cancer patient material

The OsloII study

Through a larger study called OsloII, the main hospitals of Oslo are aiming to collect tumor material, blood and clinical data from about 2000 breast cancer patients, managed by the Oslo Breast Cancer Research Consortium (OSBREAC). The data used in this thesis comprised of FV levels in blood samples of 366 breast cancer patients and FV tumor mRNA data from 152 breast cancer patients, generated on Agilent arrays.

Gene Expression-Based Outcome for Breast Cancer Online (GOBO)

GOBO is an online tool with pooled breast cancer data set from 11 public data sets, analyzed using Affymetrix U133A arrays. It can be used for a number of analyses, for example gene expression levels in subtypes of breast tumors and cell lines, identification of co-expressed genes, and association between gene expression levels and outcome. GOBO comprises of gene expression data and annotation data from tumors of 1881 breast cancer patients. In addition, gene expression levels in 51 breast cancer cell lines are available.

A number of tools were used in the clinical study of FV expression in the breast cancer materials, listed in Table 2.

Table 2: Tools for studies of the clinical breast cancer material.

Tool	Web address	Purpose
DAVID	david.ncifcrf.gov	Functional annotation of genes co-expressed with *F5*
dbSNP	ncbi.nlm.nih.gov/SNP/	SNP info, e.g. function, location, allele variants and frequencies
ensembl	ensembl.org	SNP info, e.g. genes in LD, function, location, allele variants and frequencies
Genetic GO Term Finder	go.princeton.edu	GO terms shared by *FV* co-expressed genes

GOBO	co.bmc.lu.se/gobo	FV tumor mRNA expression across breast cancer subtypes and tumor characteristics, and association to survival. Co-expressed genes and gene modules. *F5* gene expression across breast cancer cell lines.
NetAffx Query	affymetrix.com	The probe sets of FV were accounted for by Affymetrix Human Genome U133A GeneChip Array
SPSS	[not an open source tool]	Statistical analysis of FV tumor mRNA and FV plasma protein in the OsloII material
UCSC Genome Browser	genome.ucsc.edu	Composition of the gene and the probes. Visualization of SNPs.

3.2 Reagents and chemicals

Table 3: Reagents and chemicals

Reagent/chemical	**Supplier**	**Catalogue number**
10x Tris buffered Saline (TBS)	BioRad, CA, USA	170-6435
10X Tris/Glycine/SDS	BioRad, CA, USA	1610732
20% SDS solution	BioRad, CA, USA	161-0418
7,5% Mini-protean® TGX™ Gels	BioRad, CA, USA	456-1024
Agar-Agar	Merck, Darmstadt, Germany	101614
Amersham ™ ECL ™ Prime Western Blotting Detection Reagent	GE Healthcare, Little Chalfont, UK	RPN2232
BlueJuice™ Gel Loadding Buffer (10x)	Thermo Fisher Scientific, Waltham, MA, USA	10816015
Bovine Serum Albumin	Thermo Fisher Scientific, Waltham, MA, USA	A7906
Comassive Brilliant Blue	BioRad, CA, USA	1610436
DMEM Dulbecco's Modified Eagle's Medium 4.5g/L Glucose w/ L-Glutamine 500ml	Lonza, Verviers, Belgium	BE-12-604F
Dulbecco's PBS (1x)	Thermo Fisher Scientific, Waltham, MA, USA	14190
Fetal Bovine Serum Ultra-Low Endotoxin Heat Inactivated	Biowest, Nuaillé, France	Si86H-500

FV deficient plasma	Kind gift from Rikshospitalet, Norway (Instrumentation Laboratory, USA)	0020011500
GeneRuler 1kb DNA ladder	Fermentas, Vilnius, Lithuania	5M0311
Glycerol	LBH Laboratory Supplies	101184K
Glycine	BioRad, CA, USA	161-0718
Halt™ Protease & Phosphatase Inhibitor Cocktail (x100)	Thermo Fisher Scientific, Waltham, MA, USA	78440
Lipofectamine® 3000 Transfection Reagent	Thermo Fisher Scientific, Waltham, MA, USA	L3000008
Magermilchpulver	Applichem GmbH, Darmstadt, Germany	A0830
Magnesium chloride hexahydrate	Thermo Fisher Scientific, Waltham, MA, USA	M2670
Magnesium sulfate heptahydrate	Merck, Darmstadt, Germany	105886
Methanol	Merck, Darmstadt, Germany	I677909313
N-Z Amine® A (casein enzymatic hydrolysate)	Thermo Fisher Scientific, Waltham, MA, USA	C-0626
NaOH	Merck, Darmstadt, Germany	1064951000
Opti-MEM Reduced Serum Medium	Thermo Fisher Scientific, Waltham, MA, USA	31985-062
Peptone from casein (Tryptone)	Merck, Darmstadt, Germany	119311000
Ponceau S solution 0,1% (w/v) in 5% acetic acid	Thermo Fisher Scientific, Waltham, MA, USA	P7170
Precision Plus Protein™ Dual Color Standards	BioRad, CA, USA	161-0374
Reagent A100	ChemoMetec A/S	910-0003
Reagent B	ChemoMetec A/S	910-0002
Recombinant FV	Hematologic Technologies Inc., VT, USA	HCV-0100
Recombinant FVa	Hematologic Technologies Inc., VT, USA	HCV-0110
RIPA buffer	Thermo Fisher Scientific, Waltham, MA, USA	R0278
S.O.C. medium (Super Optimal broth with Catabolite Repression)	Thermo Fisher Scientific, Waltham, MA, USA	15544034

Sodium Chloride (NaCl)	Merck, Darmstadt, Germany	106404500
TaqMan® Gene Expression	Thermo Fisher Scientific, Waltham, MA, USA	4369016
Trizma® Base	Thermo Fisher Scientific, Waltham, MA, USA	TI503
Trypsin EDTA	Lonza, Verviers, Belgium	BE17-161E
Tween® 20	Thermo Fisher Scientific, Waltham, MA, USA	P1379
WST-1 Cell Proliferation Reagent	Abcam, UK	Ab155902
Yeast Extract	Thermo Fisher Scientific, Waltham, MA, USA	Y1625-250G

3.3 Kits

Table 4: Kits

Kit	**Supplier**	**Catalogue number**
Agencourt CleanSEQ	Beckman Coulter, CA, USA	A29154
Amersham™ ECL™ Prime Western Blotting Detection Reagents	GE Healthcare, Buckinghamshire, UK	RPN2232
BigDye® Terminator v3.1 Cycle Sequencing Kit	Thermo Fisher Scientific, Waltham, MA, USA	4337455
Cell Death Detection ELISA ^{Plus}	Roche Applied Science, IN, USA	11774425001
E.Z.N.A.® Plasmid DNA Mini Kit I Protocol - Spin Protocol	Omega bio-tek, Norcross, GA, USA	D6942
EndoFree ® Plasmid Maxi Kit	QIAGEN, Alameda, CA, USA	12362
High Capacity cDNA Reverse Transcription Kit	Thermo Fisher Scientific, Waltham, MA, USA	4368813
Human Inflammatory Cytokines Multi-Analyte ELISArray™ Kit	QIAGEN, Alameda, CA, USA	MEH-004A
Multi-Analyte ELISArray Kit for detection of Human Inflammatory Cytokines	QIAGEN, Alameda, CA, USA	MEH-004A
MycoAlert™ Assay Control Set	Lonza, Verviers, Belgium	T07-518
PIERCE ® BCA Protein Assay kit	Thermo Fisher Scientific, Waltham, MA, USA	23225

QuikChange II XL Site-Directed Mutagenesis Kit	Agilent Technologies, Santa Clara, CA, USA	200521
RNAqueous® Total RNA Isolation Kit	Thermo Fisher Scientific, Waltham, MA, USA	AM1912
ZymoPURE ™ Plasmid Maxiprep Kit	Zymo Research, Irvine, CA, USA	D4202
ZYMUTEST Factor V	Hyphen-BioMed, Neuville-sur-Oise, France	RK009A

3.4 Instruments and equipment

Table 5: Instruments and equipment

Instrument/equipment	**Supplier**
ABI 3730 DNA analyzer	Applied Biosystems
ABI PRISM 7900HT Sequence Detection System	Applied Biosystems
ASYS Atlantis 4	ASYS hitech gmbH
DELFIA® Plate Shake	Wallac
DNA Sequencing Analysis Software v5.1	Applied Biosystems
ImageQuant LAS 4000 imagine system	GE Heathcare
Incubator	Termaks A/S
Infors Mutlitron Incubation Shaker	Infors HT
Lucetta ™ Luminometer	Lonza
Mini-protean® TGX™ Gels 7,5%, 10 well comb, 50µl (cat. no. 456-1024)	Bio-Rad
NanoDrop® ND-1000 Spectrophotometer	NanoDrop Technologies
Nikon Eclipse TE 300 microscope	Nikon
Nitrocellulose Membrane, 0.2 µm (cat.no. 1620112)	Bio-Rad
NucleoCassette	ChemoMetec A/S
NucleoCounter® NC-100™	ChemoMetec A/S
Nunc™ Cell Culture Treated Flasks with Filter Caps (25cm^2, 75cm^2, 175cm^2)	Thermo Scientific
Nunc™ Cell Culture Treated Multidishes (6-well and 12-well)	Thermo Scientific
QuantStudio 12k Flex	Applied Biosystems
SoftMax Pro 6.4 software	Molecular Devices

Steri-Cycle CO_2 incubator	Thermo Scientific
Thermal Cycler 2720	Applied Biosystems
Veriti 96 well Thermal Cycler	Applied Biosystems
VersaMax microplate reader	Molecular Devices

3.5 Antibodies

Table 6: Primary and secondary antibodies for Western blotting

	Antibody	**Supplier**	**Catalogue number**	**Dilution**
Primary antibody	Anti-Human Factor V antibody, monoclonal mouse	Hematologic Technologies Inc.	AHV-5146	1:1000
Secondary antibody	Polyclonal, Goat α-mouse, Immunoglobin/HRP	DAKO	P0447	1:1000

3.6 Taqman/qRT-PCR assay

Assay	**Primer/probe/ID**	**Sequence 5'-3'**	**Supplier**
FV	Hs00914120_m1		Thermo Scientific
Human ribosomal protein lateral stalk subunit P0 (RPLP0)	Probe	AACGGGTACAAACGAGTCCTGGCCTT	Self-made
	Forward primer	TTGCATCAGTACCCCATTCTATCAT	Self-made
	Reverse primer	AAGGTGTAATCCGTCTCCACAGA	Self-made

3.7 Cells

Table 7: Cells

Cell type	**Supplier**	**Catalogue number**
OneShot ® TOP10 Chemically Component Cells, *Escherichia coli*	Invitrogen, Carlsbad, CA, USA	C4040-03
HEK293T	ATCC, Manassas, USA	ATCC® CRL-3216
MDA-MB-231	ATCC, Manassas, USA	ATCC® HTB-26™

The breast cancer cell line MDA-MB-231 was chosen for *in vitro* studies in this thesis due to its characteristics fitting well with the results obtained from the in silico analysis. The MDA-MB-231 cell line is basal-like and triple negative, and had previously been successfully used in transfection experiments by the research group.

The human endothelial kidney cell line HEK293T was used for initial studies. The cell line was known by the research group and had previously been successfully used in transfection experiments by the research group.

Moreover, qPCR experiments showed that both cell lines had low endogenous FV expression, hence they were considered as suitable models for overexpression of FV.

3.8 Primers

3.8.1 Primers for sequencing

Table 8: Sequencing primers

Primer*	**Sequence 5'-3'**
F5-1F	TGGGGGAGCCAAGGGACAGA
F5-2F	CTCGGGGCCAGAATTATTCTCCATTCA
F5-3F	CATCGCCTCTGGGCTAATAGG
F5-4F	AGATTTTTGAACCTCCAGAATCTACAGTCA
F5-5F	GGAGGAAAGAGTAGACTGAAGAAAAGCCA
F5-6F	ATGACTCTCTCTCCAGAACTCAGTCAG
F5-7F	GGAAGAGGTCCAGAGCAGTGAAGA
F5-8F	TGGTTTAAGGAAGATAATGCTGTTCAGCCA
F5-9F	GGCCCCTTCTGCCTGGTTCA
F5-10F	AGAGAATCAGTTTGACCCACCTATTGT
F5-11F	CCCCCAATCATTTCCAGGTTTATCCGT
F5-1R	AGGTGTATTCTCGGCCTGGAGC
F5-2R	ATGCTATAGGGGCGGCTGGC
F5-3R	CCACGCATGGGGAAGAGGGT
F5-4R	AGCCAAATGCCATCTCCCAACCA

F5-5R	AGGATCTGTGACTGGGGTCTGA
F5-6R	TCCGGGAGAAGGGTGGTGTCA
F5-7R	GGATAACATCATCCACTTCAGCTCTGA
F5-8R	ACACTCCAAGCATTATAAGATCCACCA
F5-9R	TCCCTGCTCACTGTAGTGGATGGTAT

*All primers obtained from Eurogentec

3.8.2 Primers for *in vitro* mutagenesis

Table 9: *In vitro* mutagenesis primers

Primer*	**Forward/reverse**	**Sequence 5'-3'**
rs6030	Forward	GGACAGCAACATGCCTATGGACATGAGAGAATTTGTC
	Reverse	GACAAATTCTCTCATGTCCATAGGCATGTTGCTGTCC
Novel mutation II	Forward	CATGGAATCAAAGTATTGCACTTCGCCTGGAACTC
	Reverse	GAGTTCCAGGCGAAGTGCAATACTTTGATTCCATG
rs6025	Forward	GCAGATCCCTGGACAGGCAAGGAATACAGAGGGCAGC
	Reverse	GCTGCCCTCTGTATTCCTTGCCTGTCCAGGGATCTGC
rs6028	Forward	GAAAAACCACAGTCTACCATTTCAGGACTTCTTGGGCC
	Reverse	GGCCCAAGAAGTCCTGAAATGGTAGACTGTGGTTTTTC

*All primers obtained from Eurogentec

3.9 Solutions

NZY+ broth:

1 g NZ amine-A

0,5 g yeast extract

0,5 g NaCl

MQ H_20 up to 100 ml

pH adjusted to 7,5. Autoclaved.

1,25 ml 1 M $MgCl_2$

1,25 ml 1 M $MgSO_4$

1 ml 2 M glucose

All sterilized through 0,2 µm filter

LB (Luria Broth) medium:

10 g tryptone

5 g yeast extract

10 g NaCl

MQ water to 1L

pH adjusted to 7,5. Autoclaved.

(For LB-agar plates, 15 g agar was added)

RIPA lysis buffer with inhibitors:

1 x RIPA buffer

1:100 Protease and Phosphatase Inhibitor Cocktail (x100)

1 x TGS (running) buffer - Western blot

100ml 10x TGS (Tris/Glycine/SDS)

900ml MQ water

Blotting buffer - Western blot

3 g Trizma base

14,4 g glycine

900 ml MQ water

100 ml methanol

0,01% SDS

1x TBS – Tween (TBST) buffer - Western blot

100 ml 10x TBS

1 ml Tween® 20

5% BSA

2,5 g Bovine Serum Albumin

50 ml TBST

Primary antibody

1ml 5% BSA in TBST

4 ml TBST

Antibody

Secondary antibody

200 µl 5% milk powder

4,8 ml TBST

Antibody

3.10 Vectors

Vector	Supplier	Catalogue number
pcDNA5frt-FV	Received as a generous gift from Versteeg, Netherlands	
pMT2-V	ATCC	ATCC® 40515™
pcDNA5frt Mammalian Expression Vector	Thermo Fisher Scientific, Waltham, MA, USA	V601020
pcDNA3.1/V5-His-TOPO	Thermo Fisher Scientific, Waltham, MA, USA	K4800

3.11 *F5* variants

In addition to the FV wild type (wt), the *F5* variants FV rs6025, FV rs6028 and FV A2184T (Figure 10) were studied in this thesis.

Not many studies support an association between FV rs6025 (FV Leiden) and increased risk of cancers. Nevertheless, homozygous carriers of FV Leiden are found to show aPCR, increased risk of thrombosis, and cancer-associated thrombosis (Dziewiecka et al. 2015; Rosendaal et al. 1995; Tinholt et al. 2016). With these associations as well as the general link between cancers and increased risk of thrombosis, it was of interest to study the FV rs6025 variant *in vitro* in this thesis.

The *F5* intron SNP (rs9332542) was found associated with increased risk of breast cancer (Tinholt et al. 2014). The *FV* variant rs6028 was found to be in LD with this intron SNP (r^2 = 0,9), hence it was of interest to study FV rs6028 *in vitro* in this thesis.

Moreover, a *F5* variant (FV A2184T) not previously reported, was found during initial experiments in this thesis. Differences in gene expression and protein secretion between FV A2184T and FV wt, made it a candidate variant for *in vitro* studies in this thesis. In addition, FV A2184T is located in the C-terminal end of FV, which is an area important in FV binding to membrane surfaces.

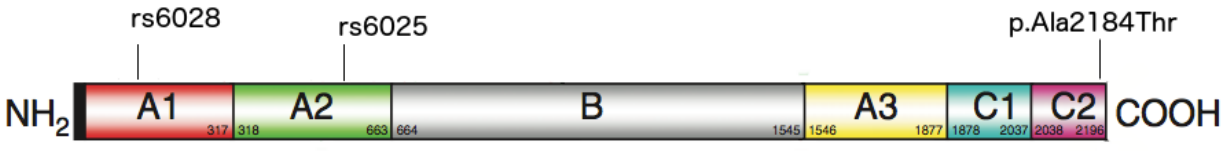

Figure 10: Schematic map of the positions of the *F5* gene variants studied in this thesis. Modified from Asselta *et al*. (2006)

4. Methods

I. Clinical studies

4.1 FV gene expression in breast cancer

Data from the breast cancer materials of the OsloII study and the GOBO dataset (described in section 3.1.1) was studied to gain insight in similarities and differences of *FV* gene expression levels between breast cancer molecular subtypes and tumor characteristics, and associations to survival.

4.1.1 FV expression levels in breast cancer molecular subtypes and tumor characteristics in OsloII and GOBO

FV tumor mRNA expression and FV plasma protein levels across breast cancer tumor characteristics in the material of the OsloII study

FV mRNA expression levels in tumors and FV protein levels in plasma were studied in the OsloII material. This was mainly done using SPSS, where the first step was to check whether the data followed normal distribution. Data of FV mRNA expression did not follow normal distribution, hence a log10 transformation was done to fit this trend. The following steps were done individually for both FV mRNA and FV protein levels. Data was distributed across breast cancer tumor characteristics, into the subgroups estrogen receptor (ER)-status, progesterone receptor (PR)-status, HER2-status, histological tumor grade, tumor status (T-status), hormone receptor (HR) negative status, triple negative status, lymph node spread and *TP53* mutation status. Independent sample t-tests and Levenes tests were performed to see whether there were significant differences in FV expression levels and FV protein levels between the subgroups within each tumor characteristic. Boxplots for FV mRNA expression and FV protein levels according to each group were made for a visual effect of variances within each group. Moreover, a distribution across PAM50 subgroups using ANOVA was made, for both FV mRNA tumor expression and FV protein plasma levels.

FV tumor mRNA expression levels across breast cancer molecular subtypes and tumor characteristics in pooled GOBO dataset

The online tool GOBO provides several options for expression analyses of breast cancer genes and was used to study the pooled GOBO dataset. The Gene Set Analysis (GSA) application of GOBO, including analyses of tumors and cell lines, was used for multiple analyses of FV mRNA expression in tumors regarding the two FV probe sets, 204713_s_at and 204714_a_st, both comprising 11 probes. Analyses of each probe set, as well as of the two probe sets merged (equal to FV) was done. From the GSA-tumor application FV tumor mRNA expression levels distributed across breast cancer subtypes were derived, visualized in boxplot format. To correlate FV tumor mRNA expression levels according to outcome, as well as breast cancer subtypes, Kaplan-Meier plots were derived. 10 years censoring endpoint for all tumors were used as parameters. In addition, queries were run with overall survival (OS), distant metastasis-free survival (DMFS) and relapse-free survival (RFS) as end-point, all with data divided into both median-cut and tertiles. The data was divided according to the FV mRNA expression level of each sample, where in the median-cut query each sample was classified above (high expression) or below (low expression) the median of all tumors within the chosen subgroup. When divided into tertiles, data in each group comprised a third of the observed data. Being the center observation the median is found in the middle of the middle group. The lower cut-point represents the third of the data with lower levels of FV mRNA expression, while the upper cut-point represents the third of the data with higher levels of FV mRNA expression. Multivariate analysis was used to adjust for covariates that may have an effect on outcome. The covariates included were ER-status, node status, histological tumor grade, age and tumor size.

4.1.2 Biological function of FV in breast cancer

FV co-expressed genes and relation to biological function

In an attempt to gain insight in what biological functions the expression of *F5* as well as genes co-expressed with *F5* are related to, the online tools GOBO, DAVID and Gene Ontology (GO) Term Finder of Lewis-Sigler Institute of Princeton University were used.

The expression levels of FV mRNA and the correlation to genes depending on their biological functions where studied. GOBO stratify genes into the following modules according to biological functions: stroma, lipid metabolism, immune response, checkpoint, M-phase, basal, early response and steroid response. The correlation was visualized in a Spearman correlation plot, stating positive or negative correlation.

A list of genes co-expressed with *F5* was derived using the Co-expressed Genes feature of the GOBO database. Pearson Correlation method for ER-negative tumors with correlation cut-off and a standard deviation cut-off at 0,4 were selected. Minimum number of connections was set to 2, but also tested for 3, 4 and 5. Both positive and negative correlations were chosen. The output gene list was used as input in the online functional annotation tool DAVID to identify biological terms associated with these co-expressed genes. The pre-set gene list for *Homo sapiens* was selected. The annotation clustering, chart and table, as well as gene ontology (GOTERM_BP_FAT, GOTERM_CC_FAT and GOTERM_FF_FAT) and pathways (KEGG) were studied. For studies of gene ontology the Genetic Ontology (GO) Term Finder of Lewis-Sigler Institute of Princeton University was used, in addition to DAVID, using GOA – *H. sapiens* (Humans) as annotation and function ontology and process ontology as aspects.

4.1.3 FV mRNA expression levels in cell lines

The GSA-cell line application was used to study FV mRNA expression levels in 51 individual breast cancer cell lines, where the cell lines were grouped according to luminal and basal breast cancer subtypes. FV mRNA expression levels were also studied in breast cancer cell lines and non-breast cancer cell lines from in-house experiments.

II. *In vitro* studies

4.2 Sanger DNA sequencing

Before the FV plasmids and FV plasmid variants were used in functional studies they were sequenced to verify their sequence.

Sanger sequencing is a DNA sequencing method that includes dideoxyribonucleotides (ddNTPs), in addition to deoxynucleotides (dNTPs), primers, DNA template and DNA polymerase. The ddNTPs lack the 3' hydroxyl group, so when DNA polymerase randomly inserts a ddNTP instead of a dNTP the sequence is terminated. This results in fragments of different lengths, with the same 5' end, but varying 3' ends. Each of the four ddNTPs are fluorescently labeled and emits light detectable at different wavelengths (Mathews et al. 2013).

FV plasmids and FV plasmid variants were sequenced using the BigDye Terminator v.3.1 Cycle Sequencing kit. Several primers were designed for the sequencing in order to cover the whole gene (listed in Table 8). One primer was used per reaction. The sequencing reactions were prepared as described in Table 10. The reactions were amplified using the Veriti 96 Well Thermal Cycler and the DNA was cleaned with CleanSEQ. The cleaned DNA was sequenced by capillary electrophoresis using the ABI 3730 DNA analyzer. The sequences were aligned with the NCBI reference sequence for FV (NM_000130.4) using the DNA Sequencing Analysis Software v5.1.

Table 10: Reagents and amounts used in one reaction for Sanger sequencing.

Reagent	**Amount**
Plasmid DNA template (100-300ng)	1 µl
5x Sequencing Buffer	2 µl
BigDye	0,25 µl
Primer (3,2 µM)	1 µl
Nuclease free water	5,75 µl
Total	**10 µl**

4.3 *In vitro* mutagenesis and microbiological techniques

4.3.1 *In vitro* mutagenesis

In vitro mutagenesis was performed to produce a FV wt and specific FV variants for use in functional studies, but first primers specific for each mutation were designed.

In vitro mutagenesis primers

Primers (listed in Table 9) were manually designed according to the specifications of the QuikChange II XL Site-Directed Mutagenesis Kit protocol.

Site-Directed Mutagenesis

The QuikChange II XL Site-Directed Mutagenesis Kit was used according to the protocol provided by the manufacturer. This site-directed mutagenesis method allows for easy editing of a plasmid DNA sequence, to study functional differences of plasmid variants. Purified plasmid DNA from miniprep and maxiprep were used. The process is divided into three steps: Synthesis of mutant strand, *Dpn1* digestion of template, and transformation.

To synthesize the mutant strand a reaction mixture containing template plasmid DNA, specific forward and reverse primers, 10x reaction buffer, dNTP mix, QuikSolution and *PfuUltra* HF DNA polymerase was made and run on the Veriti 96 Well Thermal Cycler according to the program stated in Table 11. The *PfuUltra* HF DNA polymerase has a much lower error rate than other polymerases like *Pfu* and *Taq* polymerase, and the QuikSolution facilitates replication of large plasmids.

Table 11: Cycling parameters for the QuikChange II XL Site-Directed Mutagenesis

Segment	Cycles	Temperature (°C)	Time
1	1	95	1 min
2	18	95	50 sec
		60	50 sec
		68	12 min
3	1	68	7 min

After amplification the reactions were treated with *Dpn1* restriction enzyme. *Dpn1* digests the amplification product on bases of methylation of DNA. The parental plasmid is methylated, while the newly synthesized plasmid is not, hence only the parental plasmid will be digested by the restriction enzyme. The *Dpn1* digested product was then transformed competent cells.

Transformation was performed using both One Shot® TOP10 Chemical Competent *Escherichia coli* (*E.coli*) and XL10-Gold Ultra Competent cells. Chemically competent cells are treated with calcium chloride which during incubation on ice facilitates the plasmid DNA to enter the competent cell by strengthening the interaction between the plasmid DNA and the surface of the competent cell, and by neutralizing the phospholipid bilayer of the competent cell. The heat shock ensures opening of the membrane pores, further facilitating the plasmid DNA to enter the competent cell.

One Shot® TOP10 Chemical Competent *E.coli* bacterial cells were transformed with pcDNA5frt-FV and pMT2-V, and empty vector according to the protocol of the manufacturer. In short, 1 µl of plasmid were added to the competent cells before they were heat shocked and incubated with SOC - medium. The suspensions were spread (20-150 µl) on LB agar plates containing 100 µg/ml ampicillin.

XL10-Gold Ultra Competent cells were used for transformation of FV wt and FV variants, according to the QuikChange II XL Site-Directed Mutagenesis Kit protocol. These cells contain the Hte phenotypes, increasing the transformation efficiency of large plasmid DNA. They are deficient to endonuclease and recombination, ensuring increased miniprep quality and insert stability, respectively. An aliquot of the competent cells got added 2 µl β-mercaptoethanol (β-ME) to further facilitate efficient transformation. In short 2 µl of plasmid DNA were added to the competent cell-β-ME-mixture. After incubation on ice, a heat shock and incubation with NZY+ broth followed before 20-100 µl of the cell suspension was spread on LB agar plates containing 100 µg/ml ampicillin.

Cultivation of transformed cells

Transformed competent cells were the following day cultivated to amplify the amount of plasmid before isolation. Single colonies where picked and cultured in separate tubes with 5 ml LB medium containing 100 µg/ml ampicillin. The cultures were incubated at

220 rpm and 37°C. For isolation using the E.Z.N.A.® Plasmid DNA Mini Kit I Protocol - Spin Protocol the cultures were incubated 16-24 hours, while for isolation using the ZymoPURE™ Plasmid Maxiprep Kit pre-cultures were incubated for 8 hours before the content was transferred to a 500 ml Erlenmeyer flask with 145 ml LB medium containing 100 µg/ml ampicillin and incubated for 16-24 hours.

4.3.2 Nucleic acid isolation and quantification

Isolation of plasmid DNA from transformed competent cells

Both the E.Z.N.A.® Plasmid DNA Mini Kit I Protocol - Spin Protocol (miniprep) and ZymoPURE™ Plasmid Maxiprep Kit (maxiprep) were used to extract and purify plasmids. The intermediates of the *in vitro* mutagenesis were isolated by miniprep, while plasmids used in functional studies were isolated by maxiprep due to higher yields of plasmid DNA. Both protocols follow the same principle where pellet resuspension with a RNase A containing buffer, breaks down present RNA before added lysis buffer denatures the genomic DNA as well as the plasmid. A neutralization buffer renature the plasmid DNA, before the plasmid DNA was separated by force. Consecutive washing steps removed contaminants. Finally, the DNA was eluted. All isolated plasmids were quantified and stored at 4 °C or -80 °C.

The E.Z.N.A.® Plasmid DNA Mini Kit I Protocol - Spin Protocol was performed according to the protocol provided by the manufacturer, with the following adjustments: Cultivated transformed cells were centrifuged at 4500 rcf for 15 minutes at room temperature. The neutralized solution was separated by micro-centrifuge at 12.000 rpm for 10 minutes at room temperature, and the plasmid DNA was eluted in 80 µl nuclease-free water.

The ZymoPURE® Plasmid Maxi prep was performed according to the protocol provided by the manufacturer, with the following adjustments: Cultivated transformed cells were centrifuged at 5000 rpm for 15 minutes at 4°C. The neutralized solution was separated through ZymoPURE™ Syringe Filter with applied force, and the plasmid DNA was eluted in 400 µl ZymoPURE Elution Solution.

RNA and DNA quantification

RNA and DNA yields and purity were determined using NanoDrop according to the instructions provided by the manufacture. Based on the sample absorption of UV light both purity and concentration can be estimated. The RNA or DNA concentrations of the samples were estimated by OD_{260}, since nucleic acids absorb UV light at 260nm. Proteins absorb UV light at 280nm, hence the 260/280 ratios were used to estimate purity of the sample. Pure samples should have a 260/280 ratio of 2,0 and 1,8 for pure RNA and pure DNA, respectively. Lower 260/280 ratios indicates contamination of phenol and/or protein.

4.4 Cell techniques

4.4.1 Cell culturing

All cells were handled with strict aseptic techniques in a laminar flow hood. Medium used for each cell line is stated in Table 12. For long-term storage of cells in liquid nitrogen, 5% DMSO in 10% Fetal Bovine Serum (FBS) DMEM was used. Nunc™ Cell Culture Treated Flasks were used for culturing, and cells were incubated at 37°C with 5% CO_2 in a Steri-Cycle CO_2. For visualization a Nikon Eclipse TE 300 microscope was used.

Table 12: Cell culture mediums for each cell line, with supplements and splitting ratios.

Cell line	Medium + supplements	Splitting ratio	Trypsin incubation time
HEK 293T	DMEM + 10% FBS	1:6	2 min
MDA-MB-231	DMEM + 10% FBS	1:5	3,5 min

When reached a confluence of 80-90% the cells were subcultured according to the radios provided in Table 12. In short, the medium was removed and the cells were gently washed with Dulbecco's Phosphate Buffered Saline (DPBS) to remove any traces of FBS. FBS inactivates trypsin, hence before cells are detached by addition of trypsin it is important that there are no FBS left. The trypsinated cells were incubated (see Table 12) to ensure detachment. Due to the toxic effect of trypsin, promptly addition of

medium (see Table 12) followed by pipetting to ensure homogenized cell culture was performed. The suspended cell culture was moved to a new Nunc™ Cell Culture Flask with additional fresh medium before incubation. Alternatively, the suspended cell culture was moved to appropriate tube for cell counting, see section 4.4.2.

All cell lines used were tested for mycoplasma contamination according to the MycoAlert™ Assay Control Set (Lonza) kit and protocol. Only cells negative for mycoplasma were used in this study.

4.4.2 Cell quantification

To count cells the NucleoCounter® NC-100™ was used as described by the manufacture. To an aliquot of the cell suspension an equal amount of Reagent A100 and Reagent B was added, mixing between each step. The sample was introduced to the NucleoCassette which was launched into the NucleoCounter® NC-100™, in order to count the cells. Reagent A100 ensures enzymatic lysis of the cells for easy accessible nuclei. Reagent B stabilizes the nuclei as well as raising the pH for optimized fluorescence of the propidium iodide. When the nuclei pass through the cannels of the NucleoCassette the fluorescent dye propidium iodide, found on the inside of the NucleoCassette, tags to the nuclei. During the measurement, the nuclei bound propidium iodide excite, and a total cell count can be estimated.

4.4.3 Transient transfection

The process resulting in genetically modified cells is known as transfection. The isolated plasmids were transiently transfected into HEK293T and MDA-MB-231 cell lines to study expression- and functional effects of FV overexpression.

Optimization of plasmid transfection

Optimal properties for transient transfection from previous experiments in the research group were tested and found to be suitable for FV overexpression in HEK293T and MDA-MB-231 cells. This included the amount of plasmid DNA (2,5 µg) and the ratio between plasmid DNA and Lipofectamine 3000 (1 µg : 1,5 µl) used per reaction. To find optimal cell confluence before transfection, two amounts of HEK293T ($6{,}0 \times 10^5$ and $7{,}0 \times 10^5$) and MDA-MB-231 cells ($2{,}8 \times 10^5$ and $3{,}5 \times 10^5$) were seeded in 6-well dishes.

The following day the confluence of the cells were determined by visual verification using the Nikon Eclipse TE 300 microscope.

FV overexpression in HEK293T cells

FV wt, FV variants, pMT2-V, pcDNA5frt-FV and empty vector were transfected into HEK293T cells to study the effects of FV overexpression at FV mRNA and FV protein levels, as well as cell growth, according to Lipofectamine® 3000 Reagent Protocol. In short, 6,0 x 10^5 cells were seeded in a 6-well dish the day before transfection. The next day, the cells were transfected with the respective plasmid using 2,5 µg plasmid DNA and 7,5 µl Lipofectamine3000® (1:1,5 ratio) per reaction, diluted in OptiMEM. The DNA-lipid mixture was incubated at room temperature for 5 minutes, then evenly spread in each well containing cells with fresh medium. Medium was not changed after transfection, due to the HEK293T cells easy detachment. Media and lysates were harvested for expression- and functional studies 24 - 96 hours after transfection.

FV overexpression in MDA-MB-231 cells

FV wt, FV variants, pcDNA5frt-FV and empty vector were transfected into MDA-MB-231 cells to study expression- and functional effects of FV overexpression, according to Lipofectamine® 3000 Reagent Protocol. In short, 2,8 x 10^5 cells were seeded in a 6-well dish the day before transfection. The next day, the cells were transfected with the respective plasmid using 2,5 µg plasmid DNA and 7,5 µl Lipofectamine3000® (1:1,5 ratio) per reaction, diluted in OptiMEM. The DNA-lipid mixture was incubated at room temperature for 5 minutes, then evenly spread in each well containing cells with fresh medium. Medium was removed and replaced by fresh medium 5 hours after transfection. Media and lysates were harvested for expression- and functional studies 1 - 96 hours after transfection.

4.4.4 Harvest of media and cells

Media was harvested by careful aspiration, and immediately placed on ice. After removal of media, cells harvested for RNA isolation were washed once with cold PBS and lysed in 300-600 µl Lysis/binding buffer. Cells harvested for total protein were washed gently three times with cold PBS and lysed in 300 µl RIPA buffer with inhibitor cocktail (ratio inhibitor cocktail 1: 100 RIPA buffer) and incubated on ice for 5 minutes, before the cells were scraped and collected. The harvested RNA and protein lysates were stored at -20

°C. Before the samples were used for protein techniques media were vortexed and centrifuged at 3500 rpm for 10 minutes at room temperature and protein lysates were vortexed and centrifuged at 8000 x g for 10 minutes at 4 °C.

Total RNA isolation

For total RNA isolation of cell lysates RNAqueous® Kit was used, according to the manufacture protocol. In short, the cell lysates were diluted in a 64% ethanol buffer optimizing the conditions for RNA to bind to the glass filter column, before the solution was forced through the column by centrifugation (12 000 rpm for 30 seconds). By three consecutive washing steps contaminants were extracted from the glass filter. The column was centrifuged to ensure removal of wash buffers, before the RNA was eluted twice (50 µl + 20 µl) with 70 °C elution solution. The isolated RNA samples were stored at -20 °C for further use.

4.4.5 cDNA synthesis

Isolated RNA was reverse transcribed to complementary DNA (cDNA) prior to real time quantitative reverse transcriptase polymerase chain reaction (real-time qRT-PCR), using the High-Capacity cDNA Reverse Transcription Kit according to the protocol provided by the manufacture. The cDNA reaction mix was prepared as described in Table 13, where the Random Primers binds to the RNA template, and are extended with dNTPs by the Reverse Transcriptase. Within each run all reactions had the same RNA input (479,5 ng - 2450 ng). The reaction mix was added to RNA containing wells, the plate was sealed and centrifuged before run on the 2720 Thermal Cycler according with the program in Table 14.

Table 13: Composition of cDNA reaction mix. Reagents needed for one reaction.

Reagent	Volume (µl)
10x RT Buffer	2,5
25x dNTP Mix (100mM)	1
10x RT Random Primers	2,5
MultiScribe™ Reverse Transcriptase	1,25
Nuclease-free water	5,25
RNA (same input per run)	12,5
Total	**25**

Table 14: Program for cDNA synthesis on the 2720 Thermal Cycler

	Step 1	Step 2	Step 3	Step 4
Temperature (°C)	25	37	85	4
Time	10 min	120 min	5 min	∞

4.4.6 Real time qRT-PCR

To measure and compare mRNA expression levels, real-time qRT-PCR was performed. In this thesis a two-step qRT-PCR was used. The first step was reverse transcription of RNA, as described in section 4.4.5. The second step, the PCR step, was in this thesis performed using TaqMan chemistry.

Real-time qRT-PCR is based on the principle of real time quantification of amplified cDNA product by the use of fluorescent emitted light. The DNA polymerase extends the primers, but also cleaves the TaqMan® probe. Cleavage of the TaqMan® probe results in separation of the reporter dye at the 5' end and a quencher dye at the 3' end (Figure 11A). When in close proximity they share energy transfer lowering the emitted fluorescent light of the reporter, but when separated the fluorescent light emitted increases, and can after each PCR cycle be detected real time by a camera. The number of target templates increases logarithmically, and the fluorescent light detected is proportional to the amount of target template. During the base line phase, an increase in fluorescent light cannot be detected, due to the emitted fluorescence light being under the detection limit of the detector (Figure 11B). In the exponential phase the number of

templates have increased and a steady increase of fluorescent light is detectable. The higher the number of target templates present from start, the less cycles are needed to reach the exponential phase. After a certain amount of cycles the fluorescent intensity reaches a given threshold (Figure 11B). This cycle number called the Ct value, is used in the calculations of relative quantity (RQ), according to the formulas below:

ΔC_T = C_T target gene - C_T endogenous control

$\Delta\Delta C_T$ = ΔC_T target sample - ΔC_T calibrator sample (reference in the experiment)

$RQ = 2^{-\Delta\Delta CT}$

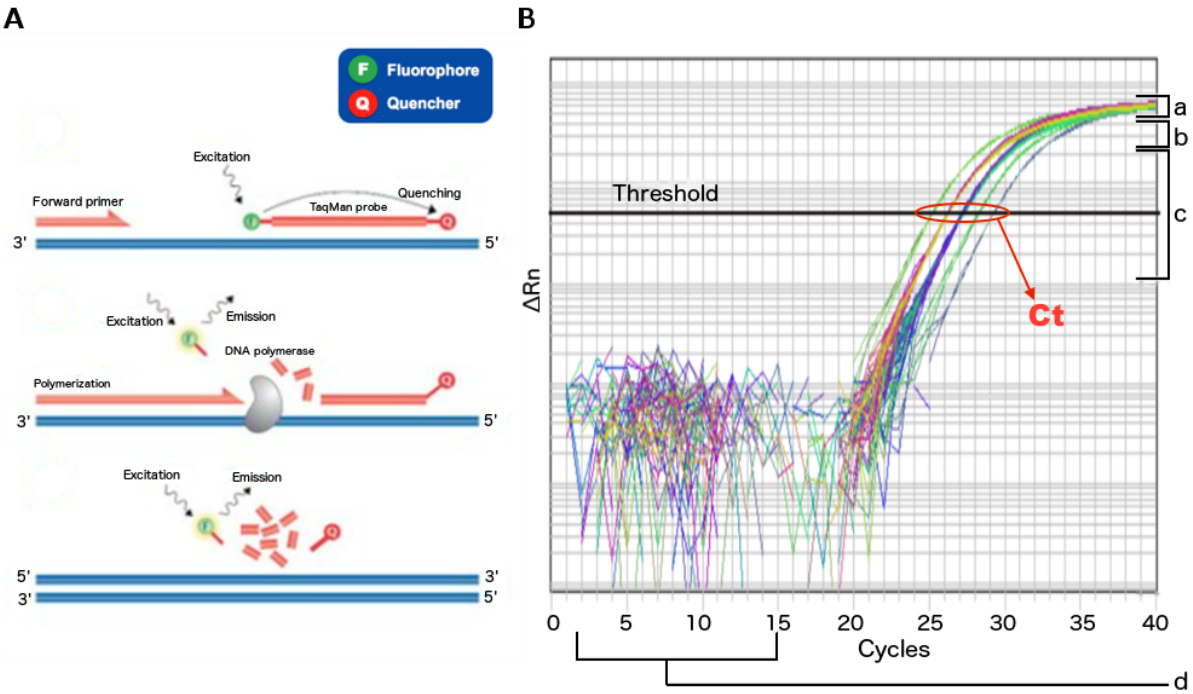

Figure 11: (A) Principle of real-time qRT-PCR. The TaqMan probe anneals to the target template between two primers. In the process of extension the DNA polymerase cleaves the TaqMan probe, resulting in emission of fluorescent light by the reporter dye (modified from http://www.garvan.org.au/research/capabilities/molecular-genetics/realtime-pcr-probes). (B) qRT-PCR amplification plot, (a) plateau phase, (b) linear phase, (c) exponential phase, (d) base line phase. Black horizontal threshold-line and Ct-values are indicated (modified from TaqMan® Gene Expression Master Mix Protocol).

The TaqMan® Gene Expression Master Mix was used according to the protocol provided by the manufacturer. The reactions were prepared on a 96-well plate according to Table 15. The cDNA was diluted in nuclease-free water to contain 90-329 ng of template, the

same amount of cDNA was used for all samples within each set up. FV and RPLP0 TaqMan assays were used. The endogenous control (RPLP0) was included to adjust for variations in RNA input and/or reverse transcriptase efficiency. The expression of the endogenous control should be equal in all samples, like a housekeeping gene, and should be unaffected by different stimuli. Also, a non-template control (NTC), where nuclease-free water substituted the cDNA template was included for verification of contamination-free assay master mixes. 10 µl of each sample reaction was added in triplicates to a 384-well plate. The plate was sealed and centrifuged at 1500 rpm for 3 minutes at room temperature before run on the ABI Prism 7900HT Sequence Detection System or the QuantStudio 12k Flex with the program described in Table 16.

Table 15: Composition of reaction mix for real-time qRT-PCR for FV overexpression. With reagents needed for one real-time qRT-PCR reaction

Reagent	**Volume (µl)**
TaqMan® Gene Expression Master Mix	5,0
Assay (FV or RPLP0)	0,5
cDNA (nuclease free water for NTC)	4,5
Total	**10**

Table 16: Program for qRT-PCR run on ABI Prism 7900HT Sequence Detection System or the QuantStudio 12k Flex

	Step 1	**Step 2**	**Step 3 (40 cycles)**	
Cycle	-	-	Part 1	Part 2
Temperature (°C)	50	95	95	60
Time	2 minutes	10 minutes	15 seconds	1 minute

TaqMan® Low Density Array Human Immune Panel was used to measure the mRNA expression levels of the human inflammatory markers IL6, IL8 and GM-CSF, according to the protocol provided by the manufacturer. The reaction mix was made according to Table 17 containing the TaqMan® Gene Expression Master Mix with FV or RPLP0 assay, and cDNA diluted in accordance with 200 ng of RNA. 100 µl were added to each reservoir, in duplicates, of the microplate precoated with the inflammatory markers of

interest. The microplate was centrifuged at 1200 rpm for 2 x 1 min, and sealed before run on the QuantStudio 12k Flex with the program described in Table 16.

Table 17: Composition of reaction mix for real-time qRT-PCR for immunogene expression. With reagents needed for one real-time qRT-PCR reaction.

Reagent	**Volume (µl)**
TaqMan® Gene Expression Master Mix with assay	50
cDNA (200ng)	6
Nuclease free water	44
Total	**100**

4.5 Protein techniques

4.5.1 Total protein quantification

The total protein levels in the cell lysates (harvested as described in 4.4.4) was measured using PIERCE® BCA Protein Assay Kit. In the first step, proteins form a chelate complex with Cu^{2+} ions (green) in an alkaline environment, and the Cu^{2+} ions are reduced to Cu^{1+}. Further, the bicinchoninic acid (BCA) reacts with the Cu^{1+} ions (purple). This BCA-Cu^{1+} complex absorbs UV light at 570nm proportional to the amount of protein in the samples.

A standard dilution series of 2x Albumin constituting six dilutions with concentrations of 0-2 mg/ml was used. 5 µl of each sample as well as each standard were plated to a 96-well flat bottom plate in triplicates. 200ul BCA working reagent (reagent A and B in a B 1:50 A ratio) was added to each well before the plate was mixed on shaker for 30 seconds. The plate was incubated at 37°C for 30 minutes, before absorbance was measured at 570nm using the VersaMax microplate reader. The total protein concentration of each sample was determined from the standard curve using the SoftMax Pro6.4 software.

4.5.2 Enzyme-linked immunosorbent assay (ELISA)

Enzyme linked immunosorbent assay (ELISA) is used to determine the presence and concentration of specific analytes in a solution. Sandwich ELISA, which was used in this thesis, is based on the principle of an analyte being 'sandwiched' between two antibodies. The microplate wells are coated with the primary antibody, which captures the analyte before the enzyme-coupled secondary antibody binds to the analyte. By adding a substrate the enzyme-linked secondary antibody will produce a product that absorbs light at a specific wavelength (Lea 2013; Thermo Fisher Scientific Inc. 2011).

FV ELISA

The ZYMUTEST Factor V, which detects human FV antigen, was used to determine the concentration of FV present in media and protein lysates (harvested as described in 4.4.4) according to the protocol from the manufacturer. A standard curve with known concentrations of FV ranging from 0-100% as well as controls with known FV concentrations were used. The microwell plate was coated with a monoclonal antibody specific for FV. The test samples were added and the plate incubated before the polyclonal secondary antibody with horse-radish-peroxidase (HRP) attached was added. After incubation, the substrate tetramethylbenzidine (TMB) with hydrogen peroxide was added to develop a blue color. After a 10 minute incubation the color reaction was stopped by addition of sulfuric acid. Absorbance was measured at 450nm using the VersaMax microplate reader. The concentrations were determined from the standard curve using the SoftMax Pro6.4 software.

ELISA for human inflammatory markers

The Human Inflammatory Cytokines & Chemokines Multi-Analyte ELISArray Kit was used for screening of inflammatory markers, according to the protocol from the manufacturer. This ELISA detects the 12 pro-inflammatory chemokines IL1A, IL1B, IL2, IL4, IL6, IL8, IL10, IL12, IL17A, IFNγ, TNFα, GM-CSF. The microwell plate was coated with 12 target-specific antibodies. The test samples were added and the plate incubated, before the labeled secondary antibody and the avidin-HRP conjugate were added. When the substrate was added, a blue color developed. After 15 minutes incubation the stop solution was added and the absorbance was measured at 450 nm using the VersaMax microplate reader. Data was obtained using the SoftMax Pro 6.4 software.

4.5.3 Western blotting

Western blotting is used to detect specific proteins and protein variants in a semi-quantitative manner by three steps: size-dependent separation by gel electrophoresis, transfer of the separated proteins to a protein-binding membrane (blotting) and detection of proteins with specific antibodies. A primary antibody specific for the target protein followed by an enzyme-linked secondary antibody (specific only for the primary antibody and not for the target protein) is used. The enzyme-linked secondary antibody will after addition of a chemiluminescent agent together form a product that with a detection system can be visualized, where light emitted will be proportional to amount of target protein (Mahmood & Yang 2012).

Gel electrophoresis

Recombinant FV and FVa as well as media from transfected cells were added to the gel with equal FV protein amounts for each blot (FV protein levels in media were corrected for variation of total protein in respective lysates). Dilutions were done in PBS. Sample buffer (0,5x of the total amount loaded onto the gel) were added to each reaction before the samples were boiled for 5 min at 97°C to denature the higher order structure of the proteins and to give them an equal overall charge. The samples and ladder (Precision Plus Protein ™ Dual Color Standards) were loaded onto the gel (7,5% Mini-protean® TGX™ Gels) placed in a tank with 1x Running Buffer. The gel was exposed to 185 Volts for 45 minutes, in order to separate the proteins by size.

Blotting

The blotting was performed in a wet manner, transferring the gel to a nitrocellulose membrane before it was sandwiched between a double layer of filter paper and a sponge. The sandwich was placed in a tank with blotting buffer and a ice-block. The proteins were transferred to the membrane by exposure of 100 Volts for 25 minutes, with constant magnetic stirring.

Detection of target protein

To prevent unspecific binding of the primary antibody, the unoccupied sites of the membrane were blocked in 5% BSA in 1xTBST with shaking for 60 minutes. The blot was washed with 1xTBST (3 x 5 min) before incubation with the monoclonal primary

antibody with constant rolling overnight at 4°C. Unbound antibody was removed by washing the membrane with 1xTBST (3 x 10 min), before the HRP conjugated polyclonal secondary antibody was added (antibodies listed in Table 6). Further, the membrane and secondary antibody were incubated rolling for 1 hour at room temperature. Washing with 1xTBST (3 x 10 min) were performed before the membrane was made ready for visualization. By introducing the Amersham™ ECL™ Prime Western Blotting Detection Reagent to the membrane a chemical reaction with HRP labeled secondary antibody results in emitting of light. The Image quant LAS 4000 Imager was used for visualization and the Image QuantTL software was used to quantify the amount of target protein present.

4.6 Functional assays

4.6.1 Cell growth

The effects of FV overexpression in FV wt and FV variants on growth in MDA-MB-231 and HEK293T cells were studied. The Cell Proliferation Agent WST-1 was used for this purpose, according to the protocol provided by the manufacturer. WST-1, a tetrazolium salt, is cleaved by mitochondrial dehydrogenases present in the test sample, to form formazan dye. Higher amounts of living cells give higher amounts of mitochondrial dehydrogenases which results in higher amounts of formazan formed. The amounts of formazan present in the test samples are proportional to the amounts of living cells.

In short, 20 µl WST-1 was added to each well containing cells transfected with the FV wt, FV variants, empty vector as well as non-transfected cells, at 1-96 hours after transfection. After 30 minutes incubation at 37°C the absorbance was measured at 450nm using the VersaMax microplate reader. Data was obtained using the SoftMax Pro6.4 software.

4.6.2 Cell death

The effects of FV overexpression in FV wt and FV variants on cell death in MDA-MB-231 cells were studied in fresh media and lysates by measuring the amount of DNA fragmentation in the cells using the Cell Death Detection ELISA^{PLUS} kit. The method was conducted as described in the protocol provided by the manufacturer, except for

preparation of the samples, which was performed as described in section 4.4.4. The assay detects low-molecular weight DNA fragments generated during cell death. The apoptotic effect was quantified in the lysates, while the necrotic effect was quantified in the media. Quantification of both types of cell death is in this method based on antibody-binding of histone-complexed DNA fragments present during both apoptosis and necrosis.

The microplate was precoated with streptavidin that binds to the biotin in the anti-histone biotin antibody. This antibody binds to histones present in the test sample, while the HRP-labeled secondary antibody specifically binds to single- and double-stranded DNA in the test sample. When the ABTS substrate was added a color developed. The reaction was stopped after 10 minutes and the absorbance was measured at 405nm with the VeraMax microplate reader.

4.7 Statistical analysis

For statistical analysis in this thesis the unpaired t-test was used to compare samples in two groups. The datasets were assumed normally distributed and independent of each other. A probability value of $P \leq 0{,}05$ was considered significant. Significance between the FV wt and the FV variants in cell growth and cell death experiments were calculated by this method.

5. Results

I. Clinical studies of FV

5.1 FV expression in breast cancer

Using data from OsloII, GOBO and in-house experiments FV expression was studied. In addition to FV expression levels in relation to breast cancer subtypes, the data was used to study possible associations to survival as well as biological functions.

5.1.1 FV mRNA expression across breast cancer subtypes

FV tumor mRNA expression and FV plasma protein levels across breast cancer subtypes

To study variances of FV expression levels the patient data was distributed according to tumor characteristics and breast cancer molecular subtypes, both for FV mRNA expression levels and FV protein plasma levels. When the data from the OsloII material was distributed across tumor characteristics the FV mRNA expression level was significantly increased in patients with ER-negative tumors (P = 0,023), PR-negative tumors (P = 0,019), HR-negative tumors (P = 0,028), triple negative (P = 0,041), and in tumors with *TP53* mutant (P = 0,001) (Table 18). The FV mRNA was also increased in patients with HER2-positive tumors, though not significant (P = 0,071). There were no significant differences in FV tumor mRNA expression levels according to grade, tumor size or lymph node spread (Table 18). A significant difference was seen for FV tumor mRNA levels across molecular breast cancer subtypes (P = 5,9e-5) (Figure 12A), with higher levels in HER2-enriched tumors and lowest in luminal A tumors.

From the pooled dataset of GOBO FV mRNA expression levels were significantly increased in ER-negative tumors (P <0,00001) (Figure 13A), and in tumors of higher grade (P = 0,00165) (Figure 13B). When distributed across molecular subtypes, FV tumor mRNA expression levels were significantly increased in the aggressive basal-like and HER2-enriched subtypes (P <0,00001) (Figure 13C).

FV plasma protein levels were not available through GOBO, but from the OsloII material a significant increase was seen in patients with tumors of higher grade (P = 0,028), while FV plasma protein levels were similar between all other subtypes, as defined in Table 18. The levels of FV plasma protein were not significantly different across the molecular subtypes (P = 0,854) (Figure 12B).

There is a tendency of increased FV mRNA levels in ER-negative tumors, basal-like tumors, HER2-enriched tumors and tumors of high grade, characteristics associated with more aggressive tumors. The same trends were not seen for FV plasma levels, though increased FV plasma levels were seen in tumors of high grade.

Table 18: Mean values for FV mRNA expression in tumors and FV protein levels in plasma according to breast cancer tumor characteristics from the OsloII material, with corresponding *P*-values. Significant *P*-values are underlined. ◆boarder line.

		mRNA expression (tumor)		Protein levels (plasma)	
Characteristic	**Groups**	**FV**	***P***	**FV**	***P***
ER status	Positive	-0,029	<u>0,023</u>	79,02	0,236
	Negative	0,147		75,77	
PR status	Positive	-0,035	<u>0,019</u>	78,47	0,957
	Negative	0,110		78,35	
HER2-status	Positive	0,138	<u>0,071</u>◆	76,59	0,574
	Negative	-0,003		78,61	
Grade	G1+G2	-0,009	0,223	80,74	<u>0,028</u>
	G3	0,059		75,61	
T-status	T1	-0,024	0,159	79,22	0,366
	T2+T3	0,003		77,19	
HR neg	Yes	0,036	<u>0,028</u>	76,15	0,328
	No	-0,024		78,91	
Triple-negative status	Yes	0,041	<u>0,041</u>	75,82	0,309
	No	-0,023		78,86	
Lymphnode spread	Yes	-0,016	0,708	78,86	0,678
	No	-0,008		77,87	
TP53 status	Wild type	-0,036	<u>0,001</u>	77,06	0,307
	Mutant	0,032		73,78	

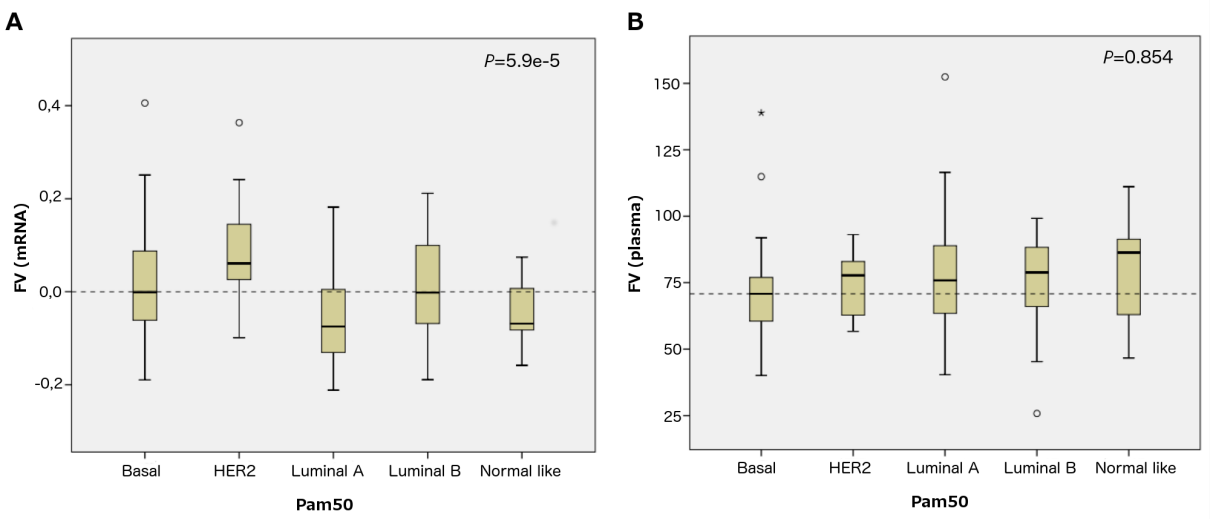

Figure 12: FV expression levels across breast cancer subtypes in the OsloII material. Box and whisker plots representing the distribution of (A) log10 transformed FV mRNA expression levels in tumors (n = 151) and (B) distribution of FV protein levels in plasma in breast cancer patients (n = 358).

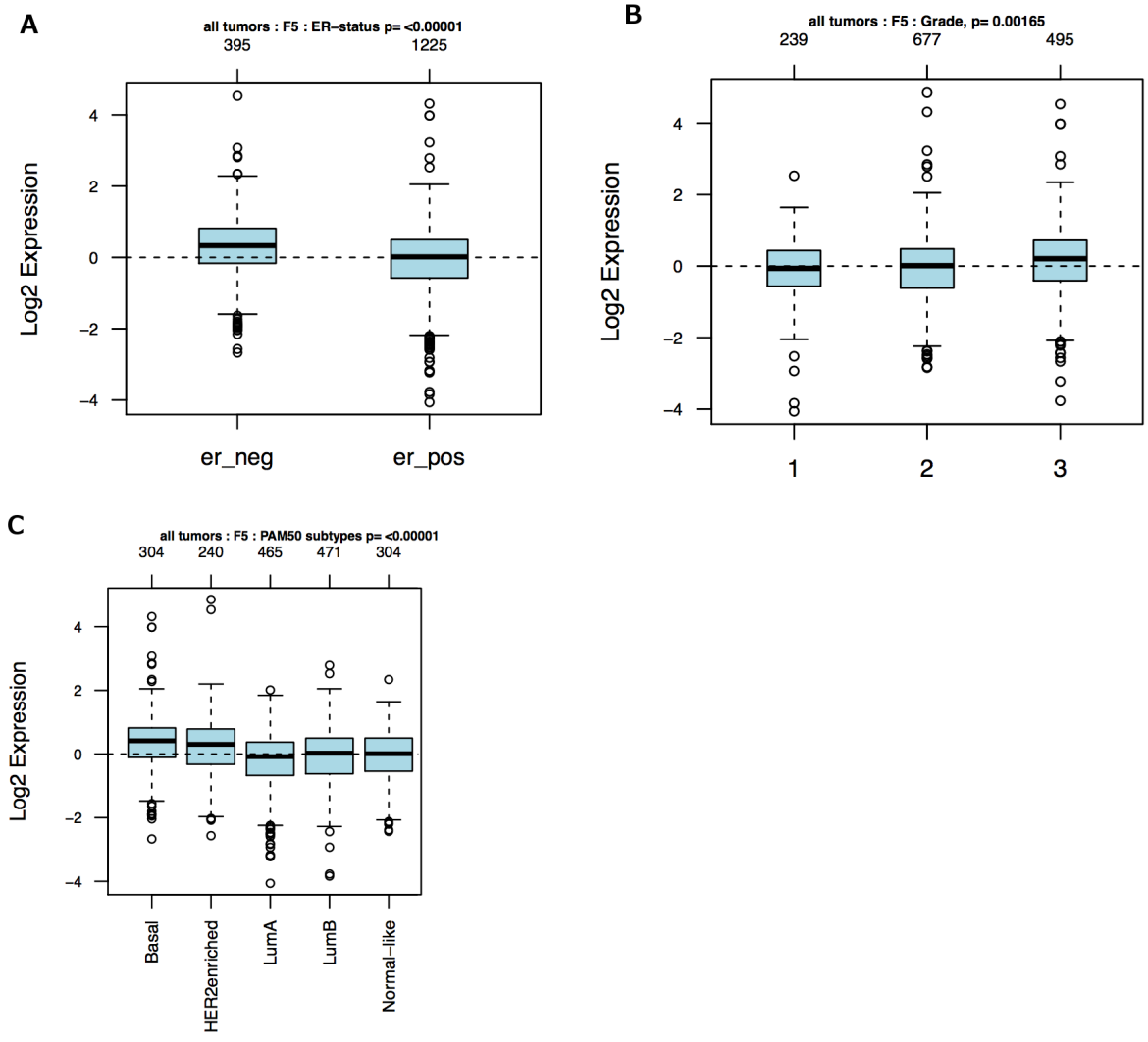

Figure 13: Tumor mRNA expression levels of FV across breast cancer tumor subtypes in the pooled GOBO dataset. Box and whisker plots showing log2 transformed FV mRNA expression levels in tumors stratified according to (A) ER-status, (B) histological tumor grade and (C) PAM50 subtypes. Data obtained using GOBO (Borg et al. 2011)

5.1.2 FV tumor mRNA expression and association to survival

From the data available for the 1881 breast cancer patients in the GOBO database, FV mRNA expression levels in tumors and possible associations to survival were studied in the different breast cancer tumor subtypes (Table S 1). Data were not available for the OsloII material. Survival was defined in subgroups of overall survival (OS), distant metastasis-free survival (DMFS) and relapse-free survival (RFS).

No significant associations between FV mRNA expression in all tumors and OS, DMFS or RFS were seen (Table S 1). Low mRNA expression levels of FV showed significant association with reduced survival for OS (P = 0.0345), DMFS (P = 9e-5) and RFS (P = 0.00837) in basal tumors (Figure 14A). Moreover, low expression levels of FV mRNA in ER-negative tumors showed significant association with reduced DMFS (P = 0.00028) (Figure 14B). Significant association between low levels of FV mRNA and reduced DMFS in grade 3 tumors was found when the data was divided into tertiles (P = 0.02549) (Figure 14C), though no significant association when divided into median-cut (Table S 1, P = 0.14839)

To adjust for other factors with potential effect on survival multivariate hazard ratio analyses with the covariates ER-status, lymph node status, histological tumor grade, age and tumor size were performed. Low FV tumor mRNA expression was an independent predictor of OS, DMFS and RFS in basal tumors (Figure 15A), for DMFS in ER-negative tumors (Figure 15B) and for DMFS in grade 3 tumors (Figure 15C). None of the other covariates showed independently significance as predictor of survival in the subgroups studied (Figure 15A-C).

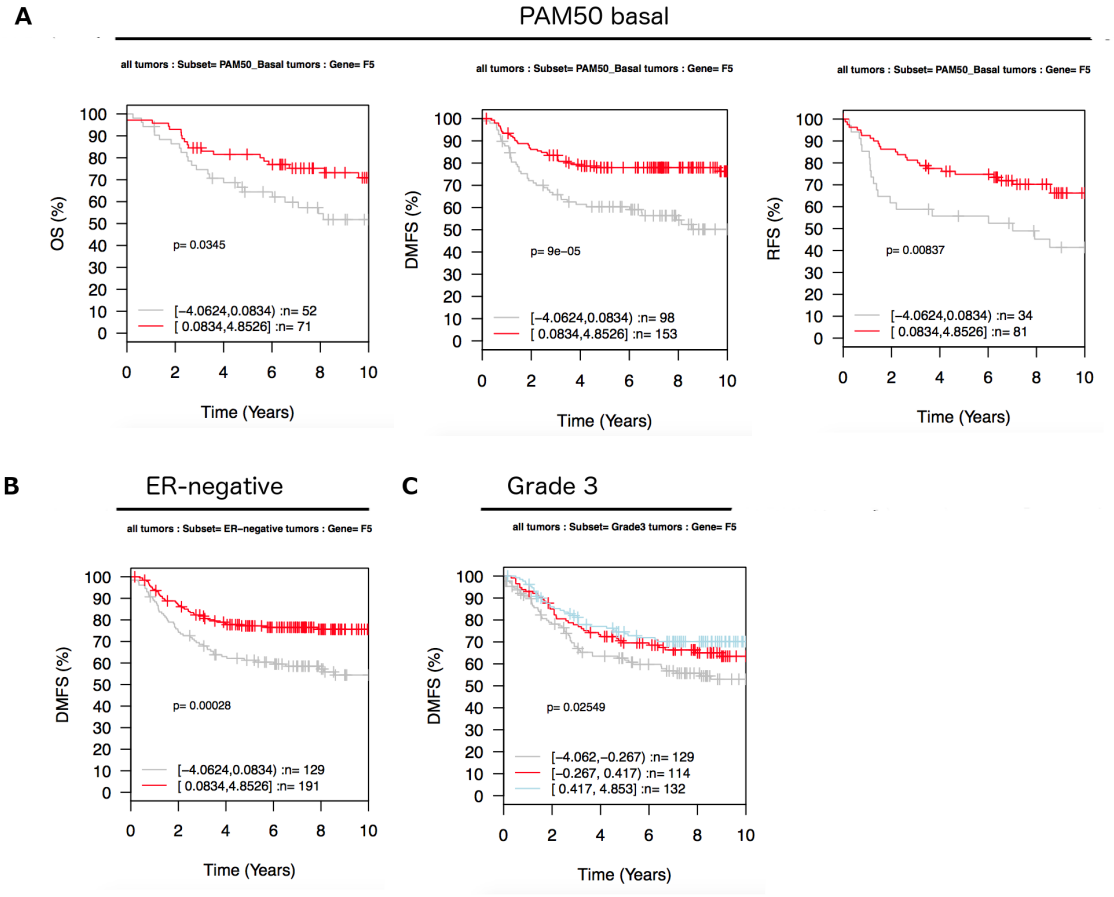

Figure 14: FV mRNA expression levels across selected breast cancer subtypes and their association to survival. FV mRNA expression levels and correlation to survival when tumors are distributed across breast cancer subtypes. Kaplan-Meier plots with 10 year censoring as endpoint in (A) PAM50 basal tumors with overall survival (OS), distant metastasis-free survival (DMFS) and relapse-free survival (RFS), (B) ER-negative tumors and (C) grade 3 tumors, both with distant metastasis-free survival (DMFS). All stratified by high (above median) and low (below median) FV mRNA tumor expression levels, except Grade 3 tumors which were divided into tertiles. Corresponding *P*-values for each plot are indicated. Data obtained using GOBO (Borg et al. 2011)

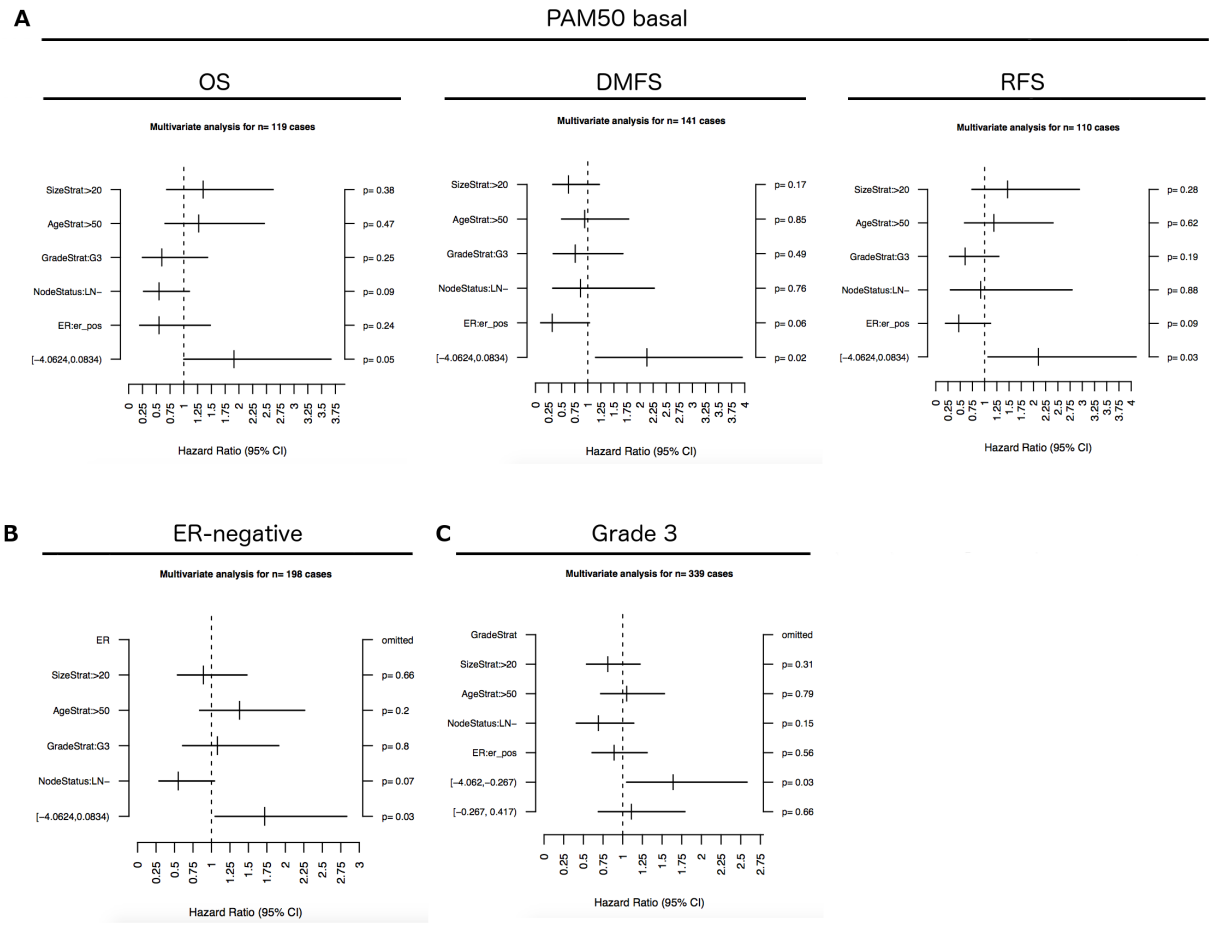

Figure 15: Multivariate hazard ratio analysis in breast cancer patients stratified by FV mRNA expression levels in breast cancer tumor subtypes. Multivariate hazard ratio analysis indicating the potential effect of covariates on FV mRNA expression and survival in (A) PAM50 basal tumors with overall survival (OS), distant metastasis-free survival (DMFS) and relapse-free survival (RFS), and (B) ER-negative tumors and (C) grade 3 tumors, both with distant metastasis-free survival (DMFS). Tumor size, age, histological grade, lymph node status and ER-status were included as covariates. Hazard ratios, 95%CIs and corresponding *P*-values are given for each covariate. Each multivariate survival analysis plot corresponds to the Kaplan-Meier plots in **Figure 14**. Data obtained using GOBO (Borg et al. 2011).

5.1.3 Biological function of FV in breast cancer

Correlation between mRNA expression levels of FV and co-expressed gene modules

To get an insight of the possible biological functions of FV in breast cancer, the correlation between FV mRNA expression levels and co-expressed gene modules mimicking biological processes were studied. FV mRNA expression was positively correlated to genes which functions within immune response, checkpoint and early

response, and negatively correlated to genes in the steroid response module (Figure 16). The strongest correlation of FV mRNA expression levels was to genes in the steroid response and immune response modules (*P*-value < 1e-10) (Figure 16), where the correlation to immune response gene module was the strongest. FV mRNA expression levels showed a lower but significant correlation to genes in the early response-module (*P*-value < 0.05), and a slightly higher correlation to genes in the checkpoint module (*P*-value < 0.00001) (Figure 16). No significant correlation between FV mRNA expression levels and genes which biological function related to lipid metabolism-, M-phase-, basal- or stroma- modules were found (Figure 16).

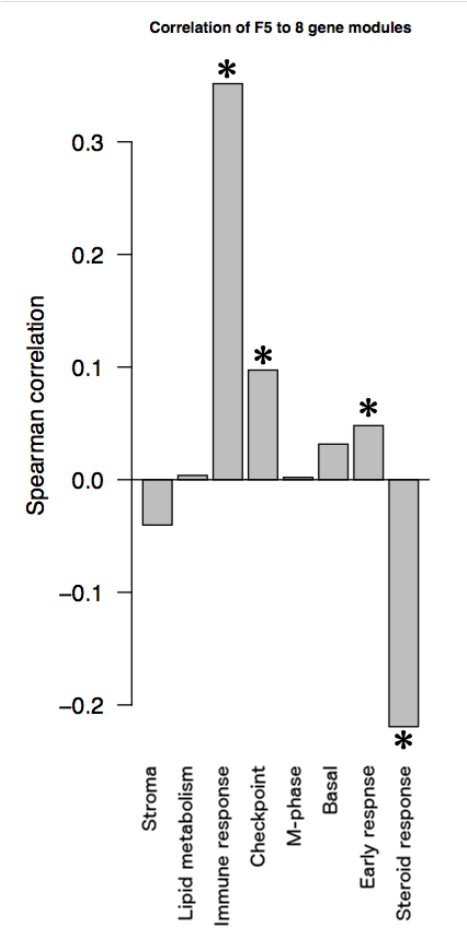

Figure 16: FV mRNA expression levels and correlation to co-expressed gene modules. FV mRNA expression levels and correlation to gene modules according to biological function. Expressed as Spearman correlation plot. **P* ≤ 0,05. Data obtained using GOBO (Borg et al. 2011)

Moreover, according to the Co-expressed Genes feature of the GOBO database, 36 genes are co-expressed with *F5* (Table 19).

Table 19: Genes co-expressed with *F5* in the pooled GOBO dataset.

GeneSymbol	Correlation	STDdev	GeneName	EntrezID
F5	1	0.878	coagulation factor V (proaccelerin, labile factor)	2153
PTPN7	0.46	0.745	protein tyrosine phosphatase, non-receptor type 7	5778
CLEC1A	0.458	0.649	C-type lectin domain family 1, member A	51267
MOSPD2	0.449	0.462	motile sperm domain containing 2	158747
RNASEH2B	0.448	0.478	ribonuclease H2, subunit B	79621
STAT4	0.438	0.595	signal transducer and activator of transcription 4	6775
SH2D2A	0.434	0.686	SH2 domain protein 2A	9047
FCRL2	0.432	1.165	Fc receptor-like 2	79368
PRKCQ	0.431	0.779	protein kinase C, theta	5588
CPA2	0.429	0.576	carboxypeptidase A2 (pancreatic)	1358
ERAP1	0.422	0.493	endoplasmic reticulum aminopeptidase 1	51752
TBC1D2B	0.421	0.492	TBC1 domain family, member 2B	23102
G6PC2	0.42	0.597	glucose-6-phosphatase, catalytic, 2	57818
KLRG1	0.42	0.524	killer cell lectin-like receptor subfamily G, member 1	10219
PIK3R5	0.419	0.533	phosphoinositide-3-kinase, regulatory subunit 5	23533
CD96	0.417	1.001	CD96 molecule	10225
SLAMF1	0.417	0.716	signaling lymphocytic activation molecule family member 1	6504
C11orf20	0.414	0.843	chromosome 11 open reading frame 20	25858
CD3E	0.414	0.781	CD3e molecule, epsilon (CD3-TCR complex)	916
A1CF	0.413	0.591	APOBEC1 complementation factor	29974
PZP	0.413	0.869	pregnancy-zone protein	5858
SELP	0.412	0.732	selectin P (granule membrane protein 140kDa, antigen CD62)	6403
IL2RB	0.411	0.64	interleukin 2 receptor, beta	3560
MCTP2	0.411	0.713	multiple C2 domains, transmembrane 2	55784
TAS2R1	0.409	1.088	taste receptor, type 2, member 1	50834
NCKAP1L	0.407	0.879	NCK-associated protein 1-like	3071
C11orf21	0.406	0.688	chromosome 11 open reading frame 21	29125
BMP15	0.405	0.646	bone morphogenetic protein 15	9210
AIM2	0.404	0.899	absent in melanoma 2	9447
SLC6A12	0.403	0.716	solute carrier family 6 (neurotransmitter transporter, betaine/GABA), member 12	6539
XKR8	0.403	0.46	XK, Kell blood group complex subunit-related family, member 8	55113
ATP2A1	0.402	1.006	ATPase, Ca++ transporting, cardiac muscle, fast twitch 1	487
GRM6	0.402	0.78	glutamate receptor, metabotropic 6	2916
LY6G5C	0.402	0.606	lymphocyte antigen 6 complex, locus G5C	80741
MAP4K1	0.402	0.742	mitogen-activated protein kinase kinase kinase kinase 1	11184
FASLG	0.401	0.633	Fas ligand (TNF superfamily, member 6)	356

Genes co-expressed with *F5* and their correlation to gene ontology (GO) terms and pathways

The genes co-expressed with *F5* outlined in Table 19 where analyzed with the online bioinformatics gene annotation tool DAVID. Several *F5* co-expressed genes were found

to be related to different enriched biological terms (GO terms) and pathways. *SELP, PRKCQ, CD3e, ERAP1* and *SH2D2A* were recurring *F5* co-expressed genes that showed association to GO terms related to biological process or molecular function (Table 20 & Table 21). Though, according to DAVID the molecular function-related GO terms were not significantly associated with the *F5* co-expressed genes (Table 20). As verification the gene list with the genes co-expressed to *F5* was run towards functional ontology and process ontology in the online tool 'Genetic Gene Ontology (GO) Term Finder' of Lewis-Sigler Institute of Princeton University (Table 21). As an example of their differences, the list derived from DAVID 'gene ontology' (Table 20) and from Princeton University's 'Generic Gene Ontology (GO) Term Finder' (Table 21) stated different *P*-values for SH3/SH2 adaptor activity related to function ontology, where DAVID stated it non significant (*P* = 0,094) and Princeton stated it significant (*P* = 1,36E-04). The genes related to the SH3/SH2 adaptor activity are *SH2D2A* and *FCRL2*. The GO terms related to process ontology stated by the Genetic Gene Ontology (GO) Term Finder were not found in the list of GO terms obtained from DAVID (Table 20 & Table 21). The only GO term with direct relation to the *F5* gene is GO:0009611-response to wounding (data not shown), which do not have a significant association (*P*=0,087), nor is it listed in the functional annotation clusters of DAVID with interesting fold enrichment values (>1.5) (data not shown).

The T cell receptor signaling pathway from KEGG PATHWAYS were found to be the most interesting pathway, with correlation to the genes *CD3e, PIK3R5* and *PRKCQ* (Figure 17). All the above-mentioned genes have a correlation to *F5* exceeding 0.4. The genes *PRKCQ* and *CD3e* are both involved in GO terms that can be connected to immune response. The T cell receptor signaling pathway with the *F5* co-expressed genes *CD3e, PIK3R5* and *PRKCQ* showed connection to cell adhesion molecules, as well as genes related to proliferation, differentiation and immune response (Figure 17).

Table 20: Enriched biological themes (GO terms) shared by the genes co-expressed with *F5*. Derived from gene ontology application of DAVID selecting GO_TERM_BP and GO_TERM_MF. Sorted by increasing *P*-values.

Terms related to biological process	***P*-value**	**Genes**
GO:0050867~positive regulation of cell activation	0.021	*SELP, PRKCQ, CD3E*
GO:0045086~positive regulation of interleukin-2 biosynthetic process	0.026	*PRKCQ, CD3E*
GO:0006509~membrane protein ectodomain proteolysis	0.030	*PRKCQ, ERAP1*
Terms related to molecular function		
GO:0005529~sugar binding	0.058	*SELP, CLEC1A, KLRG1*
GO:0008235~metalloexopeptidase activity	0.073	*ERAP1, CPA2*
GO:0005070~SH3/SH2 adaptor activity	0.094	*SH2D2A, FCRL2*

Table 21: Enriched biological themes (GO terms) shared by the genes co-expressed with *F5*. GOA – *H. sapiens* (Humans) as annotation, function ontology and process ontology as aspects. Derived from the tool Genetic Gene Ontology (GO) Term Finder of Lewis-Sigler Institute at Princeton University.

Terms related to function ontology	***P*-value**	**Genes**
GO:0005070~ SH3/SH2 adaptor activity	1,36E-04	*SH2D2A, FCRL2*
Terms related to process ontology		
GO:0048584~ positive regulation of response to stimulus	5,36E-06	*AIM2, FASLG, NCKAP1L, SH2D2A, BMP15, FCRL2, IL2RB*
GO:0007166~ cell surface receptor signaling pathway	1,32E-05	*GRM6, AIM2, FASLG, NCKAP1L, SH2D2A, BMP15, KLRG1, IL2RB*

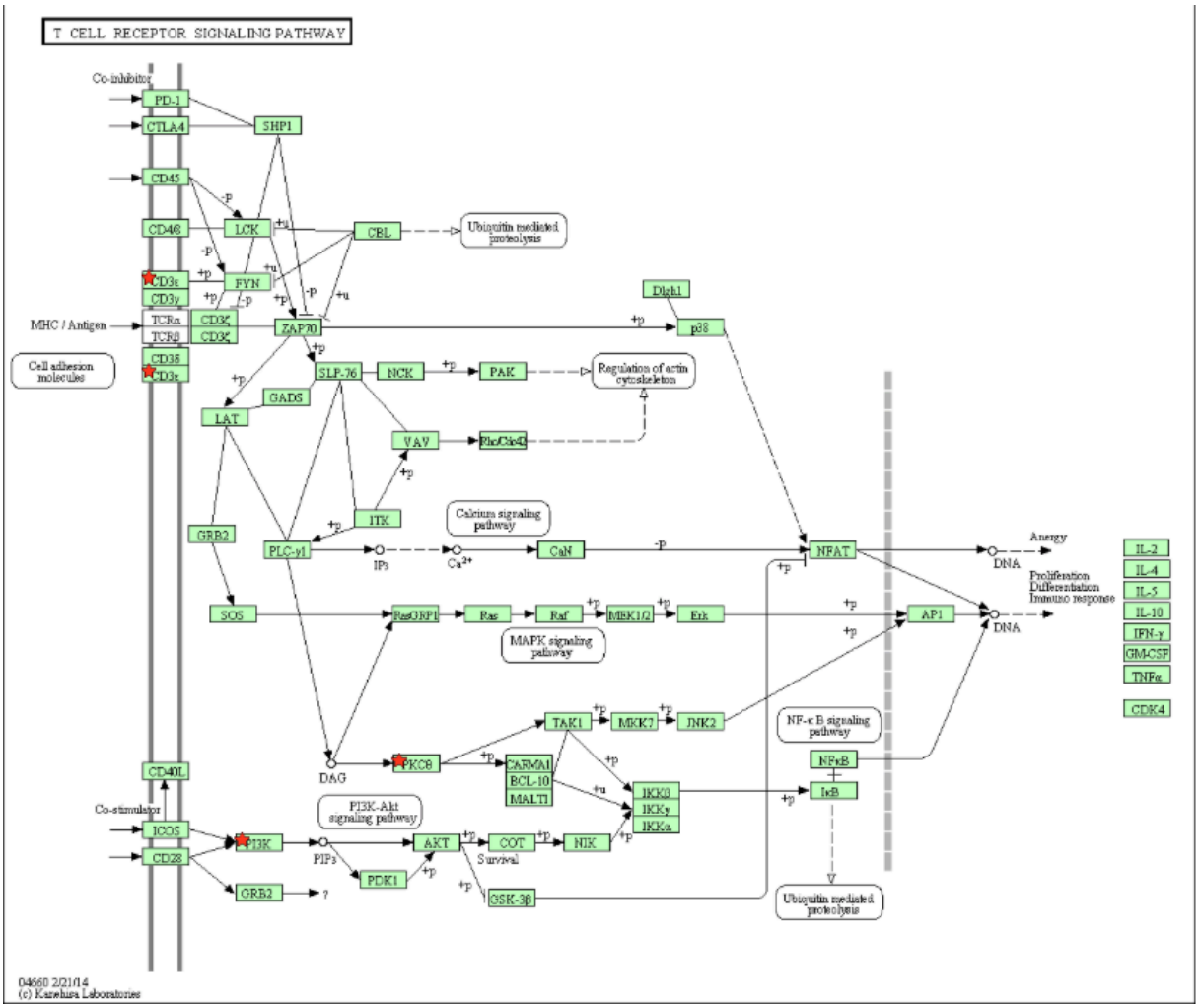

Figure 17: KEGG illustration of T cell receptor signaling pathway. Genes related to the *F5* co-expressed gene list are indicated with a red star, and are *PRKCQ, PIK3R5* and *CD3e*. Derived from KEGG pathway application in DAVID.

5.1.4 FV mRNA expression in cell lines

In addition to studying FV mRNA expression levels in breast cancer patients, studies of FV mRNA expression levels were done in regard to specific breast cancer cell lines, both from GOBO and in-house experiments (Figure 18). For comparison, FV mRNA expression levels in a selection of in-house non-breast cancer cell lines were included (Figure 18).

From the data available through GOBO a higher variation of FV mRNA expression levels was seen within the basal-like cell lines than within the cell lines of luminal character (Figure 18A), but no significant differences were seen when comparing the mean of the

two groups (data not shown). In the in-house experiments FV mRNA levels were compared to the levels of the liver cell line HepG2 with known high expression of *F5*. The non-breast cancer cell lines showed, as expected, low endogenous FV overexpression compared to HepG2 (Figure 18B). The human embryotic kidney cell line HEK293T showed higher FV mRNA levels than the endothelial and epithelial cell lines, but still 82% lower than HepG2 (Figure 18B). In the basal-like subgroup the HC1500 and SUM102 cell lines showed moderate FV overexpression (Figure 18B), while the human breast cancer cell line MDA-MB-231 had low endogenous overexpression of FV, with only 0,6% of the level seen in HepG2 (Figure 18B). The cell lines in the luminal subgroup showed in general low levels of endogenous FV overexpression (Figure 18B).

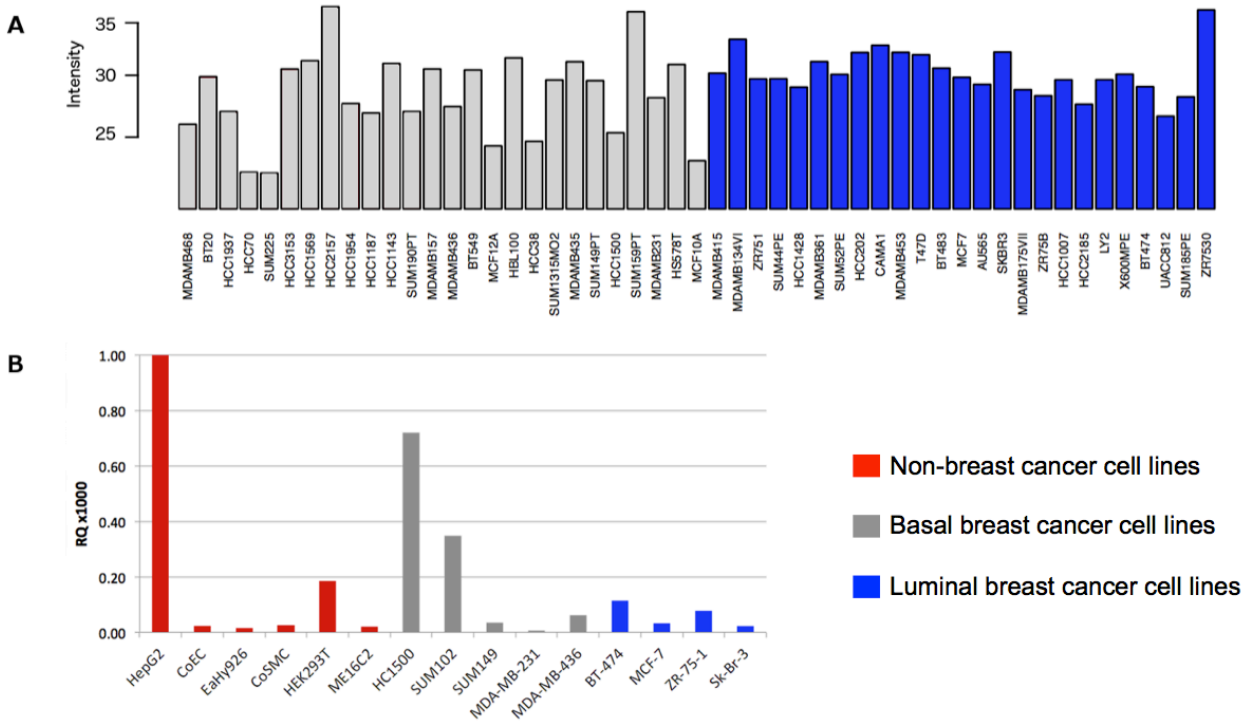

Figure 18: FV mRNA expression (A) by intensity in the 51 basal (grey) and luminal (blue) breast cancer cell lines derived from GOBO, and (B) by RQ values in luminal (blue), basal (grey) and non-breast cancer cell lines (red) from in-house experiments.

II. *In vitro* studies of FV

5.2 Optimization of the FV overexpression cell model

5.2.1 Optimization of FV mRNA overexpression in HEK293T and MDA-MB-231 cells transfected with pcDNA5frt-FV

To study the effects of FV overexpression *in vitro* a FV overexpression cell model was generated. Initially, the easy-to-transfect human embryotic kidney cells HEK293T were used to optimize overexpression of FV, transfected with the plasmid received as a generous gift from another research group, pcDNA5frt-FV, as well as an empty vector. The plasmid concentrations and Lipofectamine® 3000 ratios previous used by the research group were tested and found optimal (data not shown). The transient transfection procedure is described in section 4.4.3. The overexpression of FV was normalized against the endogenous control and compared to the empty vector.

High FV mRNA overexpression was seen in HEK293T at 24 hours (> 5 000 fold), while an even higher FV overexpression was seen at 48 hours (> 216 000 fold) (Figure 19A). With a successful overexpression of FV in HEK293T the same transfection protocol was used for transfection of MDA-MB-231 breast cancer cells. Even more efficient FV overexpression was seen in transfected MDA-MB-231 cells than in HEK293T cells. At 24 hours an extreme increase in FV overexpression was seen (> 4 500 000 fold) (Figure 19B). Though the levels of FV overexpression decreased with time, > 1 000 000 fold increase was seen even at 96 hours (Figure 19B).

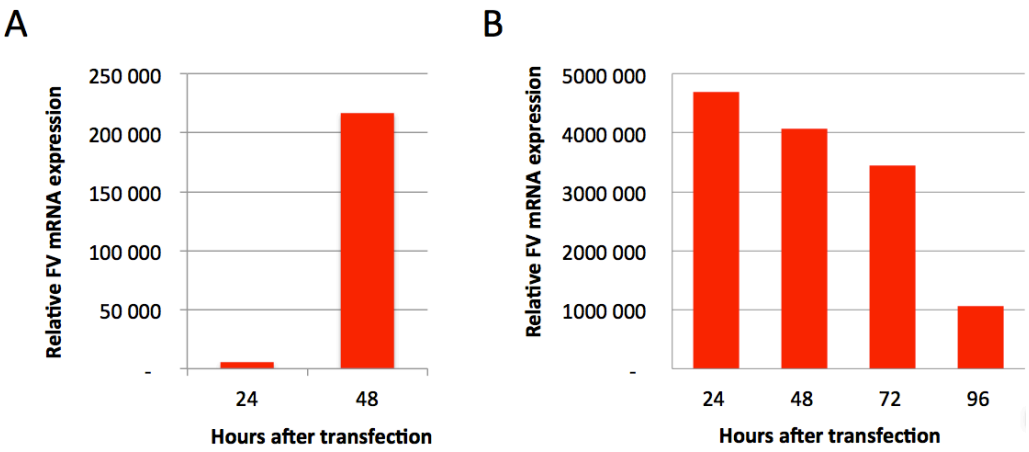

Figure 19: FV mRNA expression levels after transient transfection with pcDNA5frt-FV. (A) in HEK293T cells harvested after 24 and 48 hours. (B) in MDA-MB-231 cells harvested after 24 – 96 hours. FV mRNA expression was measured with qRT-PCR and normalized against the endogenous control. Presented as relative quantity (RQ) compared to empty vector. Mean values (n =1) from one experiment are shown.

5.2.2 FV protein levels in transfected MDA-MB-231 cells and samples with known concentrations of FV

FV protein levels in MDA-MB-231 cells transfected with pcDNA5frt-FV

After having confirmed successful FV overexpression at the mRNA level, we wanted to measure the FV protein secretion in the transfected MDA-MB-231 cells by ELISA (described in section 4.5.2). Surprisingly, all values obtained were below the lowest standard (data not shown), hence no FV protein concentrations could be estimated. To ensure the specificity of the FV ELISA, samples of known FV concentrations were run.

The FV ELISA was to measure human FV antigen levels, where 100% was stated by the manufacturer to be 10 µg/ml. FV deficiency plasma showed low levels of FV (3%), as expected (Figure 20). The normal plasma expressed 58% FV protein levels (Figure 20), which correlates with levels from other in house experiments (data not shown). Also presenting levels within expected range was the sample with a 50/50 ratio of normal plasma and deficiency plasma (31%) (Figure 20). Recombinant FVa with concentration of 10 µg/ml expressed 90% FV protein, while the level for the recombinant FVa with a concentration of 5 µg/ml were 40% (Figure 20).

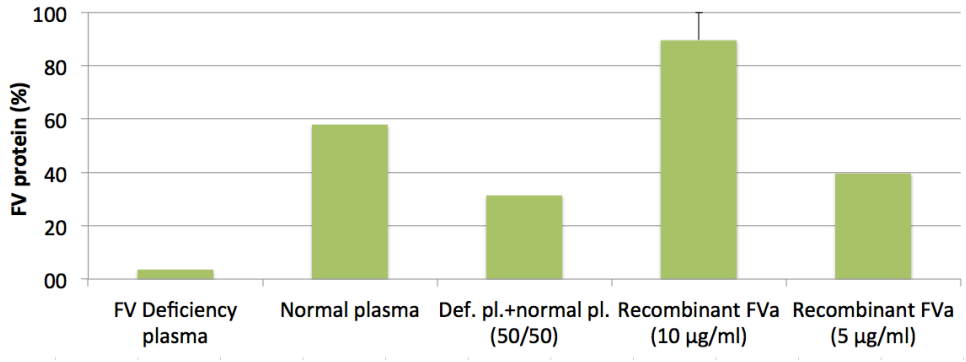

Figure 20: Specificity of FV ELISA. The FV protein levels in recombinant FV and FVa, FV deficiency plasma, normal plasma and samples of combinations were estimated to test the specificity of the ELISA assay. Mean values (n ≥ 1) + SEM from one experiment are shown.

The specificity of the FV ELISA was established. A hypothesis for the lack of FV secretion was altered protein function, and this was tested by measuring FV mRNA and FV protein secretion in HEK293T transfected with the commercially bought plasmid, pMT2-V. Again, measured at 48 hours, high FV mRNA overexpression was seen, but also undetectable FV protein secretion (data not shown). With two plasmids failing to provide detectable FV protein secretion, it was decided to sequence the two plasmids.

5.2.3 Sequencing of FV plasmids and *in vitro* mutagenesis

Sequencing of pcDNA5frt-FV and pMT2-V plasmids

The plasmids were sequenced as described in section 4.2, to study possible alterations in their sequence that could explain the lack of FV protein. The sequencing revealed several SNPs compared to the reference sequence (NM_000130.4) (Table 22). In addition, two unreported mutations were found (p.Gly1288Ala and p.Ala2184Thr) (Table 22). Except for the p.Gly1288Ala, which were only found in pcDNA5frt-FV, both plasmids comprised the same variants. The majority of the variants were located in exon 13 (Figure 21), and found to be in a LD block ($r^2 > 0,8$) (no data for p.Gly1288Ala).

Table 22: Variations in pcDNA5frt-FV and pMT2-V compared to reference sequence NM_000130.4.

SNP/mutation	Nucleotide change	Amino acid change	Exon
rs6016	c.2208C>T	Synonymous	13
rs6017	c.2235C>T	Synonymous	13
rs6021	c.2301A>G	Synonymous	13
rs4524	c.2573G>A	lys>arg	13
rs4525	c.2594G>A	his>arg	13
rs6032	c.2773A>G	arg>gly	13
rs1800594	c.3804C>T	Synonymous	13
rs9287090	c.3948T>C	Synonymous	13
Gly1288Ala *	c.3950G>C	p.Gly1288Ala	13
rs6030	c.5290A>G	met>val	16
A2184T	c.6637G>A	p.Ala2184Thr	25

* Only present in pcDNA5frt-FV

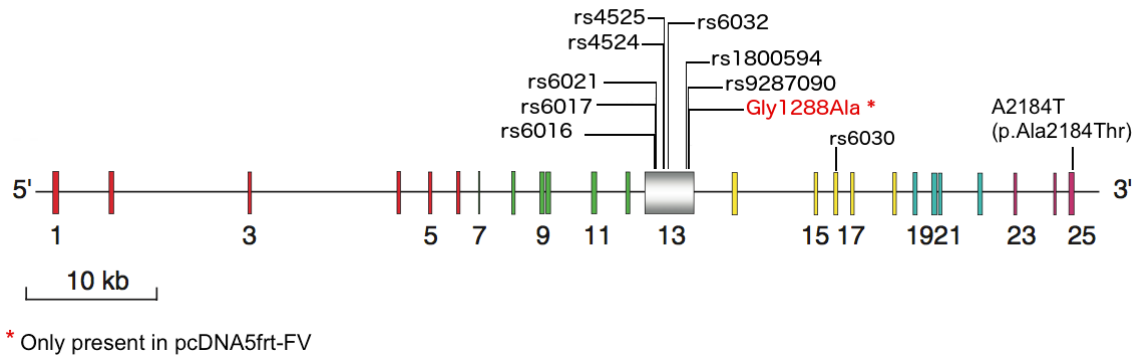

Figure 21: Variants present in pcDNA5frt-FV and pMT2-V. Location of SNPs and mutations compared to the *F5* reference sequence NM_000130.4.

We wanted to check if these variants had any effect on the FV protein secretion. The focus was set to the variants rs6030 and A2184T since exon 13 is cleaved off during activation of FV and the SNPs in LD block will express a haplotype effect. Also, it was not possible to design primers for the Gly1288Ala due to location in a highly repetitive area. Using *in vitro* mutagenesis (described in section 4.3.1) two FV plasmid variants were obtained: pMT2-V corrected for both rs6030 and A2184T (hereafter termed FV wt) and pMT2-V corrected for rs6030 (hereafter termed FV A2184T).

FV protein levels in HEK293T cells transfected with FV plasmid variants

In addition to measuring the FV protein secretion in HEK293T cells transfected with FV wt and FV A2184T, cells transfected with pMT2-V and pcDNA5frt-FV were included. Moreover, FV protein levels were also measured in the sample lysates to ensure that the protein secretion that was the flaw. All FV protein levels were performed by ELISA.

FV protein levels in the media were generally 3,8 – 6 fold higher than in the corresponding cell lysates (Figure 22). The pMT2-V and FV A2184T showed similar FV protein levels in media, both with 3-fold higher levels than pcDNA5frt-FV (Figure 22), indicating minor effect of the rs6030 SNP on FV protein secretion. The FV wt showed a 2-fold increase of FV protein in media compared to pMT2-V and FV A2184T, while compared to pcDNA5frt-FV the increase was as high as 6-fold (Figure 22). This indicates that both the unreported mutations contribute to lower FV protein secretion.

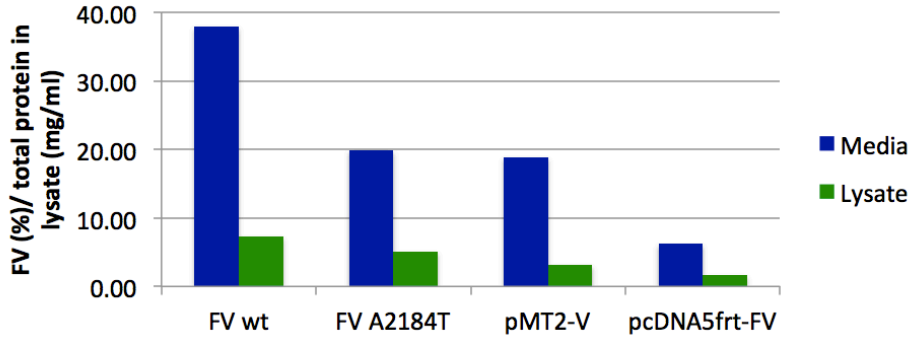

Figure 22: FV protein levels in HEK293T cells transfected with FV wt, FV A2184T, pMT2-V and pcDNA5frt-FV, and harvested after 48 hours. FV protein levels in media and lysate were corrected for total protein levels in lysate. N = 1 from one experiment is shown.

Since the FV wt showed higher (and detectable) secretion of FV protein it was concluded to use this as a FV wildtype for further studies in this thesis. Also, it was of interest to study possible functional effects of the FV A2184T variant, in addition to the planned SNPs rs6025 and rs6028.

Sequencing of *in vitro* mutagenesis products

To obtain plasmids with the *F5* SNPs rs6025 and rs6028, the FV wt was used as a template in *in vitro* mutagenesis (described in section 4.3.1) to generate the FV plasmid variants FV rs6025 and FV rs6028, respectively. The FV wt FV A2184T, FV rs6025 and

FV rs6028 were sequenced (see section 4.2) with the primers listed in Table 8, to ensure successful *in vitro* mutagenesis. Compared to the FV wt each FV variant expressed the desired SNP/mutation (Figure 23).

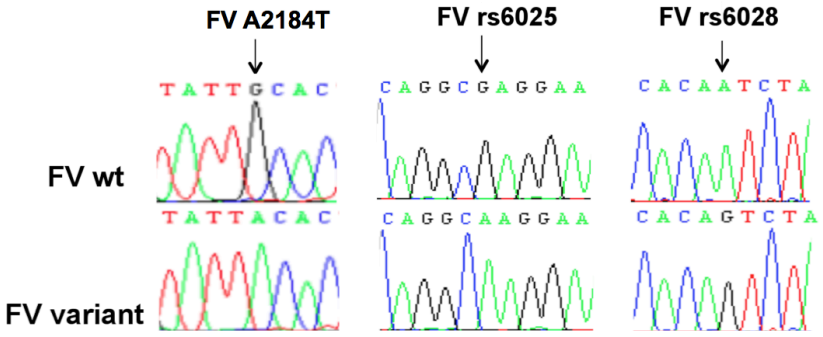

Figure 23: Sequencing of FV variants compared to FV wt, with conversion of desired mutation/SNP.

5.3 Validation of TaqMan assays

PCR Efficiency

To use the comparative Ct method for calculation of relative mRNA expression it is a prerequisite that the target and endogenous assays have a similar PCR efficiency. A 5-fold dilution series of cDNA with FV overexpression were run in triplicates for both the target assay (FV) and the endogenous control assay (RPLP0). From the obtained standard curve the slope values for each assay were calculated (Figure 24). Optimized assays must have an amplification efficiency in the range 90 – 105%, which was calculated from the slope value with the formula:

$$\text{Efficiency (\%)} = 10^{(-1/\text{slope})} - 1 * 100\%$$

Both assays passed the requirement for amplification efficiency, as well as for good linearity of the standard curves ($R^2 \geq 0{,}980$) and similarity in slope value ($\Delta\text{Slope} < 0{,}1$) (Figure 24 & Table 23). Thus, the comparative Ct method could be used to calculate the relative FV mRNA expression with RPLP0 as endogenous control in this thesis.

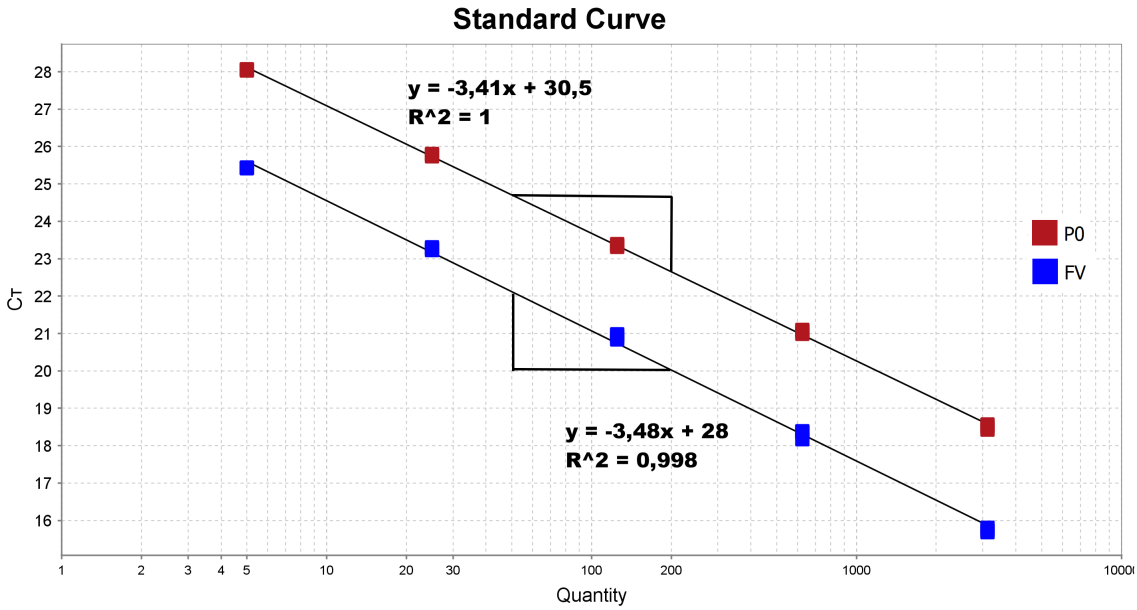

Figure 24: Standard curves for the TaqMan assays, FV and P0. The slope values, R^2 and efficiency rate are indicated, for FV assay and RPLP0 (P0) assay. Δslope = -3,48 – (-3,41) = -0,07

Table 23: TaqMan assay PCR efficiency and Δslope for the FV and RPLP0 assays.

Assay	Efficiency	Δslope
FV	93,8 %	- 0,07
RPLP0	96,5 %	

Validation of endogenous control assay

The assay of the endogenous control (RPLP0) was tested to ensure that it amplified unaffected by differences in FV levels. A curve plot of the Ct values for the endogenous control assay with cDNA from FV overexpression samples with equal RNA input was made. There are no significant differences between the Ct values between either of the samples tested (Figure 25). The endogenous control assay, RPLP0 is unaffected by differences in FV levels, and can be used in further experiments.

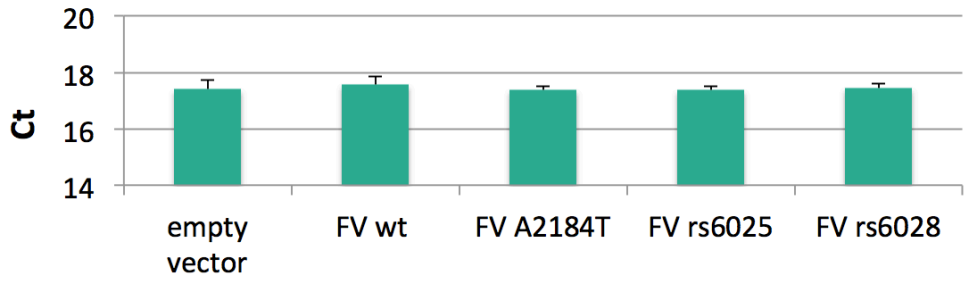

Figure 25: Ct values of the RPLP0 assay. Mean values (n = 3) + SD from three independent experiments are shown.

5.4 Overexpression of FV wt and FV variants in MDA-MB-231 cells

With an optimized FV overexpression cell model and FV plasmid variants of interest, we wanted to study the effect of FV overexpression at mRNA and protein levels in MDA-MB-231 cells transfected with empty vector, FV wt, FV rs6025, FV rs6028 and FV A2184T (see section 4.4.3), and harvested after 48 hours (see section 4.4.4) (unless else is specified).

5.4.1 Relative FV mRNA expression in FV wt and FV variants

The comparative Ct method was used to estimate the relative expression of FV mRNA in FV wt and FV variants, as described in section 4.4.6. The overexpression of FV mRNA in the FV plasmid variants were normalized against the endogenous control and compared to empty vector.

In accordance with experiments during optimization of the FV overexpression models, cells transfected with FV wt showed an extreme FV mRNA overexpression with a 230 000 fold increase compared to empty vector (Figure 26).

High FV mRNA overexpression was seen for the FV variants as well, though they showed lower levels than the FV wt. The FV rs6025 and FV rs6028 had 67% and 58% lower levels, respectively, while the difference for FV A2184T was even more evident with 89% lower FV mRNA levels than FV wt (Figure 26).

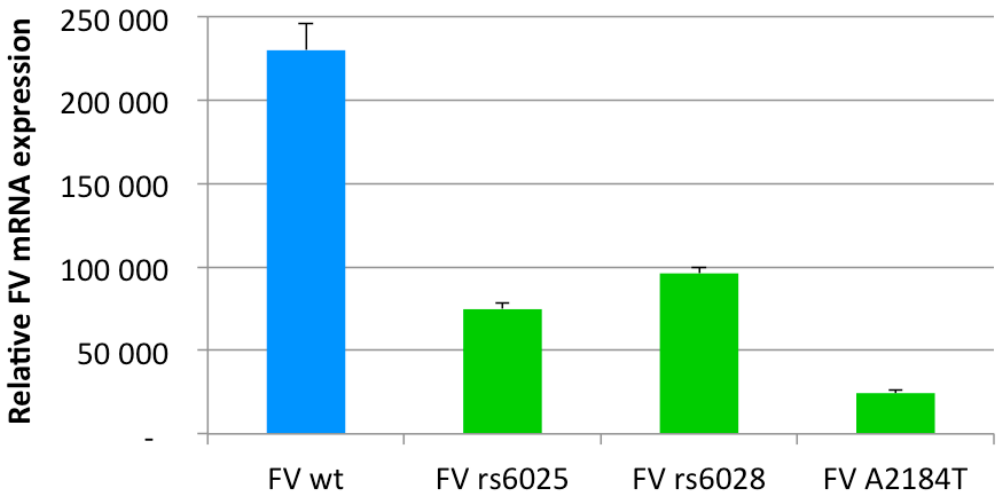

Figure 26: Relative FV mRNA expression in MDA-MB-231 cells. The cells were transfected with empty vector, FV wt and FV variants, and harvested after 48 hours. mRNA expression was measured with qRT-PCR and normalized against the endogenous control. Expressed as RQ compared to empty vector. Mean values (n = 9) + SEM from three independent experiments are shown.

5.4.2 FV protein levels in media of transfected MDA-MB-231 cells

To confirm FV overexpression at the protein level the FV protein secretion was measured by FV ELISA (described in section 4.5.2), in the cell media of MDA-MB-231 transfected with empty vector, FV wt and FV variants.

The FV protein values from media were corrected for variation of total protein in respective lysates. No FV protein was detectable for the empty vector, affecting the value corrected for total protein. Cells transfected with FV wt showed high levels of secreted FV (Figure 27).

Cells transfected with the FV variants expressed FV protein levels significantly different from those transfected with FV wt. Both FV rs6025 and FV rs6028 showed FV protein levels 29% lower than FV wt, while FV A2184T transfected cells showed 81% lower FV protein levels than FV wt (Figure 27).

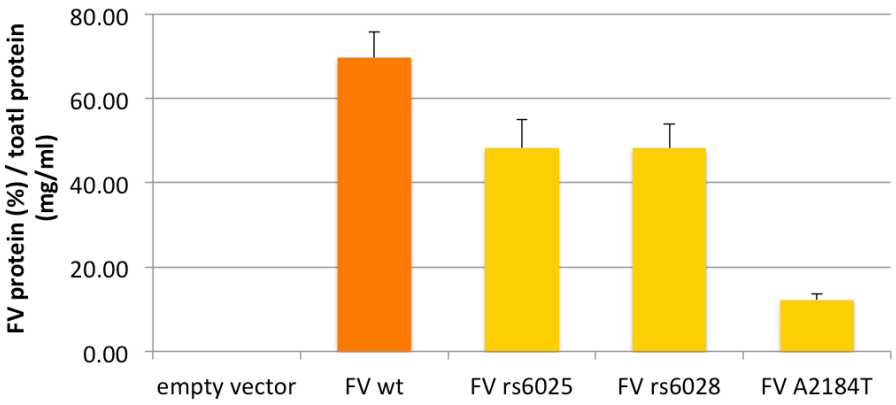

Figure 27: FV protein levels in media of transfected MDA-MB-231 cells. The cells were transfected with empty vector, FV wt and FV variants, and harvested after 48 hours. FV protein levels in media adjusted for variance in total protein in lysate. Mean values (n ≥ 6) + SEM from three independent experiments are shown.

A visual verification using Western blot analysis was performed to support the FV protein expression results described above. First, a blot with the recombinant FV and FVa was run together with a sample of HEK293T cells with FV overexpression to ensure that the right fragments were present in the transfected cells. The size of full-length FV is 330 kDa, and since the AHV-5146 antibody binds to an epitope on the heavy chain of FV and FVa, it should result in a band of 330 kDa for single chain FV and a band of 94 kDa for FVa (corresponding to the heavy chain of FV). The blot confirmed that the recombinants were indeed pure for FV and FVa (Figure 28A well 1 and 2, respectively), and that the transfected sample contained full-length FV (Figure 28A). Nevertheless, a faint band at about 150 kDa was also seen, which was most likely contamination from the media.

The media of the MDA-MB-231 transfected samples were added in accordance to the amount of total protein in lysate, assuring equal amounts in all wells (Figure 28B). As expected, a band at 330 kDa was seen, except for empty vector.

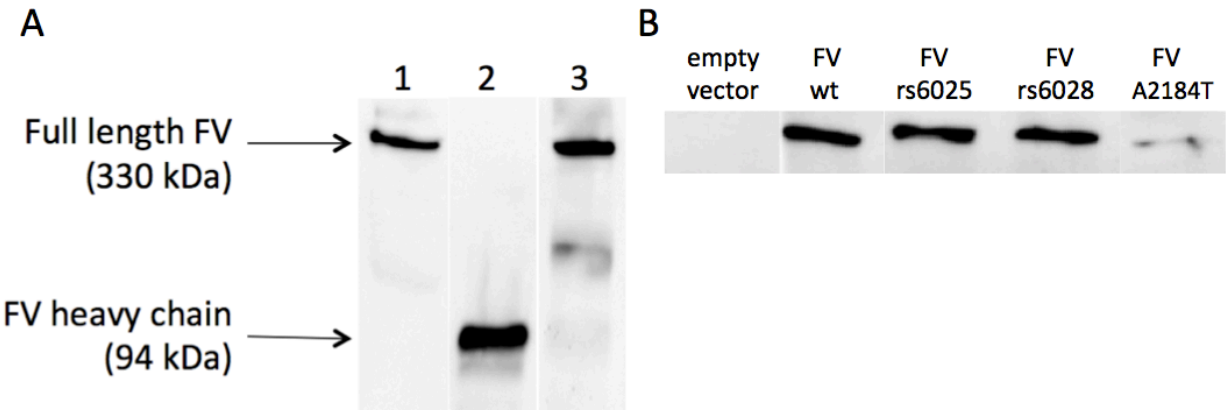

Figure 28: Western blot analysis and verification of fragment sizes in (A) recombinant FV (1), recombinant FVa (2) and media of HEK293T transfected with FV wt and (B) media from MDA-MB-231 cells transfected with empty vector, FV wt, FV rs6025, FV rs6028 and FV A2184T. Amount of sample added contained equal amounts of total protein (in lysate) for the sample within each blot. One representative blot is shown for (B).

5.5 Functional effects of FV overexpression in MDA-MB-231

A clear difference in FV overexpression at mRNA and protein levels was seen between the FV wt and empty vector and between the FV wt and the FV variants. With the findings from the clinical study of FV being associated with cell proliferation, differentiation and immune response it was of interest to study the functional effects of FV overexpression in the FV wt and FV variants. We wanted to test for possible differences in cell growth and cell death as well as inflammatory markers.

Effect of FV overexpression on cell growth in FV wt and FV variants

In the clinical study *F5* and co-expressed genes were found associated with genes related to cell growth and proliferation. Hence it was of interest to study cell growth of the FV overexpressed FV wt and FV variants *in vitro.* MDA-MB-231 cells and HEK293T cells were transfected with empty vector, FV wt and FV variants, and the WST-1 Cell Proliferation Reagent was used to measure the absorbance as an indicator of cell viability (described in section 4.6.1). Ratios of the obtained OD-values were calculated according to an equal start point, for easier comparison.

Non-transfected MDA-MB-231 cells showed a significant higher growth rate, with a steeper curve compared to the MDA-MB-231 cells transfected with empty vector (Figure

29A). Transfection with FV wt abolished the cell growth seen by a 0,5 – 1,6-fold lower growth in FV wt than empty vector. Also, the growth was similar for FV wt at 24 hours and 96 hours (P = 0,5799) (Figure 29A). The abolishing effect FV wt had on cell growth presented in Figure 29A gained strength after similar results in two new independent experiments (Figure 30), where a significantly lower growth was seen for the cells transfected with FV wt compared to empty vector at 72 hours (P > 0,0001).

MDA-MB-231 cells transfected with FV variants showed increased growth rate compared to FV wt (Figure 29A). A significant 1,4 – 2-fold increase in cell growth was seen for FV rs6025 compared to FV wt (Figure 29A). The FV A2184T-transfected cells showed the same growth pattern as FV rs6025, except at 72 hours, where a 3,5-fold increase in cell growth compared to FV wt was seen (Figure 29A). Cells transfected with FV rs6028 showed a steady increase in cell growth both compared to FV wt and empty vector (Figure 29A), with a 2,8- and 2,9-fold higher growth than FV wt at 72 and 96 hours, respectively.

Similarities can be seen between the growth curves of transfected MDA-MB-231 cells and the transfected non-breast cancer cells HEK293T (Figure 29). Cells transfected with FV wt showed lower growth than cells transfected with empty vector at 96 hours (0,6-fold reduction), while the growth was similar at 48 and 72 hours (Figure 29B).

As seen in the transfected MDA-MB-231 cells, the HEK293T cells transfected with FV rs6025 and FV A2184T shared the same growth pattern. The growth of these variants was similar to the growth seen for empty vector, while compared to FV wt a 0,5-fold increase was seen at 96 hours (Figure 29B). As seen in MDA-MB-231, the HEK293T cells transfected with FV rs6028 showed a higher increase in growth than the other variants (Figure 29B), with a significant 0,6- and 1,2-fold increase compared to FV wt at 72 and 96 hours, respectively.

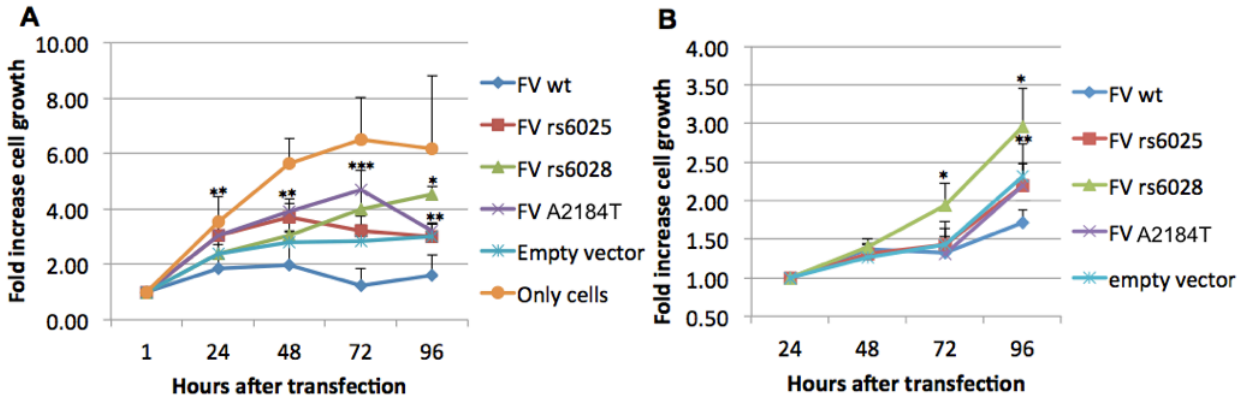

Figure 29: Time dependent cell growth, in (A) MDA-MB-231 cells transfected with FV wt, FV variants and empty vector, in addition to non-transfected cells, and (B) HEK293T cells transfected with FV wt, FV variants and empty vector. The cells were harvested at 1 - 96hrs, and OD was measured at 450nm. Mean values (n ≥ 3) of ratios to time point 1 + SD of one representative experiment are shown. The FV variants with significant difference ($P \leq 0{,}05$) to FV wt are marked with *. Only cells = non-transfected cells.

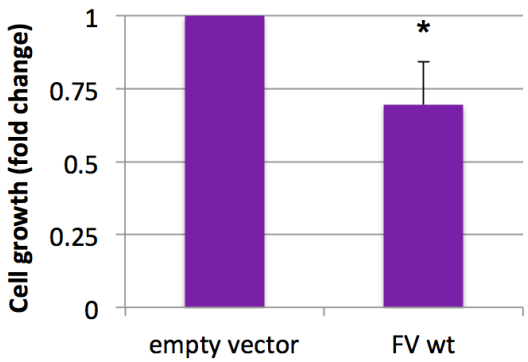

Figure 30: Cell growth in MDA-MB-231 transfected with FV wt and empty vector harvested at 72 hours. Fold change cell growth compared to empty vector. Mean values (n ≥ 5) in ratio to empty vector + SD from three independent experiments are shown. Significant difference ($P \leq 0{,}05$) marked with *.

5.5.1 Effect of FV overexpression on cell death in FV wt and FV variants

As relations in the clinical study were made to processes involving cell death, and since cell growth and cell death are highly related, the effects of FV overexpression on apoptosis and necrosis in MDA-MB-231 cells were quantified. Estimation of DNA fragmentation in cell lysates and cell media, respectively, was performed using the Cell Death Detection ELISA^{PLUS}, on transfected cells harvested after 48 hours (described in section 4.6.2).

Effect of FV overexpression on cell death in MDA-MB-231 cells transfected with FV wt

The cells transfected with FV wt showed significantly elevated apoptosis with 28% higher DNA fragmentation than the cells transfected with empty vector (Figure 31A). The apoptotic effect of cells transfected with the empty vector were more than 2,5-fold increased compared to non-transfected cells (Figure 31A), indicating how stressful the transfection was for cells. Moreover, the non-transfected cells showed similar levels of apoptosis, and both with more than a 2-fold reduction compared to empty vector (Figure 31A). This suggests that the apoptotic effect of the transfection mixture itself was minimal. On the other hand, the necrosis in cells transfected with FV wt were significantly lower than empty vector, with a 35% reduction (Figure 31B). Moreover, the necrotic effect of the transfection itself was not as great as seen for the apoptotic effect, showing a 1,4-fold increase in necrosis for FV wt compared to the non-transfected cells (Figure 31B). Similarly, the non-transfected cells with and without transfection mixture showed equal necrotic effect (Figure 31B).

Effect of FV overexpression on cell death in MDA-MB-231 cells transfected with FV variants

Studying the differences in cell death between FV wt and the FV variants, all values were adjusted against the FV protein levels in the respective cell media and compared to FV wt. Theoretically, if level of cell death is dependent only on the FV protein level the differences should be equalized. Any difference in level of cell death after adjustment can indicate differences in biological function. Cells transfected with FV rs6025 and FV rs6028 did not show any difference in either necrotic or apoptotic effect compared to cells transfected with FV wt (Figure 31C and D, respectively). An evident difference in cell death was seen for the FV A2184T variants, with a 3,5 and 2,5- fold increase in apoptotic and necrotic effect, respectively (Figure 31C and D, respectively).

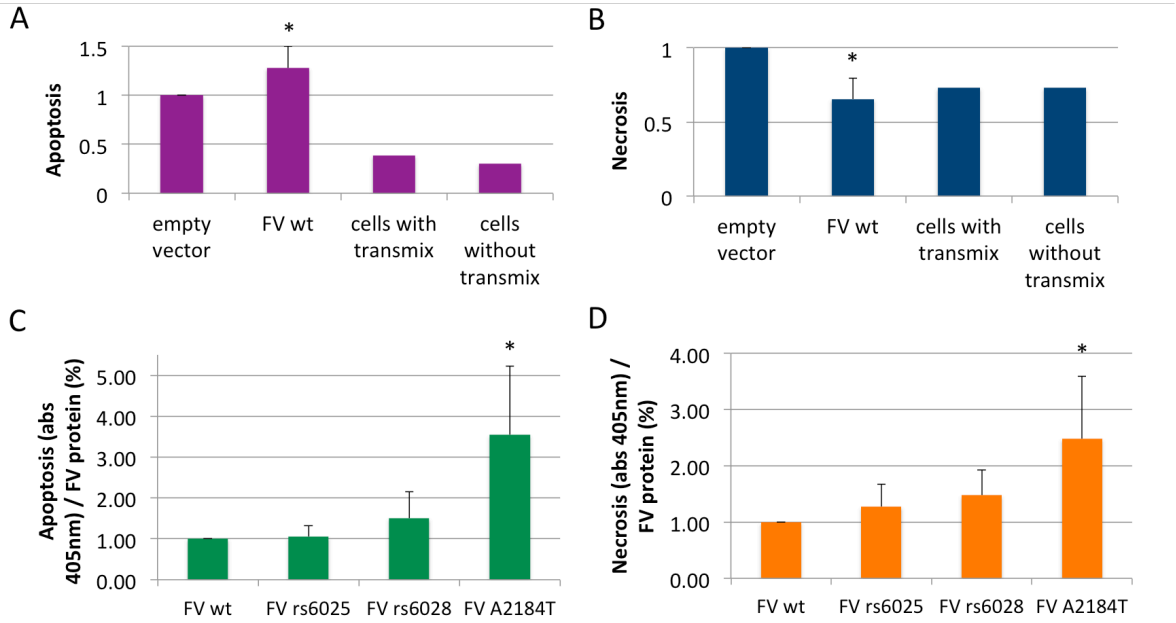

Figure 31: Effect of transfection and overexpression of FV on cell death in MDA-MB-231 cells. In addition to non-transfected cells, with added and not added transfection mixture, cells were transfected with empty vector, FV wt and FV variants. Cells and media were harvested after 48 hours. (A) Relative apoptosis compared to empty vector, (B) relative necrosis compared to empty vector, (C) relative apoptosis adjusted for FV protein levels and compared to FV wt and (D) relative necrosis adjusted for FV protein levels and compared to FV wt. Mean values (n = 6) + SD of two independent experiments are shown. Significant differences ($P \leq 0{,}05$) to empty vector (for A and B) and FV wt (C and D) marked with *.

5.5.2 Inflammatory markers induced by FV overexpressed in MDA-MB-231

Due to the correlation with genes related to immune response found in the clinical study, it was of interest to screen for possible effects on inflammatory markers. MDA-MB-231 cells transfected with empty vector, FV wt and FV variants harvested after 48 hours were used. The Multi-Analyte ELISArray was used for detection of changes in inflammatory markers at protein level, while the TaqMan® Low Density Array Human Immune Panel was used for detection at mRNA level (described in section 4.5.2).

Of the 12 pro-inflammatory cytokines present on the ELISArray, increased levels of IL6, IL8 and GM-CSF were found to be 2-fold increased in cells transfected with FV wt compared to empty vector (Figure 32A). At mRNA level a higher FV overexpression was seen for IL6 with a 5-fold increase compared to empty vector, while the increase for both IL8 and GM-CSF was 2-fold (Figure 32B).

The effect on the inflammatory markers IL6, IL8 and GM-CSF were estimated also in the MDA-MB-231 cells transfected with the FV variants. The values were adjusted for differences in FV protein levels and compared to FV wt. All FV variants showed increased levels of the tested inflammatory markers, compared the FV wt. Similar levels of the inflammatory markers were seen in FV rs6025 and FV rs6028, with 1-1,5-fold increase compared to FV wt (Figure 33). FV A2184T had the highest levels of the inflammatory markers compared to the FV variants and FV wt, with an evident 2,5-, 3- and 3,5-fold increase compared to FV wt for IL6, IL8 and GM-CSF, respectively (Figure 33).

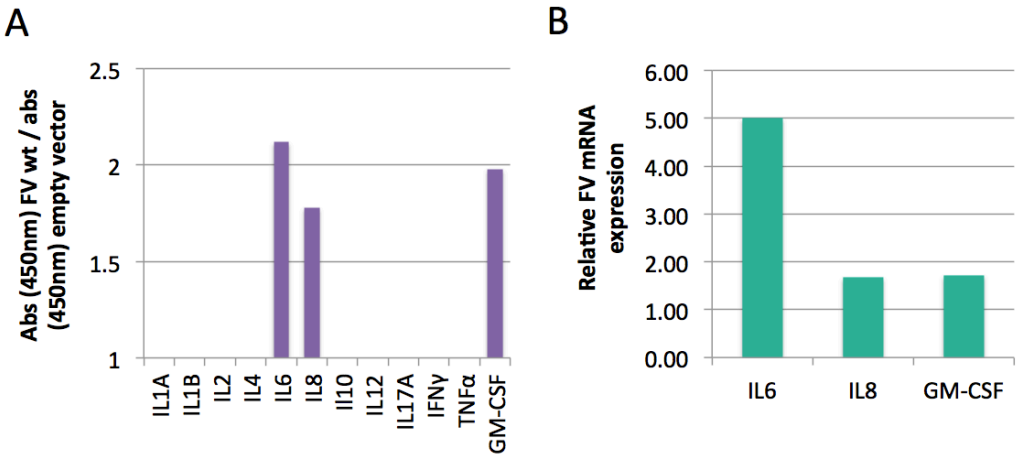

Figure 32: qRT-PCR of inflammatory markers in MDA-MB-231 cells. The cells were transfected with empty vector and FV wt, and harvested after 48 hours. (A) Media from three biological parallels of one experiment were pooled and compared to empty vector. (B) Relative FV mRNA expression levels of IL6, IL8 and GM-CSF from samples respective to A. Normalized against endogenous control and compared to empty vector. Values ($n \leq 1$) from one experiment are shown.

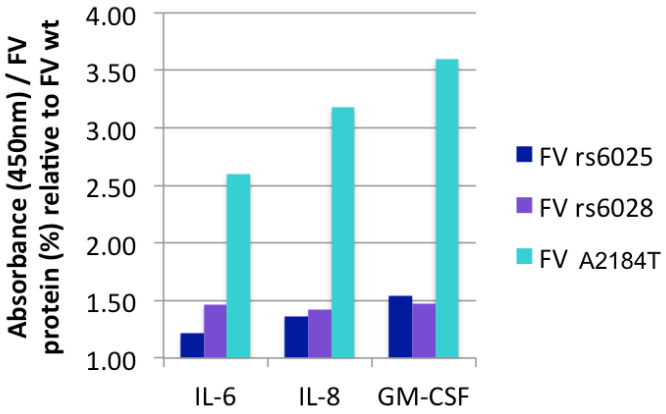

Figure 33: Relative expression of IL6, IL8 and GM-CSF in MDA-MB-231 cells overexpressed with FV variants. The cells were transfected with the FV variants and harvested after 48 hours. Media from three biological parallels of one experiment were pooled. The absorbance values were adjusted for FV protein levels in the respective samples, and compared to FV wt. Values ($n \leq 1$) from one experiment are shown.

6. Discussion

In addition to the well known link between cancer and risk of thrombotic diseases (Blom et al. 2005; Carrier et al. 2008), it is known that increased coagulation activity is associated with tumor progression (Boccaccio & Comoglio 2009; Garnier et al. 2012). Studying the underlying molecular mechanisms could lead to a better understanding of the association between cancer and hemostasis, and possibly lead to a more individualized treatment for patients suffering cancer or cancer-related thrombosis. Tinholt *et al*. (2014) found indications that activated coagulation may reflect the biology of the underlying tumor in breast cancer, which could be an important indicator of cancer progression. The role of coagulation FV in cancer has not been studied extensively, but is known to contribute to a balance in the coagulation process with both procoagulant and anticoagulant properties. In this thesis the aim was to gain a better understanding of how coagulation FV relates to breast cancer, using both a clinical breast cancer material and *in vitro* breast cancer cells.

6.1 FV expression in breast cancer tissue and cell lines

F5 gene expression was studied in tumors of breast cancer patients, i.e. in relation to breast tumor subtypes and to patient survival. In addition, *F5* expression across breast cancer cell lines and non-breast cancer cell lines was studied regarding choice of cell lines for the *in vitro* studies.

In the OsloII material increased FV tumor mRNA expression was associated with ER-negative, PR-negative, HR-negative, TN and *TP53* mutant tumors. Similarly, a significant increase in FV tumor mRNA was also seen for ER-negative tumors and in tumors of high grade, in the pooled datasets from GOBO. Across breast cancer subtypes increased FV tumor mRNA levels were associated with HER2-enriched tumors in both materials, while an increase was also seen in basal-like tumors in the pooled datasets from GOBO. The FV mRNA expression levels across breast cancer cell lines, and non-breast cancer cell lines were studied in results from previous in-house experiments and in available data from GOBO. A varying degree of FV mRNA levels were seen in both basal-like and

luminal breast cancer cell lines, corresponding well with the heterogeneity of breast cancer. Nevertheless, the larger variation within the basal-like subgroup suggests greater heterogeneity within these breast tumors than the luminal breast tumors. Non-consistency between the FV levels found in tumors and plasma suggest that the FV plasma protein concentrations not are influenced by the FV mRNA levels in tumors.

6.2 FV expression and breast cancer survival

Basal-like tumors commonly show triple negative and high grade characteristics, while the HER2-enriched tumors differ from the basal-like tumors by being HER2-positive (Kennecke et al. 2010; Sotiriou & Pusztai 2009; Vuong et al. 2014). Both basal-like and HER2-enriched tumors tend to be aggressive and fast-growing. This term comes from the resistance HR-negative tumors show towards hormone treatment and their association to a less favorable prognosis (Vuong et al. 2014). Interestingly, the patients with ER-negative tumors, high grade tumors and basal-like tumors and high levels of FV had a more favorable prognosis than patients with lower FV levels. Tissue factor (TF) constitutes an important part of the activation of the coagulation cascade and is associated with progression of tumors (van den Berg et al. 2012), for example as part of the cancer-related TF-FVIIa-PAR2 pathway (Ruf et al. 2011). This pathway can be inhibited by a reduction in coagulation activity caused by for example TF or FV, leading to reduced tumor growth. This is the first study reporting that *F5* acts as a suppressor gene, and thus its position as a possible new target gene for treatment of breast cancer.

6.3 FV expression in breast cancer and association with biological function

To obtain knowledge of how FV correlates to biological functions online annotation tools were used. When studying *F5* expression and relation to co-expressed gene modules mimicking biological functions, a negative correlation to steroid response was found. This is compatible with the poor response basal-like and HER2-enriched tumors show towards hormone treatment. A significant positive correlation to the modules immune response, checkpoint and early response were found. Out of the 36 genes found to be co-

expressed with *F5* and associated to ER-negative characteristics, genes with functions within proliferation, differentiation and immune response were prominent, which is in consistency with the co-expressed gene modules. Moreover, these functions are important in the progression of cancer (Hanahan & Weinberg 2011).

With these interesting results, pointing in the direction of *F5* having tumor suppressor effects, it was of interest to create an *in vitro* FV overexpression model to further study FV as a possible target for breast cancer treatment.

6.4 Overexpression of FV wt and FV variants in MDA-MB-231

For the overexpression of FV in the *in vitro* studies we wanted cell lines that showed low endogenous FV mRNA levels and that matched the aggressive tumor characteristics found in the clinical study. The basal-like and triple negative breast cancer cell line MDA-MB-231 matched these criteria. The non-breast cancer embryotic kidney cell line HEK293T is a commonly used cell line for initial experiments, which was also its purpose in this thesis. Also, the HEK293T cells showed low endogenous FV mRNA levels. Compared to primary cells, the use of cell lines has several advantages: They have an infinite cell division potential, can be frozen and thawed when needed, and are cost efficient. On the other hand, *in vitro* studies with cell lines involve 2D culturing on plastic, resulting in possible loss of the complexity seen in an *in vivo* system. Hence, *in vitro* studies cannot be used to draw clear conclusions regarding functions in a human body, but provides an indication for further studies.

6.4.1 Optimization of the FV overexpression cell model

As mentioned, to conduct studies of FV overexpression *in vitro* an optimal FV overexpression cell model was needed. The HEK293T cells were used as a test-cell line before the experiments were conducted in the MDA-MB-231 breast cancer cells. Transfection with the pcDNA5frt-FV plasmid resulted in high FV mRNA overexpression in both cell lines at all the time points tested. Due to the high FV overexpression at the mRNA level, it was somehow surprising that no FV protein could be detected in the cell media of the MDA-MB-231 breast cancer cells. Either, the cells did not secrete the protein as excepted, or there could be a translation problem. A second FV expression

plasmid was tested, however, the secreted FV protein levels were under the detection limit also for this plasmid. The next step was therefore to sequence both plasmids to look for alterations that might explain lack of FV protein. Sequencing revealed the presence of several common SNPs as well as unreported variants. We hypothesized that some of these variants affected the protein production. The majority of the variants were located in exon 13 which constitutes the B domain that is cleaved off during FV activation. Moreover, the SNPs in exon 13 were found to be in high LD ($r^2 > 0,8$) and thus represent one haploblock. Even though this thesis studied the non-activated form of FV, they were not changed to confer with the reference sequence. This may have affected the *in vitro* results in this thesis, hence care should be taken in drawing definite conclusions. After we had obtained plasmids corrected for the variants located outside exon 13, the FV protein production of the different FV plasmids (originals and variants) were tested. The FV protein levels in media were >3,8 fold increased compared to FV protein levels in the respective lysates, suggesting that most of the FV protein produced is secreted by the cells. FV protein levels of the FV plasmids and FV plasmid variants showed differences in protein secretion. Lower FV protein secretion in the plasmids with unreported variants suggests the importance of their presence for FV protein secretion. In the process of cell division mutations occur, and in a stressful cell environment mutations resulting in a more beneficial outcome for the cell is favored. Selecting for these two unreported variants resulting in reduced FV protein levels is most likely more beneficial for the cells. Nevertheless, for studying the effect of specific variants it is important to have a base plasmid without such variants.

With an optimal FV overexpression model and FV plasmid variants ready to be transfected we could study possible differences in FV mRNA and protein levels between the FV variants. The PCR efficiency and the specificity of the endogenous control assay were estimated and concluded to be suitable for using the comparative Ct method and RPLP0 as endogenous control, respectively.

6.4.2 Overexpression of FV wt in MDA-MB-231

The optimized FV overexpression cell model was used to study differences in gene expression and protein levels, as well as functional effects of FV wt and the FV variants. MDA-MB-231 cells transfected with FV wt and empty vector showed high FV

overexpression with more than a 230 000 fold increase compared to the empty vector. Due to post-transcriptional modifications, the mRNA levels and protein secretion derived form the same gene do not necessarily follow each other (Greenbaum et al. 2003). The increased levels of secreted FV as well as FV mRNA seen for the FV wt compared to the empty vector thus suggest minimal alterations at translational level.

6.4.3 Functional effects of FV wt overexpression in MDA-MB-231

As mentioned, during the clinical study FV was found related to proliferation, differentiation and immune response. Since uncontrolled cell growth by evading growth suppressors (Hanahan & Weinberg 2000), the ability to evade apoptosis (Kerr et al. 1972; Levine 1997), and the ability of tumor promoting inflammation (Eiró & Vizoso 2012) are characteristics of proliferating cancer cells, we wanted to study the effect of FV overexpression on cell growth, cell death and inflammatory markers.

The effect of FV wt overexpression on cell growth

Overexpression of FV wt showed a reduced cell growth over time compared to the empty vector in both HEK293T and MDA-MB-231 cells. The same trend in cell growth for the non-cancer cell line and the breast cancer cell line suggests that the effect of FV overexpression seen is a general effect. Nevertheless, only one method was used to estimate cell growth in this thesis, hence the results should be confirmed using other methods, like cell count, total protein quantification and cell cycle arrest studies. Another method to verify the cell growth is to use cell lines with high endogenous FV and downregulate FV, before studying cell growth with expectantly opposite effects. No studies focusing on effects of FV on cell growth has been reported to our knowledge. The studies conducted in this thesis indicate that elevated levels of FV wt in aggressive breast cancer tumors inhibit cell growth, and can be connected to the increased survival seen for breast cancer patients. This further suggests that *F5* acts as a tumor suppressor gene.

The effect of FV wt overexpression on cell death

Apoptosis is a programmed cell death removing unwanted cells. Upon signaling the cell undergoing apoptosis will shrink, and embedding of the membrane will eventually lead to generation of apoptotic bodies. Neighboring cells will ingest these apoptotic bodies. Necrosis has been believed to be uncontrolled cell death, but studies prove that in

resemblance to apoptosis, necrosis is a result of cellular crosstalk. Cells undergoing necrosis show features like cytoplasmic swelling and chromatin condensation. The cell will as a result burst, releasing its cellular components. In cases where the apoptotic pathways of a cell have been blocked, such as during bacterial and viral infections, necrotic cell death is crucial for extinguishing the treat (Festjens et al. 2006). Apoptosis and necrosis have traditionally been viewed as two independent processes, but new studies have shown that the two methods of cell death are linked through among others, the p53 pathway (Nikoletopoulou et al. 2013). Normally seen in a tumor environment, the tumor cells evade apoptosis, leading to proliferation. Apoptosis was significantly increased in the cells transfected with FV wt compared to cells transfected with empty vector. The higher level of apoptosis in FV wt may partly explain the reduced growth seen in these cells. Reduced growth of cancer cells could be connected to increased survival, and in this thesis breast cancer patients with elevated levels of FV were indeed found to have a more favorable outcome. More surprisingly, the necrotic effect was reduced in FV wt compared to empty vector. This suggests that increased FV levels suppress the necrotic effect of the cells. The mechanisms behind this abolishing necrotic effect requires further research.

DNA fragmentation assay, like the one used to measure levels of apoptosis and necrosis in this thesis, is a common method to measure cell death. Western blot analysis is another method to estimate cell death by DNA fragmentation. By using cell death-specific antibodies against the extrinsic apoptotic markers caspase 8 or PARP the amount of a target marker can be estimated, reflecting the level of apoptosis. In fact, Western blot analyses with caspase 8 and PARP were performed in this thesis, but due to inconsistency between parallels the results were not included. Other methods to measure cell death also exist. For example can apoptosis be measured by caspase activity, where assays can be caspase-specific differentiating between markers of the intrinsic and extrinsic apoptotic pathway, or be non-specific. Alterations in the cell membrane is a feature of cell death that can be used to measure apoptosis and necrosis (Roche Diagnostics GmbH 2008).

The elevated FV wt protein levels and the reduced cell growth seen in the *in vitro* study thus suggest that increased FV levels inhibit growth and proliferation of cancer cells.

With a reduced proliferation of cancer cells the rates of survival would assumingly be greater. Nevertheless, these *in vitro* results as well as the clinical results indicate that *F5* acts as a suppressor gene.

Inflammatory markers induced by FV wt overexpression in MDA-MB-231

Inflammation is found to facilitate tumor transformation, and 15-25% of all cancer cases have been associated with underlying infections and inflammatory reactions. Also, cancer cells have the ability to produce cytokines and chemokines that promote cancer proliferation and invasiveness (Balkwill & Mantovani 2001; Eiró & Vizoso 2012). The gene expression of the pro-inflammatory cytokines IL6, IL8 and GM-CSF were found to be increased after overexpression of FV wt in MDA-MB-231 cells.

Apoptotic bodies do not lead to any inflammatory response, due to the enclosure within a plasma membrane, but the macrophages ingesting the apoptotic bodies may generate anti-inflammatory cytokines (Roche Diagnostics GmbH 2008). The increased levels of apoptosis found thus conflict with the increased levels of inflammatory markers, but could be connected to the reduced cell proliferation in FV wt. Increase in pro-inflammatory cytokines normally leads to an increase in inflammation and thus cancer progression. However, since reduced cell proliferation was seen in this thesis it implies that FV have anti-inflammatory effects. The anticoagulant form of FV has been found to express an anti-inflammatory effect through its cofactor properties with aPC, which abolishes the EPCR dependent inflammatory PAR2 signaling by destabilizing TF-FVIIa-FXa complex (Liang et al. 2015; Sun 2015). Moreover, inhibition of the TF-FVIIa-PAR2 pathway have proven to attenuate cell growth and angiogenesis (Ruf et al. 2011). On the contrary to apoptotic cells, cells undergoing necrosis will burst and release their cellular components leading to generation of pro-inflammatory cytokines (Festjens et al. 2006). The level of necrosis seen for the FV wt was lower than the empty vector, but also than the non-transfected cells. Since an increase in pro-inflammatory cytokines should be accompanied by increased necrosis (Roche Diagnostics GmbH 2008), our results indicate that increased FV inhibits necrosis.

Several studies have reported that elevated levels of IL6 in sera of cancer patients (including breast cancer patients) were associated with advanced tumor stages and reduced survival (Bachelot et al. 2003; Goswami et al. 2012; Guo et al. 2012; Kozłowski

et al. 2002). Whilst Bachelot *et al.* (2003) found elevated IL6 levels to be associated with tumors responding poorly to hormone treatment, Goswami *et al*. (2012) did not find any differences in IL6 levels according to ER-status, PR-status or HER2-status, but it was connected to tumors of higher grade. IL6 is involved in signal transduction through the cell membrane, and through the glycoprotein 130 receptor it can alter cell behavior and also modulate the transcription of several liver-specific genes during acute inflammatory state (Guo et al. 2012; Lauta 2003). Moreover, changes in the epigenetic status of *IL6* expression was reported between the luminal MCF7 and the basal-like MDA-MB-231 breast cancer cell types (Ndlovu et al. 2009). The expression of *IL8* is important in tumor growth, angiogenesis and metastasis. Schadendorf *et al.* (1993) have reported that IL8 is associated with the progression and metastatic spread of melanoma, but also in immunomodulation. Moreover, high levels of IL8 are reported to be associated with advanced tumor stage, tumors of high grade and reduced survival in patients suffering ovarian carcinoma (Merritt et al. 2008). The same authors reported that silencing of *IL8* resulted in reduced tumor growth through antiangiogenic mechanisms (Merritt et al. 2008). GM-CSF is associated with the growth of hematopoietic cells, and Kinoshita *et al*. (1995) reported indications that GM-CSF prevents apoptosis in these cells. GM-CSF is also found to be involved in cell cycle progression in endothelial progenitor cells through the PI3K/Akt, JNK and ERK signaling pathways (Qiu et al. 2014).

6.4.4 Overexpression of FV variants in MDA-MB-231

The FV rs6025, FV rs6028 and FV A2184T showed significantly lower FV mRNA overexpression than the FV wt. The same trend was seen for FV protein secretion, suggesting that *F5* comprising each variant leads to alterations in gene expression levels and thus secreted FV protein. Variations in gene expression have shown to be of great importance for the expressed phenotype, and for risk factors such as the risk for cardiovascular diseases (Zeller et al. 2010). Noteworthy, in this thesis the FV protein levels were corrected for levels of total protein, which gives a more correct impression of FV levels when comparing samples. Alterations of gene expression can explain the decrease in FV levels seen for the FV variants. Since the trend of decrease in FV is similar on the mRNA and protein levels, the alterations on translational level are most likely minimal while alterations on transcriptional level are more likely to be the cause. DNA-

histone modifications like phosphorylation, ubiquitinylation, methylation, deacetylation and GpC islands can reduce the accessibility of sites for transcription initiation affecting the transcription efficiency (Deaton & Bird 2011; Lesk 2010). Moreover, the stability of mRNA is far lower than the stability of both DNA and proteins. MicroRNAs (miRNA) are important for RNA instability. One miRNA can silence and destabilize multiple mRNAs, achieved by cleavage or degradation of the mRNA (Alberts et al. 2015). Of interest in cancer proliferation, the expression of miRNAs are affected by inflammation and cell stress (Schetter et al. 2010). Interestingly, Duan *et al.* (2003) reported that a mutation in the human dopamine receptor D2 gene not only lead to reduced mRNA stability and decreased mRNA translation, but that the cause was due to change in the mRNA folding pattern and not by silencing of mRNA (Duan et al. 2003).

Effects of overexpression of FV variants on cell growth

The FV variants showed similar trends of cell growth in HEK293T and MDA-MB-231, all with elevated growth compared to FV wt. The higher cell growth for the FV variants can be explained by lower levels of FV, suggesting an abolished tumor suppressor effect. The cell growth was not corrected for differences in FV levels, so the most elevated cell growth seen for FV rs6028 should be interpreted with some caution. Nevertheless, with cell growth higher than empty vector, the FV rs6028 may not only abolish the suppressor effect of FV, but also promote cell growth. Thus, with the assumption of cell growth unaffected by FV levels, it could be argued that an alteration in protein function does indeed play a role. With these assumptions the results indicate that breast cancer patients with tumor of aggressive character carrying the *F5* rs6028, or the intronic proxy SNP rs9332542, which was found to be associated to risk of breast cancer (Tinholt et al. 2014), may have a less favorable outcome than patients without the gene variants. The *F5* SNP rs6028 have not been in focus of previous studies, but is an interesting polymorphism for further studies, both *in vitro* and *in vivo*. The cells transfected with FV rs6025 showed increased growth compared to FV wt, but similar to the empty vector. This indicates that the suppressor effect of this variant is inhibited, and could lead to reduced survival rates for FV Leiden carriers with aggressive tumors, compared to non-carriers, due to lower levels of FV. Even with 81% lower FV protein levels the FV A2184T showed increased cell growth compared to FV wt. The elevated cell growth is a logic consequence of lower FV levels, with the assumption of abolished FV suppressor properties. Like seen for FV rs6025, these results indicate a possible

reduced survival for cancer patients with aggressive tumors and the p.Ala2184Thr mutation, compare to non-carriers, due to lower levels of FV.

Effects of overexpression of FV variants on cell death

Apoptosis and necrosis were adjusted for differences in FV protein levels, to verify whether the possible differences could be due to FV protein levels or to FV protein function. No significant differences were seen between apoptosis and necrosis for FV rs6025 and FV rs6028, when compared to FV wt, indicating that the increased cell growth was due to lower FV protein levels. Interestingly, a significant increase in cell death was seen for the FV A2184T, suggesting that the functional effects of this variant was not only due to lower FV protein levels, but also due to altered protein function. As further discussed later, the location of p.Ala2184Thr is in an area important for FV binding to membrane surfaces.

Effects of overexpression of FV variants on inflammatory markers

From previous studies mutations in cancer-related genes are known to alter the expression of angiogenic and pro-inflammatory factors, as well as the procoagulant activity of the cancer cell (Boccaccio & Comoglio 2009; Garnier et al. 2012). In this thesis, overexpression of the FV variants rs6025 and rs6028 resulted in slightly higher expression levels of IL6, IL8 and GM-CSF compared to FV wt, whereas the FV A2184T caused an even greater increase in expression of the same markers. The known association between increased proliferation and increased levels of inflammatory markers (Eiró & Vizoso 2012) is somewhat contradicting to the elevated levels of cell death and increased levels of inflammatory markers seen for A2184T.

The FV Leiden variant (rs6025) is associated with aPCR and inhibited generation of anticoagulant FV (Castoldi et al. 2004; Lucotte & Mercier 2001). That would have a negative impact on the anti-inflammatory cofactor properties FV has towards aPC, which could result in increased levels of inflammatory cytokines. This could further be connected to increased cancer proliferation. Moreover, due to lower inactivation of FVa and FVIIIa in the absence of anticoagulant FV, it may be possible that in an *in vivo* system this would lead to increased thrombin and fibrin generation, known to cause increased inflammation as well as increased risk of thrombosis (Boccaccio & Comoglio 2009). Even though FV Leiden has been associated to not be a risk factor of breast cancer nor

the hypercoagulable state in breast cancer patients (Tinholt et al. 2016), FV Leiden carriers are known to have an increased risk of thrombosis (Rosendaal et al. 1995; Rosendaal & Reitsma 2009). Also, aPCR is associated with increased risk of breast cancer (Nijziel et al. 2003; Tinholt et al. 2014). Supported by literature, the results obtained in this thesis may explain a possible reduced survival for cancer patients who are FV Leiden carriers.

As previously mentioned, the alterations in expression- and functional effects seen for FV A2184T cannot only be explained by lower FV levels. The elevated cell growth seen is a logic consequence of lower FV levels, with the assumption of abolished FV tumor suppressor properties. Reduced cell death would also be a logic consequence of increased cell growth, which on the contrary was not the case. Focusing on the properties of necrosis, an increase in necrosis is normally followed by an increase in pro-inflammatory cytokines leading to increased inflammation and tumor proliferation. This could explain the increased levels of the pro-inflammatory markers and increased cell growth seen for FV A2184T. Due to the elevated levels of apoptosis and necrosis it is to assume that also the biological function of the protein play a role. The location of p.Ala2184Thr on the C-terminal end of the light chain of FV could explain an alteration in protein function. The C-terminal end of FV have shown important in the binding between FV and membrane surfaces due to electrostatic and hydrophobic properties of this area (Kalafatis 2005; Lecompte et al. 1994). The p.Ala2184Thr leads to a change from the hydrophobic amino acid alanine to the hydrophilic threonine, which may alter the membrane-binding properties of FV, and consequently alter the protein function. Also, phospholipid binding residues in the FV C1 and C2 domains are crucial for the binding between platelet derived FV and its carrier protein multimerin 1 (MMRN1). Platelet derived FV is bound to MMRN1 before activation, and these MMRN1 binding sites have also shown importance for the storage of the FV-MMRN1 complexes in platelets (Jeimy et al. 2008; Kalafatis 2005). The results in this thesis indicate that breast cancer patients with tumor of aggressive character carrying the *F5* p.Ala2184Thr variant may show reduced survival compared to patients without the variant, due to both lower FV protein levels and altered protein function. Whether the trends reported in this thesis will be seen in other cancer cells or *in vivo,* and affect the survival of breast cancer patients requires further research.

6.5 Limitations

Several limitations were present during the work of this thesis. Comparing experiments from different cell lines may cause differences in results since cell type specific properties may have distinct impacts on the functional effects of FV. Also, the empty vector used was not the same as the FV vector, hence their sequences vary, leaving possibility for differences in expression and functional effects. Except for the estimation of FV wt effect on cell growth and cell death in MDA-MB-231 at 72 hours, the functional studies of this thesis were not replicated adequately following the norm of at least three individual experiments, each with biological replicates. Nevertheless, all the functional studies had more than three biological replicates, but lacked repetition of independent experiments. Thus care should be taken in drawing definite conclusions from these experiments. The results regarding cell growth and cell death are derived by the use of one method, but due to results varying between methods, several methods should be performed to support each other.

7. Conclusions

In this thesis, we aimed to gain a better understanding of the role of coagulation FV in breast cancer. We studied the clinical significance of FV in breast cancer patients, and the expression- and functional effects of overexpression of FV wt and FV variants in a basal-like breast cancer cell line *in vitro*. The results suggest that *F5* acts as a tumor suppressor gene, and may be a good candidate gene for therapeutic purposes in breast cancer patients or patients suffering cancer-related thrombosis. The main conclusions are as follows:

I. The clinical significance of FV:
   - Increased FV tumor mRNA levels were associated with aggressive tumor characteristics: ER-negative tumors, basal-like tumors, HER2-enriched tumors and tumors of high grade.
   - Increased FV protein plasma levels were associated with tumors of high grade.
   - High *F5* gene expression levels were associated with increased survival in patients with the aggressive tumor subtypes ER-negative, high grade and basal-like.
   - *F5* was co-expressed with genes associated with functions related to proliferation, differentiation and immune response.

II. Expression- and functional effects of FV overexpression *in vitro*:
   - An optimized FV overexpression cell model was obtained after transient transfection with a FV plasmid in the MDA-MB-231 breast cancer cell line and the non-breast cancer cell line HEK293T. The FV overexpression was high both at the mRNA and the protein levels.

   - FV wt overexpression was associated with reduced cell growth, increased apoptosis, reduced necrosis and increased expression levels of the inflammatory markers IL6, IL8 and GM-CSF. Overall, this indicates that FV possesses tumor suppressor effect.

- Introducing the *F5* variants rs6025 (FV Leiden), rs6028, and A2184T into the FV plasmid caused a reduction in *F5* mRNA expression and protein levels compared to the FV wt plasmid, especially the *F5* A2184T.
- The FV rs6025 and FV rs6028 showed increased growth, similar cell death and increased inflammatory markers compared to FV wt, indicating an inhibition of the suppressor effect due to lower levels of FV.
- The FV A2184T showed increased growth, increased cell death and increased inflammatory markers compared to FV wt, indicating not only an inhibition of the suppressor effect caused by lower levels of FV, but also caused by altered biological function.

7.1 Further perspectives

- Further study effects of cell growth (by living cell count or total protein quantification) and cell death (by DNA fragmentation through Western blot analysis or assays measuring membrane alterations) to support the findings from this thesis.
- Further study the effects of inflammatory markers, by pro-inflammatory assays as well as siRNAs and inhibitors, with 24 hours time point as main focus. Moreover, these methods should be used to gain a better understanding of how cell death is induced, with focus on the p53 and MAPK/JNK pathways.
- Construct a new FV wild type without variants found in exon 13, and perform new experiments to study the functional impact of this haplotype.
- Use recombinant FV to study effects of FV overexpression.
- Study the effect of FV on other aspects of cancer development, such as migration and angiogenesis.

8. References

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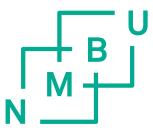
9. Supplement

Tumor characteristics	OS (%)		DMFS (%)		RFS (%)	
	Tertiles	Median-cut	Tertiles	Median-cut	Tertiles	Median-cut
All tumors	NS	NS	NS	NS	NS	NS
Basal tumors	NS	NS	0,00069	8E-05	NS	0,03115
Luminal A tumors	NS	NS	NS	NS	NS	0,01633
Luminal B tumors	NS	NS	NS	NS	NS	NS
ERBB2 tumors	NS	NS	NS	NS	NS	NS
Normal like tumors	NS	NS	NS	NS	NS	NS
ER-positive tumors	NS	NS	NS	NS	NS	NS
ER-negative tumors	NS	NS	0,00017	0,00028	NS	NS
PAM50_Basal tumors	NS	0,0345	0,00158	9E-05	NS	0,00837
PAM50_Her2 tumors	NS	NS	NS	NS	NS	NS
PAM50_Luminal A tumors	NS	NS	NS	NS	NS	NS
PAM50_Luminal B tumors	NS	NS	NS	NS	NS	NS
PAM50_normal-like tumors	NS	NS	NS	NS	NS	NS
LN neg tumors	NS	NS	NS	NS	NS	NS
LN pos tumors	NS	NS	NS	NS	NS	NS
ERpos LN neg tumors	NS	NS	NS	NS	NS	NS
Grade 1 tumors	NS	NS	NS	NS	NS	NS
Grade 2 tumors	NS	NS	NS	NS	NS	NS
Grade 3 tumors	NS	NS	0,02549	NS	NS	NS
Untreated tumors	NS	NS	NS	NS	NS	NS
TAM tumors			NS	NS	NS	NS

Table S 1: FV expression in breast cancer tumor characteristics and association to survival, in for OS, DMFS and RFS divided into tertiles and median-cut. Significant *P*-values given. OS: overall survival, DMFS: distant metastasis-free survival, RFS: relapse-free survival, NS: non significant.



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