Proteomic Signatures and Biomarkers in Early and Pro-Diagnosis of Type 2 Diabetes Mellitus

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Abstract

Advancements in proteomic technologies have significantly enhanced our understanding of human biology and opened new avenues in disease diagnosis and therapeutic development. Proteomics plays a pivotal role in unraveling the molecular mechanisms underlying various diseases, particularly metabolic disorders such as Type 2 Diabetes Mellitus (T2DM). Comparative proteomic analyses of serum samples from normal, pre-diabetic, and diabetic individuals—with or without abdominal obesity—using techniques such as two-dimensional electrophoresis (2DE) combined with MALDI-TOF mass spectrometry, followed by validation through western blotting, have revealed distinct protein expression patterns. Further analysis using network-based approaches and the STRING database has enabled the identification of key protein–protein interactions. Notably, proteins such as alpha-1-antichymotrypsin, apolipoprotein A-1, alpha-1-antitrypsin, haptoglobin, transthyretin, retinol-binding protein 4, and zinc-alpha-2-glycoprotein showed significant expression differences between normal and pre-diabetic/diabetic groups. Functional annotation through gene ontology indicated these proteins are predominantly involved in lipid transport, regulation of lipoprotein particle levels, and lipid localization. These findings suggest the potential utility of these proteins as early biomarkers for the diagnosis and progression monitoring of T2DM.

Keywords: - Proteomics, Diabetes mellitus type 2, Obesity, Biomarkers, Proteomics techniques, Diabetes

Introduction:- Diabetes mellitus type 2 (T2DM) is increasing day by day globally. One point about Diabetes mellitus type 2 (T2DM) should be noted that all suffering from diabetes mellitus 2 are not obese, some are underweighted also but all obese patients get affected by diabetes mellitus type 2 (T2DM).[1] To find out which patients are at higher risk for developing this disease. It is very important to understand their relation. Diabetes mellitus 2 is a result of insulin resistance. Both diabetes type 1 and 2 diabetes show serum lipid and lipoprotein abnormalities. Diabetes type 2 is related or connected with increase in the concentration of inflammatory reactions in serum [2, 3]. High concentration of tumour necrosis factor alpha and interleukins -6 related with type 2 diabetes and obesity might intervene with anti-inflammation of insulin [4] .so in such case large number of inflammatory cytokines can be used to detect insulin resistance and can tell increasing serum glucose [5]. Proteomics could be taken as the most reliable data set [6]. Proteome analysis of serum can be used to identify the biomarkers related to diagnosis or pro-diagnosis of diabetes and as a result, it can describe severity of disease and mechanism related to diabetes in body [7, 8, 9, 10]. More number of biomarkers is needed for better diagnosis because single protein marker usually does not fully describe severity of condition or condition with prominent clinical value [11, 12]. For quantification of disease associated alterations analytical technique can be used such as 2-DE combined with mass spectroscopy. It is used for complicated or biological sample because it can resolve hundreds to thousands of proteome forms in a single analytical run [13].

Overview of Diabetes: - Diabetes is a chronic disease which occurs due to insulin malfunction or less functioning when pancreas is not able to make insulin or body can’t use the insulin which pancreas is producing. Insulin is a hormone released by pancreas which acts like a key for glucose to let in the cell from blood stream to produce energy. All foods are broken down as glucose in blood and insulin helps the glucose to get in the cells for energy production. When pancreas is not able to produce insulin, it leads to rise in glucose level in the blood. For long term high glucose in blood can cause damage to body and failure of organs and tissues.

Type 1- Diabetes :- It can develop at any age but mostly found in children and adolescent. In this type of diabetes body produces very little or no insulin. In that case we have to give daily insulin injection to maintain glucose level in blood. It is caused by combination of genetic and environmental condition. In case of no insulin injection, person will die.

Type 2- Diabetes: - It is most occurring diabetes which is common in adults and accounts for 90% diabetes cases. In this type Body is not able to use insulin or respond to insulin and insulin can't work properly so blood glucose keeps on rising and pancreas starts releasing more and more insulin resulting in exhaustion of pancreas or pancreas malfunctioning and as a result production of insulin gets lower and lower causing high blood glucose level. It is mostly found in older adults. In 2014, 8.5% of adults aged 18 years and older had diabetes. In 2019, 1.5 million deaths were caused by diabetes. If we see a large picture then higher than optimal blood glucose causes more deaths due to cardiovascular disease, chronic kidney disease and tuberculosis [14]. Figure 1 illustrates the key signaling pathways involved in the pathophysiology of Type 2 Diabetes Mellitus within adipocytes, hepatocytes, and skeletal muscle cells. It highlights the molecular interactions and dysregulations associated with insulin resistance, glucose uptake, lipid metabolism, and inflammatory responses. This schematic provides a comprehensive overview of the cellular mechanisms contributing to disease progression and offers potential targets for therapeutic intervention. Figure 2 depicts the signaling pathways involved in Type 2 Diabetes Mellitus within pancreatic beta cells. It outlines the molecular mechanisms governing insulin synthesis and secretion, along with the factors leading to beta-cell dysfunction under diabetic conditions. The figure emphasizes the impact of oxidative stress, endoplasmic reticulum stress, and inflammatory signaling on beta-cell viability, providing insights into the cellular basis of insulin deficiency in Type 2 Diabetes.

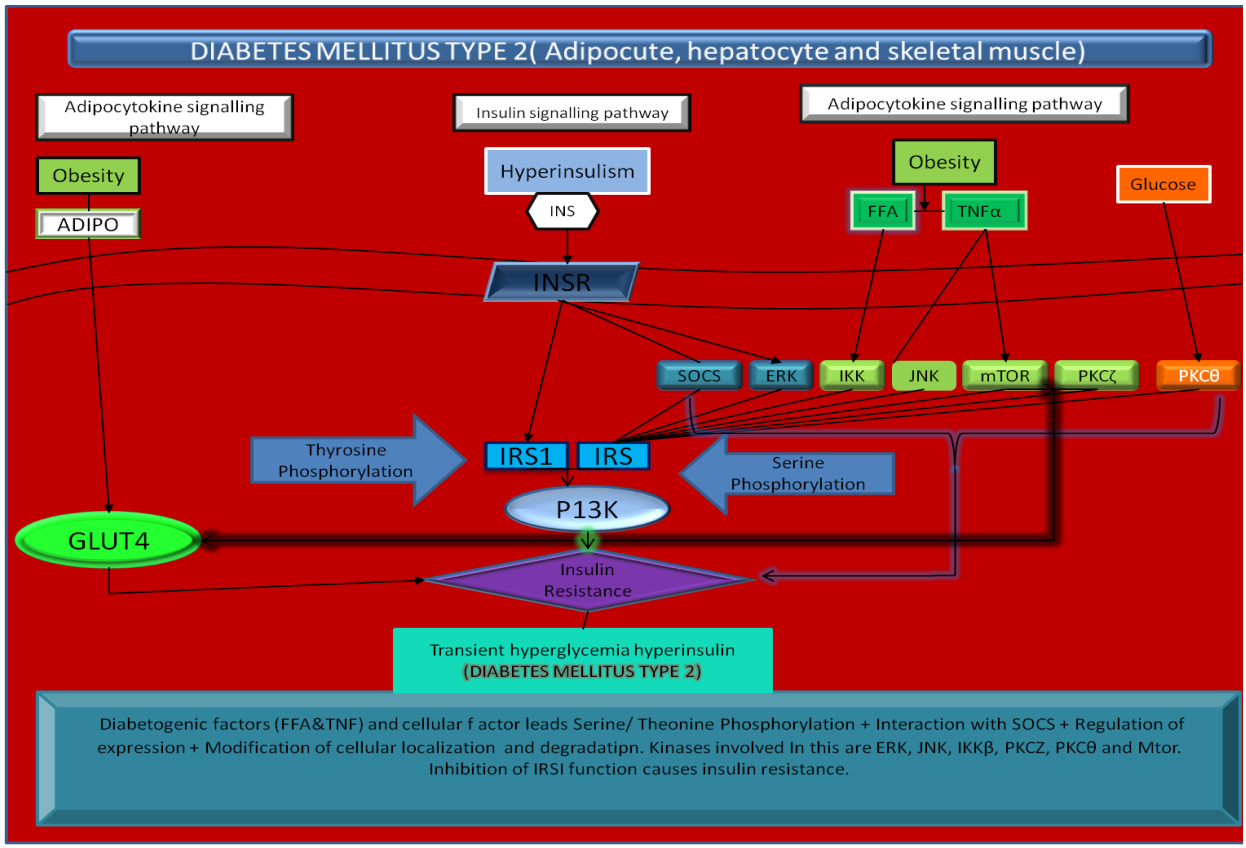

Fig-1-Diabetes mellitus type 2 signaling in adipocytes, hepatocytes and skeletal muscle cells.

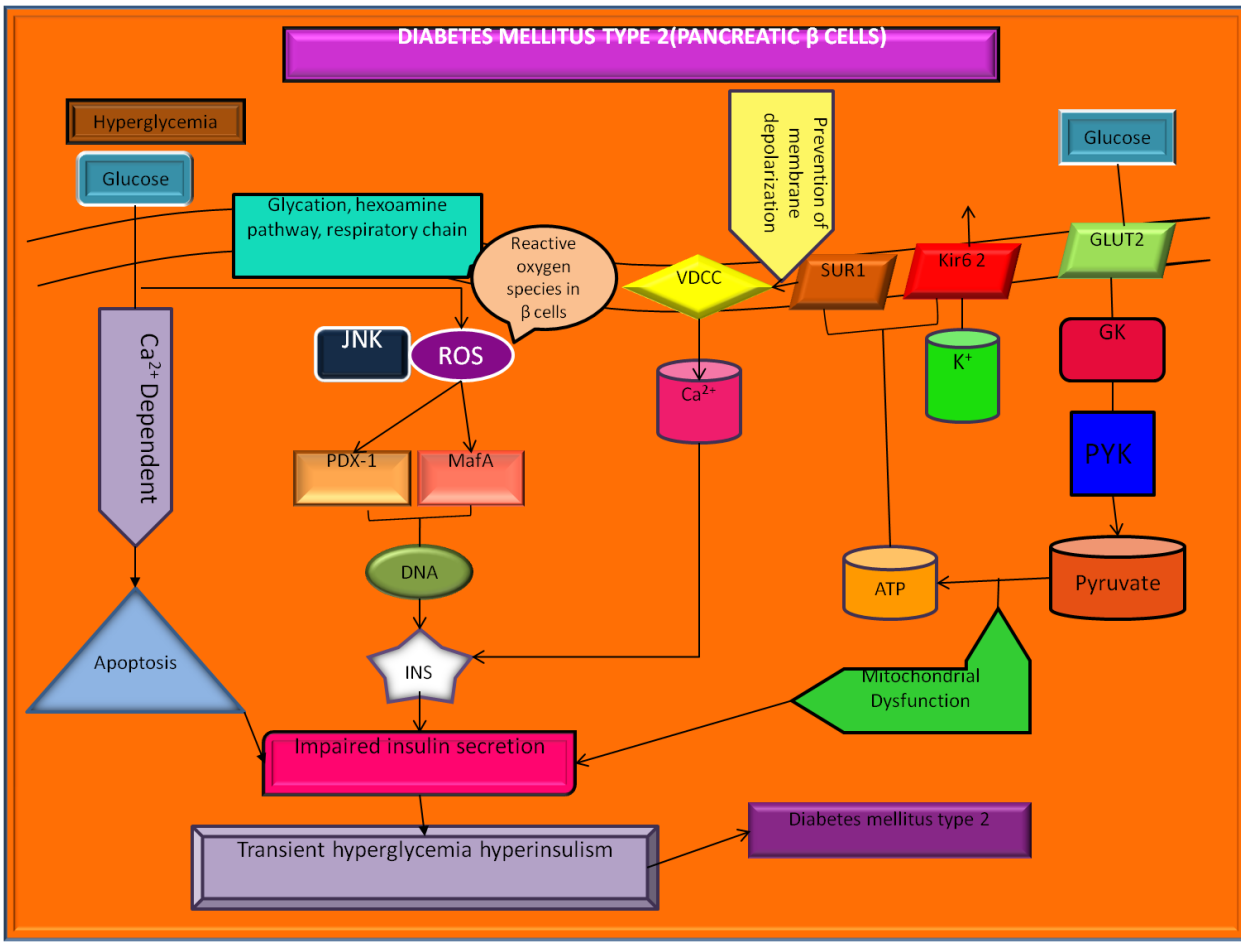

Fig-2-Diabetes mellitus type 2 signaling in beta cells.

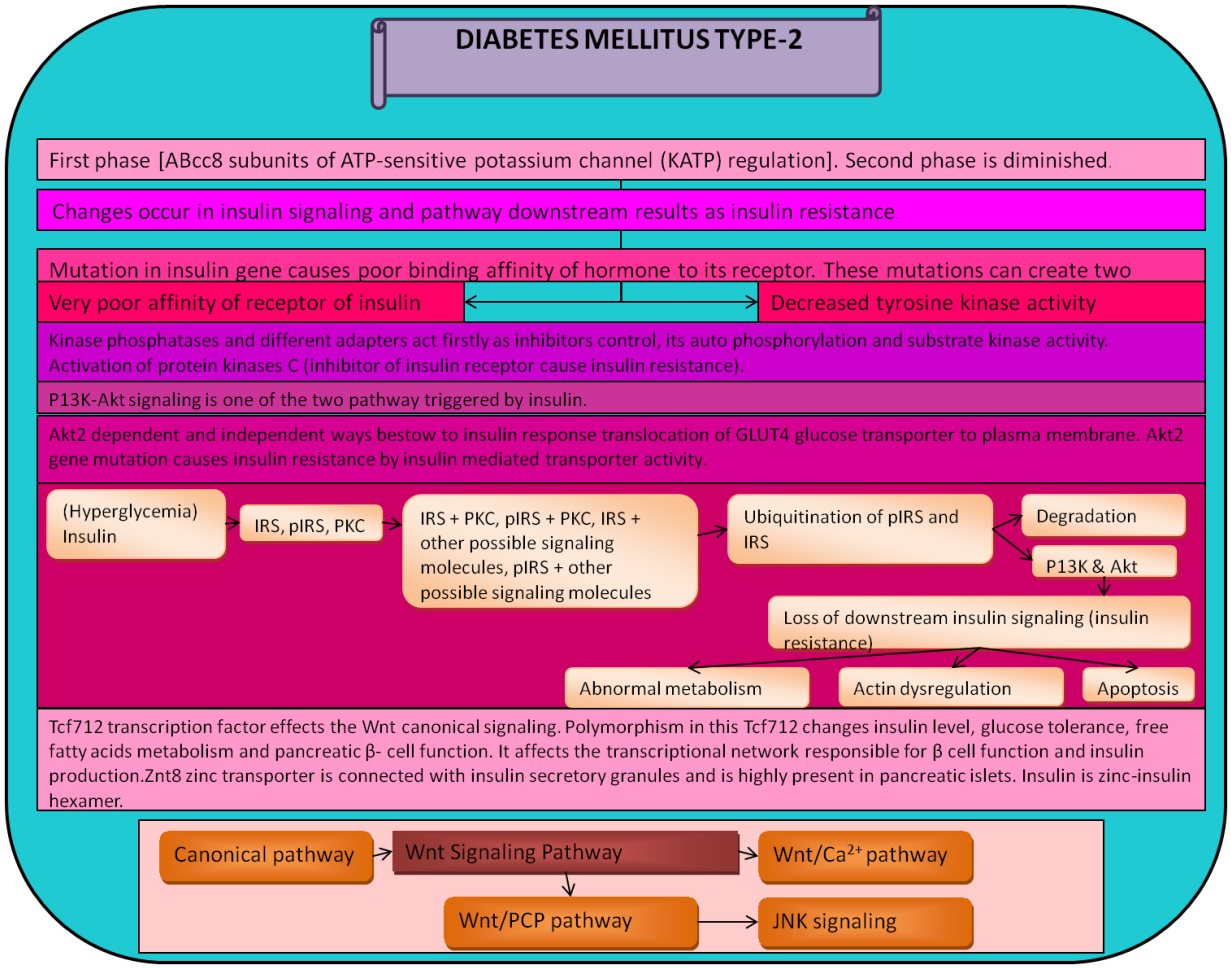

Fig 3-Diabetes mellitus type two changes cause of insulin resistance.

Overview of Proteomics:- Proteomics involve large scale study of proteins and their structure, identification of functions [15, 16]. Proteins are present in all living organism as they are most important part of cellular processes. In 1994, Mark Wilkins coined the word Proteome, which is a blend of protein and genome. He coined this term while working on the concept as a PhD student [17]. Whole set of protein produced or modified by organisms is known as Proteome. In 1997 the word Proteomics was first coined as analogous to genomics [18]. Proteomics allows the study of Proteome produced or modified by an organism or system and it varies with time and a variety of environmental factors [19]. Proteomics usually refers to a large-scale analysis of protein identification and purification. Genome is static and Proteome is dynamic. Proteome is basic component of functional genomics [20]. In Eukaryotes, post-translational modifications occur in many ways at different sites which makes them complex for proteomics

[21]. Figure 3 illustrates the physiological and molecular changes associated with insulin resistance in Type 2 Diabetes Mellitus.

Preparation of Sample (serum):- Fluids (blood, serum, urine and salivary) or tissue (for example – adipose) of body for sample preparation is very necessary pre requisite for advising robust and reproducible data through proteomics. For further analysis the Sample (body Fluids or tissue) must put to freeze at -80℃ in a bio baking system [22, 23] ordinarily aliquots are divided in many vials to prevent sample degradation from freezing and fluxing over and over again. Selection of particular area from human body while working on particular tissue (Adipose tissue) is really important. Comparable proteomics deals with small differences and comparision so for reliable resulting data proper sample preparation techniques should be applied [24]. Tissue handling is really important because different tissues require different methods and different homogenization. In Proteome assay changes can take place which can modify results. For example- contamination of protein sample (1) It can cause poor protein separation during electrophoresis, (2) Low expression of proteins in ms analysis, (3) 3-D structure can be changed due to other interaction with proteins [25]. Protein handling and storage process should be done carefully which can help in minimizing damage and maximizing the chances of an accurate result. The steps in the process that are extremely decisive are: - the use of anti-protease and anti-phosphatases and freezing of protein sample speedily after harvesting in several vials. From sample of patients the obtained protein is really low but clinical samples provide highly valuable data even with less amount. It is important to optimize the protocol for lysis of cell and tissue disruption for isolation of protein from body fluids like- serum, urine, blood and salivary fluids etc., Using reagents including protease and phosphatase inhibitor. Pre-analytical sample treatment can be used to remove extra protein [26], a lot of international laboratories are standardizing specific protocols for storing apart from analysis through proteomics and MS using regenerate protocols [27]. Albumin/IgG depletion kit may be essential at the time of serum or plasma analysis to identify protein with low concentration [28, 29,30]. Many proteins exist in isoform instead of single form. It may be formed due to PTM (Post-translational modifications) of a given protein, so for protocols the person who's following must know about isoform of protein. Selected reaction monitoring or multiple reaction monitoring assay can be used for the analysis of the proteins in the complex mixtures. Albumin and immunoglobulin are two main groups of protein which can create

difficulty in study of serum Proteome. These both comprises 95%of the total protein and remaining 5% includes cytokines, hormones, enzymes and cytoplasmic or nuclear proteins. Albumin and IgG can cover up the important changes in the target protein, both of these can be removed by affinity chromatography but in diabetes mellitus 2 Albumin has great importance [31]. Lipids have important role in cardiovascular disease and hyperlipidaemia, etc., lipids can interact with sample protein, so chylomicrons and low-density lipoprotein can be removed by fractionation (centrifugation) [32, 33, 34].

Methods used in proteomics

Methods based on Antibody: - To identify protein and their level of expression enzyme-linked immunosorbent assay and western blotting is used which depends on availability of antibodies targeted certain proteins. ELISA is used to quantitatively measure interferon γ and TNF α levels in serum sample. Capture ELISA was developed for Echinostomac. detection in infected experimental rats [35].

Methods Based on Gel: - The first proteomic techniques developed was two-dimensional electrophoresis (2DE or 2D – PAGE) in which electric current is used to separate protein in a gel. It is very useful for protein separation [36]. 2DE gel-based techniques uses two dimensions in which first dimension is separation of protein on the basis of net charge by isoelectric focusing through immobilized pH gradient gel strips and second dimension is separation on the basis of mass using SDS PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis. It measures mass to charge ratio after ionizing protein. 2DE has paired with a process in which different fluorescent dyes are used to allow the comparison of different protein samples in the same gel then that gel is laser scanned and multiple sample images acquired from single gel, this process is known as differential gel electrophoresis [37]. SDS-PAGE,2-DE, and 2D-DIGE techniques are used for complex protein sample [38, 39, 40]. These methods are used before the further analysis by mass spectroscopy.

Methods based on Chromatography: - These methods can be used to separate and purify proteins from complex mixtures. Such as Ion exchange chromatography, size exclusion chromatography and affinity chromatography [41, 42, 43]. Affinity Chromatography use reversible interactions between particular affinity ligands and their target proteins [44]. Size-exclusion Chromatography separate proteins on the basis of molecular size. Liquid

Chromatography is highly preferable for separation of proteins and it is often used with Mass Spectrometry (MS). With MS micrograms quantity is required [45]. Ion exchange Chromatography (IEX) separates protein based on charge. In this positively charge Molecules are attracted to negatively charged solid support and vice versa. There are cation exchange chromatography and anion exchange chromatography. SCX and SAX is often used with MS [46, 47]. SCX can purify peptide hormone and proteolytic fragments [48].

Microarrays: - Proteins Microarrays apply small amounts of a sample to a chip for analysis. Target Protein in compound sample is captured by particular antibodies which can be immobilized to the chip surface. If it's termed as analytical protein microarrays, these are used to calculate binding affinities and expression level of proteins. Protein functions for example protein- RNA interactions and enzyme substrate turnover are characterized by functional protein microarrays. Proteins such as untreated versus treated cells are bound to the chip, which is then probed with antibodies against the target proteins.

Protein technique based on Mass spectroscopy: - There are such protein separation techniques also which are gel free which allows quantitation and comparative proteomics such as stable isotope labelling with amino acids in cell culture (SILAC), isotope- coded affinity tag (ICAT), and isobaric tags for relative and absolute quantification (iTRAQ) [49, 50, 51, 52]. There is less quantitation technique which is multidimensional protein identification technology (MudPIT) [53].

Isobaric tag for relative able absolute quantitation: - Multiplexed isobaric chemical tagging reagent is used in this. It allows multiplexing of 2 to 8 complex sample at the same time and for 8 version of same derived tryptic peptide. It makes identical MS/MS sequencing ion [54]. Analysis is done by comparing the peak areas and ratios.113 to 119 and 121 Da reporter ions are used [55].

Stable isotope labelling with amino acids in cell culture: - For subsequent quantitative analysis *in-vivo* a label is incorporated into proteins. Its principle is based on metabolic fusion of a given form of amino acids (light or heavy) through substituted stable isotope forms into proteins [56].

Isotope - coded affinity tag: -This is in vitro isotopic labelling method. ICAT reagents are cysteine binding tags. These reagents are prior to pooling, digestion, cation and avidin Chromatography [57, 58, 59]. Quantitation is based on LC-MS peak areas of the stable isotope profile of cysteine containing peptides [60]. This technique is also used with Mass Spectrometric Proteome study [61, 62].

Sequential elution of immobilized metal affinity chromatography followed by TiO2: - Multi-phosphorylated peptides are isolated through IMAC and mono- phosphorylated peptides through TiO2. Its principle is that in both IMAC and TiO2 metal contains positive charges and link the negatively charged phosphorylated peptides. SIMAC appeared as a phosphorylated peptides enrichment tool which hands features of IMAC conjoined with TiO2 [63, 64, 65, 66, 67]. ZrO2 is another phosphorylated peptide enrichment method which is prior to MS [68].

Selected reaction monitoring or multiple reaction monitoring: -Two mass analyzers are used as static mass filter to monitor a specific fragment ion in SRM. In quantitative assay for extremely high sensitivity of resulting selectivity the two filtering stages coupled to high duty cycle. It is MS/MS based quantitative proteomics tool. In specific mass spectrometer SRM or MRM is carried out known as ion tapes and tripe quadrupole [69]. MIDAS-MRM is rapid and cost-effective technique which quantifies and validates the biomarkers without antibodies [70].

Label-free quantification: -In this technique protein mixtures are directly analyzed after this separate analysis then compared to each other. In this whole process there is no mixing of samples which gives higher proteome coverage. It is based on Mass spectrometry.

Mass spectrometry:- It is also known as mass spectroscopy which is an analytical technique in which chemical substances (in this case protein) are identified by gaseous ion sorting in electric and magnetic fields according on the basis of their mass to charge ratio. The instrument which is used is mass spectrometer. MS work flow has basically three steps: - Firstly ion source of mass spectrometer ionises proteins or peptides. Secondly mass analyzer separates resulting ions on the basis of their mass to charge ratio. Thirdly the ions are detected. At the time of gel free techniques, the resulting sample directly transferred in the mass spectrometer, while using gel-based methods the protein spots are cut out and digested before separating by LC or directly analyzed by MS. In MS, ionization sources used are-

1-Matrix associated laser desorption/ionization (MALDI) [71, 72].

2- Electrospray ionization (ESI) [73].

Less common methods are flow discharge ionization and electron impact. Four main mass analyzers are used-

1-Time-of-flight (TOF)

2-Ion trap

3-Quadrupole Fourier- transform ion cyclotron

4-Electrostatic sector and magnetic sector are two less commonly used adopted mass analyzers

MALDI is often used with TOF analyzer. This analyzer accelerates the ions in an electric field and measure time they take to reach the detector. ESI is used with quadrupole mass filter which consists of four rods made up of metal. Only one specific mass to charge ratio can be selected and reach the detector by controlling electric field (within four rods by radio frequency voltage). Both quadrupole ion trap and quadrupole filter work similarly except quadrupole ion trap catch the ions and release them sequentially.

Tandem mass spectroscopy, (MS/MS) or MS^n: -In this technique, peptides are subjected to multiple rounds of fragmentation and mass analysis. In first round of MS Peptides isolation takes place (Proteases for example trypsin break protein into peptides), In second round of MS fragmentation of peptides is done by neutral molecule through collision induced dissociation and lastly mass to charge ratio is measured. Sequence information is obtained by examining mass spectrum results. N- and C- terminal peptides are mainly generated by this technique [74, 75]. Figure-4 highlighted the different proteomics techniques.

Peptide mass fingerprinting: - Its main aim is to cleave the protein into smaller fragments and analyze in mass spectrometer. In general acetonitrile is used to extract the fragments and these fragments are dried using vacuum. The final result (Graph) obtained by MS is compared with databases.

Top-down and bottoms-up proteomics: -Top-down proteomics uses ion trapping mass spectrometer to store an isolated protein ions for mass measurement and tandem MS analysis

or other purification methods such as 2D gel electrophoresis [76, 77, 78]. In Bottoms-up proteomics proteolytic digestion of proteins is done and then result is analyzed by MS [79, 80].

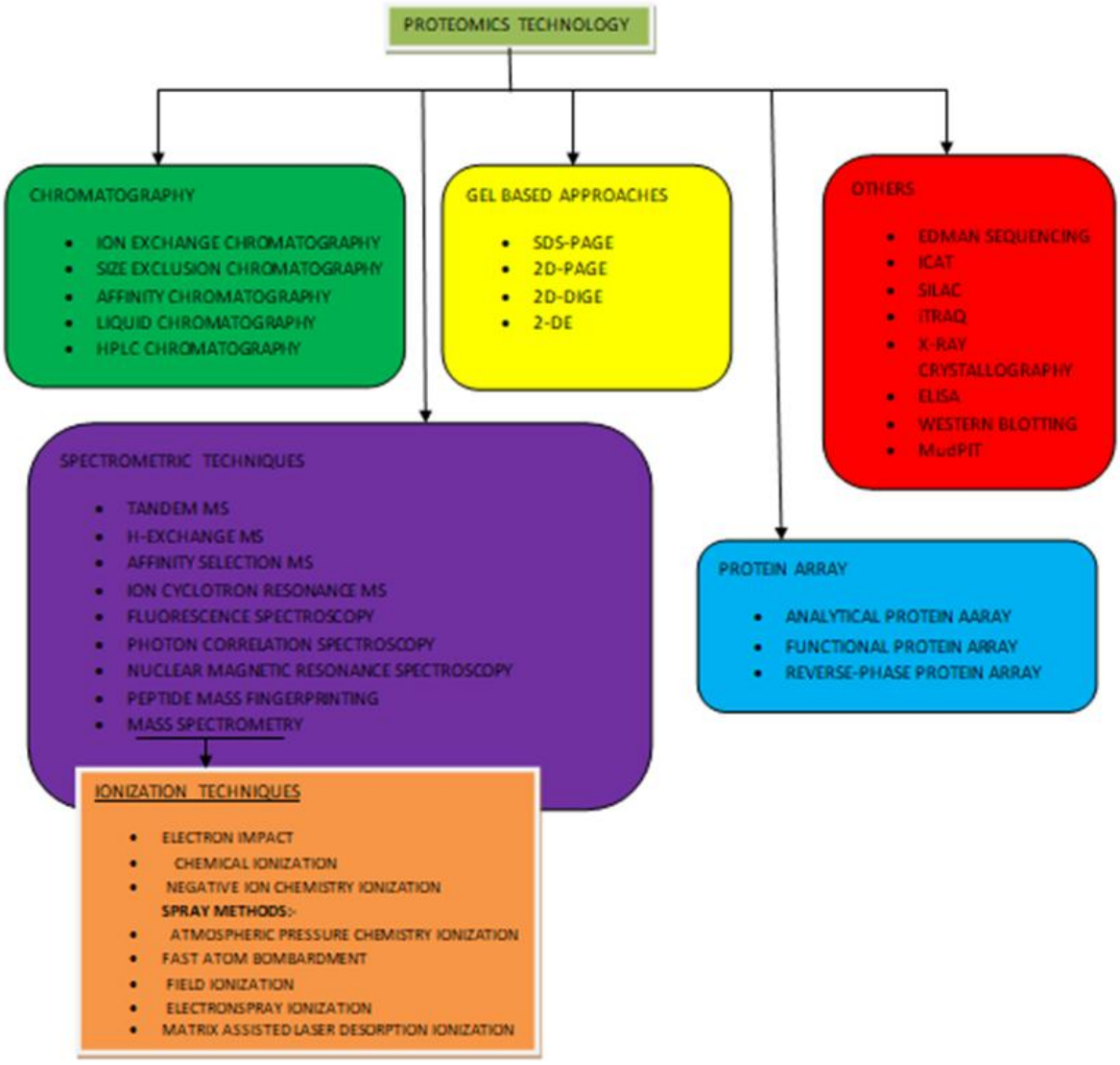

FIG-4- The proteomics techniques

Data analysis and identification of protein: -After 2-DE analysis, gel Image acquisition can be done using UMAX powerlook 1120 system and modified image master 2-D V4.95. The detected spots then can be matched with reference spots. For correct difference in gel staining relative optical density and relative volumes are calculated. Background subtraction and total spot volume normalization is used to process each spot's intensity. String V10.5 is used for analysis of differentially expressed proteins during protein analysis. String database is used to

visualize connections between differentially expressed genes and proteins with highly defined molecular networks [81]. For identifying, characterizing and quantifying MASCOT server is ordinarily used for the resulting protein after MS results. There are other servers also such as Sequest, COMET, X!tandem and Denovo. Via manual inspection of all spectra, it is possible to validate the identified protein biomarkers from the clinical proteomics research study. The proteomics data can be uploaded to the repositories that can help in database searching [82]. The databases such as BioGRID, IntACT, MINT and HRPD all the information related to the interactions in complexes are saved [83, 84, 85]. Table 1 provides a curated list of widely used servers and search tools that facilitate various aspects of proteomic research. These tools support functions such as protein sequence analysis, structural prediction and modeling, motif-based alignment, and database searching. They play a critical role in interpreting experimental data, enabling the identification and characterization of proteins with high precision.

Table 1. Some servers and search tools for Protein sequence databases, structural analysis, structure prediction, structure modelling, motif-based alignment and search tool.

NAME	TYPE
GenBank	Database
SwissProt	Database
BLASTP	BLAST
PSI-BLAST	Position Specific Iterated BLAST
DELTA-BLAST	Domain Enhanced Lookup Time Accelerated BLAST
PHI- BLAST	Pattern Hit Initiated BLAST
CD server	Protein domain servers
PATTINPROT	Protein motif search tools
Gibbs	Motif based alignment server
ModBase	Protein structure database
ConSurf	Protein structure analysis server
PredictProtein	Protein structure predictions server
EBI	Protein structure modelling server

Case study of Diabetes mellitus 2: -In research by Kim et al., [99] for finding biomarkers 36 (males) people were examined. They all were divided into 6 groups depending upon their high glucose level and glycated haemoglobin levels and waist circumference / hip circumference ratio. The optimal WHC of abdominal obesity is 0.9 in men. Patients with similar BMI were grouped. Then they performed 2-DE analysis using serum sample from each donor in triplicate. Peptide mass fingerprinting was also carried out for image analysis. Then they did image acquisition and data analysis by UMAX powerlook and modified image master. Protein analysis was done by MASCOT. The PMF acceptance criteria was based on probability scoring [-10*log (p)]. ELISA test was done, and then immunoblast analysis was carried out. Further SDS PAGE technique was used. String V10.5 was used for network analysis. One way analysis of variance (ANOVA) is used to calculate or evaluate all the results by using statistical package. 36 patients were divided into 6 groups, group1 for normal, group 2 for normal with abdominal obesity, group 3 with pre- diabetic, group 4 with pre- diabetic with abdominal obesity, group 5 for diabetic and group 6 for diabetic with abdominal obesity. Amongst 245 spots in all groups except first group 25 were highly up regulated and 11 were down regulated. Mass ranges from 6 to 240 and kDa between 4 and 7 pH. According to MALDI-TOF Identification serpin peptidase inhibitor AI (AAT/SERPINAI), Haptoglobin protein (HP), zinc-alpha 2- glycoprotein (ZAG), apolipoprotein A-I(APOA-I), and retinol binding protein 4 (RBP4) were more highly expressed in normal patients with abdominal obesity (group2), pre diabetic (group 3 and 4) and diabetic (group 5 and 6) than in normal patients without abdominal obesity (Table 2). In contrast growth- inhibiting protein 25(GIG25/AACT/SERPINA3), albumin (ALB), and transthyretin (TTR) were expressed lower levels in normal patients with abdominal obesity, pre diabetic, and diabetic than in normal patients. Serpin peptidase inhibitor A1 and A3 were associated with liver disease [86]. TTR exist in two forms tetrameric and monomeric. It has role in modulation of food intake and energy balance [87, 88, 89]. RBP is indicators of protein nutritional status [90]. According to proteomics analysis for further validation of serum proteins amongst identified proteins that were clearly related to disease were AACT, AAT, APOA-I, HP, RBP4, TTR and ZAG. Western blotting can be used to further examine the expression of these seven proteins. As a result, AAT and AACT expressions in group 4, 5 and 6 were lower than group 1 and no

difference between the normal group (1&2). The spots which were expressed higher in pre-diabetic (group 3&4) were two of APOA-I spots and RBP4. While in diabetes group expression level of all spots were decreased. In pre-diabetic HP alpha spots were down regulated, in diabetic patients three out of five spots of HP alpha expression were high than in group 1 and no difference noted between the normal groups (1&2). In group 2 and 5 TTR expressions were high than group 1 and ZAG showed no difference in these groups. In this given study high interferon γ was present in patients of group 6 than group 1&2 of normal individuals but no difference in tumour necrosis factor α amongst these six groups. Many evidences suggest that in obesity these two increases [91, 92, 93,94].

Table 2 The expression levels of these spots are high and low in comparison with group 1 of normal individuals. (Group 1 individual have all given below in normal level)

Group	High	Low
Group 2 (normal with abdominal obesity)	TTR	In comparison with group 1 no lower
Group 3 (pre-diabetes)	APO-A1	HP
	RBP4	
	INF gamma	
Group 4 (pre-diabetes with abdominal obesity)	APO-A1	AACT (GIG5, SERPINA3)
	RBP4	AAT(SERPINA)
	INF gamma	HP
Group 5 (Diabetes)	HP	AACT (GIG5, SERPINA3)
	TTR	AAT(SERPINA)
	INF gamma	
Group 6 (Diabetes with abdominal obesity)	HP	AACT (GIG5, SERPINA3)
	INF gamma	AAT(SERPINA)

Conclusion: -Proteomics encompasses a diverse array of techniques for the identification, separation, and analysis of proteins and peptides, including X-ray crystallography, hydrogen–deuterium exchange mass spectrometry, nuclear magnetic resonance (NMR), peptide mass fingerprinting, tandem mass spectrometry (MS/MS), and liquid chromatography–mass

spectrometry (LC-MS). These tools collectively provide profound insights into biological systems and are instrumental in addressing health-related challenges. Proteomics plays a crucial role in disease diagnosis, progression monitoring, and therapeutic interventions for conditions such as cancer, diabetes, and AIDS. Complementing these advances, biobanks serve as valuable repositories of biological specimens, meticulously catalogued with detailed metadata including age, sex, body mass index, and clinical background, thereby enabling comprehensive proteomic investigations. Experimental studies using animal models have demonstrated how specific treatments, such as extracellular polysaccharides or plant-derived compounds like phlorizin, can modulate the expression of diabetes-associated proteins, indicating potential pathways for therapeutic intervention and disease prevention. Additionally, phosphoproteomic analyses have revealed novel components of the insulin signaling cascade, further expanding our understanding of metabolic regulation. A wide range of molecular, separation, and structural techniques are employed in proteomics, including gene expression tools such as microarrays and RNA interference, separation methods like one- and two-dimensional electrophoresis and various chromatographic techniques, as well as protein identification methods such as Edman degradation and mass spectrometry. Structural elucidation is achieved through NMR, X-ray crystallography, and computational modeling. The choice of technique is dictated by the complexity and nature of the target proteins. With the continuous discovery and validation of disease-specific biomarkers, proteomics stands at the forefront of precision medicine, offering unparalleled potential for advancing diagnostics and therapeutics across a broad spectrum of diseases.

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