QATAR UNIVERSITY

COLLEGE OF HEALTH SCIENCE

VISUALIZATION OF FACTOR VIII WITH FLOW-CYTOMETRY AS A

TOOL FOR NOVEL GENE THERAPY APPROACH IN HEMOPHILIA A

BY

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ABSTRACT

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Title: Visualization of Factor VIII With Flow-Cytometry As A Tool For A Novel Gene Therapy Approach In Hemophilia

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Haemophilia A is a genetic X-linked disorder, characterized by coagulation Factor VIII (FVIII) deficiency and leading to pathological bleedings. The disease occurs in a rate of 1 in 5000 males' births. The treatment is the administration of plasma-derived or recombinant Factor VIII which is expensive and leads to the development of inhibitory antibodies in around 40% of patients affected by the severe form of the disease. The disease becomes for these patients life-threatening.

New approaches to treat Haemophilia include Gene Therapy (GT). Cells corrected through genetic modifications are used to produce in Haemophilia A patients FVIII protein in a sustained manner, as long-term treatment for this disorder. The cells of choice should be persistent and equipped with the machinery for large protein assembly and secretion. So far, target cells for Haemophilia gene correction are mostly liver cells, although they are highly immunogenic and exposed to immune-mediated destruction after GT.

Based on literature evidences, Bone Marrow Transplantation can correct Haemophilia A in mice, providing evidence that Hematopoietic Stem Cells (HSC) or their progeny are able to produce FVIII. We chose the approach of correcting HSC with lentiviral vectors carrying the FVIII gene cassette.

Whereas classically FVIII protein is visualized on adherent cells through immunohistochemistry staining, Flow-Cytometry (FC) literature publications are very scarce.

FC analysis is an attractive method for analysing hematopoietic cells, and in general, a versatile method for protein visualization. However, large proteins as FVIII are difficult to be carefully analysed, and the method requires several steps of optimization.

This joint project with Dr. Muhammad Elnaggar, (postdoctoral fellow) .aims to optimize a method to characterize large proteins as FVIII with a reliable FC staining protocol. To this aim we used cell lines to evaluate the expression and secretion pathways of FVIII, the intracellular requirements to fold and secrete large proteins, and the toxicities of protein accumulation, in case of GT mediated protein overexpression. To this aim, the FC experiments were performed to optimise the FC protocol for FVIII visualization, by improving blocking efficacy, antibody labelling efficacy and to ensure accuracy and validity through qPCR and FC double staining.

This FC protocol proved its validity and usefulness in visualizing and studying functionally FVIII. The project successfully facilitated safe GT protocols in HSC, moreover, addressed the need in the field of biomedical research by optimizing a reliable FC protocol of staining and visualization of large proteins like FVIII.

DEDICATION

This thesis is dedicated to my mother who has always been an essential source of care and support. I would like to give gratitude to my thesis supervisors. This work is also dedicated to the man, I am grateful for being his daughter, my father.

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CHAPTER 1: INTRODUCTION

1.1. Research Hypothesis

We hypothesized that a flow-cytometric (FC) analysis for Factor VIII visualization, would result in an improvement in the characterization of this protein, particularly in the context of Gene Therapy (GT) modification of target cells.

In order to test our hypothesis, we chose to work on different cells, that can produce FVIII in good quantity naturally.

1.2. Research aim and objectives

Recent literature suggests that Hematopoietic Stem Cells (HSC) might be an efficient target for FVIII replacement through Gene Therapy in Haemophilia A (HA), although FVIII is mainly produced in liver. Targeting HSCs as FVIII protein producers needs careful quality and safety controls.

This research project aims to optimize a reliable Flow-Cytometry (FC) analysis to characterize FVIII coagulation protein production in cells. The advantage of this method is to add functional information, otherwise impossible with currently available immunohistochemistry analyses, and to improve the specificity and granularity of data.

To this aim, the objectives of the project are to: A) Build an extensive background through the collection of literature on FVIII production and secretion. B) Validate a FC method to visualize FVIII in intracellular compartments and on cell membrane surfaces. C) Study FVIII expression and secretion pathways in different cells. D) Evaluate the capacity of FVIII protein production in different cells after FVIII gene transduction.

If successful, this method may be broadly applied to the visualization of FVIII and similar large proteins.

1.3. **Research plan:**

Table 1. The description of the timeline of the project within the year in terms of tasks and dates.

	Task description	Start Date	End date	Comment
Objective 1	**Literature background**			
Objective 2	**RNA extraction, Quantitative PCR**	**January, 2017**	**March 2019**	**-**
Objective 3	**Antibody staining of FVIII**			
Task 1	Anti-FVIII antibody labelling with Dylight 650	January, 2017	March 2019	-
Task 2	Antibodies panel designing and experimental design	February, 2017		To ensure there is no spectral overlap between antibodies used for cellular staining.
Task 3	Performing FC experiments on PBMCs and the other cell lines	March, 2017		-To stain cells for FVIII protein and other CD markers. Optimizing FVIII expression and detection by FC. -To perform functional studies to study FVIII.
Objective 4	**Results retrieval, analysis, discussion, and finalizing of the written thesis by April 2019**			

CHAPTER 2: LITERATURE REVIEW

2.1. Chapter Overview

The current chapter critically analyses the existing scholarly literature, to explore the issues related to the process of coagulation and involved pathways, the epidemiology, and pathophysiology of HA, and the specific structure of coagulation FVIII along with its role in the process of coagulation. Subsequently, an overview of the Gene Therapy pre-clinical trials to treat this disorder. Finally, how FC facilitates the characterization and visualization of proteins.

2.2. Coagulation and Clotting Factors: Coagulation Pathway

Coagulation pathways are complex and diverse and have generally been divided into the following types of cascades: intrinsic, standard and extrinsic (Krishnamoorthy et al., 2016). Extrinsic pathway of coagulation process has been characterized as the initial (first) step in the process of haemostasis, which is mediated by plasma. The process is activated by the Tissue Factor (TF), which in turn is expressed within the sub-endothelial tissue (Palta et al., 2014). Within the context of standard conditions (e.g., no trauma), vascular endothelium serves as a barrier, which prevents (or minimizes) the contact between plasma procoagulants on one side and TF. However, during the process of vascular injury, TF gets exposed and binds to calcium and Factor VIIa. Such a process triggers another critical step, which is the conversion of Factor X to its active form – Xa (Konkle et al., 2015).
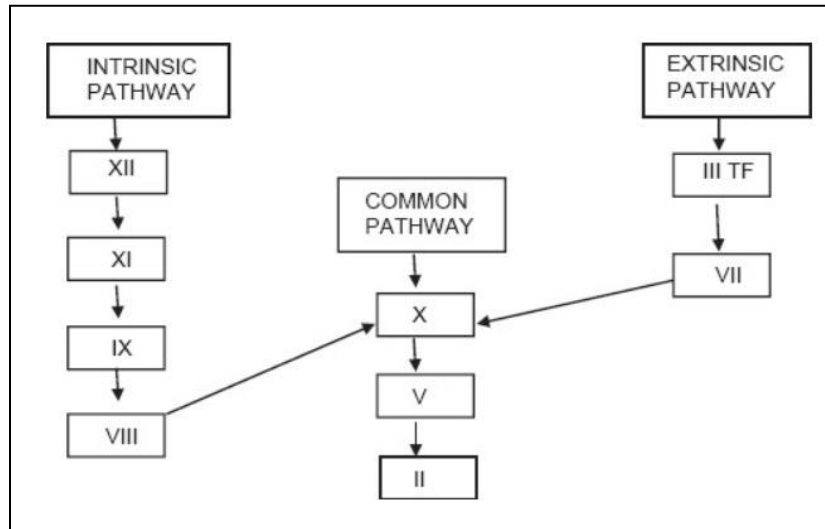
Figure 1. The discussed coagulation pathways: extrinsic, common and intrinsic. (Source: Palta, Sarao & Palta, 2014)

The intrinsic pathway of coagulation can be described as a pathway, parallel to the extrinsic one the critical feature of this pathway is the activation of thrombin by coagulation Factor XII. The pathway cascade is initiated by the following components: XII, kininogen, HMW, factor XI, and prekallikrein. Their combined action leads to the activation of factor XI Once factor XI is activated; it triggers a reaction leading to activation of factor IX. Coagulation factor IX, in close interaction with its cofactor – VIII, forms a complex on a phospholipid surface, which helps to activate coagulation factor X (Konkle et al., 2015; Palta et al., 2014).

Common coagulation pathway, finally, is the activation path of coagulation factor X with tissue phospholipids, calcium and cofactor V. Their interaction leads to the formation of the prothrombinase complex, which in turn helps transforming prothrombin into thrombin (Palta et al., 2014; Konkle et al., 2015). The obtained thrombin helps to cleave further fibrinogen, which is circulating in the bloodstream, and transforms it into an

insoluble fibrin form, thus contributing to the formation of the secondary haemostatic plug and stabilization of blood clots (Palta et al., 2014)

2.3. Clotting FVIII: Biochemistry Basis of This Clotting Factor

Blood clotting FVIII is a non-enzymatic protein serving as a cofactor to activated factor IX. It is encoded by FVIII gene in the X-chromosome and specific defects of this gene-, which mostly is an inversion mutation in intron 22 of the gene - can lead to the development of Haemophilia A disorder (Antonarakis et al., 1995).

The FVIII gene has a size of 186 kbp; however, the total length of the coding sequence is only 9 kbp (Gitschier et al., 1984).

FVIII gene has a tissue-specific expression pattern. Due to its large size and the complex machinery necessary for translation and secretion, FVIII protein is limited to specific tissues, mainly liver cells. Nevertheless, it was reported that FVIII protein has been detected not only in liver cells but also in liver resident macrophages, the so-called Kupffer cells (Do H. et al., 1999; Zanolini et al., 2015), and several other tissues in the body express FVIII mRNA (Su AI, Wiltshire T, Batalov S, et al., 2004).

FVIII maximum length polypeptide is made up of 2332 amino acids combined in six domains A1-A2-B-A3-C1-C2. Localized at the margins of A domains are the acidic sub-domains, which have a function in FVIII interaction with other proteins especially the thrombin (Gitschier et al., 1984).

FVIII is homolog to Factor V and other proteins, as MFGE8 protein (Lactadherin), which is a milk-fat protein (Figure 3). Both the C domains of FVIII and Lactadherin bind to phospholipids. The C2 domain is also crucial for binding to von Willebrand factor when

inactive (Lin et al., 2007). A1 and A2 domains are both homologous to the corresponding

A domains of Ceruloplasmin, the copper- binding protein (Figure 3) (Pasi et al., 2017).
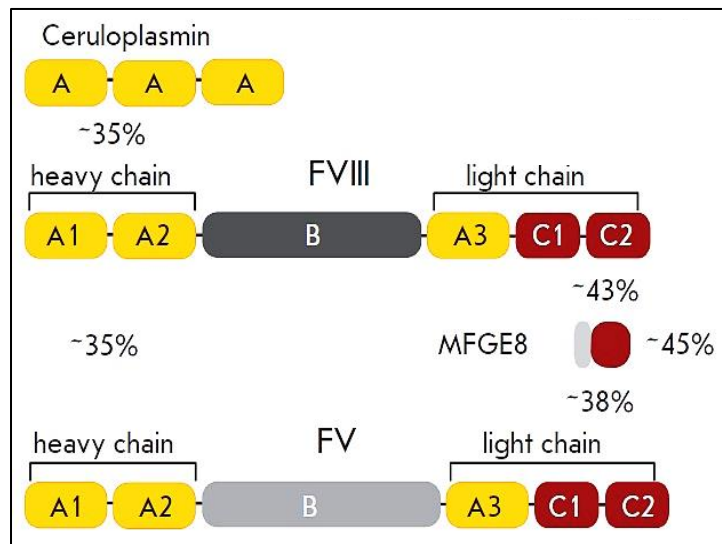
Figure 2. FVIII protein and FVIII homologs domain structure. The numbers represent homology level of amino acids for the domain groups. All proteins shown are from human origin (Source: Orlova, Kovnir, Vorobiev, Gabibov, & Vorobiev, 2013)

The metal ions - specifically copper - play an essential role in the assembly of FVIII

chains, which is impossible otherwise (Wakabayashi et al., 2001).

The transfer of the FVIII protein into the Endoplasmic Reticulum (ER) of the cell,

leads then to the formation of disulfide bonds, and the attachment of N-linked

oligosaccharides to the FVIII chain. After the occurrence of N-glycosylation, FVIII protein

binds to two lectins: Calnexin (CN) and Calreticulin (CRT), and these two lectins function

in preventing the secretion of immature FVIII proteins (Pipe et al., 1998). The immature

protein that needs to be further processed bound with Calnexin and Calreticulin is usually

transferred to the Golgi apparatus where it goes through processing (glycosylation,

sulfation and proteolytic processing) (Figure 4). The unfolded proteins remain in the

endoplasmic reticulum for re-glycosylation and undergo again through the Calnexin cycle (Pipe et al., 1998).
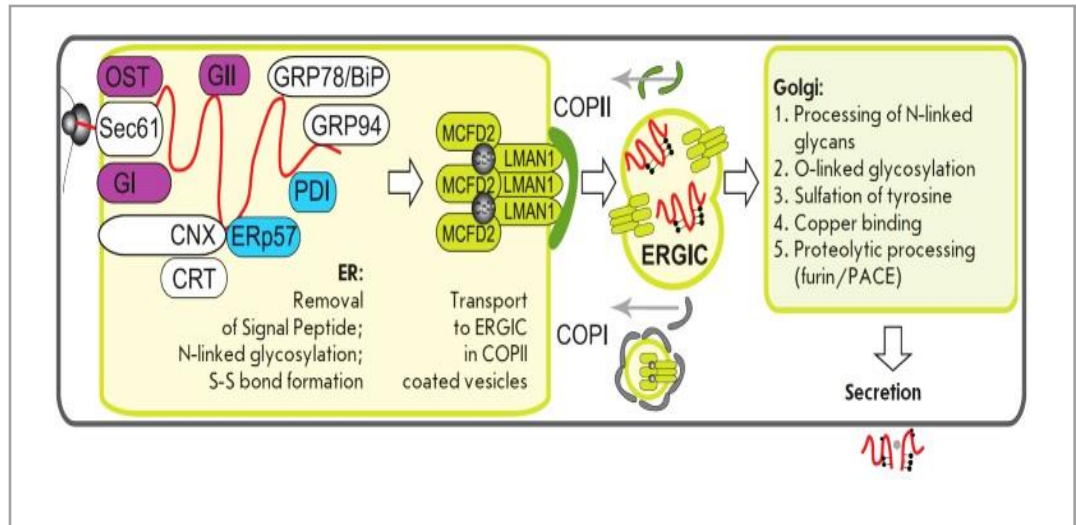
Figure 3. FVIII protein Intracellular trafficking before secreted. Membrane-protein translocators OST and Glucosidases GI and GII. Then, Calnexin and Calreticulin are bound to the chain (Source: Orlova et al., 2013).

As FVIII protein is in the lumen of Endoplasmic Reticulum, it forms a complex with the so-called Immunoglobulin Binding Protein BiP, which is a significant chaperone of the Endoplasmic Reticulum. This chaperone is a critical component of the Unfolded Protein Response signaling reaction or pathway (UPR). During cells stress, like in case of starvation of sugars/glucose where N-glycosylation is inhibited or in case of overexpression of FVIII protein, the amount of misfolded proteins inside the ER increases, and the synthesis of BiP in high amount is induced (Dorner et al., 1989; Brown et al., 2011) and the complex of BiP with FVIII protein leads to its retention in ER. The adequately folded FVIII protein is transported from the Endoplasmic reticulum to the Golgi apparatus through intermediate structures known as ER-Golgi intermediate compartment ER-GIC,

clearly shown in Figure 3. After that, FVIII protein gets ready to be secreted outside of the cell (Dorner et al., 1989).

Additionally, the production of FVIII in "professional producer" cells like endothelial cells is supported by a more organized structure: FVIII is stored in cigar-like shaped organelles, known as Weibel-Palade bodies (WPB), and released FVIII upon specific cell stimulation.

Finally, Von Willebrand factor (vWF) is a multimeric glycoprotein that plays a key role in FVIII lifecycle. The protein is synthesized mainly in the endothelium and megakaryocytes, and its synthesis contributes to WPBs formation where FVIII is stored.

VWF guides the trafficking of FVIII inside the cells (Wagner, 1990) and is secreted in the bloodstream, where it binds to FVIII, protects it from degradation, from unspecific activation, and directs FVIII to sites of platelet plugs, facilitating adhesion and subsequent clot formation (Wise RJ, Dorner AJ, Krane M, et al. The role of von Willebrand factor multimers and pro-peptide cleavage in binding and stabilization of FVIII. J Biol Chem. 1991; 266:21948–55).

2.4. Clotting FVIII: Mechanism of Action

The process of FVIII activation is central to the process of blood coagulation. FVIII is synthesized in the form of a single polypeptide forming two chains, consisting of the A1 – A2 domains (heavy chain), and the A3 – C1 – C2 domains (light chain). The chains are linked by the B domain, that is detached before secretion. The activation in FVIIIa takes place after secretion, through cleavage of the secreted protein and detachment from vWF chaperone (Figure 5 B). The C2 domain has an essential function in membrane association

through the binding of phospholipids. A deletion in this region can lead to complete abrogation of FVIII binding to the platelets surface (Konkle et al., 2015).

The coagulation cascade along with the FVIII and its role in it is graphically represented in Figure 4. As discussed earlier within the chapter, the coagulation process features two distinct pathways (intrinsic and extrinsic) (Figure 5 A), both triggered (activated) via exposure to tissue factor (TF). Activated platelet membranes can also expose phosphatidylserine groups (PS) that can also activate both pathways. After being activated via proteolytic way, for example by thrombin FXa, FVIII undergoes dissociation from VWF and creates an association with the FIXa, which is a serine protease (Palta et al., 2014). FVIII is responsible for directing the localization of the complex that has been formed to the membrane surface of platelets that have been activated. The latter are activated via interaction with the C2 domain of the complex (Figure 5 A). The complex between FIXa and FVIIIa (membrane-bound) has a crucial function: to activate Factor X via proteolytic way, which in turns triggers the activity of thrombin (Palta et al., 2014).

Within human blood, FVIII circulates as a part of a stable complex with von Willebrand Factor. Once thrombin (also known as factor IIa) is activated, it activates FVIII and the complex begins interacting with element IXa. Such a process is known as the coagulation cascade. Factor IXa takes part in the activation of another coagulation factor – X. Cofactor X has been shown to act as a cofactor to factor Va, which has the power to activate more thrombin (Pasi et al., 2017). Thrombin, furthermore, can cleave fibrinogen which transforms into fibrin. Fibrin has a unique ability for polymerization and

crosslinking (via the assistance of factor XIII), which eventually results in the formation of blood clots (Pasi et al., 2017; Krishnamoorthy et al., 2016). As FVIII is no longer attached and protected by vWF, it undergoes the process of proteolytic inactivation. Protein C and factor IXa are involved in inactivating FVIII and quickly removing it from the human bloodstream.

2.5. Clotting FVIII: Site of Production

As of now, the primary site of FVIII release is considered to be ambiguous (Pasi et al., 2017); however, it is known that it is produced and released into the human bloodstream from tubular, glomerular and vascular types of endothelium.

The liver is the main site for FVIII production, specifically in liver sinusoidal endothelial cells, that are layered at the interface with hepatocytes and the blood stream in the sinusoids of the liver (Do et al., 1999). Furthermore, Konkle et al. report that Haemophilia Type A has been known to be corrected among patients who have undergone liver transplantation procedure.

Nevertheless, based on literature evidence, there are also other sources of FVIII production or secretion, so-called extra-hepatic FVIII sources. Different tissues and organs have been identified as FVIII producers, as monocytes, macrophages, kidney, and lung (Hollestelle et al., 2000; Groth et al., 1974; Zanolini et al., 2015; Jacquemin et al., 2006). Similarly, it is proven that HSC transplantation can correct HA in mice, providing evidence that HSC or their progeny can produce functional FVIII (Follenzi et al., 2004).

2.6. Haemophilia A: Genetic alterations and pathophysiology

1. Genetic mutations in HA

HA is a genetically inherited bleeding disorder, characterized by the lack or deficiency of Coagulation FVIII. FVIII first characterization was in 1994 by Genentech scientists (Gitschier J et al., 1994). The FVIII gene is on the X chromosome at position q28. HA is an X- linked recessive disease, affecting therefore males with the overt disease, while affecting females predominantly with mild traits or a silent carrier status. The genetic aberrations are normally inherited, but may also arise from *de-novo* mutations, accounting for a 30% of sporadic (not-inherited) HA cases (Kasper & Lin, 2007).

Incorrect and abnormal biosynthesis and secretion of clotting factors can be a direct result of different defects. Importantly, gross rearrangements and deletions can lead to abnormal DNA transcription, RNA processing and alter the process of translation (Konkle et al., 2015). The deficiencies in quantity or functioning of FVIII arise from various genetic mutations, including intron 22 inversion, gross deletions, point mutations, missense substitutions and splice site defects. Such gene mutations lead to the inability of an organism to create a fully functional FVIII protein or the inability to synthesis any FVIII. Therefore, no effective and operational blood clots can be produced to repair the damaged blood vessel walls in case of an injury (Peyvandi et al., 2016).

According to Palta et al., minor gene defects such as single missense mutations can also result in defective secretion of FVIII. Empirical evidence suggests that such mutations commonly occur within a specific codon – 2307 – and are caused by the replacement of Arg by Leu or by Gln. The proteins created after translation appear to be normal as far as their function, however, display poor secretion patterns and therefore cannot effectively

contribute to the process of coagulation. Importantly, there is growing evidence that defects associated with functioning and production of the FVIII may not be limited to the FVIII gene itself.

Moreover, deficiency of factors V and VIII registered among some hemophilic patients has been linked to gene defects that lay outside of the FVIII gene, and instead – located on chromosome 18 between the genetic markers D18S1103 and DD18S849 (Konkle et al., 2015).

2. Clinical features and diagnosis.

As a clinical condition, haemophilia can be manifested via spontaneous and traumatic bleeding. The pathophysiology of the condition is closely associated with abnormally low levels of FVIII, or its significantly reduced level of activity.

The process of diagnostics of haemophilia is focused on the identification of clinical symptoms and analysis of the concentration of the coagulation factor in patient`s blood and plasma (Pasi et al., 2017). Depending on the concentration of the coagulation factor, the observed bleeding disorder can be classified as either: mild (5 – 40% of standard concentration), moderate (1 – 5% of normal concentration), and severe (less than 1% of normal concentration).

3. Treatment.

The specific treatment plans and interventions depend on the severity of the symptoms, genetic make-up, and possible complications and associated comorbidities – the co-occurrence of more than one health condition or diseases (Al-Tonbary et al., 2010).

The key modern healthcare approach towards treatment and management of HA offers a protein replacement therapy, with recombinant FVIII. Pasi et al. (2017) discuss a

common complication among patients undergoing such treatment, which is the development of neutralizing antibodies. These antibodies arise against the FVIII protein that is being introduced into a patient`s body during the treatment, in a scenario, where the immune system might recognize the protein for the first time. Collectively, nearly 30% of all the patients diagnosed with severe haemophilia, A and 3-13% of patients with mild-moderate forms develop such alloantibodies, capable of inactivating coagulation FVIII, and as a result – nullify the replacement therapy (Pasi et al., 2017). Such a phenomenon has negative consequences for the patient`s health and makes haemophilia treatment highly complex, requiring alternative long-term efficient therapies, such as cell and GT approaches (Krishnamoorthy et al., 2016).

2.7. Epidemiology of HA: Global Perspective and Gulf Region

Among the Inherited Blood Disorders (IBDs), haemophilia has been described as the most common type. The two haemophilia types A and B have been demonstrated to impact one in every 5000 and one in every 50,000 males worldwide respectively (Al-Tonbary et al., 2010). Some risk factors have been identified that determine the epidemiology of the disease. First of all, due to the genetic nature of inheritance, family history of haemophilia is a key risk factor (Pasi et al., 2017; Shen et al., 2008; Peyvandi et al., 2016). Secondly, due to the specific genetic inheritance male gender can also be regarded as a risk factor.

The phenomenon of X chromosome inactivation (or lyonization) defined as the inactivation of two out of three random X chromosomes in the human cell, contributes to such prevalence rate of HA among the female population (Wight & Paisley, 2003). Importantly, there is the third type of haemophilia known as haemophilia type C, or

"acquired haemophilia", which affects both genders and ethnic groups in equal proportions at the rate of 1/1,000,000 persons and is manifested by the body producing antibodies that target FVIII (Shen et al., 2008, Wight & Paisley, 2003 Collins PW, 2011).

The acquired form can be due to inflammatory bowel disease, hepatitis, pregnancy and the postpartum period, diabetes, etc. (Peyvandi et al., 2016)

According to the argument presented by Konkle et al. (2015), genetic factors, as well as cultural differences, may contribute to epidemiological patterns of haemophilia distribution worldwide. For example, within Egypt where the local population has reached 80 million people, consanguineous marriage is a widespread phenomenon. This leads to more individuals being born with autosomal recessive coagulation diseases and an overall high prevalence of haemophilia when compared to the rest of the world (Al-Tonbary et al., 2010; Krishnamoorthy et al., 2016).

Reports about the frequency of different types of haemophilias -including type A- in the Middle East in general and Gulf Region Countries in particular, are very scattered. For instance, Awidi conducted longitudinal research to explore the frequency and severity of various IBDs within Jordan. According to the obtained results, HA, Von Willebrand Disease, and Glanzmann thrombasthenia are the most frequent disorders in Jordan, with 10% of haemophilia A patients experiencing haemophilia inhibitors (Awidi, 2006). Abu Amero implemented the first systematic sequencing effort of various haemophilia-related gene abnormalities among Arab patients in Qatar to investigate FVIII mutations spectrum. According to the obtained results from this pilot study conducted on 20 patients, patients displayed a range of FVIII mutations, among, which 22 intron inversion was the most

common mutation impacting nearly 55% of the patient population, (11 out of total 20 HA subjects) in range with the worldwide described genetic distribution (Abu Amero, 2008).

2.8. GT approach for the treatment of HA

Due to the reasons discussed above, scientists are focusing on advancing therapeutic platforms for HA, to ensure a long lasting and efficient treatment. (Nienhuis, Nathwani, & Davidoff, 2017). GT can be a fascinating approach for treating HA for the reasons that: a) this disorder is monogenic, which makes it a good target for GT, b) different cell types in the human body are capable of synthesizing functional FVIII protein meaning that different cell types can be targeted for gene transfer, and c) GT is more economical and less time consuming than the protein replacement therapy, which is expensive, it requires multiple infusions of the recombinant protein and – as discussed before - can lead to the risk of developing anti-recombinant FVIII antibodies (Dimichele, 2002).

Several studies are focused on hepatocytes as a target for GT approach to correct hemophilia as the liver is known for being the primary source for coagulation factors production. GT targeting liver was tested by IV infusion of adeno-associated virus (AAV) vectors that successfully showed outstanding efficiency in correcting HA in dogs (Finn et al., 2010). Not only in canine models, clinical trial showed that AAV vector had a high efficacy in transducing hepatocytes and showed long term of expression of clotting factors in severe haemophilic patients (Rangarajan S et al., 2017). However, due to the immunogenicity of the liver, using IV infusion of AAV vectors directed to hepatocytes can lead to immune-mediated reactions, through T-cell responses (CD8+) directed to the AAV

capsid. The hallmark of this event is the elevation of liver enzymes (Mingozzi et al., 2007), and this alteration is tightly monitored in clinical trials, since it is the first sign of immune-rejection of the GT product. Patients with liver enzymes alterations have to undergo cycles of high-dose immune suppressive therapies, to promptly stop this immune trigger. Therefore, scientists are working on different viral vectors not only to improve the efficacy of the gene transduction but also to improve the immunogenicity profile.
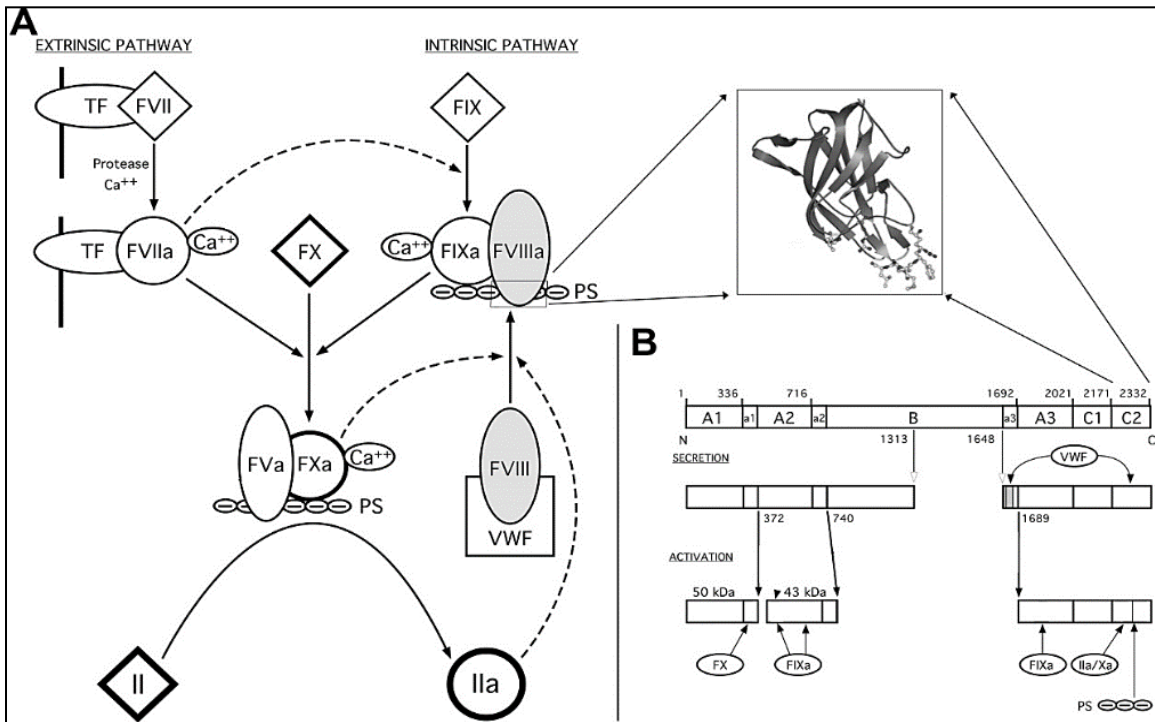
Figure 4. The coagulation cascade and the role and structure of FVIII (source: Shen et al., 2008)

Lentiviral vectors (LV) are less immunogenic in comparison with AAV, and at the same time, their capacity for transgene cassette expression is suitable for a large gene like FVIII. To solve the problem of high immunogenicity, parallel pre-clinical work is ongoing to test the gene correction through LV targeting other tissues, more immune-privileged than liver. FVIII LV mediated ex-vivo GT using mobilized peripheral blood CD34+ HSC with

a CD68 monocyte specific promoter showed a sustained FVIII production in an immunodeficient murine model without signs of toxicity (Doering, et al., 2018).

CD34+ HSC cells have been proven an optimal target of GT correction in several disease models, as ADA-SCID, Fanconi Anaemia, Wiskott-Aldrich Syndrome and Beta-Thalassemia (Donnelly, Zelterman, Sharkis, & Krause, 1999). Once gene corrected, these cells give rise to a stable gene modified progeny, that is long-lasting. So far, the longest patients' follow-up of are about 20 years in SCID diseases, but potentially the correction is lifelong.

In HA is not clear which HSC-derived lineage is producing FVIII, other than megakaryocytes, and myeloid-derived Kupffer cells, although these evidences and the scientific achievements in HA phenotype corrections in mice after CD68 targeted GT, collectively point to the myeloid lineages (Doering, et al., 2018).

While targeting the HSC population with gene modifications is necessary to maintain a stable transgene production, the choice of the best final target cell is needed in HA, since FVIII off-target expression might be toxic for the cells and for the whole body (Merlin, et al., 2017).

There are some hurdles in the field of GT, one of them is the insertional mutagenesis which is the case that the DNA is integrated in a certain spot within the genome for instance in a tumour suppressor gene, this can lead to cancer development which increases the harm to the patient. This occurred in clinical trials for X-SCID leading to some patients about 15% developing leukaemia after receiving GT treatment of stem cells transduced with curative gene (Thrasher AJ, et al. 2006). In addition to the hurdle of durability of GT

benefit. Therefore, careful monitoring of the results of GT treatment by proper analysis needs to be implemented.

Noticeably, a Flow-cytometry (FC) platform for the visualization of FVIII protein in blood cells would be the best option to advance in this field, both in the discovery of the best GT target cell, and to assess GT results in such target cells.

2.9. Flow-Cytometry: A Method to Visualize and Characterize Proteins

Flow cytometry is a clinical chemistry technology that analyses single cells based on their fluorescence and optical characteristics. Physical properties, like internal complexity and size, which affect how light is scattered in this technology, can be used to determine certain cell groups (Pandey et al., 2013). In flow cytometry, a laser emits a beam of monochromatic light through the cells in a fluid sample, and the cells further give off the light in all directions. Optics, in the flow cytometer, direct the given off light to dichroic mirrors and an array of filters that sequester specific wavelength bands, therefore, resolving cell populations (Pandey et al., 2013).

Flow-Cytometry recently started gaining popularity and preference by many cancer therapists, immunologists, and even gene-therapists. Flow-Cytometry is a complex instrumental measurement for multiple physical properties of a cell (Figure 5).

It can assess cell's size, granularity and different proteins expression and characterize therefore cells phenotype trough CD antigen markers/expression/secretion of individual molecules. All these parameters are measured simultaneously as the cells in fluid

suspension is flowing through the cytometry device (Adan, Alizada, Kiraz, Baran, & Nalbant, 2017).
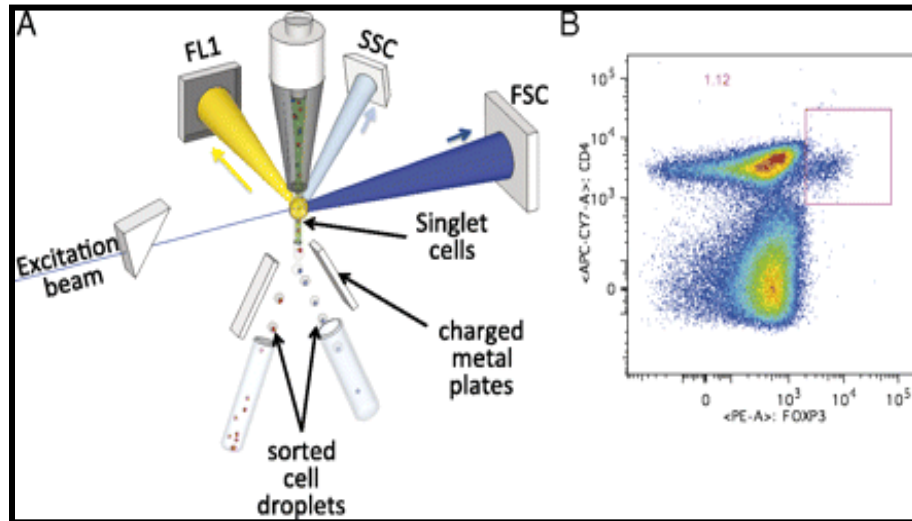
Figure 5. A scheme of FC and a sample showing Flow-Cytometric analysis of data: A) a suspension of cells moving through the sheath fluid to the flow cell in, which a laser beam hit the cells leading to light scatters (FSC: forward scatter and SSC: side scatter). B) An example of analysis of CD4+ T cells that are also positive for FOXP3+. (Source: Jaye, Bray, Gebel, Harris & Waller, 2012)

Cells are usually labelled with fluorescent dyes or with fluorescent monoclonal antibodies before being analyzed by FC. While flowing in the machine, cells move in a sheath fluid and their physical and fluorescent properties are captured by the device within small sheat-fluid droplets; each droplet is a single cell.

Forward scatter (FSC), reflects cells' size and Side scatter (SSC) reflects granularity and complexity of cells. In addition, the fluorescent light emitting from the antibodies bound to the cells are detected form the Fluorescence channels (example: FL1 in Figure 5) of the machine.

The field of FC has grown considerably for the last three decades both in the areas of research and clinical diagnostics, as shown by the number of articles in the medical literature involving FC (Figure 6).

Number of publications per year

*(1953) The Coulter Principle. Invention of A method of counting cells suspended in A fluid

*(1965) Van Dilla at Los Alamos Labs invents the cell sorter.

*(1969) Gohde develops the first fluorescent flow cytometer; Van Dilla et al use cell microfluorimetry to measure cell properties

*(1972) Herzenberg and his team at Stanford invent fluorescence activated cell sorting (FACS)

*(1975) Kohler and Millstein produce monoclonal antibodies in hybridomas

*(1979) Reinherz separate subsets of T cells using monoclonal antibodies

*Early 1980's, high speed flow cytometry used to sort chromosomes for the Human Genome Project.

*(1985) The same year the HIV/AIDS pandemic announced, new techniques for assessing cell morphology using two parameters of light scatter were developed by Smart et al.

*(2002) New fluorescent dyes with narrow excitations and emission wavelength bands

Figure 6. Representation of FC main milestones: a bar graph showing the critical FC development events. (Source: Jaye et al., 2012).

Flow-cytometry analysis as a methodology has several advantages over other methods such as immunohistochemistry or Western blotting in terms of functional assessment and characterization of all types of cells. The benefits of FC can be summarized

as flexibility, reproducibility, increased specificity and most of all, the possibility of function evaluations.

Therefore, an FC protocol optimized and validated for the visualization of FVIII protein would be an excellent option to assess the efficiency of HA Cell and GT trials, particularly for blood cells, but also for any FVIII containing cell. Of note, such method would also open unprecedented diagnostic potentials for HA patients.

CHAPTER 3: MATERIALS AND METHODS

3.1. Materials

Cells used:

U937 (human pre-monocytic) cells, HECV (human vascular endothelial) cells were received as a kind gift from Antonia Follenzi, Health Sciences Department in Piemonte Orientale University in Novara-Italy. HELA cells (cervical cancer) were purchased from American Type Culture Collection (ATCC™), USA. PBMCs purchased by AllCells (Ca, USA).

Cell culturing media for this project were IMDM, and Advanced RPMI purchased from Gibco™, USA. Foetal Bovine Serum (FBS) used is from Sigma Aldrich™, USA. Trypsin for adherent cells was TrypLE Express Enzyme from Gibco™, USA.

Antibodies used:

Anti-FVIII monoclonal antibodies (GMA8002 against the A1 domain of the FVIII protein and (GMA8024) against the A2 domain of the FVIII protein were purchased from Green Mountain Antibodies™, USA. Corresponding IgG isotype controls: IgG2a and IgG1 from Roche diagnostic™. Anti-CD19: PerCP Cy5.5, anti-CD33: PE-Cy7) anti-surface markers antibodies were from BioLegend™.

FC analysis kits used:

Two main kits for antibody labelling were used: 1) Dylight 650 NHS Ester™, Thermo scientific (the custom antibody labelling kit), 2) Zenon™ Alexa Fluor Mouse Labelling Kit (Zenon antibody labelling with different colours: AF647, AF488, and AF568), Invitrogen. Mouse serum for staining was from Life Technologies™. The FC

staining was performed based on the manufacturer's protocol of the fixing and permeabilization of cells of the BD Cytofix/Cytoperm™ staining Kit. Thermo Fisher Scientific LIVE/DEAD™ Fixable violet Dead Cell Stain kit was utilised. FcR Blocking Reagent was from Miltenyi Biotec™ and Human MFGE8 or Lactadherin was from Avivasysbio™. Lactacystin (26S proteasome inhibitor) was purchased from Sigma Aldrich™, USA.

RNA extraction and quantitative PCR kits used:

As for the RNA isolation and qPCR, for RNA extraction using Invitrogen™ RNA isolation kit, cDNA synthesis cDNA using reverse transcription GoScript™ Promega kit and qPCR was done using GoTaq kit from Promega™.

Gene transfer viral vector used:

The viral vector used is a Lentiviral Vector carrying the Beta-deleted FVIII protein gene cassette, under PGK promoter (Self-Inactivating (SIN) 3rd generation LV hPGK.hFVIIIBDD). This viral vector was a kind gift from Antonia Follenzi, University of Piemonte Orientale, Italy.

3.2. Ethical approval of the research work

This study was performed in a laboratory with an ethical approval for biosafety (IBC approval) from Qatar Biomedical Research Institute-Doha, Qatar. The protocol number of the approval is IBC/2016/009.

3.3. Cell cultures and maintenance of cell lines

Two adherent types of cell lines were used in this project: HECV, which are human vascular endothelial cells and HELA, which are cervical cancer cells. These cells were cultured in IMDM with 10% and 1% (v/v) FBS and Penicillin/Streptomycin respectively (complete medium). Cells were trypsinized once a week with this method. Media was removed by aspiration from the cells; cells were washed with 1X PBS and then detached by incubation for 5-8 minutes with 1 ml of TrypLE Express Enzyme, which is a gentle trypsinizing solution. Media was then added to cells with FBS to inhibit/stop the trypsin, and then cells were collected from flasks, counted using Trypan blue viability stain and microscopy, centrifuged at a speed of 300g for 5' and split (typically ¼ of the volume) into new flasks with fresh media.

The non-adhering human pre-monocytic cell line U937 was also used in this research. U937 cells were cultured in Advanced RPMI complete medium. Cells were counted using Trypan blue viability stain and microscopy, and a specific number of cells were passed (typically ¼ volume of cells) into new flasks with fresh medium. Cells were incubated at 37 degrees, 5% CO_2 incubator. PBMCs were used both either directly after thawing or after an overnight culture with complete medium. Cells for experiments were used at a normalized fashion to always be $1x10^6$ cells/conditions, if not otherwise specified.

Cell culture with Lactadherin (milk fat globule) was performed with 4 nM Lactadherin incubated with seeded cells in 24-well culture plates for overnight to block the cells and prevent FVIII stickiness to the cells' membrane. The next day, cells were harvested and immune-stained for FVIII to detect FVIII expression intracellularly and extracellularly.

FVIII protein degradation via proteasomal pathway was assessed by FC after proteasome inhibition by 50µM Lactacystin incubated with cells for 4 hours cells at 37 degrees, 5% CO_2. After the incubation time, FC was performed on these cells according to staining kit manufacturer's protocol.

3.4. Flow cytometry for cells

Cells were normalized for all FC experiments to be divided into 1 million cells/condition. Then, cells were washed once with PBS for LIVE/DEAD Fixable Dead Cell Staining, performed for 15' in the dark at RT. Cells were then washed with staining buffer (BSA-PBS solution) at 300g for 5'. Surface staining using (anti-CD19: PerCP Cy5.5, anti-CD33: PE-Cy7) anti-surface markers antibodies to the cells with staining buffer done with 20' in 4 degrees' fridge incubation and then cells washed once with staining buffer. Cells were thoroughly re-suspended in fixation buffer and incubated for 15-20' at room temperature.

Cells were then washed and re-suspended in permeabilization buffer along with the fluorescently labelled anti-FVIII antibody and incubated for 15' at room temperature. Cells were then washed twice with staining buffer at 300g for 5'and resuspended in paraformaldehyde and read on the flow cytometer. The staining was performed according to the manufacturer's protocol steps of the staining FIX & PERM (Molecular Probes™) kit.

For more specific details on labelling method, see also Table 2, Section Results.

3.5. RNA extraction and Quantitative PCR

Some cells were dedicated for mRNA extraction that was performed using RNA extraction from Tissues kit from Thermofisher scientific according to manufacturer's procedures. Cells harvested from the cell culture were centrifuged at 300g for 5' to pellet the cells. Media was discarded and cells were resuspended in RNALater buffer and placed in 4-degree fridge. RNA was extracted in the laboratory fume hood. After mRNA was isolated, synthesis of cDNA from RNA was achieved through using the high capacity kit for cDNA reverse transcription kit GoScript™ Kit from Promega™. Following cDNA synthesis according to the kit's protocol, cDNA was stored at 20- degrees' freezer.

Quantitative PCR was performed using 96 well plates. The plate was set up with RNAse/DNase free water, the kit's Master Mix, the deoxynucleotide Triphosphates (dNTPs), the Taq polymerase, and the primers for FVIII according to manufacturer's protocol. The following hFVIII specific primers were used: (Forward Sequence: CCAGAGTTCCAAGCCTCCAACA; Reverse Sequence: GGAAGTCAGTCTGTGCTCCAATG) for evaluating the FVIII transcripts (Carol Miao et al., 2015).

Samples were usually run in duplicate and results expression is relative to expression of GAPDH housekeeping gene (GAPDH Forward Sequence: TGCACCACCAACTGCTTAGC; Reverse Sequence: GGCATGGACTGTGGTCATGAG). Controls were utilized and samples were analysed in replicates in order to ensure results' consistency and accuracy.

3.6. Viral vector transduction

Cells were harvested and counted by Trypan blue and haemocytometer. The cells were seeded in culturing plates as 500,000 cells per well. Cells were transfected with the Lentiviral vector carrying the FVIII gene cassette at two different Multiplicity of Infection (MOI): 5 MOI and 30 MOI. The cells were transduced with the viral vector for 24 hours. On the second day, cells were washed to remove the virus and cells were cultured normally.

3.7. Data analysis

FC data were analysed using FlowJo version 8.5 and Kaluza softwares. Quantitative PCR data were analysed by Roche Light Cycler software, the data were analysed using the double delta Ct analysis using Ct values of the housekeeping and FVIII genes. Moreover, the statistical analyses were done using GraphPad Prism and Microsoft excel software in which the type of test that were used to check significance of results is by using Student's t-test to compare means and the results were shown or represented as Mean+/- SEM.

CHAPTER 4: RESULTS

4 Optimization of The Flow-Cytometry Staining Procedure for FVIII Visualization:

This project is aimed at optimizing and validating a FC staining protocol that can serve as a tool for FVIII characterization. To successfully do this, as a first step, we stained PBMCs using anti-FVIII and IgG2a antibodies labelled with Dylight 650 NHS Ester™. To visualize FVIII in cells, custom labelling was performed for GMA anti-FVIII antibodies against different FVIII domains with the labelling kit Dylight 650 NHS Ester™.

4.1. FVIII detection in PBMCs using FVIII and IgG2a Dylight 650 NHS Ester™ labelled Antibodies

Murine anti-human FVIII antibody (GMA™ 8002), specific to A1 domain of the FVIII protein in addition to a parallel IgG2a isotype control were labelled in-house using Dylight 650 NHS Ester™ labelling kit and the labelling was done based on the manufacturer's procedural protocol.

Peripheral Blood Mononuclear Cells (PBMCs) were then stained with AF647 custom labelled anti-FVIII or IgG2a control antibodies, at 1 million cells/condition both for surface (by staining without permeabilizing the cells) and intracellular staining, after 1% of Mouse serum blocking in staining buffer to reduce non-specific antibody binding. For surface staining anti-CD19: PerCP Cy5.5, anti-CD33: PE-Cy7 were added as well to distinguish PBMC sub-fractions.

After the staining, the FVIII percentage was measured using flow cytometer and staining showed high positivity of both FVIII and the IgG isotype control, suggesting high

non-specific binding of anti-FVIII antibody to the extracellular and intracellular compartments of PBMCs (Figure 7).
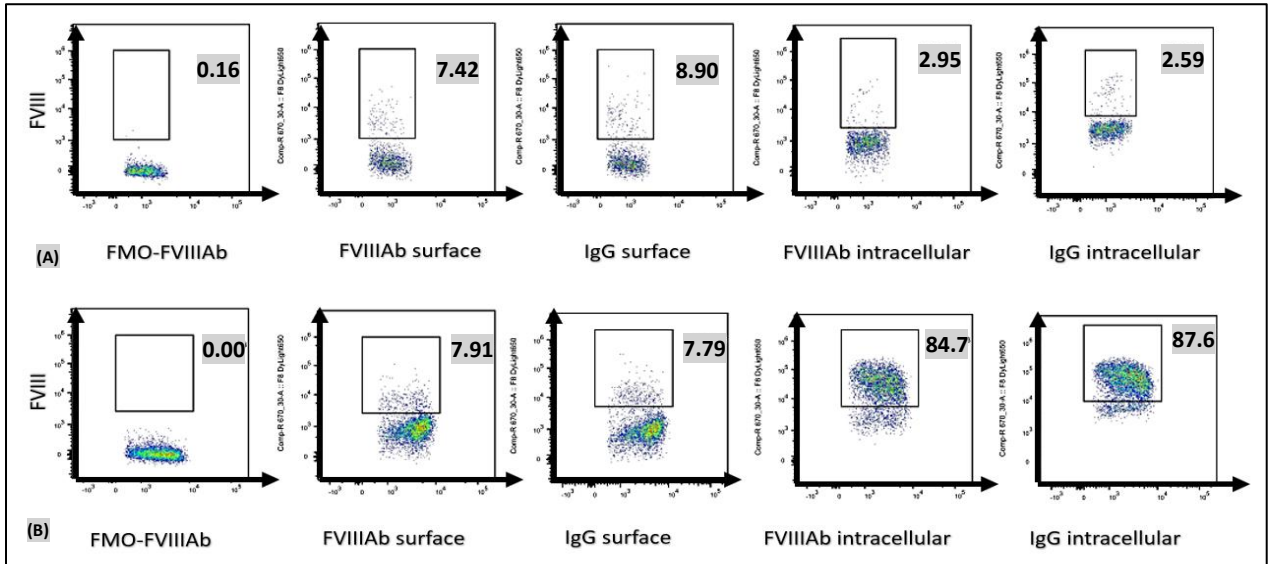

Figure 7: FC analysis for PBMCs stained for FVIII using Dylight 650 NHS labelled anti-FVIII antibodies showing un-specificity staining patterns. Cells shown here were stained for surface marker CD19 (shown the plots in panel A upper row) and CD33 (shown the plots in panel B below row).

4.2. Competition assay to assess specificity and binding (competition with recombinant FVIII protein)

Due to the results of the first experiment an experiment to assess the antibody's specificity was performed. Dylight 650 NHS-labelled anti FVIII and IgG2a antibodies were co-incubated with recombinant FVIII protein before labelling, hypothesizing a reduction in the staining due to saturation of the specific binding sites.

In this assessment experiment, PBMCs were washed once with staining buffer. Cell blocking was done with 1% mouse serum in staining buffer with cells and the other staining conditions were kept as above. The detailed scheme of experiment follows in Figure 8.

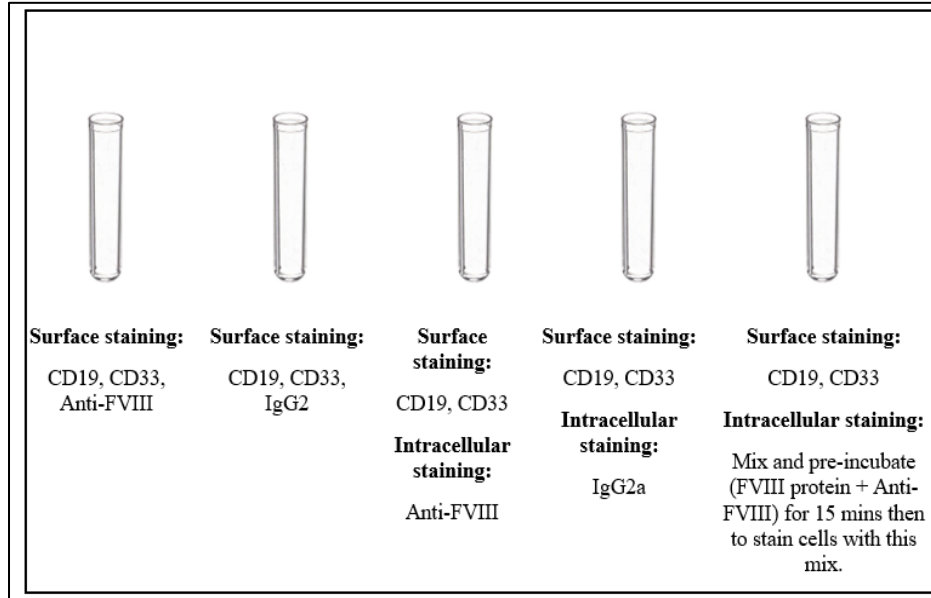| Surface staining:	Surface staining:	Surface staining:	Surface staining:	Surface staining:
CD19, CD33, Anti-FVIII	CD19, CD33, IgG2	CD19, CD33 **Intracellular staining:** Anti-FVIII	CD19, CD33 **Intracellular staining:** IgG2a	CD19, CD33 **Intracellular staining:** Mix and pre-incubate (FVIII protein + Anti-FVIII) for 15 mins then to stain cells with this mix.

Figure 8: Schematic representation of the FC experiment performed on PBMCs to study the behavior of the FVIII protein and the specificity of the antibody.

By pre-incubating the antibody with the protein, the contrary occurred than what was expected: the cells stained with the mix of anti-FVIII antibody and FVIII protein had the highest FVIII percentage of positivity (Fig 9). These data confirmed that the abundance of FVIII protein was binding non-specifically to the cellular membrane.

FVIII has domains (C2 domain mainly) that are involved in the stickiness or aggregation property of the protein to the phosphatidyl serine parts (o-phospho-L-Serine)

or (PS) of the cell membrane. Therefore, it is very likely that as cells were permeabilized and incubated with recombinant FVIII protein; the protein was aggregating to the membrane in the PS regions leading to the antibody unspecific binding as shown in the results.

Moreover, antibodies' Fc regions can bind to the PBMCs Fc receptors, very abundant especially in dendritic cells and monocyte/macrophages. This highlighted the need for proper and adequate blocking steps in order to mitigate for the un-specific binding of the antibodies and for the adhesiveness of the protein.
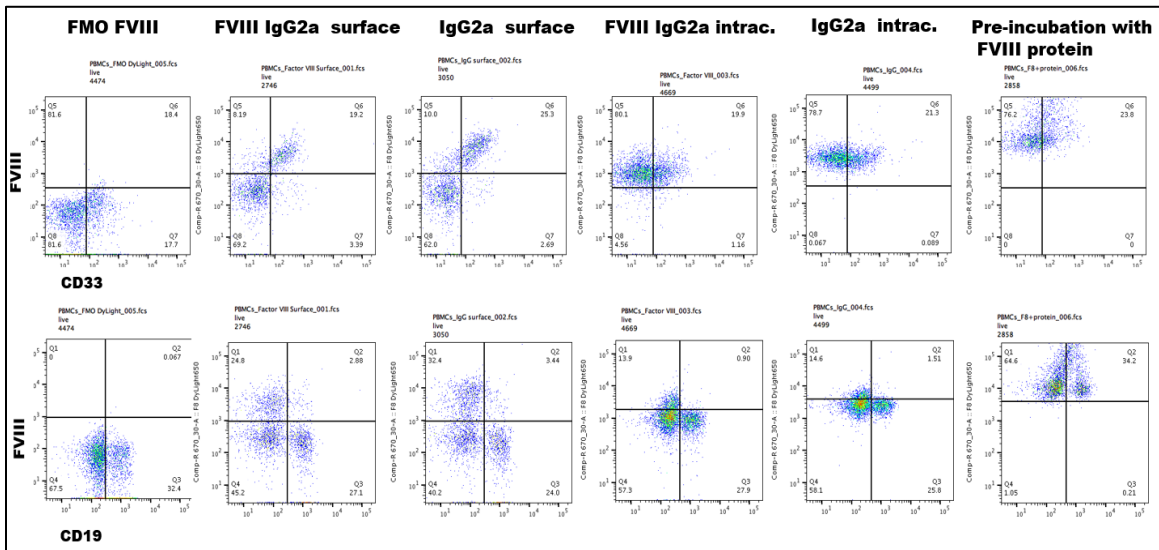

Figure 9: Dot plot of the FC experiment for FVIII assessment. Showing the data of the competition assay. As shown clearly. Pre-incubation of PBMCs with recombinant FVIII protein led to increase in staining.

4.4. Comparison between Fc Block Vs. Mouse serum block efficiencies in reducing the intracellular non-specific binding using PBMCs and use of IgG1 isotype control

The next experiment was done to mitigate the unspecific binding of antibodies; therefore, we assessed the efficiency of Fc blocking with increasing concentrations versus the efficiency of mouse serum blocking. Also, this experiment was done to compare between IgG1 isotype control versus IgG2a isotype control.

The FC was performed on PBMCs, stained using the same procedure with either: IgG2a or IgG1 isotype labelled with AF647. The Fc blocking (at different concentrations) and mouse serum blocking (10% concentration) were examined. The conditions of the experiment were: Negative (Unstained, fixed), live and dead staining only, IgG2a + mouse serum 10% (v/v) + Fc 2.5 µg; IgG2a + Fc 2.5 µg; IgG2a + Fc 5 µg; IgG2a + mouse serum 10% (v/v), IgG1 + Fc 5 µg. All conditions except the negative conditions were stained for CD14 BV421 and CD 8 PE.

Mostly monocytes were affected by high un-specificity. This is due to high autofluorescence and to the abundance of Fc receptors. Indeed, increasing concentrations of Fc blocking showed a partial correction of un-specificity (Figure 10 panel A and B).

However, addition of 10% Mouse Serum blocking showed even neater staining without FC block (panel C). Adding 10% MS to 2.5 mg FC block did not increase the efficiency of MS alone (panel D). This indicates that MS is per se efficient enough to block Fc receptors.

Finally, substituting IgG1 to IgG2 showed clearly a drastic reduction in the background staining (panel E). Thereafter, Mouse Serum 10% block was consistently added to every experiment and IgG1 preferred as control instead of IgG2.
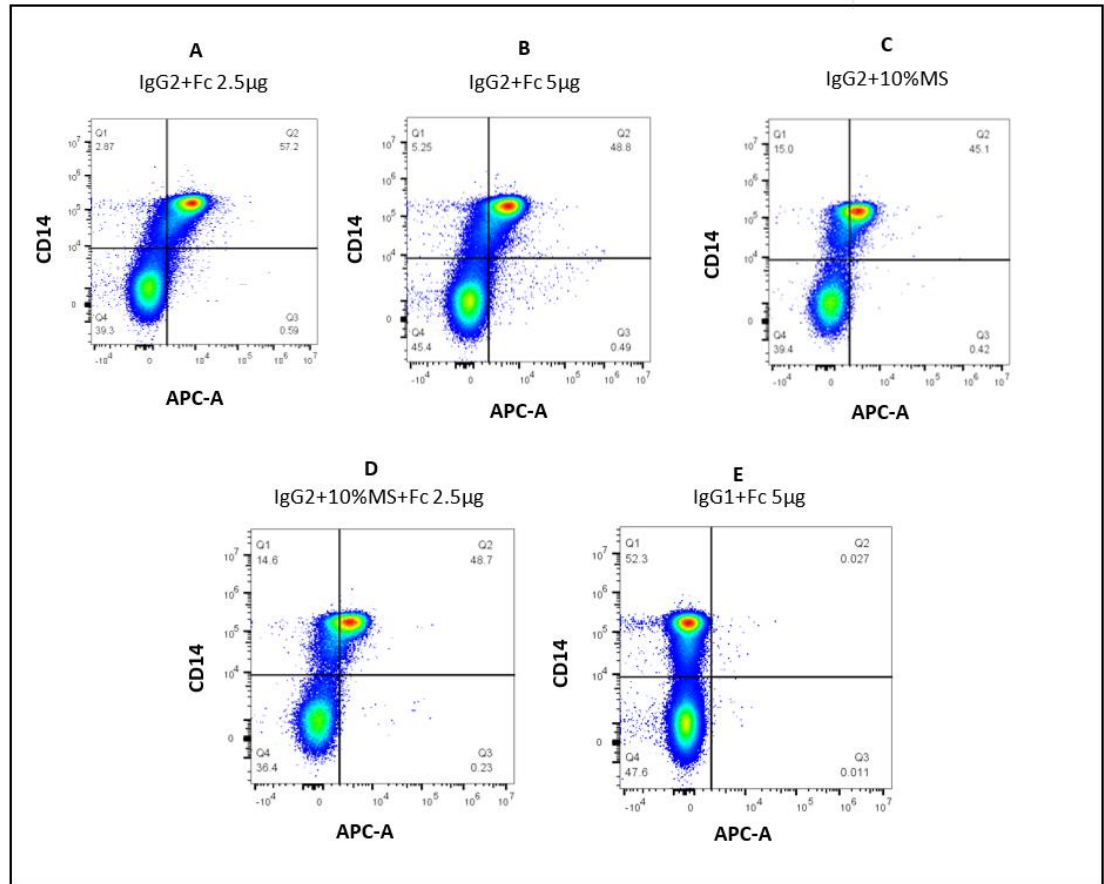

Figure 10: FC experiment for comparison of different blocking types: comparing the efficacy of different concentrations of Fc blocking (Panel A 2,5 µg/100 µl buffer, Panel B 5 µg/100 µl buffer, Panel C Mouse serum 10% final concentration, Panel D 2,5 µg/100 µl buffer and panel E IgG1 isotype control.

4.5. Zenon technology for labelling of the anti-FVIII antibody

We compared to custom labelled antibody another labelling method for anti-FVIII antibody: The Invitrogen Zenon™ Alexa Fluor 647 Mouse IgG1 Labelling Kit.

The Zenon labelling technology can provide high specificity, and a time-sparing protocol - as the labelling can be done within minutes - and reliability of results. The technology of Zenon uses Fab fragments that are fluorophore-labelled against Fc of a primary antibody (Anti-FVIII antibody) to form the labelling complex (Fig. 11).

Therefore, the labelling reagent directly stains the target antibody with fluorophore-labelled Fab fragments. The FC experiment data are showing how the staining with Zenon can be more specific than the stable labelled dye in PBMCs. (Fig. 12).
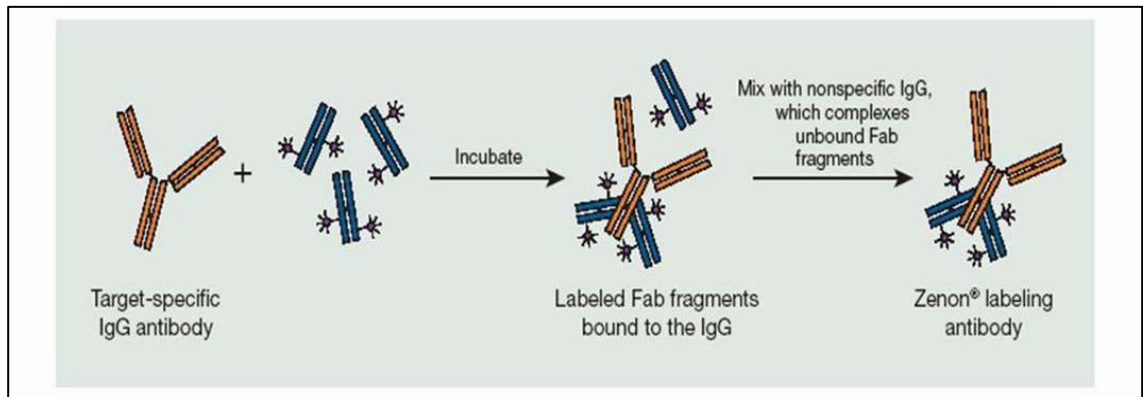

Figure 11: Zenon labelling technology for antibodies: the illustration is showing the principle of this technology of antibody labelling.
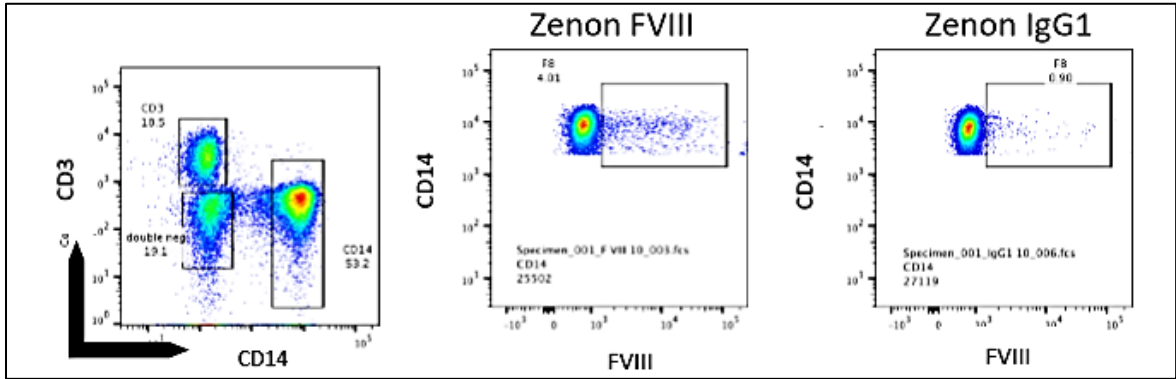
Figure 12: FC dot plots of PBMCs: PBMCs were stained with the anti-FVIII and IgG1 isotype control both labelled with the Zenon labelling kit for AlexaFluor 647. As shown, the staining quality is improved with the Zenon technology giving less un-specific binding and more clean staining of cells.

4.6. FVIII FC visualization on cell lines

To verify if FC FVIII visualization was broadly applicable to various cell types and not only restricted on blood cells, we tested different cell lines with this staining.

We chose HECV endothelial cells, as "professional" FVIII producers' cells, since FVIII is mostly produced by liver sinusoidal cells. We selected then HELA cancer cell line, being the cervical tissue rich of FVIII mRNA (Su AI, Wiltshire T, Batalov S, et al., 2004). We also included U937 cells: a pre-monocytic cell line, representative of myeloid blood cell capacity for FVIII assembly and production (Zanolini D, Haematologica 2015).

We first confirmed the mRNA content of the cell lines through quantitative RT-PCR.

FVIII mRNA was measured against the housekeeping gene primers for GAPDH. Expression of FVIII mRNA was tested after RNA isolation, cDNA synthesis and then cDNA amplification through quantitative PCR (qPCR). FVIII expression in the different

cell lines is shown below. FVIII protein was successfully visualized in all 3 cell lines, as shown in various experiments listed below.

FVIII mRNA in different cell lines

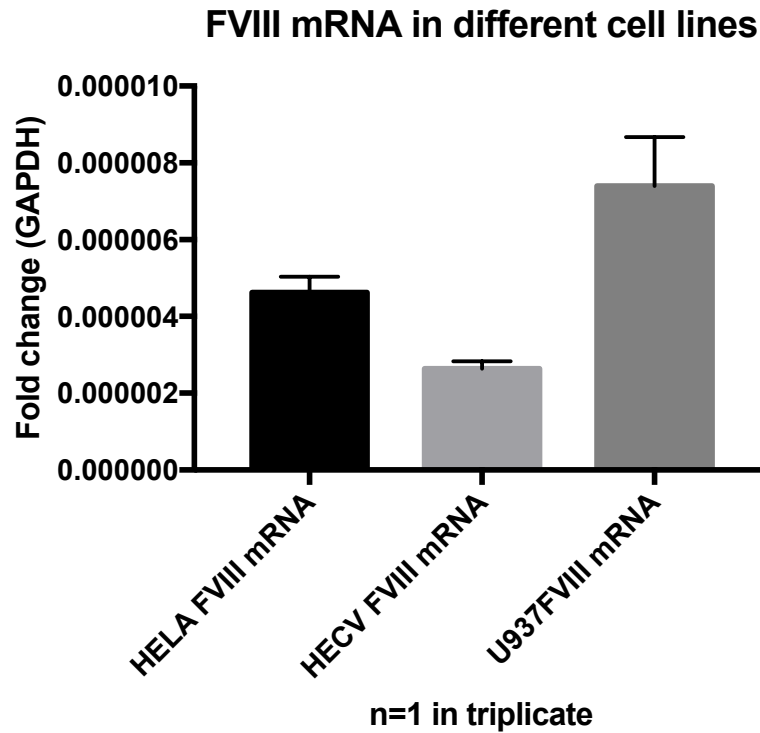
Figure 13: FVIII mRNA expression in un-transduced (UT) HECV, Hela and U937 cell lines.

4.7. FVIII Visualization Gating Strategy

FVIII protein visualization was as well enhanced by consistently using the following stringent logical gating strategy which is illustrated clearly in the below figure of FVIII visualization in U937 cells (Fig. 14). The logical gating starts by defining the cell

populations strictly on the FSC/SSC parameters, then to exclude doublet cells and select

singlets only on FSC-A/FSC-H parameters, and further exclude dead cells/debris,

enriched of cell membranes non-specifically bound to FVIII molecules through PS

exposed sites. Finally, FVIII positivity gets compared to the appropriate IgG isotype
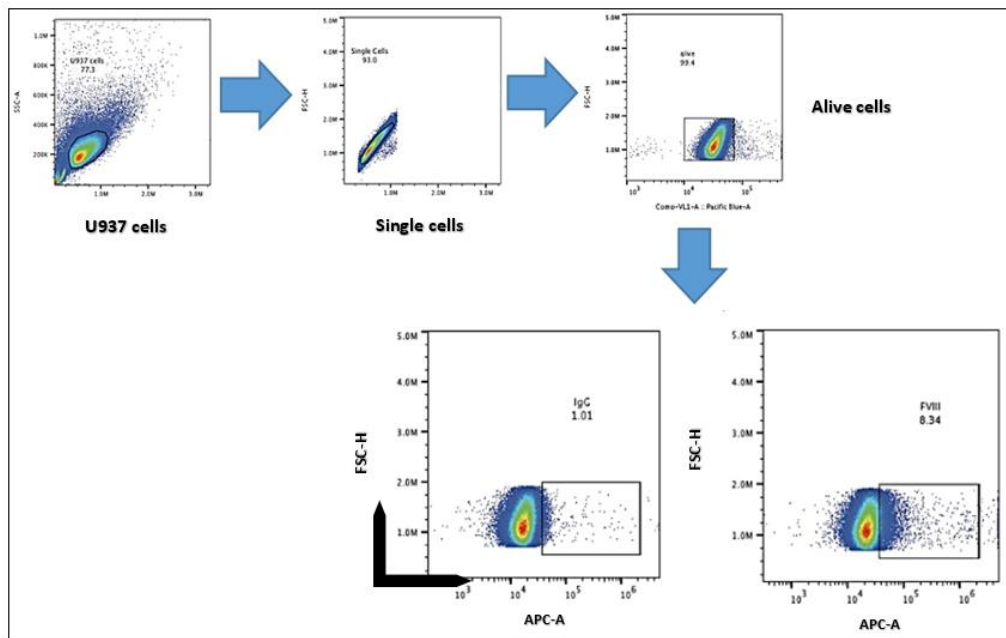
control.

Figure 14: Gating strategy for FC analysis for FVIII. Illustration of the gating strategy used for FC analysis of U937 cells stained with anti-FVIII antibody labelled with Zenon AF647.

4.8. Different cell confluence interplay with FVIII expression in HECV and HELA

Cell confluence can have its impact on the expression of proteins by cells such as FVIII protein because high confluence may inhibit protein production. The confluence of cells and its interplay with FVIII expression was carefully assessed using 2 adherent cell-lines: HECV, and HELA cells. The cells were divided into several culture flasks with different confluence rates: 50% HECV cell culture flask, 50% HELA cell culture flask, 80% HECV cell culture flask, and 80% HELA cell culture flask.

Then the different flasks were harvested and analysed by FC for FVIII expression detection using the using Zenon labelling method.

This experiment was vital for understanding the most suitable confluence for FVIII cell production (Fig. 15). The experiment confirmed – as expected - that expression of FVIII was relatively higher in cells with 50% confluence than in cells with 80% confluence, both in HECV and HELA (fig. 16).

Figure 15: Cell confluence and FVIII expression rate. FC dot plots of HELA cells stained with Zenon anti-FVIII antibody. (a) and (b) 50% confluent HELA FVIII and IgG. (c) and (d) 80% confluence. This figure shows the gating strategy shown in the ancestry plots. N=3
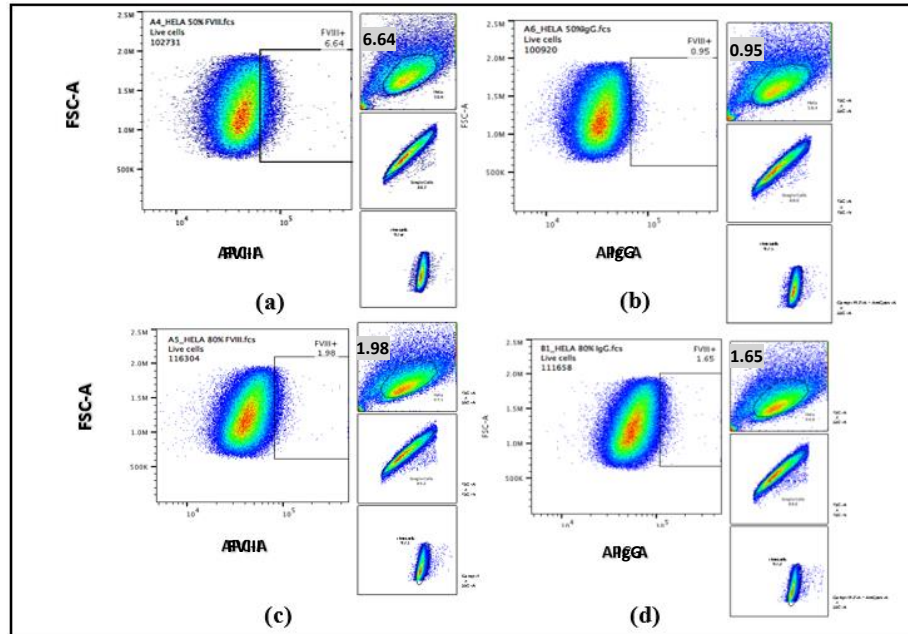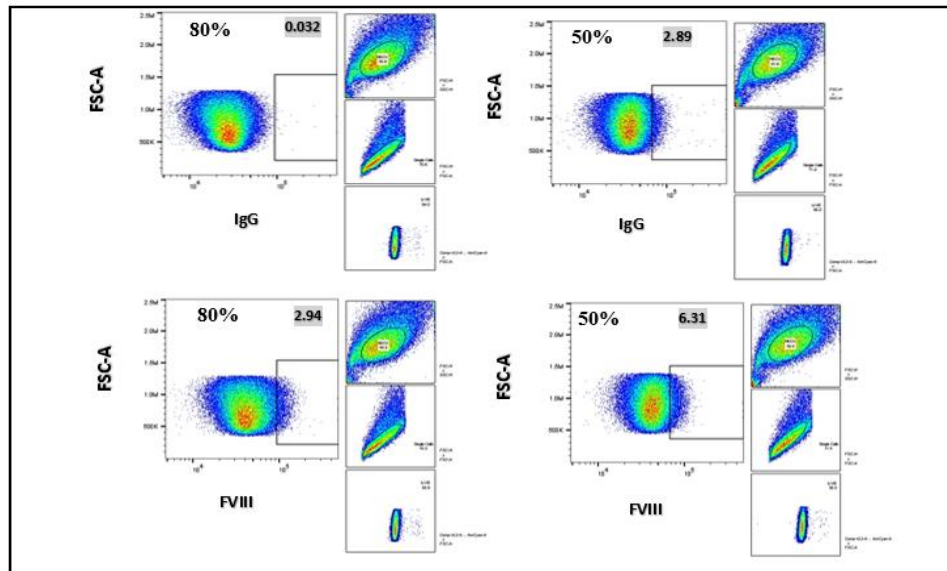

Figure 16: Cell confluence and FVIII expression rate. FC dot plots of HECV cells stained with Zenon anti-FVIII antibody. (a) and (b) 50% confluent HECV FVIII and IgG. (c) and (d) 80% confluence. This figure shows also the gating strategy shown in the ancestry plots. N=3

4.9. Assessment of Lactadherin (MFGE8) blocking's effect on expression of FVIII

FVIII binds to phosphatidylserine sites on the cellular membrane. Due to this binding property, FVIII proteins secreted by bystander cells may attach to the cell membrane and confound measurements of cell-specific FVIII.

Different blocking measures can be tested to decrease this binding and increase therefore the specificity of antibody staining. In this project the human recombinant milk protein MFGE8 (milk fat globule-EGF factor 8) or Lactadherin was used for this purpose.

Lactadherin was incubated with seeded cells (U937 cells) at a concentration of 4 nM in 24 well-culture plates overnight. The next day, cells were harvested and stained to detect FVIII using Zenon labelled anti-FVIII antibody labelling kit either intracellularly (after cell permeabilization) or extracellularly (by surface staining without permeabilizing the cells). The results of the of this experiment (Fig. 17) confirmed that Lactadherin competed with FVIII on binding on the surface and this was clearly shown by the noticeable reduction of FVIII percentage in the cells incubated with Lactadherin overnight.

When Lactadherin was tested on intracellular staining, it did not affect the % of FVIII positive cells, showing that the surface unspecific binding was not a confounding element in the intracellular staining procedure. Therefore, we did not include Lactadherin treatment in the blocking steps of the intracellular FVIII visualization protocol.
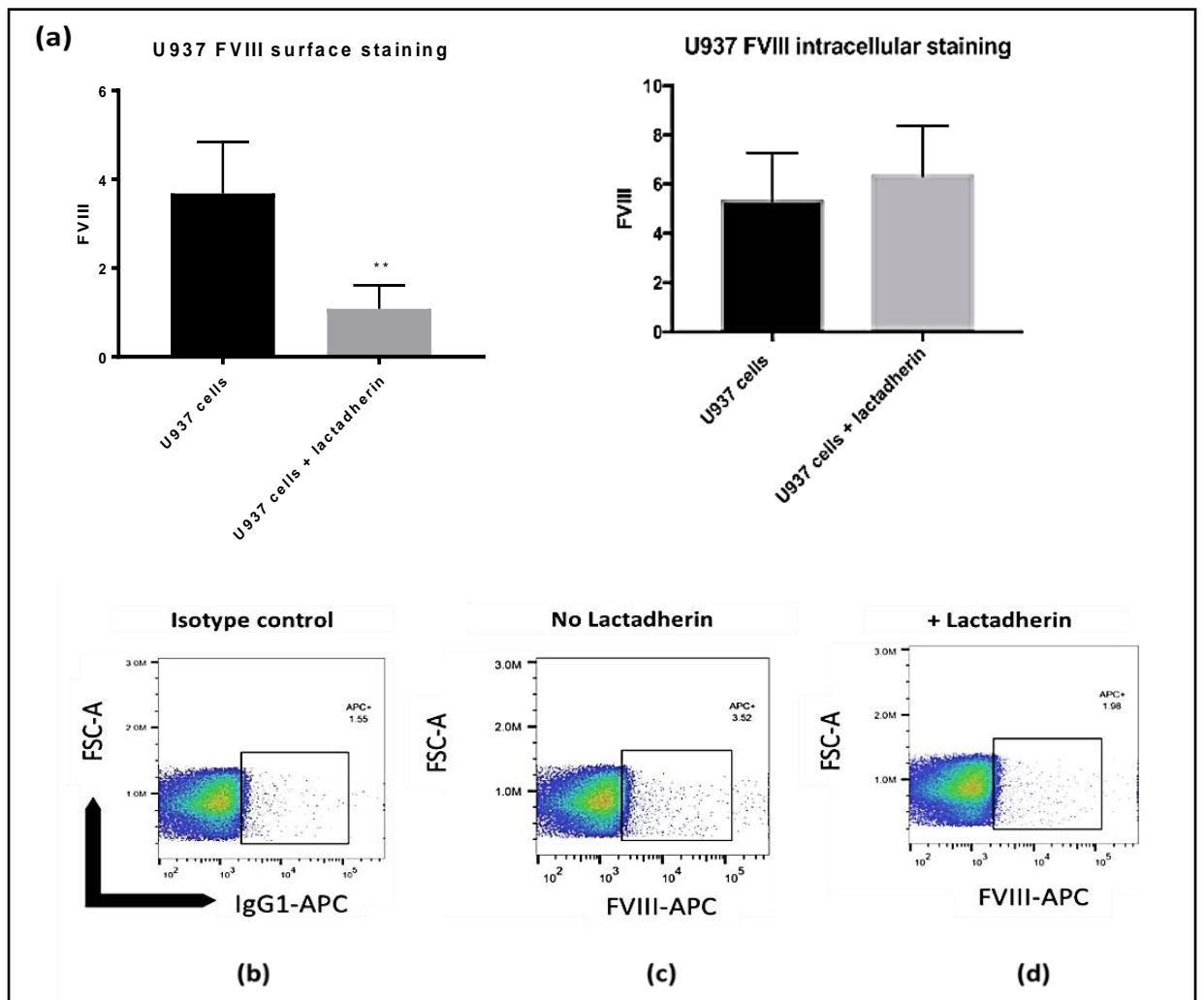
Figure 17: FC for Lactadherin Competition assay: (a) Bar-chart showing FVIII% of U937 cells without Lactadherin vs with Lactadherin overnight (12 hrs) incubation (Mean+/- SEM of N=4, P-Value=0.0039) and intracellular staining . (b)(c)(d) are dotplots of FC analyses of FVIII% in U937 cells (without Lactadherin incubation=3.52% and with Lactadherin incubation=1.98%).

4.10.   The proposed optimized FC staining protocol for FVIII characterization

Table 2. The optimized protocol of FC analysis for FVIII visualization with Zenon and with custom labelled Abs

Step		Details	Interval
1	**Harvesting of cells**	Wash cells once with PBS (Centrifuge). Harvest cells and count using Trypan blue and hemocytometer and divide cells as one million cells per condition.	Centrifuge at 300xg, for 5 min at RT.
2	**Live and dead staining**	In 1 ml of cells in PBS, add 1 µl of live/dead dye (Incubate), according to manufacturer protocol (LIVE/DEAD™ Viability/Vitality Kit, Molecular Probes)	Incubate for 15 min in the dark at RT.
3	**Staining buffer wash**	Wash cells in 3 ml of staining buffer: 0.1% BSA-PBS solution (Centrifuge).	Centrifuge at 300xg, for 5 min at RT.
4	**CD markers or FVIII (surface staining)**	Cells are re-suspended in X µl of staining buffer for surface staining, desired labelled surface Abs are added (total volume for surface staining should be 100 µl) (Incubate).	Incubate for 30 min in the dark at RT.
5	**Staining-buffer wash**	Wash cells in 3 ml of staining buffer: 0.1% BSA-PBS solution (Centrifuge).	Centrifuge at 300xg, for 5 min at RT.
6	**Cells Fixation**	Re-suspend cell pellet in 100 µl Fixing buffer (Incubate) according to manufacturer protocol (Invitrogen™ FIX & PERM™ Cell Permeabilization Kit).	Incubate for 15 min at RT.
7	**Staining-buffer wash**	Wash cells in 3 ml of staining buffer: 0.1% BSA-PBS solution (Centrifuge).	Centrifuge at 300xg, for 5 min at RT.
8	**Cells Permealization**	Re-suspend the cells in 100µl of perm buffer. Add 10% final concentration of Mouse Serum (Incubate).	Incubate for 15 min at RT.
9	**FVIII (Intracellular staining ɫ)**	- Zenon staining: Label anti-FVIII Abs and IgG isotype control with Zenon labelling dye (according to manufacturer protocol of Zenon™ Antibody Labelling Kits Thermofisher) and add the labelled anti-FVIII Ab to the cells (Incubate). - Stably labelled anti-FVIII Abs: add to cells the labelled Abs at titrated concentration. (Incubate)	Incubate for 20 min in the dark at RT.
10	**Staining-buffer wash**	Wash cells in 3 ml of staining buffer which is 0.1% BSA-PBS solution (Centrifuge).	Centrifuge at 300xg, for 5 min at RT.
11	**Final Cells Fixation**	Re-suspend the cells in 200 µl of 2% paraformaldehyde-PBS.	_
12	**Data acquisition**	Acquire the results on Flow-cytometry instrument	_

ɫ During the incubation time, compensation beads are as well prepared for further FC acquisition, using UltraComp eBeads™ from Invitrogen™ with adding equivalent colors used for cellular staining.

The optimized protocol was then used to proof the FC analytic method of FVIII in different biological scenarios.

4.11. Lentiviral Vector -mediated gene transfer of FVIII in cell lines

A 3rd generation Self Inactivating (SIN) Lentiviral vector, carrying the FVIII human gene under a Phosphoglycerate Kinase (PGK) promoter was used to transduce cell lines at different viral concentrations, measured as MOI (Multiplicity of Infection Units) to represent the number of viral particles added to the cells in culture. MOIs of 5 to 40 (meaning 5 to 40 viral particles/cell) were used for the experiments.

After a day of incubation, the viruses were removed, and the cells were grown in fresh culture media. Gene transfer experiments were done in the lab by Muhammed Elnaggar and other colleagues.

Transduced cell lines with FVIII gene were tested for FVIII expression. As shown below, the genetic modification of the cells resulted in increasing FVIII expression, according to the MOI used, and plateauing at the highest MOI. This result demonstrated that FVIII transgene-derived protein can be efficiently visualized by FC.
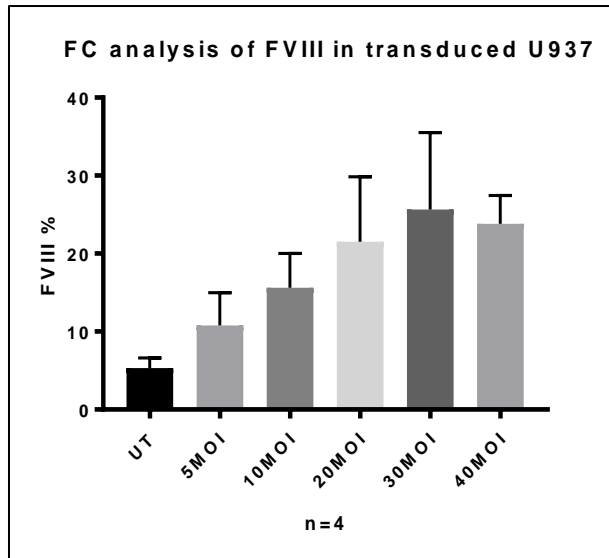
Figure 18: FC FVIII analysis of U937 cells before (UT) and after transduction with LVV-FVIII at different MOIs: FVIII% increases with increasing transduction rate/MOI, plateauing at MOI 30. Mean+/- SEM of N=4.

4.12. Functional study of FVIII kinetics (production and trafficking inside cells) by studying the effect of Lactacystin proteasome inhibition

Un-transduced and transduced cell lines were used to study FVIII protein accumulation effects in the cells. This is important in the field of GT, to exclude toxic effects in the target cells due to overexpression of the transgene.

The first cellular response to protein accumulation is Endoplasmic Reticulum (ER)-stress. This is associated with Ubiquitin-Proteasome System (UPS) and Unfolded Protein Response (UPR) pathways, leading eventually to cell autophagy if the ER stress is prolonged.

To study FVIII accumulation inside the cells, we blocked the proteosomal pathway using Lactacystin which is a 16S proteasome inhibition protein. Lactacystin was incubated at 50μM concentration for 4 hours prior to FC analysis with U937 cells un-transduced or transduced at 5 & 30 MOIs. Proteasome inhibition leads to accumulation of misfolded proteins in the cells.

After proteasome inhibition FVIII was higher both in un-transduced and transduced cells (fig. 19). FVIII sharp increase after proteasomes inhibition proves that normally a good portion of FVIII is routed to proteasome for degradation. This happens both in un-transduced and transduced cells and in different cell lines.
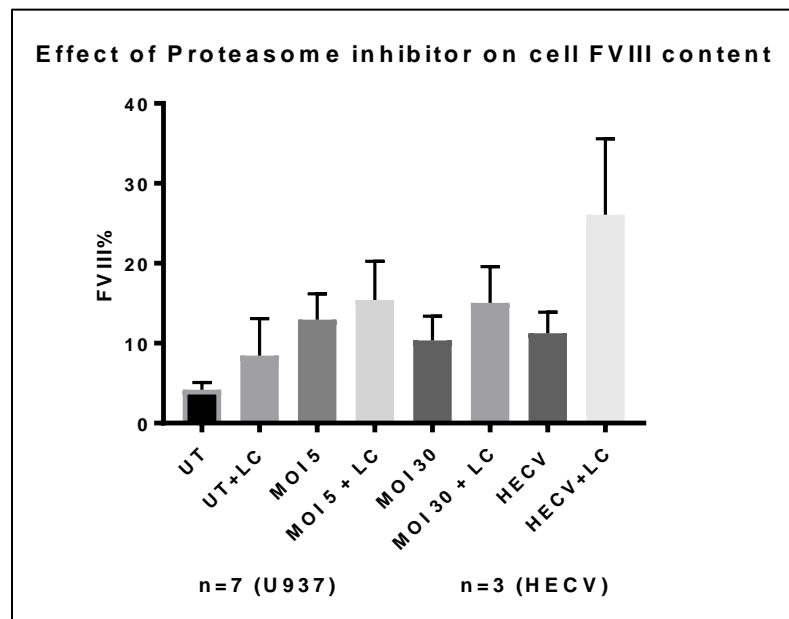

Figure 19: Functional studies of FVIII. Bar graph of FC data showing that transduced U937 cells had higher FVIII than un-transduced cells (effect of gene transduction using PGK-FVIII-LVV (Mean+/- SEM). Adding Lactacystin (LC) to cells shows the accumulation of misfolded proteins inside the cells (effect of LC on FVIII level).

The rates of FVIII in cells is directly proportional to the rate of transduction by FVIII-LV vector (until MOI 30, as shown in Figure 18).

Of note, even un-transduced cells (U937 and HECV) are active in the degradation of existing FVIII misfolded proteins, so this suggests that careful dose-limiting analyses have to be calculated before administering FVIII GT products in order to avoid UPR pathway engulfment and consequent cell apoptosis.

This set of experiments shows the efficiency of the optimized protocol to capture fine functional differences otherwise impossible to highlight with conventional FVIII staining techniques.

4.13. FC analysis validation: Double staining of FVIII with two antibodies against two different domains

To validate the optimised FC staining method and to confirm that staining positivity is truly of FVIII protein being detected, a double staining of cells was performed with 2 Abs targeting 2 different FVIII domains: light chain and A2 domains. Abs were labelled with different colours using Zenon (fig. 20).

This result confirmed the specificity of FVIII FC Ab staining by showing the co-expression of FVIII staining of the 2 different domains.
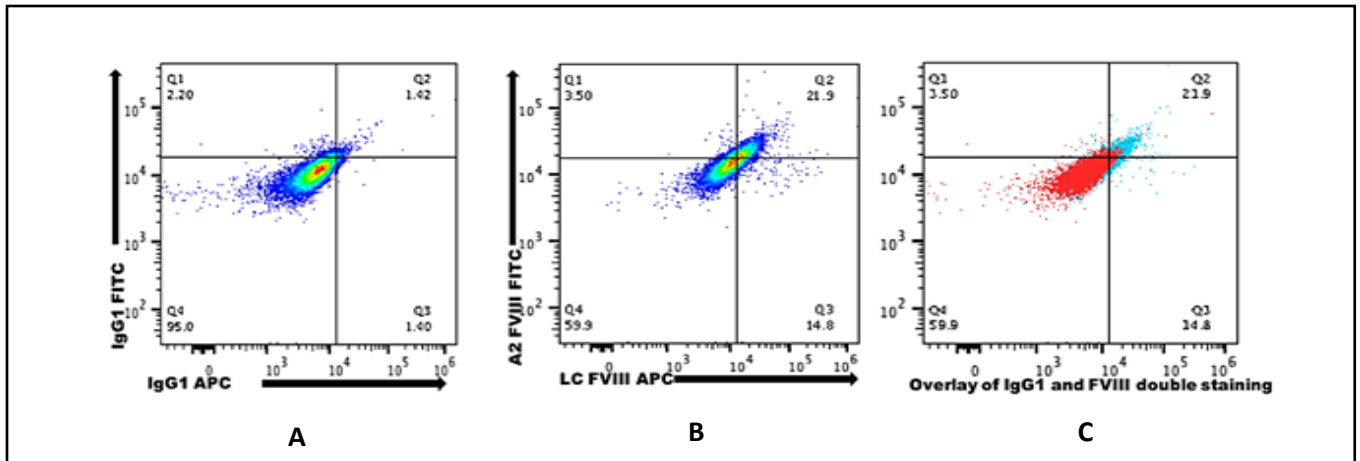
Figure 20: IgG1 anti A2 domain, and -anti LC domain antibodies with IgG1 isotype, were used for a comparison of single and double staining on U937 transduced at 30 MOI with PGK-FVIII-LVV.(A) (B) and (C) FC dot plots showing the double FVIII staining with respect to the isotype control. n=1 in duplicate, means and SEM shown in graph D.

This is demonstrated by the overlay plot (C) that shows FITC-labelled FVIII A2-domain

co-expressed in the upper right quadrant with APC-labelled FVIII Light Chain-domain.

Note: Experimental results listed above were achieved in collaboration with Dr.

Muhammad Elnaggar and other colleagues in the lab.

CHAPTER 5: DISCUSSION

In the field of GT, FC shows its clinical relevance when applied to the determination of gene transfer level on target cells. The outcome of this project suggests that FC technique can be adopted for assessment of GT efficiency, as it achieved a reliable measurement of intracellular and extracellular levels of FVIII protein in different subsets of cells. Such measurement of FVIII protein is not possible by currently used methods. The use of a FC protocol for FVIII characterization is novel and useful after the development of GT approaches for HA. Indeed, currently, no commercial pre-labelled anti-FVIII monoclonal antibody is available to perform FC analysis of cells.

FVIII detection in current clinical diagnostics is performed mainly through plasma level measurements which does not enable FVIII functional studies. The recent development of GT approaches requires a more sophisticated analysis platform, to address specific issues of GT efficiency, toxicity and transgene permanence directly on target cells. An FC analysis able to quantify and functionally measure the transgene in target cells would be a useful reliable and novel tool for GT approaches on HA. Although FC is strongly emerging in the field of clinical research, a validated FC protocol for FVIII protein analysis is still missing and this increases the necessity to this project. To this aim, we sought to optimize in this study a protocol for FC FVIII visualization.

We realized, after the first FC experiments on PBMCs stained with anti-FVIII antibodies labelled with Dylight 650 NHS ester dye, that high non-specificity patterns in FVIII antibodies and IgG control had to be systematically reduced, to avoid unrealistic high positivity results of FVIII in cells. We confirmed by a competition assay with recombinant

FVIII proteins that FVIII itself was un-specifically binding to membrane surfaces, and aggregating labelled antibodies falsely enhancing the positive threshold.

Stickiness of FVIII protein was supposed by the natural behavior of FVIII protein, as pro-coagulant factor, and described by previous scientists who successfully addressed that FVIII has a PS of membrane binding capacity similarly to Annexin 5 (Engelke H et al., 2011) and Lactadherin (Dasgupta, Guchhait, & Thiagarajan, 2006). To address this, different blocking approaches were tested.

PBMCs especially phagocytes like monocytes have Fc binding receptors (Swanson JA et al., 2004). These receptors were hypothesized to be interfering with the un-specificity observed as these receptors bind antibodies. To address this, Fc block and mouse serum block were assessed for their blocking efficacy. We observed that 10% mouse serum blocking gives cleaner staining of PBMCs by blocking un-specific binding sites on cells. Monocytes in PBMCs benefitted from mouse serum and concentrated Fc receptor block; however, the best specificity result was achieved by using IgG1 isotype control instead of IgG2a. Non-specificity in flow cytometry staining is a common issue and was under investigation in order to be tackled. Similarly, to what we concluded, Anderson et al. postulated in a technical research work in which they used PBMCs that erroneous results in immunostaining can be eliminated by proper isotype control and sufficient blocking at high concentrations (Andersen, Al-Karradi, Kragstrup, & Hokland, 2016). In our project, we observed that specifically mouse serum in 10% (v/v) concentration blocking is crucial to eliminate non-specificity due to non-specific binding sites on cells.

Non-specificity of staining was further improved after shifting to Zenon technology for antibody labelling. Antibody labelling with NHS dyes led to high leakage of color and

high fluorescence. Zenon unlike custom labelling, proved itself in all experiments performed to be a more specific labelling method. This is labelling method binds commercially fluorescent-labelled Fab-fragments to Fc of target antibodies; the staining happens outside the cell, and unbound Fab labeled fragments are quenched and washed away, therefore improving the specificity. As observed in PBMCs' FC analysis, this immune-labelling method, provided enhanced reproducible labelling specificity and reduced labeling leakiness issues we faced in the early FC experiments.

The body's sites of FVIII production are still unclear. Apparently, there are many cell types other than hepatic sinusoidal cells and PBMCs, that can produce FVIII, as several cells are rich of FVIII RNA as proven previously in the literature (Su AI, Wiltshire T, Batalov S, et al., 2004). In this project, HECV, HELA and U937 cells were tested by quantitative PCR and confirmed their positivity for FVIII. With an aim to show the applicability of FC analysis on different cell types, these cells were utilized in FC analyses and confirmed their positivity also for FVIII protein by FC analysis.

Cell confluence can impact cell expression pattern. Previous data showed that contact inhibition reduces secretion of proteins as confluent cells become stressed and subsequently enter quiescence which is a state of cell inactivity/dormancy as was described by Hayes et al. that cell confluency's effect on cells in terms of inducing quiescence and affecting cell's functions (Hayes et al. 2005). This shows the high impact of cell confluence on cell activities especially protein production. As noticed in HECV and HELA cells, it is also true for FVIII, that showed an enhanced expression when cells were less confluent as compared to highly confluent cells, giving insight that FVIII production is best visualized at FC at low cell concentration.

FVIII adhesiveness to the plasma membrane was studied using FC analysis. FVIII adhesiveness to PS parts of the membrane of cells was shown through a competition assay between FVIII and Lactadherin. Lactadherin adherence to membranes was previously described by Shi et al. and a similar property was also observed in Annexin 5 (Shi et al., 2004; Dasgupta, Guchhait, & Thiagarajan, 2006). Lactadherin block only affected FVIII extracellular adherence but did not reduce/affect intracellular FVIII levels; thus, Lactadherin block was not implemented in the FC analysis protocol as the main interest of this study was to visualize intracellular FVIII.

FVIII FC protocol after optimization, served as a tool to perform FVIII functional studies; and to investigate FVIII expression in different scenarios. Such FC FVIII studies were the first to be performed in literature.

The first scenario was the LVV gene-transduction mediated FVIII production. We observed that FVIII expression gradually increased after increasing the transduction rate (MOI) showing that gene transfer is effective in making cells produce FVIII in high amounts. It can be suggested that, with very high viral load, cells can be stressed and FVIII expression can be reduced. This was inferred by the evidence of FVIII plateauing at 40 MOI of transduction.

Protein being routed to proteasomal degradation pathway occurs in normal cells and most importantly in transduced cells as a result of protein overload. Cellular stress due to FVIII protein accumulation inside cells, can lead to FVIII degradation and if overloading the UPR machinery, to cell apoptosis. This study showed that FC analysis visualizes FVIII expression in transduced and un-transduced cells, and FVIII misfolded protein accumulation by proteasome inhibition through Lactacystin.

FVIII intracellular accumulation due to AAV mediated gene transduction was studied previously by Sabatino et al. similarly showing that FVIII accumulation inside the cells can activate UPR pathway as Sabatino and his colleagues were able to show by different experiments mainly through quantitative PCR showing that cells after gene-transfer induced protein production can lead to accumulation and activation of Unfolded protein response (Sabatino et al., 2017).

To confidently show that FVIII positivity in the staining was truly to FVIII and not staining artefacts, double staining FC analysis was done to target 2 different FVIII domains with 2 different fluorescence. The data confirmed that staining is specific towards FVIII and not staining artefacts, by showing co-expression of the 2 domains.

It is important to note that the staining protocol was confirmed by multiple lab operators performing the steps and achieving reproducible results and multiple samples being processed showing also consistent results.

CHAPTER 6: CONCLUSION & FUTURE DIRECTIONS

We demonstrated that, after deep and thorough optimization, a robust FC analysis on large proteins as FVIII is possible and is of great importance. This analysis enables measurements that were impossible so far, like functional quantification of intracellular FVIII, potentially applicable as an advanced diagnostic tool both in HA patients and in HA patients treated with GT.

We succeeded in optimizing this protocol in PBMCs and in different cell lines, transduced and un-transduced. This suggests that FC visualization of FVIII can be broadly applied to any FVIII producing cells.

Future directions would be to implement this FC protocol in real clinical practice i.e. for diagnosis of HA patients, and for follow up of HA patients receiving GT, by performing FC analysis to assess GT efficiency and eventual toxicity on target cells.

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Qatar Biomedical Research Institute (QBRI)
Institutional Biosafety Committee Research Registration/Approval Application

W.QM5.01.Feb19.V3

Research involving any of the agents listed below must be approved by the QBRI (Qatar Biomedical Research Institute) Institutional Biosafety Committee (IBC) prior to initiation:

- Pathogens and potential pathogens of humans, animals or plants;
- Materials potentially containing human pathogens (including human blood, tissue, and cell lines; non-human primate blood, tissue, and cell lines);
- Recombinant DNA (and RNA) including creation or use of transgenic plants and animals;
- Any material requiring a Health institutes import license or a customs permit;
- Any material that is considered extremely toxic or a hazardous substance that requires work at a Biosafety Level 3;
- Addition of new *in vivo* work not previously approved by the IBC (previous approval was for *in vitro* work only).

Submission guideline:

- The Principal Investigator (PI) of the research project is responsible for completing all appropriate parts of this registration document and for notifying the IBC when information submitted in this document changes, such as laboratory location, research personnel, procedures, funding, biosafety level (either upgrade or downgrade), etc. If such changes occur, the PI will be required to fill out the Amended application;
- Any submission received after the 10th of each month will be postponed to the next month;
- Only typed forms will be accepted, the application must be completed, signed by all appropriate personnel and approved by the department director and submitted to the **IBC coordinator;**
- At the time of submission, you are asked to also submit all grant proposals pertaining to your research and a copy of certification for Safety training and biosafety cabinets Failure to provide all information requested, including requested signatures, will lead to a delay in processing your request. If further instructions are necessary.

Annual renewal reporting:
To maintain continuing IBC approval, protocol update reports are submitted annually as follow:

- The Investigators should complete the whole application;
- The Investigators are responsible for updating contact information with the IBC Office, and certification for Safety training and biosafety cabinets;
- Institutional focal point should send *courtesy* renewal notices via email prior to expiration of the approval for the IBC office and the Investigators;
- Unmodified renewal application should be reviewed and approved by the Institutional focal point and will be expedited from full committee review.

Termination:
Protocols that are not renewed on the day of the expiration are automatically expired at 11:59 pm that same day.

- Expired or Terminated protocols cannot be "re-started" once the expiration is in effect;
- To reactivate an expired protocol, a new application must be completed (refer to the above renewal reporting).

For IBC Office use only			Principal Investigator:
Date:	Protocol Number:	IBC/2016/009	Sara Deola

A. Submission Information
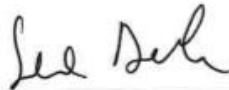
Project Submission	Indicate Yes/No in check boxes	Yes	No
1. Is this an existing (approved) IBC application? • If *NO* (e.g. this is a New application) skip to the next section		✔	☐
2. Provide the protocol number for the existing/renewing IBC approved project:			
3. Have there been any changes in the *investigator contact information* since the last IBC review interval? • If *YES*, section B must be updated to reflect the new Principle Investigator's information		☐	✔
4. Have there been any changes in the *alternate contact information* since the last IBC review interval? • If *YES*, Section B must be updated to reflect the new information		☐	✔
5. Have there been any changes to the *funding* of this research since the last IBC review interval? • If *YES*, Section D question 7 must also be updated to reflect the new information		✔	☐
6. Have there been any changes in the *location of the research facilities* since the last IBC review interval? Did the lab relocate to another building or room? • If *YES*, Section O must be updated to reflect the new information		✔	☐
7. Have there been any changes or additions to the recombinant or synthetic nucleic acid molecules or vectors since the last IBC review interval? • If *YES*, Section F and K must be updated to reflect the new information included with the previously described materials and procedures • Highlight the updated information in the section		✔	☐
8. Have there been any changes or modifications to any linked IACUC protocols since the last IBC review interval? • If *YES*, Sections D and M must be updated, as applicable		☐	✔
9. Are there any changes in the title(s) for this continuing IBC protocol? • If *YES*, Section D must be updated to reflect the new title information		✔	☐
10. Have there been any reported injuries/exposures since the last IBC review interval? • If Yes attach a copy of the report , If *NO*, skip to question, below		☐	✔

B. Principal Investigator Information

Principal Investigator:	Sara Deola
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C. Investigator Assurance

- I attest that the information contained in this registration is accurate and complete.
- I agree to comply with all QBRI- IBC requirements regarding research involving biohazardous and / or recombinant materials.
- I agree not to initiate any research subject to IBC approval unless I have received such approval.
- I agree to notify the IBC immediately of incidents involving biohazardous and / or recombinant nucleic acid agents
- I acknowledge my responsibility for the conduct of this research in accordance with the QBRI IBC safety guidelines and policies.
    a. I have the knowledge and training required to safely handle the materials described.
    b. I agree to train all of my laboratory personnel according to the BSL of the laboratory.
    c. Entry doors to the laboratory will be closed and locked when the laboratory is unattended.
- I agree to provide all personnel working in the laboratory notification, information and training on the hazards, laboratory security and emergency policies and procedures associated with working in my laboratory. **All personnel are further advised that working in a laboratory that conducts experiments using live microorganisms could increase their risk of infection and be hazardous to their health.**

Seel Deel	13/3/19	Sara Deola
Signature of Principal Investigator	**Date**	**Typed/Printed Name**
C.Graell	18/3/19	Christoff Von Kalle
Signature of Center Director	**Date**	**Typed/Printed Name**