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Saliva for Diabetes' Complications: Non-Invasive Diagnostics and Monitoring

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Abstract: Type 2 Diabetes Mellitus is a chronic metabolic disease that poses a significant long-term health risk due to its debilitating complications. While the diagnosis and monitoring of diabetes are well-established, the monitoring of its numerous complications presents a challenge, which can compromise patients' prognosis and quality of life. We analyzed the proteome of ten saliva samples, five from healthy individuals and five from patients with Type 2 Diabetes Mellitus. The aim was to identify potential biomarkers of Type 2 Diabetes Mellitus and its complications. We used PANTHER and FUNRICH tools to conduct a functional analysis and enrichment approach. Our findings revealed 622 proteins involved in 16 disrupted biological processes, which are linked to Type 2 Diabetes Mellitus. Metabolic processes, response to stimuli, processes of the immune system, and signaling are notable due to their known relation with Type 2 Diabetes Mellitus. Enrichment analysis has revealed that there are eleven biological processes that have been deregulated. These processes involve twenty different salivary proteins that are associated with multiple complications in diabetes. Our findings demonstrate that saliva is a dependable source of possible biomarkers that can be used to monitor diabetes and its complications.

Keywords: Saliva; Proteomics; Biomarkers

1. Introduction

Type 2 Diabetes Mellitus is a chronic metabolic disease, defined by hyperglycaemia caused by abnormalities in insulin secretion, insulin action, or both. Diabetes is a major risk to health due to its characteristic long-term complications [1] with the International Diabetes Association estimating a prevalence of 537 million people living with diabetes worldwide in 2021 and a projected increase of 46% by 2045 [1].

Frequently, Diabetes Mellitus leads to several health complications. Those can be grouped in "microvascular diseases" (due to damage to small blood vessels) and "macrovascular diseases" (due to damage to the arteries) [2]. Microvascular complications include retinopathy, nephropathy, and neuropathy [2]. The major macrovascular complications include accelerated cardiovascular disease resulting in myocardial infarction and cerebrovascular disease manifesting as strokes [2]. In opposite, diabetic ketoacidosis from exceptionally hyperglycaemia and coma as the result of hypoglycaemia can be fatal [3]. Type 2 Diabetes Mellitus is also characterized by comorbidities such as obesity and cardiovascular diseases.

Type 2 Diabetes Mellitus has an established screening and diagnostic protocol, either by the haemoglobin A1C criteria or plasma glucose concentration (after 12h fasting or 2-hour plasma glucose) [4]. After a diagnosis of Diabetes Mellitus, the patients require self-monitoring of plasmatic glucose concentrations, regularly. The estimation of plasmatic glycated haemoglobin, and of lipid levels are also necessary [4] for the evaluation of disease progression. Although the diagnosis of diabetes and blood glucose monitoring are well established, monitoring the many complications of diabetes remains a challenge [2].

Saliva is being addressed as a relevant biofluid for diagnostics/prognosis since it is safe and easy to collect, its collection is non-invasive, and it contains several blood and systemics molecules frequently used as biomarkers of diagnosis [5]. Although salivary diagnostics is increasingly being considered, monitoring pre- and established diabetes and its complications via saliva is yet to be validated [6]. The identification of markers of prognosis could support the development of "point-of-care" tests that a physician could use for patient follow up and alert for behavioral changes or adjustment of diabetes control medication.

The aim of this study was to propose biomarkers in saliva that allow monitoring of diabetes and its complications. We hypothesize that saliva provides the biological information – proteins – to identify characteristic proteome changes induced by Type 2 Diabetes Mellitus.

2. Results

We hypothesized that Type 2 Diabetes Mellitus induces characteristic proteome changes detectable in saliva. We used a methodological approach for the functional analysis of altered salivary proteins identified by mass spectrometry to point out or clarify molecular changes associated with Type 2 Diabetes Mellitus.

Analysis of the 10 saliva samples (Type 2 Diabetes Mellitus and Healthy) resulted in the identification of 917 proteins (Table S1) with a 5% normalized FDR, of which 622 were quantified with a 1% normalized FDR (Table S2). PANTHER gene list analysis has successfully mapped 611 from the 622 proteins participating in 16 biological processes (Figure S1).

2.1. Protein enrichment analysis

Enrichment analysis of the 622 proteins quantified revealed 185 proteins involved in 11 disrupted biological processes. The fold change of proteins involved in the enriched biological processes is represented as follows: 112 proteins are increased, while 73 proteins are decreased in the saliva of patients with Type 2 Diabetes Mellitus.

Eleven biological processes are significantly altered (enrichment analysis, Biological Process Enrichment, Figure 1). In a summary assessment of the enrichment analysis, the mechanism most affected is inflammatory response (124 proteins). Other mechanisms altered in DMT2 patients are Detection of chemical stimulus involved in sensory perception of bitter taste (7 proteins), Cellular response to starvation (4 proteins), Maintenance of mitochondrion location (1 protein) and Oligosaccharide metabolic process (1 protein).
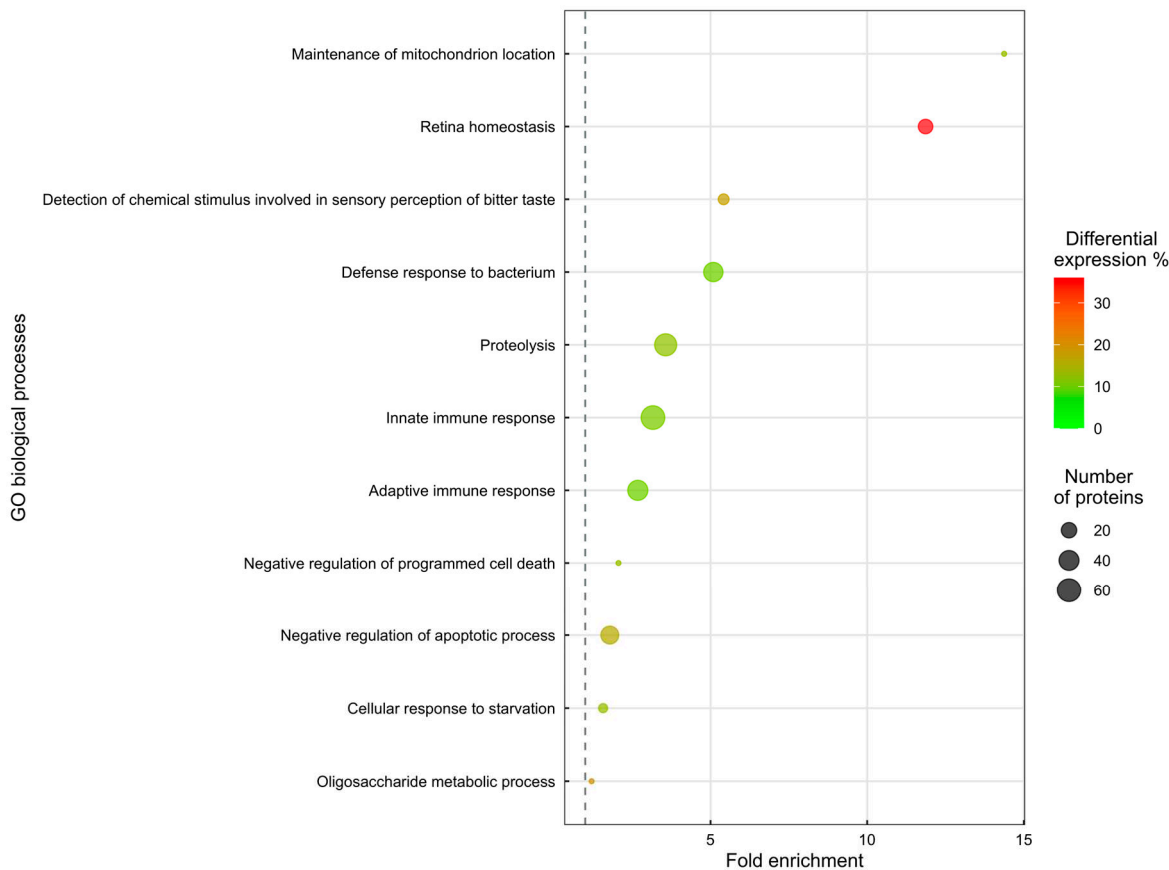
Figure 1. Biological Processes enrichment of Healthy and Type 2 Diabetes Mellitus saliva proteins relative to the percentage of expression analysis against the UniProt background database. The most representative and significant biological processes are represented and are sorted by fold enrichment. The dot size indicates the number of proteins associated with which process and the dot colour indicates the differential quantities compared to the background. The vertical grey dashed line represents a fold enrichment of 1.

3. Discussion

Despite the high prevalence of diabetes Mellitus type 2, the molecular mechanisms underlying the development and progression of this disease are still not fully understood. Researchers have focused on identifying biomarkers that can facilitate the diagnosis, monitoring, and treatment of Type 2 Diabetes Mellitus. One potential source of biomarkers is saliva, which contains proteins that can reflect changes in the body's metabolic state. We investigated whether Diabetes Mellitus type 2 induces characteristic proteome in saliva.

3.1. Retina homeostasis

Retina homeostasis, related to retinopathy (one of the most well-known complications of diabetes) is dysregulated in Type 2 Diabetes Mellitus. We identified 9 proteins related to retina homeostasis (Table 1).

Table 1. List of proteins related with dysregulated processes in the saliva of Type 2 Diabetes Mellitus patients (p value <0.05). Shown are the UniProtKB accession numbers (AC), protein and gene names, fold change (Type 2 Diabetes Mellitus/Healthy), the variable importance projection (VIP) score defined by the PLS-DA analysis and the dysregulated processes identified in the enrichment analysis. Only proteins with a VIP > 1 are shown. Fold changes calculation was done according to: ($fold\ change\ Disease$) / ($fold\ change\ Health$) = fold change ($\geq +1$); For the fold changes lower than 1: 1/ ($fold\ change$ ($< +1$)) = fold change (-).

Dysregulated processes	UniProtKB AC	Protein name	Gene name	Fold change	VIP
Retina homeostasis	P04792	Heat shock protein beta-1	HSPB1	-2.49	1.08
	P25311	Zinc-alpha-2-glycoprotein	AZGP1	-1.56	1.6
	P02787	Serotransferrin	TF	1.66	1.49
	P02768	Albumin	ALB	2.23	1.59
	P01860	Immunoglobulin heavy constant gamma 3	IGHG3	2.87	1.05
	P02788	Lactotransferrin	LTF	5.25	1.48
	Q96DA0	Zymogen granule protein 16 homolog B	ZG16B	5.7	1.27
	P31025	Lipocalin-1	LCN1	6.02	1.44
Negative regulation of apoptotic process	Q04760	Lactoylglutathione lyase	GLO1	-2.73	1.48
	P09211	Glutathione S-transferase P	GSTP1	-2.71	1.09
	P04792	Heat shock protein beta-1	HSPB1	-2.49	1.08
	P11021	Endoplasmic reticulum chaperone BiP	HSPA5	-2.48	1.31
	O95865	Dimethylarginine dimethylaminohydrolase 2	DDAH2	-2.44	1.42
	P63104	14-3-3 protein zeta/delta	YWHAZ	-2.34	1.15
	P05120	Plasminogen activator inhibitor 2	SERPINB2	-1.62	1.08
	P16070	CD44 antigen	CD44	1.16	0.99
	P07996	Thrombospondin-1	THBS1	1.86	1.32
	Q8NBS9	Thioredoxin domain-containing protein 5	TXNDC5	1.9	1.21
	P02768	Albumin	ALB	2.23	1.59

	P04040	Catalase	CAT	2.45	1.38
	P14780	Matrix metalloproteinase-9	MMP9	2.75	1.32
	P21333	Filamin-A	FLNA	2.87	1.13
	P05164	Myeloperoxidase	MPO	4.72	1.11
	P02788	Lactotransferrin	LTF	5.25	1.48
Adaptative and innate immune response	O00584	Ribonuclease T2	RNASET2	-2.68	1.69
	P01871	Immunoglobulin heavy constant mu	IGHM	-2.34	1.02
	P01782	Immunoglobulin heavy variable 3-9	IGHV3-9	-2	1.38
	A0A0B4J1X5	Immunoglobulin heavy variable 3-74	IGHV3-74	-1.94	1.15
	P01721	Immunoglobulin lambda variable 6-57	IGLV6-57	-1.47	1.33
	P06331	Immunoglobulin heavy variable 4-34	IGHV4-34	-1.31	1.03
	P17931	Galectin-3	LGALS3	-1.13	1.15
	Q14508	WAP four-disulphide core domain protein 2	WFDC2	1.29	1
	A0A0C4DH25	Immunoglobulin kappa variable 3D-20	IGKV3D-20	1.35	1.27
	P05109	Protein S100-A8	S100A8	1.38	1.03
	P01700	Immunoglobulin lambda variable 1-47	IGLV1-47	1.41	1.44
	P01601	Immunoglobulin kappa variable 1D-16	IGKV1D-16	1.5	1.12
	P05156	Complement factor I	CFI	1.62	1.08
	P31151	Protein S100-A7	S100A7	1.68	1.25
	P02743	Serum amyloid P-component	APCS	1.73	1.66
	P0C0L4	Complement C4-A	C4A	1.74	0.99

P06702	Protein S100-A9	S100A9	1.77	1.11
P01859	Immunoglobulin heavy constant gamma 2	IGHG2	1.9	1.26
P0DOX5	Immunoglobulin gamma-1 heavy chain		1.99	1.31
A0A0C4DH36	Probable non-functional immunoglobulin heavy variable 3-38	IGHV3-38	2.03	1.25
P02671	Fibrinogen alpha chain	FGA	2.11	1.06
Q9UGM3	Deleted in malignant brain tumors 1 protein	DMBT1	2.43	1.34
P10909	Clusterin	CLU	2.5	1.27
P01860	Immunoglobulin heavy constant gamma 3	IGHG3	2.87	1.05
P80188	Neutrophil gelatinase-associated lipocalin	LCN2	2.89	1.94
O75594	Peptidoglycan recognition protein 1	PGLYRP1	2.91	1.32
P0DOY2	Immunoglobulin lambda constant 2	IGLC2	3.08	1.86
P12724	Eosinophil cationic protein	RNASE3	3.25	2.02
P49913	Cathelicidin antimicrobial peptide	CAMP	3.35	1.22
P15814	Immunoglobulin lambda-like polypeptide 1	IGLL1	3.38	1.38
P05089	Arginase-1	ARG1	3.71	1.1
P04003	C4b-binding protein alpha chain	C4BPA	3.78	1.65
P02675	Fibrinogen beta chain	FGB	4.06	1.38
P26583	High mobility group protein B2	HMGB2	4.32	1.62
P02788	Lactotransferrin	LTF	5.25	1.48
Q9NP55	BPI fold-containing family A member 1	BPIFA1	5.84	0.99
P05155	Plasma protease C1 inhibitor	SERPING1	6.58	1.55

	P0C0L5	Complement C4-B	C4B	7.02	1.55
	P17213	Bactericidal permeability-increasing protein	BPI	14.99	1.49
Defence response to bacterium	P15515	Histatin-1	HTN1	-4.52	1.46
	P01871	Immunoglobulin heavy constant mu	IGHM	-2.34	1.02
	P01782	Immunoglobulin heavy variable 3-9	IGHV3-9	-2	1.38
	A0A0B4J1X5	Immunoglobulin heavy variable 3-74	IGHV3-74	-1.94	1.15
	P06331	Immunoglobulin heavy variable 4-34	IGHV4-34	-1.31	1.03
	P05109	Protein S100-A8	S100A8	1.38	1.03
	P06702	Protein S100-A9	S100A9	1.77	1.11
	P01859	Immunoglobulin heavy constant gamma 2	IGHG2	1.9	1.26
	A0A0C4DH36	Probable non-functional immunoglobulin heavy variable 3-38	IGHV3-38	2.03	1.25
	P01860	Immunoglobulin heavy constant gamma 3	IGHG3	2.87	1.05
	P80188	Neutrophil gelatinase-associated lipocalin	LCN2	2.89	1.94
	P0DOY2	Immunoglobulin lambda constant 2	IGLC2	3.08	1.86
	P49913	Cathelicidin antimicrobial peptide	CAMP	3.35	1.22
	P15814	Immunoglobulin lambda-like polypeptide 1	IGLL1	3.38	1.38
	P00738	Haptoglobin	HP	4.43	1.54
	P05164	Myeloperoxidase	MPO	4.72	1.11
	P08246	Neutrophil elastase	ELANE	8.02	1.14
Proteolysis	P55786	Puromycin-sensitive aminopeptidase	NPEPPS	-2.34	1.22

Q9BQR3	Serine protease 27	PRSS27	-2.22	1.24
Q9H4A4	Aminopeptidase B	RNPEP	-2.09	1.03
P28838	Cytosol aminopeptidase	LAP3	-1.87	1.28
P21128	Uridylate-specific endoribonuclease	ENDOU	-1.58	1.19
Q9UBR2	Cathepsin Z	CTSZ	-1.39	1.14
Q9UBX7	Kallikrein-11	KLK11	-1.34	1.16
P48147	Prolyl endopeptidase	PREP	-1.27	1.23
Q9NY33	Dipeptidyl peptidase 3	DPP3	-1.25	1.06
Q14508	WAP four-disulfide core domain protein 2	WFDC2	1.29	1
P05156	Complement factor I	CFI	1.62	1.08
P31944	Caspase-14	CASP14	1.77	1.19
Q9UKR3	Kallikrein-13	KLK13	1.88	1.02
P07339	Cathepsin D	CTSD	1.94	1.56
O60235	Transmembrane protease serine 11D	TMPRSS11D	1.97	1.54
P08311	Cathepsin G	CTSG	2.01	1.2
P22894	Neutrophil collagenase	MMP8	2.35	1.63
P14780	Matrix metalloproteinase-9	MMP9	2.75	1.32
Q9UL52	Transmembrane protease serine 11E	TMPRSS11E	2.79	1.2
Q9GZN4	Brain-specific serine protease 4	PRSS22	2.91	1.15
P08603	Complement factor H	CFH	3.3	1.19
P08519	Apolipoprotein(a)	LPA	3.48	1.21
P01024	Complement C3	C3	3.62	1.68

P02788	Lactotransferrin	LTF	5.25	1.48
P31025	Lipocalin-1	LCN1	6.02	1.44
P08246	Neutrophil elastase	ELANE	8.02	1.14
P24158	Myeloblastin	PRTN3	9.49	1.41

Retina requires a healthy choroid to nourish the different outer retinal layers. Lactotransferrin (LTF), present in the human retina, has been successful used in the treatment of lesions in choroidal neovascularization in mice and, in parallel, when coated, demonstrated to be efficient on the treatment of the dry eye [9], due it is including iron-transferring, anti-inflammatory, antioxidant, antimicrobial, and anticancer activities [10]. We showed that LTF is more abundant in Type 2 Diabetes Mellitus saliva samples (FC = 5.25) than in control samples, being one of the protein contributors to the separation of study groups (VIP = 2.02). Although LTF has been associated to diabetes before, some researchers report elevated concentrations while others report a decreased concentration of LFT in diabetic patients [11].

Heat shock protein beta-1 (HspB1) is upregulated upon optic nerve injury [12]. This suggests that HspB1 might have a protective value for retinal ganglion cells [12]. In a similar perspective, HspB1 has been shown to play a vital role in maintaining the integrity of retinal capillary endothelial cells. Proinflammatory cytokines are generally upregulated in the diabetic retina, reducing the levels of HspB1 in retinal capillary endothelial cells, which could lead to their apoptosis in diabetic retinopathy [13]. This reduction was observed in our results with HspB1 being less abundant on Type 2 Diabetes Mellitus saliva samples (FC=-2.49) than in control samples.

We identified an increase in Lipocalin-1 (LCN1) concentration on Type 2 Diabetes Mellitus saliva samples (FC=6.02). To our knowledge, this is the first report of an association between LCN1 and Type 2 Diabetes Mellitus. Lipocalin-1 belongs to a family of transport proteins and scavenges lipids from the corneal surface [14]. Lipocalin-2 (LCN2, suggested as being an upstream regulator of apoptosis of retinal degeneration) levels are significantly increased in the whole retinae of rats after induction of retinal degeneration, compared to the controls [15]. LCN2 is instrumental for the progression of diabetic complications like encephalopathy and peripheral neuropathy [15]. The LCN2 upregulation observed after the retinal degeneration can be correlated with the LCN1 increased concentration on our Type 2 Diabetes Mellitus samples.

Albumin (ALB), previously suggested as diabetic retinopathy biomarker in serum [16], is also more abundant (FC=2.23) in Type 2 Diabetes Mellitus saliva. Increased levels of albumin have been related to the disruption of the retinal barrier that occurs in diabetes [17].

3.2. Negative regulation of apoptotic processes

Apoptotic processes are involved in the dynamic regulation of pancreatic β-cell mass which are directly linked to insulin production. Hyperplasia of β-cells may be related to active replication and/or neogenesis. This increase is balanced by the reduction of the number of cells via cell death resultant from necrosis and/or apoptosis. It is apoptosis that plays a critical role in the reduction of β-cell mass occurring in patients with Type 2 Diabetes Mellitus and subsequent progressive worsening of glycaemic control. The negative regulation of apoptotic process is dysregulated in Type 2 Diabetes Mellitus saliva samples (Table 1).

Lactotransferrin promotes apoptosis and the elevated levels have been related with neutrophil dysfunction in Type 2 Diabetes Mellitus [18]. In fact, LTF has been suggested as a biomarker to monitor the development or progression of diabetes and its complications [18]. On the opposite side, Endoplasmic reticulum chaperone BiP (HSPA5) is suggested to be a protector against ER stress,

required for β-cells dysregulated apoptosis and consequent Type 2 Diabetes Mellitus development [19].

It can be hypothesized that a failure of the negative regulation of apoptotic process can lead to necrosis of β-cells. We show that Lactotransferrin is more abundant on Type 2 Diabetes Mellitus saliva samples (FC = 5.25) sustaining the assumptions of previous authors [18] that LTF promotes the neutrophil dysfunction in Type 2 Diabetes Mellitus. HSPA5, identified as protector of ER, is less abundant on Type 2 Diabetes Mellitus saliva samples (FC = -2.48), which corroborates the previous assumption.

It is important to point out that the negative regulation of programmed cell death process is dysregulated as well (Figure 1). Expansion (neogenesis, replication and hypertrophy) and involution (apoptosis, necrosis, and atrophy) processes are related since both may contribute to a unfunctional equilibrium in the β-cell mass and consequent glycaemic control. Albumin (ALB), that plays a role as a negative inflammation biomarker, was found elevated on Type 2 Diabetes Mellitus saliva samples (FC = 2.23) suggesting an active inflammatory status [20].

3.3. Cellular response to starvation

Starvation is one of the earliest forms of insulin resistance and is associated with increased lipid metabolism molecules (lipolysis, plasma fatty acid, ketone bodies and intramyocellular lipids). This increased lipid load is related with reduced insulin sensitivity and consequently with Type 2 Diabetes Mellitus, but the related mechanisms remain unclear [21]. Our data shows that the negative regulation of cellular response to starvation is altered in Type 2 Diabetes Mellitus (Figure 1).

Glutamine synthetase (GLUL) is involved in the catalysis of glutamate to glutamine. Glutamate is directly dependent of leptin to be up taken in astrocytes in a time-dependent manner. The rapid glutamate capture by astrocytes indicates that leptin could reduce the stimulatory effects of glutamate at nearby synapses, thereby reducing appetite. The rapid rise of insulin with feeding promotes uptake and storage of ingested energy in fat and muscle. The rapid fall of insulin with starvation promotes catabolism [22]. Both leptin and insulin play key roles in signaling the physiologic transition from energy sufficiency to deficient energy/starvation [22]. We show that GLUL is less abundant in saliva (FC = -1.62) of Type 2 Diabetes Mellitus patients which can be a consequence of starvation stimulus dysregulation.

3.4. Maintenance of mitochondrion location

Type 2 Diabetes Mellitus is characterized by mitochondrial dysfunction and consequent homeostasis disruption, with elevated production of reactive oxygen species (ROS) and low levels of ATP. Downregulation of genes involved in mitochondrial biogenesis and oxidative phosphorylation has been observed in Type 2 Diabetes Mellitus [23]. The maintenance of mitochondrion location is characterized by two processes: the conservation of specific location within a cell and prevention from moving elsewhere.

The link between the levels of Albumin and the maintenance of mitochondrion location are not well established yet. In the context of Type 2 Diabetes Mellitus, albumin levels may be influenced by several factors, including mitochondrial oxidative stress increase that leads to a change in mitochondrial function [24]. In saliva of patients with Type 2 Diabetes Mellitus, albumin is increased (FC = 2.23). Being a marker of the oxidative stress characteristic of diabetes [25] a preventive monitorization of Albumin could avoid this microvascular disease appearance.

3.5. Detection of chemical stimulus involved in sensory perception of bitter taste

Taste receptors in the gustatory system are expressed in the gastrointestinal tract and influence nutrients intake. Different flavors stimulate specific taste receptors that release metabolic hormones, modulating insulin expression with impact on glucose homeostasis. In Type 2 Diabetes Mellitus, some taste receptors are altered, and this is linked to glucose and insulin dysregulation. The detection

of the chemical stimulus involved in sensory perception of bitter taste seems to be dysregulated, as suggested by the enrichment analysis (Figure 1).

Cystatin-SN (CST1) is more abundant in saliva of individuals experiencing bitter tastes [26]. In Type 2 Diabetes Mellitus, Cystatin-SN is up-regulated in patients with burn mouth sensation, attributed to diabetic peripheral neuropathy [27]. This could reflect a defensive reaction against an on-going inflammation [27]. In our study, CST1 is less abundant (FC = -2.18) in the saliva of Type 2 Diabetes Mellitus patients, which is contrary to that found previously [26].

Zinc-alpha-2-glycoprotein (AZGP1) has a direct relation with sensory perception of bitter taste. AZGP1 gene has been suggested as a potential obesity gene that may predispose to the development of obesity [28]. AZGP1 plays a role in the regulation of adipose tissue mass, stimulating lipolysis, inhibiting lipid accumulation in adipose tissue, regulating serum lipid values, and influencing the secretion of other adipokines [29]. AZGP1 is less abundant (FC = -1.56) in Type 2 Diabetes Mellitus saliva samples than in control samples.

Carbonic anhydrase 6 (CA6) is a key factor responsible for the reduction of hepatic glucose production and has been suggested as a marker for several complications of diabetes, namely retinopathy, kidney disease, neuropathy, and cardiovascular disease [30]. In saliva, CA6 was more abundant (FC = 10.52) in Type 2 Diabetes Mellitus patients, in agreement with previous studies [30].

3.6. Adaptative and Innate immune response

Recent research suggests that both innate and adaptive immunity play a critical role in the pathogenesis of Type 2 Diabetes Mellitus, with evidence pointing to the abnormal differentiation of immune system components as a key factor in the development of insulin resistance. Alterations in the proliferation of T cells and macrophages, and impairment in the function of NK and B cells are frequent phenomena in individuals with Type 2 Diabetes Mellitus [31].

Enrichment analysis revealed a dysregulation of the adaptative and innate immune response (Figure 1, Table 1).

Fibrinogen alpha chain (FGA) and Fibrinogen beta chain (FGB) are involved in the disruption of the adaptative and innate immune responses. Fibrinogen is a crucial modulator of haemostatic balance and inflammatory processes in diabetes [32]. In our study FGA and FGB were found elevated in saliva of the patients with Type 2 Diabetes Mellitus (FC = 2.11 and FC = 4.06), in consonance with previous fundings that show that Fibrinogen is increased in blood in different moments of Type 2 Diabetes Mellitus: before the onset of Type 2 Diabetes Mellitus, and during the transition from pre-Type 2 Diabetes Mellitus to Type 2 Diabetes Mellitus [33].

The bactericidal permeability-increasing protein (BPI) is an endogenous protein that in Type 2 Diabetes Mellitus can reduce inflammation [34]. In saliva, BPI was more abundant in patients with Type 2 Diabetes Mellitus than in healthy subjects (FC = 14.99), probably a reflection of the body's attempt to fight the inflammatory state characteristic of diabetes.

3.7. Defense response to bacterium

In Diabetes, insulin deficiency and hyperglycaemia cause the disruption of immune system mechanisms such as cytokine production, immune cells function and phagocytosis [35]. The defense response to bacteria is dysregulated in patients with Type 2 Diabetes Mellitus (Figure 1, Table 1).

These immune alterations can affect the production and activity of immunoglobulins, reducing their levels in saliva [36]. A compromised immune response may also impact the expression and secretion of histatin-1, increasing the susceptibility to infections [37]. We found that, in saliva, Histatin-1 is less abundant (FC = -4.52), as in other studies [36,37]. It's possible to assume a direct link between these factors and an increased risk of oral infections and oral health complications in individuals with T2DM.

We found that, in saliva of diabetic patients, Neutrophil elastase (NE) is more abundant (FC = 8.02) supporting the link with defense response to bacteria dysregulation in Type 2 Diabetes Mellitus.

Diabetic foot ulcer is characterized by the unbalance of the immune system and is a common complication in Type 2 Diabetes Mellitus individuals. Open wounds are an entry for different

pathogens that take advantage of a compromised immune system. Although the underlying pathophysiological mechanisms of the diabetic foot are still unclear, the relation between S100-A8 and S100-A9 protein levels in serum and the healing process was reported [38,39]. The data is not consensual, as the levels of both proteins in serum were lower in Type 2 Diabetes Mellitus individuals [39] or higher depending on the technology used to detect them: proteomics or immune-specific quantitative assays [38]. In saliva of Type 2 Diabetes Mellitus patients, both S100-A8 and A9 were more abundant: S100-A8 (FC = 1.38) and A9 (FC = 1.79). This upregulation can stimulate the inflammatory response, causing the release of proinflammatory cytokines, and preventing the normal wound cicatrisation [39].

3.8. Proteolysis

Insulin resistance of Type 2 Diabetes Mellitus patients leads to an increase in proteolysis and a decrease in protein synthesis. Enrichment analysis shows that proteolysis is dysregulated in Type 2 Diabetes Mellitus saliva (Figure 1, Table 1).

Type 2 Diabetes Mellitus results from a reduction in the ability of insulin to stimulate glucose utilization (insulin resistance) and an inadequate pancreatic β-cell insulin secretion in response to hyperglycaemia [40]. Matrix mettaloproteinase-9 is upregulated in plasma of Type 2 Diabetes Mellitus individuals exhibiting an increased proteolytic activity [40]. In saliva of Type 2 Diabetes Mellitus patients MMP-9 is more abundant than in control saliva.

3.9. Oligosaccharide metabolic process

Functional oligosaccharides play a positive role in the prevention of diabetes, including improvement of pancreas function, α-glucosidase inhibition, the relief of insulin and leptin resistance, anti-inflammatory effects, regulation of gut microbiota and gut hormone, and intervention of diabetic risk factors [41].

We show that the oligosaccharide metabolic process is dysregulated (Figure 1). This process has the carbohydrate metabolic process as its ancestor. Alpha-amylase 1A, the protein contributing to this dysregulation, is produced primarily in the salivary glands and pancreas and is responsible for breaking down complex carbohydrates, including starch and glycogen, into smaller sugar molecules such as oligosaccharides [42]. Some studies have shown that individuals with Diabetes Mellitus type 2 may have higher salivary Alpha-amylase activity compared to individuals without diabetes [43]. Nonetheless, our data show a decrease on the amount of Alpha-amylase in saliva of type 2 Diabetes Mellitus patients (FC = -2.14), which may be explained by the method of saliva collection under fasting or non-fasting conditions [44].

3.10. Additional protein analysis

In addition to the proteins involved in enriched biological processes, we addressed the role of proteins with VIP>1. Thirty-eight proteins with a VIP > 1 (Figure 2), but that do not account for the enrichment of the biological processes are listed in the supplementary Table S3.

Basic salivary proline-rich protein 1 (PRB1) is expressed in large amounts in parotid glands. In saliva, PRB1 is much less abundant in the saliva of diabetes patients than in healthy subjects (FC = -20), which is correlated with a lower oral health typical of diabetic patients [45].

Alpha-2-macroglobulin-like protein 1 (A2ML1) was suggested as a potential biomarker for diabetic retinopathy and other diabetic complications [46]. A2ML1 is upregulated in the plasma of diabetic patients with retinopathy [46]. In our sample group, A2ML1 was less abundant in the saliva samples of Type 2 Diabetes Mellitus patients (FC = -2.78).

Alpha-1-antitrypsin (SERPINA1), a defense response protein involved in the pro-inflammatory immune response, has been associated with Type 2 Diabetes Mellitus, and is also increased in proliferative diabetic retinopathy [47]. In saliva, this protein was also found more abundant in the saliva of Type 2 Diabetes Mellitus patients (FC = 2.75).

Hemopexin (HPX), a scavenger of heme, is more concentrated in the saliva of patients with proliferative diabetic retinopathy [48] and in the urine of uncontrolled diabetes patients [49]. In our study this protein was found more abundant in the saliva of diabetic patients (FC = 3.38).

It is important to point out that both Hemoglobin subunits (alpha and beta) were identified in the saliva of diabetic patients, which surely can be related with the common oral manifestation of diabetes such as periodontitis [characterized by bleeding gums [50]].
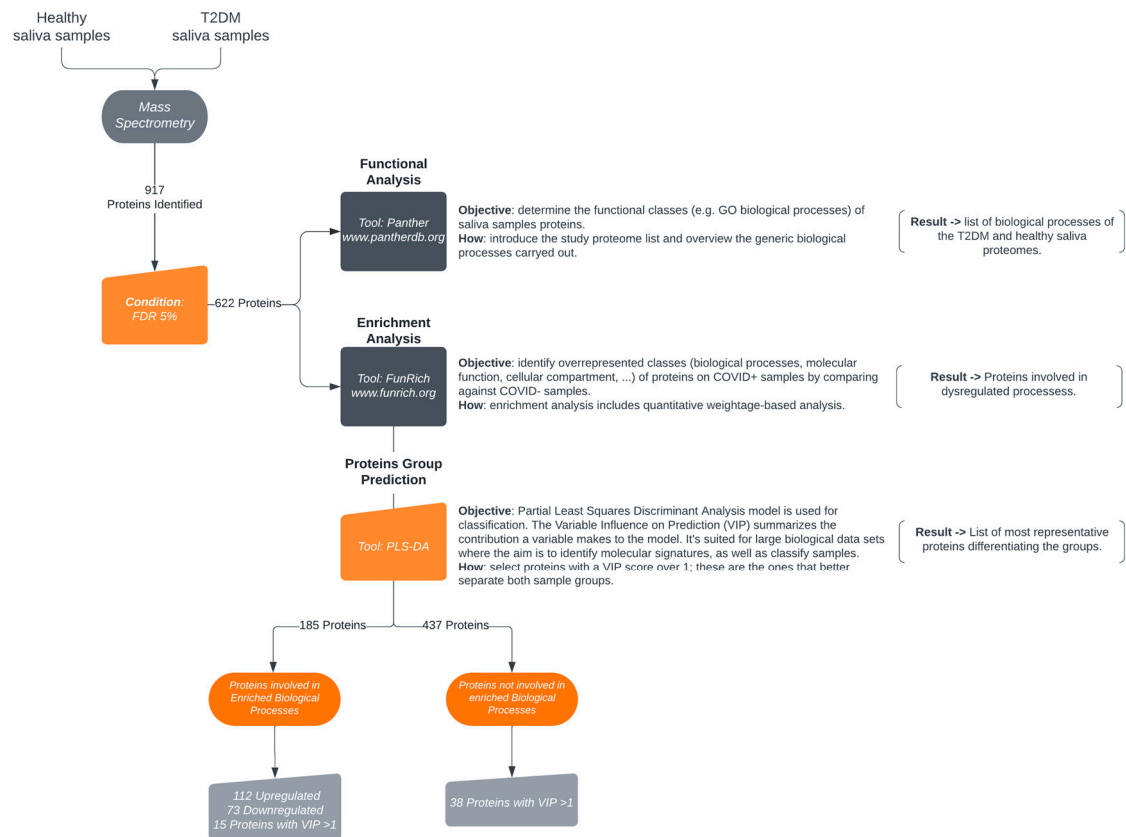
Figure 2. Functional analysis workflow. Proteins were identified by LC-ESI-TOF mass spectrometry. An initial dataset of 917 proteins was narrowed down filtered by an FDR 5%, resulting in 622 salivary proteins then descripted according to GO biological process by PANTHER tool followed by an enrichment analysis by FunRich tool. Group analysis by PLS-DA model defined 53 proteins with a VIP score>1, 15 of which are also included in the enriched biological processes. The enrichment analysis resulted in 185 proteins, with dysregulated processes with a gene expression >10%. Adapted from Esteves et al. 2022 [7].

4. Materials and Methods

4.1. Ethical Statement

This study was carried out in accordance with the Helsinki Declaration and the Oviedo Convention. Saliva samples were collected following the guidelines for the protection of personal data and ethical issues in use at the Biobank of the Institute of Molecular Medicine (IMM) of the Faculty of Medicine, University of Lisbon (http://biobanco-imm.biobanco.pt/documents). Written informed consent including the purpose of the study, data confidentiality, rights of participants, and the right to withdraw from the study at any time was provided by every participant or by their legal representatives or guardians before study enrolment.

4.2. Participant Enrolment

Five saliva samples (3 females and 2 males aged 69 to 82) of a cohort of Type 2 Diabetes Mellitus patients from Centro de Saúde Dão Lafões, Portugal, were collected. Saliva samples from five healthy subjects (3 females and 2 males aged 8 to 39) showing no evidence of oral and systemic pathologies after clinic evaluation, were also collected.

4.3. Saliva Collection

Unstimulated saliva samples were collected with two sterile cotton rolls placed under the tongue, for 2 minutes. The cotton rolls were placed inside a 15 mL sterile plastic tube with a sterile 100 mL pipette tip in the bottom to facilitate saliva collection by centrifugation (10,000 × g; 10 minutes; 4 ℃). Samples were then refrigerated and inactivated using 1% Triton X-100. Whole saliva was centrifuged (10,000 × g; 10 min; 4 °C). The supernatant was aliquoted and stored (80 °C) until analysis.

4.4. Sample preparation and LC-MS methodology

Protein concentration was determined using the Pierce BCA assay kit (ThermoFisher Scientific), according to the manufacturers' instructions and adjusted to 50 mg/mL. Saliva was analyzed by capillary electrophoresis (Experion™ automated capillary electrophoresis system, Bio-Rad Laboratories), and compared to the in-house profiles to confirm sample integrity (data not shown).

Sample preparation for data-dependent acquisition (DDA) experiments and data-independent acquisition (DIA), as well as LC-MS methodology [protein identification and relative quantification of proteins (SWATH-MS)] was performed as described previously [7].

4.5. Protein functional analysis

Functional analysis of salivary proteins in Type 2 Diabetes Mellitus identified by mass spectrometry was performed according to Figure 2.

Narrowed down proteome biological processes classification was obtained via the PANTHER Gene List Analysis (PANTHER V 17.0) (http://www.pantherdb.org/). Only the PANTHER GO-SLIM biological processes were considered. The dataset followed an enrichment analysis of biological processes' using FunRich tool against UniProt curated database as background (http://www.funrich.org/). Selected biological processes enriched with a minimum of 10% of differential expression were plotted using an adapted script as described in Bonnot et al., 2019 [8] within the ggplot2 package version 3.4.0 in RStudio version 2022.12.0.353.

PLS-DA model, based on variable importance projection (VIP) values, was used to select the proteins capable to classify the 2 groups of samples.
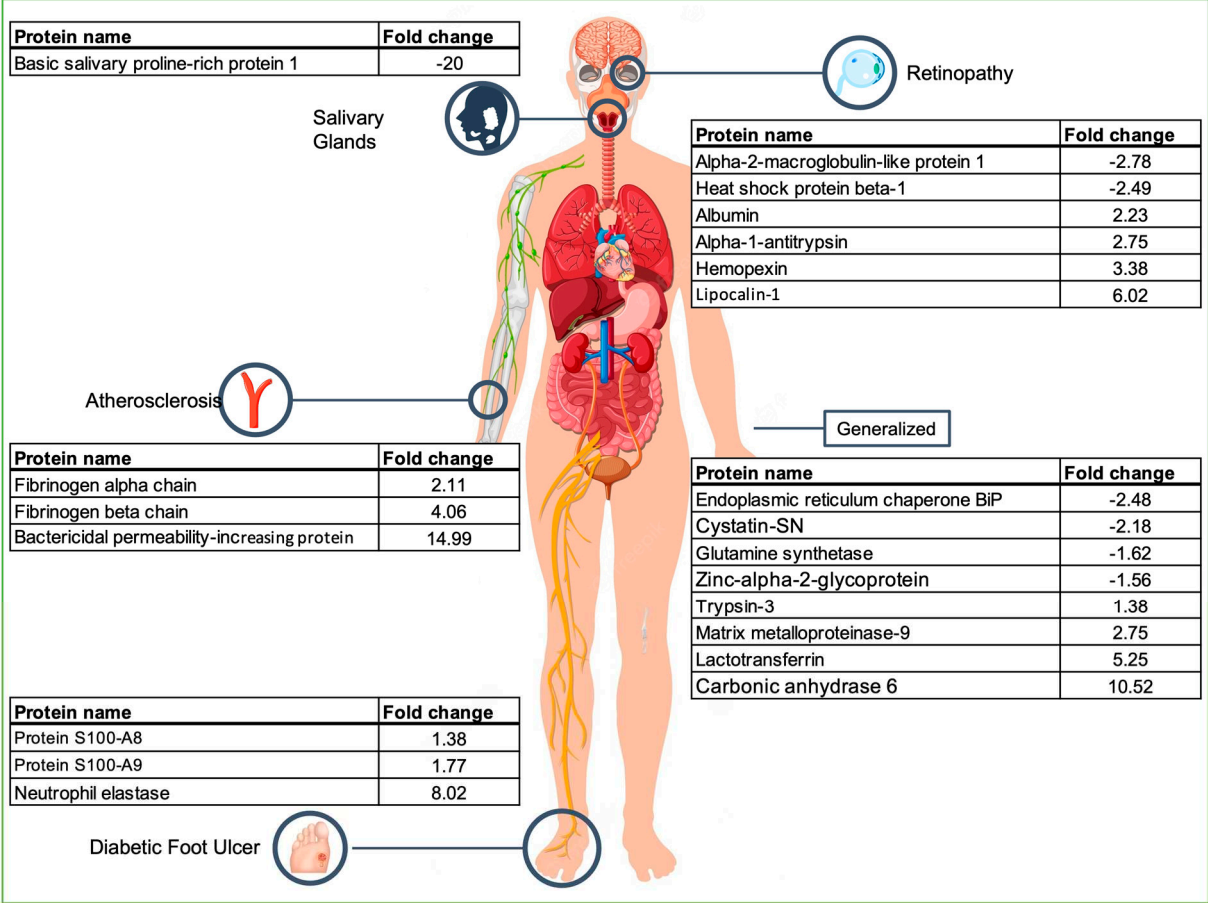
4.6. Statistical analysis

The Mann-Whitney test was used to select the proteins statistically different between the Type 2 Diabetes Mellitus and Healthy samples. The multivariate analysis was performed in MetaboAnalyst as previously described [7].

5. Conclusions

This study shows the enormous potential of saliva as a diagnostic fluid. We have identified 622 proteins altered in patients with Type 2 Diabetes Mellitus, highlighting twenty three that reflect conditions dispersed for different body locations: arteries (atherosclerosis), eye (retinopathy), diabetic foot ulcer (foot), metabolic syndrome (generalized), insulin resistance (generalized), molecular impact of glucose and insulin homeostasis dysregulation (generalized), haemostatic balance and inflammatory processes (generalized), protein catabolism (generalized) and salivary gland function (salivary glands) (Figure 3). These proteins may be indicative of complications resulting from the disease and therefore we propose them as (potential) biomarkers for diabetes complications and prognosis. The proposed panel of biomarkers of diabetes complications, includes both Albumin and Lactotransferrin that are common to some of these processes. Therefore, these

proteins can be considered more generic indicators of diabetes complications but not specific markers of one of these complications.

The identification of a panel of biomarkers for Type 2 Diabetes Mellitus complications in saliva is a pilot study that needs validation in a larger number of samples allowing a stronger correlation of the molecular data with patient complications. These results can serve as a basis for the development of new tests to support a more complete diagnosis of diabetes and its complications, and a point-of-care for close monitoring and providing frequent data to the physician.

Protein name	Fold change
Basic salivary proline-rich protein 1	-20

Salivary Glands

Retinopathy

Protein name	Fold change
Alpha-2-macroglobulin-like protein 1	-2.78
Heat shock protein beta-1	-2.49
Albumin	2.23
Alpha-1-antitrypsin	2.75
Hemopexin	3.38
Lipocalin-1	6.02

Atherosclerosis

Protein name	Fold change
Fibrinogen alpha chain	2.11
Fibrinogen beta chain	4.06
Bactericidal permeability-increasing protein	14.99

Generalized

Protein name	Fold change
Endoplasmic reticulum chaperone BiP	-2.48
Cystatin-SN	-2.18
Glutamine synthetase	-1.62
Zinc-alpha-2-glycoprotein	-1.56
Trypsin-3	1.38
Matrix metalloproteinase-9	2.75
Lactotransferrin	5.25
Carbonic anhydrase 6	10.52

Protein name	Fold change
Protein S100-A8	1.38
Protein S100-A9	1.77
Neutrophil elastase	8.02

Diabetic Foot Ulcer

Figure 3. Proposed salivary biomarkers of diabetes complications according to the localization of the altered process.

Supplementary Materials: Table S1: LC-MS identification table with protein summary (Protein FDR Summary); Table S2: Proteins identified with an FDR 5% and p < 0.05; Table S3: List of proteins with a VIP score > 1 not listed in the enrichment analysis. These are the proteins that better differentiate Type 2 Diabetes Mellitus and Healthy samples. Shown are the UniProtKB accession number (AC), protein names, gene names, fold change (Type 2 Diabetes Mellitus /Healthy) and the variable importance projection (VIP) score defined by the PLS-DA analysis; Figure S1: PANTHER Biological processes of saliva proteins. Box size follows the number of proteins involved in each biological process. 1 – Cellular process (297 proteins); 2 – Metabolic process (226 proteins); 3 - Biological regulation (132 proteins); 4 – Response to stimulus (129 proteins); 5 – Localization (78 proteins); 6 – Immune system process (72 proteins); 7 – Signaling (51 proteins); 8 – Biological process involved in interspecies interaction between organisms (34 proteins); 9 – Multicellular organismal process (32 proteins); 10 – Developmental process (31 proteins); 11 – Locomotion (14 proteins); 12 – Biological adhesion (14 proteins); 13 – Growth (6 proteins); 14 – Reproductive process (2 proteins); 15 – Reproduction (2 proteins) and 16 – Biomineralization (1 protein).

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