DIFFERENTIAL PROTEOMICS STUDY IN HEMATOLOGICAL DISORDERS

By

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DECLARATION

I, hereby declare that the investigation presented in the thesis has been carried out by me. The work is original and has not been submitted earlier as a whole or in part for a degree / diploma at this or any other Institution / University.

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List of Publications arising from the thesis

Journal

1. Differential regulation of plasma proteins between members of a family with homozygous HbE and HbEβ-thalassaemia, Suchismita Halder, Tridip Chatterjee, Amit Chakravarty, Sudipa Chakravarty, Abhijit Chakrabarti, *Thalassemia Reports*, 2014, *Vol.* 4(s1):1837. (doi:10.4081/thal.2014.s1.1837)

2. Fractional Precipitation of Plasma Proteome by Ammonium Sulphate: Case Studies in Leukemia and Thalassemia, Sutapa Saha, Suchismita Halder, Dipankar Bhattacharya, Debasis Banerjee and Abhijit Chakrabarti, *J Proteomics Bioinform*, 2012, *Vol.* 5(8), 163-171. (doi.10.4172/jpb 1000230).

Conferences

1. 1ST Proteomics Society (India) seminar on "INDIAN **Proteomics: User's Perspective**", July 2010, Kolkata.

2. 51ST Annual Conference of Indian Society and Transfusion Medicine "**HAEMATOCON 2010**", November 2010.
   Presented a poster titled "**Plasma Proteomics in Hematological Disorders**".

3. Indian Proteomics Conference "**IPCON 2011**", April 3rd-5th 2011, New Delhi.
   Presented a poster titled "**Plasma Proteomics of Thalassemia**".

4. International Conference on "**Omics Meets Disease**" and 3rd Annual Meeting of Proteomics Society (India), December 2011, Kolkata.

   Presented two poster- a) titled "**Urinary Proteomics in Urothelial Neoplasm**" and

   b) titled "**Lipodomics in E-beta Thalassemia**".

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   Presented a poster titled "**Lipodomics in E-beta Thalassemia**".

6. IUBMB 10TH International Symposium on "**Biochemical Role of Cell Surface Macromolecules**" organised by Saha Institute of Nuclear Physics during 20-24th January 2014.

   Presented a poster titled "**Lipodomics in E-beta Thalassemia**".

Others

1. Proteomics study of hemoglobinopathy, Abhijit Chakrabarti, Suchismita Halder and Shilpita karmakar, 28th ISMAS Symposium cum Workshop on Mass spectrometry held at Parwanoo, March 9 – 13, 2014; IT-8.

2. Differential Expression of Red Cell Proteins in Hemoglobinopathy, Abhijit Chakrabarti Dipankar. Bhattacharya, Avik Basu, Sumanta Basu, Sutapa Saha, Suchismita Halder, *Proteomics Clin Appl*, 2011, *Vol* 5, 98-108. (doi: 10.1002/prca.201000063).

3. Plasma Proteomics in Hematological Disorders, Suchismita Halder, Sutapa Saha, Debashis Banerjee, Sudipa Chakravarty, Amit Chakravarty, Abhijit Chakrabarti, *Ind J of Hematol Blood Trans*, 2010, *Vol.* 26 (4); 167.(doi 10.1007/s12288-010-0045-z).

4. Differential Regulation of Urine Proteins in Urothelial Neoplasm, Suchismita Halder, Ranjan Kumar Dey, Anadi Roy Chowdhury, Palash Bhattacharyya, Abhijit Chakrabarti. (communicated).

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DEDICATIONS

Dedicated to all those children who are diagnosed with thalassemia and are heavily dependent on transfusion for survival, and also to their parents who undergo a mental and financial battle for the well being of their children.

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CONTENTS

SYNOPSIS

DIFFERENTIAL PROTEOMICS OF HEMATOLOGICAL DISORDERS

INTRODUCTION

The HbE-β is one of the commonest forms of hemoglobinopathies worldwide [(1)]. The HbE mutation is located near the junction between the first exon and the first intron of the β-chain gene. Nucleotide sequence change near the consensus splice site region activates a cryptic splice site, which is not normally used for mRNA processing. This new splice site competes with the normal splice site. Some mRNAs are still processed using the normal splice site and thus produce a protein with a Lys instead of a Glu at position 26. The variant (HbE) is thus innocuous in its homozygous states [(2)]. The primary clinical importance of HbE trait arises when the β^E allele interacts with other β-thalassaemia mutations leading to a moderate to severe anemia known as HbEβ-thalassaemia [(3)].

Although the spectra of clinical pathophysiology vary depending on coinheritance of other genetic modifiers, the underlying pathology among the types of thalassemia is similar. This pathology is characterized by decreased Hb production and erythrocytes survival. The excess of unaffected globin chain, which form unstable homotetramers that precipitate as inclusion bodies causes marked erythrocytes damage and severe hemolysis associated with ineffective erythropoiesis (IE) and extramedullary hemolysis [(4)].

The only definitive form of treatment for thalassaemia is bone marrow transplantation, which is possible only when there is a matching donor relative. Symptomatic treatment involves regular blood transfusion and the use of iron-chelating drugs to remove the excess iron that results from transfused blood. Hence more studies are required to frame a better

disease management program. Therefore a lot of attention being given to the disease monitoring as this is of prime importance for the welfare of the patients.

OBJECTIVE

Our objective has been to initiate proteomics studies of the body fluids such as plasma and urine keeping in mind the present disease management system. We have also done lipidomics study of plasma, erythrocytes and erythrocytes membrane. The objectives and scope of the present study would be elaborated in the following three Chapters:

1. **Chapter 1: Plasma Proteomics of Eβ Thalassemia**- conducting a 2D gel electrophoresis (2DGE) based study of the differential expression of proteins of Eβ thalassemic samples as compared to normal samples.
2. **Chapter 2: Urinary Proteomics of Eβ Thalassemia** - 2DGE based study of the changes in the urine proteome of the Eβ thalassemic samples as compared to normal.
3. **Chapter 3: Lipidomics Study of Eβ Thalassemia** - Study the lipidome of plasma, erythrocytes and erythrocytes membrane fractions and analyze the changes in lipidome of the Eβ thalassemia patients as compared to normal samples. We also aim to analyze the levels of oxidized lipids as oxidative stress is a well known condition in Eβ thalassemia.

Appendix: Urinary Proteomics of Urothelial Neoplasm: We've also initiated study to find changes in urinary proteome of patients suffering from urothelial neoplasm which are classified based on their p53 immunohistochmistry (IHC).

METHODS

Sample preparation

Plasma was separated from the 2ml of blood sample collected from normal as well as Eβ thalassemic patients using self forming 75% percoll gradient. This plasma fraction was then subjected to 20% ammonium sulphate precipitation to deplete the high abundant

proteins[5]. The precipitate was dialysed and dissolved in 2D rehydration buffer after protein estimation. This was then used to run 2 DGE.

Urine samples (Eβ thalassmeic, urothelial neoplasm and normal) (~30ml) were centrifuged to remove cellular debris [6]. The supernatant was then centrifuged in Amicon ultra centrifugal filter units with 5kDa cut off membrane concentrated to a volume of 2ml and proteins were precipitated using 75% ethanol. The precipitate was directly solubilised in 2D rehydration buffer and stored for further analysis.

The plasma and erythrocytes were separated according to density using 75% percoll. A fraction of the erythrocytes was further lysed and the erythrocyte membranes were taken separately. Lipid extraction was done using methyl tertiary butyl ether (MTBE) as described in [7]. The samples were then vortexed for 1hour and subsequently centrifuged. 800 µL of the upper organic phase was transferred into a new vial and stored at -20°C until analysis.

2DGE, mass spectrometry and western blot analysis

The solubilised samples (plasma and urine) were separated first on the basis on pI on 17cm pH 3-10 IPG strips and then on the basis of molecular weight by 2DGE. Gels were stained either with colloidal Coomassie [8] or sypro ruby according to manufacturer's instructions. Densitometry analyses were done on Versa Doc series 3000 imaging system using PDQuest software (version 7.1, Bio -Rad). Spot volumes (intensity) of the desired spots were normalized as parts per million (ppm) of the total spot volume in gels, to calculate the relative abundance of a spot in a sample. The protein spots from 2D gels of normal as well as diseased samples were excised, and annotated using MALDI ToF/ToF (AB 4700, Applied Biosystems) following published protocol [9].

Few differentially regulated proteins were further analysed by western blot analysis and student t-test performed. For plasma β tubulin was used as loading control. In the absence of

any proper loading control, in case of urine samples, we have stained the total blot and the intensity of each band is normalised against the total intensity of the lane.

Mass spectrometry of lipids

For mass spectrometric analysis, dilutions were made of the extract with $CHCl_3$/MeOH/2-propanol 1/2/4 (v/v/v) containing 7.5 Mm ammonium acetate. Mass spectrometric analysis was performed on a LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific) equipped with a robotic nanoflow ion source using chips with 4.1 mm nozzle diameter. The ion source was controlled by chipsoft 6.4. software (Advion BioSciences) and operated at the ionization voltage of 0.95 kV and gas pressure 1.25 psi. MS survey scans were acquired in positive and negative ion mode using the Orbitrap analyzer operated under the target mass resolution of 100,000 FWHM (Full Width at Half Maximum). Targeted MS^n experiments were performed using pulsed Q-dissociation (PQD) for positive ion mode and high energy collisional dissociation (HCD) for negative ion mode using the LTQ Orbitrap machine.

The raw data files acquired were converted to *.mzXML format using MS converter from ProteoWizard [(10)]. Mass spectra were further processed by Lipidxplorer software [(11)] and lipids annotated by matching the m/z of their monoisotopic peaks to the corresponding elemental composition constraints using molecular fragmentation query language (mfql) [(12)]. The mfql for around 19 major lipid classes were used. To analyse the oxidized species hydroxyl, keto, hydroperoxide, epoxy and polyhydroxy derivatives of PC as long chain oxidized PCs and saturated or unsaturated aldehyde and carboxylic acid derivatives of truncated PCs were only considered. Hierarchical clustering (single linkage) study was done on the entire data set of the three fractions separately using Cluster 3.0 [(13)]. The similarity protein expressions data was measured by correlation (centered). Further PCA was applied to

the peak lists produced from all samples analyzed in each fraction separately using XLSTAT (version 2014.3.01) software.

RESULTS AND DISCUSSIONS

1. Plasma Proteomics of Eβ Thalassemia

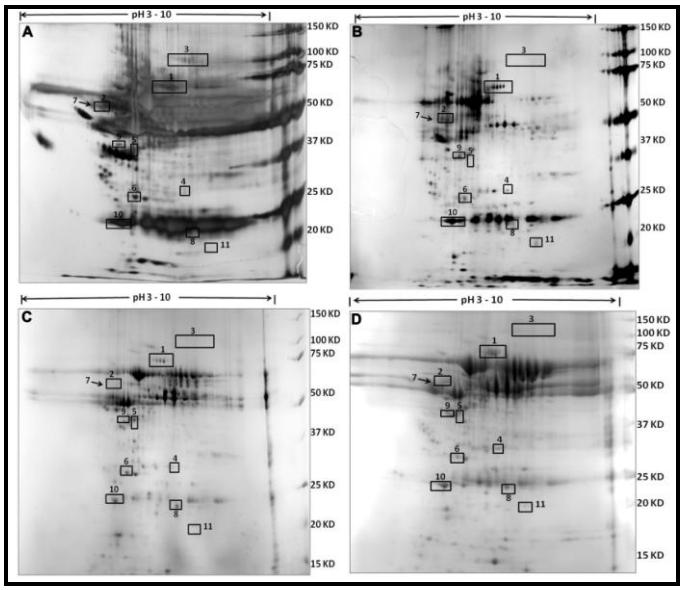

Figure 1: Representative gels of the four categories analysed. ***A*** *- Normal sample,* ***B*** *- Eβ thalassemic sample,* ***C*** *- EE homozygous sample and* ***D*** *- high HbF percent without any diseased condition.* ***1*** *= Transferrin;* ***2*** *= alpha 1 antitrypsin;* ***3*** *= plasminogen;* ***4*** *= apolipoprotein A IV precursor;* ***5*** *= haptoglobin precursor;* ***6*** *= alpha 1 microglobulin/ inter alpha trypsin inhibitor precursor;* ***7*** *= vitronectin;* ***8*** *= adenylate kinase 1;* ***9*** *= apolipoprotein A IV;* ***10*** *= apolipoprotein A I; 11 = glutathione S transferase A2.*

We have further compared these changes with plasma samples of homozygous EE condition as well as with another non-thalassemic condition where the patient exhibits high level of fetal haemoglobin (HbF).

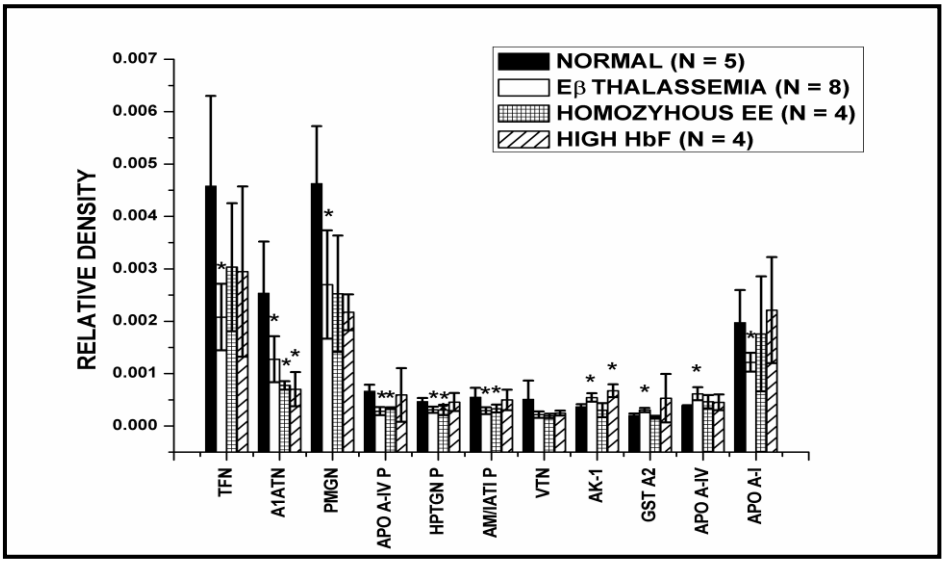

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Figure 2: A bar plot representating the changes in the protein levels in the four categories. Significant ($p \leq 0.05$) results are marked by "" and N is the number of samples processed.*

TFN: Transferrin; A1ATN: alpha 1 antitrypsin; PMGN: plasminogen; APO AIV P: apolipoprotein AIV precursor; HPTGN P: haptoglobin precursor; A1M/IATI P: alpha 1 mroglobulin/ inter alpha trypsin inhibitor precursor; VTN: vitronectin; AK-1: adenylate kinase 1; GST A2: glutathione s-transferase A2; APO A-IV; apolipoprotein A IV; APO A-I: apolipoprotein A1.

In the Eβ thalassemic plasma samples transferrin (TFN), alpha 1 antitrypsin (A1ATN), plasminogen (PMGN), apolipoprotein A-IV precursor (APO A-IV P), haptoglobin precursor (HPTGN P), alpha 1 microglobulin/ inter alpha trypsin inhibitor precursor (A1M/IATI), Vitronectin and apolipoprotein A-I (APO A-I) shows a decrease in protein levels whereas adenylate kinase 1 (AK-1), apolipoprotein A-I (APO A-I) and glutathione s transferase A2 (GST A2) show an increase in protein level compared to normal. Proteins such as TFN, AK-1 and apo A IV shows a very different trend compared to EE homozygous as well as high HbF conditions thereby indicating the specificity of the changes observed (Figure 2).

As most of the de-regulated proteins participate in multiple physiological processes like proteolysis, cargo-transport and iron homeostasis, their de-regulation might enlighten clinical manifestation. Eβ thalassemic patients irrespective of transfusion requirement, are exposed to oxidative stress and hypercoagulable state. Therefore we see decrease in the level of proteins such as HPTGN, TFN, APO A-I, A1M/IATI, VTN and PMGN. Those who have chronic anemia and are transfusion dependent undergo severe hemolysis, iron overload, hypercoagulability, thrombolytic events. Therefore the changes so observed in proteins such as HPTGN and AK-1, changes according to the severity and transfusion dependency of the patients [(14)]. These 11 proteins if studied in greater details could be used collectively to monitor the condition of a patient and determining the effect of transfusion.

2. Urinary Proteomics of Eβ Thalassemia

In an attempt to establish a noninvasive method of disease monitoring we shifted our paradigm to urine proteomics. When we compared the urinary proteome of Eβ thalassemic samples compared to normal samples we found proteins such as A1ATN and APO A IV which shows a significant decrease in case of Eβ thalassemic samples similar to what we have seen in case of plasma. So far, proteins such as A1BG, A1ATN and TTN A show significant decrease in levels in case Eβ thalassemia. Interestingly proteins such as albumin (ALB), TFN and PMGN which are present in considerable amount in normal urine 2D gels were not detected in the 2D gels of Eβ thalassemic samples. A plausible explanation for this might be due to the fact that Eβ thalassemic patients utilises the TFN and PMGN to combat severe iron overload as well as hypercoagulability.

3. Lipidomics of Eβ Thalassemia

A comprehensive characterization of the major abundant lipid classes was performed leading to the identification of around 260 lipids in total distributed among 19 lipid classes in the plasma, erythrocytes and erythrocyte membrane fractions on combining the lipids identified in the positive as well as the negative ion mode.

In the plasma fractions of normal individual, lipids such as triacylglycerides (TAGs), cholesteryl esters (chol esters), lysophosphatidylcholines (LPCs) and phosphatidylcholines (PCs) are the most intense. Greater amount of LPCs and TAGs species were detected in the plasma fractions as compared to the other two fractions. Cholesteryl esters and diacylglycerides (DAGs) were only detected in the plasma fractions. Whereas, in case of erythrocytes and erythrocytes membrane fractions, lipids like PCs, sphingomyelin (SMs) and lysophosphatidylethanolamine (LPEs) are the most intense. Phosphatidic acids were observed only in case of erythrocytes and erythrocytes membrane. On comparing the lipidome of the Eβ thalassemic patients to that of normal, we have observed an increase in the ceramides

(Cer), and ethers of phosphatidylcholine (PC-O) populations in all three fractions. Lipids such as LPCs and LPEs showed a decrease in erythrocyte and erythrocyte membrane fractions whereas an increase in the plasma fraction. PCs and SMs show a decrease in all the fractions. The hydroxyl, keto, hydroperoxide, epoxy and polyhydroxy derivatives of long chain oxidized PCs were also detected in huge amounts in all three fractions, but a consistent increase in the Eβ thalassemic samples were observed only in case of erythrocyte and erythrocyte membrane fractions. The most abundant lipid species reported here also correlates with earlier studies [(15)].

The changes observed clearly indicate that the erythrocytes are in a proapoptotic condition in the diseased samples. The premature eryptosis leading to acute anemia is one of the key pathological features of Eβ thalassemia. The changes in the lipidome combined with our already vast knowledge of the changes in the proteome in plasma as well as the erythrocytes may help us discover new insights so as to prolong the survival of the diseased erythrocytes. A detailed quantitative study of these changes might enable a "fingerprinting" approach towards a better understanding of the disease. This area has a huge potential in the therapeutic level as well as diagnostic level of this disease.

- Urinary Proteomics of Urothelial Neoplasm

Albumin (ALB), Alpha 1 antitrypsin, Apolipoprotein A1, haemoglobin β subunit (Hbβ) and Transthyretin show an increase in expression in the urothelial neoplasm samples irrespective of their grade. Whereas proteins such as Transferrin show a greater increase in the high grade samples and Haptoglobin shows an increase in the low grade neoplasm samples. On the other hand, Inter alpha trypsin inhibitor heavy chain precursors show a decrease in both the grades. In certain cases, such as TTN and HP, the age matched control samples show an opposite trend suggesting that the changes observed in the neoplasm samples has no relevance to aging or medications.

CONCLUSION

From our proteomic study of two different body fluids (plasma and urine) we can see that proteins such as transferrin, plasminogen, apolipoprotein A-IV and alpha 1 antitrypsin play a significant role in maintaining homeostasis. The differential regulations of these proteins are suggestive of the stress the patient is undergoing. This study gives a set of proteins which can be further studied to help establish a robust disease and transfusion management system. Urine samples, which are easily obtainable, will help in a frequent and regular sample collection for monitoring disease conditions in a cost effective way.

Our lipidomics study shows the role that the lipids might play in the disease. The changes observed clearly indicate that the erythrocytes are in a proapoptotic condition in the diseased samples. The premature eryptosis leading to acute anemia is one of the key pathological features of Eβ thalassemia. The changes in the lipidome combined with our previous knowledge of the changes observed in the plasma proteome as well as the erythrocytes may help us discover new insights so as to prolong the survival of the diseased erythrocytes. A detailed quantitative study of these changes might enable a "fingerprinting" approach towards a better understanding of the disease. This area has a huge potential in the therapeutic level as well as diagonistic level of this disease.

HbEβ-thalassaemia because of changing phenotypes and variable medical interventions, it is difficult to accurately characterize the severity of this disease. Its remarkable phenotypic diversity is still not well understood[16]. We believe our study auguements the knowledge already available.

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CHAPTER I

INTRODUCTION

1.1 HEMATOLOGICAL DISORDERS

Hematopoiesis is the formation of blood cellular components from a population of pluripotential stem cells, which are formed in embryonic life and persist thereafter through self-regeneration. When stimulated by hematopoietic growth factors such as cytokines, these precursor cells give rise to progenitor cells. These progenitor cells, through a series of divisions and maturational changes, give rise to myeloid or lymphoid mature cells in the circulating blood.[1] Now depending on cell type in which there is a defect, the hematological diseases can be classified as erythrocyte disorders such as G6PD deficiency, thalassemia, sickle cell disease, hereditary spherocytosis, thrombocyte disorder such as thrombocythemia, thrombocytopenia and white blood cell disorders such as leukopenia and leukocytosis.

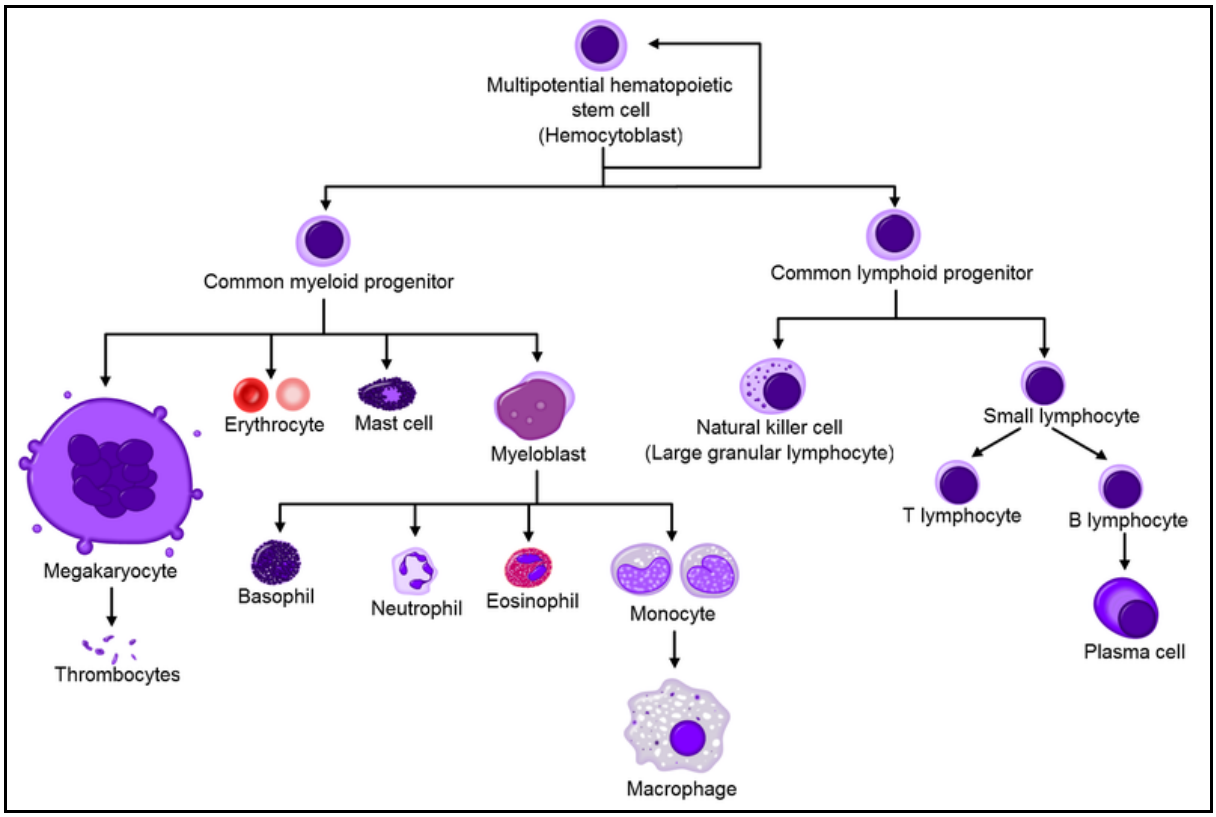


Figure 1.1: A diagrammatic representation of the process of hematopoesis[2]

Erythrocyte disorders may result in a decrease or an increase in the number of erythrocytes in the blood. Polycythemia may be defined as an abnormal increase in the erythrocyte count in the peripheral blood, usually accompanied by an increase in hemoglobin and hematocrit. Whereas anemia is present whenever there is a decrease in the normal amount of circulating hemoglobin. This reduction in hemoglobin may result from blood loss, as in common iron deficiency anemia; from increased destruction of red blood cells, as in the hemolytic anemias; from decreased production of red cells, as in pernicious and folic acid deficiency anemias.[1]

Hemolytic anemia can be further divided into two groups: intravascular and extravascular. Intravascular hemolytic anemia is the destruction of erythrocytes in the circulation with the release of cell contents into the plasma. Mechanical trauma from a damaged endothelium, complement fixation and activation on the cell surface, and infectious agents may cause direct membrane degradation and cell destruction. The more common extravascular hemolytic anemia is the removal and destruction of erythrocytes with membrane alterations by the macrophages of the spleen and liver. The general classification of hemolytic anemia is either inherited (hemoglobinopathies and membranopathies) or acquired (infection and immune-mediated) (Table 1.1).[3]

With approximately 7% of the worldwide population being carriers, hemoglobinopathies are the most common monogenic diseases, with more than 1,000 different mutant alleles having been identified on the molecular level and one of the world's major health problems.[4-6] They are a group of autosomal abnormalities, the majority of which are recessive and which are characterized by the production of structurally abnormal hemoglobin (Hb) variants like the sickle cell disease (SCD) or by an imbalance in the rate of synthesis of the globin chains like the thalassemias; less frequently, both phenotypes may be present in the same individual (concomitant reduced synthesis and structural variation) [3, 7-9]

The areas in which such abnormalities were originally most common extend from Africa over the Mediterranean basin and the Near and Middle East to Southeast Asia and the Indian subcontinent.[10]

Table 1.1: Overview of Hemolytic Anemia [3]

Type	*Etiology*	*Associations*	*Diagnosis*	*Treatment*
Acquired*				
Immune-mediated	Antibodies to red blood cell surface antigens	Idiopathic, malignancy, drugs, autoimmune disorders, infections, transfusions	Spherocytes and positive DAT	Treatment of underlying disorder; removal of offending drug; steroids, splenectomy, IV gamma globulin, plasmapheresis, cytotoxic agents, or danazol (Danocrine); avoidance of cold
Microangiopathic	Mechanical disruption of red blood cell in circulation	TTP, HUS, DIC, pre-eclampsia, eclampsia, malignant hypertension, prosthetic valves	Schistocytes	Treatment of underlying disorder
Infection	Malaria, babesiosis, Clostridium infections		Cultures, thick and thin blood smears, serologies	Antibiotics
Hereditary†				
Enzymopathies	G6PD deficiency	Infections, drugs, ingestion of fava beans	Low G6PD activity measurement	Withdrawal of offending drug, treatment of infection
Membranopathies	Hereditary spherocytosis		Spherocytes, family history, negative DAT	Splenectomy in some moderate and most severe cases
Hemoglobinopathies	Thalassemia and sickle cell disease		Hemoglobin electrophoresis, genetic studies	Folate, transfusions

DAT = direct antiglobulin test; IV = intravenous; TTP = thrombotic thrombocytopenic purpura; HUS = hemolytic uremic syndrome; DIC = disseminated intravascular coagulation; G6PD = glucose-6-phosphate dehydrogenase.

**—Other select causes of acquired hemolysis include splenomegaly, end-stage liver disease/spur cell (acanthocyte) hemolytic anemia, paroxysmal cold hemoglobinuria, paroxysmal nocturnal hemoglobinuria, insect stings, and spider bites.*

†—Other select causes of inherited hemolysis include Wilson's disease and less common forms of membranopathy (hereditary elliptocytosis), enzymopathy (pyruvate kinase deficiency), and hemoglobinopathy (unstable hemoglobin variants).

1.1.1 THALASSEMIA

The thalassaemias result from inherited defects in the synthesis of the globin chains of hemoglobin. Humans have different hemoglobins at various stages of development. Normal adults have a major hemoglobin (Hb) called HbA, comprising about 90% of the total, and a minor component, HbA_2, which accounts for 2–3%. The main hemoglobin in fetal life is HbF, traces of which are found in normal adults.

All these different hemoglobins are tetramers of two pairs of unlike globin chains. Adult and fetal hemoglobins have α chains associated with β (HbA, α2β2), δ (HbA_2,α2δ2), or γ chains (HbF, α2γ2). Each individual globin chain has a heme moiety attached to it, to which oxygen is bound.

There are two common types of thalassaemia, α and β, which result from defective synthesis of α or β globin chains. The thalassaemias are inherited in a mendelian recessive fashion. The severe, homozygous form of the disease is called thalassaemia major, and the carrier state, in which only one defective globin gene is inherited, is called the trait. The disease is very heterogeneous from the clinical viewpoint; many patients are encountered who fall between these extremes. These latter disorders are called 'thalassaemia intermedia'.[11]

More than 180 different mutations of the β globin genes have been found in patients with β thalassaemia. They may affect gene function at any level between transcription, processing of the primary messenger ribonucleic acid transcript, translation, or post-translational stability of the gene product. Rarely, β thalassaemia may result from a partial or complete deletion of the β globin gene. Some of these mutations cause an absence of β-chain production and the resulting disease is called β^0 thalassaemia, whereas others result in a reduced output of β chains, β^+ thalassaemia.

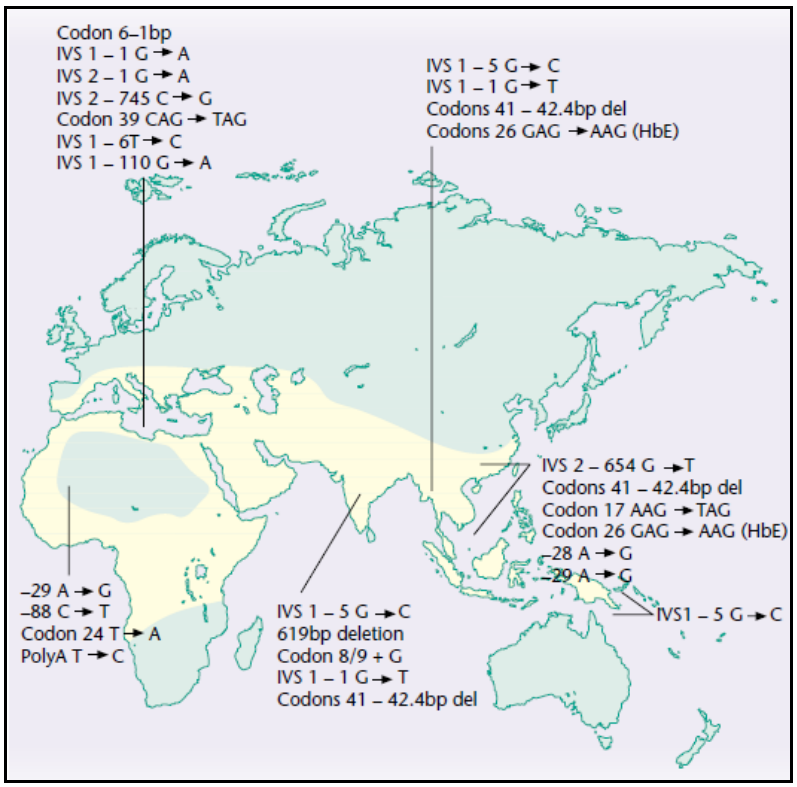


Figure 1.2: World distribution of the β thalassaemias. Each population has a different set of mutations. These are described either by the nucleotide base position in introns (IVS 1 or 2) or in the particular codons in exons. Mutations that are given the prefixes are those in the 5' noncoding regions of the β globin genes. Those marked polyA are mutations in the 3' noncoding regions. bp, Base pair.[11]

Hemoglobin E (Hb E), the most common structural hemoglobin variant, occurs widely throughout the eastern half of the Indian subcontinent, Myanmar, and east and Southeast Asia. It occurs at varying frequencies but in some parts of Asia, notably the northern parts of Thailand and Cambodia, called the "hemoglobin E triangle," up to 70% of the population are carriers.[12] Hb E results from a G→A substitution in codon #26 of the β globin gene.[13] Because there is also a high frequency of different β thalassemia alleles in these populations, the coinheritance of HbE and β thalassemia, HbEβ thalassemia, occurs very frequently.[12]

1.1.2 HEMOGLOBIN Eβ THALASSEMIA

Globally, approximately half of the clinically important forms of β thalassemia result from the compound heterozygous inheritance of hemoglobin (Hb)E and β thalassemia.[14] Hb Eβ-thalassaemia results from co-inheritance of a β-thalassaemia allele from one parent and the structural variant Hb E from the other. The G→A substitution in codon # 26 of the β globin gene produces a structurally abnormal hemoglobin as well as activates a cryptic splice site, resulting in abnormal messenger RNA (mRNA) processing. The level of normally spliced mRNA, βE, is reduced [15] and, because a new stop codon is generated, the abnormally spliced mRNA is non functional. Hence, Hb E is synthesized at a reduced rate, and behaves like a mild form of β-thalassaemia.[13]

Although clinical spectra vary depending on coinheritance of other genetic modifiers, the underlying pathology among the types of thalassemia is similar. This pathology is characterized by decreased Hb production and erythrocyte survival, resulting from the excess of unaffected globin chain, which forms unstable homotetramers that precipitate as inclusion bodies. α-Homotetramers in β-thalassemia are more unstable than β-homotetramers in α-thalassemia and therefore precipitate earlier in the erythrocyte life span, causing marked erythrocyte damage and severe hemolysis associated with ineffective erythropoiesis (IE) and extramedullary hemolysis[16] (Figure 1.3). In severe β-thalassemia, IE results in expanded marrow cavities that impinge on normal bone and cause distortion of the cranium, and of facial and long bones. In addition, erythroid activity proliferates in extramedullary hematopoietic sites, causing extensive lymphadenopathy, hepatosplenomegaly, and, in some cases, extramedullary tumors.[17]

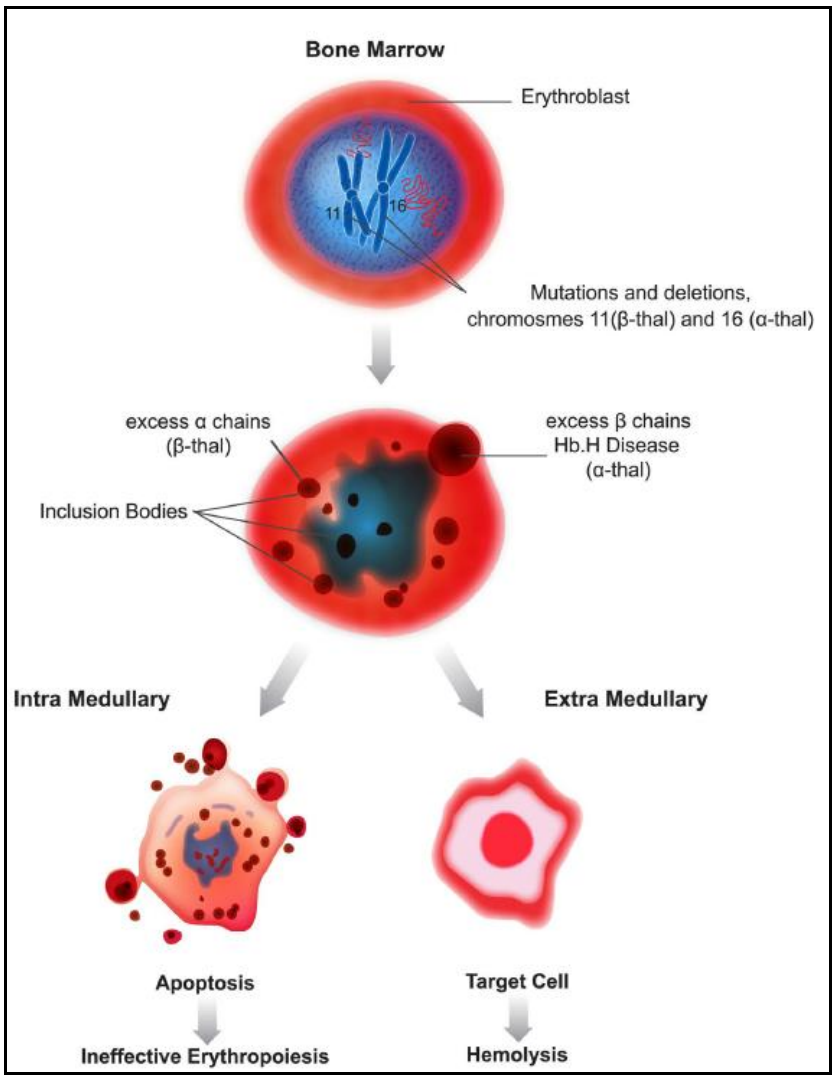


Figure 1.3: Mechanism of IE and hemolysis in thalassemia.[17]

Severe IE, chronic anemia, and hypoxia also cause increased gastrointestinal (GI) tract iron absorption. Without transfusion support, ~ 85% of patients with severe homozygous or compound heterozygous β-thalassemia will die by 5 years of age because of severe anemia.[18] However, transfusions lead to progressive iron accumulation because of inadequate excretory pathways. When serum transferrin saturation exceeds 70%, free iron species, such as labile plasma iron, have been found in the plasma as well as labile iron pool in the erythrocytes. These iron species are mainly responsible for generating reactive oxygen species[19] with eventual tissue damage, organ dysfunction, and death. There have been attempts to ameliorate oxidative stress in thalassemic blood cells using antioxidants, but so

far they have not met with clinically significant success.[20, 21] Iron chelation therapy has proven to be the only option to reduce morbidities and prolong survival into the fourth and fifth decades of life. [17]

Abnormalities in the levels of coagulation factors and their inhibitors have been reported, resulting in what can be defined as a chronic hypercoagulable state. Erythrocyte-membrane abnormalities contribute to hypercoagulability.[22] Membrane lipid peroxidation increases the surface expression of anionic phospholipids such as phosphatidylserine (PS).[23] Exposure of PS on the erythrocyte was highly correlated with the expression of platelet activation markers.[22] Erythrocytes that are exposed to phosphatidylserine may also contribute directly to the vascular damage observed in thalassemia. [24]

One of the most striking features of Hb Eβ thalassemia is its remarkable clinical heterogeneity. At one end of the spectrum, there are patients whose clinical course is almost indistinguishable from that of severe β-thalassemia major; whereas at the other end, there are patients who grow and develop normally without the need for blood transfusion, albeit often at a relatively low hemoglobin level. In patients with severe thalassemia subsequent complications may include spenomegaly, viral, bacterial and fungal infections, hepatomegaly, growth retardation, thromboembolism, hypertension and cardiac disease.[12]

1.1.3 TREATMENT

- Transfusion therapy - Regular blood transfusion remains the main conventional treatment modality for Thalassaemia major. Before the advent of transfusion treatments in the 1960s, patients died of severe anaemia at a very early age.[25, 26] It is now generally appreciated that no patient with Hb Eβ thalassaemia should be placed on a regimen of regular transfusions without an extended period (of at least 3-6

months without intercurrent illness) in which growth, pubertal development if applicable, quality of life, symptoms and signs of anemia including changes in spleen size, are monitored.[13]

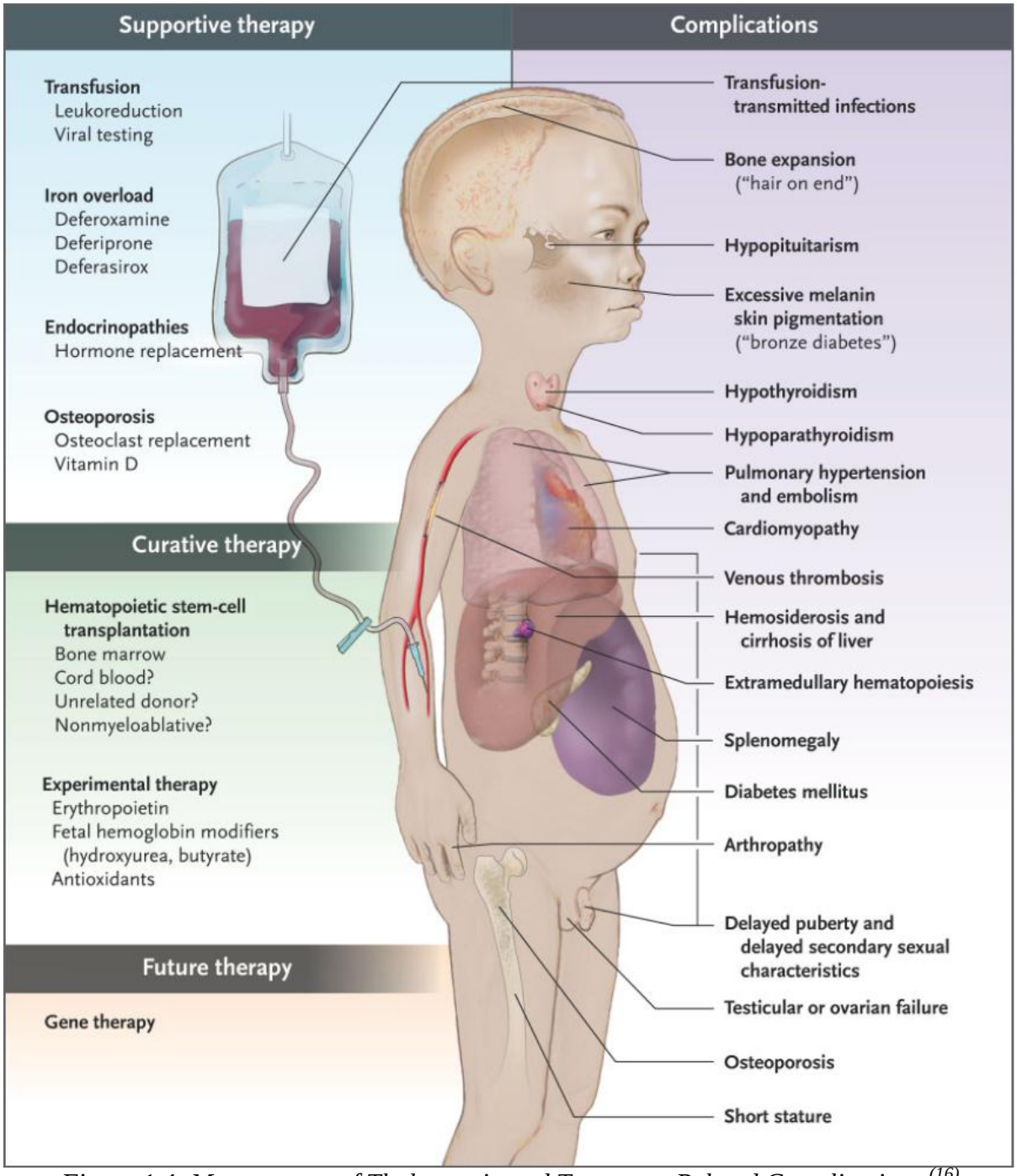


Figure 1.4: Management of Thalassemia and Treatment-Related Complications.[16]

- Chelation therapy - The management of severe forms of Hb Eβ Thalassaemia entails regular blood transfusion with chelation therapy to prevent the effects of iron accumulation. Iron removal in transfusional iron overload is achieved using chelation therapy with chelating drugs like Desferrioxamine (DF) and Deferiprone (L1). Effective chelation therapy in chronically transfused patients is achieved when iron chelators remove sufficient amounts of iron, equivalent to that accumulated in the body from transfusion, to be able to maintain the body iron load at a non- toxic level. For this, chelating drugs have to be administered daily in high doses.[25, 26]
- Hemopoitic stem cell transplantation - is the conventional curative option for thalassaemia patients. This therapy infuses the Thalassaemic patients with stem cells harvested from a compatible donor. If engraftment occurs, these normal stem cells will then re-populate the recipient's marrow and proliferate to produce normal red blood cells. If the treatment is successful, the patient is no longer transfusion dependent. The sources of stem cells include bone marrow (compatible sibling or matched unrelated donor), cord blood (sibling or cord blood registry) and peripheral blood (sibling or unrelated donor). As families with Thalassaemia tend to have fewer children, the chances of obtaining a normal and compatible sibling donor is about 15-25%. However, stem cell transplantation is recommended for patients with compatible sibling donors.[25]

Other treatments include inducing the production of HbF again in patients with thalassaemia offers the prospect of a better quality of life without the need for blood transfusions. The drugs used for this are Hydroxyurea, 5-Azacytidine etc., which inhibit the proliferation of cells, having the same effects as those used in cancer treatment. Nutrition, vitamin support and psychology therapy are other modalities used in the treatment of

thalassaemia patients. Vitamin C is only given to those with established depletion and those who are on chelation therapy.

As we see that the mainstay of treatment of thalassaemia is blood transfusion combined with iron chelation therapy therefore monitoring of body iron load is crucial for the well being of these patients. The methods of body iron measurements used nowadays are mentioned below:-

- Ferritin level: Ferritin is the most frequently used measure as it is inexpensive, widely available. However, in individuals the predictive value of ferritin is limited by inflammation and vitamin C deficiency. As a result, even patients with low ferritin levels experience elevated rates of heart disease as they age. Furthermore, the predictive value of ferritin is not documented in diseases other than thalassemia, such as myelodysplastic syndromes and sickle cell disease.

  Transferrin saturation (TSAT): Nontransferrin-bound iron (NTBI) appears in the blood when transferrin is highly saturated so its presence can be predicted by TSAT values [27]. NTBI is toxic to the liver, heart, and other endocrine tissues and increased blood levels may indicate developing organ toxicity in iron-overloaded patients. Quantification of TSAT is readily available, however, inter-laboratory assay variability, rapid physiologic modulation by inflammation, and nonlinearity with respect to total body iron levels limit the practical usefulness of this measure.
- Liver biopsy: Liver biopsy is the only direct assessment of liver iron and remains the standard of care in institutions without access to noninvasive surrogate techniques for iron measurement. However, liver biopsy is expensive and carries a risk for serious bleeding [28]. Post-procedure discomfort limits patient acceptance and sampling variability of the procedure is relatively high at 12–15% overall [29, 30].

- Superconducting quantum interference device (SQUID): SQUID was among the first noninvasive techniques used to measure body iron loading [31]. SQUID is a machine which is used to perform a radiologic study of the iron status in the body. SQUID uses the magnetic field to measure the amount of iron stored in the body. Due to its sensitivity patients with pace-makers, artificial joints or other metallic clips or staples, or patients who weigh less than 25kg or is below the age of 5 years may not be eligible.
- Computed tomography (CT): CT is well-tolerated by patients and relatively inexpensive, so it has the potential for wide clinical use. However, application of this technique has been critically limited by lack of validation in humans, poor sensitivity in patients with low iron loads, and exposure to ionizing radiation.
- Magnetic Resonance Imaging (MRI): MRI measures iron content in all organs, is widely available, and has been validated for measuring liver iron content.

Therefore a more **accurate**, preferably **noninvasive** method of disease monitoring is crucial for the evaluation and management of transfusion and chelation therapy. Hence we have focused on the proteomic study of body fluids (plasma and urine) in search of protein(s) that might help in disease monitoring and management. We have further studied the lipid population of the erythrocytes and plasma for a better understanding of the disease.

1.2. PROTEOMICS

Proteomics is the large-scale study of total proteins expressed in a specific cell or tissue at a specific time point. Proteomic study of clinical samples aims at the better understanding of physiological and pathological conditions, as well as the discovery of diagnostic and

prognostic markers for the latter. Quantitative and/or qualitative variations of body fluid proteome may reflect health- or disease-associated events connected to the adjacent or distant body regions of the fluid production/secretion/excretion and/or systemic reactions to the presence of disease.[32]

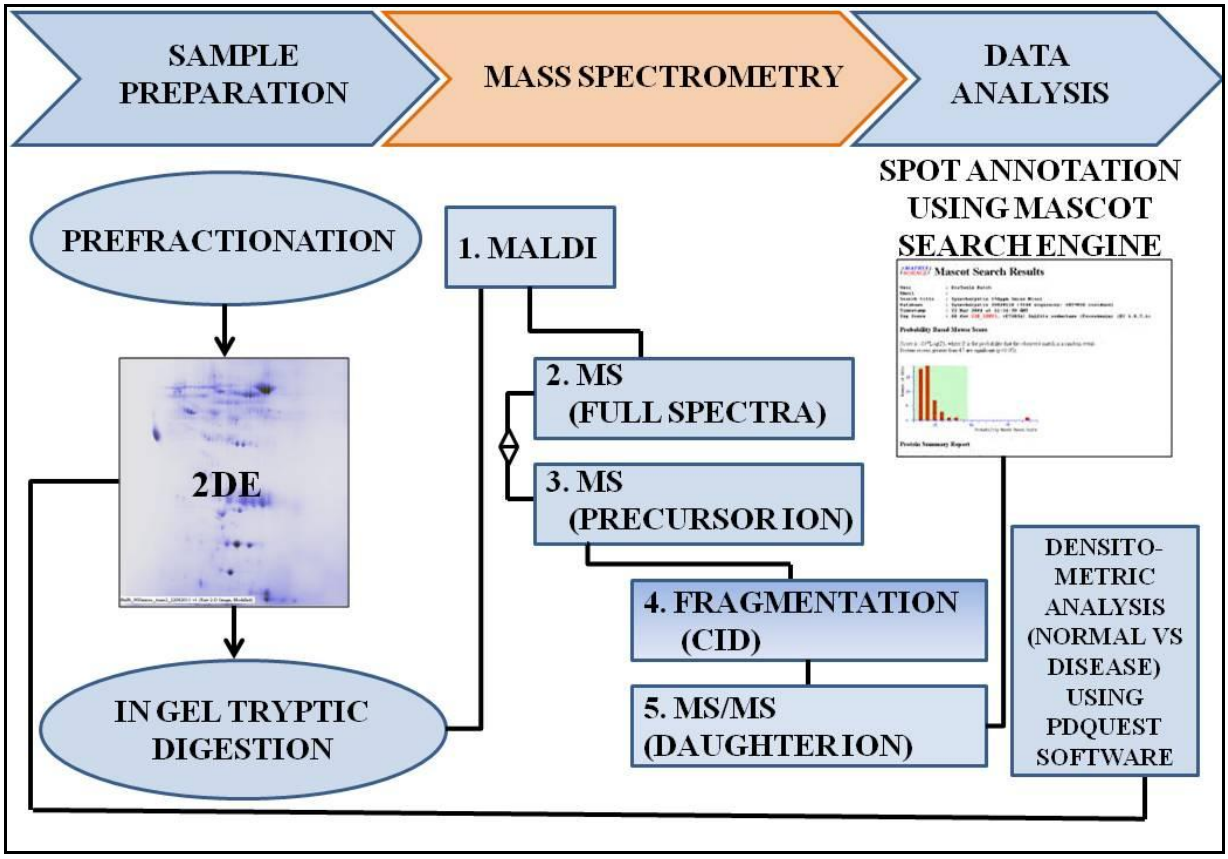


Figure 1.5: workflow of two dimensional gel based proteomics study using MALDI.

Researchers have long studied human biological materials—such as cells or body fluids collected in research projects, biopsy specimens obtained for diagnostic purposes, and organs and tissues removed during surgery—to increase knowledge about human diseases and to develop better means of preventing, diagnosing, and treating these diseases. However, of utmost importance is how we prepare the samples. Here we have followed a two dimensional gel (2DG) based approached for the analysis of the plasma and urine samples of

thalassemic samples and compared them with those of the normal samples. The following sections will deal with each step followed in the 2DG based proteomic study as shown in figure 1.5

1.2.1 GEL BASED PROTEOMICS

Two high-resolution electrophoretic procedures (isoelectric focusing and SDS–polyacrylamide gel electrophoresis) are combined to provide much greater resolution than either of the individual procedures. [33] When taking proteomics as the large-scale analysis of proteins from biological samples be it cells or body fluids, 2-DE has dominated the scene for the last 20 years. 2-DE was introduced in the mid-70's [33-35], but its true importance as a useful technique was realised after the development of microanalytical techniques able to identify proteins at the amounts available from 2-D gels. Mass spectrometry has greatly increased the power of this microcharacterization step, as it is much more productive and sensitive MS than the classical methods.[36-38] This combination between mass spectrometry and 2-DE has made the turn which resulted in the word proteomics in the mid-90's. Since then, multiple pieces of work using this approach in order to tackle various biological questions have appeared in the literature. [39]

In spite of the recent popularity of LC based mass spectrometry, two dimensional gel electrophoresis is still regarded as a good technique for protein annotation as well as comparative proteomics. The steps involved are i) sample preparation, (ii) first dimension (IEF), (iii) second dimension (SDS-PAGE), (iv) 2D protein pattern visualization, (v) 2D pattern analysis and (vi) spot picking and digestion of protein spots for MS identification.[40]

1.2.1.1 SAMPLE PREPARATION

As we can see in fig. 1.5 sample preparation is the first step. A sample preparation is still the most critical step in 2DE and should be optimized for each type of sample. The aim of sample preparation for 2DE is to convert the native sample into a suitable physicochemical state for first dimension IEF while preserving the native charge and *Mr* of the constituent proteins. In many cases this means that the proteins of the sample need to be solubilised, disaggregated, denatured and reduced.

Plasma is known to be a difficult sample having a dynamic range of proteins that result in the masking of the low abundant protein. Excess of salt and very low concentration of proteins makes the 2DE analysis of urine samples complicated. Therefore prefractionation of sample prior to 2DE involves enrichment techniques, depletion of high abundant proteins, salt removal by precipitation methods etc. Detailed sample preparation for each sample type that has been used here will be discussed in the later sections.

1.2.1.2 FIRST DIMENSION OR ISOELECTRIC FOCUSING

The principle of Isoelectric focussing (IEF) is that proteins placed in a medium with a pH gradient and subjected to an electric field, will initially move toward the electrode with the opposite charge. During migration through the pH gradient, the proteins will either pick up or lose protons. As they migrate, their net charge and mobility will decrease and the proteins will slow down. Eventually, the protein will arrive at the point where the pH gradient is equal to their pI. Being uncharged, they will stop migrating (fig. 1.6). If a protein should happen to diffuse to a region of lower pH, it will become protonated and be forced back toward the cathode by the electric field. If, on the other hand, it diffuses into a region of pH

greater than its pI, the protein will become negatively charged and will be driven toward the anode. In this way, proteins condense, or are focused, into sharp bands in the pH gradient at their individual characteristic pI values.[41]

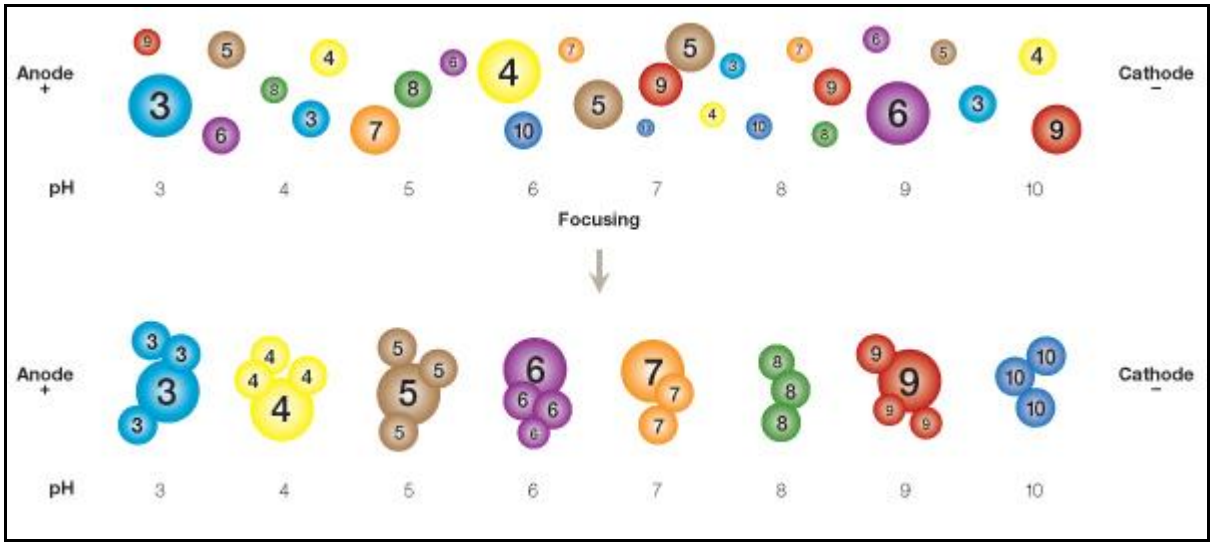


Figure 1.6: ISOELECTRIC FOCUSSING: in IEF, a mixture of proteins is resolved on a pH 3–10 IPG strip according to each protein's pI and independently of its size.

Immobilized pH gradient (IPG) strips of different lengths from 7-24 cms and varying pH ranges ranging from pH 3 to pH 11 including ultra narrow range (≤ 1 pH unit) and overlapping pH ranges are available commercially. In some of these strips, >1 mg of protein could be loaded. The IEF cells have also been devised where stepwise voltage gradients could be applied. For a typical 18 cm long IPG strip, a final voltage of 10000V is often required. Before the second dimension it is essential to equilibrate the IPG strips so that the proteins interact fully with SDS. Glycerol and urea are added in equilibrium buffer for efficient liberation of proteins from strip to gel. It is often done in 2 steps where in the first step a reducing agent like DTT is added for disulfide bond reduction and in the second step, the free sulphydril ends are deactivated by iodoacetamide. Iodoacetamide also alkylates any

free DTT left; otherwise the free DTT migrates with the protein in SDS-PAGE resulting protein streaking.

1.2.1.3 SECOND DIMENSION OR GEL ELECTROPHORESIS

The second-dimension of 2-D gel electrophoresis is a standard polyacrylamide gel. The proteins resolved by IEF on IPG strips in the first dimension are applied to an SDS polyacrylamide gel and separated by size (molecular weight) in a direction perpendicular to the first dimension. The pores of the second-dimension gel sieve proteins according to size because SDS coats all proteins with a negative charge essentially in proportion to their mass. The net effect is that proteins migrate as ellipsoids with a uniform negative charge-to-mass ratio, with mobility related logarithmically to mass.[42]

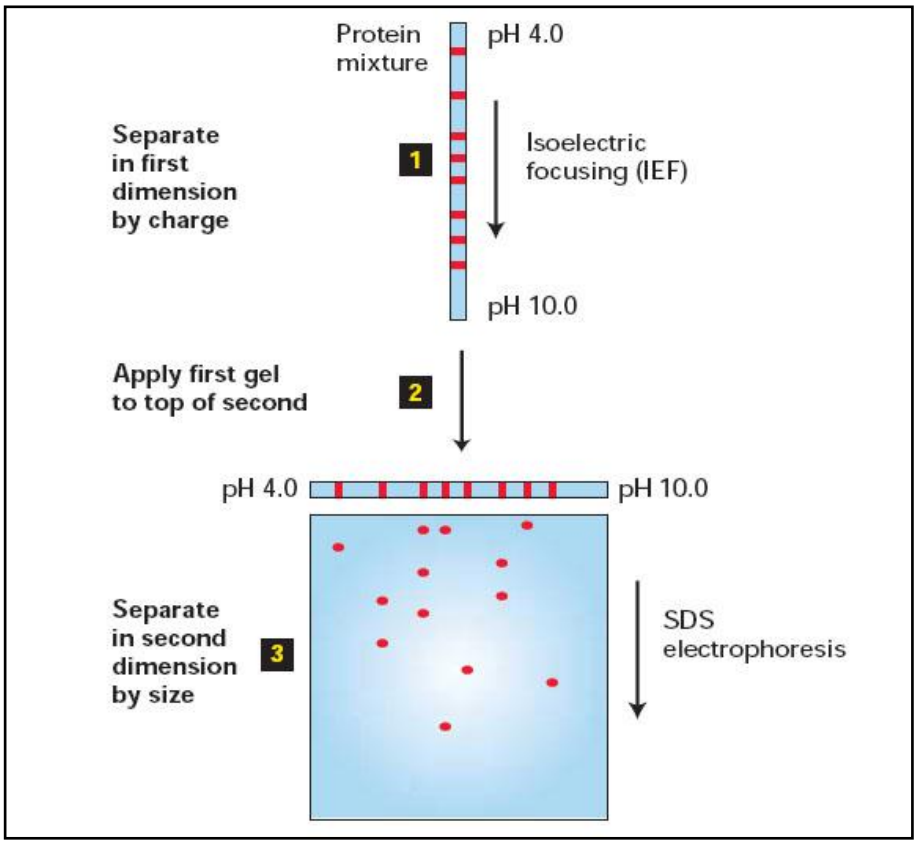


Figure 1.7: Two dimensional gel electrophoresis.

1.2.1.4 GEL STAINING AND IMAGING

Gels are run for either analytical or preparative purposes. The intended use of the gel determines the amount of protein to load and the means of detection. It is most common to make proteins in gels visible by staining them with dyes or metals. Each type of protein stain has its own characteristics and limitations with regard to the sensitivity of detection and the types of proteins that stain best. Depending on the protein load we have used 3 kinds of stains for staining purpose. They are listed below:

- Blue silver: developed by Candiano *et al*.[43] Blue silver is more sensitive than the other colloidal CBB-G stains, with a detection limit approaching 1 ng for BSA.
- SYPRO RUBY dyes: They have high sensitivity (1–10 ng: similar to silver-staining methods) and are fully compatible with MS techniques.[44, 45]
- Silver stain: Silver-staining protocols are the most sensitive.[46] These are based upon deposition of metallic silver on proteins.[46, 47] Typically they are multistep, time-consuming procedures that can, however, detect 100 pg to 1 ng protein per spot.[48] In general silver stain is not compatible with MS, which is always true for ammoniacal silver stains.[46, 49]

1.2.2 MASS SPECTROMETRY

1.2.2.1 MATRIX ASSISTED LASER DESORPTION IONISATION

MALDI mass spectrometer was developed by Karas & Hillenkamp in 1988.[50] In MALDI the sample is embedded into a crystalline compound, and a laser operating at a

wavelength at which the matrix strongly absorbs, is used to desorb and ionize the sample.[51] When the laser hits the matrix in high vacuum and at high accelerating voltage, the pulsed nature of laser radiation produces ions in pulses. As the samples are solid, MALDI samples are co-crystallized with a very large molar excess of a matrix, typically 1/1000. MALDI is often characterized as a "softer" ionization technique because most of the laser energy is spent reacting with the matrix leaving the polymer intact as it carried intact with the vaporized matrix into the drift tube of the Tof. Effective matrix compounds in MALDI have been developed on a largely empirical basis, but the matrices such as 2, 5-dihydroxybenzoic acid (DHB) and α-cyano-4-hydroxycinnamic acid (CHCA) are commonly used. Both can readily form co-crystal with peptides, absorb laser energy and get volatilized along with the peptide and in the vapour state one proton transfer takes place from the matrix to the analyte molecule resulting into primarily uni-positive peptide ions.[52]

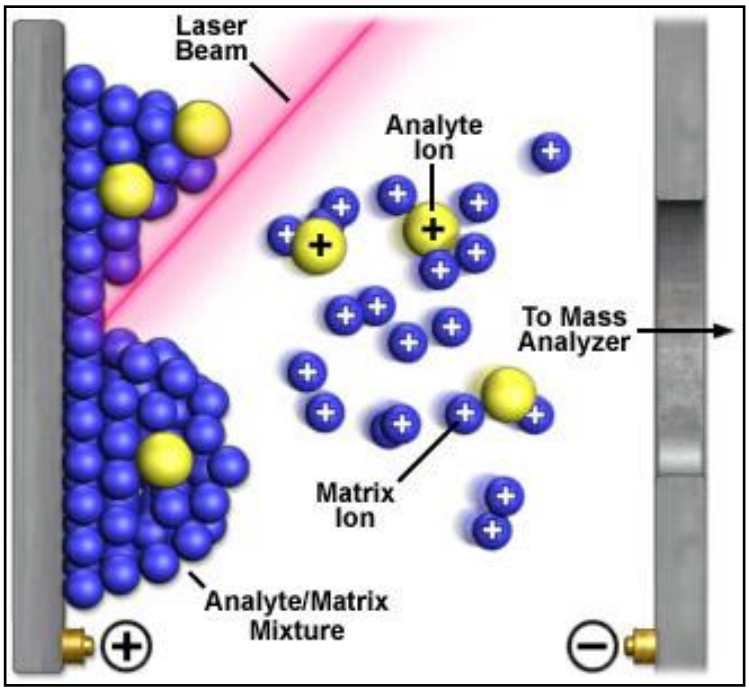


Figure 1.8: MALDI Principle and Molecular Ion Formation (adapted from MAGNET LAB)[53]

1.2.2.2 TIME OF FLIGHT (TOF) AND TOF-TOF ANALYZERS

TOF mass spectrometry is based on the fact that ions with the same energy but different masses travel with different velocities. The ions are formed in the ionization region of the source. They are then accelerated out of the source towards the collector by either one or a series of constant electric fields. [54] For an ion accelerated by a voltage V, the resulting velocity is proportional to the mass-to-charge ratio. In a time-of-flight (TOF) mass spectrometer, ions are separated on basis of the time t needed to travel a path L.

$$t = [\sqrt{m/(2zeV)}].L$$

Ions of very high mass-to-charge (several hundreds of kD) may be recorded after an appropriate length of time. [55] A reflectron situated at the far end of the flight tube provides a linear potential gradient from ground to just above the accelerating voltage. This slows the ions and then reverses their direction of flight, accelerating them back toward a detector situated at a slightly offset angle. The fastest ions penetrate further into the reflectron and travel a longer distance before reaching the detector than the slowest ions. Through a suitable choice of geometry, it is possible to have all ions of each unique mass arriving at the detector simultaneously, even though they have slightly different velocities. With the combination of the ion source and the reflectron, modern high-performance MALDI-ToF instruments with suitable calibration allows mass measurements to be made with an accuracy of better than 10 ppm.[56]

Alongside the new breakthroughs in the field of ionisation methods such as MALDI and ESI (discussed in later sections) protein mass fingerprinting (PMF) was also evolving. The logic behind PMF is that, a protein may be defined as a set of amino acids arranged in a specific sequence to yield a defined activity or property. Although some proteins may have a high degree of homology—sequence similarity—with other

proteins, some, if not many portions of any one protein's sequence are unique. If a protein could be cut in a predictable manner, the sizes of the pieces should form a fingerprint for that protein. Further, if each entry in a database of protein sequences could be cut in the same manner *in silico*, the fingerprint would serve to identify the protein. [57] In this case the proteins are cleaved using trypsin and the peptides are run through the TOF analyzer to generate the PMF. After the initial mass determination, specific ions are selected (For example the 15 most intense peaks are selected from the PMF) and subjected to fragmentation through collision induced dissociation (CID).[58]

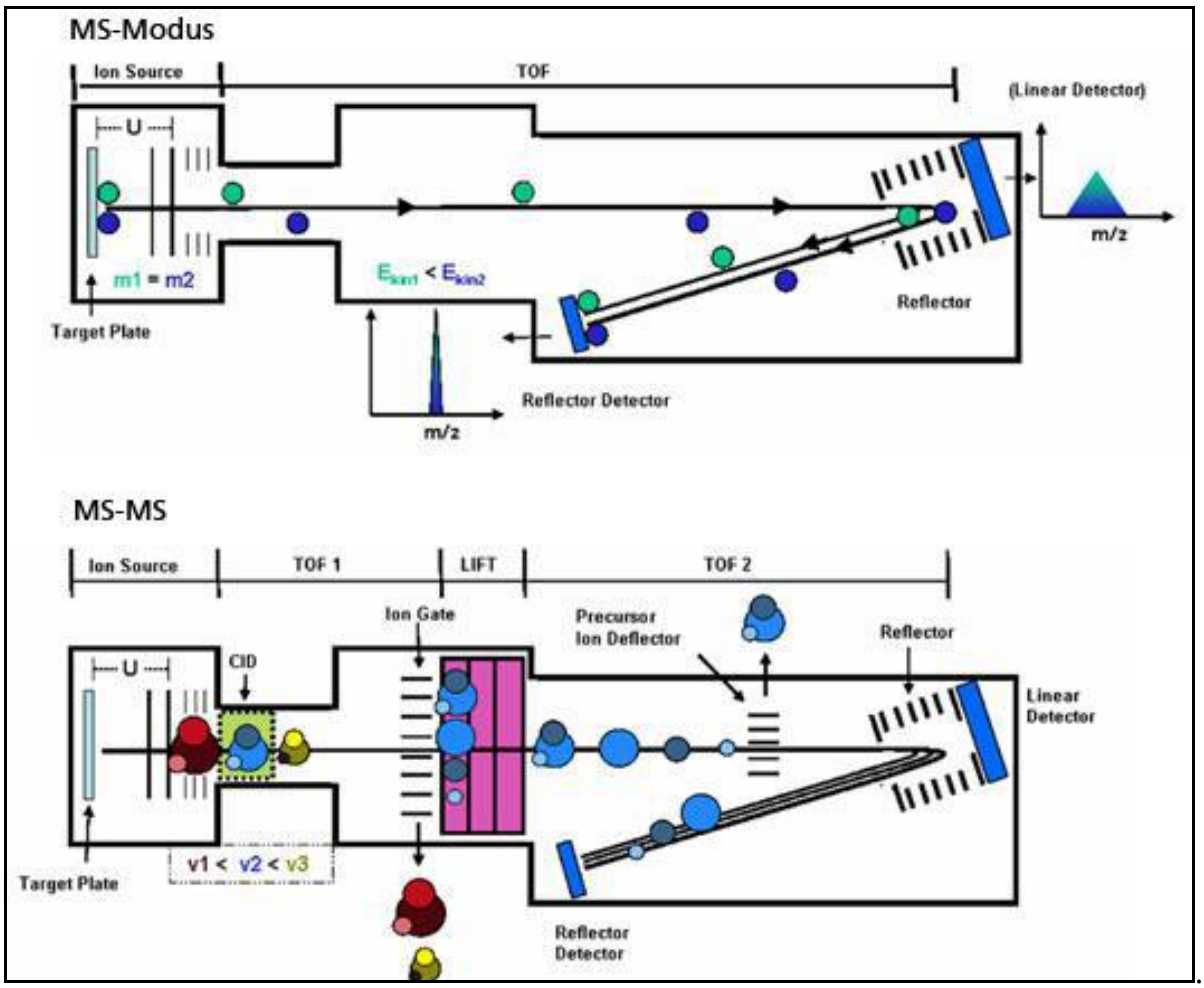


Figure 1.9: ToF and ToF-ToF Principle (adapted from AB SCIEX 4700 Manual.)

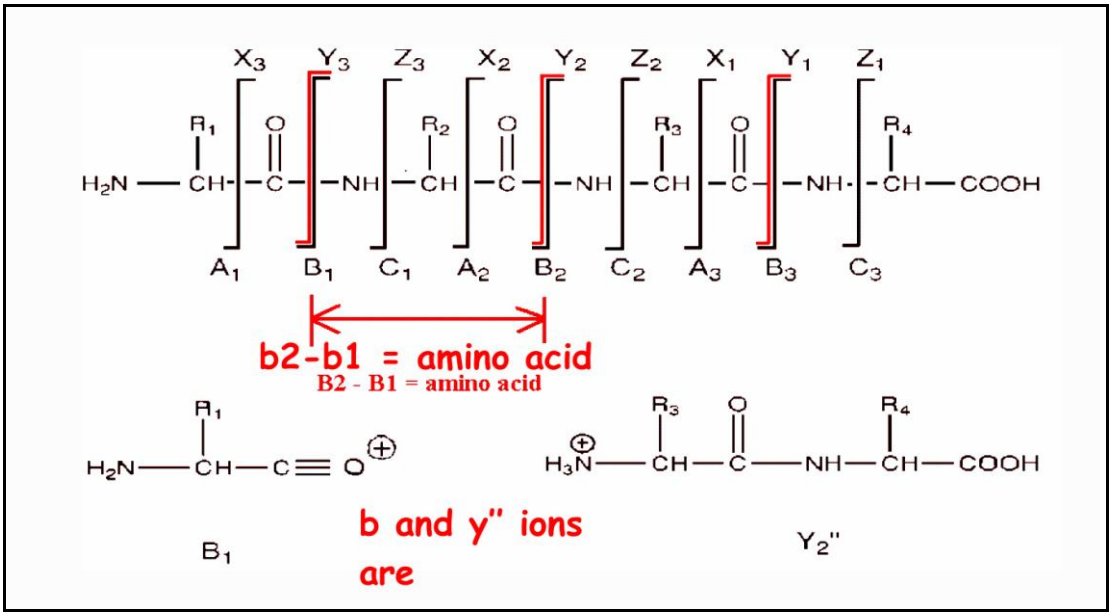


Figure 1.10: *Peptide backbone cleavages- formation of b ions and y ions.*

Fragmentation at an amide (or peptide) bond giving a "b" ion (N-terminal fragment) or a "y" ion (C-terminal fragment) (Figure 1.10) is a relatively low-energy process which takes place in majority.

1.2.3 MASCOT

MASCOT is a proprietary identification program available from Matrix Science. Mascot is a powerful search engine which uses mass spectrometry data to identify proteins from primary sequence databases.

While a number of similar programs available, Mascot is unique in that it integrates all of the proven methods of searching. These different search methods can be categorised as follows:

- Peptide Mass Fingerprint in which the only experimental data are peptide mass values, (tutorial)

- Sequence Query in which peptide mass data are combined with amino acid sequence and composition information. A super-set of a sequence tag query,
- MS/MS Ion Search using uninterpreted MS/MS data from one or more peptides.

The sequence databases that can be searched on the Matrix Science free, public Mascot server are Swissprot, NCBInr, EMBL EST divisions, contaminants and cRAP. As of recent version it supports peptide quantitation methods compatible like Isotope Coded Affinity Tag (ICAT) or Isobaric tag for relative and absolute quantification (iTRAQ) methods, in addition to the identification features.[59]

1.2.4 PLASMA PROTEOMICS

Plasma proteome represents an important subproteome, as it harbors proteins secreted from almost all tissues [60-62]. In addition to classical blood proteins, plasma contains proteins secreted by various cells, glands and tissues along with proteins derived from commensal and infectious organisms and parasites residing inside the body.[60] The plasma proteome comprises 22 highly abundant proteins including albumin, immunoglobulins, transferrin and haptoglobin, which make up 99% of total protein abundance in plasma. The remaining fraction is composed of proteins of much lower abundance including proteolytically cleaved protein fragments.[63] This wide dynamic range of protein abundance, greater than 10 orders of magnitude, makes plasma proteome a challenging proteome to analyze. Reduction of sample complexity is thus an essential first step in the analysis of plasma proteome.[64]

Despite the analysis of plasma not being straightforward from an analytical standpoint, it is the most investigated body fluid in clinical diagnostics. Components of plasma including circulating tumor cells, cell-free RNA and DNA, metabolites, electrolytes and proteins are all considered as molecular markers for early detection of diseases, disease

monitoring and prognosis.[65-67] When compared with other body fluids such as cerebrospinal fluid, gastric juice, bile and synovial fluid, plasma is more readily accessible and requires a simple collection procedure.[60, 68, 69] Thus, detection and quantitation of proteins present in plasma of a healthy as well as diseased patient could be used to determine the physiological and pathological states of an individual.[70] Several plasma or serum proteins have already been identified as potential biomarkers of diseases including cardiovascular diseases, autoimmune diseases, infectious diseases and neurological disorders.[71-78]

Owing to the importance of plasma proteins, several proteomic efforts have been carried out to explore human plasma proteins. There have been three main methods of depleting abundant proteins from serum samples: affinity removal method [64, 79-85]; membrane filtration method to separate low-mass proteins from high-mass ones [86, 87]; and multidimensional chromatographic fractionation [79, 88, 89]. But all these methods are expensive, laborious and time consuming, as depletion of multiple abundant proteins from each plasma sample requires multiple technical steps. Our lab has used the simple concept of salting out of proteins using 20% ammonium sulphate.[90] Fountoulakis and coworkers have earlier reported fractionation of plasma proteins with 50% and 70% ammonium sulphate to reduce concentrations of high-abundance components and enrich lower abundant components in plasma 2DGE profiles, thereby facilitating the identification of disease markers.[91]

A lot of efforts are being put into developing a complete proteome map of the plasma. In 2002, Anderson and Anderson compiled immunoassay- and 2D gel electrophoresis-based investigations of plasma proteome and reported the presence of 289 proteins.[60] Adkins et al. used two different separation techniques followed by mass spectrometry (MS) for characterization of proteins from depleted serum and reported 490 proteins.[92] Human Proteome Organization (HUPO) initiated a pilot phase of a major community initiative—the Human Plasma Proteome Project (HPPP)—in 2002 to determine the human plasma or serum

protein constituents.[93] With the involvement of 35 laboratories across the globe, this led to the identification of 3020 plasma proteins.[94] As a part of this initiative, the group of Akhilesh Pandey and T. S. Keshava Prasad have developed a web-based resource called the Plasma Proteome Database (PPD).[95] Recent incorporation of depletion strategies to remove high-abundance proteins and multiple fractionation strategies coupled to LC-MS/MS approaches and high-resolution MS have resulted in a substantial increase in the number of proteins identified from plasma. For example, a study by Liu et al. coupled two different fractionation methods to MS and identified 9087 proteins in plasma, which is the largest data set on plasma proteins reported so far.[96] Farrah et al. have analyzed raw MS/MS data from plasma/serum, submitted to the Proteomics Identifications Database (PRIDE) and Human Plasma PeptideAtlas using Trans-Proteomic Pipeline and reported a set of high-confidence 1929 proteins.[97-99] These newly described human plasma proteins are systematically documented and made them available for the biomedical community through the updated version of PPD.

1.2.5 URINE PROTEOMICS

The urine is one of the ideal biological samples for the discovery of noninvasive biomarkers for human diseases, because it is available in almost all patients and its collection is simple and does not require any invasive procedures. It can be viewed as modified ultrafiltrate of plasma combined with proteins derived from kidney and urinary tract, with protein concentration approximately 1000-fold lower than in plasma itself.[100] Urinary proteomics has thus become one of the most attractive subdisciplines in clinical proteomics, particularly with the aim for biomarker discovery and clinical diagnostics.[101, 102] However, there are few complications in urinary proteome analysis. Urine has low protein

concentration, high levels of salts or other interfering compounds, and more importantly, high degree of variations (both intra-individual and inter-individual variabilities).[103]

A number of studies have been carried out to identify urinary markers in various disease conditions and candidate biomarkers have been identified for several kidney related disorders including diabetic nephropathy,[104, 105] acute renal injury and obstructive nephropathy.[106] In addition to these renal disorders, potential biomarkers for systemic illnesses have also been identified from urine including prostate cancer,[107] bladder cancer[108], breast cancer[109], colon cancer[110] , tuberculosis[111] and major depressive disorder[112].

A lot of work has been done in standardizing the process of sample collection and storage, sample preparation methods for concentrating or isolating urinary proteins, removal of interfering compounds, intra-individual and inter-individual variabilities and data normalisation. The methods of sample preparation reported in various studies are ultrafiltration, precipitation with either ethanol, acetone or a combination of acetone and TCA, ultracentrifugation etc.[113-117] An international urine collection protocol created as a result of joint consensus of European Kidney and Urine Proteomics (EuroKUP)[118] and the Human Kidney and Urine Proteome Project (http://eurokup.org) [119] and the availability of a standard human urine sample [120] allows comparing samples from different hospitals and institutions, making the conclusions of the urine proteomic studies more relevant and independent of the sample source. We have got the best results by concentrating the samples using membrane filters followed by 75% ethanol precipitation. Following this method we could visualise ~300 spots, however we could annotate only 44 unique proteins. This was a pilot study and we feel a need to use other enrichment techniques to see past the high abundant proteins of the plasma such as albumin, transferrin and haptoglobins which are also present in the urine.

In 1979, the Anderson's group published the first studies by two-dimensional electrophoresis (2-DE) on normal urine [121, 122] in which they identified only the major components, in general the same ones present in plasma and some low molecular mass peptides. Up to the end of the century no more improvements were obtained [123] when it became clear that more than one technique should be utilized for improving sensitivity. Thus several groups analyzed the "normal human urinary proteome" by combining liquid chromatography with tandem mass spectrometry (LC–MS) [124, 125] and/or utilizing two methods of sample fractionation, e.g. acetone precipitation and ultracentrifugation. [115, 126-128]. These new approaches notably improved sensitivity, so that in 2011 the total normal urinary proteome listed about 2000 proteins [129-135]. Santucci et al. could only identify 1176 proteins from unfractionated normal urine, however the increased this number to about 3429 using ultracentrifugation, butanol precipitation and CPLL methods as shown in the flowchart (fig 1.11) .[136]

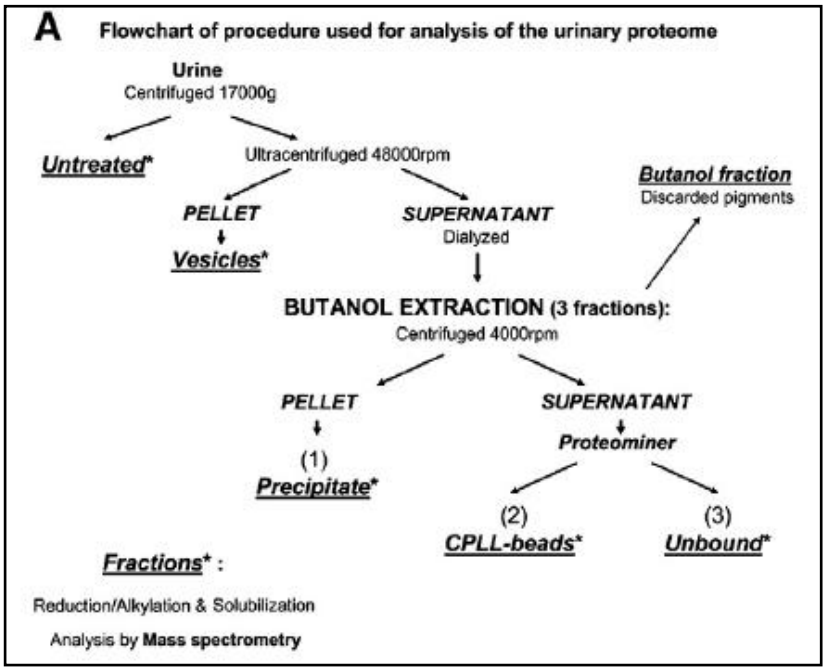


Figure 1.11: A: Flowchart of the procedure used for analysis of the urinary proteome. After obtaining vesicles from the pellet, the supernatant was treated with a butanol precipitation and with ProteoMiner followed by Tris & SDS–DTE elution. The 5 fractions thus obtained (untreated, vesicles, precipitate, CPLL-beads eluate and unbound) were processed by mass spectrometry analysis.[136]

1.3 LIPIDOMICS

Lipidomics is a sub-discipline of metabolomics and is defined as the large-scale study of non-water-soluble metabolites (lipids and lipidome) that utilize system-level analysis to characterize lipids and their interacting moieties.[137] Chemically, lipids are broadly defined as hydrophobic or amphiphilic small molecules that originate either entirely or in part from two distinct types of building blocks: ketoacyl and isoprene groups (Fig. 1.12) [138]. Using this approach, lipids have been divided into eight categories: fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, saccharolipids and polyketides (derived from condensation of ketoacyl subunits); and sterol lipids and prenol lipids (derived from condensation of isoprene subunits) (Table 1).[138, 139] Yetukuri et. al have estimated that this number is of the order of 200 000 [140]. While glycerophospholipids are the main structural

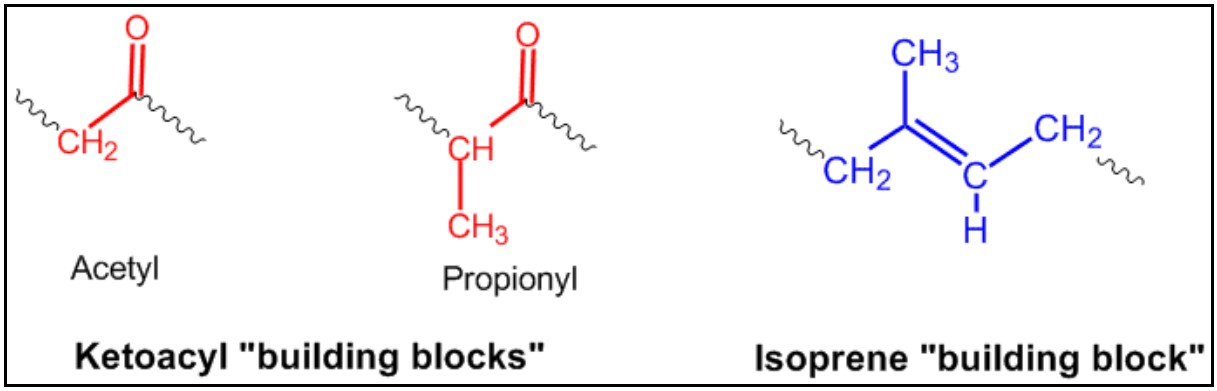
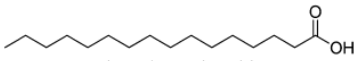
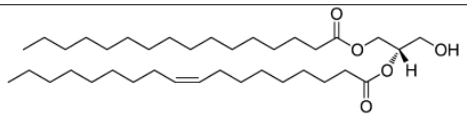
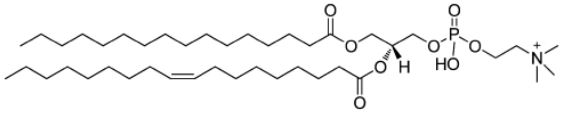
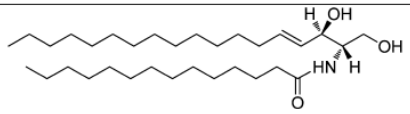
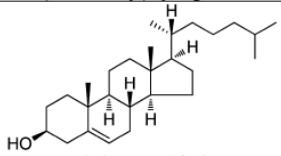
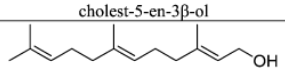
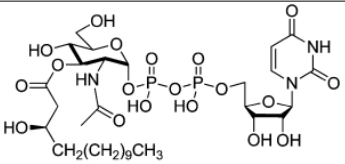
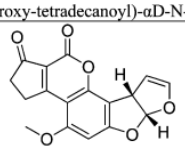


Figure 1.12: Building blocks of lipids

component of biological membranes, such as the plasma membrane of cells and the intracellular membranes of organelles; other non-glyceride lipid components such as sphingomyelin and sterols (mainly cholesterol in mammalian cell membranes) are also found in biological membranes. Triacylglycerols, stored in various cells, but especially in adipose

(fat) tissue, are a major form of energy storage in mammals. Furthermore, in recent years, considerable evidence has emerged demonstrating that lipid signalling is a vital component of cell signalling. There are many examples of important signalling lipids including sphingosine-1-phosphate, a sphingolipid derived from ceramide that is a potent messenger molecule involved in regulating calcium mobilization, cell growth, and apoptosis; diacylglycerol

Table 1.2: Example of eight categories of lipids

Categories	Structures Examples	Typical Classes: Subclasses
Fatty acyls, FA	 hexadecanoic acid	Fatty acids: straight-chain fatty acids Eicosanoids Fatty alcohols Fatty esters Fatty amides
Glycerolipids, GL	 1-hexadecanoyl-2-(9Z-octadecenoyl)-*sn*-glycerol	Monoradylglycerols: monoacylglycerols Diradylglycerols: diacylglycerols Triradylglycerols: triacylglycerols
Glycerophospholipids, GP	 1-hexadecanoyl-2-(9Z-octadecenoyl)-*sn*-glycero-3-phosphocholine	Glycerophosphocholines Glycerophosphoethanolamines Glycerophosphoserines Glycerophosphoglycerols Glycerophosphoglycerophosphates Glycerophosphoinositols Glycerophosphoglycerophosphoglycerols
Sphingolipids, SP	 N-(tetradecanoyl)-sphing-4-enine	Sphingoid bases Ceramides Phosphosphingolipids Neutral glycosphingolipids Acidic glycosphingolipids
Sterol lipids, ST	 cholest-5-en-3β-ol	Sterols Cholesterol and derivatives Steroids Bile acids and derivatives
Prenol lipids, PR	 2E,6E-farnesol	Isoprenoids Quinones and hydroquinones Polyprenols
Saccharolipids, SL	 UDP-3-O-(3R-hydroxy-tetradecanoyl)-αD-N-acetylglucosamine	Acylaminosugars Acylaminosugar glycans Acyltrehaloses Acyltrehalose glycans
Polyketides, PK	 aflatoxin B1	Macrolide polyketides Aromatic polyketides Nonribosomal peptide/polyketide hybrids

(DAG) and the inositol phosphates dervived from the phosphatidylinositolphosphates (PIPs), involved in calcium-mediated activation of protein kinase C; the prostaglandins, which are one type of fatty-acid derived eicosanoid involved in inflammation and immunity; the steroid hormones such as estrogen, testosterone and cortisol, which modulate a host of functions such as reproduction, metabolism and blood pressure; and the oxysterols such as 25-hydroxy-cholesterol that are liver X receptor agonists.[141-149]

As lipids comprise a very significant part of the metabolome and play pleiotropic roles in cellular functions, lipidomics has become an important tool to practice systems biology. As an increasing number of disorders are linked to lipid metabolism, lipidomics is used to search for biomarkers, understand disease mechanism and follow the efficacy of therapeutic options.

Mass spectrometry (MS), nuclear magnetic resonance (NMR), and other spectroscopic approaches have become powerful approaches for lipid characterization. The direct infusion MS strategy does not need any previous separation, which makes it less time-consuming. Developments in mass spectrometry [150, 151] and analytical methods forged lipidomics into a recognized scientific discipline [152]. Of the ionization techniques, the most popular and the most successful are electrospray ionization (ESI) [153, 154] and matrix-assisted laser desorption/ionization coupled to time-of-flight analyzers (MALDI-TOF) [155, 156]. In contemporary lipidomics, MS has been deployed in two ways, namely (*a*) targeted lipidomics, which focuses on the identification and quantification of a single lipid or subset of lipids in a tissue or cellular extract, and (*b*) so-called global lipidomics, which aims to identify and quantify all the lipids in a system [157, 158]. Lipid changes have been examined in clinical biopsies and animal models of various diseases, including Alzheimer's disease [159, 160], Parkinson's disease [161], hepatic liver diseases [162], and diabetes [163]. Moreover, the lipomes of pathogenic organisms have been analyzed to attempt to identify novel targets,

including Mycobacterium tuberculosis [164, 165] and human cytomegalovirus virions [166]. While these and other exciting discoveries are emerging, the field has yet to reach its full potential by identification of novel mechanisms that truly underlie human diseases.[167]

1.3.1 LIPID EXTRACTION

In order to obtain satisfactory results, extraction of lipids from complex biological matrixes, which aims at removing interfering agents in analysis of lipids, such as proteins, saccharides, or other small molecules, is usually indispensable before analysis. Generally, there are mainly two extraction methods, liquid−liquid extraction (LLE) and solid phase extraction (SPE) for sample preparation in lipidomics analysis.

The most widely used LLE method was developed by Folch and co-workers[168] in 1957 using chloroform/methanol (2:1, v/v) as the extraction solvent, which was improved later by Bligh and Dyer[169] through adding some water or other modifying agent, such as acetic acid, to increase the recovery and to prevent the degradation of lipids. Another LLE method is based on a hexane/isopropanol system, with a typical ratio of hexane to isopropanol of 3:2.[170] Compared with the Folch method, the solvents in this method are much less toxic, but this method is not well accepted due to its relatively low extraction efficiency.

Matyush et al. in 2008 [171] reported methyl tertbutyl ether (MTBE)-based LLE method to extract lipids specifically developed for shotgun profiling of complex lipidomes from samples with excessive amounts of biological matrices. In 2013, Chen and co workers [172] developed a methyl tertbutyl ether (MTBE)-based LLE method to extract lipids and different classes of metabolites simultaneously. This novel approach realized a comprehensive analysis of metabolites including lipids in the same experiment after a single

extraction, thus opening the way for a more complete characterization of lipids metabolism in health and diseases.

Besides the extraction of regular samples, such as blood, tissue, or cells, Gregory et al. in 2013 reported a full fecal lipidome LLE method.[173] This method utilized two separate, complementary extraction chemistries, one is dichloromethane (DCM) and another is a MTBE/hexafluoroisopropanol mixture, along with high pressure cycling.

As for SPE method [174-176] normal-phase stationary materials, such as bared or boned silica gel with −CN, −NH2, or diol groups, are preferred in lipidomics analysis, with methanol, hexane, and chloroform as elution solvents. In comparison with LLE methods, SPE reduces the consumption of solvents and time. However, when dealing with a large volume of samples, the recovery will be significantly reduced due to the low peak capacity of SPE.

Besides LLE and SPE methods, many new extraction methods have been utilized in lipidomics analysis in recent years, e.g., ultrasound-assisted extraction (UAE),[177] solid-phase microextraction (SPME),[178-180] pressurized fluid extraction (PFE),[181] and dispersive liquid−liquid microextraction (DLLME).[182] They all showed high efficiency, especially in targeted lipidomics.[183]

1.3.2 Electro Spray Ionization (ESI)

The recent progress in high-resolution MS techniques, such as LTQ Orbitrap MS [184-187] and Fourier transfer ion cyclotron resonance mass spectrometry (FTICRMS), [188-191] has significantly influenced the research of lipidomics, which especially facilitated direct infusion ESI-MS for the simultaneous analysis of multiple lipid classes without the need for prior separation and even for extensive MS/MS analysis.[192] The ultrahigh resolution (≥100 000) provides the ability to separate lipid ions with the same nominal m/z values. Combined with

higher energy collision dissociation (HCD) as a complementary fragmentation tool, high-resolution MS improves the confidence of molecular species assignment and accuracy of their quantification, especially for low-abundance lipid species.[193, 194]

ESI involves spraying a continuous stream of dilute solution of analyte from a needle held at a high potential into a chamber at atmospheric pressure. Formation of droplets and evaporation/desolvation of the solvent produces a continuous stream of ions, and are applied to mass analyzers that work in a continuous mode.

1.3.3 IONTRAP AND ORBITRAP ANALYZER

An ion trap is a combination of electric or magnetic fields that captures ions. An ion trap mass spectrometer may incorporate a Penning trap (Fourier transform ion cyclotron resonance) [195], Paul trap [196] or the Kingdon trap on which the current Orbitrap is based [197]. Briefly, an orbitrap is an ion trap mass analyzer consisting of an outer barrel-like electrode and a coaxial inner spindle-like electrode that traps ions in an orbital motion around the spindle [198]. The image current from the trapped ions is detected and converted to a mass spectrum using the Fourier transform of the frequency signal. Thermo Fisher Scientific first commercially introduced this analyzer as a part of hybrid LTQ Orbitrap instrument in 2005. Now the Orbitrap analyzer can be interfaced to a linear ion trap (LTQ Orbitrap family of instruments), quadrupole mass filter (Q Exactive family) or directly to an ion source (Exactive instrument. These instruments have been widely used in proteomics, lipidomics and metabolomics studies and along with associated softwares [199]. The resolution, mass accuracy and acquisition speed, provided by the Orbitrap are the most important vantages of this analyzer. High mass accuracy and resolution in particular help to overcome the uncertainty, low specificity of low resolution mass spectrometry.

1.3.4 LIPIDXPLORER SOFTWARE

The characterization of lipids from ample MS and MS/MS spectra requires functional analysis software. The software should be able (a) to read out MS and MS/MS spectra, (b) to identify lipids, (c) to perform isotopic correction for quantification, and (d) to process in batch-mode for larger studies. These aspects are sufficiently fulfilled by LipidProfiler or LipidView (from ABSciex) and the in-house developed LipidXplorer software [(200)]. LipidProfiler or LipidView are exclusively applicable for spectra acquired on instruments of the same manufacturer. In contrast, LipidXplorer is compatible to data formats of different manufacturers and includes also the analysis of high resolution spectra that are generated by Orbitrap mass spectrometer [(200)]. It relies upon a flat file database (MasterScan) that organizes the spectra dataset acquired in the entire lipidomics experiment. To identify and quantify lipids, the MasterScan is then probed via queries written in the molecular fragmentation query language (MFQL), which supports any lipid identification routine in an intuitive, transparent and user-friendly manner independently of the instrumentation platform.[(200)] Basically, each query defines what structure-specific ‘‘signature’’ ions (Fig 1.13) and/or their Boolean combinations should be recognized in MS and MS/MS spectra within the MasterScan. Both precursor and fragment ions could serve as ‘‘signatures’’ leading to unequivocal identification of species in complex lipid extracts.[(201-203)]

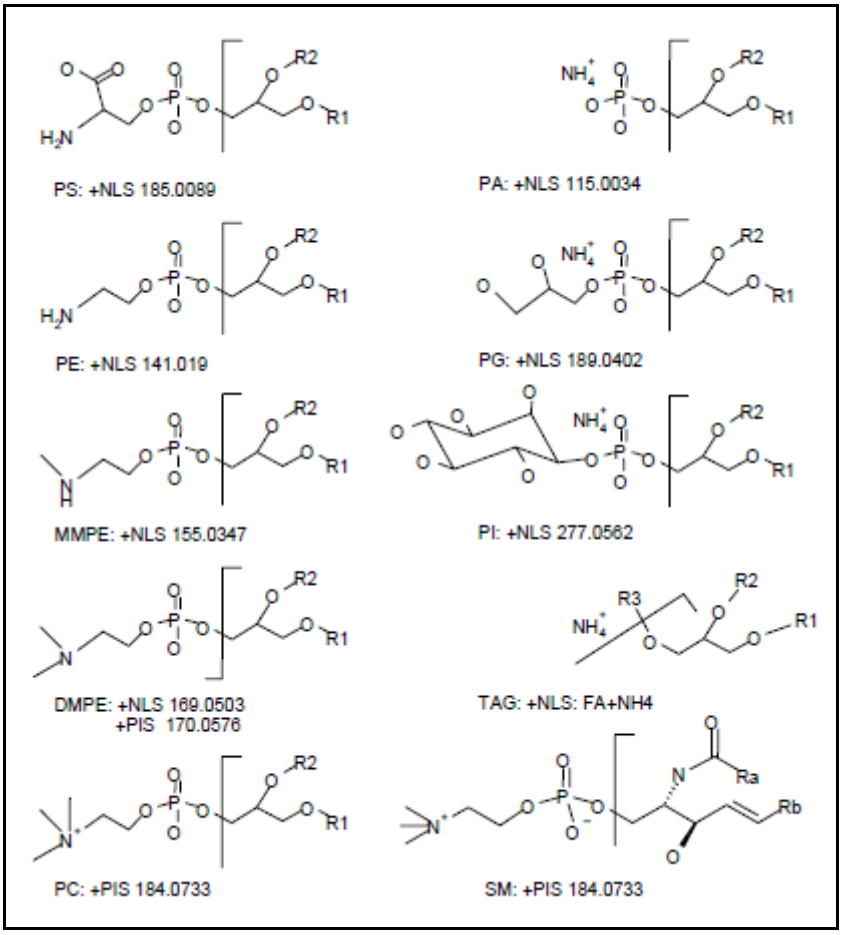


Figure 1.13: Generic chemical structures of lipid classes. The structures and calculated m/z of lipid class specific ions and neutral losses (NLS) are indicated. R1 designates a fatty acid, ether or vinyl ether moiety; R2 and R3– fatty acid moieties; Ra, Rb – alkyl radicals)[204]

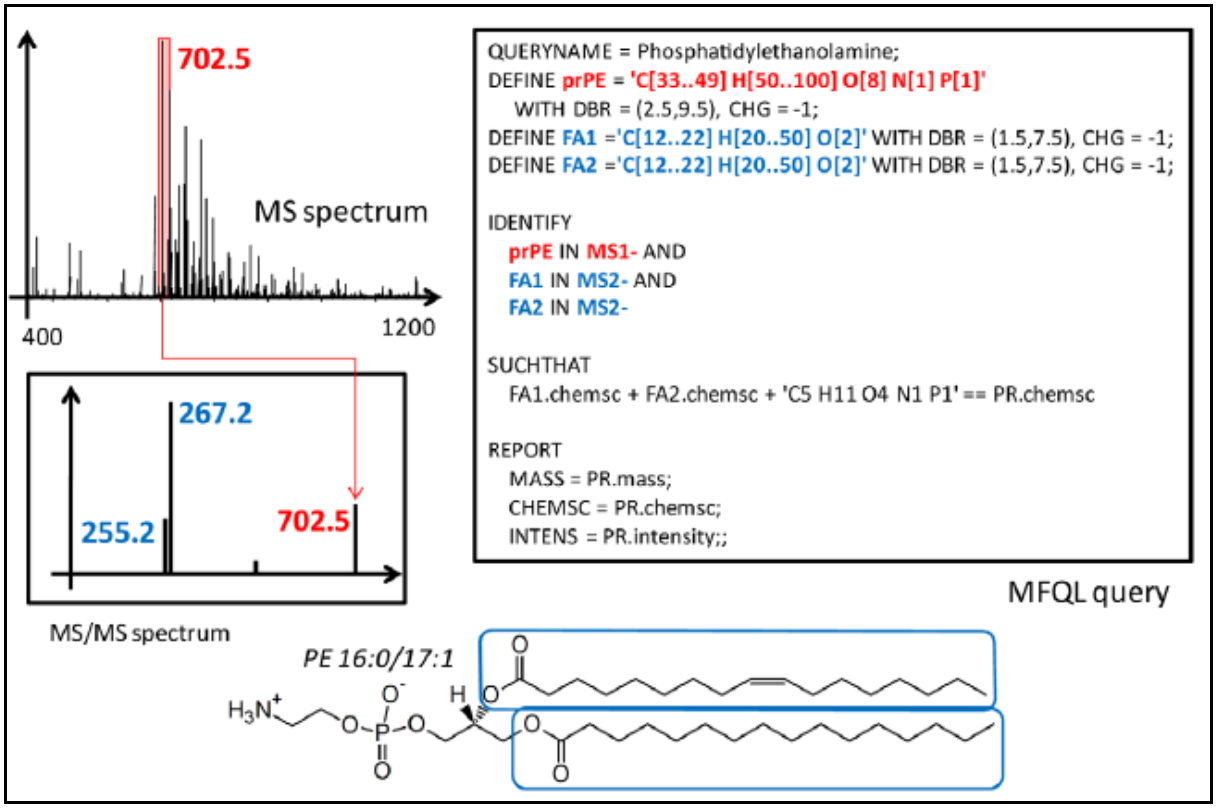


Figure 1.14: MFQL query for identifying phosphatidylethanolamine (PE) species in MS/MS spectra. PE molecules consist of a glycerol backbone to which the phosphoethanolamine head group and two fatty acid moieties are attached via phosphoether and ester bonds, respectively. The chemical structure of PE 16:0/17:1 in its zwitterion form is shown at the bottom panel with two fatty acid moieties (16:0 and 17:1) boxed; other PE species may differ by their fatty acid moieties. If identified by precursor m/z and/or lipid-class specific fragment (for example, originating from the phosphoethanolamine head group), lipids are annotated by their class and total number of carbon atoms and double bonds in both fatty acid moieties (in this case, PE 33:1). However, by identifying both fatty moieties the analysis may recognize the individual molecular species (here PE 16:0/17:1). Usually the location of fatty acid moieties at the glycerol backbone (sn-1 or sn-2) could be inferred from the relative abundance of corresponding acyl anions. In this example, PE molecular anion having m/z 702.5 was detected in the survey MS spectrum (here termed as MS1-) and then its MS/MS

spectrum (here termed as MS2-) was acquired. The latter was dominated by abundant acyl anion fragments (m/z 255.2 and 267.2) originating from 16:0 and 17:1 fatty acid moieties, respectively. Other species of PE class will fragment similarly. During the shotgun experiment all peaks of plausible PE precursors will be fragmented. To identify PE species, we first DEFINE the sum composition constraints (rather than exact values of expected masses) for intact PE molecules (prPE) and acyl anion fragments (FA1 and FA2); we also expect them to be singly charged (CHG =21) and that their unsaturation (expressed as the double bond equivalent range, DBR) should be within 1.5 to 7.5. The next section requests to IDENTIFY previously DEFINE(d) precursors in MS1- spectrum and fragments in corresponding MS2- spectra SUCHTHAT sum compositions of both fatty acid moieties (FA1.chemsc and FA2.chemsc), together with the phosphoethanolamine head group and glycerol backbone (C5 H11 O4 N1 P1), add up to the sum composition of the intact precursor (PR.chemsc). Next, the 'REPORT' section describes the data output format. Here the query requests to report the masses (MASS), sum compositions (CHEMSC) and intensities (INTENS) of all matched PE precursors (prPE.intensity); more elaborate queries may also name the identified species according to a user-defined convention and report intensities of relevant fragment ions along with corresponding mass measurement errors. Further details on MFQL format and syntax are provided at the LipidXplorer wiki site at: https://wiki.mpicbg.de/wiki/lipidx/index.php/LipidXplorer_MFQL.[(203)]

1.3.5 IDENTIFICATION OF OXIDISED LIPIDS

In the 21st century the role of lipid peroxidation in disease is still a subject of considerable research, and within this area there is growing interest in the roles of oxidized phospholipids, which have been found in pathological conditions, often at raised levels

compared to normal physiology [205, 206]. Research on oxidation of phospholipids goes back to very early studies by Thunberg in 1910 observing "respiration" of phospholipid suspensions and noting that iron salts accelerated the uptake of oxygen in tissues [207]. The term "oxidized phospholipids" was reported for the first time in 1939 by Frederik and Mary Bernheim, following the studies on the action of vanadium salts as catalysts in the oxidation of phospholipids from heart and brain tissues [208]. The free radical nature of the process was identified and a number of low molecular weight fragmentation products, such as malonaldehyde, pentanal and 4-hydroxynonenal were identified [209].

However, until the late 1980s knowledge about the oxidized phospholipid products was somewhat limited by the available methods of structural analysis. The most structurally-informative method was gas chromatography coupled with mass spectrometry detection (GC–MS), but this required saponification and derivatisation protocols in order to generate volatile analytes. This resulted in the loss of information about the original esterified structure, as well as the possibility of artefact generation through extensive sample manipulation. The introduction of fast atom bombardment (FAB) and subsequently ESI paved the way for analysis of less volatile biomolecules and reduced thermal degradation, thus enabling structural determination of many oxidized phospholipids. At the end of 1980s, a phosphatidylcholine derivative that was a substrate for the platelet-activating acetylhydrolase enzyme found in human plasma was synthesized [210], and found to be 1-palmitoyl-2-(5-oxo-pentanoyl)-3-glycero-PC (POVPC). Later this compound and other structurally similar ones were found in minimally oxidized low density lipoproteins (LDL) and atherosclerotic plaques [211-213], leading to an association of oxPL with the process of vascular dysfunction and atherosclerosis, thus prompting expansion of this new field of research. Full understanding of the role of phospholipids and their oxidized counterparts is only recently emerging with the development of user-friendly and robust mass spectrometers

that enable the complete mapping of phospholipid species present in biological samples, and are attractive to a wider range of researchers such as biologists, biochemists, pharmaceuticals, and chemists.

The unsaturated fatty acid chains present in phospholipids are the main targets of oxidation [206, 214]. Oxidation reactions involving phospholipids produces a wide variety of compounds that can be classified according to the nature of the modifications:

i. long-chain products, which are products that preserve the phospholipid skeleton;
ii. short chain or truncated products, formed by cleavage of the fatty acyl chains (unsaturated fatty acid); and
iii. adducts, formed by reaction between oxidation products and/or molecules containing nucleophilic groups.

Phospholipid adducts include the products usually formed by cross-linking reactions between phospholipid oxidation products with carbonyl groups and amino groups present in neighboring biomolecules, such as peptides, proteins and ethanolamine phospholipids (PE) [212, 215-218]. In figure 1.15 an example of all these structures, changes that are observed on lineloyl–palmitoyl-containing phospholipid are shown.

Phosphatidylcholines are the most abundant lipid in the plasma as well as in the erythrocytes as seen in our study and they also show a decrease in case of thalassemic samples. Therefore as far as oxidized lipids are concerned we have limited our study to the oxidized products of PCs. Furthermore, among the unsaturated fatty acid chains occurring in biological samples, oleic, linoleic and arachidonic acids predominate. Owing to the double bond these fatty acids are susceptible to oxidative damage due to reactive oxygen species (ROS).[219] From the list of PCs generated after lipidxplorer run we have restricted the search

for three categories of oxidized PCs (Fig. 1.15) to only those containing 14,16,18,20 and 22 fatty acid chains with a maximum of 8 double bonds in the parent PC.

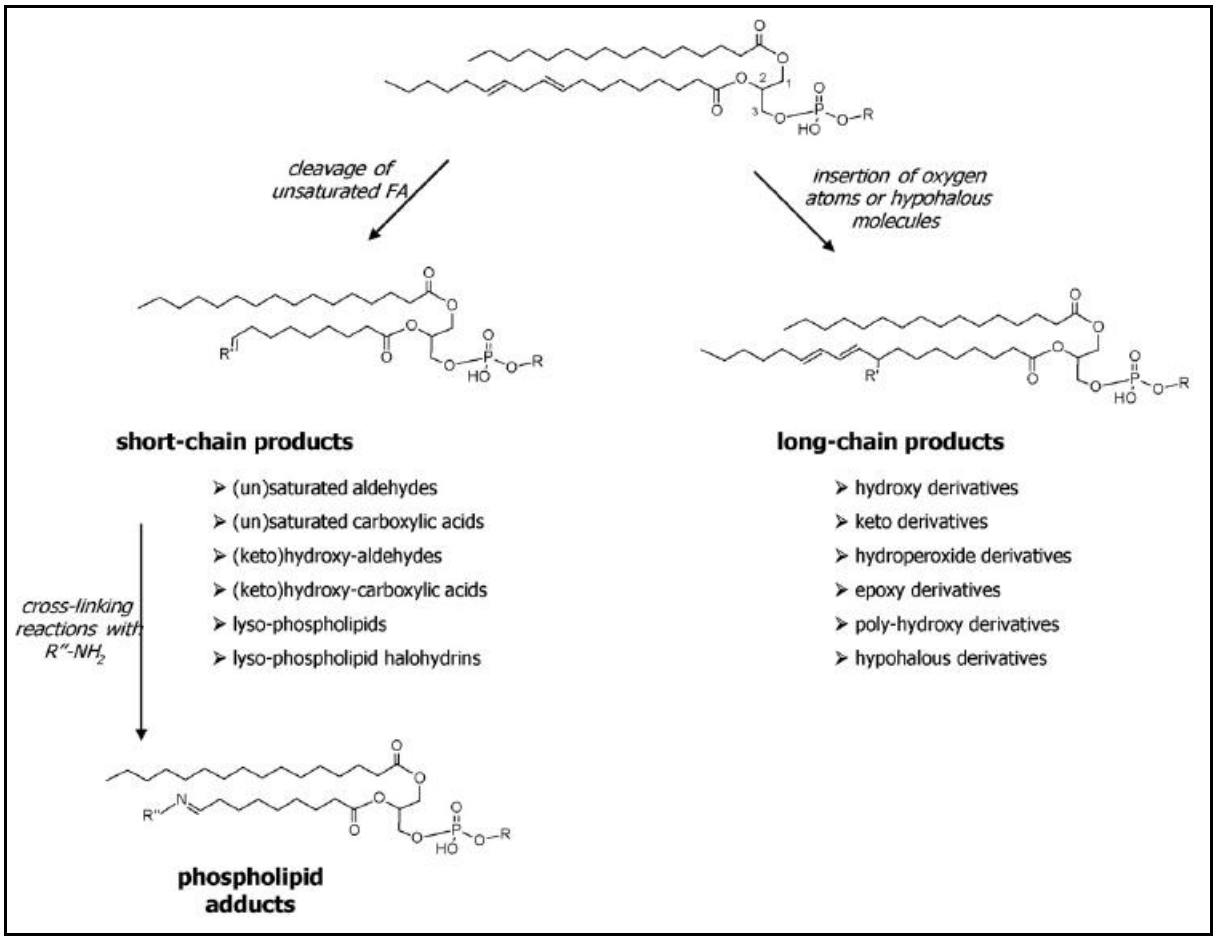


Figure 1.15: Oxidative modifications in phospholipids[220]

1.3.6 PLASMA LIPIDOMICS

Considerable attention has been paid to the links between diseases and the levels of blood plasma lipids, in particular between metabolic disease and cholesterol. Nevertheless, only partial knowledge of the lipids present in human blood plasma has been available, and until 2010 no study was attempted to systematically analyze the full range. The LIPID MAPS

Consortium has developed innovative lipidomic techniques based on liquid chromatography coupled to mass spectroscopy to probe biological systems [221], and has undertaken the task of analyzing the NIDDK/NIST SRM (National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) in collaboration with the National Institute of Standards (NIST) produced a human plasma standard reference material (SRM 1950) for metabolite analysis) by systematically identifying and quantifying the lipid molecular species of the mammalian lipidome [138, 139]. They have reported an in-depth lipid profile of human plasma that reveals the enormous structural diversity of lipids comprising the six major lipid categories as defined by LIPID MAPS.[222]

Since then a number of groups have used plasma lipidomics to study the pathophysiology in a number of diseases. Lipidomic analyses of both whole plasma and lipoprotein subfractions are integral to the current push to understand the relationships between lipoprotein composition and function and how these are affected by disease and treatment. Graessler et al. showed that hypertension was specifically associated with reduced levels of free plasma cholesterol and ether lipids, in particular with ether phosphatidylcholines (PC-O) and ether phosphatidylethanolamines (PE-O).[223] Altered lipid metabolism involved in osteoarthiristis was shown by Castro-Perez et. al in 2010 using liquid chromatography coupled with ESI mass spectrometry.[224] Donovan et al. has shown an upregulation of ether-lipids in case of obese individuals.[225] Meikle et. al. (2014) has shown that lipidomics of biological samples such as plasma could have potential in risk prediction and therapeutic monitoring for diabetes and cardiovascular disease.[226]

1.3.7 ERYTHROCYTE LIPIDOMICS

In 1959, Phillips and Roome provided a preliminary portrait of the human erythrocyte phospholipidome [(227)]. However, it was only in 1960 that Hanahan and colleagues described a more complex scenario, also by including species-specifc differences between human and bovine erythrocytes [(228)]. Four years later, Ways and Hanahan reported a detailed lipid class composition of normal human erythrocytes, indicating the following percentages: cholesterol 25%, choline glycerophosphatides 30%, sphingomyelin 24%, ethanolamine glycerophosphatides 26%, and serine glycerophosphatides 15% [(229)]. Farquhar and Ahrens [(229)] had showed that 67% of the PE, 8% of the PS, and 10% of the lecithin of human erythrocytes are in the plasmalogen form. As early as 1967, Dodge and Philips described a silicic acid thin-layer chromatography strategy to investigate the phospholipid and phospholipid fatty acids and aldehydes in human erythrocytes [(230)]. Thirty three fatty acids and five aldehydes were separated and tentatively classifed into lipid classes, including phosphatidylethanolamine (PE), phosphatidyl serine (PS), lecithin, and sphingomyelin (SM) 24:0 and 24:1, while fatty acid moieties were tentatively attributed.[(231)] Different instrumentations and techniques have been tested for the improvement of lipid analysis. During the last two decades, big technological strides have prompted the dissemination of chromatography separation and mass spectrometry-based lipidomics studies of erythrocytes. [(231-237)].

The human erythrocyte membrane is an object pathological investigation because it can provide a convenient and reliable indication of a range of pathological conditions. The lipid molecular species composition of the erythrocyte is preserved within relatively narrow limits throughout the lifetime of the cell. Retailoring of the constituent lipids is accomplished by uptake of fatty acids from the plasma which, in turn, reflects the intake of lipids in the

diet. This intake can be highly variable so that the process of lipid turnover must be selective in that the fatty acids incorporated into complex membrane lipids are tightly regulated. Nevertheless, changes in membrane lipids provide useful indicators of a range of haemoglobinopathies. [238] It may be expected that phospholipid turnover and repair is higher in hemoglobinopathies because damage to the membrane resulting from free radical reactions is often a feature of these conditions. A number of reports indicate evidence of lipid oxidation in RBCs from sickle cell or thalassemia patients, [239, 240] suggesting that phospholipid repair is not efficient enough to maintain the proper lipid molecular species composition in these cells.

1.4 OBJECTIVES

Keeping in mind the present disease monitoring and management system of Eβ thalassemia our objective is to do a proteomics study of the body fluids such as plasma and urine. Our lab has already reported a proteomics study of erytrhocytes in Eβ thalassemia [241]. To complete our total knowledge we have also done a lipidomics study of plasma, erythrocytes and erythrocytes membrane. The objective and scope of the present study is discussed elaborately below:

1. **Chapter 1: Plasma Proteomics of Eβ Thalassemia**- our objective is to conduct a 2-dimensional gel electrophoresis (2DGE) based study of the differential expression of proteins of Eβ thalassemic samples as compared to normal samples.
2. **Chapter 2: Urinary Proteomics of Eβ Thalassemia** - the objective is to do a 2-DGE based study of the changes in the urine proteome of the Eβ thalassemic samples as compared to normal.

3. **Chapter 3: Lipidomics Study of Eβ Thalassemia** - Finally our objective is also to study the lipidome of plasma, erythrocytes and erythrocytes membrane fractions and analyse the changes in lipidome of the Eβ thalassemia patients as compared to normal samples. We also aim to analyse the levels of oxidized lipids as oxidative stress is a well known condition in Eβ thalassemia.

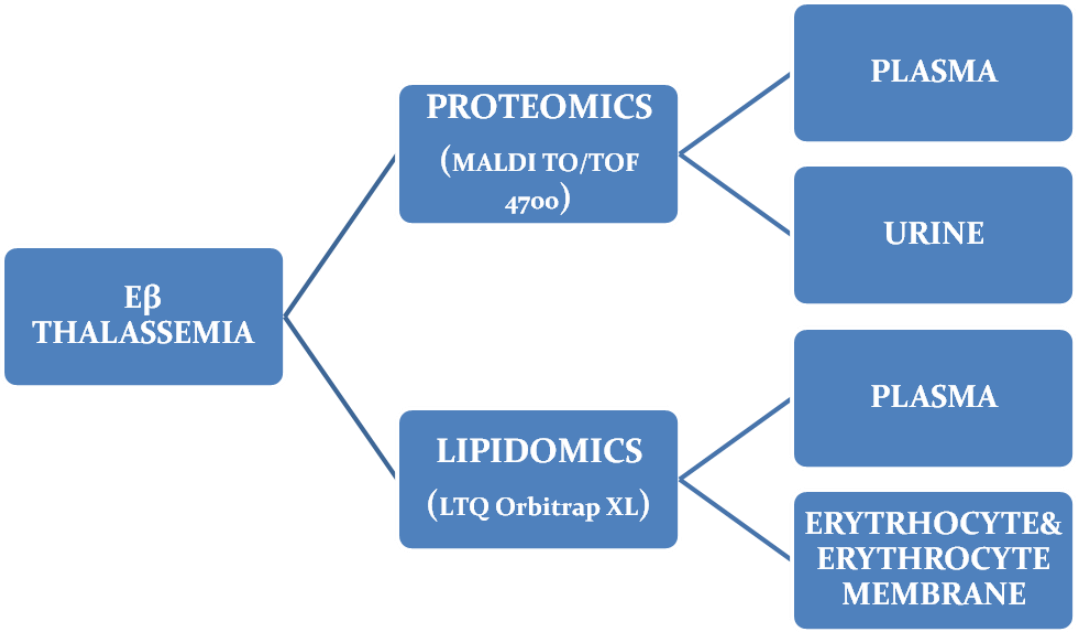


Figure 1.16: Scheme of work

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CHAPTER II

PLASMA PROTEOMICS OF Eβ THALASSEMIA

2.1 INTRODUCTION

The hemoglobin (Hb) E-β is one of the commonest forms of hemoglobinopathies worldwide.[1] The HbE mutation is located near the junction between the first exon and the first intron of the β-chain gene. Nucleotide sequence change near the consensus splice site region activates a cryptic splice site, which is not normally used for mRNA processing. This new splice site competes with the normal splice site. Some mRNAs are still processed using the normal splice site and thus produce a protein with a Lys instead of a Glu at position 26. The variant (HbE) is thus innocuous in its heterozygous and homozygous states.[2] The primary clinical importance of HbE trait arises when the βE allele interacts with other β-thalassemia mutations leading to a moderate to severe anemia known as HbEβ-thalassemia.[3]

The only definitive form of treatment for thalassaemia is bone marrow transplantation, which is possible only when there is a matching donor relative. Symptomatic treatment involves regular blood transfusion and the use of iron-chelating drugs to remove the excess iron that results from transfused blood. Hence more studies are required to frame a better disease management program. Therefore a lot of attention being given to the disease monitoring as this is of prime importance for the welfare of the patients.

Blood plasma is a rich source of biochemical products that can indicate physiological or clinical status of a patient.[4] It is the most valuable specimen for protein biomarker determination because it is readily obtainable and contains thousands of protein species secreted from cells and tissues.[5, 6] In case of HbEβ thalassemia the RBCs releases a pool of proteins which participate in cell to cell communication as well as act as signal molecules for WBCs and platelets. Hence, plasma will be that fraction of the human blood which will give us an insight into the crosstalk between various cells in the blood. The discovery of protein biomarkers in plasma for diseases is challenging and requires a highly parallel display and

quantization strategy for proteins [7-9] like two dimensional gel electrophoresis (2DGE). The protein content of plasma however, is dominated by a handful of proteins such as albumin, immunoglobulins (IgG), and lipoproteins present across an extraordinary dynamic range of concentration. This exceeds the analytical capabilities of traditional proteomic methods, making detection of lower abundance serum proteins extremely challenging. Reduction of sample complexity is thus an essential first step in the analysis of plasma proteome [10].

In our study we have employed 20% ammonium sulphate precipitation process to deplete the high abundant proteins followed by 2DGE.[11] Here we report that proteins involved in transportation or with catalytic activity, such as the apolipoprotein A1 (APO A1) and E (APO E), transferrin (TNF) and adenylate kinase 1 (ADK 1) show changed levels in diseased conditions. The 11 proteins reported here, if studied in greater details could be used collectively to monitor the condition of a patient and determining the effect of transfusion.

2.2 MATERIALS AND METHODS

SAMPLE COLLECTION

Peripheral blood samples were collected from patients with Eβ thalassemia (n = 10), homozygous EE (n = 4) and with high (>80%) HbF (n = 4) and normal volunteers (n = 10) in vials containing 5 mM ethylenediaminetetraacetic acid (EDTA). Details of the samples are given in the table below (Table 2.1). Written consent was taken from adults and in case of children it was taken from the parents as per guidelines of institutional ethical committee. The handling of all human blood samples was carried out in accordance with the guidelines established by the Local Ethical Committee. For transfusion dependent patients peripheral blood was taken after transfusion gap of minimum 45 days gap.

Plasma and erythrocytes were separated using 75% percoll for the Fluorescence Activated Cell Sorting (FACS) and proteomic studies, as described earlier [12, 13].

Table 2.1: Sample Details

PATIENT	CONDITION	AGE	GENDER	Hb F	Hb A	HbA_2/ HbE
N1	Normal	38 years	Male	0.6%	87.7%	2.7%
N2	Normal	28years	Male	0.4%	88.1%	2.6%
N3	Normal	49years	Female	0%	88.1%	2.5%
N4	Normal	26 years	Female	0.1%	87.5%	2.5%
N5	Normal	26 years	Male	0.4%	89%	3.1%
N6	Normal	26 years	Female	0.3%	88.3%	2.6%
N7	Normal	30 years	Male	0.9%	85.4%	2.4%
N8	Normal	26 years	Male	0.3%	87.1%	3.1%
N9	Normal	25 years	Female	0.7%	86.7%	2.6%
N10	Normal	35 years	Female	0.5%	88.8%	3.2%
Eβ1	HbEβ thalassemia	40 years	Male	5.3%	8.1%	83.2%
Eβ2	HbEβ thalassemia	6 months	Male	4.0%	70.3%	17.8%
Eβ3	HbEβ thalassemia	11 months	Male	41.4%	6%	50.9%
Eβ4	HbEβ thalassemia	6 years 6 months	Male	68.4%	0.7%	27.5%
Eβ5	HbEβ thalassemia	3 years	Male	39.7%	4.5%	54.4%
Eβ6	HbEβ thalassemia	13 years	Male	58.6%	3.4%	45.5%
Eβ7	HbEβ thalassemia	27 years	Female	21.8%	3.2%	81.7%
Eβ8	HbEβ thalassemia	7 years	Female	5.6%	4.2%	80.5%
Eβ9	HbEβ thalassemia	7 years 6 months	Female	16.3%	6.9%	66.0%
Eβ10	HbEβ thalassemia	3 years	Female	38.2%	3.3%	53.1%
EE1	Homozygous EE	36 years	Female	2.1%	3.6%	73.6%
EE2	Homozygous EE	8 years	Male	1.7%	2.1%	90.3%

EE3	Homozygous EE	32 years	Female	1.5%	6.18%	86.2%
EE4	Homozygous EE	12 years	Male	2.3%	3.8%	83.9%
HPFH1	Consistent with heterozygous state of hereditary persistence of fetal hemoglobin	11 years	Male	14.3%	3.0%	74.1%
HPFH2	Consistent with heterozygous state of hereditary persistence of fetal hemoglobin	40 years	Male	15.4%	2.8%	73.5%
HPFH3	Consistent with heterozygous state of hereditary persistence of fetal hemoglobin	32 years	Female	16.4%	2.8%	72.8%
HPFH4	Consistent with heterozygous state of hereditary persistence of fetal hemoglobin	16 years	Male	88.9%	6.2%	2.2%

ANNEXIN BINDING

The erythrocytes were suspended in the buffer provided in the kit to a final concentration of 1 x 10^{6} cells/ml and incubated in presence (test set) and absence (control set) of FITC conjugated annexin V (AV-FITC, BD Biosciences Pharmingen). The erythrocyte populations were defined by size in forward and side scatter plots. Fluorescence intensities were expressed in logarithmic scale. The control sample incubated without FITC-AV was used to set the region for positive fluorescence such that the fraction of cells with positive (auto-) fluorescence was lower than 0.2% of total. The population of cells labelled

with FITC-AV above background was determined from the fraction of cells in this region in excess of that obtained with the (unlabeled) control [14].

PLASMA FRACTIONATION

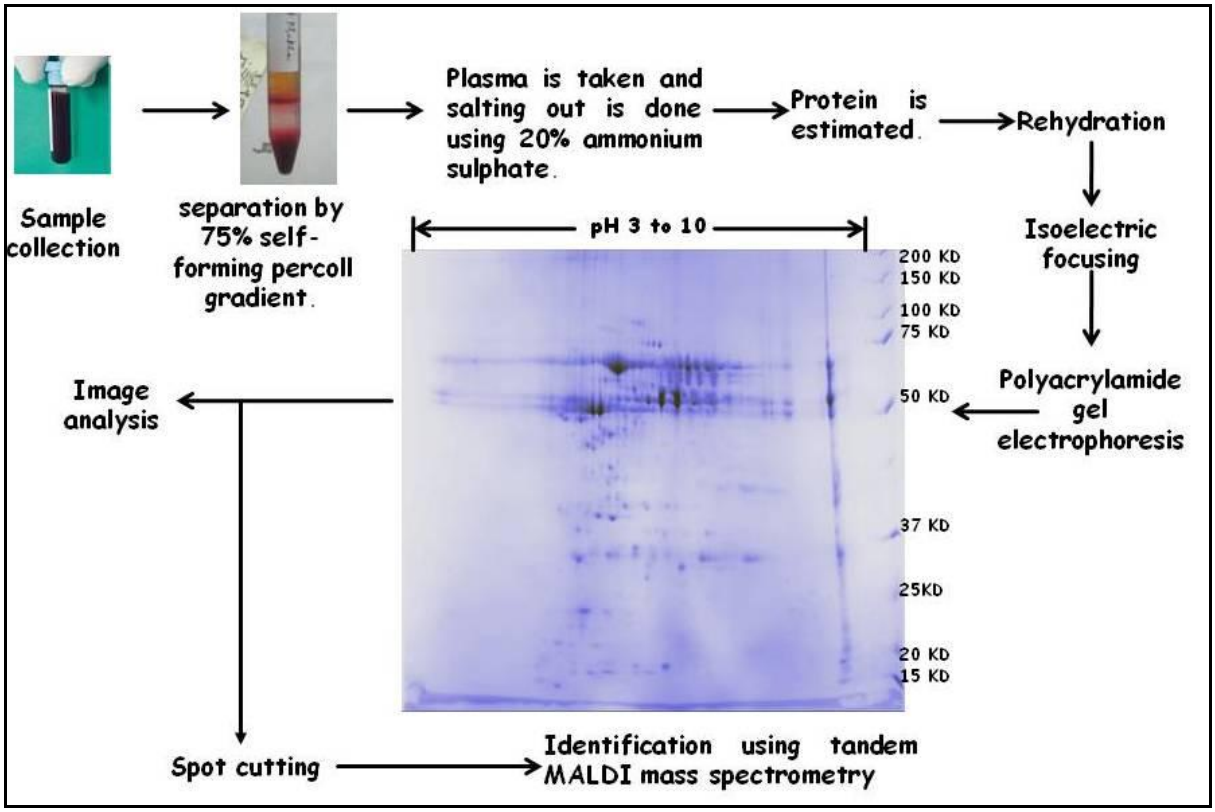


Figure 2.1: schematic representation of the protocol followed for proteomic study of plasma samples.

Plasma samples were centrifuged at 12000g, 4°C, for 30 minutes and the supernatants diluted with PBS (2.7mM KCl, 1.5mM KH_2PO_4, 137mM NaCl, 8.1mM Na_2HPO_4, pH 7.4) to protein concentration of ≤ 20mg/ml. Salting out with 20% ammonium sulphate was done to deplete the high abundant proteins from the diluted plasma fraction as described in our earlier work [11]. The precipitate was dissolved in minimum volume of solubilisation buffer (5mM

sodium phosphate, 20mM KCl, 1mM EDTA, 0.2mM DTT and pH 8.0). The solubilised ammonium sulphate precipitate was dialysed overnight against 10mM Tris, 5mM KCl, pH-7.5 at 4°C.

2DGE OF THE PLASMA FRACTION

After dialysis the samples protein estimation was done using Bradford protein assay reagent (Bio-Rad, Hercules, CA). The samples were mixed with 2D rehydration buffer containing 8M urea, 2% (w/v) CHAPS, 0.05% Bio-lyte 3-10 ampholyte, 20mM DTT (Bio-Rad, Hercules, CA) and protease inhibitor (Roche Diagnostics) to bring it to a final volume of 350µl. 17cm pH 3-10 IPG strips (Bio-Rad) were passively rehydrated with the plasma samples. Isoelectric focussing (IEF) was carried out in a Protean IEF cell (Bio-Rad), stepwise up to 120000 Volt-Hours. Equilibration of the IPG strips after IEF was performed following the [15]. The second dimension was run on 8-16% acrylamide gradient gels in a Protean II XL electrophoresis module (Bio-Rad). According to the total protein load gels were stained with either silver (600-900µg) following the method of Rabilloud[16], SYPRO-RUBY (~1.2mg) or Blue Silver Coomassie (~1.8mg) [17]. Image was taken and densitometric analysis was done on Versa doc series 3000 imaging system using PDQuest software (version 7.1, Bio-Rad). Densitometry analysis of the gel spots of interest was performed using the density tool of PDQuest. Spot volume (density) of the desired spot(s) was normalized as parts per million (ppm) of the total spot volume using the spots that were present in all the gels, to calculate the relative density of the spot in the sample.

IN-GEL TRYPTIC DIGESTION AND MASS SPECTROMETRY

Protein spots from gels, stained with Coomassie and Sypro-Ruby from normal and diseased samples, were excised. Spots were destained with 50% acetonitrile and 25mM ammonium bicarbonate followed by in-gel tryptic digestion. Peptides were then extracted and run on a MALDI ToF/ToF (AB 4700, Applied Biosystems) following published protocol [13]. CHCA (Waters) was used as matrix. MS of the digested peptides was done positive ion mode. Autotryptic and common keratin peaks were excluded from the MS/MS analysis. 12 most intense peptides from each spot was subjected to MS/Ms analysis. GPS explorer V3.6 was used to generate the peak list using MS and MS/MS data. The data was searched against human MSDB and Swiss-Prot databases using MASCOT V2.1 (Matrix Science) server and MOWSE score (with $p<0.05$) was considered to determine significant hits. For homologous proteins having similar MOWSE scores, preference was given to the protein with best match between theoretical and experimental molecular weights and pI. All MS experiments were repeated atleast 3 times with spot excised from different gels from different samples. The database search parameters included one missed cleavage, error tolerance of ±100 ppm for PMF and ± 1.2 Da for MS/MS ion search and variable modifications like carbamidomethyl cysteine, methionine oxidation and N-terminal acetylation.

WESTERN IMMUNOBLOTTING

Raw plasma samples (25µg) were suspended in SDS-PAGE buffer (2% mercaptoethanol (v/v), 1% glycerol, 50mM Tris-HCl and a trace amount of bromophenol blue) to bring it to a final volume of 30µl and heated to 95°C for 5minutes, cooled and loaded directly onto a 12% gel. SDS-PAGE was performed in a MINI Protean III-cell (Bio-Rad)

using tris-glycine with 0.1% SDS, following manufacturer's protocol. Proteins separated in the gel were blotted onto PVDF membranes and subsequently blocked with tris-buffer-saline (TBS) and 5% non fat dry milk for 2 hours at room temperature. Primary antibodies (Abcam) were diluted in TBS/0.1% Tween (TBST) following manufacturer's instructions. B-Tubulin was used as the loading control. Anti-rabbit or anti-mouse HRP-conjugated IgGs were used as secondary antibodies (Abcam). Membranes were washed with TBST and detected by ECL (Pierce) with Versa Doc imager (Bio-Rad).

2.3 RESULTS

ANNEXIN BINDING

The percentage of phosphatidylserine (PS) asymmetry of normal erythrocytes and the patients was defined by the percentage of annexin V-FITC binding in the young and aged RBC population of peripheral blood. On comparing the percentage PS asymmetry in the total erythrocyte population amongst the four categories, the erythrocytes from the Eβ thalassemic patients exhibited maximum PS exposure (6.3 ± 2.8) followed by HPFH samples (1.81 ± 0.56) and patients with homozygous EE condition (1.78 ± 0.32). Finally the least PS exposure was observed in case of the normal samples (1.04 ± 0.23). Student's t test was performed and the changes were found to be significant with a p value ≤ 0.01.

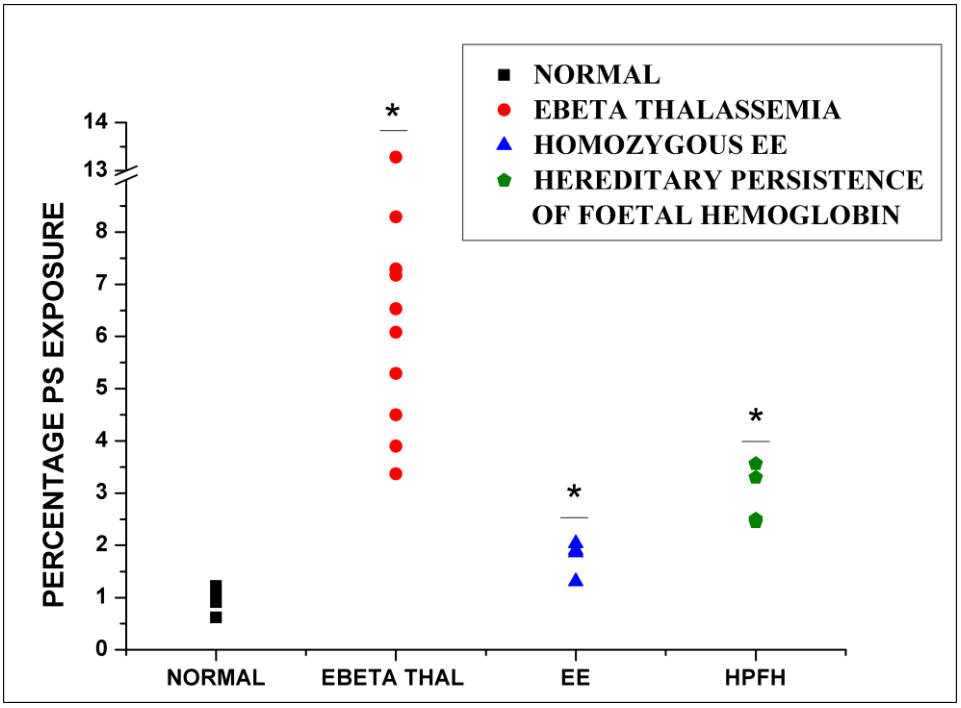


Figure 2.2: scatter plot representing the percentage of PS exposure in the erythrocyte membrane in the four categories.

DIFFERENCES IN THE PLASMA FRACTIONATED PROTEOME

The clinical features of the samples in the four categories are given in table 2.1. The hereditary persistence of fetal haemoglobin (HPFH) samples were taken as positive control as other than the fact that these samples have very high percentage of fetal haemoglobin, they have no disease phenotype. Furthermore the FACs data shows that these samples along with homozygous EE condition have low annexin V-FITC binding similar to the normal samples.

On comparing the normalised intensities of the protein spots of the gels in the four categories (figure 2.3) as stated above ten proteins have shown differential regulation. These proteins are the ones showing more than 1.5 fold change in the mean ppm spot volume compared to normal. Proteins involved in blood coagulation and iron and cholesterol metabolism shows a differential regulation. Transferrin (TFR), alpha 1 antitrypsin (A1AT),

plasminogen (PLMN), apolipoprotein A IV precursor (APOA4 precursor), haptoglobin precursor (HPT), α-1 microglobulin/ inter α-trypsin inhibitor precursor (AMBP), apolipoprotein A1 (APOA1) shows a decrease whereas adenylate kinase 1 (ADK1), glutathione S transferase α 2 (GSTA2) and apolipoprotein A IV (APOA4) shows an increase in their levels in the plasma. Almost in all cases the 10 proteins from samples with HPFH showed the same trend as normal samples except in case of TFR, PLMN and ADK1. In case of the homozygous EE condition TFR, AIAT, PLMN, APOA4 precursor, HPT precursor and AMBP also shows a decrease as in case of the Eβ thalassemic samples however showing a smaller fold change (Table 2.2, next page).

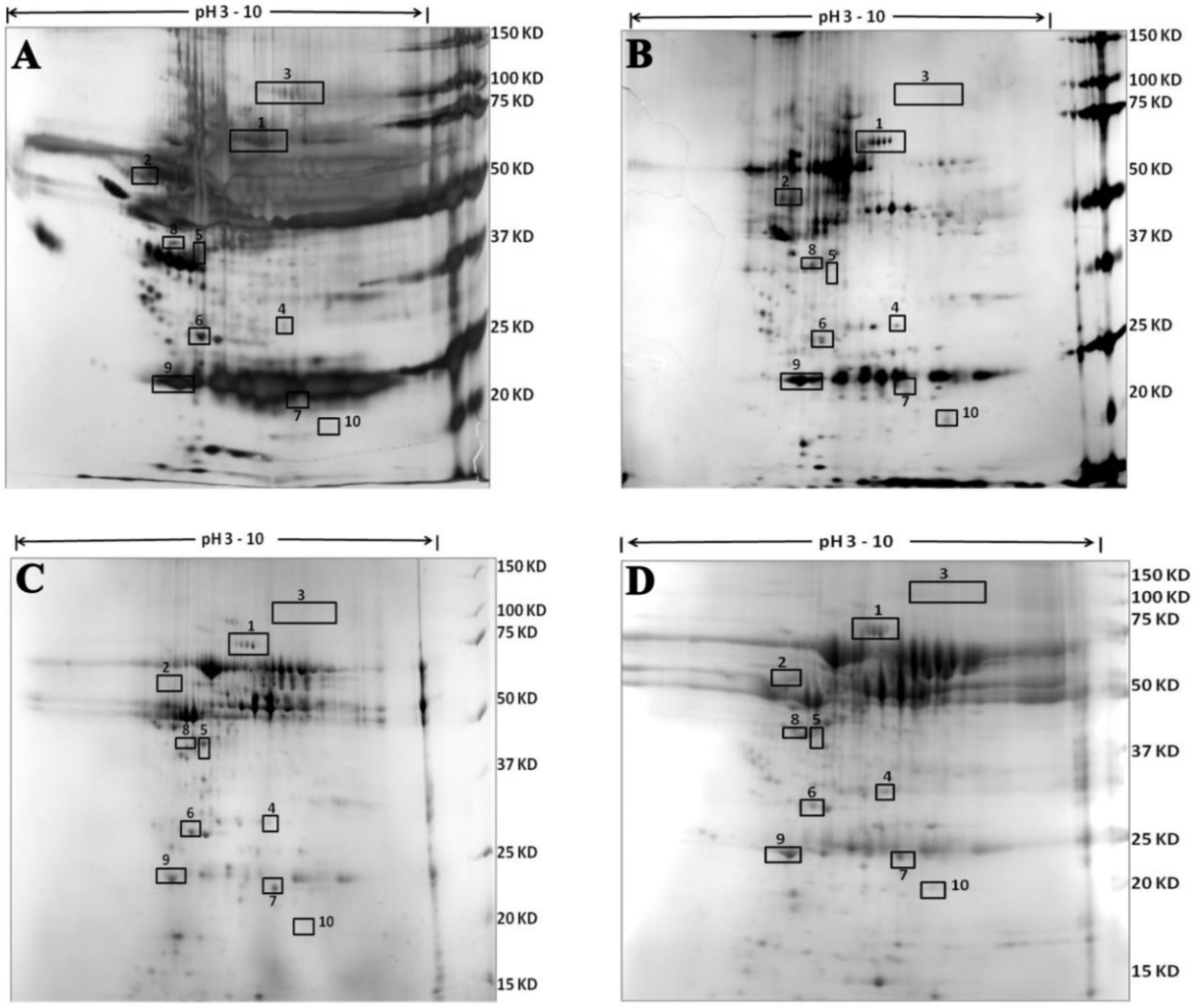


Figure 2.3: representative 2D gels from each category showing the 10 protein spots; A: normal samples; B: Eβ thalassemia; C: EE homozygous; D: Consistent with heterozygous state of hereditary persistence of foetal haemoglobin.

***1** = Transferrin; **2** = alpha 1 antitrypsin; **3** = plasminogen; **4** = apolipoprotein A IV precursor; **5** = haptoglobin precursor; **6** = alpha 1 microglobulin/ inter alpha trypsin inhibitor precursor; 7 = adenylate kinase 1; 8 = apolipoprotein A IV; 9 = apolipoprotein A I; 10 = glutathione S transferase A2.*

TABLE 2.1: SPOT ANNOTATIONS

S. NO.	PROTEIN NAME	A. NO.	M.WT. PI	SCORE	SQ COV	FOLD CHANGE		
						Eβ	EE	HPFH
1	TRANSFERRIN PRECURSOR	Q53H26_ HUMAN	77,030 (6.68)	163 (64)	46%	-2.2	-1.51	-1.55
2	ALPHA-1-ANTITRYPSIN	AAB59495	46,677 (5.43)	90 (64)	55%	-2.17	-2.39	-1.04
3*	PLASMINOGEN	Q5TEH4_ HUMAN	90,510 (7.04)	90 (64)	40%	-2.62	-1.52	-1.8
4*	APOLIPOPROTEIN A-IV PRECURSOR	LPHUA4	45,307 (5.23)	107 (64)	58%	-2.36	-1.98	-1.12
5	APOLIPOPROTEIN A-IV	AAA51748	43,358 (5.22)	182 (64)	47%	1.6	1.2	1.18
6	α-1 MICROGLOBULIN / INTER α-TRYPSIN INHIBITOR PRECURSOR	HCHU	38,974 (5.95)	70 (64)	26%	-1.88	-1.66	-1.104
7*	ADENYLATE KINASE 1	Q5T9B7_ HUMAN	23,396 (8.78)	29 (64)	29%	1.54	-1.26	1.64
8	HAPTOGLOBIN PRECURSOR	HPHU1	38,427 (6.13)	89 (64)	39%	-1.52	-1.54	-1.03
9	APOLIPOPROTEIN A I (FRAGMENT)	LPHUA1	30,759 (5.56)	624 (64)	81%	-1.63	-1.13	1.12
10*	GLUTATHIONE S TRANSFERASEA2	GSTA2_ HUMAN	25,531 (8.54)	33 (64)	85%	1.5	-1.22	1.09

(Where C = Control, LG = Low Grade and HG = High Grade, S. SNO. = Spot number, A. NO. = accession number, M.WT = molecular weight, PI = isoelectric pH, SQ COV = sequence coverage); *For the proteins spots marked with "*" the protein identification*

supported by published SWISS-2D-PAGE plasma map and other proteomic studies of blood plasma as described in text. (MS and MSMS data given in supplementary).

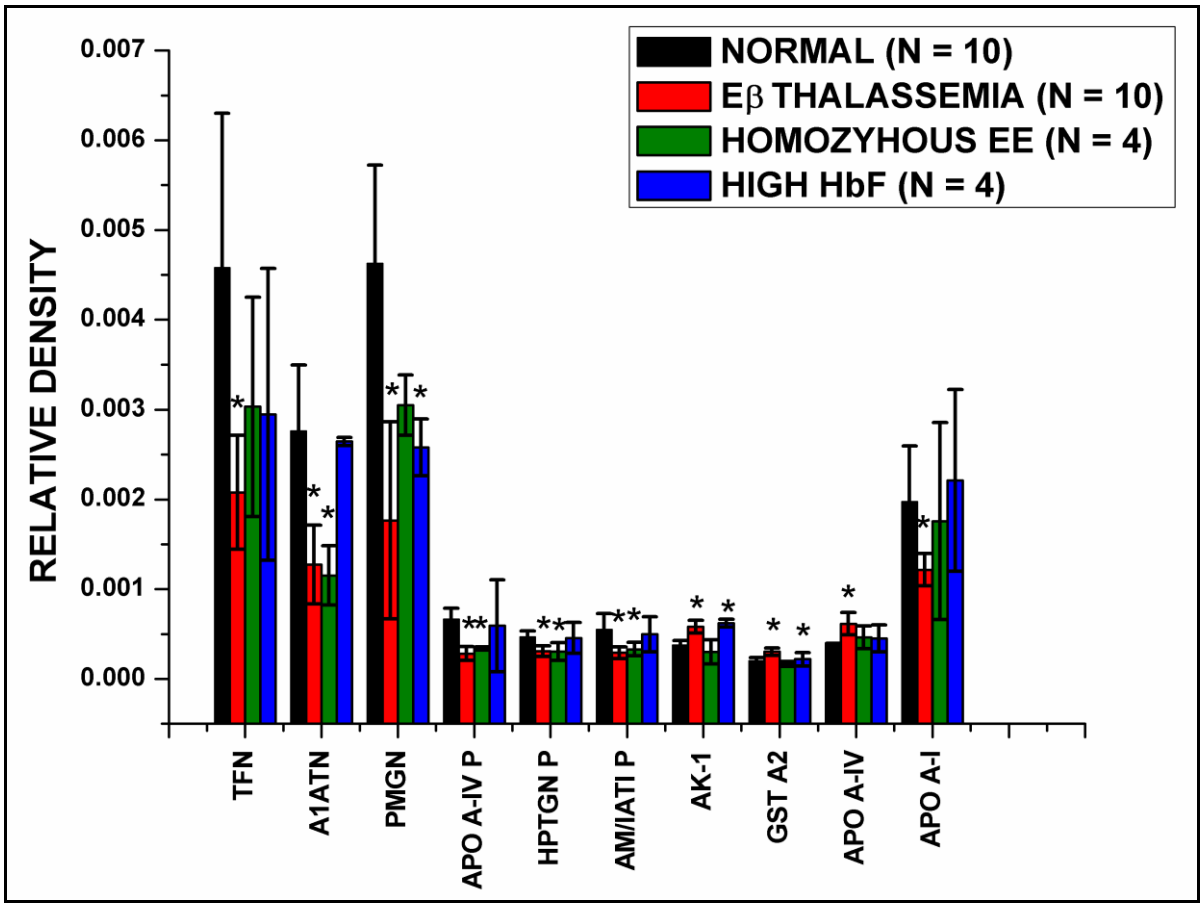


Figure 2.4: Bar plot showing the relative density of the 10 proteins for the 4 categories. Statistically significant ($p \leq 0.05$) changes are marked with "".*

TFN: transferrin; A1ATN: alpha 1 antitrypsin; PMGN: plasminogen; APO A-IV P: apolipoprotein A IV precursor; HPTGN P: haptoglobin precursor; AM/IATI P: alpha-1 microglobulin/ inter α-trypsin inhibitor precursor; AK-1; adenylate kinase 1; GST A2: glutathione S transferase A2; APO A IV: apolipoprotein A IV; APO A-I: apolipoprotein A I.

VALIDATION BY WESTERN IMMUNOBLOTTING

To confirm the results obtained from 2DGE experiments, we quantitated the amounts of five differentially regulated proteins in raw plasma, obtained from a separate set of 3 normal controls and 4 Eβ thalassemic patients, using western immunoblotting. Figure 2.5 shows the immunoblots for 5 proteins with β-tubulin as loading control. The immunoblots clearly support results from 2DGE experiments.

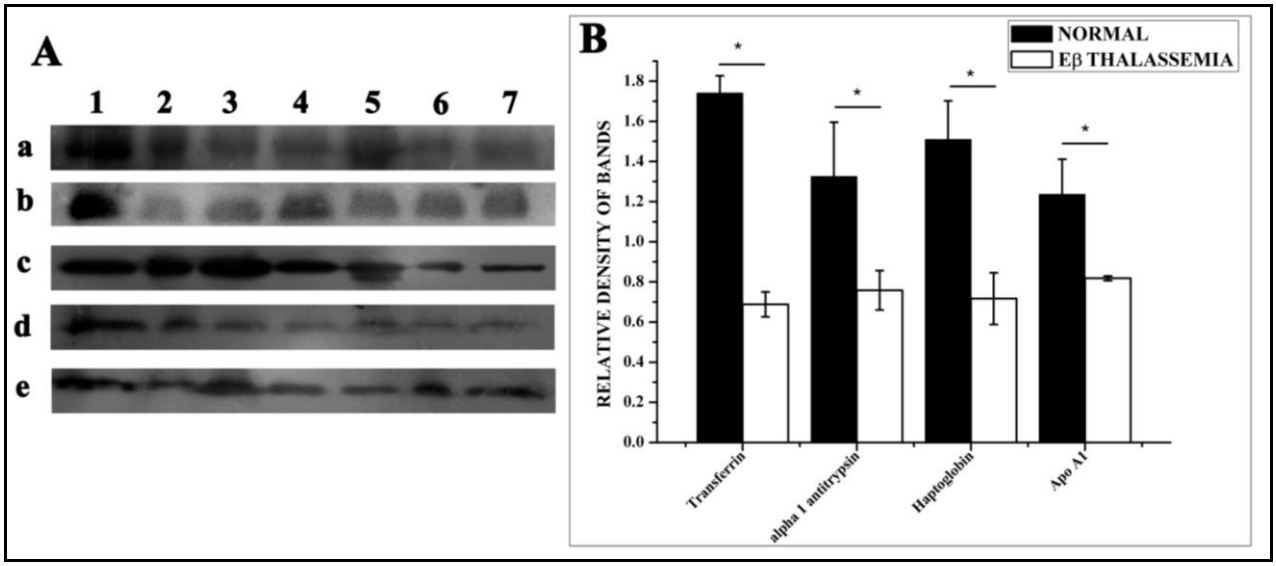
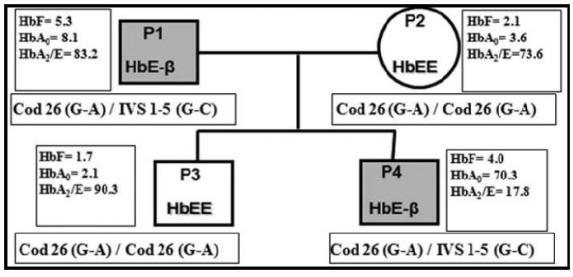


Figure 2.5:Immunoblot of four proteins showing differential regulation. A shows the immunoblots of 5 proteins where lanes 1-3 are normal samples and 4-7 are Eβ thalassmeic samples. a- transferrin; b- alpha 1 antitrypsin, c- haptoglobin; d- apolipoprotein A1 and e- β tubulin as loading control. B is the bar plot of normalised band densities of the normal and Eβ thalassemic samples for the four proteins.

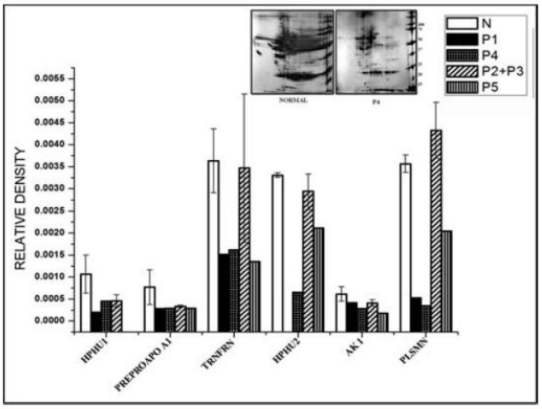
Differential regulation of plasma proteins between members of a family with homozygous HbE and HbEβ –thalassemia: A case study

This study is based in a classical case which shows that, not only different β-thalassemia mutations can give rise to variety of clinical consequences, but also same β-thalassemia mutation upon interacting with βE allele can lead to variety of clinical manifestation of the disease condition. Here we've compared the plasma protein profiles of 4 individuals in a family. Father and the younger son both are HbEβ-thalassaemic {Cod 26 (G-A)/IVS 1-5 (G-C)}, but the father never requires transfusion, whereas the younger son requires monthly blood transfusion. Mother and the elder son are HbEE {Cod 26 (G-A)/Cod 26 (G-A)} without any history of transfusion.



Pedigree of the family along with major clinical and mutational data. Hb, hemoglobin.

Proteomic study revealed that few plasma proteins, involved in hemoglobin scavenging, hemolysis or hypercoagulation show differential changes between the strongly transfusion dependent patients, P4 and P5 (similar clinical features as that of the P4) from those of normal individuals and the ones who do not need blood transfusions, P1, P2 and P3 indicating the changes to be specific to the disease conditions. Erythrocytes from the P4 and P5 undergo oxidative stress, are prone to hemolysis and found to have tendencies of hypercoagulation.



Histogram plots showing change in relative densities of the 6 differentially regulated proteins in the plasma fraction. Deviations from the mean of two normal (empty bar) and homozygous E (in slanted striped bar) samples are shown. The inset shows a 2D gels of normal and P4. N, normal; P1, father; P4, younger son; P2+P3, averaged P2 (mother) and P3 (elder son); P5, unrelated HbE-β-thalassemic sample; HPHU1, haptoglobin precursor 1, PREPROAPO A1, preproapolipoprotein A1, TRNFRN, transferrin; HPHU2, haptoglobin precursor 2; AK 1, adenylate kinase 1; PLSMN, plasminogen.

Taken together, this study establishes the relation between the differences in the levels of plasma proteins with the progression of the disease phenotype, manifested in the extent of transfusion dependence of the patient.

2.4 DISCUSSION

There are reports that chronic hemolytic anemias such as β thalassemia, sickle cell disease, paroxysmal nocturnal hemoglobinuria, autoimmune hemolytic anemia and unstable hemoglobinopathies, are characterized by a hypercoagulable state [18]. In addition to increased thrombin and fibrin generation, increased tissue factor activity, and increased platelet activation, patients with hemolytic anemias manifest thrombotic complications, including venous thromboembolism, *in situ* pulmonary thrombosis and stroke [18-23]. The abnormal phospholipid membrane asymmetry present in the red blood cells with resultant phosphatidylserine exposure appears to play a significant role in the aetiology of the observed hypercoagulable state [24]. The anticoagulant pathways that regulate blood coagulation include the protein C anticoagulant mechanism, the serine protease inhibitors in plasma, and the Kunitz-like inhibitors, tissue factor pathway inhibitor and protease nexin 2. Finally, the fibrinolytic mechanism that comprises the activation of plasminogen into plasmin prevents excessive fibrin accumulation by promoting local dissolution of thrombin and promoting wound healing by reestablishment of blood flow.

Furthermore, plasma extracellular hemoglobin (ECHb) liberated by intravascular hemolysis has deleterious effects on the vasculature. ECHb scavenges nitric oxide (NO) and promotes the pathogenesis of several clinical events including pulmonary hypertension, priapism and non-hemorrhagic strokes. ECHb reduces the bioavailability of NO which down-regulates platelet activation, leading to platelet aggregation and vascular clot formation [25].

Among the 10 proteins reported we see (fig 2.6) that all the proteins are directly or indirectly involved in blood coagulation or haemoglobin scavenging. We have seen the downregulation of 2 serine proteases, namely haptoglobin and plasminogen, where haptoglobin is known as a hemoglobin scavenger [26] thus fighting against ECHb and

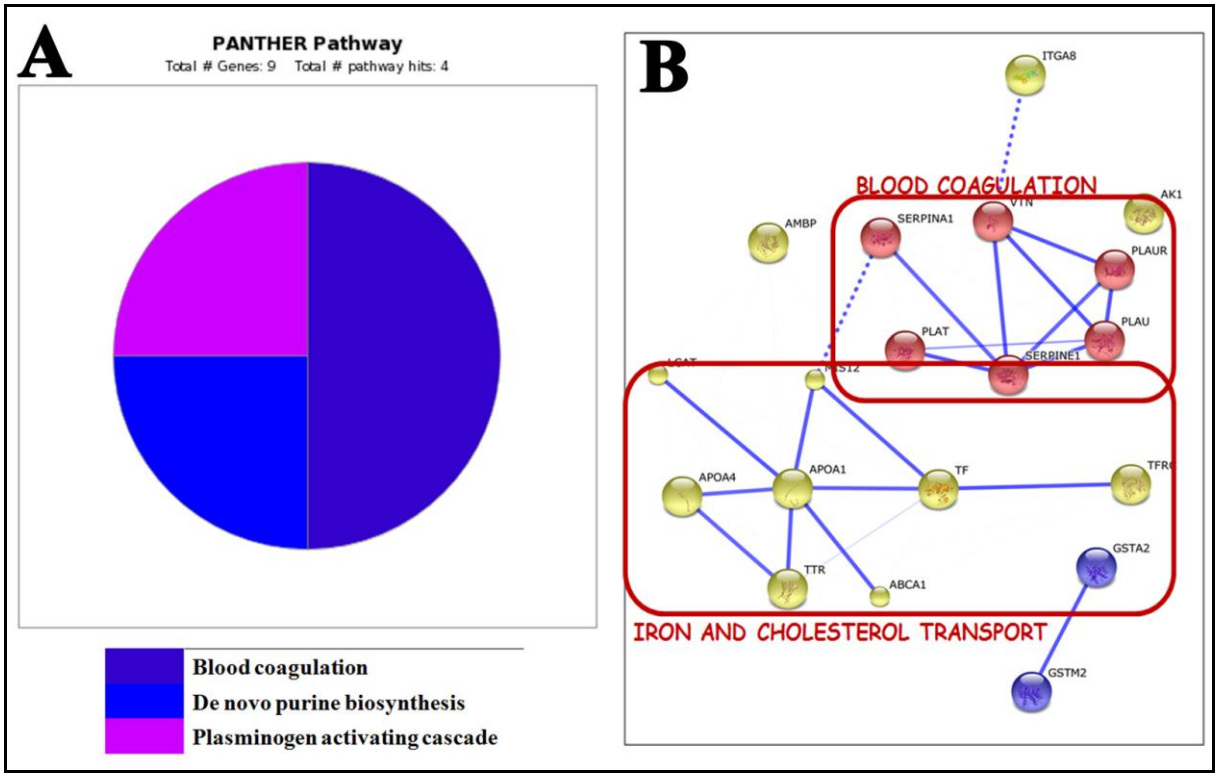


Figure 2.6: A is a representation of the distribution of the proteins according to the pathways in which they participate using Panther Classification System and B is a protein-protein interaction network of the proteins as obtained from STRING.

plasminogen is the zymogen of plasmin which participates in fibrinolysis, i.e. degradation of fibrin [(27)]. Haptoglobin is a serum protein that functions as an antioxidant by virtue of its ability to bind to hemoglobin [(28)] and thereby to prevent the oxidative tissue damage that may be mediated by free hemoglobin [(29)]. It has already been reported that the haptoglobin level gets depleted in various haemolytic anemia [(30, 31)] which we observe in Eβ thalassemia too.

We further have 2 proteins which act as serine protease inhibitors, namely α-1-antitrypsin and α-1 microglobulin/ inter α-trypsin inhibitor. α-1-antitrypsin is the inhibitor of serine proteases. Its primary target is elastase, but it also has a moderate affinity for plasmin and thrombin [(32)]. On the other hand, α-1 microglobulin/ inter α-trypsin inhibitor are two

chains of the protein alpha-1-microglobulin/bikunin precursor [33]. A1M binds and degrades heme, is a radical scavenger as well as a reductase [34] whereas Inter-alpha-trypsin inhibitor inhibits trypsin, plasmin, and lysosomal granulocytic elastase [33]

Hypocholesterolemia and lowering plasma apolipoprotein levels with or without hypertriglyceridemia have long been known to be lowered in β thalassemia and SCD [35], [36]. First, anemia, frequently observed in thalassemia is expected to induce the secretion of erythropoietin, which stimulates the differentiation of the erythroid progenitor cells in the bone marrow and promotes their proliferation, leading to a mild erythroid hyperplasia. Now, cell proliferation is associated with an increased requirement for cholesterol, a way to meet these requirements is to increase the expression of the LDL-R (i.e., the number of the LDL-Rs on the cell surface). The second mechanism may be related to the activation of the monocyte/macrophage system in various districts of the body. It is well established that chronic inflammation causes hypocholesterolemia through a reduction of LDL and, to some extent, HDL [37]. The most abundantly significant of these is apolipoprotein A-I (apoA-I). The protein promotes cholesterol efflux from tissues to the liver for excretion, and it is a cofactor for lecithin cholesterolacyltransferase (LCAT) which is responsible for the formation of most plasma cholesteryl esters [38]. However we have observed an increase in the levels of apoliporpotein A IV in the diseased condition. Apo A IV plays a part in the activation of LCAT and hence also participates in cholesterol efflux [39]. In the general population, low apoA-I and HDLC levels are a risk factor for the development of atherosclerosis, a proliferative vasculopathy primarily affecting the coronary and cerebral vasculature, promoting the risk of myocardial infarction and stroke [40].

The other two proteins showing an upregulation in the diseased condition are adenylate kinase 1 and glutathione s transferase A2. We have already seen that there are proteins which act as hemoglobin scavengers and are responsible for the removal of

hemoglobin from circulation. We have seen a downregulation in such proteins however, adenylate kinase 1 has been reported a marker for the diagnosis of hemolysis [41]. The increase in the level of adenylate kinase in the plasma can be attributed to the erythrocyte adenylate kinase released during hemolysis. Adenylate kinase is always present in small amounts in platelet free plasma, although largely originating from damaged erythrocytes. The aggregation of platelets by adenosine diphosphate (ADP) is thought to play a part in both homeostasis and thrombosis. Adenylate kinase accelerates reversal of the following process:

$$^{2}ADP^{3-} \rightleftharpoons AMP^{2-} + ATP^{4-}$$

Degradation of ADP is responsible for the spontaneous reversal of aggregation [42]. On the other hand, glutathione s transferase A2 has peroxidase activity thereby protecting the cells from reactive oxygen species and the products of peroxidation [43]. Thus the increase of these two proteins indicates that Eβ thalassemic patients are under severe oxidative stress leading to extensive hemolysis and hypercoagulability state.

Finally, iron overload, which maybe the directly or indirectly responsible for all the pathophysiological conditions discussed above, is one of the results of extensive hemolysis which is further aggravated by regular and frequent blood transfusions. Transferrins are iron binding transport proteins which can bind two Fe^{3+} ions in association with the binding of an anion, usually bicarbonate. It is responsible for the transport of iron from sites of absorption and heme degradation to those of storage and utilization [44]. Therefore a downregulation in transferrin level in the plasma is a clear sign of oversaturation of free transferrin.

As most of the de-regulated proteins participate in multiple physiological processes like proteolysis, cargo-transport and iron homeostasis, their de-regulation might enlighten clinical manifestation. Eβ thalassemic patients irrespective of transfusion requirement, are exposed to oxidative stress and hypercoagulable state. Therefore we see decrease in the level

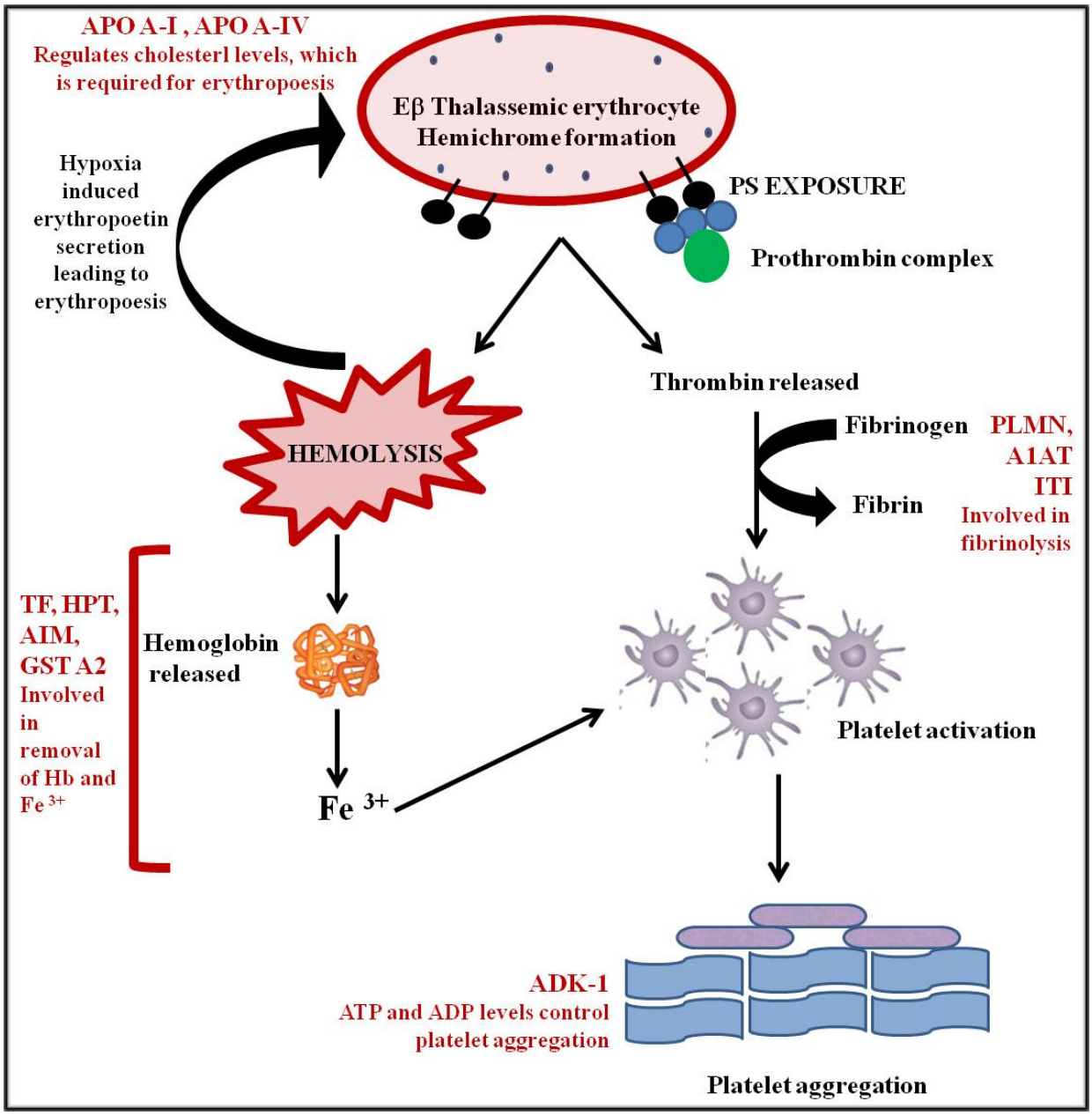


Figure 2.7: Schematic representation of the physiological events in Eβ thalassemia that lead to hypercoagulability and oxidative stress.

TF- transferrin; HPT- haptoglobin; AIM- α 1 microglobulin; GST A2- glutathione S transferase A2; PLMN- plasminogen; A1AT- alpha 1 antitrypsin; ITI- inter α-trypsin inhibitor; ADK-1- adenylate kinase 1; APO AI- apolipoprotein A I; APO A IV- apolipoprotein A IV.

of proteins such as haptoglobin, transferrin, apolipoprotein A1, α-1 microglobulin/ inter α-trypsin inhibitor and plasminogen (Fig 2.7). Those who have chronic anemia and are transfusion dependent undergo severe hemolysis, iron overload, hypercoagulability, thrombolytic events. Therefore the changes so observed in proteins such as haptoglobin and adenylate kinase 1, changes according to the severity and transfusion dependency of the patients [45].

These 10 proteins if studied in greater details could be used collectively to monitor the condition of a patient and determining the effect of transfusion.

2.5 CONCLUSION

Transfusion therapy, which is the mainstay of treatment, allows for normal growth and development and suppresses ineffective erythropoiesis for thalassemic patients. While blood transfusion has the advantage of prolonging the patient's life, it causes iron over-load. Iron overload results both from transfusional hemosiderosis and excess gastrointestinal iron absorption. Iron deposition in the heart, liver, and multiple endocrine glands results in severe damage to these organs, with variable endocrine organ failure [46]. Iron removal in transfusional iron overload is achieved using chelation therapy with chelating drugs like Desferrioxamine (DF) and Deferiprone (L1). However, it is estimated that less than 10% of patients requiring iron chelation therapy worldwide are able to receive Desferrioxamine, because of its high cost, low compliance of patients, and toxicity in some cases.

Besides blood transfusion, another curative option is the use of haemopoietic stem cell transplantation. The sources of stem cells include bone marrow (compatible sibling or matched unrelated donor), cord blood (sibling or cord blood registry) and peripheral blood (sibling or unrelated donor). As families with Thalassaemia tend to have fewer children, the

chances of obtaining a normal and compatible sibling donor is minimal. However, stem cell transplantation is recommended for patients with compatible sibling donors.[47]

Due to the limitations mentioned disease management and monitoring is of prime importance. We believe that the 10 proteins reported in this study can provide us the platform to establish a way to monitor the patients' condition and transfusion requirements. As the proteins reported are abundant in the plasma of any individual, developing methods to monitor such proteins might prove to be a little less complicated. Monitoring based on collective protein levels should be more reliable than the present methods such as ferrintin measurements and MRI of the liver etc.

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CHAPTER III

URINE PROTEOMICS OF Eβ THALASSEMIA

3.1 INTRODUCTION

Urine is an easily accessible body fluid and plays an important role in clinical diagnostics. Urine may contain information not only from the kidney and the urinary tract but also from more distant organs via plasma obtained by glomerular filtration. In healthy individuals, 70% of the urinary proteome originates from the kidney and the urinary tract, whereas the remaining 30% represents proteins filtered by the glomerulus [(1)]. Hence, urine can be viewed as modified ultrafiltrate of plasma and proteins derived from the organs involved in its production and excretion (kidney and urinary tract), with protein concentration approximately 1000-fold lower than in plasma itself [(2)].

Several properties make urine a favourable source for proteomic biomarkers: 1) Urine can be obtained in large quantities using non-invasive procedures and trained personnel are not required for its collection. Due to the non-invasive nature of the procedures, patients are generally willing to donate samples on consecutive occasions; hence, urine is perfectly suited for non-invasive monitoring of disease progression or response to a therapy. 2) Urine contains proteins and peptides of low molecular weight which can be analyzed by avoiding excessive manipulation, even tryptic digestion. 3) Urine is a highly stable body fluid, probably because it is stored for hours in the bladder; hence proteolytic degradation by endogenous proteases is essentially complete at the time of voiding. The urinary proteome does not change fundamentally if urine is stored for several years at −20 °C [(3)] and the urine protein profile does not change significantly when stored for 3 days at 4 °C or after repeated freeze–thaw cycles [(4)].

Even though the urinary proteome is much less complex than the plasma proteome, it should not be underestimated. The report by Kentsis et al. constitutes the largest comprehensive catalog of urine proteins reported to date, with 2362 proteins identified by

extensive fractionation of the urine proteome using ultracentrifugation, gel electrophoresis, ion exchange, and reverse-phase chromatography coupled to high resolution MS [(5)].

We have already discussed the effects that can be seen in the plasma proteome in the previous chapter. Iron overload causes most of the mortality and morbidity associated with thalassemia. Both transfusional iron overload and excess gastrointestinal absorption are contributory. Accurate, preferably non-invasive measurement of iron stores is crucial for the evaluation and management of chelation therapy [(6)]. The recent methods followed for measurement of the body iron load are as follows (discussed in details in chapter I):

- Ferritin level
- Transferrin saturation (TSAT)
- Liver biopsy
- Superconducting quantum interference device (SQUID)
- Computed tomography (CT)
- Magnetic Resonance Imaging (MRI)

Therefore aggressive monitoring of body iron burden is the key to the survival and well being of chronically transfused patients. Here we have studied the urine proteome of Eβ thalassemic patients undergoing regular blood transfusions and compared them with the urine proteome of normal individuals for the better understanding of the pathophysiological changes. Urine being easily obtainable, this study might help to build a platform for developing a monitoring method for the heavily transfused patients.

3.2 MATERIALS AND METHODS

SAMPLES

Urine samples were taken from Eβ thalassemic patients undergoing regular blood transfusions (n=8). These patients were transfusion dependent to maintain a haemoglobin level of 8 to 9 g/dl. The sample details are given in the table below (table 3.1). Normal samples (n=4) were collected from healthy volunteers within our institute. Written consent was taken from adults and in case of children it was taken from the parents as per guidelines of institutional ethical committee.

Table 3.1: Sample details

PATIENT	CONDITION	AGE	GENDER	Hb F	Hb A	HbA_2/ HbE
NU_1	Normal	25 years	Female	0.6%	87.7%	2.7%
NU_2	Normal	27years	Male	0.4%	88.1%	2.6%
NU_3	Normal	28years	Male	0%	88.1%	2.5%
NU_4	Normal	27 years	Female	0.1%	87.5%	2.5%
EβU_1	HbEβ thalassemia	6 years 6 months	Male	68.4%	0.7%	27.5%
EβU_2	HbEβ thalassemia	27 years	Female	21.8%	3.2%	81.7%
EβU_3	HbEβ thalassemia	25 years	Female	10.1%	3.9%	81%
EβU_4	HbEβ thalassemia	8 years	Male	1.7%	2.1%	90.3%
EβU_5	HbEβ thalassemia	8 years	Male	12.3%	5.9%	72.0%
EβU_6	HbEβ thalassemia	22 years	Male	1.6%	6.8%	85.2%
EβU_7	HbEβ thalassemia	13 years	Male	58.6%	3.4%	45.5%
EβU_8	HbEβ thalassemia	6 years 6 months	Male	68.4%	0.7%	27.5%

The handling of all human blood samples was carried out in accordance with the guidelines established by the Local Ethical Committee. For transfusion dependent patients peripheral blood was taken after transfusion gap of minimum 45 days gap.

SAMPLE PREPARATION

Urine samples (~25ml) were centrifuged to remove cellular debris [7]. The supernatant was then centrifuged in Amicon ultra centrifugal filter units with 5kDa cut off membrane concentrated to a volume of 2ml and then proteins were precipitated using 75% ethanol. The precipitated proteins were directly solubilised in 2D rehydration buffer.

2DGE OF THE URINE SAMPLES

After dialysis the samples protein estimation was done using Bradford protein assay reagent (Bio-Rad, Hercules, CA). 17cm pH 3-10 IPG strips (Bio-Rad) were passively rehydrated with the plasma samples. Isoelectric focussing (IEF) was carried out in a Protean IEF cell (Bio-Rad). Equilibration of the IPG strips after IEF was performed following the published protocol [8]. The second dimension was run on 8-16% acrylamide gradient gels in a Protean II XL electrophoresis module (Bio-Rad). According to the total protein load gels were stained with either silver (600-900µg) following the method of Rabilloud[9], SYPRO-RUBY (~1.2mg) or Blue Silver Coomassie (~1.8mg) [10]. Image was taken and densitometric analysis was done on Versa doc series 3000 imaging system using PDQuest software (version 7.1, Bio-Rad). Densitometry analysis of the gel spots of interest was performed using the density tool of PDQuest. Spot volume (density) of the desired spot(s) was normalized as parts

per million (ppm) of the total spot volume using the spots that were present in all the gels, to calculate the relative density of the spot in the sample.

IN-GEL TRYPTIC DIGESTION AND MASS SPECTROMETRY

Protein spots from gels, stained with Coomassie and Sypro-Ruby from normal and diseased samples, were excised. Spots were destained followed by in-gel tryptic digestion. Peptides were then extracted and run on a MALDI ToF/ToF (AB 4700, Applied Biosystems) following published protocol [(11)]. GPS explorer V3.6 was used to generate the peak list using MS and MS/MS data as discussed in the earlier chapter. The data was searched against human MSDB and Swiss-Prot databases using MASCOT V2.1 (Matrix Science) server and MOWSE score (with $p<0.05$) was considered to determine significant hits. For homologous proteins having similar MOWSE scores, preference was given to the protein with best match between theoretical and experimental molecular weights and pI. All MS experiments were repeated atleast 3 times with spot excised from different gels from different samples. The database search parameters included one missed cleavage, error tolerance of ±100 ppm for PMF and ± 1.2 Da for MS/MS ion search and variable modifications like carbamidomethyl cysteine, methionine oxidation and N-terminal acetylation.

3.3 RESULTS

IDENTIFICATION OF PLASMA PROTEINS BY TANDEM MASS SPECTROMETRY

From coomassie and SYPRO-RUBY stained 2D gels of normal plasma, we have identified around 150 protein spots out of which a total of 44 unique proteins were identified

by performing MS+MS/MS combined searches as elaborated in Figure 3.1 and Table 3.2. All MS and MS/MS spectra together with a detailed description of the identified proteins/peptides are provided as supplementary.

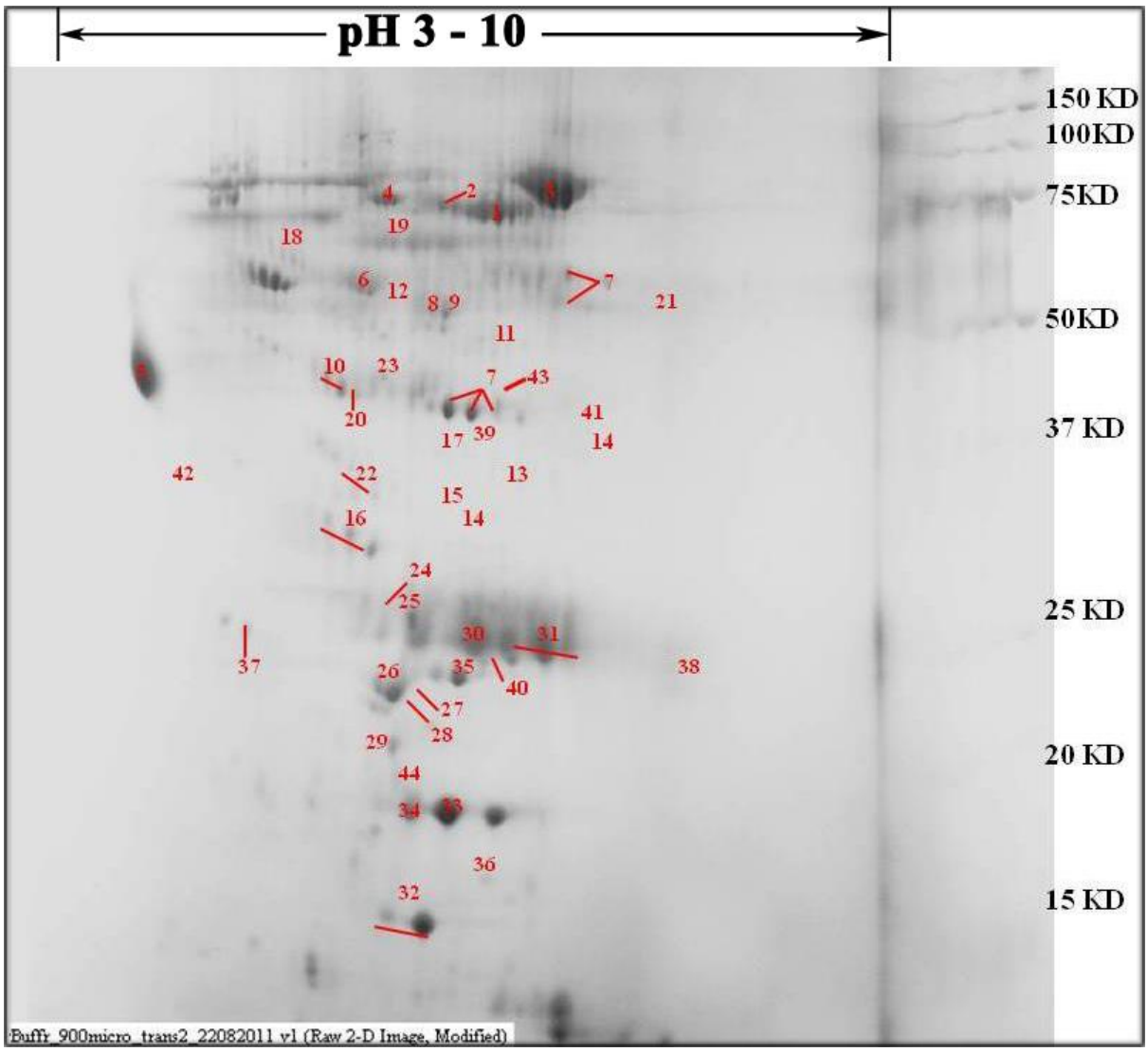

Figure 3.1: representative 2D gel of a normal urine sample stained with Blue Silver Coomassie with the 44 unique proteins marked marked by numbers 1-44. The details of each protein are given in the following table.

Table 3.2: Protein annotations

Spot no.	Name of the protein/ polypeptide	Accession ID	M. wt. pI	Score	Sequence coverage	Number of MSMS match
1	SERUM ALBUMIN, CHAIN A, HUMAN	1A06A	65695D 5.63	434 (64)	47%	8
2	HUMAN HEMOPEXIN	AAA52704	51512D 6.57	165 (64)	38%	4
3	TRANSFERRIN PRECURSOR	Q53H26	77000D 6	240 (64)	51%	7
4	ALPHA 1 B GLYCOPROTEIN	OMHU1B	51908D 5.65	305 (64)	27%	6
5	ALPHA -1-ACID GLYCOPROTEIN 1 PRECURSOR	OMHU1	23497D 4.93	295 (64)	42%	5
6	ALPHA-1-ANTITRYPSIN	Q53H26	46678D 5.51	206 (64)	39%	6
7	FIBRINOGEN BETA CHAIN PRECURSOR	FGHUB	55892D 8.54	545 (64)	47%	9
8	FIBRINOGEN GAMMA A CHAIN PRECURSOR	FGHUG	49465D 5.7	124 (64)	36%	3

9	HOMO SAPIENS MAP 4q28 FIBRINOGEN ALTERNATIVE SPLICE PRODUCT	AAB59531	49450D 5.61	130 (64)	36%	6
10	ZINC ALPHA-2 GLYCOPROTEIN, CHAIN A – HUMAN	1ZAGA	31539D 5.7	380 (64)	44%	7
11	HUMAN FIBRINOGEN	AAB59531	49450D 5.61	376 (64)	41%	6
12	ALB PROTEIN (GROWTH INHIBITING PROTEIN 20)	Q86YG0_ HUMAN	47330D 5.97	218	37%	6
13	FIBRINOGEN FRAGMENT D, CHAINB – HUMAN	1FZAB	35875D 7.66	326 (64)	68%	8
13	FIBRINOGEN FRAGMENT D, CHAINB – HUMAN	1FZAB	35875D 7.66	326 (64)	68%	8
14	TRANSFERRIN N_TERMINAL LOBE, CHAIN C	1BP5C	36832D 6.54	75 (64)	41%	2
15	TRANSTHYRETIN (also called prealbumin) COMPLEX WITH 3',5'-DIBROMO-	1THCA	12937D 6.08	307 (64)	67%	5

	2',4,4',6-TETRA-HYDROXYAURONE, CH					
16	ALPHA-1-MICROGLOBULIN/INTER-ALPHA-TRYPSIN INHIBITOR PRECURSOR	HCHU	38974D 5.95	175 (64)	43%	8
17	HUMAN APOA IV	AAA51748	43358D 5.22	231 (64)	55%	7
18	KININOGEN 1 VARIANT. - HOMO SAPIENS (HUMAN).	Q53EQ0_ HUMAN	47823D 6.29	78 (64)	41%	2
19	VINCULIN	VINC_HUMAN	123591D 5.51	159 (64)	34%	5
20	ZINC-ALPHA-2-GLYCOPROTEIN, CHAIN D	1ZAGD	30547D 6.03	157 (64)	42%	4
21	IMMUNOGLOBULIN LAMBDA HEAVY CHAIN PRECURSOR	CAA75032	52694D 8.74	65 (64)	25%	3
22	INTER-ALPHA-TRYPSIN INHIBITOR HEAVY CHAIN RELATED PROTEIN PRECURSOR_HUMAN	JX0368	103321D 6.5	117 (64)	19%	5

23	APOLIPOPROTEIN A IV PRECURSOR	LPHUA4	45307D 5.23	297 (64)	58%	7
24	PROSTAGLANDIN D2 SYNTHASE 21KD (BRAIN) (FRAGMENT)	A44455	22821D 9.92	96 (64)	28%	3
25	Ig KAPPA CHAIN V-C REGION (Fab fragment 3D6), CHAIN L	1DFBL	23233D 8.24	110 (64)	47%	2
26	APOA1 PROTEIN FRAGMENT	CAA00975	28061D 5.27	335 (64)	74%	7
27	PERLECAN PRECURSOR	A38096	468525D 6.05	253 (64)	9%	6
28	PEROXIREDOXIN 2	PRDX2_HUMAN	21747D 5.67	92 (64)	27%	1
29	RETINOL BINDING PROTEIN, CHAIN E	1QABE	20403D 4.94	143 (64)	45%	4
30	IMMUNOGLOBULIN KAPPA LIGHT CHAIN VLJ REGION (FRAGMENT)	BAC01703	29465D 6.73	140 (64)	30%	3
31	Ig KAPPA CHAIN NIG26, PRECURSOR	JE0204	23504D 5.46	128 (64)	32%	3

32	HAPTOGLOBIN ALPHA AND BETA CHAIN (FRAGMENT)	CAA25248	41499D 6.25	67 (64)	32%	2
33	TRANSTHYRETIN, CHAIN A	1BM7A	12470D 5.26	99 (64)	42%	1
34	TRANSTHYRETIN, CHAIN B	2ROYB	13026D 5.26	64 (64)	73%	2
35	HSU22961 NID: - HOMO SAPIENS Human mRNA clone with similarity to L-glycerol-3-phosphate:NAD oxidoreductase and albumin gene sequences	AAA64922	52048D 5.69	93 (64)	32%	4
36	COMPLEMENT C3 PRECURSOR		187046D 6.02	109 (64)	21%	4
37	BETA GLOBIN CHAIN (FRAGMENT)	Q4TWB7	11480D 5.9	148 (64)	73%	4
38	HEMOGLOBIN BETA SUBUNIT VARIANT_HOMO SAPIENS	AAF00489	15972D 6.75	151 (64)	76%	6
39	ALPHA 2 HS	WOHU	39300D	158	40%	5

	GLYCOPROTEIN, CHAIN A		5.43	(64)		
40	HUMAN ALPHA FETOPROTEIN	E973181	66358D 5.67	106 (64)	41%	3
41	TRANSFERRIN N_TERMINAL LOBE, CHAIN C	1BP5C	36832D 6.54	75 (64)	41%	2
42.	GP3 6B GLYCOPROTEIN	AK222609	40222D 6.6	89 (64)	14%	2
43	GELSOLIN PRECURSOR, PLASMA	FAHUP	85644D 5.9	80 (64)	21%	4
44.	GM2 ACTIVATOR PROTEIN	Q6LB5_HUMAN	20350D 5.17	115 (64)	36%	1

GO Panther analysis was using PANTHER classification system database [12, 13] done with the 44 identified proteins and they can be classified into the following molecular functions, biological process they are involved in and the protein classes they belong to as shown in fig. 3.5 A, B and C respectively. We see that the most common molecular functions of these set of proteins are enzymatic activity, catalytic activity and binding. The biological processes the proteins are in cellular processes such as cell communication, cell-cell adhesion, cell - matrix adhesion or transportation such as retinol binding protein, hemopexin and transthyretin involved in vitamin transport, also proteins such as hemoglin involved in oxygen transport and transferrin in iron transport. Proteins such as alpha 2 HS glycoprotein,

alpha-1-microglobulin/inter-alpha-trypsin inhibitor and alpha 1 antitrypsin are involved in mmetabolic processes. Furthermore proteins such as complement c3, haptoglobin,

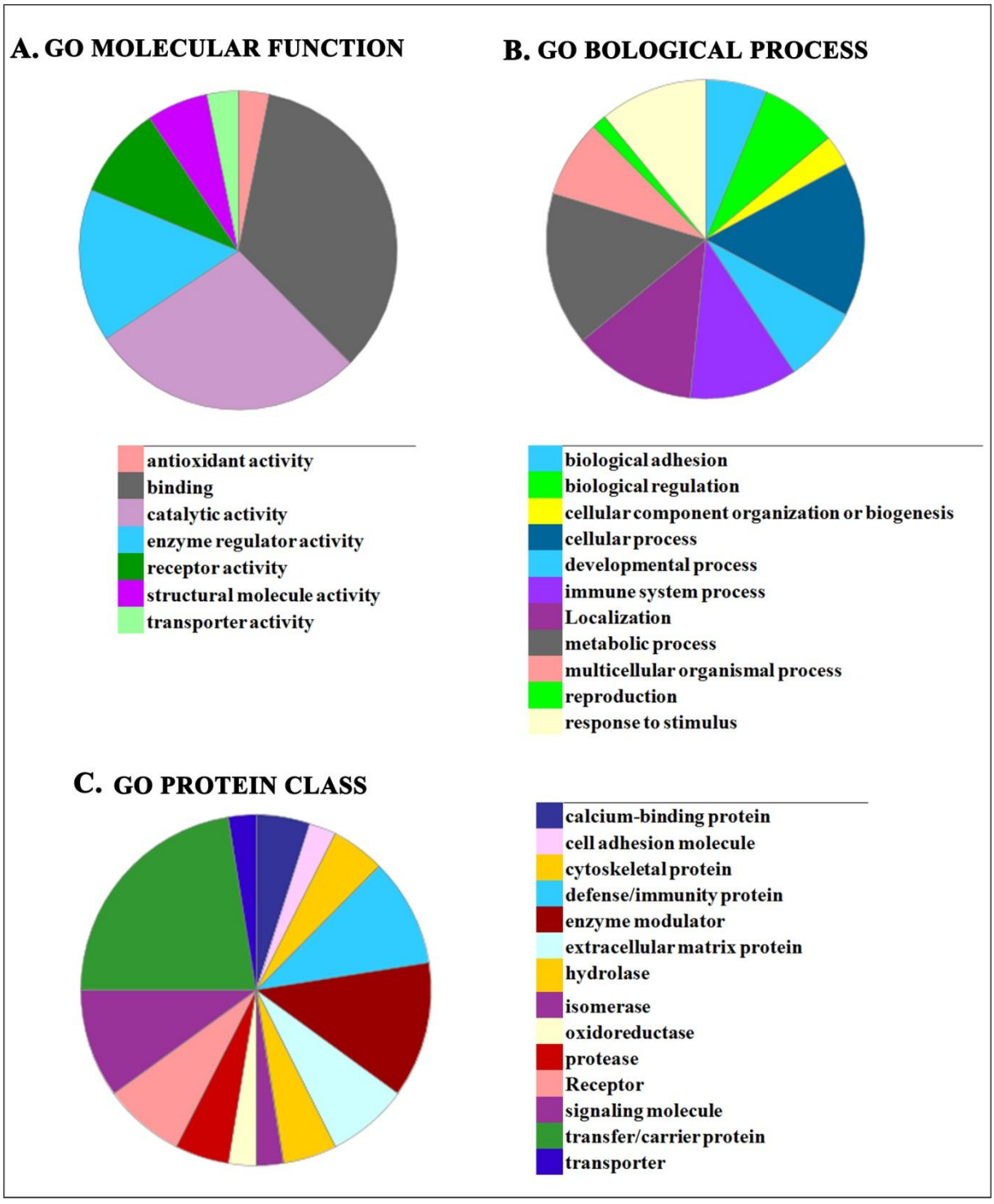

Figure 3.2: GO Panther analysis of the 44 annotated proteins on the basis of molecular function (A), biological process (B) and protein class (C).

immunoglobulins and peroxiredoxin 2 involved in immune response were also identified in the urine samples.

DISPLAY OF DIFFERENTIALLY REGULATED PROTEINS IN Eβ THALASSEMIC SAMPLES

On comparing the normalised intensities of the 44 protein spots of the gels in the two categories (fig 3.3) as stated above 9 proteins have shown differential regulation (inset of fig.3.4). These proteins are the ones showing more than 1.5 fold change in the mean ppm spot volume compared to normal and could be seen atleast in 75% of the gels. However, amongst these 9 proteins the fold change of only 4 proteins were found to be significant ($p \leq 0.05$) (fig 3.4). This is due to the small sample size (normal = 4 and Eβ = 4). Interestingly, the point in the gel of Eβ thalassemic samples where one would expect to find albumin, transferrin and plasminogen were blank (marked in red circle in fig. 3.3).

The four proteins which were showing significant differential regulation are alpha 1B glycoprotein (A1BG), alpha 1 acid glycoprotein (AIAG), alpha 1 antitrypsin (A1AT) and transthyretin chain A (TTR A). Transthyretin as a whole do not show any significant changes however chain A of the transthyretin shows a downregulation.

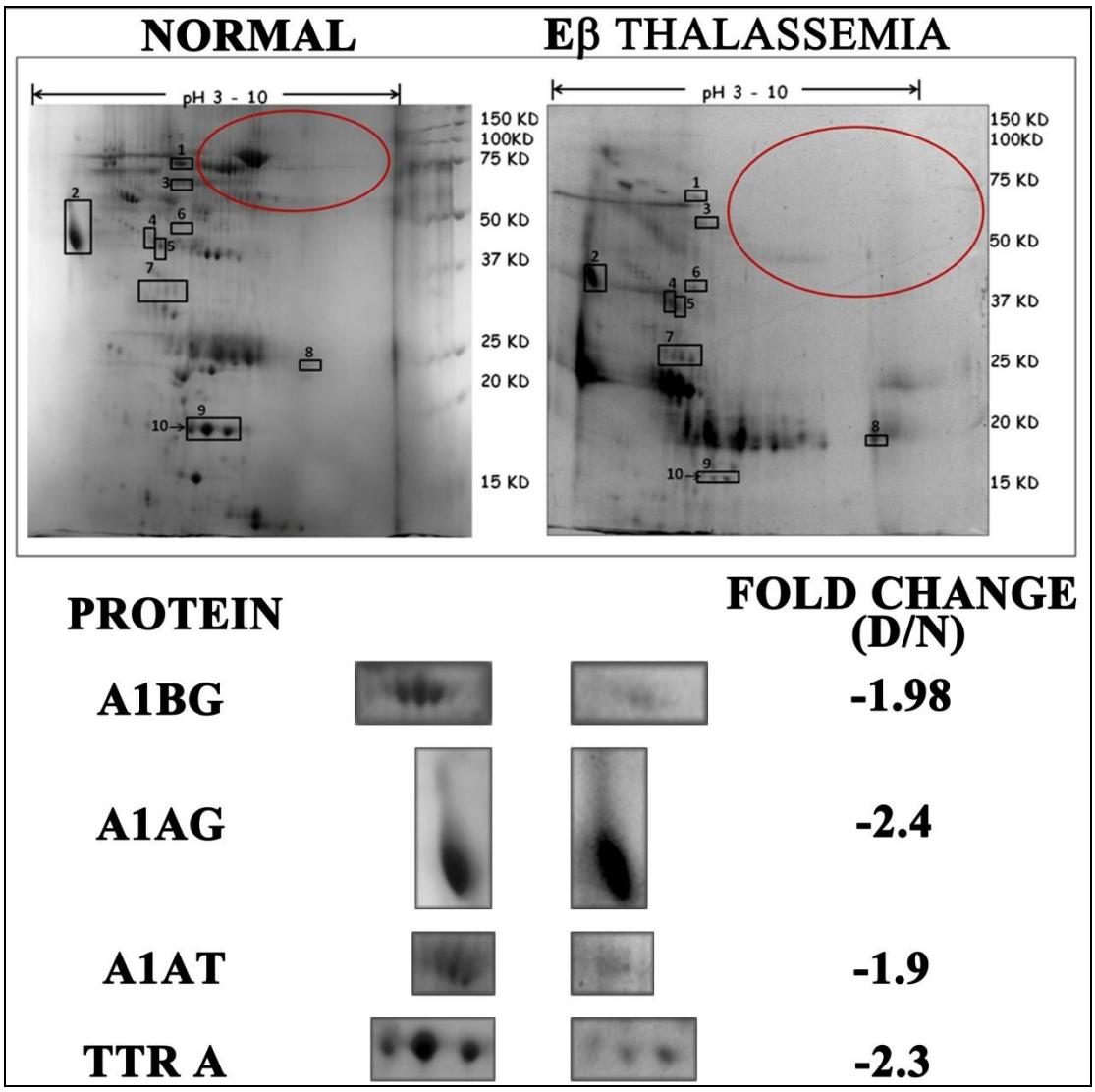

Figure 3.3: representative 2D gels of normal and Eβ thalassemic samples with the nine spots marked. The spots showing statistically significant fold change (normal/ diseased) are shown separately along with their fold change where the negative sign indicates a downregulation in case of diseased condition. The red circles in the two gels indicates the region where the spots are absent in the Eβ thalassemic sample gels.

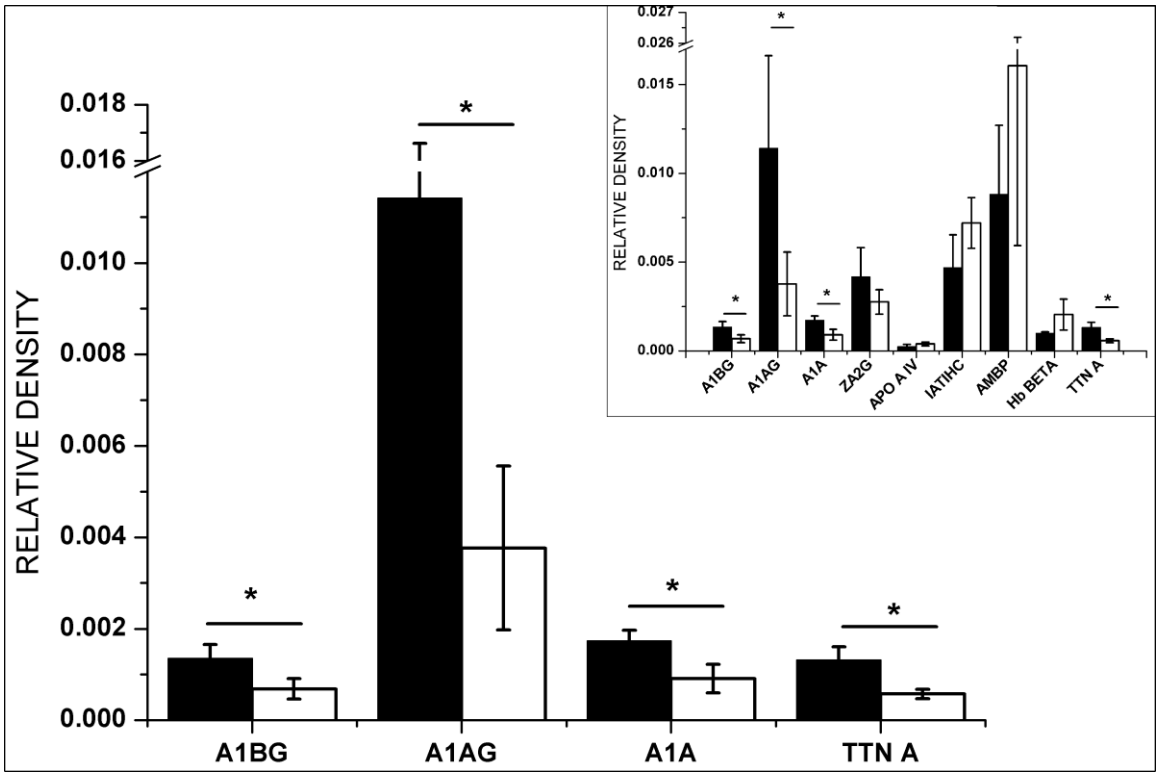

Figure 3.4: bar plot of the 4 proteins showing significant changes in the Eβ thalassemic samples (hollow bars) as compared to normal samples (black bars). Inset shows the bar plot of all 9 proteins which shows a dysregulation in the diseased condition but were not statistically significant.

3.4 DISCUSSION

The human kidney (Fig. 3.5) is composed of 1 million functional units called nephrons, which can be divided in two functional parts: the glomerulus, which filters the plasma yielding the so-called "primitive" urine, and the renal tubule, which reabsorbs most of the primitive urine. More than 99% of this primitive urine is reabsorbed. The remainder (the "final" urine) exits the kidney via the ureter into the bladder. Therefore urine may contain information not only from the kidney and the urinary tract but also from more distant organs via plasma obtained by glomerular filtration. In healthy individuals, 70% of the urinary

proteome originates from the kidney and the urinary tract, whereas the remaining 30% represents proteins filtered by the glomerulus. [1, 14]

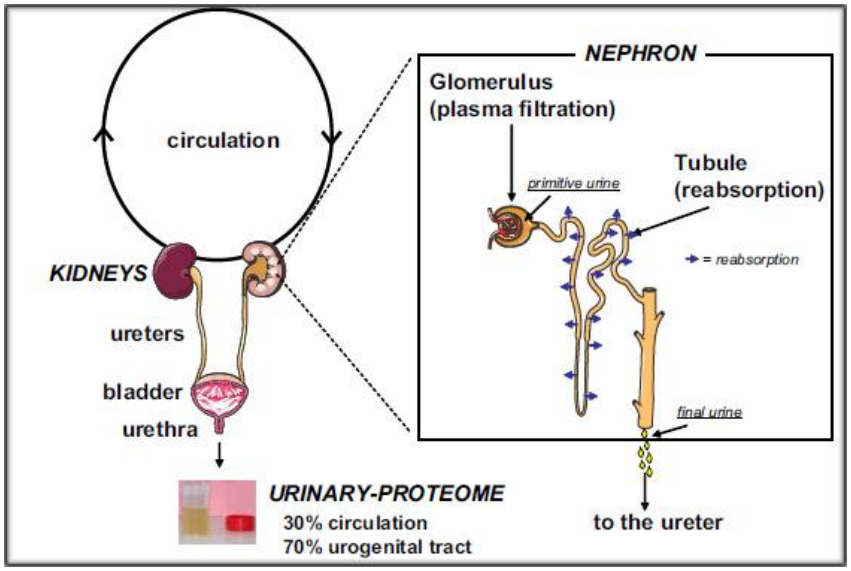

Figure 3.5: Urine production in the kidney. 90% of the "Primitive urine" produced from the glomerulus filtration of the plasma is reabsorbed in the renal tubule [(1)].

Therefore the 9 proteins showing dysregulation in the urine proteome are proteins which are secreted into the plasma. The four proteins which show a statistically significant downregulation in case of Eβ thalassemic samples all secreted into the plasma. In the previous chapter we have already reported a downregulation in A1AT and an upregulation in case of APO AIV. We had also seen a downregulation in case of transferrin and plasminogen in the plasma fractions (Chapter II) and here we see a complete absence of these proteins from the gel of the Eβ thalassemic samples. Therefore we can see that the changes observed in the plasma are manifested in the urine samples as well.

A1BG and A1AG are both plays a part in the immune response [15, 16]. Immune response is likely affected by iron at several levels. Unfortunately, blood transfusions are associated with alloimmunization, risk of exposure to infectious pathogens, and the accumulation of iron. Iron has specific effects on both the adaptive and the innate immune systems [17-19]. Iron is unique among metals because of its central role in oxygen transport.

However, excess iron also promotes microbial growth and can suppress immune function [20]. Significant immunologic abnormalities have been observed in thalassemia in relationship to the number of lifetime transfusions and iron overload [21, 22]. A1BG belongs to the immunoglobulin receptor family and is involved in activation of natural killer cells and cell-cell signalling [23]. A1AG is expressed in the liver and secreted in to the plasma and its synthesis is controlled by glucocorticoids, interleukin-1 and interleukin-6. It appears to function in modulating the activity of the immune system during the acute-phase reaction [16]. The downregulation of these 2 proteins can be due to either of the two possibilities – 1) the synthesis of these proteins are downregulated. However we did not find any such downregulation in the proteomic study of the plasma samples in chapter II (although majority of the samples were either transfusion independent or required transfusion after long gaps). Furthermore upregulation of pro inflammatory cytokines have been reported in case of thalassemia [24],which in turn might increase the level of A1AG. Therefore, the other possibility is 2) that these patients might be producing normal amounts of these proteins which are maximally getting used up and hence we see a downregulation in the urine samples.

Vitamins and trace minerals represent key buffers against oxidative damage. However, chronic demands on oxidative buffering capacity may produce conditional deficiencies in key amino acids and enzymatic cofactors. Fat soluble vitamin A is significantly decreased in thalassemic patients due to high consumption as antioxidants against iron overload with subsequent lipid peroxidation [25]. Transport of vitamin A to the target cells is mediated by the retinol-binding protein. In plasma, RBP is found in a complex with its carrier protein Transthyretin (TTR) [26]. Binding of RBP to TTR was thought to prevent its glomerular filtration, since the apparent molecular mass of this protein–protein complex increases to 76 kDa [27]. Furthermore TTR is a 55-kDa homotetrameric protein

involved in the transport of T_4 in the plasma. Thyroid hormones are needed for normal development and metabolism. Nevertheless, TTR serves only a backup role in the transport of T4 in the human plasma, as TBG is the principal carrier of thyroid hormones [28, 29]. Although we did not see a downregulation of the total protein but only a chain of it, therefore the significance of this dysregulation has to be studied further for the understanding of it implications.

Taking into account all the 9 proteins irrespective of their statistical significance we can divide them according to their molecular function (Fig 3.6, A), biological processes (B**)** the proteins are involved and the protein classes (C) they can be divided in are given below.

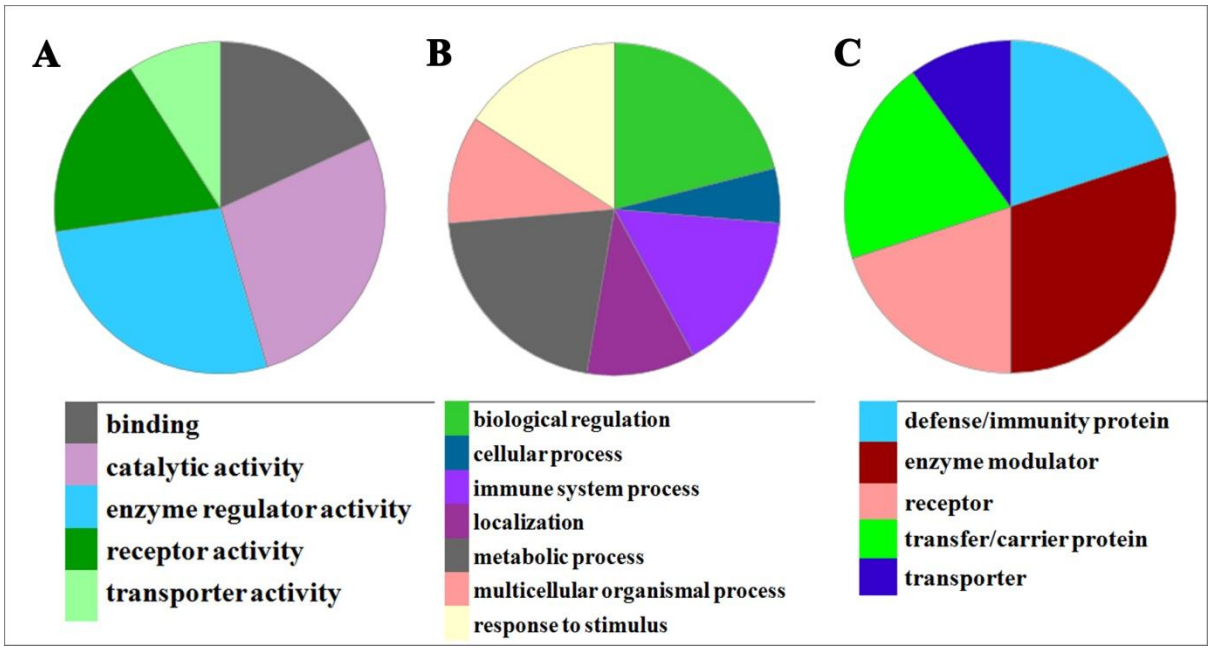

Figure 3.6: GO Panther analysis of the 9 dysregulated proteins on the basis of molecular function (A), biological process (B) and protein class (C).

From the Go Panther analysis we can see that these proteins are involved in immune response, metabolic processes, biological regulation such as catalytic activity, proteolysis etc. However, due to the small sample size, the participation of these proteins in the disease

phenotype cannot be established. We must study these proteins to a greater extent with a wider and larger sample pool to conclude this study.

3.5 CONCLUSION

Due to the lack in the sample size this work could not be completed. More samples need to be processed and the number of proteins analysed (44 in this case) should also be increased. This is a very preliminary data successfully establishes the fact that urine samples do have the promised potential to act as a tool for disease monitoring and management. If a particular protein or a set of proteins can be identified which are differentially regulated in case of transfusion dependent patients then it would help to develop a non-invasive method of monitoring the transfusion requirement of the patients as well as the physiological conditions of the patient who are on regular and frequent transfusions.

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CHAPTER IV

LIPIDOMICS STUDY OF BLOOD PLASMA AND ERYTHROCYTES FROM PATIENTS OF Eβ THALASSEMIA

4.1 INTRODUCTION

Phospholipids are the major building blocks of a cell membrane. They not only provide a permeability barrier to water and solutes but also act as anchors for macromolecules and an interface for cell-cell communication [(1)]. These phospholipids can be divided into specific classes depending upon the polar head groups. The heterogeneity of this mixture is further complicated by the number of individual fatty acyl substituents that are esterified to two additional hydroxyl groups of the glycerol backbone [(2)] . These asymmetric cell membranes maintain a particular composition and organization of the phospholipid population. In stressed conditions this asymmetry is perturbed leading to premature removal of erythrocytes from the circulation by phagocytosis by macrophages of the reticulo-endothelial system [(3)].

Furthermore, due to the presence of double bonds in the lipids they are susceptible to oxidative damage by the reactive oxygen species (ROS). The lipid peroxidation process is a radical reaction that leads to the formation of oxidized intact phospholipids, known as long-chain products, such as hydroxy and/or hydroperoxide derivatives, phospholipids that contain a truncated lipid at the sn-2 position and lysophospholipids or formyl-phospholipids generated on plasmalogen oxidation [(1)],[(4)]. It has already been shown that lipid peroxidation is increased in certain diseases like cancer, rheumatoid arthritis, drug-associated toxicity, and post ischemic reoxygenation injury, as well as in the degenerative processes associated with aging [(5)].

In our study for the first time we are studying the changes in the lipid population that occur in the plasma and erythrocyte membrane in HbEβ thalassemia. Thalassaemias as a whole belongs to the family of autosomal recessive blood disorders which are caused due to mutations in the globin chain gene giving rise to defective globin chains [(6)]. Eβ thalassemia is the most

common form of thalassemia which is prevalent in the Indian subcontinent and south-east Asia. This form is associated with the most common variant of hemoglobin, HbE, caused by the single mutation in β-globin gene (β26 Glu→Lys). HbE does not result in clinical severity, but its combination with β- thalassemia is responsible for the pathophysiological severity of the disease. These patients suffer from varying phenotypes ranging from severe transfusion dependent thalassemia major to thalassemia intermedia that mainly include ineffective erythropoiesis, faster aging and premature hemolysis of the red blood cells [7].

Due to the defective synthesis of the β globin chain there is an accumulation of the α globin chain. The excess α globin chains form unstable homotetramers. These homotetramers precipitate as inclusion bodies and the iron is released leading to the oxidative damage of membrane lipids [6, 7].

Most studies have focused on end products of lipid peroxidation such as aldehydes and volatile hydrocarbons. However, recent focus has shifted to the oxidatively modified lipids as they may have significant biological activities [8]. In our study apart from the lipid populations we have tried to monitor this population of oxidatively modified lipids in the three fractions.

4.2 MATERIALS AND METHODS

SUBJECTS:

Blood samples, collected in EDTA vials (BD Biosciences), from six normal healthy volunteers and six patients of HbEβ thalassemia, diagnosed for the first time who did not receive any blood transfusion, were used for the lipidomic studies (table I). Blood samples were

Table 4.1: sample details.

PATIENT	CONDITION	AGE	GENDER	Hb F	Hb A	HbA_2/ HbE	% PS EXPOSURE
2011_N	normal	38 years	Male	0.6%	87.7%	2.7%	0.62
2011_N1.	normal	28years	Male	0.4%	88.1%	2.6%	1.18
2011_N2	normal	49years	Female	0%	88.1%	2.5%	1.19
2012_N1	normal	26 years	Female	0.1%	87.5%	2.5%	ND
2012_N2	normal	26 years	Male	0.4%	89%	3.1%	ND
2012_N3	normal	26 years	Female	0.3%	88.3%	2.6%	ND
2011_Eβ	HbEβ thalassemia	27years	Female	21.8%	3.2%	81.7%	3.39 %
2011_Eβ1	HbEβ thalassemia	3years 6 months	Female	25.5%	3.3%	70.4%	3.6 %
2011_Eβ2	HbEβ thalassemia	11 months	Male	41.4%	6%	50.9%	3.9 %
2012_Eβ1	HbEβ thalassemia	6 years 6 months	Female	68.4%	0.7%	27.5%	3.11 %
2012_Eβ2	HbEβ thalassemia	7 years	Female	45.8%	3.2%	50.3%	8.45 %
2012_ Eβ3	HbEβ thalassemia	4 years	Female	6%	47.2%	40.7%	4.5 %

obtained from Ramakrishna Mission Seva Pratishthan, with informed written consent following the guidelines of the Institutional Ethical Committee of Vivekananda Institute of Medical Sciences, Ramakrishna Mission Seva Pratishthan, Kolkata 70026, INDIA and Institutional Animal & Bio ethics committee of Saha Institute of Nuclear Physics. The Institutional Ethical Committee of Vivekananda Institute of Medical Sciences, Ramakrishna Mission Seva Pratishthan, and Institutional Animal & Bio ethics committee of Saha Institute of Nuclear Physics have also specifically approved the current study.

ANNEXIN BINDING

Erythrocytes were labeled using FITC Annexin V Apoptosis Detection Kit (BD Biosciences). The erythrocytes were suspended in the buffer provided in the kit to a final concentration of 1×10^6 cells/ml and incubated with FITC-annexin. FITC-annexin binding was measured with respect to unlabeled samples. Flow cytometry of FITC-annexin labeled erythrocytes were performed in FACs caliber flow cytometer (Becton Dickinson). Acquisitions were taken following the protocol described in our previous work [(9)].

LIPID EXTRACTION

The plasma and erythrocytes were separated according to density. Erythrocytes were further lysed and the erythrocyte membranes were taken separately. Lipid extraction was done as described in [(10)]. Briefly, to the plasma, and the erythrocyte membranes 1 mL of MTBE was added and the mixture was vortexed at 20°C for one hour. Then 250 µL of water was added and

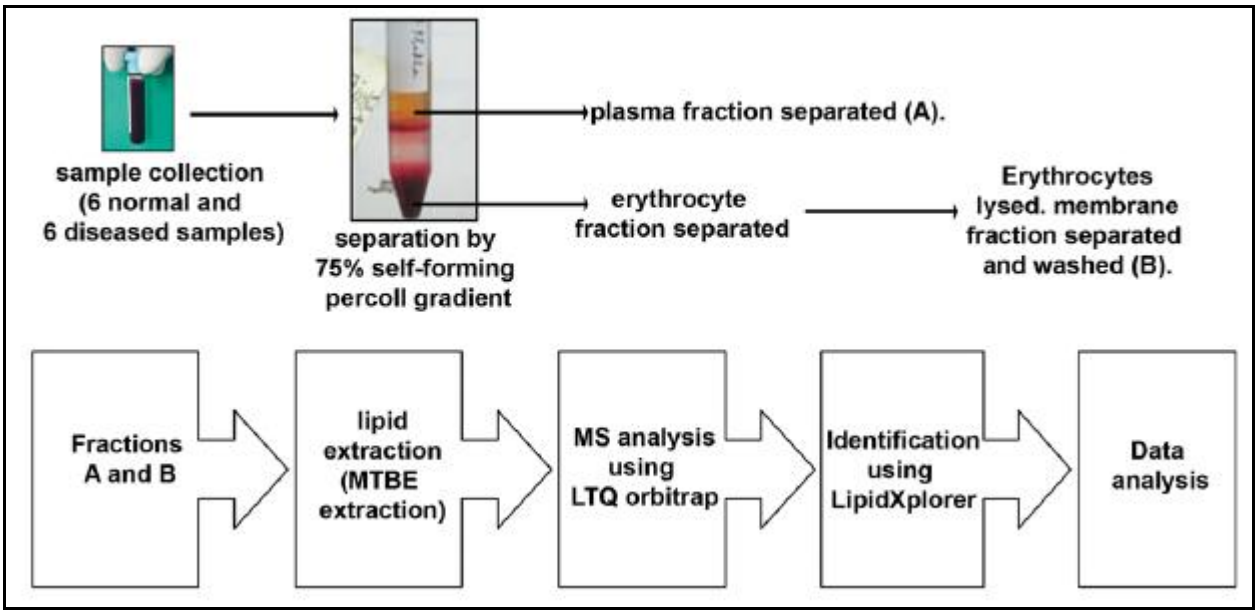

Figure 4.1: A schematic representation of the protocol from lipid extraction to data analysis.

thoroughly vortexed. After centrifuging for 1 minute at 4000 rpm 800 µL of the upper organic phase was transferred into a new vial and stored at -20°C until analysis. For mass spectrometric analysis, tenfold dilutions were made of the extract with $CHCl_3$/MeOH/2-propanol 1/2/4 (v/v/v) containing 7.5 Mm ammonium acetate in 96 well plate and then sealed with aluminum foil. The lipids of the two fractions were then analyzed with a LTQ Orbitrap XL mass spectrometer enabling to perform a lipidomics screen.

MASS SPECTROMETRIC ANALYSIS

Mass spectrometric analysis was performed on a LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a robotic nanoflow ion source using chips with 4.1 mm nozzle diameter. The ion source was controlled by chipsoft 6.4. software (Advion BioSciences) and operated at the ionization voltage of 0.95 kV and gas

pressure 1.25 psi. MS survey scans were acquired in positive and negative ion mode using the Orbitrap analyzer operated under the target mass resolution of 100,000 FWHM (Full Width at Half Maximum). Targeted MS^n experiments were performed using pulsed Q-dissociation (PQD) for positive ion mode and high energy collisional dissociation (HCD) for negative ion mode using the LTQ Orbitrap machine.

LIPID ANALYSIS

The raw data files acquired were converted to *.mzXML format using MS converter from ProteoWizard [(11)]. Mass spectra were further processed by Lipidxplorer software [(12)] and lipids annotated by matching the m/z of their monoisotopic peaks to the corresponding elemental composition constraints using molecular fragmentation query language (mfql) (given in supplementary) [(13)]. Peaks recognized in blank controls with an average intensity more than 0.1 fold compared to the averaged intensities of all the acquisitions for every lipid species were discarded. Further lipid species with less than 50% occupancy were also ignored in the analysis. The mfql for around 19 major lipid classes were used : cholesterol (chol), cholesteryl ester (chol-FA), ceramides (cer in positive mode, cer-adduct in negative ion mode), glucosylceramide (glcCer), diacylglycerides (DAGs), triacylglycerides (TAGs), glycerophospholipid diethers (GPL diethers), lysophosphophatidylcholine (LPC), phosphatidylcholines (PC, PC-O), lysophosphatidylethanolamines (LPE), phosphatidylethanolamines (PE, PE-O), lysophosphatidic acid (LPA), phosphatidic acid (PA), lysophosphatidylinositol (LPI), phosphatidylinositol (PI), phosphatidylserine (PS) and sphingomyelin (SM).

For analyzing the oxidized lipids we have limited our search to the PCs only. From the list of PCs generated after lipidXplorer run as described above we have restricted the search for oxidized PCs to only those containing 14,16,18,20 and 22 fatty acid chains with a maximum of 8 double bonds in the parent PC. The oxidized species covered are the hydroxyl, keto, hydroperoxide, epoxy and polyhydroxy derivatives of PC as long chain oxidized PCs and saturated or unsaturated aldehyde and carboxylic acid derivatives of truncated PCs. After matching the m/z of their monoisotopic peaks to the corresponding elemental composition constraints using mfql (query files in the supplementary 1) and processed as discussed before.

STATISTICAL ANALYSIS

Hierarchical clustering (single linkage) study was done on the entire data set of the two fractions separately using Cluster 3.0 [(14)]. The similarity protein expressions data was measured by correlation (centered). Further PCA was applied to the peak lists produced from all samples analyzed in each fraction separately using XLSTAT (version 2014.3.01) software.

4.3 RESULTS

CHARACTERISTIC OF THE SAMPLE POPULATION

The percentage PS exposure is measured in terms of percentage binding of annexin V – FITC to the exposed PS on the erythrocyte membranes. It was found to be greater in case of the diseased samples as compared to normal samples. This value for the normal was found to be 0.997 (±0.33) whereas for the disease it was found to be 4.49 (±2). This data shows that the

diseased samples taken in this study have a large percentage of erythrocytes with PS externalization.

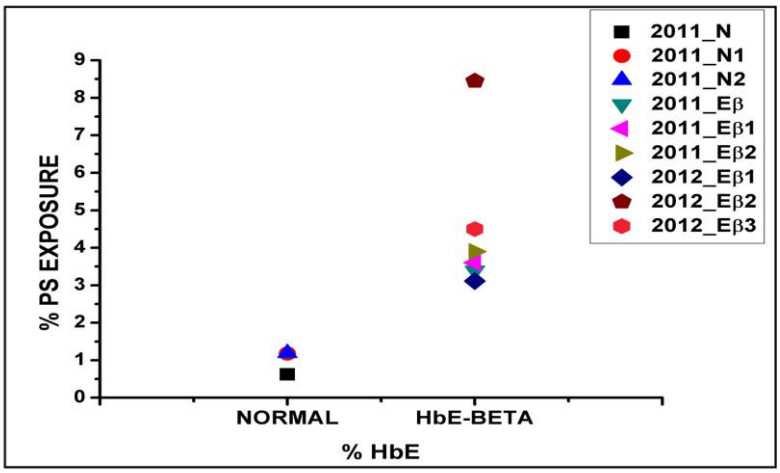

Fig 4.2: Scatter plot representing the percentage PS exposure of the samples.

LIPID IDENTIFICATION

A comprehensive characterization of the major abundant lipid classes was performed leading to the identification of around 260 lipids in total distributed among 19 lipid classes in the plasma, erythrocytes and erythrocyte membrane fractions on combining the lipids identified in the positive as well as the negative ion mode. Representative spectra of the plasma, erythrocyte and erythrocyte membrane fractions are shown in figure 4.3 A, B and C respectively.

In the plasma fractions, as evident from the spectra in positive ion mode (figure 4.3 A) lipids such as TAGs, chol esters, LPCs and PCs are the most intense. Greater amount of LPCs and TAGs species were detected in the plasma fractions as compared to the other two fractions. Cholesteryl esters and DAGs were only detected in the plasma fractions (figure 4.3 D).

Whereas, in case of erythrocyte and erythrocyte membrane fractions PCs, SMs and LPEs are the most intense (Figure 4.3 B, C, E and F).

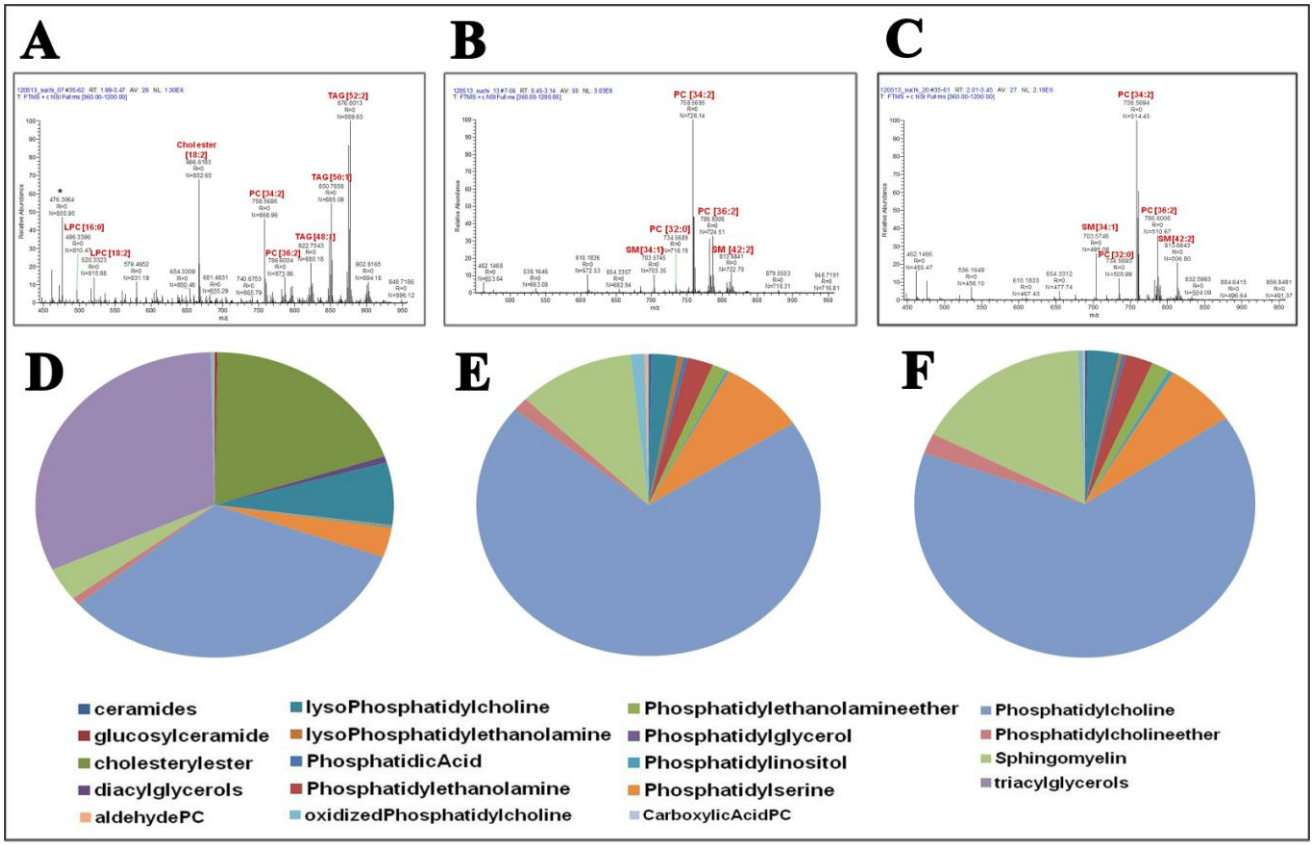

Figure 4.3: A, B and C are the representative spectra of the plasma, erythrocyte and erythrocyte membrane fractions in the positive ion mode respectively. D, E and F are pie charts showing the distribution of the lipid populations in plasma, erythrocyte and erythrocyte membrane respectively.

Amongst the PCs the species PC (34:2) was the most abundant in the three fractions. Cholesteryl ester (18:2) was the most abundant in the plasma. LPCs 16:0 and 18:0 were the major LPCs in all three fractions. In case of LPE, species 20:4 is the most abundant in the erythrocyte and erythrocyte membrane. In case of PI species 38:4 is the most abundant in all three fractions with PI (36:2) being the second abundant in case of plasma. PS (38:4) is the most abundant in the erythrocytes and erythrocyte membrane fractions. SM (34:1) and SM (42:2) being the most abundant in all three fractions.

Clustering studies clearly show that the lipid populations of normal and Eβ thalassemic samples show changes in the lipid levels in the three fractions (Fig 4.4 A, B and C). However the changes observed in the erythrocyte fraction does not clearly indicate the difference between the normal and diseased samples, but the plasma and erythrocyte membrane fraction clearly show the separate clustering of the normal and diseased samples in the figure

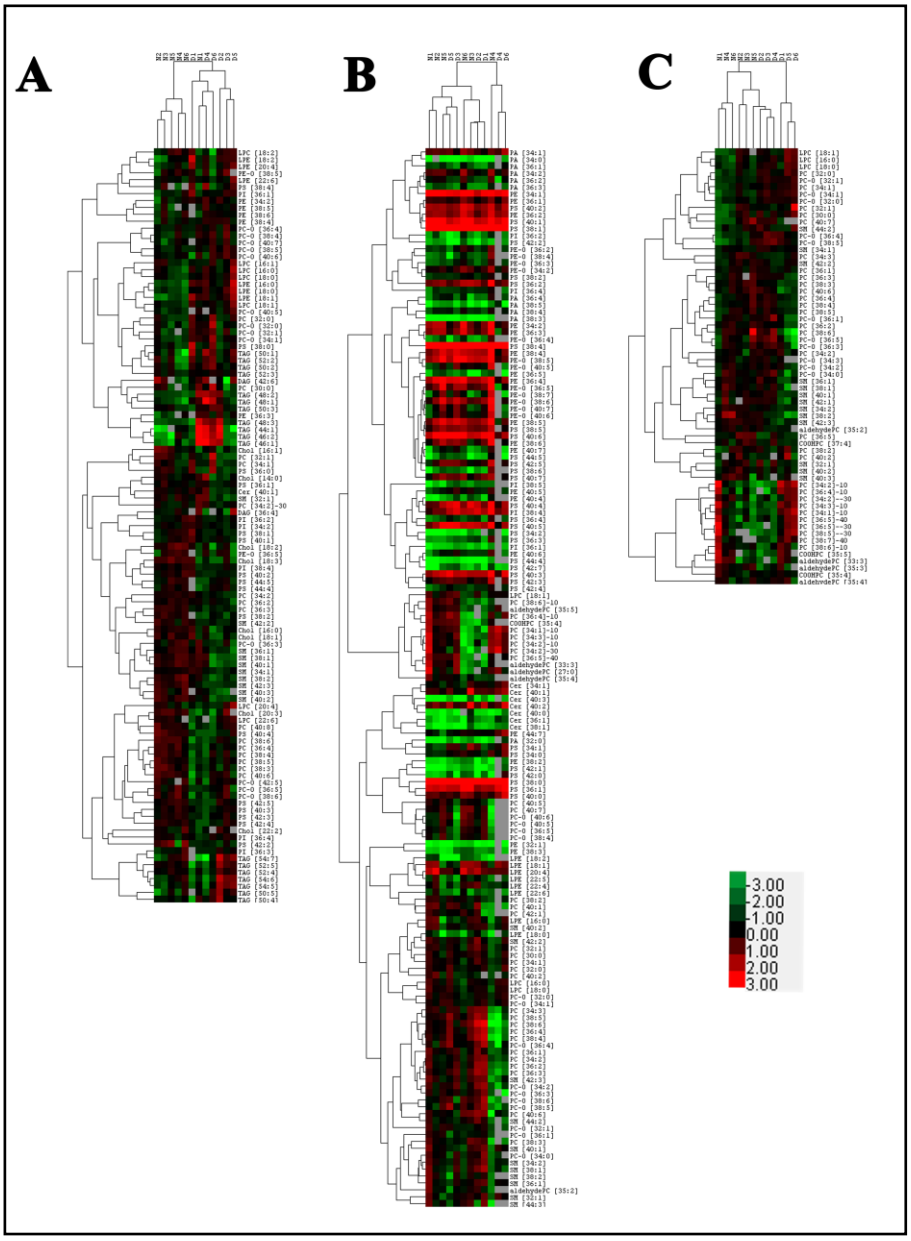

Figure 4.4: *Hierarchical clustering (single linkage) using Cluster 3.0 shows the changes in the level of lipid species in diseased condition with respect to normal (A, B and C) in the three fractions.*

. **PLASMA**

The primary function of plasma is transportation of the blood cells as well as nutrients, hormones, proteins and lipids. Hence, lipids which act as secondary messengers such as ceramides (Fig 4.6 A) and lyophospholipids (LPE and LPC) (Fig 4.6 C) show changes in their levels in case of diseased samples. Ceramides and LPEs show an increase in case of Eβ thalassemia whereas LPCs show a decrease (Fig 4.5 A and C). Phosphatidylcholines (Fig 4.6 D) and sphingomyelins (Fig 4.6 B) show a decrease; however PC-Os (Fig 4.6 E) show an increase in case of the diseased plasma samples. The PCA plots of all the lipid classes are shown in figure 4.5 (A – H) in the next page.

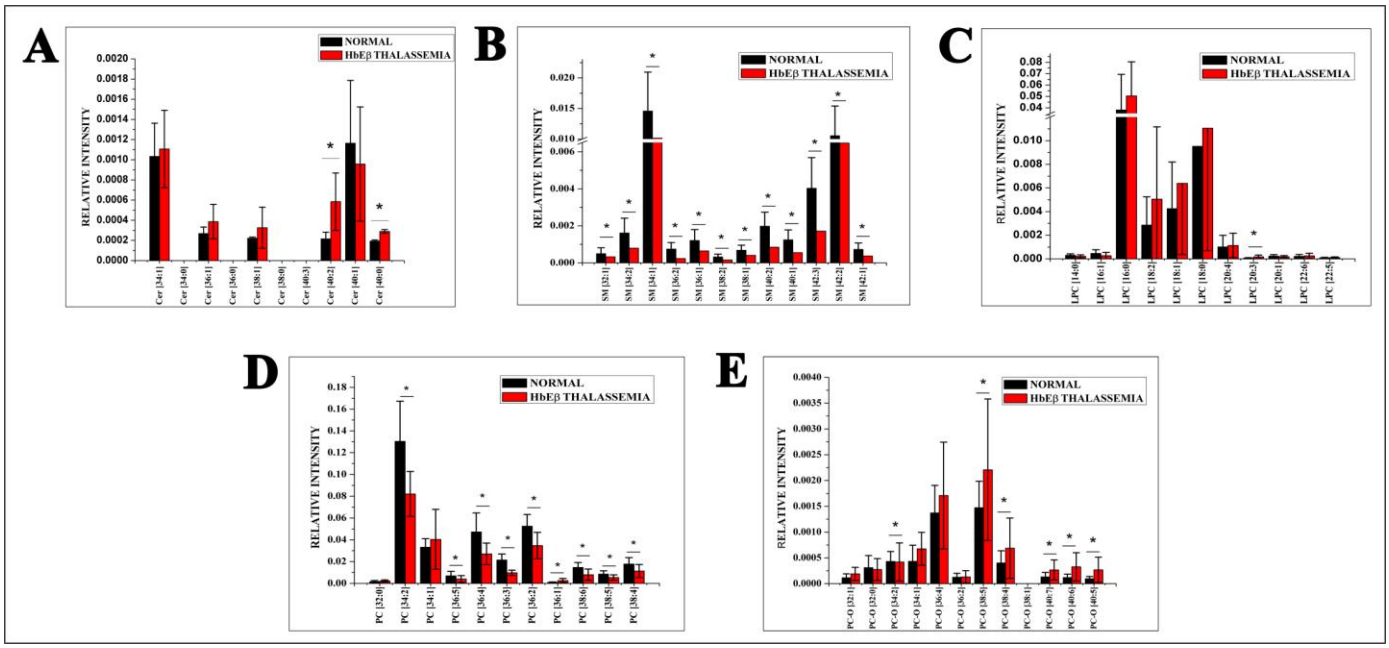

Figure 4.6: Bar plot showing the differential expression of representative lipids of cer (A), SMs (B), LPCs (C), PCs (D) and PC-Os (E) of the plasma fraction.

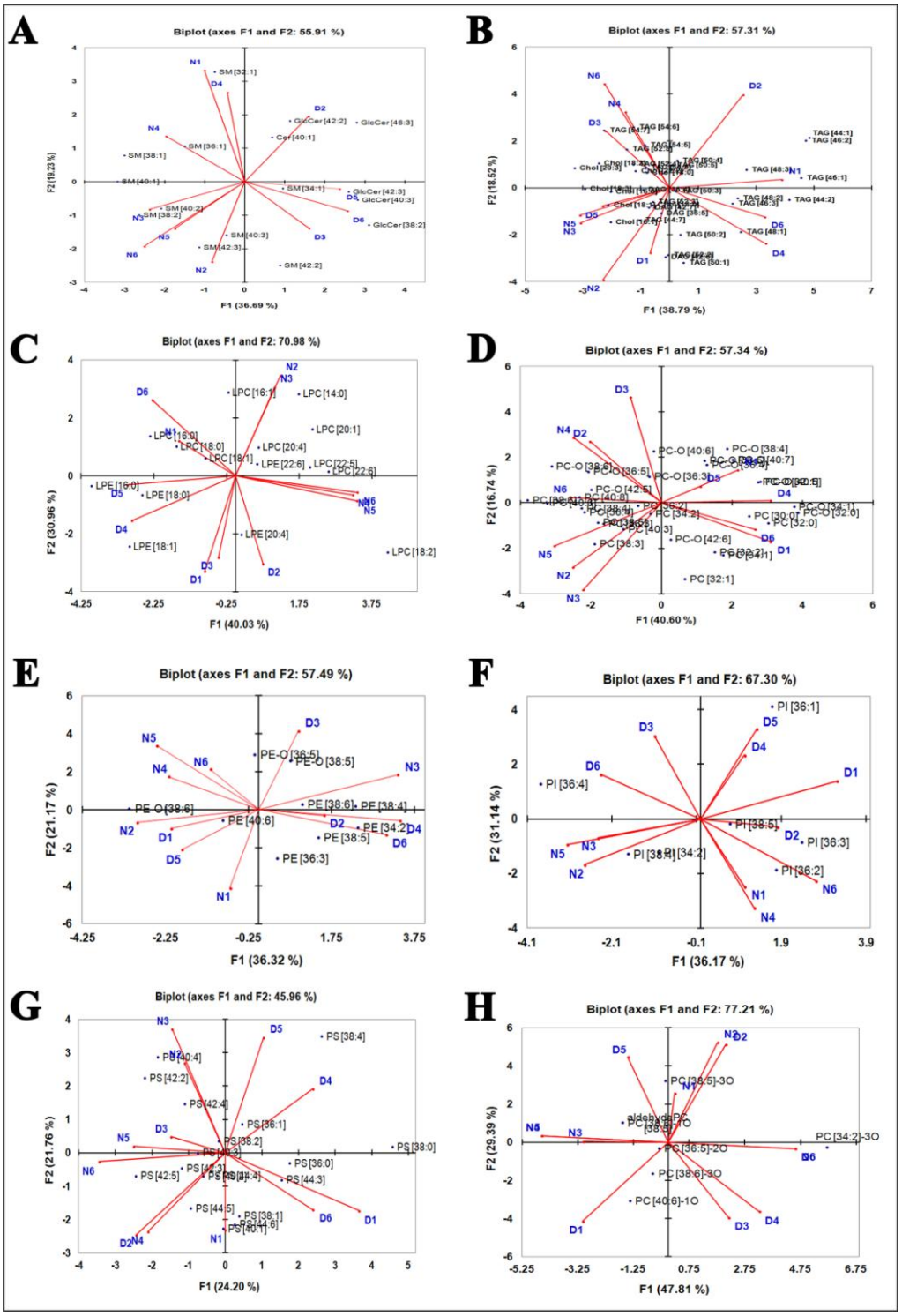

Figure 4.5: A – H PCA analysis of plasma samples of lipid classes Cer and SMs (A), TAGs and DAGs (B), and LPCs and LPEs (C), PCs and PC-Os (D), PEs and PE-Os (E), PI (F), PS (G), oxidized lipids (H).

ERYTHROCYTES

PCA analysis of the erythrocyte fractions did not reveal any change in pattern between the normal and diseased samples (Fig. 4.7). This might be due to some sort of contamination which might have occurred during the separation of the whole erythrocyte fraction from the blood. However the on comparing few representative lipids (Fig 4.8) of each classes shows that PCs and the PC-Os follows the same pattern as described in the plasma fraction and erythrocyte membrane fraction.

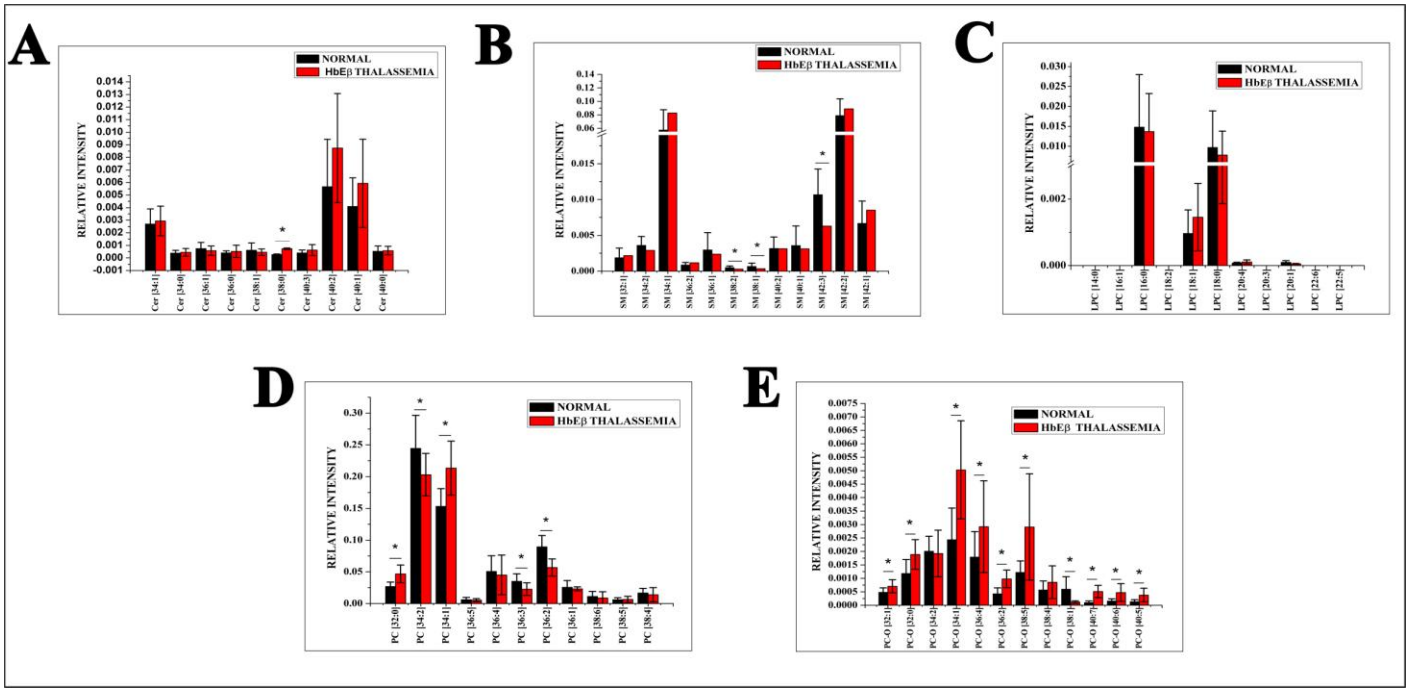

Figure 4.8: *Bar plot showing the differential expression of representative lipids of cer (A), SMs (B), LPCs (C), PCs (D) and PC-Os (E) of the erythrocyte fraction.*

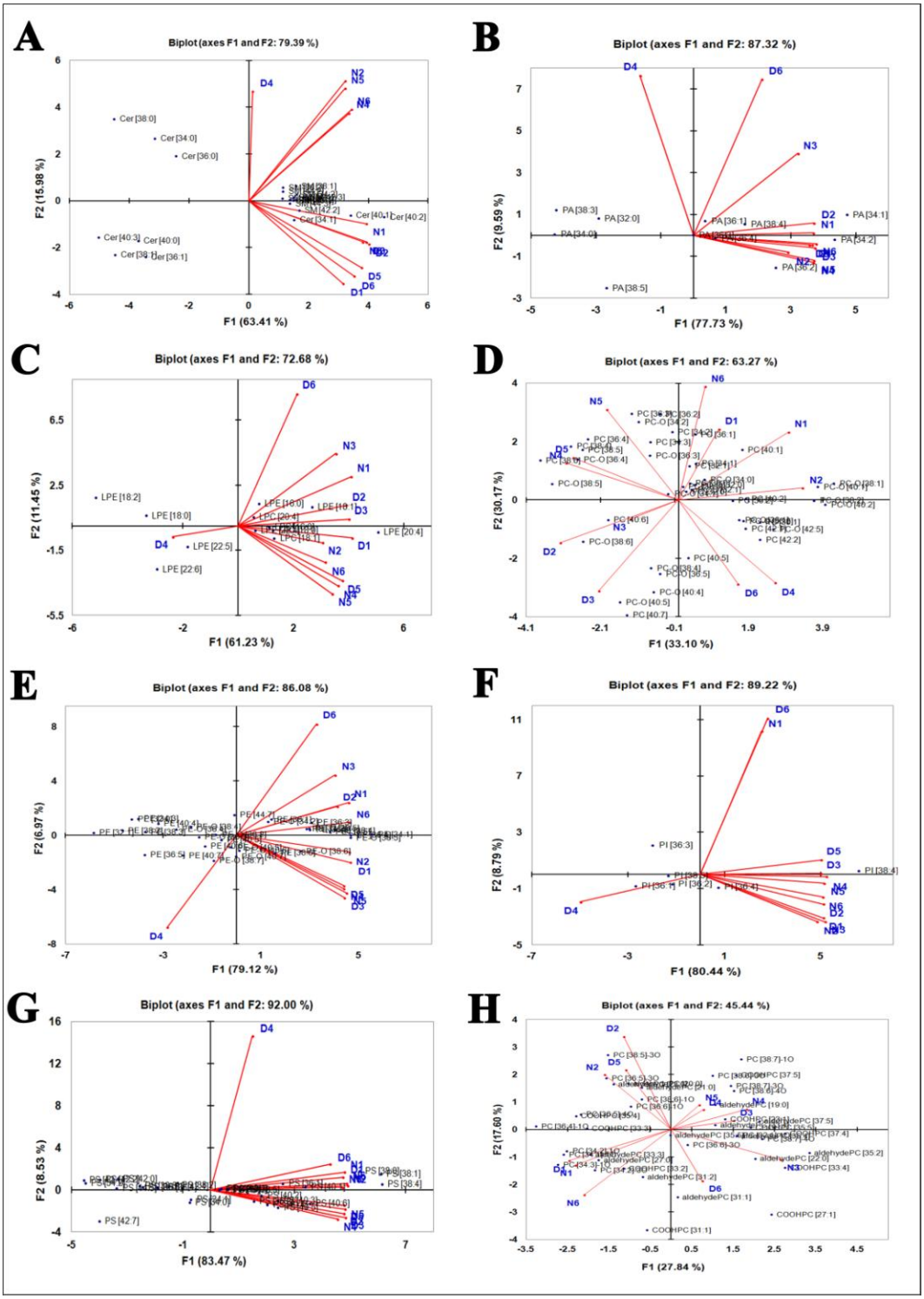

Figure 4.7: A – H PCA analysis of erythrocyte samples of lipid classes Cer and SMs (A), PAs (B), and LPCs and LPEs (C), PCs and PC-Os (D), PEs and PE-Os (E), PI (F), PS (G), oxidized lipids (H).

ERYTHROCYTES MEMBRANE

From our annexin V binding study we have already seen that a huge percentage of erythrocytes are proapoptotic. This can be initiated by the huge oxidative stress that the erythrocyte population has to undergo. Therefore we see an increase in ceramides (Fig 4.10 A) and ethers of PC (PC-O) (Fig 4.10 E), whereas a decrease in case of the sphingomyelins (Fig 4.10 B), lysophospholipids (LPE and LPC) (Fig 4.9 C and 4.10 C) and PCs (Fig 4.10 D). The PCA plot for each lipid class is shown in figure 4.9 in the next page.

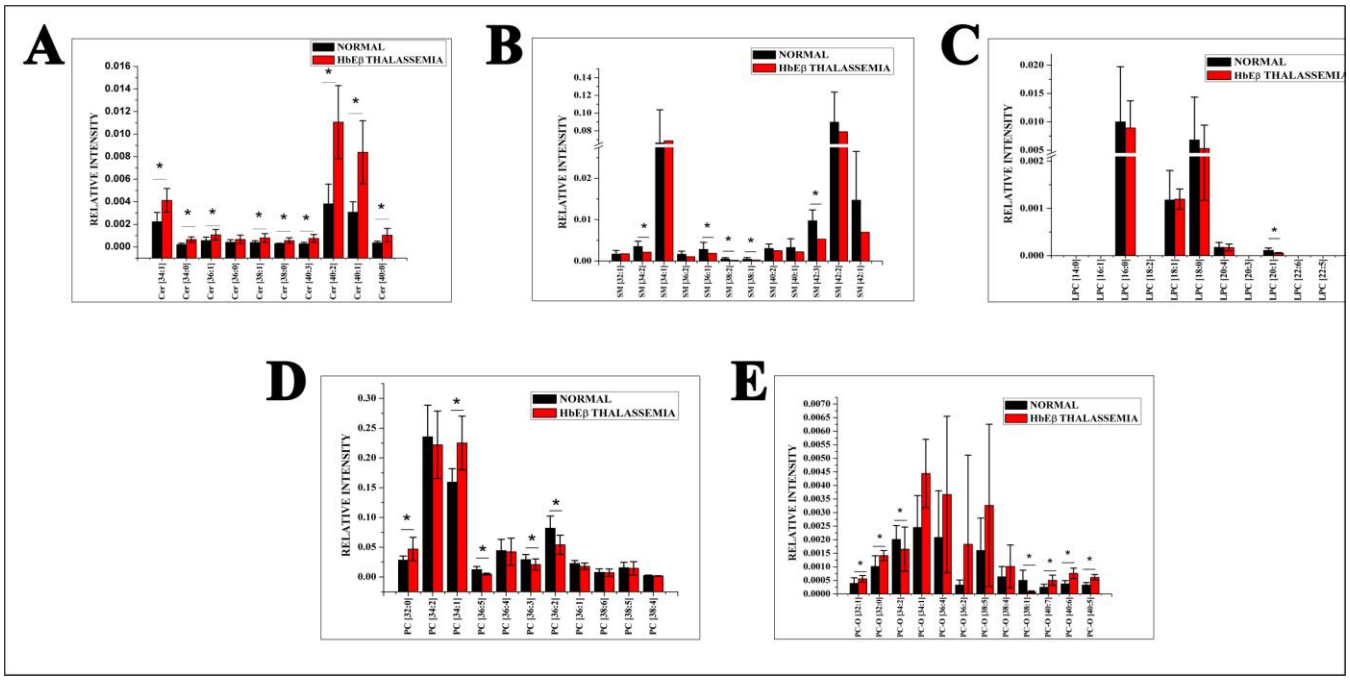

Figure 4.10: *Bar plot showing the differential expression of representative lipids of cer (A), SMs (B), LPCs (C), PCs (D) and PC-Os (E) of the erythrocyte membrane fraction.*

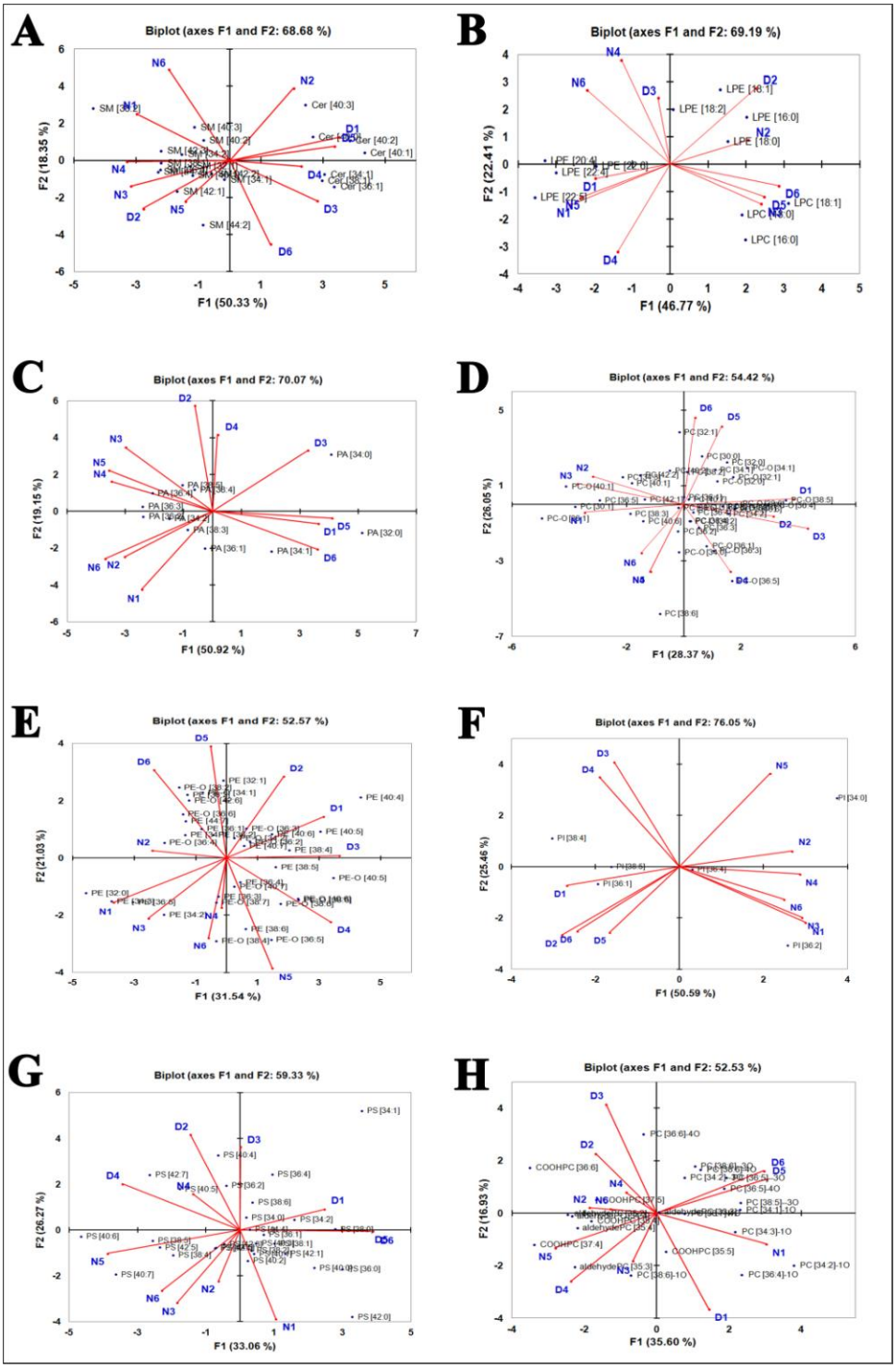

Figure 4.9: A-H PCA analysis of erythrocyte membrane samples of lipid classes Cer and SMs (A), PAs (B), and LPCs and LPEs (C), PCs and PC-Os (D), PEs and PE-Os (E), PI (F), PS (G), oxidized lipids (H).

On looking into the oxidized lipid populations the saturated and unsaturated aldehydes and carboxylic acid derivatives of truncated PCs show an increase in the plasma and erythrocyte membrane fractions but the number of these species detected in the plasma fraction "(Fig 4.5 H) is much less compared to that of the other fraction (Fig 4.9 H). The hydroxyl, keto, hydroperoxide, epoxy and polyhydroxy derivatives of long chain oxidized PCs were also detected in huge amounts in both the fractions, but a consistent increase in the Eβ thalassemic samples were observed only in case of erythrocyte membrane fraction.

4.4 **DISCUSSION**

The lipids identified so far are the major lipids in the eukaryotic system. As we know that the lipid population is tissue specific, hence the population and their abundance are unique to our sample type. The most abundant lipid species reported here also correlates with earlier studies [(15)]. We also see a difference between the populations of lipids in the plasma and erythrocyte fractions. This difference is due to the fact that the plasma fractions derive lipids from the diet and the lipid population is very sensitive to dietary changes.

It has already been reported in many studies that the most common fatty acid (FA) in animals, plants and microorganisms is palmitic acid (16:0). Stearic acid (18:0) is a major FA in animals. Amongst the unsaturated FA Oleic acid (18:1; ω – 9) is the common monoenoic acid in plants and animals. Linolenic acid (18:2; ω – 6) is a major FA derived from dietary plant oils in animals. Arachidonic acid (20:4; ω – 6) is a major component of the membrane phospholipids throughout the animal kingdom, but very little is found in the diet [(16)]. Therefore we see an abundance of phospholipids composed of such fatty acid chains, such as PC (34:2) was the most

abundant in the three fractions. Cholesteryl ester (18:2) was the most abundant in the plasma. LPCs 16:0 and 18:0 and LPE 20:4, PI (38:4) were the most abundant. PS (38:4) is the most abundant in the erythrocytes and erythrocyte membrane fractions. SM (34:1) and SM (42:2) being the most abundant in all three fractions.

It has been reported that in diseased conditions mature erythrocytes undergo rapid self destruction process leading to increased intracellular calcium content, reduced deformability, metabolic disruption, membrane protein modification and PS externalization [17]. The increase in ceramide with a decrease in sphingomyelin and lysophospholipids in erythrocytes indicate a regulative role of lysophospholipids and ceramides for cell-to-cell communication during premature eryptosis.

CERAMIDES AND SPHINGOMYELINS

Ceramides have been implicated in suicidal death of erythrocytes and is greatly studied in cancer therapy [18-20]. The increase in ceramides and a decrease in sphingomyelins clearly indicate the activation of the sphingomyelinase which is responsible for the hydrolysis of sphingomyelin to ceramides [21]. This sphingomyelinase may be expressed in the plasma membrane of erythrocytes or released into the plasma [19].

Another potential source of ceramides is the reversal of synthesis. Sphingomyelin synthase converts diacylglycerols and sphingomyelin to ceramide and phosphatidylcholine. Furthermore, the ratios, PC/SM and DAG/Cer are said to be intrinsically related and may have a role in cross-talk between glycerolipids and sphingolipid signaling [19, 22].

In our case the plausible explanation is that a phospholipase mediated release of platelet-activating factor (PAF) stimulates sphingomyelinase which in turn converts sphingolipids to ceramides and choline [20]. Simultaneously the oxidative stress activates the Ca^{2+} cation channels, thus increasing the Ca^{2+} levels. The concerted effect of elevation of ceramides and Ca^{2+} levels play a role in PS externalization.

It has also been reported that when ceramides increase in large quantities they can also affect membrane physical properties such as deformability. Ceramides displace cholesterol from lipid umbrellas and drive its esterification [23].

LYSOPHOSPHOLIPIDS

Signaling induced hydrolysis of glycerophospholipids lead to the formation of lysophospholipids. Lysophospholids are also generated due to the oxidation of phospholipid ethers [1]. These lysophospholipids are known to carry only one aliphatic chain and hence readily leaves the plasma membrane. This explains why we have seen an increase of LPEs in the plasma fraction but a decrease in the erythrocyte membrane fractions (Fig 3). The lysophospholipids act as messenger lipids and act through membrane receptors in contrast to ceramides which remain in the membrane and employs cytosolic proteins for signaling [23].

However we see a decrease in case of LPCs in both the fractions. This can be correlated to the fact that the PCs are undergoing extensive oxidation. Therefore there is a tremendous pressure to maintain the PC levels in both the fractions and hence the LPCs are being continuously used up showing a decrease in their levels.

PHOSPHATIDYLCHOLINE AND ITS ETHER

Phosphatidylcholines are the most abundant glycerophospholipids present in the eukaryotic system accounting for more than 50% of the total membrane lipids [23]. There have been a lot of reports on the identification of oxidized products of PCs in a number of diseases [1, 8, 24, 25]. Being the most abundant it is greatly exposed to reactive oxygen species (ROS) and is easily oxidized. Thus there is a decrease in the levels of intact PCs in all three cases, however in case of the erythrocyte and erythrocyte membrane fractions the changes were not so pronounced compared to the plasma fraction. This might be due to the increased inward movement of PCs in the thalassemic erythrocytes [26]. Intact PCs have other than making up the major bulk of the membrane also acts as the biosynthetic precursor for lipids such as SMs [27, 28], LPCs [29], PAs [30] and as well as PSs [28] (Fig 4.11) . Hence with an increase in Cer, LPCs and oxidized PCs (as discussed later) and a decrease in sphingomyline followed by PS externalization it is inevitable that the level of PCs decreases.

Contrary to earlier findings in case of neurological diseases or metabolic and inflammatory disorder, the levels of ethers of PCs in our case show an increase. However, long chain ethers species containing arachidonic acid (20:4) or docosahexanoic acid (22:6) shows a decrease. The phospholipid ethers have very short life span, about 30mins in case of PC ethers. Hence the populations of PC ethers are greatly adapted to changing environmental conditions. Phospholipid ethers are reported to have antioxidant function as well as they act as reservoirs of long chain fatty acids especially arachidonic acids [31]. Arachidonic acid in turns acts as precursor in the biosynthetic pathways of all lipids. Hence alteration in these ethers not only indicates the change in the oxidative state but also alters other lipid metabolism. Ethers also give rise to a

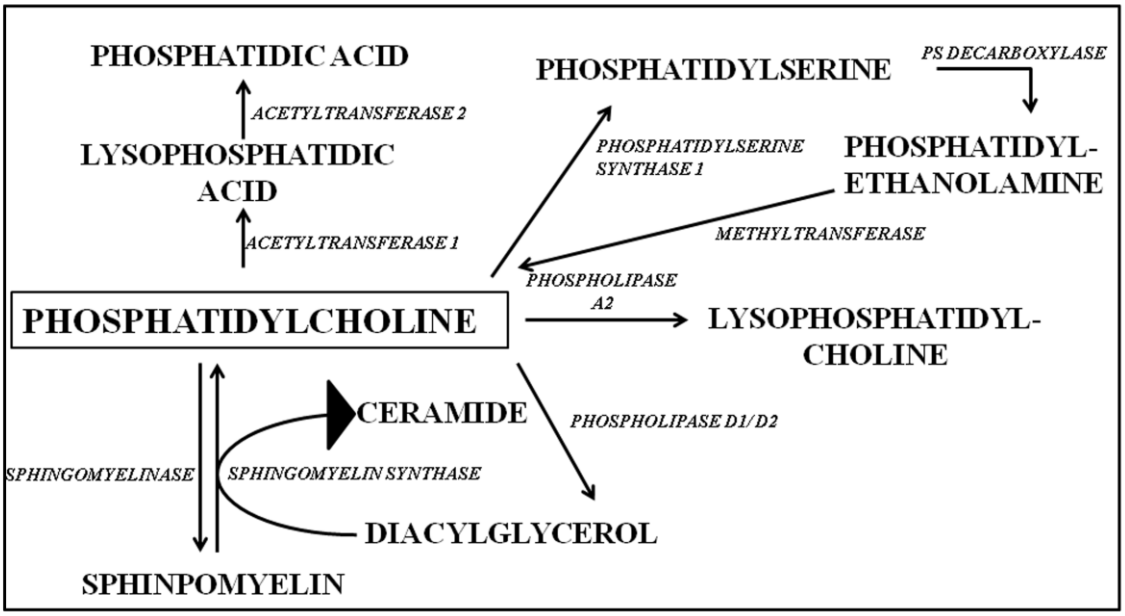

Figure 4.11: Schematic description of the role that phosphatidylcholine plays in the biosynthesis of other lipid classes.

number of second messengers in the signaling cascades [1, 31]. Keeping all this in mind we can assume that the erythrocytes which show an elevation of ether population are the ones battling against the oxidative stress that is induced in the diseased condition and the ones who could not synthesize the ethers as per demand are the ones who have lost the battle and perished. Hence we can only see those erythrocytes who have survived the test. In other words the elevation of the ethers levels might denote the defense mechanism of the body.

OXIDISED PHOSPHATIDYLCHOLINES

We have also observed an increase in the levels of oxidized PCs. There was a huge amount of long-chain products, such as hydroxy and/or hydroperoxide derivatives in both the

fractions in normal as well as diseased condition. As reported in other studies [32], we also detected polyhydoxy derivatives which have been generated in case of polyunsaturated acyl chains where the addition of oxygen molecules occurred several times. The increase that we observe in case of the thalassemic samples reinforces our claim of a condition of extreme oxidative stress. We have also shown an increase in the population of aldehyde and carboxylic derivatives of the truncated PCs. However, compared to the erythrocyte membrane fractions the plasma fractions did not show very consistent changes. This might be due to the fact that the susceptibility of lipid peroxidation alters as the lipid environment changes as well as the oxidants present [32, 33]. The increase in the oxidized species population further explains the decrease in the level of PCs.

4.5 CONCLUSION

The premature eryptosis leading to acute anemia is one of the key pathological features of Eβ thalassemia. The pathophysilogical symptoms observed in the diseased condition can be linked to the changes observed in the lipid population that have been reported in this study, which in turn can be due to the increase in reactive oxygen species (ROS) as shown in Fig 4.12. The changes in the lipidome combined with our already vast knowledge of the changes in the proteome in plasma as well as the erythrocytes may help us discover new insights so as to prolong the survival of the diseased erythrocytes. A detailed quantitative study of these changes might enable a "fingerprinting" approach towards a better understanding of the disease. This area has a huge potential in the therapeutic level as well as diagnostic level of this disease.

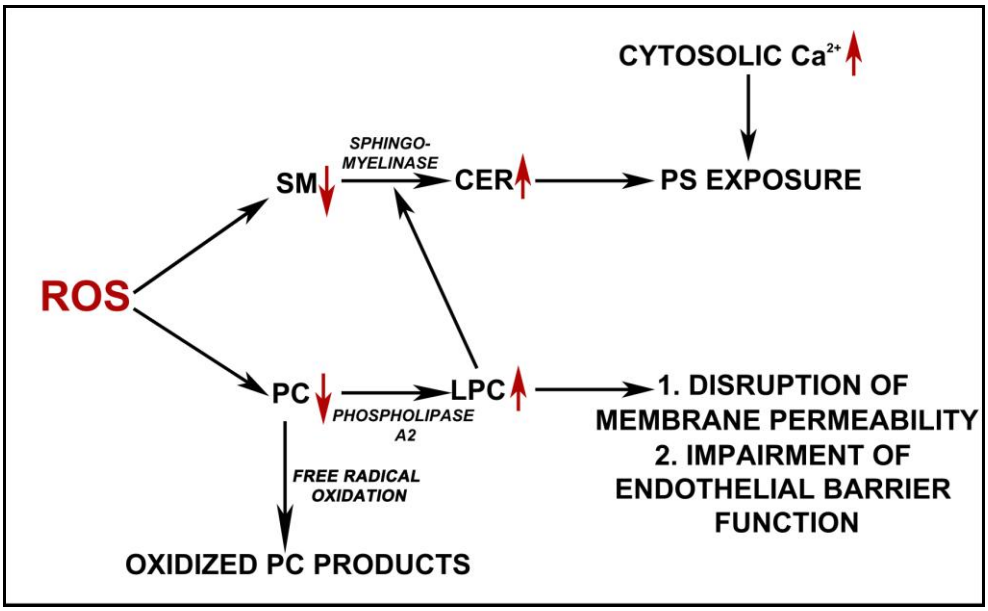

Figure 4.12: Schematic representation of the effect of ROS on the levels of lipid classes and the physiological changes that occur in turn due to the changes in their levels.

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CHAPTER V

CONCLUSION

CONCLUSION

The most remarkable feature of the human erythrocyte is its durability, given that it is an anucleated cell without the vital organelles that are considered necessary for the survival and function of most other cell types. They are devoid of mitochondria that ensure efficient oxidative metabolism, ribosomes for generation of damaged proteins, and a nucleus to regulate the regenerative process. *De novo* synthesis of lipids is also precluded, because of a very limited metabolic repertoire. The erythrocyte shows extreme deformability under normal physiologic circumstances, mainly because of the plasticity and viscosity of the membrane. Therefore as discussed before iron overload, followed by generation of reactive oxidative stress leading to eryptosis.

From our proteomic study of two different body fluids (plasma and urine) we can see that proteins which play a significant homeostasis are altered. The levels of proteins participating in cholesterol metabolism, iron transport, coagulation, hemoglobin scavenging and redox regulation were altered (fig. 5.1). This finding is also supported by our earlier work on erythrocyte proteomics study [(1)]. Recent studies indicated that endothelial dysfunction, the precipitating factor in the atherosclerotic process, is an important cardiovascular risk determiner in β thalassemia patients.[(2)] Among the numerous factors known to confer increased susceptibility to atherosclerosis, iron and cholesterol merit particular consideration in thalassemic patients.

Cholesterol metabolism and transportation is affected in thalassemia and has been reported in earlier studies by measuring the level of total cholesterol, low density lipoproteins (LDL), high density lipoprotein (HDL) and triacylglycerides (TAGs). [(2-4)] Despite the fact that hypocholesterolaemia in thalassaemia was first described many years ago, there is no definitive explanation for the mechanism underlying this clinical condition in severe forms of

thalassaemia. The two main pathogenetic mechanisms that have been proposed are (discussed in details chapter II): the presence of enhanced cholesterol consumption required for cell membrane formation,[5-7] and the presence of a hyperplastic and overactive reticuloendothelial system, which may be responsible for an increased uptake of LDL.[8, 9]

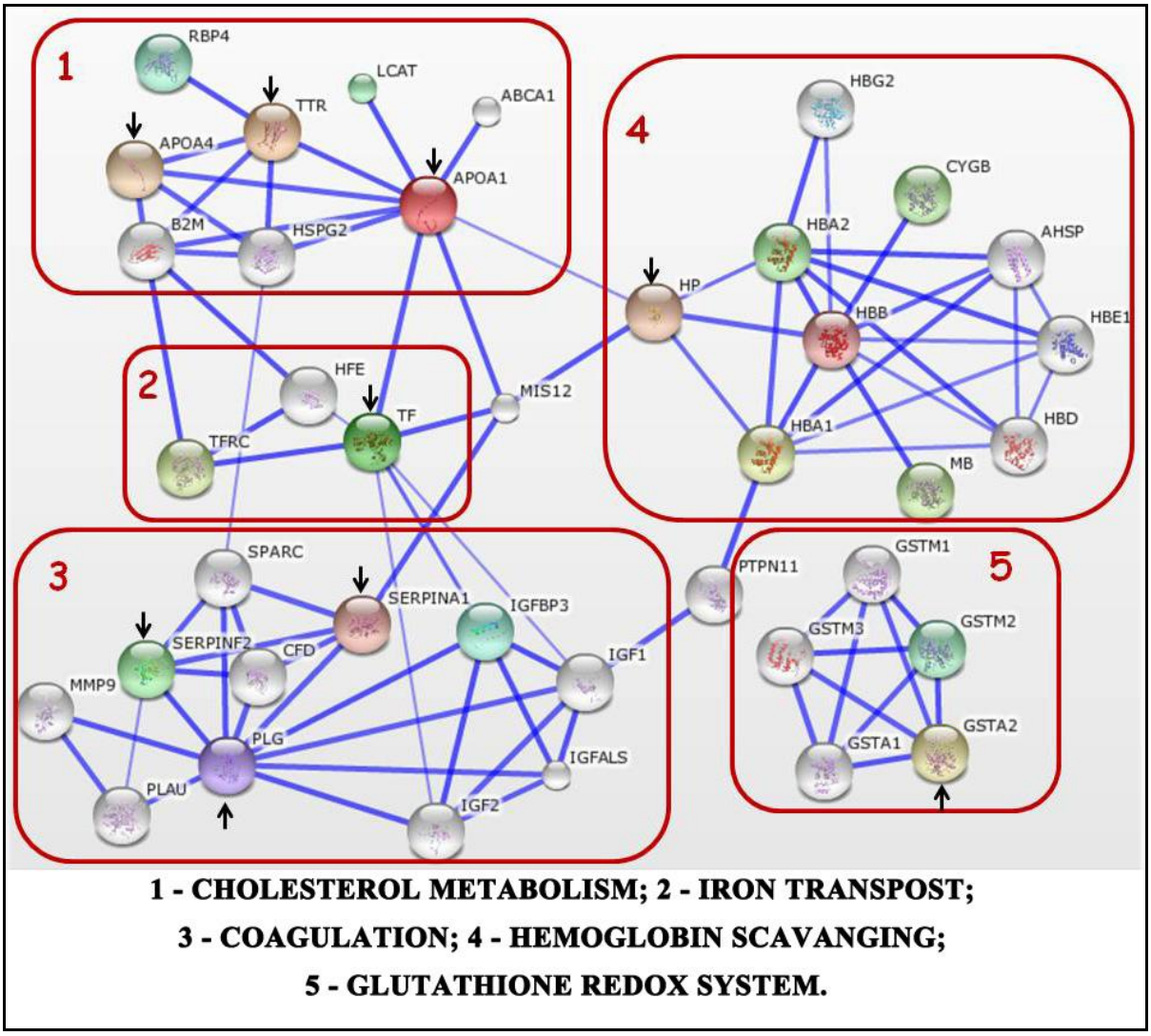

Figure 5.1: protein interaction network using STRING 9.1 software. The black arrows point the proteins which have shown differential regulation in our plasma and/or urine proteomics study.

All the processes shown above are inter-related and triggering one of them leads to the successive initiation of the other processes. For instance, the defective erythrocytes undergo severe hemolysis leading to the release of hemoglobin into the plasma. This activates the hemoglobin scavengers such as haptoglobin so that this hemoglobin is quickly removed. The iron attached to the heme of the hemoglobin however is responsible for iron overload in patients undergoing transfusion. The labile unbound iron is responsible for the oxidative stress. This again stimulates the antioxidant machinery such as glutathione transferase redox system. The oxidative stress also leads to lipid peroxidation which will affect lipid metabolism and transportation. It has also been described that homotetramer deposition of the alpha globin chains on the erythrocyte membrane is one of the reasons for PS externalization and subsequent thrombolytic events, thus affecting the coagulation pathway.

The proteins which have shown differential regulation in our study are shown by black arrows in fig 5.1. These proteins will not only help in disease monitoring but may also help as drug target if analysed in further details.

Our lipidomics study shows that the lipids also play a role in the disease. The changes observed clearly indicate that the erythrocytes are in a proapoptotic condition in the diseased samples. The premature eryptosis leading to acute anemia is one of the key pathological features of Eβ thalassemia. Lipids such as lysophospholipids play role in the pathogenesis of atherosclerosis and inflammation [(10)]. Platelet activation factor (PAFs) stimulates sphingomyelinase which is responsible for the generation of ceramides from sphingomyelins and ceramides in turn play a role in the eryptosis [(11)]. Oxidative stress produced in the erythrocytes lead to lipid peroxidation. The lipid species to be most affected has been the phosphatidylcholines (PCs). Being the most abundant lipid species they are the most exposed to lipid oxidation. PCs are also involved in the metabolism of other lipid classes as has been discussed earlier (Chapter IV, fig. 4.11). The ethers of PCs have been implicated in

cholesterol transport and membrane trafficking, they act as sink for polyunsaturated fatty acids and also act has antioxidant capacity [12, 13]. We see that the aforementioned processes such as cholesterol trafficking, coagulation and antioxidant machinery are also seem to be affected from our lipidomics study. The changes in the lipidome combined with our knowledge of the changes observed in the plasma and urine proteome as well as the erythrocytes may help us discover new insights so as to prolong the survival of the diseased erythrocytes. A detailed quantitative study of these changes might enable a "fingerprinting" approach towards a better understanding of the disease. This area has a huge potential in the therapeutic level as well as diagnostic level of this disease.

Hb Eβ-thalassaemia because of changing phenotypes and variable medical interventions, it is difficult to accurately characterize the severity of this disease. Its remarkable phenotypic diversity is still not well understood[14]. We believe our study auguements the knowledge already available.

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APPENDIX

DIFFERENTIAL REGULATION OF URINE PROTEINS IN UROTHELIAL NEOPLASM

5.1 INTRODUCTION

Urothelial neoplasms of the upper urinary tract account for approximately 5% of all epithelial tumors of the urinary tract only, whereas urinary bladder tumors are the most common and are the fourth most common form of cancer. This disease is mainly diagnosed in the elders, approximately at the age of 65 years and is more common in men than in women [(1)]. It is a major challenge in clinical oncology due to its high frequency. Although the incidence varies in different countries, bladder cancer constitutes a worldwide public health problem. Roychowdhury and co-workers showed that, p53 protein expression is associated with high grade urothelial neoplasm and advanced stage of the disease [(2)] .

For diagnosis doctors collect data about a cancer to determine its stage. This information comes from the various tests used to identify staging in different types of cancer. These tests can include:

- **Physical examinations** that can provide clues as to the extent of the cancer. The physical exam may determine the location and size of the tumor(s) and provide additional information on whether the cancer has spread to the lymph nodes and/or to other organs.
- **Imaging tests** such as x-rays, CT scans, and MRI scans can show the location of the cancer, the size of the tumor, and whether the cancer has spread.
- **Laboratory tests** that provide information on blood, urine and other fluids and tissues removed from the body.
- **Pathology reports** that can provide information about the size of the tumor, the growth into other tissues and organs, the type of cancer cells and the grade of tumor (how closely the cancer cells resemble normal tissue). Pathology reports often confirm the diagnosis of cancer, as well as the stage.

- **Surgical reports** from samples removed during surgery can determine the size and appearance of a tumor and provide insights about lymph node and organ involvement.[3]

The TNM system is one of the most widely used cancer staging systems. This system has been accepted by the Union for International Cancer Control (UICC) and the American Joint Committee on Cancer (AJCC). Most medical facilities use the TNM system as their main method for cancer reporting.

The TNM system is based on the size and/or extent (reach) of the primary tumor **(T)**, the amount of spread to nearby lymph nodes **(N)**, and the presence of metastasis **(M)** or secondary tumors formed by the spread of cancer cells to other parts of the body. A number is added to each letter to indicate the size and/or extent of the primary tumor and the degree of cancer spread.

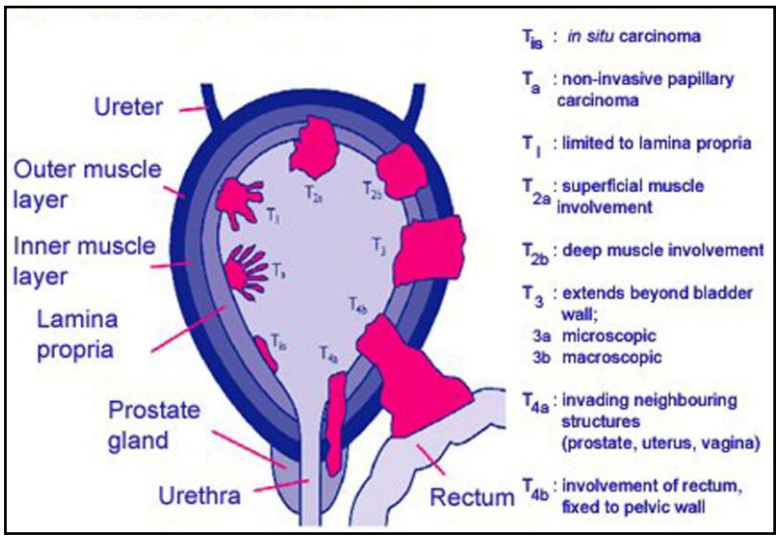

Figure 5.1: T staging of bladder cancer.

Primary Tumor (T) TX: Primary tumor cannot be evaluated

T0: No evidence of primary tumor

Tis: Carcinoma in situ (CIS; abnormal cells are present but have not spread to neighbouring tissue; although not cancer, CIS may become cancer and is sometimes called preinvasive cancer) **T1, T2, T3, T4**: Size and/or extent of the primary tumor.

Regional Lymph Nodes (N)

NX: Regional lymph nodes cannot be evaluated

N0: No regional lymph node involvement

N1, N2, N3: Degree of regional lymph node involvement (number and location of lymph nodes)

Distant Metastasis (M)

MX: Distant metastasis cannot be evaluated

M0: No distant metastasis

M1: Distant metastasis is present[4]

Because each cancer type has its own classification system, letters and numbers do not always mean the same thing for every kind of cancer. Once the T, N, and M are determined, they are combined, and an overall *stage* of 0, I, II, III, IV is assigned. Sometimes these stages are subdivided as well, using letters such as IIIA and IIIB.

Table 5.1: Cancer Staging

Stage	Definition
Stage 0	Carcinoma in situ
Stage I, Stage II, and Stage III	Higher numbers indicate more extensive disease: Larger tumor size and/or spread of the cancer beyond the organ in which it first developed to nearby lymph nodes and/or tissues or organs adjacent to the location of the primary tumor
Stage IV	The cancer has spread to distant tissues or organs

Currently, many bladder tumor markers are being researched, but only a few are commercially available US Food and Drug Administration approved products. Some of the FDA approved Bladder tumor markers are enlisted below:

Table 5.2: FDA approved bladder tumor markers and their functions.

S. no.	PROTEIN NAME	FUNCTION
1	Nuclear Matrix Protein 22 (NMP22)	NMPs are an important part of the structural framework of the nucleus. These proteins have important roles in DNA replication, transcription, and regulation of gene expression. NMP22 is specific for the nuclear mitotic apparatus protein that is involved with the proper distribution of chromatids to daughter cells.
2	Bladder Tumor Antigen (BTA)	This antigen is a human complement factor H–related protein similar to human complement factor H. BTA interacting with complement factor C3b interrupts the complement cascade, potentially conferring a selective growth advantage to cancer cells.
3	Immunocyt	utilizes monoclonal antibodies to detect exfoliated urothelial cancer cells in the urine. Three fluorescent-labelled monoclonal antibodies target the M344, LDQ10, and 19A211 antigens, which are specific for bladder carcinoma
4	Telomerase	Telomerase is involved with the synthesis of these tandem "TTAGGG" repeats and typically inactivated in mature somatic cells. Alteration of this enzyme can result in the

		immortalization of cell lines in certain malignancies, such as bladder cancer.
5	Hyaluronic Acid (HA)	HA is an extracellular glycosaminoglycan that supports tumor cell adhesion and migration, and offers some protection from immune system surveillance in tumor tissues. Small fragments of HA stimulate angiogenesis and are produced by hyaluronidase (HAase). The small HA fragments have been identified in the urine of patients with bladder cancer.
6	BLCA-4	A nuclear matrix protein specific to bladder cancer tissues, termed BLCA-4, has been identified. It holds great promise as a potent urine-based bladder tumor marker. This protein has been found to effect the pathogenesis of bladder cancer by increasing IL-1α (which enhances proliferation and invasion), thrombomodulin (which maintains blood flow for cell survival), and IL-8 (which aids in angiogenesis).

However search is still on for markers which has high prognostic value, are non invasive and are easily obtainable. Urothelial neoplasm occurs at different sites in the urinary bladder with varying frequency. Vigilant monitoring of patients after definitive treatment for urothelial neoplasm is essential owing to the high rate of multifocality and recurrence. However, there are no clear cut ways of predicting which urothelial carcinomas would subsequently recur or progress or which muscle invasive tumors would progress following treatment. In this work, for the first time, we have tried to establish a correlation between

different tumor grades and changes in the protein profile of urine samples of urothelial neoplasm patients. Urine being the body waste is easily obtainable for monitoring at various stages of the disease and hence will help in establishing a non-invasive disease and post-treatment monitoring method.

Apart from the standard diagnostic procedures we have also performed p53 immunohistochemical studies. Mutations of p53 are found to be present in 40–45% of cancers, including all sites combined. Indeed, p53 mutation is the most frequent genetic event demonstrated to date [(5)]. These point mutations lead to the loss of its tumor suppressing function. The wild-type p53 protein has a short half-life of 15 to 30 minutes, whereas the mutated p53 gene results in a protein with a prolonged half-life, which is the basis of its nuclear accumulation that is detectable by immunohistochemistry (IHC). The accumulated p53 has been associated with the progression of bladder cancer and might play a role in the evolution of the tumours to a higher grade, shown in earlier studies [(2, 6)].

Therefore in this study we take three parameters under consideration –

1) Standard staging of the tumors,
2) p53 IHC and
3) Proteomic study of urine samples.

5.2 MATERIALS AND METHODS

MATERIAL

Amicon ultra centrifugal filter units with 5kDa cut off membrane and PVDF membrane were obtained from Millipore. Ethanol from Merck, 2D rehydration buffer, 17cm pH 3- 10 IPG strips, Isoelectric focussing system, two dimensional gel electrophoresis (2DGE) system and western blot transfer setup were purchased from Bio-Rad. Colloidal

Coomassie from Sigma and sypro ruby stain from Invitrogen. Sequence grade trypsin was purchased from Promega, in-gel tryptic digestion kit from Pierce Biotechnologies and α-Cyano-4-hydroxycinnamic acid (CHCA) was purchased from Waters. Primary antibodies for the two proteins and anti-mouse HRP-conjugated IgGs used as secondary antibodies were purchased from abcam. All other reagents, if not mentioned otherwise, were purchased locally and were of analytical grade.

SAMPLES

Urine samples were taken from pre-operative, non-azotemic patients suffering from urothelial neoplasm (n=12). These samples were further categorised into low grade (n=6) and high grade (n=6) depending on p53 immunohistochemistry [(2)]. These patients also suffered from various age related and lifestyle related disorders such as bronchial asthma and hypertension (Table 5.3). We collected urine samples from non-cancer individuals who are under medication for bronchial asthma or hypertension and are above the age of 50 years as our control samples (n=3). Normal samples (n=4) were collected from healthy volunteers within our institute. Urine samples were obtained from RG Kar Medical College, Kolkata with informed written consent following the guidelines of the Institutional Ethical Committee of RG Kar Medical College, Kolkata 700037, India and Institutional Animal & Bio ethics committee of Saha Institute of Nuclear Physics. The Institutional Ethical Committee R. G. Kar Medical College, Kolkata 700037, India and Institutional Animal & Bio ethics committee of Saha Institute of Nuclear have also specifically approved the current study.

SAMPLE PREPARATION

Urine samples (~30ml) were centrifuged to remove cellular debris [(7)]. The supernatant was then centrifuged in Amicon ultra centrifugal filter units with 5kDa cut off membrane

Table 5.3: Sample details

NAME	AGE	SEX	ASSOCIATED ILLNESS	DRUG HISTORY	HISTO-PATHOLOGICAL EXAMINATION	p53 IHC
N_1	25	F	None	None	-	-
N_2	27	M	None	None	-	-
N_3	26	M	None	None	-	-
N_4	28	M	None	None	-	-
C_1	60	F	Hypertension, Bronchial asthma	Bronchodialator	-	-
C_2	57	M	Bronchial asthma	Bronchodialator	-	-
C_3	52	M	Brionchial asthma	Bronchodialator	-	-
LG_1	75	M	Bronchial asthma	Bronchodialator	PUNLMP, Ta	<5%
LG_2	62	F	None	None	Low grade, Ta	10%
LG_3	35	M	Hypertension	Anti-hypertensive	Low grade, T1	10%
LG_4	60	M	None	None	PUNLMP, Ta	5%
LG_5	39	F	None	None	Low Grade, T1	10%
LG_6	58	M	Hypertension	enalapril	PUNLMP, Ta	10%
HG_1	53	M	Bronchial asthma	Bronchodialator	High grade Ta	-

HG_2	85	M	COPD, Hypertension	Bronchodialator	High grade, T2, squamous differentiation-90%	90%
HG_3	60	M	None	None	High grade, T1	90%
HG_4	76	M	Hypertension	Enalapril	High grade, T2	80%
HG_5	56	M	Diabetes mellitus 2	Oral hyppo-glycemic	High grade, T2	99%
HG_6	72	M	Hypertension	Anti-hypertensive	High grade, T2	100%

concentrated to a volume of 2ml and then proteins were precipitated using 75% ethanol. The precipitated proteins were directly solubilised in 2D rehydration buffer.

2DGE AND MASS SPECTROMETRY

The solubilised samples were separated first on the basis on pI on 17cm pH 3-10 IPG strips and then on the basis of molecular weight by 2DGE. Gels were stained either with colloidal Coomassie [8] or sypro Ruby according to manufacturer's instructions. Each sample was processed in duplicates. Images of the stained gels were taken and densitometry analyses were done on Versa Doc series 3000 imaging system using PDQuest software (version 7.1, Bio -Rad). Spot volumes (intensity) of the desired spots were normalized as parts per million (ppm) of the total spot volume using the spots that were present in all gels, to calculate the relative abundance of a spot in a sample. Student t-test was performed to find the significance

of the densitometric changes. The protein spots from Coomassie and Sypro -Ruby stained 2D gels of normal as well as diseased samples were excised. Spots were destained followed by in-gel tryptic digestion. Peptides were then extracted and run on a MALDI ToF/ToF (AB 4700, Applied Biosystems) following published protocol [9].

WESTERN BLOT ANALYSIS

Two of the proteins which were differentially regulated were further analysed by western blot analysis and student t-test performed. In the absence of any proper loading control we have stained the total blot and the intensity of each band in normalised against the total intensity of the lane.

5.3 RESULTS

From the 2D gels 44 spots were analyzed out of which 8 proteins were found to exhibit differential expression in the urine samples of the patients with respect to normal and control samples. These proteins are the ones showing more than 1.5 fold change in the mean ppm spot volume in any of the three categories compared to normal. These proteins were identified as albumin, transferrin, alpha 1-antitrypsin, apolipoprotein A1, haemoglobin β subunit, transthyretin chain A and B, inter alpha trypsin inhibitor heavy chain related protein precursor and haptoglobin. The protein annotations along with their fold change are given in Table 5.4 along with the spot positions in the representative gels of the four categories, shown in Figure 5.2. As no enrichment protocol was followed the proteins identified are mostly the abundant proteins in the urine of a healthy individual.

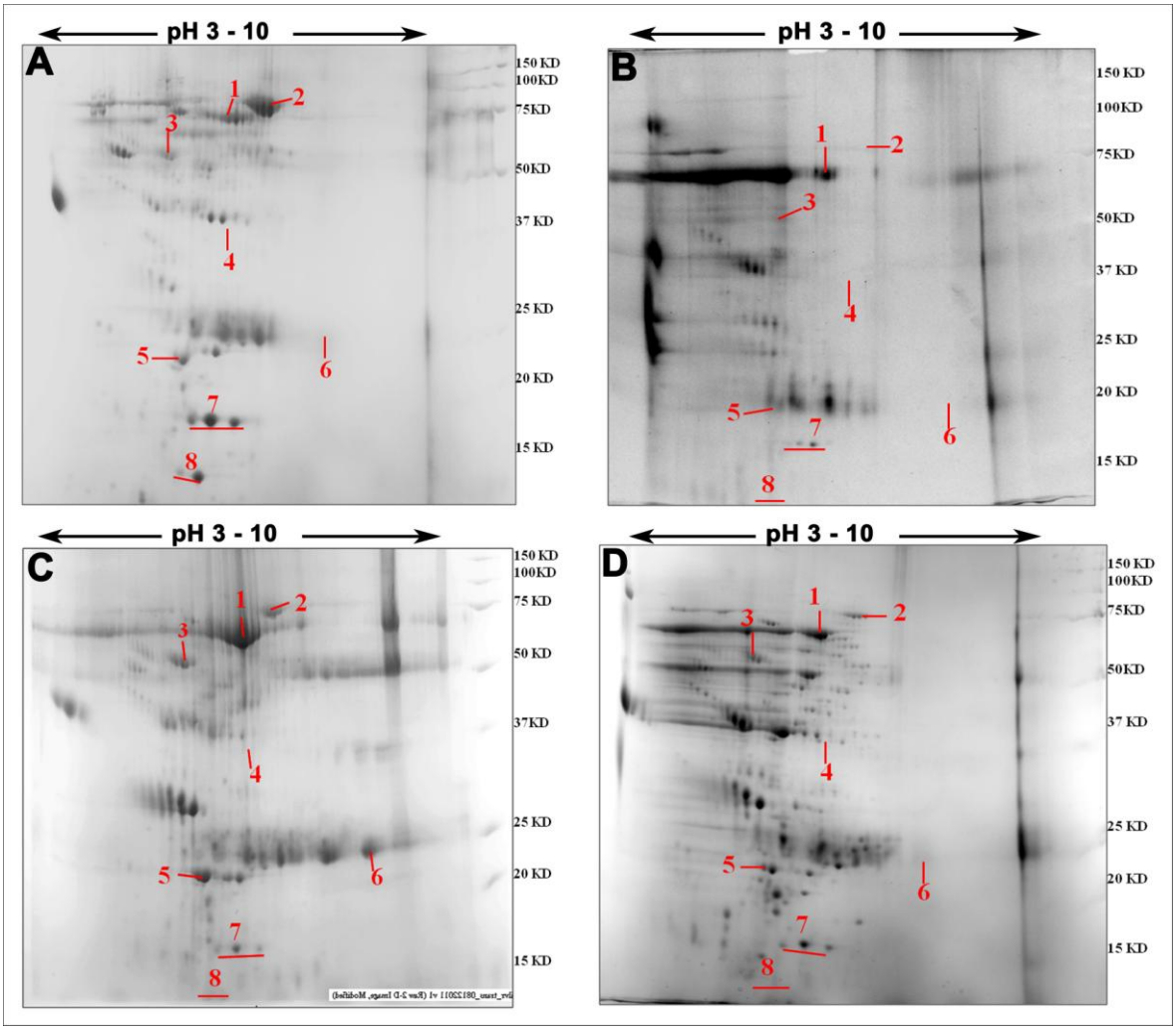

Figure 5.2*: Representative 2D gels of the four categories of urine samples involved in this study. (A) Normal volunteer, (B) Control samples with hypertension and bronchial asthma, (C) Patients with low grade urothelial neoplasm and (D) Patients with high grade urothelial neoplasm. 1-8 are the protein spots showing changes with respect to normal.*

1*-Albumin,* ***2****-transferrin,* ***3****-alpha 1 antitrypsin,* ***4****-inter alpha trypsin inhibitor heavy chain related protein,* ***5****-apolipoprotein A1,* ***6****- hemoglobin β subunit,* ***7****- tranthyretin (chain A and B),* ***8****- haptoglobin.*

Table 5.3: Spot Identification

S. NO.	PROTEIN NAME	A. NO.	M.WT. PI	SCORE	SQ COV	FOLD CHANGE		
						C	LG	HG
1	SERUM ALBUMIN HUMAN	IBJ5	66130D 5.73	316	37%	-1.2	3.17	2.9
2	TRANSFERRIN PRECURSOR	Q53H26	77000D 6	240	51%	-1.27	-1.2	4.07
3	ALPHA-1-ANTITRYPSIN	AAA51547	46678D 5.51	206	39%	1.1	1.7	2.2
4	INTER-ALPHA-TRYPSIN INHIBITOR HEAVY CHAIN RELATED PROTEIN PRECURSOR_ HUMAN	JX0368	103321D 6.5	117	19%	1.02	-2.7	-2.3
5	APOA1 PROTEIN FRAGMENT	CAA00975	28061D 5.27	335	74%	-1.47	3.4	2.6
6	HEMOGLOBIN BETA SUBUNIT VARIANT_HOMO SAPIENS	AAF00489	15972D 6.75	151	76%	-1.6	1.64	2.04

10	TRANSTHYRETIN, CHAIN A	1BM7A	12470D 5.26	99	42%	-1.8	1.24	1.64
11	TRANSTHYRETIN, CHAIN B	2ROYB	13026D 5.26	64	73%			
9	HAPTOGLOBIN ALPHA AND BETA CHAIN (FRAGMENT)	CAA25248	41499D 6.25	67	32%	-1.6	1.5	1.17

(Where C = Control, LG = Low Grade and HG = High Grade, S. SNO. = Spot number, A. NO. = accession number, M.WT = molecular weight, PI = isoelectric pH, SQ COV = sequence coverage)

The scatter plots in Figure 5.3 (next page) clearly shows that proteins such as Albumin (ALB) (Fig 5.3A), Alpha 1 antitrypsin (A1AT) (Fig 5.3C), Apolipoprotein A1 (APO A1) (Fig 5.3E), hemoglobin β subunit (Hbβ) (Fig 5.3F) and Transthyretin (TTN) (Fig 5.3G) show an increase in expression in the urothelial neoplasm samples irrespective of their grade. Whereas proteins such as Transferrin (TF) (Fig 5.3B), show a greater increase in the high grade samples and Haptoglobin (HP) (Fig 5.3H) shows an increase in the low grade neoplasm samples. On the other hand, Inter alpha trypsin inhibitor heavy chain precursors (ITIH) (Fig 5.3D) show a decrease in both the grades.

In certain cases, such as TTN and HP, the age matched control samples show an opposite trend suggesting that the changes observed in the neoplasm samples has no relevance to aging or medications.

Western blot analysis of apolipoprotein A1 and transferrin were also done to further validate the densitometric data, shown in fig. 5.4, indicating concurrence with those from densitometry.

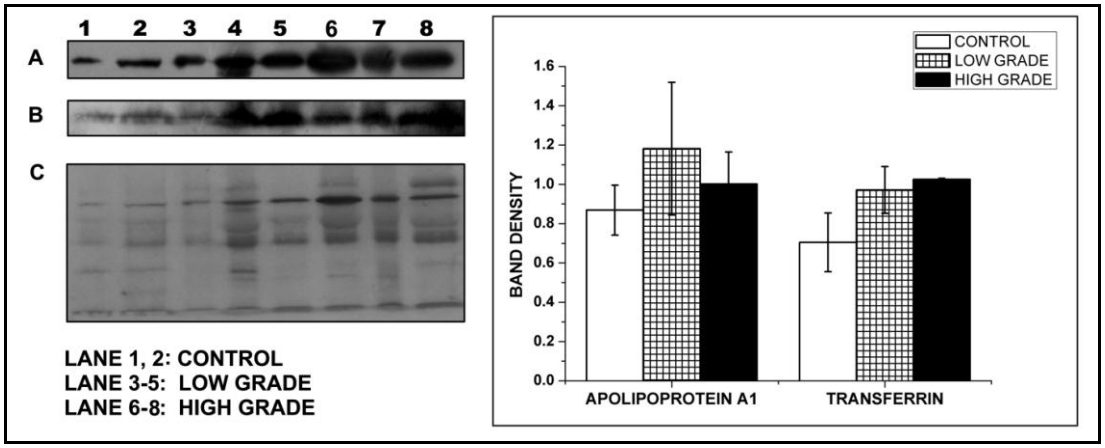

***Figure 5.4**: **A** and **B** are the developed films for transferrin, apolipoprotein A1 and **C** is the entire blot stained by Coomassie used as control. **D** is the bar diagram represents the normalised band density for the two proteins.*

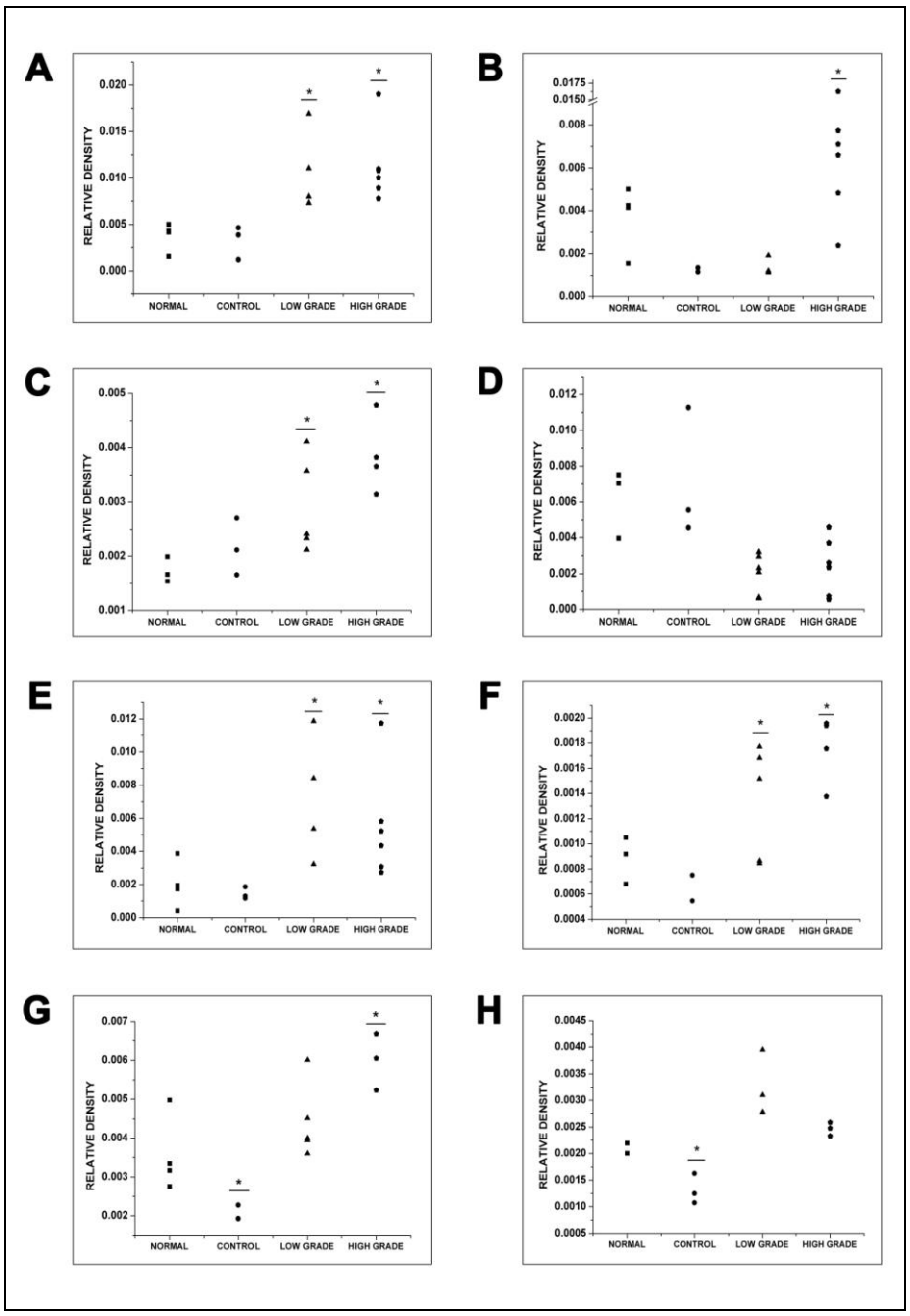

Figure 5.3: Scatter plot representing normalised spot intensities of the samples in the 4 categories. ('')mark denotes significant changes with $p \leq 0.05$)*

***A**-albumin, **B**- transferrin, **C**- alpha 1 antitrypsin, **D**- inter alpha trypsin inhibitor heavy chain related protein, **E**- apolipoprotein A1, **F**- hemoglobin β subunit, **G**- Transthyretin, **H**- haptoglobin.*

■ *-normal* ● *- control* ▲ *- low grade* ◆ *- high grade*

5.4 DISCUSSION

No enrichment technique was used for protein samples and hence, most of the proteins showing changes in urine levels are the highly abundant proteins e.g. albumin, transferrin, alpha 1 antitrypsin, apolipoprotein A1, transthyretin and haptoglobin. The differentially regulated proteins are mainly catalytic, enzymatic or transporters (Fig. 5.5).

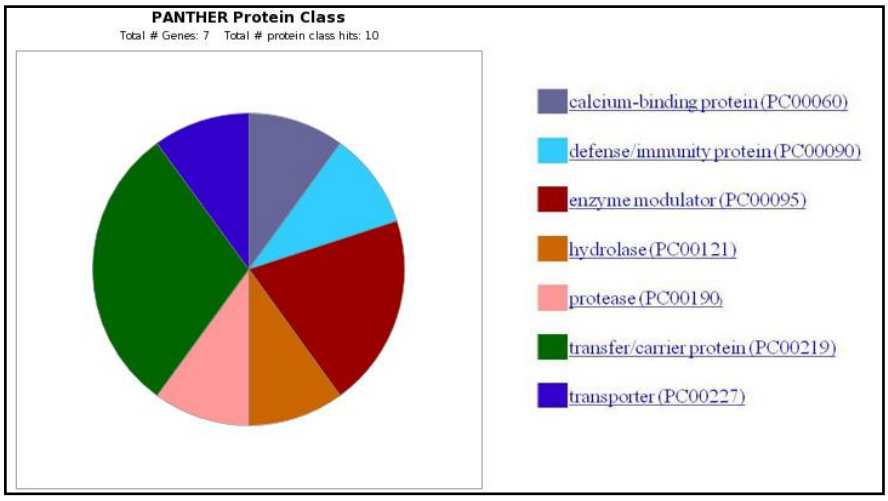

Figure 5.5: Panther protein classification of the 8 differentially regulated proteins based on protein class.

Albuminuria has been reported in different types of cancer such as lungs, breast, renal and colon/rectal. It has been suggested to be a nonspecific marker of malignancy reflecting a microvascular response to tumor cell cytokines [(10)].

Alpha 1 antitrypsin is upregulated during acute phase response to tissue necrosis, inflammation and secondary hypertension seen in few of the patients [(11, 12)]. It has been reported to be related to the expression of vascular endothelial growth factor (VEGF) which in turn is one of the most potent angiogenic molecules, regulating both angiogenesis and vascular permeability, and hence promotes tumour progression and development [(13)].

Transferrin is a major plasma protein involved in iron transport [(14).] It binds to the free iron in circulation and the diferric TF then binds to the TF receptors (TFR) on the cell surface

and both are internatilized in clathrin - coated pits through receptor - mediated endocytosis. The decrease in pH facilitates the release of iron form the TF/TFR in the endosome. The apo-TF/TFR complex returns to the cell surface where apo-TF is released [15]. It has been seen that highly proliferative cells have an increased need for iron as a co factor of the ribonucleotide reductase enzyme involved in DNA synthesis. It has also been reported and studied to a great extent that TFR increases in bladder transitional cell carcinoma and is further correlated with the tumour grade or prognosis. Hence it will not be very unjustified to expect an increase in the TF levels in the plasma as well as urine and we can further see from this study that a significant increase in observed in the samples we have categorised as high grade [16, 17].

ApoA-I, which have shown an increase in levels in the urine samples of neoplasm patients, mediates the reverse transport of cholesterol from peripheral cells to the liver for excretion [18]. Apo A1 is the major High Density Lipoprotein (HDL) component in plasma of the HDL particle. In the lipid-bound state, Apo A1 governs lipid transport, receptor recognition and other function including activation of lecithin - cholesterol acetyltransferase (LCAT). Apo A1 has been reported to be downregulated in hepatocellular cancer and ovarian cancer, whereas it has been found that it is overexpressed in patients with recurrent head and neck squamous carcinoma, indicating that it may indirectly promote tumour survival [18-20].

Transthyretin (formerly called prealbumin) acts as a transporter of thyroxine and retinol binding protein (RBP) [21]. It has been extensively used as a marker for studying the nutritional status. Recent studies have reported TTN as a biomarker for breast cancer, ovarian cancer, lung cancer [22] as well as bladder cancer [18]; [23]. However, in our study can see an increase in transthyretin level in the urine samples. This study only makes it more evident that further study is required for transthyretin to be used as a cancer marker. Since it is

affected by the dietary habits of an individual therefore the sensitivity and specificity of this marker is questionable.

Haptoglobin is synthesized predominantly in liver in response to cytokines such as interleukin-6, interleukin-1and tumor necrosis factor. Haptoglobin is a haemoglobin scavenger [(24)], which shows increase in the low grade neoplasm as well as in other cancers [(25, 26)]. Hematuria is very common in urothelial neoplasm. The severity of hematuria increases with severity of neoplasm explaining presence of lower levels of haptoglobin in the high grade samples with excess haemoglobin. Also we see an increase in the haemoglobin β subunit variant in the urine samples of the patients. Hbβ has also been reported to show changes in its levels in the plasma ovarian cancer and breast cancer patients [(27)].

The ITIH chains stabilize the extracellular matrix and (ECM) have been implicated in tumor invasion and metastasis. The hyaluronic acid (HA) degradation products may induce angiogenesis. Since HA linking and ECM stability is strongly dependent on ITIH chains, deregulation of ITIH family members should influence the vascularisation process during tumour development [(28, 29)].

It has also been reported that HP and TTR also interacts with Apo A1 and in turn impairs Apo A1 stimulation of Lecithin-Cholesterol acyl transferase (LCAT) enzyme.[(30, 31)] It has been observed that cholesterol levels are upregulated in highly proliferative cells with a subsequent decrease in the HDL-cholesterol level in the plasma. The efficiency of HDL to collect the cholesterol stored in the lipid bilayer of cells is achieved by the activation of LCAT [(32)]; [(33)]. The requirement for cholesterol by the cancer cells can be the answer as to why we see an upregulation of proteins such as HP and TTR. We also know that Hbβ subunit, an interactor of HP, also gets upregulated. Due to the cross talk between these

proteins (Fig. 5.6) it is only logical that a change in one of them will also influence the expression of the other. Therefore a collective approach towards the study of these proteins might prove to be beneficial.

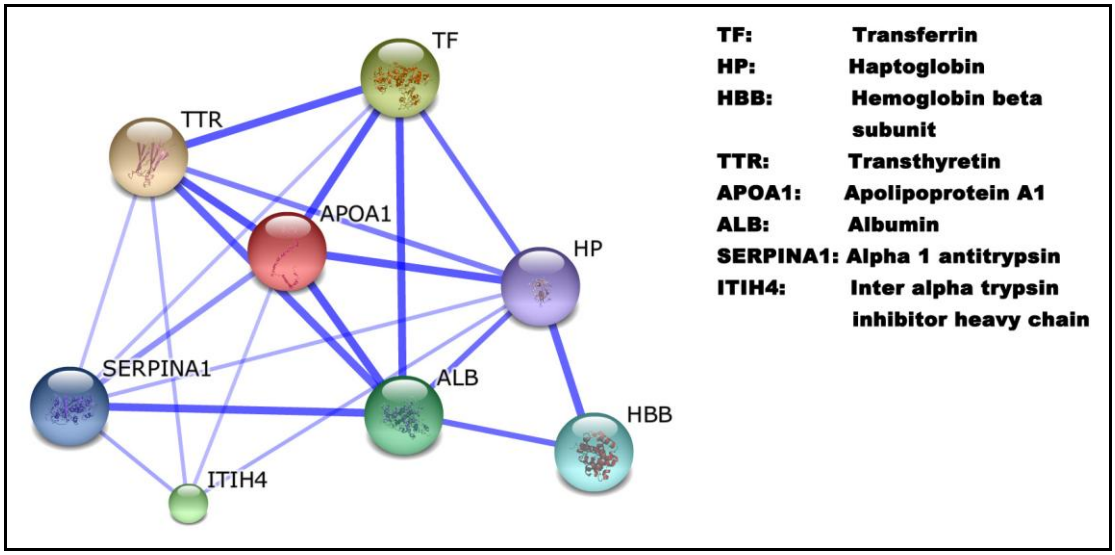

Figure 5.6: STRING interactome of the differentially regulated proteins observed in the urine.

Taken together, we report for the first time a urine proteomic study in cases of urothelial neoplasm showing differential levels of few proteins which could be correlated to the severity of the neoplasm associated to p53 immunohistochemical staining. We are showing that the gradation of the sample set based on the p53 IHC staining is also reflected in the protein changes observed in this study. We are also emphasising the fact that neoplasm prognosis can be made based on urine protein expression levels. An interaction study of these proteins with each other should also be done to obtain the bigger picture. This study also shows that the protein set obtained are the ones which are high abundant and hence are easily determined in the urine samples of any individual as well as show significant changes in neoplasm samples. These set of proteins taken together might provide a robust system to

follow patient condition in a non-invasive method. Few proteins like Apo A1 and transferrin have already been reported as biomarkers for bladder cancer [17, 18] and hence the role of these proteins together with alpha 1 antitrypsin, haptoglobin and ITIH show potential to act as prognostic tools.

5.5 CONCLUSION

The most common prognostic marker used to monitor cancer progression is p53 immunohistochemistry, which is an invasive method. In this study we have been able to report urinary proteins which show differential regulation in case of the disease with varying severity. We believe that these proteins should be studied in more details and with a wider and bigger sample pool as they have the potential to become diagnostic markers or therapeutic targets which could be easily collected and provide a non-invasive method for disease monitoring.

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Differential regulation of plasma proteins between members of a family with homozygous HbE and HbEβ-thalassemia

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Abstract

In this report we've compared the plasma protein profiles of 4 individuals in a family. Father and the younger son both are hemoglobin (Hb) Eβ-thalassemic {Cod 26 (G-A)/IVS 1-5 (G-C)}, but the father never requires transfusion, whereas the younger son requires monthly blood transfusion. Mother and the elder son are HbEE {Cod 26 (G-A)/Cod 26 (G-A)} without any history of transfusion. Proteomic study was done on the plasma fraction of the blood following ammonium sulphate precipitation. Proteins were separated by 2D-gel electrophoresis, expression of proteins compared by densitometry and proteins identified by tandem MALDI mass spectrometry. Proteins responsible in hemolysis, hypercoagulation and hemoglobin scavenging have shown differential regulation, establishing the relation between the differences in the levels of plasma proteins with the progression of the disease phenotype, manifested in the extent of transfusion dependence of the patient.

Introduction

The hemoglobin (Hb) E-β is one of the commonest forms of hemoglobinopathies worldwide.[1] The HbE mutation is located near the junction between the first exon and the first intron of the β-chain gene. Nucleotide sequence change near the consensus splice site region activates a cryptic splice site, which is not normally used for mRNA processing. This new splice site competes with the normal splice site. Some mRNAs are still processed using the normal splice site and thus produce a protein with a Lys instead of a Glu at position 26. The variant (HbE) is thus innocuous in its heterozygous and homozygous states.[2] The primary clinical importance of HbE trait arises when the β^E allele interacts with other β-thalassemia mutations leading to a moderate to severe anemia known as HbEβ-thalassemia.[3] In this paper we are presenting a classical case which shows that, not only different β-thalassemia mutations can give rise to variety of clinical consequences, but also same β-thalassemia mutation upon interacting with β^E allele can lead to variety of clinical manifestation of the disease condition. Here, two members of the family (the father and the younger son) even after having identical β chain mutation, shows marked difference in terms of clinical severity and transfusion requirement.

Materials and Methods

We describe clinical details of a thalassemic family showing an unusual and interesting pattern of blood transfusion requirement between two members of identical β globin mutational background. In the family, the father (P1, Table 1) is HbE-β-thalassemic with no history of blood transfusion. Though his hemoglobin was found to be less than normal (6.8 g/dL) never experienced any clinical complications and physiological manifestation of anemia, even after doing vigorous physical work. Mother (P2, Table 1) is HBEE and has no history of blood transfusion. Elder son (P3, Table 1) is also HbEE, like mother and never had blood transfusion. As per classical symptoms of HbEE patients, the mother (P2) and the elder son (P3) both maintain good hemoglobin level and are asymptomatic in terms of expression of anemic features. Younger son (P4, Table 1), is HBE-β thalassemic like his father, however, is highly anemic and requires monthly blood transfusion, starting from an age of 8 months. We have also included a non-related HbE-β thalassemic sample (P5) having similar clinical features as that of the P4 to validate or confirm the plasma proteome of P4.

Peripheral blood samples were collected from every member of the above-mentioned family, patient and normal volunteers in vials containing 5 mM ethylenediaminetetraacetic acid. Written consent was taken from adults and in case of children it was taken from the parents as per guidelines of institutional ethical committee. The handling of all human blood samples was carried out in accordance with the guidelines established by the Local Ethical Committee. For the patients P4 and P5 who are on regular blood transfusion, peripheral blood was taken after transfusion gap of 45 days, the maximum gap they could withstand. Plasma and red blood cells were separated using 75% percoll for the proteomic studies, as described earlier.[4]

As per protocol all members of the affected family were evaluated for baseline clinical investigations, which are as follows: fetal hemoglobin (HbF), HbA_0, HbA_2, hemoglobin, mean cell volume, mean cell hemoglobin, mean cell hemoglobin concentration, red cell distribution width and hematocrit. The hematologic data (Hb and complete blood count) were obtained by automatic analysis (Cell Counter: Medonic 530, cell counter: Medonic 530; EMerck, Darmstadt, Germany) and Hb variants (HbA, HbF and HbA_2/E) were estimated by high performance liquid chromatography (HPLC) (Bio-Rad Lab., Hercules, CA, USA) using manufacturer's protocol.

DNA was isolated from white blood cells, using a DNA isolation kit for mammalian blood (Qiagen, Venlo, The Netherlands). Patients were screened for five common β-thalassemia mutations of Eastern India[5-7] like IVS1-1 (G-T), IVS1-5 (G-C), codon 8/9 (+G), codon 26 (G-A), and Fr. 41/42 (–TCTT). The screening was performed by polymerase chain reaction (PCR) based technique, amplification refractory mutation system (ARMS) as described by Old.[8] Direct DNA sequencing of the β-globin gene was also done to further confirm the mutations. Salting out with 20% ammonium sul-

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Key words: HbE-β thalassemia, blood transfusion, differential proteomics.

Contributions: SH, TC contributed equally to this work. SH did all the proteomics work; SH, TC prepared the first draft of the manuscript; TC did all the mutation experiments and collected the clinical data; SC, AC, worked as clinical collaborators and corrected the manuscript; AC supervised the entire work and corrected the final version of the manuscript.

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Table 1. Clinical data of the members of the family in study.

Clinical parameters	P1 (father)	P2 (mother)	P3 (elder brother)	P4 (younger brother)	P5 (unrelated sample)
Hemoglobin (g/dL)	6.8	11.2	11.4	4.1	5.7
MCV (fL)	48.5	54.2	50.3	57.0	79.9
MCH (pg)	17.2	21.1	21.2	16.6	27.8
MCHC (%)	35.4	38.9	42.2	29.2	34.9
Rdw (%)	29.0	23.6	22.2	26.4	32.4
Hct (%)	19.3	26.8	27.1	14.2	16.6
HbA_2/E (%)	83.2	73.6	90.3	17.8	28.1
HbA0 (%)	8.1	3.6	2.1	70.3	36.8
HbF (%)	5.3	2.1	1.7	4.0	33.6
P3 (%)	3.3	5.3	5.1	4.7	ND
History of BT	No	No	No	1st BT on 8 months, then on monthly BT	1st BT on 1½ years, then on monthly BT

MCV, mean cell volume; MCH, mean cell hemoglobin; MCHC, mean cell hemoglobin concentration; Rdw, red cell distribution width; Hct, hematocrit; ND, not detected; BT, blood transfusion.

phate was done to deplete the high abundant proteins from the plasma fraction as described in our earlier work.[9] The depleted plasma fraction was then dialyzed against 10 mM Tris, 5 mM KCl, pH 7.5, at 4°C, which was then used for 2D gel experiments. The protein sample was then separated by 2D gel electrophoresis (Bio-Rad) and the gels were stained following the protocol as described in our earlier work and densitometry analysis done using the PDQuest (V 7.1) software package (Bio-Rad).

Results and Discussion

Mutational studies

The clinical features of the family members are given in Table 1. The HPLC results indicate that the father (P1), the younger son (P4) and the non-related sample P5 are HbE-β-thalassemics and the mother (P2) and the elder son (P3) are HbEE. These data were reconfirmed by the results obtained from ARMS-PCR. The genotype of the four members on the basis of β globin mutations are following: P1 and P4 are Cod 26 (G-A) / IVS 1-5 (G-C), P2 and P3 are Cod 26 (G-A) / Cod 26 (G-A).

P2 and P3 are homozygous for HbE, hence no other β mutations were found in them, which confirm their homozygous state of HbE. P1 and P4 have another β mutation IVS 1-5 (G-C) along with Cod 26 (G-A), responsible for making them compound heterozygous *i.e.* HbE-β-thalassemia. These mutational studies also confirm that, P4, who is the main concern of this study, inherits HbE [Cod 26 (G-A)] allele from mother (P2) and other mutated β [IVS 1-5 (G-C)] allele from father (P1) (Figure 1).

Differences in the fractionated plasma proteome

On comparing the normalized intensities of

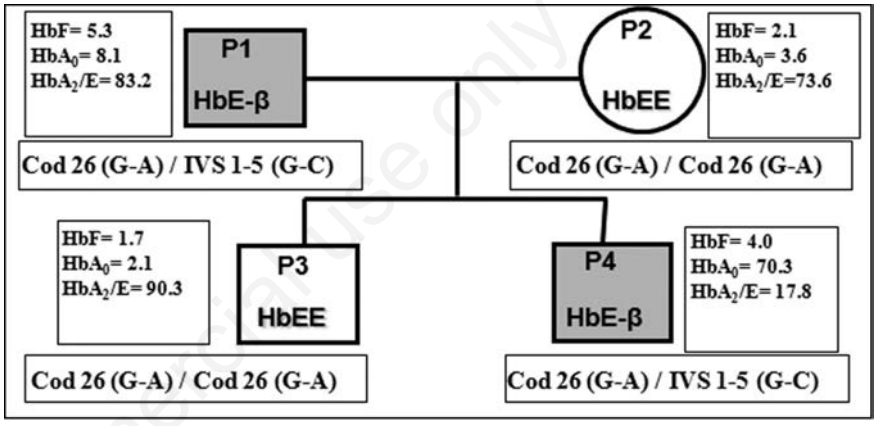

Figure 1. Pedigree of the family along with major clinical and mutational data. Hb, hemoglobin.

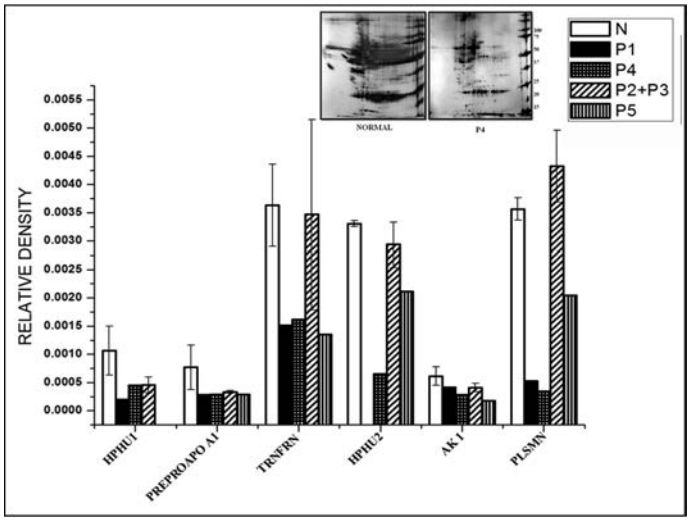

Figure 2. Histogram plots showing change in relative densities of the 6 differentially regulated proteins in the plasma fraction. Since this was a case study, statistical analysis on the test samples were not done. Deviations from the mean of two normal (empty bar) and homozygous E (in slanted striped bar) samples are shown. The inset shows a 2D gels of normal and P4. N, normal; P1, father; P4, younger son; P2+P3, averaged P2 (mother) and P3 (elder son); P5, unrelated HbE-β-thalassemic sample; HPHU1, haptoglobin precursor 1, PREPROAPO A1, preproapolipoprotein A1, TRNFRN, transferrin; HPHU2, haptoglobin precursor 2; AK 1, adenylate kinase 1; PLSMN, plasminogen.

the protein spots of the gels in the five individual samples, few proteins have shown differential expression. Haptoglobin precursor 1 (HPHU1) and preproapolipoprotein A1 (LPHUA1) shows decrease in all five samples compared to the normal samples. On the other hand, the samples P4 and P5 who are heavily transfusion dependent show down regulation of haptoglobin precursor (HPHU2) and adenylate kinase 1 (Q5T9B7). HPHU1 and LPHUA1 show down regulation in all. The plausible explanation for the down regulation of haptoglobins could be due to the fact that erythrocytes are exposed to varying degrees of oxidative stress, leading to differential hemolysis. Haptoglobins in the plasma scavenge for free hemoglobin released due to hemolysis, thus leads to a decrease in the level of haptoglobin in these samples. Preproapolipoprotein A1 is found primarily on high-density lipoprotein cholesterol. It has been observed that the down regulation of apolipoprotein A1 is related to the increase of pulmonary hypertension, which is a manifestation of acute or chronic hemolysis[10,11] (Figure 2).

Transferrin (Q53H26) and plasminogen (Q5TEH4) show down regulation in the P1, P4 and P5. The extent of down regulation is lesser in P5, which could be attributed to the presence of very high level of HbF (33%).[12] The lowering of the transferrin levels could be due to iron overload, commonly resulting from frequent blood transfusion.[13] Plasminogen is an inactive precursor of plasmin, and is involved in fibrinolysis. Chronic hypercoagulable state is observed in thalassemic patients undergoing regular transfusion, like in case of P4. Lowering of the plasminogen level could be a manifestation of this state.[14] Since father does not take any transfusion, we can only assume that the decrease in plasminogen in father is due to the maintenance of homeostasis from oxidative stress produced in the blood.

The haptoglobin precursor (HPHU2) and adenylate kinase 1 (Q5T9B7_HUMAN) are the two proteins, which is seen to be down regulated only in P4 and P5 as compared to normal. In this case, haptoglobin precursor (HPHU2) can be explained in the same light as haptoglobin precursor (HPHU1). Adenylate kinase 1 in plasma has been reported to play a role in the irreversible breakdown of any adenosine diphosphate (ADP) accessible to it. The aggregation of platelets by ADP, in turn is thought to play a role in homeostasis and thrombosis. It is inferred that degradation of ADP leads to the reversal of aggregation. Hence the down regulation of adenylate kinase 1 is seen in case of the transfusion dependent patients, who are prone to thrombolytic events.[15] As shown in earlier work,[4] redox regulator proteins like, peroxiredoxin 2 (prdx2), thioredoxin, Cu-Zn superoxide dismutase and chaperones like hsp70 and α hemoglobin stabilizing protein show marked increase in erythrocyte cytosol and the extent of increase depends on the extent of HbE percentage present in the samples. It is seen in our earlier proteomic study[4] that patients having HbE levels ranging in between 50-60% show higher changes in these proteins compared to those with >80% HbE, as observed here in case of P1 with 83.2% HbE.

To summaries, we have seen that the genomics study of the family showed that P1 and P4 has the exactly identical mutation in their β globin gene, but the father showed no clinical manifestation, whereas the younger son is severely anemic and requires blood transfusion every month. Proteomic study revealed that few plasma proteins, involved in hemoglobin scavenging, hemolysis or hypercoagulation show differential changes between the strongly transfusion dependent patients, P4 and P5 from those of normal individuals and the ones who do not need blood transfusions, P1, P2 and P3 indicating the changes to be specific to the disease conditions. Erythrocytes from the P4 and P5 undergo oxidative stress, are prone to hemolysis and found to have tendencies of hypercoagulation. Taken together, this report shows the importance of differential protein expressions in the clinical presentation of the disease as well as the modulators of globin synthesis.[16] The combination of both genomic and proteomic approaches could provide a better understanding of the disease progression and pathophysiology.

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Research Article Open Access

Fractional Precipitation of Plasma Proteome by Ammonium Sulphate: Case Studies in Leukemia and Thalassemia

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Abstract

Human plasma proteome is a comprehensive source of disease biomarkers. However, the >10 orders-wide dynamic concentration range of its constituent proteins necessitates depletion of abundant proteins from plasma prior to biomarker discovery. Our objective has been to develop a simple method that would deplete the most abundant proteins e.g. albumin and immunoglobulins, effectively facilitating identification of differentially regulated proteins in plasma samples. We employed ammonium sulphate based pre-fractionation of plasma followed by two-dimensional gel electrophoresis (2DGE) for comparison of normal proteins with those from the plasma samples of the patients, after identification of proteins by MALDI-TOF/TOF tandem mass spectrometry. Fractional precipitation of the plasma samples by 20% ammonium sulphate from raw plasma doubled the number of protein spots after 2DGE and led to identification of 87 unique proteins, including several low-abundance proteins. Case studies done with fractional precipitation of the plasma samples of patients suffering from hematological diseases e.g. leukemia and thalassemia indicate the utility of such pre-fractionation in the detection of differentially regulated proteins.

Keywords: Depletion technique; Blood plasma; Differential proteomics; Hematological malignancy; Hemoglobinopathy; 2DGE

Introduction

Blood plasma is a rich source of biochemical products that can indicate physiological or clinical status of a patient [1]. It is the most valuable specimen for protein biomarker determination because it is readily obtainable and contains thousands of protein species secreted from cells and tissues [2,3]. The discovery of protein biomarkers in plasma for diseases is challenging and requires a highly parallel display and quantization strategy for proteins [4-6] like two dimensional gel electrophoresis (2DGE). The protein content of serum however, is dominated by a handful of proteins such as albumin, immunoglobulins (IgG), and lipoproteins present across an extraordinary dynamic range of concentration. This exceeds the analytical capabilities of traditional proteomic methods, making detection of lower abundance serum proteins extremely challenging. Reduction of sample complexity is thus an essential first step in the analysis of plasma proteome [7].

There have been three main methods of depleting abundant proteins from serum samples: affinity removal method [1,4,8-10]; membrane filtration method to separate low-mass proteins from high-mass ones [7]; and multidimensional chromatographic fractionation [3,4,6]. But all these methods are expensive, laborious and time-consuming, as depletion of multiple abundant proteins from each plasma sample requires multiple technical steps. Besides, all these studies were mainly concentrated on the depletion of high-abundance proteins, primarily albumin and IgG, with little attention to detection of low-abundance biomarker proteins. We have employed 20% ammonium sulphate precipitation for rapid depletion of abundant proteins from plasma. Fountoulakis and coworkers have earlier reported fractionation of plasma proteins with 50% and 70% ammonium sulphate to reduce concentrations of high-abundance components and enrich lower abundant components in plasma 2D profiles, thereby facilitating the identification of disease markers [11]. Unlike other albumin-depletion studies, we checked the efficiency of our method in detecting differentially regulated plasma proteins in hematological malignancies like B-cell acute lymphoblastic leukemia (B-ALL), acute myeloid leukemia (AML) and in the hemoglobinopathy, HbEβ–thalassemia. We could detect differential regulation of several proteins in leukemic and thalassemic plasma samples compared to normal controls which includes many differentially regulated proteins in leukemic plasma samples also identified earlier.

Materials and Methods

Fractionation of plasma proteins using ammonium sulphate

Blood plasma samples of healthy normal volunteers, and B-ALL, AML and HbEβ-thalassemia patients on *de novo* diagnosis, were collected from R.K. Mission Hospital and Clinical Hematology Service, Kolkata. Clinical details of normal individuals and patients are summarized in Supplementary material 1. Written consent was obtained from all of the participants, and the study was conducted in accordance with the principles of the Helsinki Declaration with the approval of the institutional ethics committee. Complete protease inhibitor cocktail (Roche Diagnostics, Germany) was added whenever plasma was stored at -80°C for later use. Plasma samples were centrifuged at 12000 g, 4°C, for 30 minutes, and the supernatants diluted with PBS (2.7 mM KCl, 1.5 mM KH_2PO_4, 137 mM NaCl, 8.1 mM Na_2HPO_4, pH 7.4) to protein concentrations ~20 mg/ml. Diluted plasma samples were distributed into 1 ml aliquots. Next, 55, 113, 144, 176, 208, 242, 277, 314, and 351 milligrams of $(NH_4)_2SO_4$ were added to different aliquots for attaining 10%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, and 55% salt concentrations respectively, and incubated on ice for

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30 minutes with occasional mixing. The solutions were centrifuged at 12000 g, 4°C for 25 minutes, the supernatants taken in fresh tubes and the precipitate dissolved in minimum volume of solubilization buffer (5 mM sodium phosphate, 20 mM KCl, 1 mM EDTA, 0.2 mM DTT, and pH-8.0). The starting plasma, the supernatant and the solubilized ammonium sulphate precipitate, all three were dialysed overnight against 10 mM Tris, 5 mM KCl, pH-7.5, at 4°C.

Two dimensional gel electrophoresis and image analysis

After dialysis, the starting plasma, the supernatant and the solubilized ammonium sulphate precipitate, all three were mixed with equal volume of 2D sample buffer containing 8 M urea, 2% (w/v) CHAPS, 0.05% Bio-lyte 3-10 ampholyte, 20 mM DTT (Bio-Rad, Hercules, CA) and Protease inhibitor (Roche Diagnostics). The

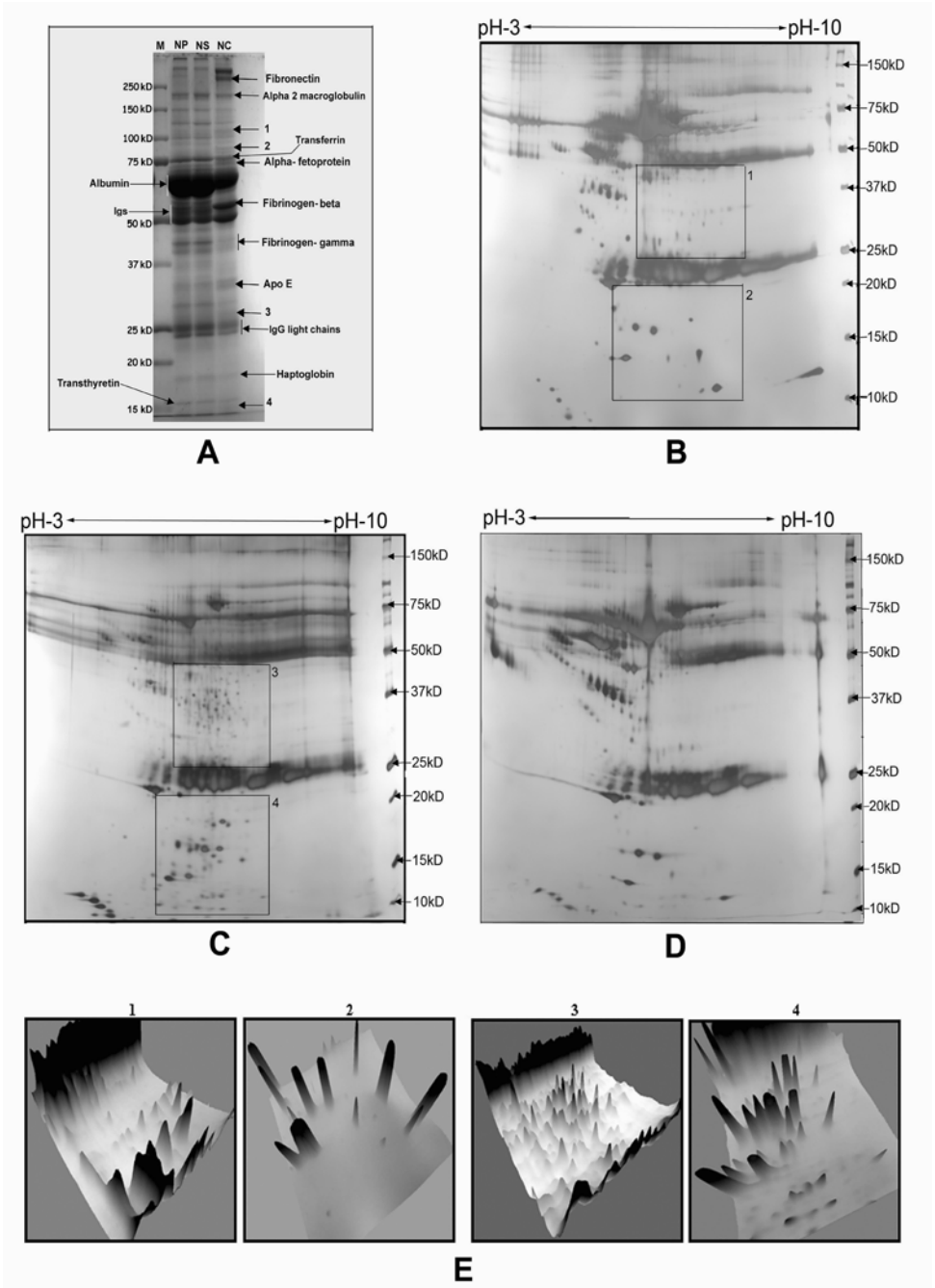

Figure 1: 1A. Coomassie stained 1D SDS-PAGE of raw plasma, the ammonium sulphate precipitate and supernatant. Lane1(from left)- MW marker (M); Lane2- raw normal plasma (NP); Lane 3- supernatant left after 20% $(NH_4)_2SO_4$ precipitation from normal plasma (NS), Lane 4- 20% $(NH_4)_2SO_4$ precipitate/cut from normal plasma (NC).
1B. Silver stained 2D profile of raw normal plasma.
1C. Silver stained 2D profile of 20% $(NH_4)_2SO_4$ precipitate from normal plasma.
1D. Silver stained 2D profile of supernatant left after 20% $(NH_4)_2SO_4$ precipitation.
1E. 3D view of boxed regions in 1B and 1C

protein concentrations of the samples were estimated using RC DC protein estimation kit (Bio-Rad), and an absolute amount of 1.8 mg for Coomassie staining, or 600 µg for silver staining, or 1.2 mg for SYPRO RUBY staining, was taken in a final volume of 350 µl. 17 cm pH 3-10 IPG strips (Bio-Rad) were passively rehydrated or cup-loaded with the plasma samples. IEF was carried out in a Protean IEF cell (Bio-Rad), stepwise up to 120000 Volt-Hours. Equilibration of the strips post IEF was performed following published protocol [12]. The second dimension was run on 8-16% polyacrylamide gradient gels in a Protean II XL electrophoresis module (Bio-Rad). Gels were stained either with Blue Silver Coomassie [13] or SYPRO-RUBY (Sigma) according to manufacturer's instructions, or Silver stain according to the method of Rabilloud [14]. Image captures and analyses were done on Versa Doc series 3000 imaging system using PDQuest software (version 7.1, Bio-Rad). Densitometry analysis of the gel spots of interest was performed using the density tool of PDQuest. Spot volume (intensity) of the desired spot(s) was normalized as parts per million (ppm) of the total spot volume using the spots that were present in all gels, to calculate the relative abundance of a spot in a sample.

In-gel tryptic digestion and mass spectrometry

Sequencing grade trypsin was purchased from Promega (Madison, WI). All other reagents were purchased from Pierce (Rockford, USA). The protein spots from Coomassie and SYPRO-RUBY stained 2D gels of normal plasma were excised using a robotic spot-cutter (Bio-Rad). The gel pieces were de-stained with 50% acetonitrile, 25 mM ammoniun bicarbonate. Subsequent in-gel tryptic digestion, peptide elution, acquisition of MS and MS/MS spectra and database searches were done following our published protocol [15]. Recrystallized CHCA and 2, 5-DHB (Sigma) were used as matrices. MS of the digested peptides was done in positive reflector mode in a MALDI-TOF/TOF tandem mass spectrometer (Applied Biosystems, AB 4700). Autotryptic and common keratin peaks were validated and subsequently excluded from MS/MS analysis. Twelve most intense peptides from each spot were subjected to MS/MS analysis. Peak lists were prepared from MS and MS/MS data using GPS explorer V3.6 (Applied Biosystems) software and noise reduction and de-isotoping were performed using default settings. Resulting PMF and MS/MS data were searched against human MSDB and Swiss-Prot databases using in-house MASCOT V2.1 (Matrix Science, UK) server and MOWSE score (with $p<0.05$) was considered to determine significant hits. For homologous proteins having similar MOWSE scores, preference was given to the protein with best match between theoretical and experimental molecular weight and pI. All MS experiments were repeated at least thrice, with spots excised from three separate gels. The database search parameters included one missed cleavage, error tolerance of ± 100 ppm for PMF and ± 1.2 Da for MS/MS ion search and variable modifications like carbamidomethyl cysteine, methionine oxidation, and N-terminal acetylation.

Western immunoblotting

Plasma protein samples (25 µg) were re-suspended in 30 µL SDS-PAGE buffer (2% mercaptoethanol (v/v), 1% SDS, 12% glycerol, 50 mM Tris-HCl and a trace amount of bromophenol blue), heated at 95°C for 5 min, cooled and loaded directly onto 12% gel. 1D-SDS-PAGE was performed in a Mini Protean III-cell (Bio-Rad) using Tris-glycine with 0.1% SDS, following manufacturer's instructions. Proteins separated on gel were blotted onto PVDF membranes and subsequently blocked with Tris-buffer-saline (TBS), 5% non fat dry milk for 2h at room temperature. Primary antibodies (Abcam) were diluted in TBS/0.1% Tween (TBST) following manufacturer's protocol. β-Tubulin was used as loading control. Anti-rabbit or anti-mouse HRP-conjugated IgGs were used as secondary antibodies (Abcam). Membranes were washed with TBST and detected by ECL (Pierce) with either the VersaDoc imager (BioRad) or on X-ray film development.

Results

Separation of pre-fractionated plasma proteins using 2DGE

1D-SDS-PAGE profiles of raw plasma, sub-fractions after $(NH_4)_2SO_4$ precipitations and respective supernatants showed more number of protein bands only in sub-fractions after 20% and 45% $(NH_4)_2SO_4$ precipitations (Supplementary material 2). We've chosen the sub-fraction after 20% $(NH_4)_2SO_4$ precipitation for further 2DGE analysis, which appeared to precipitate the maximum proportion of lower abundance proteins leaving most of the abundant proteins in solution. From 20 mg protein in raw plasma, 3.5 ± 0.8 mg was obtained in the 20% $(NH_4)_2SO_4$ precipitate while the supernatant retained the rest of it (15 ± 1.6 mg estimated). Both 1D and 2D profiles of raw diluted plasma, the fraction after 20% $(NH_4)_2SO_4$ precipitation and the supernatant after precipitation together revealed that $(NH_4)_2SO_4$ precipitates only a fraction of the whole plasma proteome. The particular fraction contained reduced load of abundant plasma proteins and was enriched with various minor proteins leaving the gel electrophoresis profile of the supernatant almost identical to that of raw plasma (Figure 1). The 1D-SDS-PAGE showed depletion of abundant proteins like albumin and IgG and enrichment/appearance of several low-abundance proteins including a tissue leakage protein, α-fetoprotein in the precipitated fraction (Figure 1A). The high percentage of albumin was found to be depleted (>80%) ensuring resolution of other proteins that were obscured by albumin in 2D gels, and minor proteins that were initially hidden by co-migration with albumin or smears became visible (Figures 1B-1D). The number of spots visible in 2D gels was

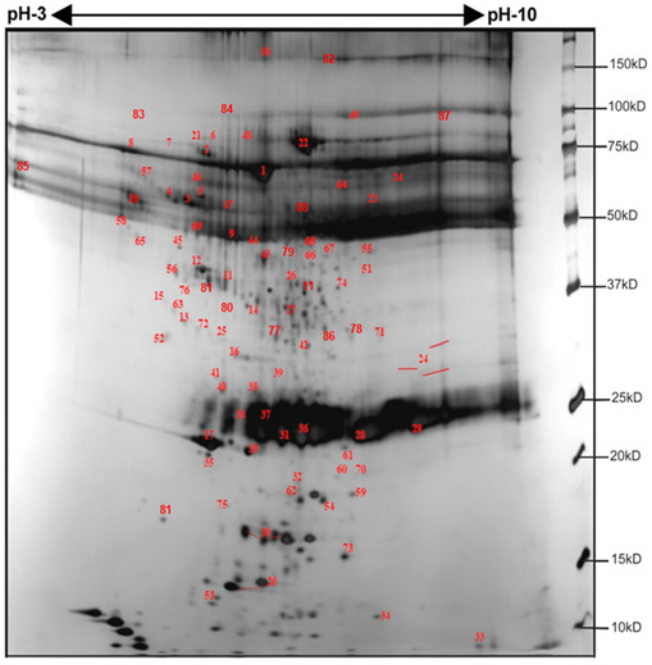

Figure 2: Annotated proteins in the fraction after 20% ammonium sulphate precipitation from normal plasma. Mass spectrometry details appear in Table 1.

doubled from 348 in raw plasma to 617 in the fraction precipitated by 20% $(NH_4)_2SO_4$ with various new spots appearing in the pI region 4.5-6.5 and between 10 kDa and 50 kDa molecular mass (Figure 1E).

We compared the 2DGE profiles of the fraction precipitated by 20% $(NH_4)_2SO_4$ and the albumin-depleted plasma after treatment with commercially available albumin depletion kit (ProteoPrep Blue Albumin & IgG Depletion kit, Sigma, St.Louis, MO). Supplementary material 3 provides the 2D gel images that shows better performance 20% $(NH_4)_2SO_4$ to justify the choice of conventional salting-out for enrichment of minor proteins in addition to depletion of abundant proteins from plasma, and preliminary screening of clinical samples. Although the ProteoPrep Blue Albumin and IgG Depletion kit specifically depleted albumin and IgG, the number of spots visible upon albumin-depletion did not increase appreciably as seen from the 2DGE profile.

Identification of plasma proteins by tandem mass spectrometry

Post $(NH_4)_2SO_4$ precipitation based pre-fractionation, a total of 87 proteins were identified from coomassie and SYPRO-RUBY stained 2D gels of normal plasma, by performing combined searches (MS + MS/MS) against MSDB, NCBI, Swissprot databases, as shown in Figure 2 and elaborated in Table 1. Of these 64 had significant scores ($p \leq 0.05$) in the combined searches. Many of the rest 24 protein identifications were supported by either the published SWISS-2D-PAGE map of human plasma (marked with asterisk '*' in Table 1), or ion score ≥ 20 of at least one MS/MS fragment, or other proteomic studies of blood plasma/serum [4,6,7] (marked with '**'). The list included many low abundance proteins that were undetectable in normal plasma prior to $(NH_4)_2SO_4$ precipitation. Low-abundance proteins present in amounts five to nine orders of magnitude lower than albumin, like

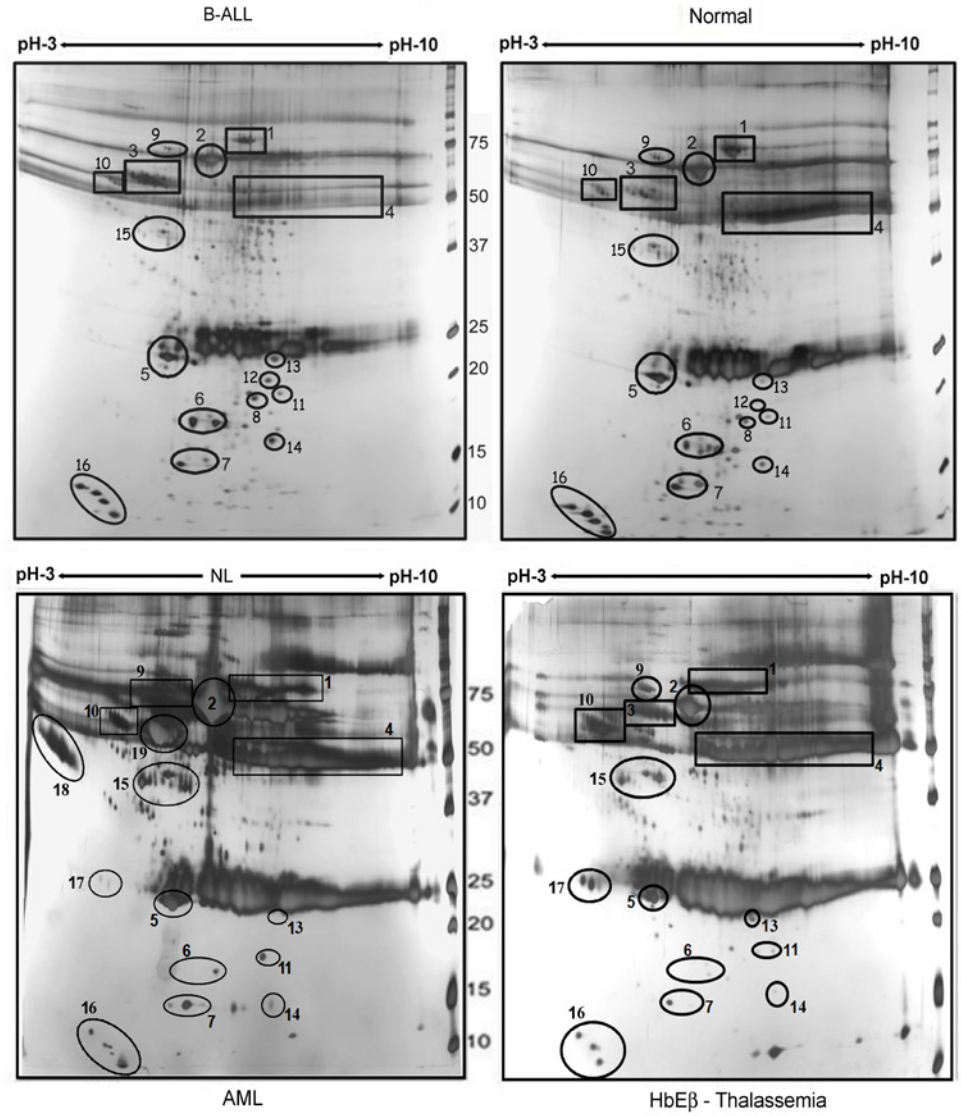

Figure 3: Display of differentially regulated proteins in the blood plasma of patients suffering from B-ALL / AML / HbEβ-thalassemia compared to normal control.

1:Transferrin, 2:Albumin, 3: α1-antitrypsin, 4:IgG heavy chains, 5:Apo-A1, 6:Haptoglobin α-chains, 7:Transthyretin, 8:Retinol binding protein 9:Interferon-β, 10:α1-B glycoprotein, 11:α2-HS glycoprotein, 12:Glutathione-S-transferase, 13:SET domain bifurcated, 14:Adenylate kinase-1, 15:T-cell receptor α-chain, 16:Haptoglobin α-chains, 17:Apo-E, 18:Apo-D, 19: Orosomucoid, 20: Fibrinogen α-chain.

Spot No.	Name of the Protein/Polypeptide	Accession Id.	Mr	pI	Mascot Score	Sequence Coverage	No. of MS/MS matches
1.	Serum Albumin-Human	1BKE	65,993	5.69	191(64)	64%	9
2.	Alpha-1-B-Glycoprotein-Human	Q68CK0_HUMAN	54,220	5.56	160(64)	48%	7
3.	Alpha-1-antitrypsin-Human	AAB59495	46,677	5.43	110(64)	36%	8
4.	Vitronectin precursor-Human	SGHU1V	54,328	5.55	112(64)	23%	5
5.	Kininogen, HMW precursor	KGHUH1	71,900	6.34	115(64)	40%	6
6.	Prothrombin-Human	Q4QZ40_HUMAN	69,920	5.70	202(64)	48%	8
7.	Plasma protease C1 inhibitor (fragment)-Human	Q59EI5_HUMAN	56,695	5.98	116(64)	27%	7
8.	Complement C1 inhibitor precursor-Human	ITHUC1	55,119	6.09	105(64)	29%	5
9.	Vitamin D binding protein-Human	Q53F31_HUMAN	52,916	5.34	221(64)	51%	8
10.	Alpha-2-HS-glycoprotein precursor-Human	WOHU	39,300	5.43	158(64)	40%	5
11.	Haptoglobin precursor-Human	HPHU1	38,427	6.13	242(64)	29%	5
12.	Human apolipoprotein-A-IV	AAA51748	43,358	5.22	494(64)	61%	9
13.	Complement component C3d-Human	1C3D	32,845	6.34	219(64)	44%	6
14.	Haptoglobin precursor	HPHU1	38,427	6.13	75(64)	14%	1
15.	Complement component C3b-Human	S27041	25,280	4.49	49(64)	55%	4
16.	1-microglobulin	HCHU	38,974	5.95	95(64)	35%	5
17.	APOA1 protein (fragment)-Human	CAA00975	28,061	5.27	470(64)	81%	11
18.	Serum amyloid P-Human	YLHUP	25,371	6.10	181(64)	30%	4
19.	Preproapolipoprotein A1-Human	LPHUA1	30,759	5.56	173(64)	59%	7
20.	Transthyretin chain A-Human	2TRYA	13,829	5.35	216(64)	94%	5
21.	Human α fetoprotein	E973181	66,358	5.67	264(64)	49%	6
22.	Transferrin-Human	Q53H26_HUMAN	77,030	6.68	339(64)	51%	8
23.	Fibrinogen β chain-Human	FGHUB	55,892	8.54	592(64)	49%	10
24.	Fibrinogen α chain-Human	FGHUA	69,714	8.23	508(64)	38%	8
25.	Fibrinogen β chain fragment d-Human	1FZAB	35,875	7.66	253(64)	79%	5
26.	Voltage gated Ca channel α23 subunit-Human	Q8IZS8_HUMAN	122,933	5.53	65(64)	29%	0
27.	Fibrinogen α chain extended splice form-Human	D44234	94,914	5.70	204(64)	35%	4
28.	Immunoglobulin κ light chain VLJ region-Human	BAC01677	27,574	7.53	160(64)	33%	3
29.	Immunoglobulin κ chain V-III region (B6)–Human	K3HUB6	11,628	9.34	59(64)	16%	1
30.	Haptoglobin precursor	HPHU2	45,177	6.13	71(64)	53%	5
31.	Immunoglobulin κ chain NIG26 precursor-Human	JEO242	23,504	5.46	203(64)	48%	3
32.	Immunoglobulin λ light chain variable region (fragment)-Human	AAD16673	11,505	5.67	32(64)	25%	1
33.	Hemoglobin α chain (fragment)-Human	Q9BX3_HUMAN	10,703	7.07	87(64)	60%	3
34.	Hemoglobin β chain-Human	2HBSB	15,827	7.26	143(64)	82%	6
35.	Ig light chain VLJ region (fragment)-Human	BAC01701	29,183	8.84	127(64)	39%	4
36.	Anti RhD monoclonal T125 κ light chain precursor-Human	Q5EFE6_HUMAN	25,682	8.70	56(64)	41%	2
37.	Fibrinogen fragment d, chain C-Human	1FZEC	34,457	5.68	64(64)	56%	2
38.	Fibrinogen fragment d, chain B-Human	1FZAB	35,875	7.66	137(64)	53%	5
39.	Fibrinogen fragment d, chain F-Human	1FZEF	34,343	5.68	109(64)	54%	4
40.	Fibrinogen fragment d, chain C-Human	1FZAC	35,144	5.57	91(64)	66%	3
41.	Fibrinogen beta chain precursor	FIBB_HUMAN	55,892	8.54	107(53)	48%	5
42.	Fibrinogen γ A chain precursor-Human	FGHUG	49,465	5.70	313(64)	55%	9
43.	ALB protein (Growth inhibiting protein 20)-Human	Q86YGO_HUMAN	47,330	5.97	170(64)	39%	8

44.	Apolipoprotein-A-IV precursor-Human	LPHUA4	45,307	5.23	107(64)	58%	5
45.	α1-antitrypsin precursor-Human	ITHU	46,707	5.37	248(64)	55%	8
46.	Fibrinogen γ B chain precursor-Human	FGHUGB	51,479	5.37	343(64)	64%	8
47.	Coagulation factor XIII chain b precursor-Human	KFHU13	75,442	5.97	94(64)	34%	3
48.	Plasminogen-Human	Q5TEH4_HUMAN	90,510	7.04	90(64)	40%	6
49.	Fibronectin1-Human	Q60FE4_HUMAN	252,848	5.66	224(64)	26%	13
50.	HUMPIS NID (CDC2-related protein kinase)-Human	AAA60092	35,549	9.02	65(64)	52%	1
51.	Replication licensing factor MCM2-Human	S42228	99,174	5.72	68(64)	21%	0
52.	(P02735) Serum amyloid A protein precursor-Human	SAA_HUMAN	13,524	6.28	157(64)	52%	2
53.	(P01574) Interferon beta precursor (IFN-beta) (Fibroblast interferon)-Human	IFNB_HUMAN	22,279	8.93	53(53)	28%	0
54.	(P61011) Signal recognition particle 54 kDa protein (SRP54)	SRP54_HUMAN	55,668	8.87	53(53)	16%	0
55.	Hypothetical protein DKFZp779N0926-Human	Q7Z664_HUMAN	45,064	5.76	547(64)	46%	6
56.	Collagen alpha 1(XI) chain precursor - human	CGHU1E	181,029	5.11	53(64)	21%	2
57.	Nicotinic acetylcholine receptor epsilon chain precursor - human	S34775	54,581	5.09	43(64)	16%	1
58.	Glutathione S-transferase A2	GSTA2_HUMAN	25,531	8.54	33(64)	85%	1
59.	SET domain, bifurcated 1-Human	Q5SZD8_HUMAN	27,685	4.85	45(64)	24%	1
60.	Adenylate Kinase 1-Human	Q5T9B7_HUMAN	23,396	8.78	29(64)	29%	2
61.	Interleukin-14 precursor –Human	A48203	54,723	9.32	26(64)	27%	1
62.	3' Histone mRNA exonuclease1	THEX1_HUMAN	39,907	6.32	45(64)	43%	1
63.	AB009303 NID membrane-type matrix metalloproteinase 3	BAA23742	69,451	8.72	58(64)	34%	1
64.	Leucine-rich PPR motif-containing protein-Human	Q7Z7A6_HUMAN	157,805	5.81	53(64)	17%	1
65.	BC015875 NID-Human (Selenoprotein P)	AAH15875	43,157	7.59	51(64)	26%	1
66.	1-Phosphatidylinositol-4-phosphate 5-kinase-Human	A55967	46,163	7.70	24(64)	9%	1
67.	HSP63G13 NID (p63 protein)-Human	AAG45609	55,652	6.41	60(64)	25%	1
68.	(Q9UPY3) Endoribonuclease Dicer-Human	DICER_HUMAN	217,490	5.45	47(53)	11%	1
69.	(P58340) Myeloid leukemia factor 1	MLF1_HUMAN	30,608	9.46	37(53)	34%	2
70.	Matrix metalloprotease MMP-27	Q9H306_HUMAN	58,986	8.83	36(64)	23%	2
71.	Apolipoprotein E precursor (ApoE)	APOE_HUMAN	36,132	5.65	284(64)	50%	7
72.	T-cell receptor β-chain precursor	CAA71260	15,318	6.07	46(64)	43%	1
73.	Homeobox protein CHX10	CHX10_HUMAN	39,386	7.11	33(53)	17%	1
74.	Ephrin-A1 precursor	EFNA1_HUMAN	23,756	6.49	41(53)	36%	1
75.	Intestinal alkaline phosphatase precursor	PPBI_HUMAN	56,776	5.53	36(53)	18%	1
76.	Ficolin 3 precursor	FCN3_HUMAN	32,868	6.20	96(64)	22%	4
77.	Complement component C4 fragment	Q5ST68_HUMAN	32,378	8.50	146(64)	37%	4
78.	Fibrinogen gamma chain precursor	FIBG_HUMAN	51,479	5.37	226(64)	30%	6
79.	Transthyretin precursor / Prealbumin (multimer)	TTHY_HUMAN	15,877	5.52	214(64)	81%	3
80.	Adrenocorticotrophic hormone (ACTH) (glycosylation shifts Mr and pI)	CAA00890	4,692	8.34	112(64)	78%	1
81.	Alpha-2-macroglobulin precursor (Alpha-2-M)	A2MG_HUMAN	163,175	6.00	74(53)	18%	2
82.	Inter-alpha-trypsin inhibitor heavy chain H4 precursor	ITIH4_HUMAN	103,294	6.51	73(53)	26%	3
83.	Complement C3 precursor	CO3_HUMAN	187,046	6.02	80(53)	25%	3
84.	Alpha-1-acid glycoprotein 1 precursor (Orosomucoid 1)	A1AG1_HUMAN	23,497	4.93	130(53)	31%	3
85.	histidine-rich glycoprotein precursor – human (glycosylation shifts pI)	KGHUGH	59,541	7.09	137(64)	22%	4
86.	Sodium/hydrogen exchanger 2 (NHE-2)	SL9A2_HUMAN	91,461	9.20	62(53)	13%	2
87.	C4A2 (C4A3)	Q6U2F0_HUMAN	58,393	5.67	77(64)	35%	6

* - protein identifications supported by the published SWISS-2D-PAGE map of human plasma
** - protein identifications supported by other proteomic studies of blood plasma/serum (Ref. 4, 6, 7)

Table 1: Protein Identifications from 20% $(NH_4)_2SO_4$ precipitate of normal plasma by 2DGE-MALDI ToF/ToF Tandem Mass Spectrometry.

serum amyloid P, vitamin D binding protein, interleukins, interferons, tissue leakage proteins (e.g. α-fetoprotein), ion channels and hormones were detected and identified from the fraction after 20% $(NH_4)_2SO_4$ precipitation, separated on 2D gels. This reflects a significant gain in the dynamic range of plasma proteins visualized in 2-D gels following 20% $(NH_4)_2SO_4$ precipitation. We could detect and identify some important blood plasma constituents, like fibrinogen-γ chain and immunoglobulin λ-light chain, that were absent from Anderson and co-workers' report of an exhaustive list of proteins detected and/or identified in plasma [16]. All the 87 proteins were searched for their molecular function, biological process and localization in the PANTHER classification system database17 indicating the identified proteins to be involved in multiple biological processes like blood coagulation, cargo transport, proteolysis, signal transduction, cell-adhesion, immunity/defense, etc.

Display of differentially regulated proteins in patient plasma

The clinical features of the B-ALL, AML, HbEβ-thalassemia patients and the normal controls are summarized in Supplementary material 1. As shown in Figure 3, a comparison between the fraction of raw plasma proteome fraction after 20% $(NH_4)_2SO_4$ precipitation, obtained from normal, B-ALL, AML and HbEβ-thalassemia samples revealed ~20 differentially regulated proteins. Differences in mean ppm spot volumes between normal controls and patient samples for all protein spots were subjected to unpaired two-tail student's t-test. Due to the inherent complexity of a 2D gel-based proteomic studies, we have only concentrated on the spots which were significantly different ($p \leq 0.01$) between normal and patient plasma sub-proteomes. We observed down-regulation of transferrin, albumin, immunoglobulin heavy chains, apolipoprotein A1 (Apo-A1), transthyretin, α1-B-glycoprotein, α2-HS-glycoprotein (AHSG); and up-regulation of α1-antitrypsin, haptoglobin, interferon-β (INF-β), glutathione-s-transferase (GST), SET domain bifurcated (SETDB), adenylate kinase-1 (AK-1), T-cell receptor-β (TCR-β) in the plasma of B-ALL patients as compared to normal plasma; shown as histogram plots in Figure 4. 2D profiles of samples from AML (hatched bars in Figure 4) and HbEβ-thalassemia (hollow bars in Figure 4) patient plasmas indicated opposite trend of differential regulation of most of these proteins, pointing towards the specificity of the observations.

Validation by western immunoblotting

To confirm the results obtained from 2DGE experiments, we quantitated the amounts of four differentially regulated proteins in raw plasma, obtained from a separate set of 3 normal controls and 4 B-ALL patients, using western immunoblotting. Supplementary material 4 shows the immunoblots for 5 proteins with β-tubulin as loading control, and histogram plot of the band intensities. All data were subjected to unpaired two-tail student's t-test and the changes were found to be significant ($p \leq 0.05$). The immunoblots clearly supported results from 2DGE experiments. The four proteins: transferrin, α1-antitrypsin, Apo-A1 and albumin, were chosen as representatives for proteolysis-modulating, carrier and acute phase proteins exhibiting differential regulation in B-ALL plasma 2D profiles.

Discussion

Since proteins differ markedly in their solubility at high ionic strength, salting-out has been the most efficient, time-tested and useful procedure for protein enrichment. The advantage of $(NH_4)_2SO_4$ is its high water-solubility leading to high ionic strength, and low heat of solvation protecting most proteins from denaturation [17]. This simple inexpensive fractionation of plasma proteins with depletes most of the high-abundance proteins e.g. albumin leading to an increase in low-abundance components, as also observed earlier [11]. As evident in this study, 20% $(NH_4)_2SO_4$ precipitation led to a representative fraction of the plasma proteome effectively facilitating detection of differentially-regulated protein markers in patient plasma samples with identification of several low-abundance proteins. The composition of the plasma proteome fraction after 20% $(NH_4)_2SO_4$ precipitation depends primarily on the quantity and solubility of the constituent proteins initially present in the sample, irrespective of the source or nature of the starting material. The fact that $(NH_4)_2SO_4$ does not differentially deplete plasma proteins from sample to sample has been apparent from the immunoblots of raw undepleted plasma samples, shown in Supplementary material 4. Our investigation of differential regulations in two different-lineage hematological malignancies, i.e. ALL & AML, and an unrelated blood disorder with similar symptoms *viz.* HbEβ-thalassemia, establishes the specificity of the observed de-regulations with respect to the disease. The specificity of the observations adds an extra line of evidence to the suitability of 20% $(NH_4)_2SO_4$ precipitation for detection of disease biomarkers in patient plasma samples. Immunoaffinity-based chromatography effectively depletes high-abundance proteins from the plasma, but even these expensive, laborious and time consuming commercially available methods fail to completely remove high-abundance components and suffer from

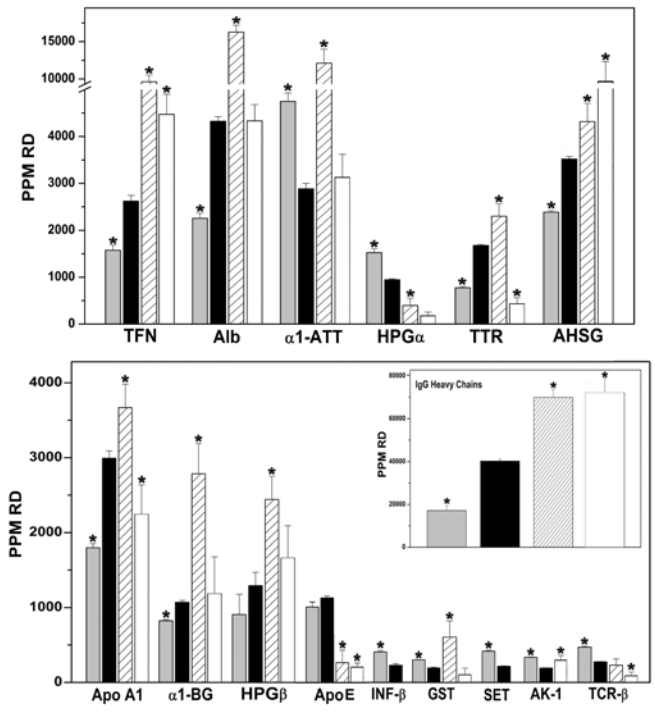

Figure 4: Histogram plots showing change in ppm relative densities (PPM RD) of the 15 differentially-regulated proteins. All data were subjected to unpaired two-tail student's t-test and significant ($p \leq 0.01$) changes from normal samples are marked with asterisk.

Gray Bars: B-ALL (n=8), Black bars: Normal (n=8), Hatched bars: AML (n=3), Hollow bars: HbEβ-thalassemia (n=4), TFN: Transferrin, Alb: Albumin, α1-ATT: α1-Antitrypsin, HPGα: Haptoglobin α-chain, TTR: Transthyretin, AHSG: α2-HS Glycoprotein precursor, Apo A1: Apolipoprotein A1, α1-BG: α1-B Glycoprotein, HPGα: Haptoglobin α-chain, Apo E: Apolipoprotein E, INF-α: Interferon α, GST: Glutathione-S-Transferase, SET: SET Domain Bifurcated, AK-1: Adenylate Kinase 1, TCR-α: T-cell receptor α-chain.

their own limitations of specificity [18-21]. Our approach has been to use a simple, cost-effective method to obtain plasma fractions with reduced content of abundant proteins and maximum number of well-resolved spots on 2D gels. Although $(NH_4)_2SO_4$ fractionation does not specifically deplete or remove a particular protein or class of proteins, however, it also does not show preference towards a particular protein mixture, irrespective of sample type (normal or patient). It treats two different types of body fluid samples e.g. plasma & urine, differently, but remains unbiased towards the source e.g. from patients or from normal volunteers. Thus, it could be effectively used for differential proteomics in clinical studies. Use of combinatorial peptide ligand libraries for depletion of abundant proteins and accessing low-abundance biomarkers in clinical proteomics studies of blood plasma [22-24] further supports our notion that any pre-fractionation strategy for plasma could come handy to increased access to disease-markers apart from depletion of high abundance components.

Many of our observations in disease plasma were supported by earlier reports. Haptoglobin up-regulation in AML, CML, and multiple myeloma has also been reported in earlier studies [25-27]. While 2D profiles support the up-regulation of haptoglobin β-chain in AML plasma [26], we emphasize on haptoglobin α-chain that exhibits opposite trends of de-regulation in AML/HbEβ-thalassemia and B-ALL. This further highlights the application of $(NH_4)_2SO_4$ precipitation for preliminary screening of patient plasma samples. AHSG is reported to be down-regulated in AML, ALL, NHL and multiple myeloma patients [25,26,28]. We observed down-regulation of AHSG in B-ALL plasma in contrast to an up-regulation in AML/HbEβ-thalassemia plasma (Figure 4). Since lymphoblasts fail to mature into antibody-secreting plasma cells in B-ALL, the patients show significant down-regulation of immunoglobulin heavy chains in plasma. In contrast, immunoglobulin heavy chains are up-regulated in AML/HbEβ-thalassemia patient sera [26]. Additionally, Apo-A1 is down-regulated in B-ALL plasma contrary to the observation in AML (Figure 4). Hence IgG and Apo-A1 can serve as important biomarkers of B-ALL. Up-regulation of transferrin, AHSG and AK-1, *vs.* down-regulation of transthyretin, Apo-A1, Apo-E and TCR-β in HbEβ-thalassemia plasma, compared to normal controls, are all preliminary reports that warrant further investigations with increased sample size. As most of the de-regulated proteins participate in multiple physiological processes like proteolysis, cargo-transport and iron homeostasis, their de-regulation might enlighten clinical manifestation of the disease. The western immunoblots qualitatively supported the 2DGE results but showed quantitative discrepancies in the degrees of deregulation of the proteins, most likely attributable to differences in the protein loads and detection limits of the two techniques. Moreover, immunoblot confirmation using a separate set of B-ALL patients and normal controls further emphasizes on the prospects of the reported de-regulations as potential diagnostic and prognostic indicators of the respective diseases, and that the differences do not arise out of the plasma pre-fractionation technique used. In conclusion, 20% ammonium sulphate precipitation shows prospects of accelerating the preliminary screening and detection of disease biomarkers in blood plasma. We further emphasize upon the assets of proteomic studies over single protein detection assays in revealing differential regulation of different classes of proteins, simultaneously in a disease, which might be a step ahead in cutting through the complexity diseases and explaining their pathophysiology and clinical manifestation. This study also reports for the first time a 2DGE based proteomic investigation of B-ALL and HbEβ-thalassemia.

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