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The salivary glands and saliva of *Anopheles gambiae* as an essential step in the *Plasmodium* life cycle: a global proteomics study

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The salivary glands and saliva of *Anopheles gambiae* as an essential step in the
Plasmodium life cycle: a global proteomics study

Valérie Choumet^{1*}, Annick Carmi¹, Christine Laurent², Pascal Lenormand², Jean-Claude Rousselle², Abdelkader Namane², Charles Roth¹ and Paul T. Brey¹

¹ Unité de Biochimie et de Biologie Moléculaire des Insectes, Institut Pasteur, 28 rue du Dr Roux, 75724 Paris cedex 15

² Plate-forme de protéomique, Institut Pasteur, 28 rue du Dr Roux, 75724 Paris cedex 15

* to whom correspondance should be addressed: tel: 33145688630; fax: 33140613471; email: vchoumet@pasteur.fr

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SUMMARY

Proteins synthesized in the salivary glands of the *Anopheles gambiae* mosquito are thought to be important in the life cycle of the malaria parasite *Plasmodium*. To describe *Anopheles gambiae* salivary gland and saliva contents, we combined several techniques: 1-DE, 2-DE and LC MS/MS. This study has identified five saliva proteins and 122 more proteins from the salivary glands, including the first proteomic description for 89 of these salivary gland proteins. Since the invasion and sporozoite maturation take place during the process of salivary glands ageing, the effect of salivary gland age on salivary component composition was examined. LC MS/MS profiling of young versus old salivary gland proteomes suggests that there is an over-representation of proteins involved in signalling and proteins related to the immune response in the proteins from older mosquitoes. iTRAQ labelling was used for a comparative proteomic analysis of salivary gland samples from infected or *Plasmodium berghei*-free mosquitoes. The expression levels of five secreted proteins were altered when the parasite was present. These observations will serve as a basis for future work concerning the possible role of these proteins in the interaction between *A. gambiae*, *Plasmodium* and the mammalian host.

INTRODUCTION

Malaria is a parasitic disease that affects 200 million people worldwide and causes 1.5 to 2.7 million deaths per year. Of the 300-500 million clinical cases annually, nearly 90% are in the sub-Saharan countries of Africa where the malaria parasite, *Plasmodium falciparum*, is primarily transmitted by the mosquito *Anopheles gambiae*. The increasing resistance of the parasite to inexpensive drugs and the resistance of mosquitoes to insecticides have created an urgent need for innovative methods that block parasite transmission during its development within the insect. The *Anopheles* mosquito not only carries the parasite from infected to uninfected people, but also plays a vital role in the parasite life cycle [1]. Mosquito saliva and salivary glands are central to the interaction between parasite, vector and mammalian host. Sporozoite maturation in the mosquito salivary glands before its transmission to vertebrates is a key stage for the effective transmission to humans since it increases the sporozoite's ability to infect vertebrate hepatocytes [2]. Additionally, sporozoites are injected into the vertebrate skin with nanolitre volumes of saliva, a complex biologically active solution, which, in addition to other activities, serves as the "transmission fluid" for the malaria parasite.

The salivary glands and their diversified protein contents are essential for overcoming the challenges posed by the host: pain and itch responses, immune defences and haemostasis [3]. There is convincing evidence that the pharmacological activity of arthropod saliva affects pathogen transmission. Salivary gland lysate from the sand fly *Lutzomia longipalpis* facilitates the infection of mice by the protozoan parasite *Leishmania major* [4, 5]. However, there has been little work on the role of mosquito salivary gland proteins in promoting infection of *Plasmodium* species in vertebrate hosts. During the last 3 years, there have been several studies on the

transcriptome and the proteome of salivary glands of arthropod vector saliva [6-11]. Kalume *et al.* [12] identified 67 proteins from *Anopheles gambiae* salivary glands, an initial step towards the cataloging of the hundreds of proteins and peptides in the salivary proteome. However, no attempts have been made to study the proteome of *Anopheles gambiae* saliva in the presence of malaria parasite.

This communication presents an expanded investigation of saliva and salivary proteins in blood-fed *A. gambiae* mosquitoes determined by several proteomics approaches. These techniques ensured good coverage of salivary gland proteins of varied pIs and molecular weights. The iTRAQ labelling technology was used to quantitate differences in the proteomes of *Plasmodium berghei*-infected and non-infected *A. gambiae* salivary glands.

MATERIALS AND METHODS

Reagents

Mosquitoes

Yaounde strain adult *A. gambiae* females were reared in insect rooms at $26\pm 0.5^{\circ}\text{C}$, 70% relative humidity, with a 16h/8h light : dark photoperiod. The adult female mosquitoes used in these experiments were either aged between 5 and 8 days or between 18 and 21 days and had blood meals 3 to 5 days after emergence. *Plasmodium berghei* NK65 strain parasites, transformed to express GFP at the sporozoite stage, were injected into mice by intraperitoneal injection; seven days later, female mosquitoes aged 2-3 days were fed on the infected mice. All mosquitoes were maintained on a diet of 10% Karo syrup solution. Salivary glands from either 5-8 day old or at 18-21 day old mosquitoes were dissected in 150 mM NaCl with protease inhibitors (Complete, Roche Diagnostics, Mannheim, Germany) at 4°C and stored at -80°C . Saliva was collected using artificial feeders. After lyophilisation, saliva components were re-suspended in water and stored at -80°C .

Salivary gland extract preparation

Salivary glands were disrupted by ultrasound (Cup horn, Sonics & Materials Inc., Newton, CT, USA) for 20 min at maximum amplitude. Salivary gland homogenates were then centrifuged for 30 min at $130,000g$ and protein was quantified using the BCATM protein assay (Pierce, Rockville, IL, USA). Aliquots of salivary gland extracts were stored at -80°C until use.

SDS PAGE

SG samples of 10 μg or 36 μg of salivary gland were dissolved in Laemmli sample buffer, and boiled for 5 min. After centrifugation (14000rpm, 10 min), 20 μl samples were loaded onto a 12% acrylamide, 1mm-thick SDS PAGE Bis-Tris minigel, and subjected to electrophoresis on a Novex apparatus (Invitrogen, Carlsbad, CA, USA). Protein molecular weight markers (Precision Plus Protein standard all blue, Bio-Rad, Hercules, CA, USA) were run on the same gel. The gel was stained with Bio-SafeTM Coomassie (Bio-Rad) or silver nitrate (PlusOneTM, GE Healthcare, Uppsala, Sweden). Two methods were used to isolate proteins from the gel for mass spectrometry. One method consisted of cutting out all bands visible after Coomassie or silver staining. The other method consisted of cutting the gel into 1 mm-thick slices. The plugs obtained were analyzed by mass spectrometry.

2-DE

Samples of salivary gland supernatant, corresponding to 50 or 120 μg of protein, were used for 2-D gel analysis. To improve 2-D gel profiles, samples were treated using a ReadyPrep 2-D Cleanup kit (Bio-Rad, Hercules, CA, USA). The pellet recovered after the last centrifugation step was dissolved in 15 mM NaCl, 0.5% SDS (final concentration), and 2% Triton X100 (final concentration). The sample was heated at 95°C for 3 min, flash-frozen in liquid nitrogen and lyophilized. The lyophilized material was dissolved in 2-DE sample buffer (7M urea, 2M thiourea, 4% CHAPS, 150 mM DTT, and 2% ampholytes).

SG samples (30 μl) were loaded onto IEF 18cm gels containing ampholines of pH ranging from 4 to 8 (Bio-Rad), and run for 20000 Vhrs. The second dimension was carried out on 12.5% acrylamide 22cm slab gels. Resolved proteins were detected by SYPRO®Ruby (Invitrogen). For

each type of salivary gland extract (young blood-fed, 21 day blood-fed, infected), at least three independent sample preparations were used, and at least three independent gel analyses were carried out.

Mass spectrometry

MALDI-TOF-MS and database searches

Mass spectrometry was performed using a MALDI-TOF instrument (Voyager-DE-STR, Applied Biosystems, Framingham, MA) operated in positive ion reflector mode. Sample preparation for in-gel digestion was carried out as described previously [13]. Bands and spots of interest were cut out using the Investigator ProPic robot (Genomic Solutions). Plugs were washed with 100 mM ammonium bicarbonate (Sigma) and proteins reduced with 10 mM 1,4-dithiothreitol (Sigma, Saint-Louis, MO, USA), S-alkylated with 55 mM iodoacetamide (Sigma) and in-gel digested at 37°C for 4 hours with modified porcine trypsin (Promega) using the Investigator ProGest robot (Genomic Solutions, Ann Arbor, MI, USA). Peptide mixtures were desalted on ZipTip C18 (Millipore) and directly eluted onto the Maldi target using the Investigator ProMS robot (Genomic Solutions). The elution solvent consisted of a six-fold dilution of a saturated solution of CHCA (10mg/ml, Sigma) in 70% ACN (J.T. Baker) containing 0.1% TFA (Sigma). Each mass spectrum (700-3000 m/z) was acquired in automatic mode (12 sub-spectra of 50 laser shots were accumulated). Trypsin autolysis peptides were used as internal calibratants (fragment 108-115: $[M+H]^+ = 842.5100$ and fragment 58-77: $[M+H]^+ = 2211.1046$). A local copy of MS-FIT 3.2 software, part of the Protein Prospector package (University of California, San Francisco) was used to search the NCBI or *Anopheles* Ensembl databases. Search parameters were set as follows:

only monoisotopic masses were used, a maximum peptide mass error of 50 ppm was allowed and one incomplete cleavage *per* peptide and a possible oxidation of methionine were considered. Moreover, no restrictions on M_r or pI were made, and a minimum of four matching peptides covering a minimum of 15% of the protein sequence, were required for protein identification. If necessary, MALDI-TOF-PSD experiments were carried out to reach protein identifications using MS-TAG software (part of Protein Prospector package).

LC MS/MS

Protein digestion before identification by LC MS/MS

Proteins were reduced, alkylated with 10 mM iodoacetamide, and digested with porcine trypsin (ratio 1:100) overnight at 37°C. The trypsin digests were desalted with C₁₈ tips (OMIX, Varian), and stored at -80°C before LC MS/MS analysis.

LC MS/MS analysis

Prior to reverse phase nanobore liquid chromatography tandem mass spectrometry (nanoLC MS/MS) analysis, samples were dissolved in Solvent A containing 5% acetonitrile and 0.1% formic acid. The nanobore LC system was from LC Packings (Amsterdam, The Netherlands), and consisted of a Famos autosampler and an Ultimate Nano LC system. It was interfaced with a QqTOF mass spectrometer, QSTAR XL (AB/MDS Sciex, Foster City, CA), using a nanoelectrospray source (Protana Engineering A/S, Odense, Denmark). Reverse phase LC was performed using a PepMap column (75- μ m inner diameter x 150-mm long, LC Packings, Dionex) equilibrated with Solvent A. The peptides were eluted using a linear gradient of 5% to 40% solvent B (95% acetonitrile, 5% H₂O, and 0.1% formic acid) in 90 min with a flow rate of 200 nl/min. This binary gradient was used for protein identification and iTRAQ experiments. We

operated the QSTAR XL mass spectrometer in an information-dependent-acquisition (IDA) mode; each full MS scan was followed by two MS/MS scans where the two most abundant peptide molecular ions were dynamically selected for CID, and dynamic exclusion was used to prevent repetitive selection of the same ions within a preset time. Collision energies were set to automatically adjust according to the charge state of the precursor ions.

iTRAQ Sample Preparation Procedure.

We denatured 40 μ g of each sample protein and blocked the cysteines as described in the iTRAQ protocol (Applied Biosystems, Foster City, CA). Each sample was then digested with trypsin solution overnight at 37 °C, and labelled with the iTRAQ tags as follows: non infected salivary glands, iTRAQ114; infected salivary glands by *P. falciparum*, iTRAQ116 or iTRAQ 117. The labelled samples were pooled and acidified for strong cation exchange (SCX) chromatography. The eluted peptides were then lyophilised and stored at -81°C before analysis.

Database search and relative quantification

MS/MS data were analyzed using ProID protein identification software version 1.1 (AB/MDS Sciex, Foster City, CA) using *A. gambiae* ORF database (Ensembl) [14]. In ProID, the peptide tolerance and the MS/MS tolerance were set to 0.15 Da. We manually inspected the MS/MS spectra to validate the identified peptides.

ProQUANT 1.1 (AB/MDS Sciex, Foster City, CA) and the *A. gambiae* ORF database (Ensembl) were used to analyze data from the iTRAQ experiments. The confidence cut off was 95. The tolerances set for peptide identification in ProQUANT searches were 0.15 Da for MS and 0.1 Da for MS/MS. We manually validated all identifications. Relative protein quantification in iTRAQ experiments was performed on the MS/MS scans and was the ratio of the areas under the peaks of

iTRAQ reagent tags at 114, 116, and 117 Da. The quantification results were normalized using the overall ratio obtained for all tagged peptide pairs in the sample.

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RESULTS AND DISCUSSION

Analysis of salivary gland, saliva and saliva components of 8 day-old blood-fed *Anopheles gambiae*

One-dimensional electrophoresis

Salivary gland extracts

Two series of experiments were performed. In the first series, 12% SDS-PAGE gels were run with 10 µg of protein extract obtained from salivary glands of 8 day-old females. After Coomassie staining, protein bands were excised and the tryptic digests were analyzed by MALDI-TOF mass spectrometry. In the second series, 12% SDS-PAGE gels were run with 36 µg of protein extract. After Coomassie staining, 1 mm-thick plugs were cut from the gel (Figure 1, supplementary Table 1). Protein identification was performed as described in Methods and seventy percent of the bands were identified (Table 1).

Saliva

A total of 18 saliva samples each from 400 female 8 day-old blood-fed *A. gambiae* were collected in water. After lyophilization, saliva components were resuspended in water and analyzed by SDS-PAGE and stained with silver nitrate (Figure 2). The stained gel bands were cut and analyzed by mass spectrometry. Five proteins were thereby identified (Table 1).

Two-dimensional analysis

After 2-D gel electrophoresis of 120 μg of salivary gland proteins, the trypsin-digested spots were analyzed by peptide mass fingerprinting, using Maldi-Tof, or by PSD Maldi-Tof. From the total set of 204 spots (Figure 3), 29 proteins were identified and described (Table 1, supplementary Table 2). MS identification showed that 37% of these proteins produced several spots during electrophoresis. Spots at varying pIs were found for the putative 5' nucleotidase precursor in the 62-kDa region of the gel (spots 13 to 33 in Figure 3) as well as for the D7 precursor allergen AED A2 in the 30-kDa region (spots 114-119, 121-125) and for D7 related-4 protein precursor in the 16 kDa region (spots 171-176). The profile of the 30 kDa protein was of particular interest with its intense spot at 32.5 kDa and a trail of spots with molecular weights between 30 to 20 kDa (Figure 3). According to the Ensembl database (release 35), two forms of the protein exist, including a long mature form of 24732.75 kDa (ensangp000000028522), and a short mature form of 13786.59 kDa (ensangp000000022344); however, only the short form remained in the Ensembl release 43. The proteomic data are consistent with a larger form of the D7 precursor that is processed by proteolytic cleavage. Several other spots identified as being secreted proteins had apparent M_r smaller than expected according to their genomic predicted M_r in Ensembl (Table 1). This was the case for the 5' nucleotidase precursor protein that was identified in spots of apparent molecular weights ranging from 62 kDa to 29 kDa (Table 1, Figure 3). To determine whether this range of sizes is due to an artifact that occurs before or during sample preparation, 2-DE profiles of salivary gland extracts obtained after several freeze/thaw cycles were examined. These profiles did not differ from those of extracts obtained after our normal sonication and centrifugation procedure (data not shown). Additionally, the heating stage was not responsible for proteolysis since the numbers of spots observed with heated salivary

gland extracts and those not heated were similar (data not shown). These observations indicate that several secreted proteins may present sequence divergence or be extensively processed and/or post-translationally modified in *A. gambiae* salivary glands. This idea is supported by the following points: 1) several proteins were only identified after post source decay; 2) an extensive processing of the human saliva proteome has been described [15].

Identification of salivary gland components using LC MS/MS

LC MS/MS is an alternative strategy for large-scale protein identification that bypasses the initial protein separation step. It consists of enzymatic cleavage of a complex protein mixture and separation of the resulting peptides by chromatography before tandem mass spectrometry identification. This gel-free strategy has worked for large-scale protein identification of several biochemical systems [16].

Using this system, 30 proteins were identified with confidence (ProtScore cutoff > to 95%). Of these 30 proteins, 15 proteins (50 %) were matched with two or more peptides and the other 50% were identified by a single peptide hit (Table 1). Using this technique, we were able to confirm that there is a problem in the ensangp00000022344 annotation corresponding to our 30 kDa protein, since only two of the five peptide sequences identified by LC MS/MS were present in the current ensangp00000022344 sequence (Ensembl release 43).

Proteome coverage of 8 day-old blood-fed Anopheles gambiae salivary gland

Together, the three technologies characterised 55 different proteins, four of which (ensangp00000028522, ensangp00000026134, ensangp00000027538, ensangp00000015472) are no longer present in the latest genome annotation (Ensembl release 43). LC MS/MS and 2-DE-MS identified a similar number of proteins and both appear more effective than 1-DE-MS. Thirty

percent of the proteins identified are secreted. Ensangp00000013568, which is predicted to have aspartic-protease activity, is one of the newly identified proteins. Blast analysis has shown that this protein has 88% sequence identity with protein AAEL006169-PA in the *Aedes aegypti* genome and is also similar to cathepsin D enzymes of other insects such as *Drosophila melanogaster* and *Bombyx mori*. Insect cathepsins D have been shown to be involved in metamorphosis [17] and their levels are modulated in pathogen-infected insect tissues [18].

Analysis of salivary gland components of 21 day-old blood-fed *Anopheles gambiae* salivary glands and comparison of salivary gland components from young (8 days) and old (21 days) blood-fed female mosquitoes

Plasmodium berghei development in *A. gambiae* takes about 14 days from the infective bloodmeal until the parasite is ready to infect its mammalian host. Thus, the proteomic profile of salivary glands may be affected by ageing. To identify the molecular changes that may occur in salivary gland cells, the proteomic profile of 21 day-old female salivary glands was analyzed by LC MS/MS (Table 2). A total of 41 different proteins were characterised (Table 2). Nineteen of these proteins were described at a proteome level for the first time. Ensangp00000029528 (apolipoprotein D precursor), a protein identified as an infection-responsive protein in the *Anopheles* midgut [19], was one of these proteins described for the first time. iRNA silencing of the midgut transcript encoding APOD resulted in increased *Plasmodium* levels. Also among the newly identified intracellular proteins, ensangp00000029324 deserves particular attention. This protein belongs to the family of α 2-macroglobulins and has 64% sequence identity with TEP15 in a FASTA comparison. These thioester-containing proteins are protease inhibitors that can play an important role in immune responses. Ensangp00000029324 has 39.42% sequence identity

with a protein described in *Ornithodoros moubata* [20]. This *O. moubata* protein is expressed in tick salivary glands, haemocytes and Malpighian tubules and its expression is enhanced in response to a blood meal. Using gene expression screening for immune response genes in the *A. gambiae* transcriptome, Oduol *et al.* [21] identified an $\alpha 2$ -macroglobulin-related molecule that responded strongly to malaria parasite infection. Thus, one could propose that ensangp00000029324 is involved in the defence against pathogens such as parasites, bacteria or fungi.

Using only the LC MS/MS identified proteins, the level of salivary components after 8 days was compared to those after 21 days of salivary gland development. Their functions were compared (Figure 4). The composition of young salivary glands was less diverse than that of older salivary glands: i.e. fewer proteins could be identified in young salivary glands (29) than in older glands (42). Eighteen proteins were common to both salivary gland extracts and most were secreted proteins (30 kDa, apyrase, 5' nucleotidase, D7 precursor allergen AED A2, D7r1, D7r2, D7r3 and D7r4, maltase precursor, peroxidase precursor, GSG6, GSG7, putative gVAG); among the other identified proteins, three were glycolytic enzymes (phosphoglycerate mutase, malate dehydrogenase, triosephosphate isomerase), one was an RNase and another one was an actin-binding protein (ensangp00000012938). All the secreted proteins identified in 8 day-old salivary glands were also found in 21 day-old salivary glands, whereas 6 additional, secreted proteins (D7 r5, GSG5, lysozyme and the hypothetical proteins 8.8, 10 and 10.2 kDa) were specific to the 21 day-old salivary glands. Protein functions, including transcription, signalling and metabolism, assigned to some of the housekeeping proteins that were found in 8 day-old salivary glands were also identified in 21 day-old salivary glands, although a larger variety of proteins were associated

with signalling and transcription regulation. Additionally, apolipoprotein D, lysozyme and α 2-macroglobulin, involved in the response to pathogens, were detected in 21 day-old salivary glands, but were not detected by LC MS/MS in 8 day-old salivary glands. A partial list of age-related mammalian protein variation from the study of ageing mammalian organs [22] includes proteins involved in: (i) telomere repair, (ii) stress response, (iii) anti-oxidant defence, (iv) nicotinamide deamination, (v) insulin/insulin-like growth factor-1 signalling, (vi) histone deacetylation, and (vii) regulation of the transcription of specific proteins, such as those involved in pituitary development. Specific age related signatures in the transcriptome of *Drosophila* body parts have also been investigated [23]. That study showed the presence of up-regulated mRNA levels in the aged thorax, where salivary glands are located, for immune response genes, genes linked to cellular morphogenesis as well as those for actin filament-based processes. Cellular components of the endoplasmic reticulum and the proteasome complex were also over-represented. Thus, our observations indicating an increased level of a subset of salivary gland proteins is consistent with the transcriptional results observed in *Drosophila*. Interestingly, proteins involved in lipid metabolisms were only identified in 21 day-old salivary glands. Lipids are known to be important for parasite metabolism. Rosinski-Chupin *et al.* [11] showed that genes involved in lipid metabolism were up-regulated by *Plasmodium berghei*. This observation suggests that the maturation of sporozoites may require happening in ageing salivary glands.

Comparison of infected and non-infected salivary gland composition

iTRAQ, an isotope labelling approach, was used for the quantitative study of gene expression at the proteome level. This approach is based on chemical isobaric tagging of the N-terminus of peptides generated from trypsin digests of proteins isolated from cells or tissues in different

states. The labelled samples are combined, fractionated together by strong cation exchange chromatography and analysed by nanoLC mass spectrometry. The labelled peptides and hence the corresponding proteins are then identified by database searching using the MS/MS data. The fragmentation of the tag attached to the peptides generates a low molecular mass reporter ion which is unique to the tag used. Comparison of the intensities of these reporter ions gives relative protein quantification. Table 3 shows the list of proteins identified and quantified using iTRAQ.

Twelve identical, secreted proteins were found in uninfected and infected salivary glands during three separate comparisons (Table 3). The ratios of reporter ion peaks of infected versus non-infected salivary glands varied between 0.65 and 1.97. From the ratio values, it was deduced that the expression of five of the proteins, was altered. The level of gVAG is increased two-fold in infected salivary glands, whereas the levels of GSG6, apyrase, D7 related-1 protein precursor and D7 precursor allergen AED A2 are decreased with ratios ranging from 0.67 to 0.77 for these proteins (Table 3). The presence of pathogens in salivary glands has been reported to induce modifications in insect behaviour and/or modifications in saliva composition. gVAG is a protein of the antigen 5 family and it has similarities with the mammalian cysteine-rich secretory proteins, vespilid antigen 5 and plant-pathogenesis-related proteins [27]. The precise function of these secreted proteins is unknown. The level of gVAG mRNA was shown to be increased in the midgut of mosquitoes infected with *Plasmodium falciparum* compared to level in uninfected midguts [19]. The silencing of this gene resulted in increased *Plasmodium* levels, suggesting that gVAG is a defence-related protein [19]. We therefore expect a similar role of gVAG in *A. gambiae* salivary glands.

The level of apyrase was reduced by a factor of 1.5 in *P. berghei*-infected *A. gambiae* salivary

glands. Apyrase inhibits ADP-induced platelet aggregation and, therefore, affects blood-feeding. The level of apyrase influences the probing time of *Anopheles gambiae* [28]. The reduction of apyrase abundance by 85% in salivary glands from *A. gambiae* due to the injection of double-stranded apyrase encoding RNA was correlated with increased probing time. Decreased apyrase levels in *Plasmodium gallinaceum* infected *Aedes aegypti* salivary glands caused an increase in mosquito probing time [24]. An increase in probing time has also been observed for *Anopheles gambiae* infected with *Plasmodium falciparum* [29]. Additionally, transcription of the apyrase encoding gene appears to be repressed in *P. berghei*-infected *A. gambiae* mosquitoes [11]. Our observation is, therefore, consistent with these data and we can expect an increase probing time for *P. berghei*-infected *A. gambiae*.

The levels of D7 precursor allergen AED A2 and D7 related-1 protein precursor proteins were decreased in infected salivary glands by a factor of 1.3 and 1.5 respectively. The D7 short proteins bind serotonin with high affinity, as well as histamine and norepinephrine, thus antagonizing the vasoconstrictor, platelet-aggregating, and pain-inducing level of these factors [30]. The decreased production of D7 related-1 protein precursor may induce an increased local inflammatory response to mosquito bites, thus modifying the immune response to the parasite. Although we did not observe a change in D7 related-4 protein precursor protein levels in our analysis, Rosinski-Chupin *et al.* [11] observed variable D7 related-4 protein precursor gene expression using SAGE. The proteins D7 precursor allergen AED A2 and GSG6 have no known function, thus we cannot anticipate the consequence of reducing their expression on parasite development and transmission.

Table 3 also shows that the iTRAQ technique identified forty three proteins not observed using

by LC MS/MS analysis of salivary gland extracts from insects of the same age. This result is consistent with the previous observations that better fragmentation is obtained using this technology, giving more peptides per protein and allowing the identification of less abundant proteins [31, 32]. One of the newly identified proteins was a homolog of “translationally controlled tumour protein” (Tctp). Tctp homologues have been described in mammals and in many other species, including plants, earthworm, parasites, hydra and yeast [33-38]. They are heat stable, calcium-binding proteins [39] and their expression is induced in response to various stimuli within cells [38]. Tctps also bind haem, and tubulin [40]. Tctps induce the release of histamine [41] and the secretion of interleukin-4 [42] from basophils. Despite having a ubiquitous tissue distribution, multiple specific potencies [43] and highly conserved amino acid sequences, their primary physiological role remains unclear [40]. The *A. gambiae* protein has the highest identity scores with its *Aedes albopictus* and *Aedes aegypti* homologs (85%). An identity score of 44% was observed with the ticks *Dermacentor* and *Ixodes* salivary histamine-releasing factors (HRF) [44, 45]. The tick HRF recombinant protein induced histamine secretion from a rat basophilic leukaemic cell line, in a dose-dependent manner. We suggest that the *Anopheles gambiae* Tctp homolog is present in saliva and contributes to the allergic inflammation associated with the *Anopheles gambiae* bite. Thus, if it is similarly able to trigger cutaneous mast cell histamine release, as observed with the *Schistosoma mansoni* Tctp homolog [46], the resulting vasodilation could facilitate *Plasmodium* sporozoite migration into blood vessels.

Our data also identify Serpin 9 (ensangp00000016680) at the proteomic level for the first time. Serpins are a very large family of serine protease inhibitors with various biological functions that are found in all higher eukaryotes and viruses [47]. The mosquito genome contains 14 annotated serpin genes, 10 of which are inhibitory protease substrates. Some of these serpins are involved

in immune signal amplification cascades. Serpin 9 is involved in the arthropod immune response and during *Staphylococcus aureus* infection, it is only induced late in infection [48]. In contrast, during the *Plasmodium* life cycle in mosquitoes, serpin 9 is primarily activated when the midgut epithelium is invaded by ookinetes [48]. However, a tag corresponding to Serpin 9 was identified in the *A. gambiae* salivary gland using SAGE [11], but the level of this tag was not modified by *Plasmodium* infection.

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CONCLUDING REMARKS

In this study, complementary proteomic approaches were used to catalogue 122 *Anopheles gambiae* salivary gland proteins from blood-fed 8 day-old and 21 day-old females (supplementary Table 3). The most acidic proteins identified were the 30 kDa protein (pI, 3.8) and calmodulin (pI, 3.9) and the most basic proteins were retrovirus-related pol polyprotein (pI, 11.28) and ensangp00000015472 (pI, 10.38). The smallest proteins identified were hypothetical 8.8 kDa (M_r , 8.8 kDa) and retrovirus related pol polyprotein (M_r , 9.6 kDa) and the largest was Ryanodin receptor 1 (M_r , 577.8 kDa). Our approach confirmed the presence of seven proteins identified in earlier Ensembl annotations but not listed in the latest version (version 43). This observation emphasizes the complementarity of proteomic and genomic approaches for accurate genome annotation, an idea previously suggested by Kalume *et al.* [49].

LC MS/MS was clearly the most powerful technique (Figure 6A). iTRAQ labelling led to the identification of 78 proteins, 39 of which were not identified by classical LC MS/MS, illustrating the value of using the two technologies in parallel for maximum proteome coverage. The proteins identified in this study were sorted into functional categories based on their annotations in the database and the results are summarised in Figure 6B. A large proportion of the identified proteins are involved in energy pathways, blood or sugar feeding, protein folding, modification and in amino acid metabolism, but the largest group (37%) is composed of proteins with no known function. The same situation is also encountered in the proteomic analysis of human saliva [50]. In *Anopheles gambiae*, twenty five percent of the identified proteins are predicted to have a signal sequence and are, therefore, putatively present in saliva. The largest category of peptide sequences was that derived from secreted proteins, demonstrating that they are the most

abundant proteins in salivary gland extracts. This observation is consistent with the findings of Kalume *et al.* [12].

Seventy-five percent of the 122 proteins reported here are identified in an *Anopheles gambiae* salivary gland proteomic study for the first time. Most of these newly identified proteins are housekeeping proteins and only few, such as GSG5, GSG3, ensangp00000029324, serpin 9, hypothetical 10 kDa and apolipoprotein D precursor, are secreted. The 2-D gel analysis suggests that some secreted proteins, including 5' nucleotidase, D7 precursor allergen AED A2, D7 related-4 protein precursor and 30 kDa, are extensively processed, although the consequence of such modifications on their activity is unknown. LC/MS-MS profiling of young versus old salivary gland proteomes suggests that there is an over-representation of proteins involved in signalling, proteins implied in carbohydrate and lipid metabolism and proteins related to the immune response in older glands. As the invasion and the maturation of sporozoites occurs during the ageing process of salivary glands, it would be interesting to know whether the age of the salivary gland affects parasite transmission. Finally, we detected a change in the level of five salivary proteins in the presence of *Plasmodium berghei* sporozoites. These observations will serve as a basis for future work to determine the possible role of these proteins in the interaction between *A. gambiae*, *Plasmodium* and the mammal host.

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LEGENDS TO FIGURES

Figure 1: SDS-PAGE of salivary gland extracts from 8 day-old blood-fed *A. gambiae*

Salivary components were separated by a 12% NU-PAGE Bis-Tris gel under denaturing and reducing conditions. Molecular mass markers are shown on the left. After Coomassie staining, the gel was cut into millimeter slices as indicated by the numbers on the right side of the figure. The plugs obtained were analyzed by mass spectrometry as described in the Methods section.

Figure 2: SDS-PAGE of 8 day-old blood-fed *A. gambiae* saliva

Saliva was collected from 7200 females using artificial feeders. After lyophilisation, saliva components were re-suspended in water and aliquots were analyzed by SDS-PAGE. Following silver nitrate staining, the numbered protein bands were analyzed by mass spectrometry.

Figure 3: 2-DE analysis of salivary gland extracts from 8 day-old blood-fed *A. gambiae*

Salivary gland extracts were purified by ReadyPrep 2D Cleanup kit and 120 µg of proteins were solubilized in 2D sample buffer, as described in the Methods section. Proteins were separated in the first dimension using carrier ampholyte gradient gels between pH 4 and pH 8. Separation in the second dimension was performed using 12.5% SDS acrylamide gel. The gel was stained using SYPRO® Ruby.

Figure 4: Comparison of 8 day-old and 21 day-old salivary component functional annotations.

A) 8 day-old salivary gland components; B) 21 day-old salivary gland components.

Figure 5: Functional annotation of the 122 salivary components identified in 8 day-old and 21 day-old blood-fed *Anopheles gambiae*.

A) Contribution of various proteomic approaches to protein identification; B) Biological processes in which they are involved.

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Table 1 Proteins identified in salivary gland extracts of 8 day-old blood fed *Anopheles gambiae*

Ensembl Identification (Ensembl release 43)	Protein Family/Description	Predicted M_r/pI	Identification	1-DE-MS % coverage	2-DE-MS spot number	2-DE-MS % coverage ^{a)}	LC MS/MS Peptide sequence	Comments	Subcellular localization ^{b)}	Found in other proteomic (P) or transcriptomic (T) studies
Ensangp00000028522 ^{c)}	30 kDa protein	26.9/3.8	2-DE-MS	-	84, 184-188, 190, 192-193, 196-199, 201-202	PSD	EQELSDCIVDKR IKECFSSLDK ELDDGLIEREQELSDCIVDK LMNPTIDLVSSTIEKYSK ECFSSLDKDVSAAMVK KDDAEEDSEEGGEEGGDGASG G EGGEKESPR	GE rich salivary gland	secreted	P [12], [9]
Ensangp00000018590	5 aminolevulinate synthase erythroid specific mitochondrial precursor *	46.31/7.54	2-DE-MS	-	110	25	-	Metabolism of amino acid	mitochondria	this work
Ensangp00000015067	Ambiguous*	35.7/10.4	2-DE-MS	-	186	16	-	?	mitochondria	this work
Ensangp00000015256	Ambiguous/candidate odorant receptor*	44.85/7.01	LC MS/MS	-	-	-	AQRPVGTAGK	Olfactory receptor (drosophila)	membranar	this work
Ensangp00000022917	Ambiguous*	72.38/10.16	LC MS/MS	-	-	-	GRPILPLLKTQSYK	Tropomyosin domain	intracellular	this work
Ensangp00000024702	Ambiguous*	30.31/9.58	LC MS/MS	-	-	-	IHDGVTHAAK	?	?	this work
Ensangp00000026134 ^{c)}	Ambiguous*	23.01/10	2-DE-MS	-	169	PSD	-	?	?	this work
Ensangp00000015382	Apyrase	61.79/8.6	1-DE-MS, LC MS/MS,	20%	-	-	AAEEGDTCIAGIAR LNVAQVAGLR GDITNEEAIGASPFNTVDLLT LR	Anti-platelet	secreted	P [12]
Ensangp00000011707	Aspartate amino transferase*	44.71/6.78	2-DE-MS	-	95	17	-	Metabolism of amino acid	cytoplasmic	this work
Ensangp00000024137 and/or Ensangp00000016868 ^{d)}	ATP synthase subunit beta mitochondrial precursor	22.69/4.9 and/or 19.72/5.27	2-DE-MS, LC MS/MS	-	66-67	(31-37)	IINVIGEPIDER LVLEVAQHLGENTVR	Catalyzes ATP synthesis	mitochondria	P [12]
Ensangp00000018543	Chromosome associated polypeptide C XCAP C homolog	156.83/5.34	LC MS/MS	-	-	-	LQTELIELKR	Structural maintenance of chromosome ABC transporter	nuclear	this work

Ensangp00000003518	CoA carboxylase mitochondrial precursor*	130.5/6.67	1-DE-MS	15%	-	-	-	related domain Key enzyme in the catabolic pathway of odd-chain amino acids□ isoleucine, threonine, methionine and valine	mitochondria l matrix	this work
Ensangp00000026391	Cofilin	16.93/7.28	2-DE-MS, LC MS/MS	-	170	42	LFLMSWCPDTAK	Binds actin and assists in translocation of actin from the cytoplasm to the nucleus essential for cytokinesis, endocytosis and other cell processes that require rapid turnover of actin filaments	cytoplasmic	T [9]
Ensangp00000022538	Creatine kinase	26.4/5.18	2-DE-MS	-	90	30	-	Phosphorylation	cytoplasmic	P [12]
Ensangp00000025174 and/or Ensangp00000018280 ^d	D7 precursor allergen AED A2	35.57/5.7 and/or 32.7/5.1	1-DE-MS, 2-DE-MS, LC MS/MS	42%	114-119, 121-125, 149-151, 154, 169	(19-33) PSD	ALDPPEAWYVYER BVLIGLQLYEEK NYELSGSSQFK SADYAFLLR SANYGYLAMGK SDLEPEVR SVLASCTGTQAYDYYSCLLNS PVK DYELADSAEFR IYHGTVDVSAK NAFYFHELK NAMDCVFR	?	secreted	P [12], [9]

Ensangp00000018340*	D7 related-1 protein precursor	18.73/9.57	1-DE-MS, LC MS/MS	20%	-	-	BLVESTSGEAFK KLPALSQYSSVVDK KVFDTVELVK CLVESTSGEAFK	Anti-inflammatory Scavenger of biogenic amines	secreted	P [12], [9]
Ensangp00000018371*	D7 related-2 protein precursor	18.46/4.8	1-DE-MS, 2-DE-MS, LC MS/MS	28%	181-183	PSD	ANTFYTCFLGTSSLAGFK ESVLLELLQR HMQBVLEVVGFDGNGEVK KANTFYTCFLGTSSLAGFK MQTSDPFDMMNR NAVDYNELLK QYTPVSSDDMDK	Anti-inflammatory	secreted	P [12], [9]
Ensangp00000018330	D7 related-3 protein precursor	19.7/4.38	1-DE-MS, 2-DE-MS, LC MS/MS	33%	180-181	PSD	ANTFYTCFLGTSSAQAFK AGKLDMGTTFNAGQVSALMK LDMGTTFNAGQVSALMK YAVDYVELLR	Anti-inflammatory	secreted	P [12], [9]
Ensangp00000018328	D7 related-4 protein precursor	19.29/7.4	2-DE-MS, LC MS/MS	30%	171-176	(22-40)	LYDPLNIIELDK CIGECVQVPTSER RYEIIIEGPEMDK YTAEFVQIMK VFDLMELK	Anti-inflammatory	secreted	P [12]
Ensangp00000027211	Disulfide isomerase precursor	54.31/5.47	2-DE-MS	-	52	15	-	Catalyzes the rearrangement of -s-s- bonds in proteins	intracellular	P [12]
Ensangp00000014287	Electron transfer flavoprotein alpha subunit mitochondrial precursor*	34.14/8.62	2-DE-MS	-	113	33	-	Participates in catalyzing the initial step of the mitochondrial fatty acid beta-oxidation	mitochondria	this work
Ensangp00000003806	Facilitated glucose transporter	16.83/8.48	LC MS/MS	-	-	-	HISQIVPLVAKGFSSKPLVP	Sugar transporter	membranar	this work T [9]
Ensangp00000000937	probable Fatty acid binding protein	19.37/9.59	LC MS/MS	-	-	-	LGGGFDEETVDGR	Fatty acid binding protein	cytoplasmic	this work
Ensangp00000016366	Precursor	45.95/9.43	2-DE-MS	-	142	23	-	Involved in energy pathways	cytoplasmic	this work
Ensangp00000011661	Glutathion S transferase (class theta)	23.78/6.51	2-DE-MS	-	155	33	-	Key role in cellular detoxification	cytoplasmic and nuclear	This work P [50]
Ensangp00000024808	Glutathion S transferase	23.44/6.26	2-DE-MS	-	156	23	-	«□	«□	this work
Ensangp00000010081	Glycogen phosphorylase	96.48/6.33	1-DE-MS	18%	-	-	-	Carbohydrate metabolism	cytoplasmic	this work

Ensangp00000009988	GSG3	20.01/4.34	2-DE-MS	-	75, 76	PSD	-	?	secreted	this work T [51]
Ensangp00000019455	GSG6	13.05/5.15	1-DE-MS, LC MS/MS	36%	-	-	EPLPYMYACPGTEPCQSSDR ETREPLPYMYACPGTEPCQSS DR	?	secreted	P [12], [9]
Ensangp00000021970	GSG7	16.29/8.46	LC MS/MS,	-	-	-	SMHDVLCDRIDQAFLEQ TLAETAQCMR TLAETAQCLR YGVQNQLR	?	secreted	P [12]
Ensangp00000005326	Guanine nucleotide releasing factor	137.53/9.17	LC MS/MS	-	-	-	LIEKALIYK	May play a role in intracellular signaling cascade	M embrane- associated	this work
Ensangp00000021028*	putative gVAG protein precursor	28.9/8.96	1-DE-MS, LC MS/MS	43%	-	-	DGQMDVYYFVBNYSFTNIMD R FPYAGQNIATQFFGYR FVSSWWSEYLDARPEHVR GGPHVGCNPPSSSGGPTCQGK KYPSSYSGKPIGHFTQIASDR MPTLTWDPELASLADANAR VGCSMWYWK	Allergen Belongs to the CAP family: protease inhibitors or proteolytic activity, probably inhibiting host coagulation or complement activity Defence-related protein	secreted	P [12], [9]
Ensangp00000017720	3 Hydroxyisobutyrate dehydrogenase mitochondrial	34.31/9.27	LC MS/MS	-	-	-	VFADIVNASTGR	Involved in amino acid catabolism pathway	mitochondria 1	this work
Ensangp00000016660	Isocitrate dehydrogenase	46.96/7.59	1-DE-MS	32%	-	-	-	Plays a key role in cellular defense against oxidative stress- induced damage	mitochondria 1	this work
Ensangp00000020184	Malate dehydrogenase	35.27/9.52	LC MS/MS	-	-	-	ANTFVGEAAGVDPQK	Metabolic enzymes which catalyse the last step in anaerobic glycolysis	mitochondria 1	P [12]
Ensangp00000011006	Malate dehydrogenase	35.37/6.95	2-DE-MS	-	96	PSD	DDLFTNASIVR	Participates in the citric acid cycle	cytoplasmic	this work
Ensangp00000017682	Maltase	67.21/5.87	1-DE-MS, 2-	27%	6-8, 12	(17-43)	AMPSGAIANWVLGNHNSR	Carbohydrate	secreted	P [12],

			DE-MS, LC MS/MS				DQPETYDMVHQWR ELNVAAQLAAPR GITQTIDYLK	digestion Converts sucrose in nectar to glucose and fructose			
Ensangp00000015067	Mitochondrial carrier	35.74/10.4	2-DE-MS	-	186	16	-				
Ensangp00000011253	Nucleoside diphosphate kinase	19.01/8.46	1DE-MS,	32%	-	-	GDLCVQVGR	Maintenance of cellular pool of nucleoside triphosphates	cytoplasmic and plasma membrane	T [9]	
Ensangp00000012716*	putative 5' Nucleotidase precursor	63.47/7.01	1-DE-MS, 2- DE-MS, LC MS/MS	20%	10, 13- 33, 38- 51, 54, 57, 60, 64, 65, 77-82, 85-87, 130-134, 140-141, 144-145	(15-30) PSD	APFPLTLIHINDLHAR DQIYYVVVPSYLADGKDGFA MK ECIAGIAR GLAPYLAELEK LGTQVIGTTEVFLDRESCR LSGADLWSAIDHSFTLDDEFR MKIPTVVANLEK NVNIIVVLSHCGLDGDK QLAEEAGDLIDVIVGAHSHSLL LNK	Anti-platelet	secreted	P [12]	
Ensangp00000028058	Peroxidase precursor	24.99/8.23	1-DE-MS, LC MS/MS	16%	-	-	AFAGAININDHMFNPTVLER CFAIPVRPDDPVLSAGGIQCLD LVR LLPAEYGDGVYVPR SNITPELTILHVAFLR TTLVNMQFQQLVAHDMGLR WEDFVELR	Vasodilator	secreted	P [12], [50]	
Ensangp00000012460	Phosphoglycerate kinase	43.84/7.54	2-DE-MS	-	109	27	-	Glycolysis	cytoplasmic	this work	
Ensangp00000015800	Phosphoglycerate mutase	28.7/6.8	2-DE-MS, LC MS/MS	-	148	25	YGEEQVLIWR	Involved in energy pathways	cytoplasmic	this work	
Ensangp00000012492	Precursor	12.39/8.75	1-DE-MS	22%	-	-	-	EGF-like domain	?	this work	
Ensangp00000013568	Precursor	41.83/5.4	2-DE-MS	-	75	-	-	Aspartic protease A1	secreted	this work	
Ensangp00000016366	Precursor	45.95/9.43	2-DE-MS	-	142	23	-	Glucose- methanol-choline oxidoreductase Involved in energy pathways	cytoplasmic	this work	
Ensangp00000019046	Precursor	28.47/5.04	LC MS/MS	-	-	-	ANDRAMVK	EGF-like domain	?	this work	

Ensangp00000020734	Pterin 4 alpha carbinol amine dehydratase	21.20/10.23	LC MS/MS	-	-	-	LAQFLDQAAAVAK	Transcriptional activator/pterin dehydratase	?	this work
Ensangp00000027538 ^o	Retrovirus related pol polyprotein	9.51/11.28	2-DE-MS	-	181, 183	PSD	-	?	nuclear	this work
Ensangp00000021077	Ribonuclease	14.41/8.04	LC MS/MS	-	-	-	ALAPYNQAIVADR	Inhibits protein synthesis by cleavage of mRNA	?	this work
Ensangp00000027418*	Salivary gland 1-like 3	44.51/6.04	1-DE-MS	30%	-	-	-	?	secreted	P [12]
Ensangp00000018041	Toll precursor	16.69/4.51	2-DE-MS	-	152	17	-	Toll IA Involved in signal transduction pathways in response to pathogens	plasma membrane	P [50]
Ensangp00000018152	Triosephosphate isomerase	26.3/6.2	1-DE-MS, 2-DE-MS, LC MS/MS	30%	-	-	AIFGETDELIAEK DWSNVVIAYEPVWAIGTGK SLLPETIGVAAQNCYK DLGLGWVILGHSER	Central enzyme in the glycolytic pathway Plays an important role in several metabolic pathways	cytoplasmic	this work P [52]
Ensangp00000012072	Unknown	29.21/4.43	2-DE-MS	-	135	20	DSTLIMQLLR	14-3-3 protein. Family of conserved regulatory molecules that bind a multitude of functionally diverse signaling proteins	cytoplasmic	P [12]
Ensangp00000015472 ^o	Unknown	15.64/10.38	1-DE-MS	20%	-	-	-	InterPro Zn-finger, C2H2 type nucleic acid-binding protein	nuclear	this work P [50] , T [9]
Ensangp00000019887	Unknown	70.9/5.1	2-DE-MS	-	9	18	-	Heat shock 70 region May be involved in response to	cytoplasmic and organelles	P [12]

Ensangp00000028294	Unknown	15.18/4.57	LC MS/MS	-	-	-	GSTINLTBAVK	stress Immunoglobulin- Membrane* this work like domain Involved in cell adhesion
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^{a)} When several spots corresponded to the same protein, the percentage range of the sequence coverage is indicated in parenthesis. ^{b)} Subcellular localization is inferred from sequence or structure similarity with orthologous proteins. ^{c)} Identification was performed using Ensembl database v35 of november 2005. ^{d)} Cases where the same peptides match more than one genomic sequence. Shaded lines: proteins identified for the first time by a proteomic approach. * means that the proteins were also identified in saliva. References underlined correspond to proteins found in human saliva.

Table 2 : Proteins identified by LC MS/MS in salivary glands of 21 day-old blood-fed *Anopheles gambiae*

Ensembl Identification (Ensembl release 43)	Protein Family/Description	Predicted Mr/pI	Peptide sequence	Comments	Subcellular Localization ^{a)}	Found in other proteomic (P) or transcriptomic (T) studies
Ensangp00000028522 b), c), d)	30 kDa protein	26.90/3.8	EQELSDCIVDKR IKECFSSLDK ELDDGLIEREQELSDCIVDK LMNPTIDLVSSTIEKYSK ECFSSLDKDV SAMVK	GE rich salivary gland	secreted	P [12] P [9]
Ensangp00000018525	Aconitate hydratase mitochondrial precursor	82.65/8.63	FDQNVYLPYEK ISILGLNMFAPGK	Iron-sulphur proteins that function as electron carriers biosynthesis of aminoacid	mitochondrial	this work
Ensangp00000016546	Ambiguous	25.56/9.94	KGIGTHLMITLVLAR	GCN5-related N-acetyltransferase Putative role in transcription and DNA repair	?	this work
Ensangp00000026066 b)	Ambiguous	25.13/7.06	MSDKVVSFLR	?	?	this work
Ensangp00000027299	Ambiguous	339.53/6.98	EILYDDIERPILQTK LAGVFTPQEPLMNYVISCWVR QIVTFPDEER TAYLYDPQDVQLSVDGIVFR TFDETWATLAVR YPFGAGGEPFR LYFFASK	Subtilase serine protease domain ? proteasome	cytoplasmic	this work
Ensangp00000029258	Apolipoprotein D precursor	26.11/4.55	QSDVGRAVVAFPDES PLEAK	Extracellular ligand-binding proteins displaying high specificity for small hydrophobic molecules response to pathogens	secreted	this work
Ensangp00000015382 ^e)	Apyrase	61.79/8.6	AAEEGDTCIAGIAR LNVAQVAGLR GDITNEEAIGASPFNTVDLLTLR	Anti-platelet	secreted	P [12]
Ensangp00000026391 b),c)	Cofilin	16.93/7.28	LFLMSWCPDTAK	Binds actin and assists in translocation of actin from the cytoplasm to the nucleus essential for cytokinesis, endocytosis and other cell processes that require rapid	cytoplasmic	T [9]

			turnover of actin filaments			
Ensangp00000025174 ^c)	D7 precursor allergen AED A2	35.57/5.7 ou 32.7/5.1	ALDP EEAWVYVER BVLIGLQLYEEK NYELSGSSQFK SADYAFLLR SANYGYLAMGK SDLEPEVR SVLASCTGTQAYDYYSCLLNSP VK DYELADSAEFR IYHGTVDVSAK NAFYFHELK NAMDCVFR	?	secreted	P [12], P [9]
Ensangp00000018340 ^C)	D7 related-1 protein precursor	18.73/9.57	BLVESTSGEAFK KLPALSQYSSVVDK KVFDTVELVK CLVESTSGEAFK	Anti-inflammatory Scavenger of biogenic amines	secreted	P [12], P [9]
Ensangp00000018371 ^c)	D7 related-2 protein precursor	18.46/4.8	ANTFYTCFLGTSSLAGFK ESVLELLQR HMQBVLEVVGFDGNGEVK KANTFYTCFLGTSSLAGFK MQTSDPFDMMNR NAVDYNELLK QYTPVSSDDMDK	Anti-inflammatory Scavenger of biogenic amines	secreted	P [12], P [9]
Ensangp00000018330 ^c)	D7 related-3 protein precursor	19.66/4.46	ANTFYTCFLGTSSAQAFK AGKLDMGTTFNAGQVSALMK LDMGTTFNAGQVSALMK YAVDYVELLR	Anti-inflammatory Scavenger of biogenic amines	secreted	P [12], P [9]
Ensangp00000018328 ^c)	D7 related-4 protein precursor	19.29/7.4	LYDPLNIIELDK CIGECVQVPTSER RYEIIIEGPEMDK YTAEFVQIMK VFDLMELK	Anti-inflammatory Scavenger of biogenic amines	secreted	P [12]
Ensangp00000018321	D7 related-5 protein precursor	18.79/5.82	SGSFFSCMLR	?	secreted	P [12]
Ensangp00000003578	GSG5 precursor	38.2/6.42	TYFQNEFVEYR	?	secreted	T [51]
Ensangp00000019455 ^c)	GSG6	13.05/5.15	EPLPYMYACPGTEPCQSSDR ETREPLPYMYACPGTEPCQSSDR SMHDVLCDRIDQAFLEQ	?	secreted	P [12], P [9]
Ensangp00000021970 ^c)	GSG7	16.29/8.46	TLAETAQCMR TLAETAQCLR	?	secreted	P [12]

Ensangp00000021028 ^o)	putative gVAG protein precursor	28.9/8.96	YGVQNQLR DGQMDVYYFVBNYSFTNIMDR FPYAGQNIATQFFGYR FVSSWWSEYLDARPEHVR GGPHVGCNPPSSSGGPTCQGK KYPSSYSGKPIGHFTQIASDR MPTLTWDPELASLADANAR VGCSMWYWK	Allergen Belongs to the CAP family: protease inhibitors or proteolytic activity, probably inhibiting host coagulation or complement activity Defence-related protein	secreted	P [12], P [9]
Ensangp00000009655	Homolog	118.45/6.27	DGKELDLVCMQK	C2 domain (cellular proteins ? involved in signal transduction or membrane trafficking) Cytochrome c heme-binding site (electron-transfer proteins) ?		this work
Ensangp00000018375	Hypothetical 10 kD protein	10/6.22	LSLQLEEFVAVCK AISDLQQGLFDLNLHCTK	?	secreted	this work
Ensangp00000018379	Hypothetical 10.2 kD protein	10.13/4.52	LQQMVEDFTACR	?	secreted	P [12]
Ensangp00000004315	Hypothetical 8.8 kDa	8.82/4.05	DKPIDIPVDFLVDVIK	?	secreted	P [12]
Ensangp00000020384	Low density lipoprotein receptor	17.3/5.04	CISRAGICDGK	Lipid metabolism	membranar	P [50]
Ensangp00000022875	Lysozyme precursor	15.33/8.56	NGSTDYGFQINNK YWBDSGYGSNDCK NLLNDDITDDIK KLPNVSSCF	Immunity related Antibacterial enzyme	secreted	P [12], P [50]
Ensangp00000020184 ^c)	Malate dehydrogenase	35.27/9.52	ANTFVGEAAGVDPQK	Metabolic enzymes which catalyse the last step in anaerobic glycolysis	mitochondrial	P [12]
Ensangp00000017682 ^o)	Maltase	67.21/5.87	AMPSGAIANWVLGNHDNSR DQPETYDMVHQWR ELNVAAQLAAPR GITQTIDYLK	Sugar digestion Converts sucrose in nectar to glucose and fructose	secreted	P [12]
Ensangp00000004215	Mitogen activated kinase kinase kinase	159.64/10.04	NIATYYGAFIK	Protein kinase ATP binding	cytoplasmic	this work
Ensangp00000003978	N acylneuraminate cytidyltransferase	21.1/5.67	HLTLARILLGME	Forms CMP-NeuAc, the nucleotide sugar donor used by sialyltransferases (modification may be important in pathogenesis)	cytoplasmic	this work
Ensangp00000021120	NADPH dependent carbonyl reductase	27.05/7.73	MDFTGKVVLTGASSGIGASTAK	Sugar metabolism	cytoplasmic	this work

Ensangp00000012716 ^c)	Putative 5' nucleotidase precursor	63.47/7.01	APFPLTLIHINDLHAR DQIYYVVVPSYLADGKDGFM K ECIAGIAR GLAPYLAELEK LGTQVIGTTEVFLDRESCR LSGADLWSAIDHSFTLDDEFR MKIPTVVANLEK NVNIIIVVLSHCGLDGDK QLAEEAGDLIDVIVGAHSHSLLL NK	Anti-platelet	secreted	P [12]
Ensangp00000020778	Peptidyl prolyl cis trans isomerase	18.29/8.97	FFDMTVDNQPLGR IVIELRPDVVVK HVVFSGSVVEGMDVVVR	Accelerates protein folding	cytoplasmic	this work
Ensangp00000028058 ^c)	Peroxidase precursor	24.99/8.23	AFAGAININDHMFNPTVLER CFAIPVRPDDPVLSAGGIQCLDL VR LLPAEYGDGVYVPR SNITPELTILHVAFLR TTLVNMQFGQLVAHDMGLR WEDFVELR YGEEQVLIWR	Vasodilator	secreted	P [12], P [50]
Ensangp00000015800 ^c)	Phosphoglycerate mutase	28.7/6.8	YGEEQVLIWR	Involved in energy pathways	cytoplasmic	this work Table 1
Ensangp00000029324	Precursor	25.94/4.8	TLTFVLKPTK	Alpha 2 macroglobulin domain	intracellular	this work
Ensangp00000021077 ^c)	Ribonuclease	14.41/8.04	ALAPYNQAIVADR	Inhibits protein synthesis by cleavage of mRNA	?	this work Table 1
Ensangp00000019607	Ryanodine receptor 1	577.53/5.18	YFDMFLKLLK	Ca2+ release channels involved in secretory pathways ?	membranar	this work
Ensangp00000008103	Stromal interaction molecule precursor	54.49/6.36	DVEGLLKAEVALK	?	membranar	this work
Ensangp00000028309	Trans enoyl COA isomerase mitochondrial precursor	30.18/7.13	ALEQAVAFLLNR	Fatty acid metabolism	mitochondrial	this work
Ensangp00000018152 ^c)	Triosephosphate isomerase	22.52/5.09	AIFGETDELIAEK DWSNVVIAIYEPVWAIGTGK SLLPETIGVAAQNCYK DLGLGWVILGHSER	Central enzyme in the glycolytic pathway Plays an important role in several metabolic pathways	cytoplasmic	this work Table 1
Ensangp00000000334 ^b	Unknown	39.57/7.29	SPILLDDIFDK	ATP/GTP-binding site motif A	intracellular	this work

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Ensangp00000011593	Wilm's tumor 1 associating WT1 associated splicing regulator female lethal 2-D homolog	32.55/4.78	FTPDSNTGKR	(P-loop) Potential role in transcriptional regulation Involves in alternative splicing regulation	nuclear	this work

^{a)} Subcellular localization is inferred from sequence or structure similarity with orthologous proteins. ^{b)} Identification was performed using Ensembl database v35 of november 2005. ^{c)} proteins identified from salivary gland extracts of young blood-fed females. Shaded lines: Proteins identified for the first time by a proteomic approach.

Table 3 : List of proteins identified in salivary gland extract of 21day-old blood-fed *Anopheles gambiae* using iTRAQ

Ensembl Identification (Ensembl release 43)	Protein Family/Description	Predicted Mr/pI	Ratio 117/114 ^{a)}	Peptide sequence	Comments	Subcellular Localization ^{b)}	Found in other proteomic (P) or transcriptomic (T) studies
Ensangp00000028522 c),d)	30 kDa protein	26.90/3.8	1.3 ± 0.5	EQELSDCIVDKR IKECFSSLDK ELDDGLIEREQELSDCIVDK EGEEGAGSDDAVSGADDETEES KDDAEEDSEEGGEEGGDGASGG EGGEKESPR LMNPTIDLIVSTIEKYSK ECFSSLDKDVSA MVK	GE rich salivary gland	secreted	P [12], P [9]
Ensangp00000022344	30 kDa protein	18.7/3.7	-	EGEEGAGSDDAVSGADDETEES KDDAEEDSEEGGEEGGDGASGG EGGEKESPR	GE rich salivary gland		
Ensangp00000018525	Aconitate hydratase mitochondrial precursor	82.65/8.63	-	FDQNVYLPYEK ISILGLNNFAPGK	Iron-sulphur proteins that function as electron carriers biosynthesis of amino acid	mitochondrial	this work Table 2
Ensangp00000019171	Acyl-coA-binding protein	9.85/9.45	-	RPSDAELLELYALFK	May act as an intra-cellular carrier of acyl-CoA esters	intracellular	this work
Ensangp00000031876	Acyl-coA-binding protein	9.65/7.35	-	NLNATPADADLLEIYGLFJ	«	«	this work
Ensangp00000017843	Alanine amino transferase 2	52.54/7.79	-	ANIGDCHAMGQPPITFIR	Metabolism of amino acid	cytoplasmic	this work
Ensangp00000026558 ^{e)}	Ambiguous*	124.54/8.43	-	STTAALLISVLVR	?	?	this work
Ensangp00000027299	Ambiguous	339.53/6.98	-	EILYDDIERPILQTK LAGVFTPQEPLMNYVISCWVR QIVTFPDEER TAYLYDPQDVQLSVDGIVFR TFDETWATLAVR YPFGAGGEPFR LYFFASK	Subtilase serine protease ? proteasome	cytoplasmic	this work Table 2
Ensangp00000015145 and/or Ensangp00000012963 ^{e)}	Annexin	35.57/4.31 and/or 27.25/4.11	-	LLTMIIVGAR	Inhibit PLA2 activity, involved in exocytosis calcium-dependent phospholipid-binding proteins	intracellular	this work

Ensangp00000015382	Apyrase	61.79/8.6	0.71±0.11	AAEEGDTCIAGIAR LNVAQVAGLR GDITNEEAIGASPFSTVDLLTLR GAEISAILEER	Anti-platelet	secreted	P [12]
Ensangp00000024604	ATP synthase subunit alpha mitochondrial precursor	59.45/9.5 2	-		Catalyzes ATP synthesis	mitochondrial	P [12]
Ensangp00000024137 and/or Ensangp00000016868 ^{e)}	ATP synthase subunit beta mitochondrial precursor	22.69/4.9 and/or 19.72/5.2 7	*	IINVIGEPIDER LVLEVAQHLGENTVR	Catalyzes ATP synthesis	mitochondrial	P [12]
Ensangp00000012700	Calmodulin	17.25/3.9 9	-	EAFSLFDKDGDTITTK VFDKDGNGFISAAELR GQNPTEAELQDMINEVDADGNG T TTKELGT IDFPEFLTM ADGNGTIDFP GTITTKELGTV EEVDEMIREAD IDFPEFLTMMAR ADQLTEEQIAEFK DMINEVDADGNGT QVNYEARILHLIK FSLFDKDGDTITT DADGNGTIDFPEFL AFSLFDKDGDTITTK	Calcium binding protein	intracellular	T [53]
Ensangp00000026391	Cofilin	16.93/7.2 8	-	LFLMSWCPDTAK	Binds actin and assists in translocation of actin from the cytoplasm to the nucleus essential for cytokinesis, endocytosis and other cell processes that require rapid turnover of actin filaments	cytoplasmic	This work Tables 1 and 2 T [9]
Ensangp00000022538	Creatine kinase	26.4/5.18	*	AVQQQLIDDHFLFK TFLVWCNEEDHLR	Phosphorylation	cytoplasmic	P [12]
Ensangp00000020091	Cytochrome c	11.78/10. 17	-	GDLIAYLK	Electron transporter	Mitochondrial membrane	this work

Ensangp00000025174 and/or Ensangp00000018280 ^{e)}	D7 precursor allergen AED A2	35.57/5.7 and/or 32.7/5.1	0.77±0.05	ALDPEEAWYVYER BVLIGLQLYEK NYELSGSSQFK SADYAFLLR SANYGYLAMGK SDLEPEVR SVLASCTGTQAYDYYSCLLNSP VK DYELADSAEFR IYHGTVDVSVAK NAFYFHELK NAMDCVFR	?	secreted	P [12], P [9]
Ensangp00000018340	D7 related-1 protein precursor	18.73/9.5 7	0.67±0.07	BLVESTSGEAFK KLPALSQYSSVVDK KVFDTVELVK CLVESTSGEAFK	Anti-inflammatory Scavenger of biogenic amines	secreted	P [12], P [9]
Ensangp00000018371	D7 related-2 protein precursor	18.46/4.8	0.92±0.08	ANTFYTCFLGTSSLGAFK ESVLELLQR HMQBVLEVVGFDGNGEVK KANTFYTCFLGTSSLGAFK MQTSDPFDMMNR NAVDYNELLK QYTPVSSDDMDK	Anti-inflammatory Scavenger of biogenic amines	secreted	P [12], P [9]
Ensangp00000018330 and/or Ensangp00000025580 ^d , e)	D7 related-3 protein precursor	19.66/4.4 6 and/or 18.6/4.5	0.95±0.15	ANTFYTCFLGTSSAQAFK AGKLDMGTTFNAGQVSALMK LDMGTTFNAGQVSALMK YAVDYVELLR	Anti-inflammatory Scavenger of biogenic amines	secreted	P [12], P [9]
Ensangp00000018328 ^d)	D7 related-4 protein precursor	19.29/7.4	0.9±0.05	LYDPLNIIELDK CIGECVQVPTSER RYEIIIEGPEMDK YTAEFVQIMK VFDLMELK	Anti-inflammatory Scavenger of biogenic amines	secreted	P [12]
Ensangp00000018321	D7 related-5 protein precursor	18.79/5.8 2	-	SGSFFSCMLR	?	secreted	P [12]

Ensangp00000018385 ^c	Disulfide isomerase precursor	54.31/5.4 7	-	QGETDAVFLFR	Catalyzes the rearrangement of -s-s- bonds in proteins	intracellular	P [12]
Ensangp00000026077	Disulfide isomerase precursor	55.41/4.6 1	*	ELETVEAAEEFLK ILEFFGMK ILEFVQSFLDGK	Catalyzes the rearrangement of -s-s- bonds in proteins	endoplasmic reticulum lumen	P [12]
Ensangp00000002028	DNA2 helicase	117.99/8.45	-	EKLIIIGDR	ATP binding	?	this work
Ensangp00000014287 ^d	Electron transfer flavoprotein subunit alpha mitochondrial precursor	34.14/8.6 2	*	FTHIVAGATAFGK	Participates in catalyzing the initial step of the mitochondrial fatty acid beta-oxidation	mitochondrial	this work
Ensangp00000018531	Enolase	46.62/6.9	*	AAVPSGASTGVHEALELR EALNLIQDAIAK GNPTVEVDLVTDLGLFR	Glycolytic enzyme	cytoplasmic*	P [12]
Ensangp00000010297	Enzyme	79.47/9.6 7	-	LTSIPTALDLALTGK	Includes enoyl coA hydratase involved in fatty-acid metabolism	mitochondrial	this work
Ensangp00000024159	Fructose biphosphate aldolase	39.18/7.7 2	*	KPTAQEIALATVTALR IVPIVEPEILPDGDHDLER	Glycolytic enzyme	?	P [12], P [50]
Ensangp00000020828	Fumarase mitochondrial precursor	50.22/7.5 5	-	IADAIALAADDVISGK	Amino acid metabolism ?	mitochondrial	this work
Ensangp00000017396	Fumaryl acetoacetase	45.64/6	-	GTKQVSLAGGETR	Last enzyme of the tyrosine catabolic pathway	cytoplasmic	this work
Ensangp00000029040	Glutathion S transferase	19.16/7.5	*	LYFDMGTLYQR	«	«	this work
Ensangp00000010360	Glyceraldehyde phosphate dehydrogenase	35.46/8.5 5	-	AGAEYVVESTGVFTTTEK WRDG KLTGM GCLVVN ASVVAI IIPAATG HATTATQKT AFRVTPNVS LSKPATYDQI GAAKAVGKVIP	Plays an important role in glycolysis and gluconeogenesis	cytoplasmic	P [12], P [50]

Ensangp0000003578	GSG5	38.2/6.42	-	TYFQNEFVEYR	?	secreted	This work Table 2
Ensangp00000019455	GSG6	13.05/5.15	0.65±0.05	EPLPYMYACPGTEPCQSSDR ETREPLPYMYACPGTEPCQSSDR SMHDVLCDRIDQAFLEQ	?	secreted	T [51] P [12], P [9]
Ensangp00000021970	GSG7	16.29/8.46	-	TLADETAQCMR TLADETAQCLR YGVQNQLR	?	secreted	P [12]
Ensangp00000021028	putative gVAG protein precursor	28.9/8.96	1.97± 0.6	DGQMDVYYFVBNYSFTNIMDR FPYAGQNIATQFFGYR FVSSWWSEYLDARPEHVR GGPHVGCNPPSSSGGPTCQGK KYPSSYSGKPIGHFTQIASDR MPTLTWDPELASLADANAR VGCSMWYWK	Allergen Belongs to the CAP family: protease inhibitors or proteolytic activity. probably inhibiting host coagulation or complement activity	secreted	P [12], P [9]
Ensangp00000014839	60 kDa heat shock protein mitochondrial precursor	60.77/5.28	-	VEFQDALVLFSEK	Protein refolding	mitochondrial	this work
Ensangp00000003808	Histone acetyltransferase GCN5	85.65/8.9	-	SIPIESIPGLR	Control of amino acid synthesis	nuclear	this work
Ensangp00000009655	Homolog	118.45/6.27	-	DGKELDLVCMQK	C2 domain (cellular proteins involved in signal transduction or membrane trafficking) Cytochrome c heme-binding site (electron-transfer proteins)	?	this work Table 2
Ensangp00000004315	Hypothetical 8.8 kDa protein	8.82/4.05	*	DKPDIDPVDFLVDVIK	?	secreted	P [12]
Ensangp00000018375	Hypothetical 10 kDa protein	10/6.22	-	LSLQLEEFVCK AISDLQQLFDLNHCTK	?	secreted	this work Table 2
Ensangp00000018379	Hypothetical 10.2 kDa protein	10.13/4.52	-	LQQMVEDFTACR	?	secreted	P [12]
Ensangp00000013285	3 Ketoacyl coA thiolase	41.67/8.47	*	AALDAAGLKPQVDSVNIGQVL VLSSTDGAF LPR LACAGELGLDINKLNL NGAQDILVGAAH TAGTASGI ASGSRITG	Involved in biosynthetic pathways such as poly beta-hydroxybutyrate synthesis or steroid biogenesis	intracellular	this work
Ensangp00000010689	Kinase	74.81/9.33	-	SLDLLSMLVLDP PGSEDLSGEEDIGSPLLPSNRDTI QNLTPSG REIKILRQ	Protein phosphorylation	cytoplasmic	this work

Ensangp00000020132	Low density lipoprotein receptor	179.24/6.29	-	AGINMM GGAGAPAG DGTERVLIVSQNL GSQRVELITK IVTAEIQAPDG SPDDAPADHVCACPQGLMLLK GRTN	Lipid metabolism	membranar	this work
Ensangp00000022875	Lysozyme precursor	15.33/8.56	*	NGSTDYGIFQINNK YWBDSGYGSNDCK NLLNDDITDDIK KLPNVSSCF	Immunity related Antibacterial enzyme	secreted	P [12], P [50]
Ensangp00000020184	Malate dehydrogenase	35.27/9.52	-	ANTFVGEAAGVDPQK	Metabolic enzymes which catalyse the last step in anaerobic glycolysis	mitochondrial	P [12]
Ensangp00000017682	Maltase	67.21/5.87	1.3±0.2	AMPSGAIANWVLGNHDNSR DQPETYDMVHQWR ELNVAAQLAAPR GITQTIDYLK	Sugar digestion Converts sucrose in nectar to glucose and fructose	secreted	P [12]
Ensangp00000003748	Myosin	121.89/10.23	-		Contractile protein	cytoplasmic	this work P [52]
Ensangp00000026137	Nucleolar RNA associated protein	117.56/7.05	-	LSSETIDELEK	Appears to be associated with ribosome biogenesis	cytoplasmic	this work
Ensangp00000011253	Nucleoside diphosphate kinase	19.01/8.46	-	GDLCVQVGR	Maintenance of cellular pool of nucleoside triphosphates	cytoplasmic and plasma membrane	this work Table 1 T [9]
Ensangp00000012716	Putative 5' nucleotidase precursor	63.47/7.01	0.92±0.24	APFPLTLIHINDLHAR DQIYYVVVPSYLADGKDFAMK ECIAGIAR GLAPYLAELEK LGTQVIGTTEVFLDRESCR LSGADLWSAIDHSFTLDDEFR MKIPTVVANLEK NVNIIIVVLSHCGLDGDK QLAEEAGDLIDVIVGAHSHSLLL NK	Anti-platelet	secreted	P [12]
Ensangp00000020778	Peptidyl prolyl cis trans isomerase	18.29/8.97	-	FFDMTVDNQPLGR IVIELRPDVVPK HVVFGSVVEGMDVVR	Accelerates protein folding	cytopasmic	this work
Ensangp00000028058	Peroxidase precursor	24.99/8.23	0.95±0.15	AFAGAININDHMFNPTVLER CFAIPVRPDDPVLSAGGIQCLDL VR LLPAEYGDGVYVPR	Vasodilatator	secreted	P [12], P [50]

				SNITPELTILHVAFLR TTLVNMQFGQLVAHDMGLR WEDFVELR			
Ensangp00000020634	Peroxisomal targeting signal 2 receptor	36.41/6.2 2	-	VSGSGDGSVQLWNT FTTNR TNLAS SVQLWNTNLASN SQFYGLAGGGT SASEVQDDKCK	Family of potein implicated in a variety of functions ranging from signal transduction and transcription regulation to cell cycle control and apoptosis	?	this work
Ensangp00000024749	Pheromone/general odorant binding protein OBP56	27.15/5.5 2	-		?	?	this work
Ensangp00000013993	Phosphatidylethanolamine-binding protein	24.17/6.6 7	-	YVFLVYK	Proteinase inhibitor	?	this work P [52]
Ensangp00000020531	Precursor	200.9/4.5	-	ERTGEIMLLQR AGTIVGNVSALDEDVGPNG TRDARLDRDTNPESYAI GTIFVNSTLNINYAAVI VERQLDYEE VSGVLD RFTVEMQERLANANLE LS	Cadherin	membranar	this work
Ensangp00000031578	Precursor	58.96/9.6 8	-	DMPNITLLNLDGNQLSR NLLQNLDLALFVAMPQLLNLN ASSPV ANNLT SAPIA PVTGR PNITLLN VSAPIGL NKITTFNIT	Leucine rich repeat Putatively involved in protein-protein interaction	?	this work
Ensangp00000021077	Ribonuclease	14.41/8.0 4	-	ALAPYNQAIVADR	Inhibits protein synthesis by cleavage of mRNA	?	this work
Ensangp00000006850	DNA directed RNA polymerase	68.25/8.1 8	-	LSYISALGMMTR	Transcription	nuclear	this work
Ensangp00000017327	Putative salivary protein GSG1b	46.6/7.37	-	DYESYLGAMFAADAFHVVYEA D GK	?	secreted	P [12]
Ensangp00000032098 ^d)	Salivary D3 protein	?	-	AAAGPAPDPSSQFCQQLDDAQ R	Saglin	secreted	P [12]
Ensangp00000020530	Serine protease precursor	25.2/4.57	-	NGQNDIALQLDRK VITSAQCTTDEGNGIPSVVRLGG TK	Involved in immunity or in coagulation cascade	secreted	this work

Ensangp00000016680	Serpin 9	46.36/7.0 1	-	SVLFAVL LIWDSVV ALLQLDRKIIN TTDEGNGIPSVVR LAAETDILHEVVNEGISR	Serine protease inhibitor Involved in immunity	secreted	T [11]
Ensangp00000009988	SG3	4.3/20.01 3	-	ATGPLFLPHFGQGR RGQQ LIFLAA SVERNPA ATIAVASAAT ASPTTAEA QQRQQVQR RPLSAYMLWLNSAR	Mucin	secreted	T [51]
Ensangp00000009009	Fact complex subunit facilitates chromatin transcription	71.65/6.2 8	-	RPLSAYMLWLNSAR	Recombination signal sequence recognition T160	nuclear	this work
Ensangp00000016164	Superoxyde dismutase	15.67/5.4 5	-	SLVVHADPDDLGVGGHELK	Metalloprotein that prevents damage by oxygen-mediated free radicals	intracellular	this work
Ensangp00000021085	Translationally controlled tumor protein TCTP	19.54/4.4 2	-	LVDDVMYEVYGK	Histamine-releasing factor	?	T [9], T [11]
Ensangp00000017522	Trio protein	43.78/6.4	-	SMYDLIGQLVQSSK	?	secreted ?	P [12]
Ensangp00000025045	Trypsin precursor	28.65/6.4 9	-	QIGIVSWGDTQCVGT RGGSSTL NETDLTVR RLALTAGH NGNFVPL PAPARATGRIV	Proteolytic enzyme	secreted	this work
Ensangp00000008105	E3 Ubiquitin ligase	201.24/6. 46	-	GLAMADLDRLEK QQLCIKP NPDN SEHRNHK GTYHSVN TQASQQQAPL LRDGSRVMMMG	Involved in protein degradation pathway	cytoplasmic	this work
Ensangp00000012822	Unknown	74.9/7.88	*	DVQASHISRLGTSSIVSYTP TLRNGTPQASNSI YCTLRNGT NVSMC PDTIDSD	Immunoglobulin-like	?	this work
Ensangp00000012893	Unknown	72.74/4.9	-	ELEDIVQPIIAK	Hsp70 and tropomyosin	ER ?	this work

Ensangp00000016832	Unknown	2 19.42/4.8	-	QQAAAAAETTSQAAGTLMDDHA K	domains Anti-freeze protein	?	this work
Ensangp00000017135	Unknown	8 85.43/8.6	-	IKCGLLLEGVR	?	?	this work
Ensangp00000019537	Unknown	4 90.81/7.4	*	KLMSDYYSVVASTN EMQSLFLPSS QREH MAHSQ TGSTT	?	?	this work
Ensangp00000028177	Unknown	1 36.81/10.03	-	LGIGSSSINGSGAVVRK	Basic helix-loop-helix dimerisation region		this work
Ensangp00000029447	Unknown	4 20.35/6.2	-	EQQLALDVR	?	secreted	this work

^{a)} Ratios indicated in bold correspond to a significant increase or decrease of protein expression in the presence of *Plasmodium*. ^{b)} Subcellular localization is inferred from sequence or structure similarity with orthologous proteins. ^{c)} Identification was performed using Ensembl database v35 of november 2005. ^{d)} The part of the sequence in bold is that described in Ensembl 43. ^{e)} Cases where the same peptides match more than one genomic sequence. * means that the protein was quantified one time. Shaded lines : Proteins newly identified by iTRAQ. References underlined correspond to proteins found in human saliva.

Supplementary Table 1 : List of proteins identified by 1-DE-MS according to their slice number

Slice number	Ensembl identification	Protein family /description
1	No signal	
2	Ensangp00000003518	CoA carboxylase
3	Ensangp00000010081 and Ensangp00000012716	Glycogen phosphorylase and putative 5' nucleotidase precursor
4	Ensangp00000012716 and Ensangp00000017682	putative 5' nucleotidase precursor and Maltase
5	Ensangp00000012716	putative 5' nucleotidase precursor
6	Ensangp00000017682	Maltase
7	Ensangp00000012716	putative 5' nucleotidase precursor
8	Ensangp00000012716 and Ensangp00000015382	putative 5' nucleotidase precursor and Apyrase
9	Ensangp00000016660	Isocitrate dehydrogenase
10	NI	
11-12	Ensangp00000025174	D7 precursor allergen AED A2
13	Ensangp00000027418	Salivary gland 1-like 3
14-15	Ensangp00000018280/25174	D7 precursor allergen AED A2
16	Ensangp00000018280/25174 and Ensangp00000021028	D7 precursor allergen AED A2 and Putative gVAG protein precursor
17	Ensangp00000018280	D7 precursor allergen AED A2
18	NI	
19	Ensangp00000018152	Triosephosphate isomerase
20-22	Ensangp00000011253 and Ensangp00000021028 and Ensangp00000018328	Nucleoside diphosphate kinase and putative gVAG protein precursor and D7 related-4 protein precursor
23	Ensangp00000018328 and Ensangp00000018330 And Ensangp00000018340	D7 related-4 protein precursor and D7 related-3 protein precursor and D7 related-1 protein precursor
24-25	Ensangp00000018371 and Ensangp00000018330	D7 related-2 protein precursor and D7-related-3 protein precursor
26-27	Ensangp00000018371 and Ensangp00000018330 and Ensangp00000019455	D7 related-2 protein precursor and D7 related-3 protein precursor and GSG6

28-29	Ensangp00000018371 and Ensangp00000018330	D7 related-2 protein precursor and D7 related-3 protein precursor
30-31	Ensangp00000018371 and Ensangp00000012492	D7 related-2 protein precursor and precursor

NI : non-identified protein

For Peer Review

Supplementary Table 2 : List of proteins identified by 2-DE-MS according to their spot

number

Spot number	Ensembl identification	Protein family /description
1-5	NI	
6-8	Ensangp00000017682	Maltase
9	Ensangp00000019887	unknown
10	Ensangp00000012716	putative 5' nucleotidase precursor
11	NI	
12	Ensangp00000017682	Maltase
13-33	Ensangp00000012716	putative 5' nucleotidase precursor
34-37	NI	
38-51	Ensangp00000012716	putative 5' nucleotidase precursor
52	Ensangp00000027211	Disulfide isomerase precursor
53	NI	
54	Ensangp00000012716	putative 5' nucleotidase precursor
55-56	NI	
57	Ensangp00000012716	putative 5' nucleotidase precursor
58-59	NI	
60	Ensangp00000012716	putative 5' nucleotidase precursor
61-63	NI	
64-65	Ensangp00000012716	putative 5' nucleotidase precursor
66-67	Ensangp00000024137 and/or Ensangp00000016868	ATP synthase subunit beta mitochondrial precursor
68-74	NI	
75	Ensangp00000013568 and Ensangp00000009988	Precursor and GSG3
76	Ensangp00000009988	GSG3
77-82	Ensangp00000012716	putative 5' nucleotidase precursor
83	NI	
84	Ensangp00000028522	30 kDa
85-87	Ensangp00000012716	putative 5' nucleotidase precursor
88-89	NI	
90	Ensangp00000022538	Creatine kinase
91-94	NI	
95	Ensangp00000011707	Aspartate amino transferase
96	Ensangp00000011006	Malate dehydrogenase
97-108	NI	
109	Ensangp00000012460	Phosphoglycerate kinase
110	Ensangp00000018590	5 aminolevulinate synthase
111-112	NI	
113	Ensangp00000014287 and Ensangp00000025174	Electron transfer flavoprotein alpha subunit and D7 precursor allergen AED A2
114-119	Ensangp00000025174	D7 precursor allergen AED A2
120	NI	
121-125	Ensangp00000025174	D7 precursor allergen AED A2

126-129	NI	
130-134	Ensangp00000012716	putative 5' nucleotidase precursor
135	Ensangp00000012702	Unknown
136-139	NI	
140-141	Ensangp00000012716	putative 5' nucleotidase precursor
142	Ensangp00000016366	Glucose dehydrogenase precursor
143	NI	
144-145	Ensangp00000012716	putative 5' nucleotidase precursor
146	NI	
147	NI	
148	Ensangp00000015800	Phosphoglycerate mutase
149-151	Ensangp00000025174	D7 precursor allergen AED A2
152	Ensangp00000018152	Triose phosphate isomerase
	and	and
	Ensangp00000018041	Toll precursor
153	NI	
154	Ensangp00000025174	D7 precursor allergen AED A2
155	Ensangp00000011661	Glutathion S transferase
156	Ensangp00000024808	Glutathion S transferase
157-168	NI	
169	Ensangp00000026134	Ambiguous
	and	and
	Ensangp00000025174	D7 precursor allergen AED A2
170	Ensangp00000026391	cofilin
171-176	Ensangp00000018328	D7 related-4 protein precursor
177-179	NI	
180	Ensangp00000018330	D7 related-3 protein precursor
181	Ensangp00000018371	D7-related-2 protein precursor
	and	and
	Ensangp00000027538	Retrovirus related pol polyprotein
	and	and
	Ensangp00000018330	D7 related-3 protein precursor
182-183	Ensangp00000018371	D7 related-2 protein precursor
	and	and
	Ensangp00000027538	Retrovirus related pol polyprotein
184-185	Ensangp00000028522	30 kDa
186	Ensangp00000015067	Mitochondrial carrier
	and	and
	Ensangp00000028522	30 kDa
187-202	Ensangp00000028522	30 kDa
203-205	NI	

NI : non-identified protein

Supplementary Table 3: Proteins identified in salivary gland extracts of *A. gambiae* blood-fed females

Ensembl Identification (Ensembl release 43)	Protein Family/Description	Predicted M _r /pI	Identification	1DE-MS % coverage	2-DE-MS spot number	2-DE-MS % coverage ^{a)}	LC MS/MS Peptide sequence	Comments	Subcellular Localization ^{b)}	Found in other proteomic (P) or transcriptomic (T) studies
Ensangp00000028522 ^{c)} Ensangp00000022344 ^{d)}	30 kDa protein	26.90/3.8 18.7/3.7	2-DE-MS LC MS/MS iTRAQ	-	84, 184- 188, 190, 192,- 193, 196- 199, 201- 202	PSD	EQELSDCIVDKR IKECFSSLDK ELDDGLIEREQELSDCIVDK LMNPTIDLVSSTIEKYSK ECFSSLDKDVSAAMVK EGEEGAGSDDAVSGADDETE ESKDDAEEDSEEGGEEGGDG ASGGEGGEKESPR	GE rich salivary gland	secreted	P [12], P [9]
Ensangp00000018525 ^{e)}	Aconitate hydratase mitochondrial precursor	82.65/8.6 3	LC MS/MS iTRAQ	-	-	-	FDQNVYLPYEK ISILGLNNFAPGK	Iron-sulphur proteins that function as electron carriers biosynthesis of amino acid	mitochondrial	this work
Ensangp00000019171 ^{e)}	Acyl-coA -binding protein	9.85/9.45	iTRAQ	-	-	-	RPSDAELLELYALFK	May act as an intra-cellular carrier of acyl-CoA esters	intracellular	this work
Ensangp00000031876 ^{e)}	Acyl-coA -binding protein	9.65/7.35	iTRAQ	-	-	-	NLNATPADADLLEIYGLFJ	«	«	this work
Ensangp00000017843 ^{e)}	Alanine aminotransferase 2	52.54/7.7 9	iTRAQ	-	-	-	ANIGDCHAMGQPPITFIR	Metabolism of amino acid	cytoplasmic	this work
Ensangp00000016546 ^{e)}	Ambiguous	25.56/9.9 4	LC MS/MS	-	-	-	KGIGTHLMITLEVLR	GCN5-related N-acetyltransferase	?	this work
Ensangp00000022917 ^{d)}	Ambiguous	72.38/10. 16	LC MS/MS	-	-	-	GRPILPLLKTVQSYK	Tropomyosin domain	intracellular	this work
Ensangp00000024702 ^{d)}	Ambiguous	30.31/9.5 8	LC MS/MS	-	-	-	IHDGVTHAAK	?	?	this work
Ensangp00000026066 ^{c)}	Ambiguous	25.13/7.0 6	LC MS/MS	-	-	-	MSDKVVSSFLR	?	?	this work

Ensangp00000026134 ^c) _d	Ambiguous	23.01/10	2-DE-MS	-	169	PSD	-	?	?	this work
Ensangp00000026558 ^c)	Ambiguous	124.54/8.43	LC MS/MS	-	-	-	STTAALLISVLVR	?	?	this work
Ensangp00000027299 ^e)	Ambiguous	339.53/6.98	LC MS/MS, ITRAQ	-	-	-	EILYDDIERPILQTK LAGVFTPQEPLMNYVISCWVR QIVTFPDEER TAYLYDPQDVQLSVDGIVFR TFDETWATLAVR YFPGAGGEPFR LYFFASK	Subtilase serine protease	cytoplasmic	this work
Ensangp00000018590 ^d)	5 Aminolevulinate synthase erythroid specific mitochondrial precursor	46.31/7.54	2-DE-MS	-	110	25	-	Metabolism of amino acid	mitochondrial matrix	this work
Ensangp00000015145 and/or Ensangp00000012963) _{e,f}	Annexin	35.57/4.31 and/or 27.25/4.11	iTRAQ	-	-	-	LLTMIIVGAR	Inhibit PLA2 activity, involved in exocytosis calcium-dependent phospholipid-binding proteins	intracellular	this work
Ensangp00000029258 ^e)	Apolipoprotein D precursor	26.11/4.55	LC MS/MS	-	-	-	QSDVGRAVVAFPDESPLEAK	Extracellular ligand-binding proteins displaying high specificity for small hydrophobic molecules	secreted	this work
Ensangp00000015382 ^d) _e	Apyrase	61.79/8.6	1-DE-MS LC MS/MS iTRAQ	20%	-	-	AAEEDGTCIAGIAR LNVAQVAGLR GDITNEEAIGASPFNTVDLLT LR	Anti-platelet	secreted	P [12]
Ensangp00000011707 ^d)	Aspartate aminotransferase	44.71/6.78	2-DE-MS	-	95	17	-	Metabolism of amino acid	cytoplasmic	this work
Ensangp00000024604 ^e)	ATP synthase subunit alpha	59.45/9.52	iTRAQ	-	-	-	GAEISAILEER	Catalyzes ATP	mitochondrial	P [12]

Ensangp00000024137 and/or Ensangp00000016868 d),e),f)	mitochondrial precursor ATP synthase subunit beta mitochondrial precursor	22.69/4.9 and/or 19.72/5.2 7	LC MS/MS iTRAQ	-	66-67	(31-37)	IINVIGEPIDER LVLEVAQHLGENTVR	synthesis Catalyzes ATP synthesis	mitochondrial	P [12]
Ensangp00000012700 ^e)	Calmodulin	17.25/3.9 9	iTRAQ	-	-	-	EAFSLFDKDGDTITTK VFDDKDGNGFISAAELR GQNPTEAELQDMINEVDADG NGT TTKELGT IDFPEFLTM ADGNGTIDFP GTITTKELGTV EEVDEMIREAD IDFPEFLTMMAR ADQLTEEQIAEFK DMINEVDADGNGT QVNYEARILHLIK FSLFDKDGDTITT DADGNGTIDFPEFL AFSLFDKDGDTITTK	Calcium binding protein	intracellular	this work
Ensangp00000018543 ^d)	Chromosome associated polypeptide C XCAP C homolog	156.83/5. 34	LC MS/MS	-	-	-	LQTELIELKR	Structural maintenance of chromosome ABC transporter related domain	nuclear	this work
Ensangp00000003518 ^d)	CoA carboxylase mitochondrial precursor	130.5/6.6 7	1-DE-MS	15%	-	-	-	Key enzyme in the catabolic pathway of odd-chain fatty acids : isoleucine, threonine, methionine and valine	mitochondrial matrix	this work
Ensangp00000026391	Cofilin	16.93/7.2 8	2-DE-MS LC MS/MS	-	170	42	LFLMSWCPDTAK	Binds actin and assists in	cytoplasmic	T [9]

			iTRAQ						translocation of actin from the cytoplasm to the nucleus essential for cytokinesis, endocytosis and other cell processes that require rapid turnover of actin filaments		
Ensangp00000022538 ^d ,e)	Creatine kinase	26.4/5.18	2-DE-MS iTRAQ	-	90	30	AVQQQLIDDHFLFK TFLVWCNEEDHLR	Phosphorylat ion	cytoplasmic	P [12]	
Ebsangp00000020091 ^e)	Cytochrome c	11.78/10. 17	iTRAQ				GDLIAYLK	Electron tranporter	mitochondrial membrane	this work	

Ensangp00000025174 and/or Ensangp00000018280 d),e),f)	D7 precursor allergen AED A2	35.57/5.7 and/or 32.7/5.1	1-DE-MS 2-DE-MS LC MS/MS iTRAQ	42%	113- 119, 121- 125, 149- 151, 154, 169	(19-33) PSD	ALDPEEAWYVYER BVLIGLQLYEEK NYELSGSSQFK SADYAFLLR SANYGYLAMGK SDLEPEVR SVLASCTGTQAYDYYSCLLNS PVK DYELADSAEFR IYHGTVDVSVAK NAFYFHELK NAMDCVFR	?	secreted	P [12], P [9]
Ensangp00000018340 *,d),e)	D7 related-1 protein precursor	18.73/9.5 7	1-DE-MS LC MS/MS iTRAQ	20%	-	-	BLVESTSGEAFK KLPALSQYSSVVDK KVFDTVELVK CLVESTSGEAFK	Anti- inflammatory Scavenger of biogenic amines	secreted	P [12], P [9]
Ensangp00000018371 *,d),e)	D7 related-2 protein precursor	18.46/4.8	1-DE-MS 2-DE-MS LC MS/MS iTRAQ	28%	181- 183	PSD	ANTFYTCFLGTSSLAGFK ESVLELLQR HMQBVLVGVGVDGNGEVK KANTFYTCFLGTSSLAGFK MQTSDPFDMNR NAVDYNELLK QYTPVSSDDMDK	Anti- inflammatory Scavenger of biogenic amines	secreted	P [12], P [9]
Ensangp00000018330 and/or Ensangp00000025580 d), e),f)	D7 related-3 protein precursor	19.66/4.4 6 and/or 18.6/4.5	1-DE-MS 2-DE-MS LC MS/MS iTRAQ	33%	180- 181	PSD	ANTFYTCFLGTSSAQAFK AGKLDMGTTFNAGQVSALMK LDMGTTFNAGQVSALMK YAVDYVELLR	Anti- inflammatory Scavenger of biogenic amines	secreted	P [12], P [9]
Ensangp00000018328 ^d ,e)	D7 related-4 protein precursor	19.29/7.4	1-DE-MS 2-DE-MS LC MS/MS iTRAQ	30%	171- 176	22-40	LYDPLNIIELDK CIGECVQVPTSER RYEIIIEGPEMDK YTAEFVQIMK VFDLMELK SGSFFSCMLR	Anti- inflammatory Scavenger of biogenic amines	secreted	P [12]
Ensangp00000018321 ^e)	D7 related-5 protein precursor	18.79/5.8 2	LC MS/MS iTRAQ					?	secreted	P [12]

Ensangp00000026077 ^e	Disulfide isomerase precursor	55.41/4.6 1	iTRAQ	-	-	-	ELETVEAAEEFLK ILEFFGMK ILEFVQSFLDGK QTVPTCTPAPSVPRIPQIPAT LSPTRVQFSCVPCPLNQRK GSVDPTLETVVPRSSVI LAPEYAKAAKVLADKESNIKL QGETDAVFLFR	Catalyzes the rearrangement of -s-s-bonds in proteins	endoplasmic reticulum lumen	P [12]
Ensangp00000027211	Disulfide isomerase precursor	54.31/5.4 7	2-DE-MS iTRAQ	-	52	15	ELETVEAAEEFLK ILEFFGMK ILEFVQSFLDGK QTVPTCTPAPSVPRIPQIPAT LSPTRVQFSCVPCPLNQRK GSVDPTLETVVPRSSVI LAPEYAKAAKVLADKESNIKL QGETDAVFLFR	Catalyzes the rearrangement of -s-s-bonds in proteins	intracellular	P [12]
Ensangp0000002028 ^e	DNA helicase	117.99/8. 45	iTRAQ	-	-	-	EKLIIIGDR	ATP binding	?	this work
Ensangp00000014287 ^d ,e)	Electron transfer flavoprotein subunit alpha mitochondrial precursor	34.14/8.6 2	2-DE-MS iTRAQ	-	113	33	FTHIVAGATAFGK	Participates in catalyzing the initial step of the mitochondrial fatty acid beta-oxidation	mitochondrial	this work
Ensangp00000018531	Enolase	28.68/9.9 3	iTRAQ	-	-	-	AAVPSGASTGVHEALELR EALNLIQDAIAK GNPTVEVDLVTDLGLFR LTSIPTALDLALTGK	Glycolytic enzyme	cytoplasmic*	P [12], P[50]
Ensangp00000010297 ^e	Enzyme	79.47/9.6 7	iTRAQ	-	-	-	LTSIPTALDLALTGK	Includes enoyl coA hydratase May be involved in fatty-acid metabolism	mitochondrial	this work
Ensangp00000021863 ^e	Epilepsy holoprocencephaly candidate	29.13/6.9 2	iTRAQ	-	-	-	YEELIRTNR	oxidoreductase activity	?	this work
Ensangp00000003806 ^d	Facilitated glucose transporter	16.83/8.4 8	LC MS/MS	-	-	-	HISQIVPLVAKGFSSKPLVP	Sugar transporter	membranar	this work
Ensangp00000000937 ^d	Probable Fatty acid binding protein	19.37/9.5 9	LC MS/MS	-	-	-	LGGGFDEETVDGR	Fatty acid binding protein	cytoplasmic	this work
Ensangp00000024159 ^e	Fructose biphosphate	39.18/7.7 2	iTRAQ	-	-	-	KPTAQEIALATVTALR IVPIVEPEILPDGDHDLER	Glycolytic enzyme	?	P[12], P[50]

Ensangp00000020828 ^e)	aldolase Fumarase mitochondrial precursor	50.22/7.5 5	iTRAQ	-	-	-	IADAIALAADDVISGK	Generation of precursor metabolites and energy	mitochondrial	this work
Ensangp00000017396 ^e)	Fumaryl acetoacetase	45.64/6	iTRAQ	-	-	-	GTKQVSLAGGETR	Last enzyme of the tyrosine catabolic pathway	cytoplasmic	this work
Ensangp00000029040 ^e)	Glutathion S transferase	19.16/7.5	iTRAQ	-	-	-	LYFDMGTYLQR	Key role in cellular detoxification	cytoplasmic and nuclear	this work
Ensangp00000011661 ^e)	Glutathion S transferase (class theta)	23.78/6.5 1	2-DE-MS	-	155	33	-			this work P [50]
Ensangp00000010360 ^e)	Glyceraldehyde phosphate dehydrogenase	35.46/8.5 5	iTRAQ	-	-	-	AGAEYVVESTGVFTTTEK WRDG KLTGM GCLVYN ASVVAI IIPAATG HATTATQKT AFRVPTPNVS LSKPATYDQI GAAKAVGKVIP	Plays an important role in glycolysis and gluconeogenesis	cytoplasmic	P[12] , P[50]
Ensangp00000024265 ^e)	Glycin cleavage system H protein mitochondrial precursor	13.52/4.2	iTRAQ	-	-	-	LMSEEQYTEFLK	Catalyses the catabolism of glycine in eukaryotes	mitochondrial	this work
Ensangp00000010081 ^d)	Glycogen phosphorylase	96.4/6.33	1-DE-MS	18%	-	-	-	Carbohydrate metabolism	cytoplasmic	this work

Ensangp000000998 ^{e)}	GSG3	20.01/4.34	2-DE-MS	-	75, 76	PSD	-	?	secreted	T [51]
Ensangp0000003578 ^{e)}	GSG5 precursor	38.2/6.42	LC MS/MS iTRAQ	-	-	-	TYFQNEFVEYR	?	secreted	T [51]
Ensangp00000019455 ^{d),e)}	GSG6	13.05/5.15	1-DE-MS LC MS/MS iTRAQ	36%	-	-	EPLPYMYACPGTEPCQSSDR ETREPLPYMYACPGTEPCQSSDR SMHDVLCDRIDQAFLEQ	?	secreted	P [12], P [9]
Ensangp00000021970 ^{d),e)}	GSG7	16.29/8.46	LC MS/MS iTRAQ	-	-	-	TLADETAQCMR TLADETAQCLR YGVQNLQR	?	secreted	P [12]
Ensangp0000005326 ^{d)}	Guanine nucleotide releasing factor	137.53/9.17	LC MS/MS	-	-	-	LIEKALIIK	May play a role in intracellular signaling cascade	membrane-associated	this work
Ensangp00000021028 ^{*,d),e)}	putative gVAG protein precursor	28.9/8.96	1-DE-MS LC MS/MS iTRAQ	43%	-	-	DGQMDVYYFVBNYSFTNIMDR FPYAGQNIATQFFGYR FVSSWWSEYLDARPEHVR GGPHVGCNPPSSSGGPTCQGK KYPSSYSGKPIGHFTQIASDR MPTLTWDPELASLADANAR VGCSMWYWK	Allergen. Belongs to the CAP family	secreted	P [12], P [9]
Ensangp00000014839	60 kDa heat shock protein mitochondrial precursor	60.77/5.28	iTRAQ	-	-	-	VEFQDALVLFSEK	Protein refolding	mitochondrial	this work
Ensangp0000003808 ^{e)}	Histone acetyltransferase GCN5	85.65/8.9	iTRAQ	-	-	-	SIPIESIPGLR	Control of amino acid synthesis	nuclear*	this work
Ensangp0000009655 ^{e)}	Homolog	118.45/6.27	iTRAQ	-	-	-	DGKELDLVCMQK	C2 domain (cellular proteins involved in signal transduction or membrane trafficking) Cytochrome c heme-	?	this work

Ensangp00000017720 ^d	3 Hydroxyisobutyrate dehydrogenase mitochondrial	34.31/9.2 7	LC MS/MS	-	-	-	VFADIVNASTGR	binding site (electron-transfer proteins) Involved in amino acid catabolism pathway	mitochondrial	this work
Ensangp00000018375 ^e	Hypothetical 10 kDa protein	10/6.22	LC MS/MS iTRAQ	-	-	-	LSLQLEEFVCK AISDLQQGLFDLNHCTK	?	secreted	this work
Ensangp00000018379 ^e	Hypothetical 10.2 kDa protein	10.13/4.5 2	LC MS/MS iTRAQ	-	-	-	LQQMVEDFTACR	?	secreted	P [12]
Ensangp00000004315 ^e	Hypothetical 8.8 kDa protein	8.82/4.05	LC MS/MS iTRAQ	-	-	-	DKPDIDPVDFLVDVIK	?	secreted	P [12]
Ensangp00000016660 ^d	Isocitrate dehydrogenase	46.96/7.5 9	1-DE-MS	32%	-	-	-	Plays a key role in cellular defense against oxidative stress-induced damage	mitochondrial	this work
Ensangp00000013285 ^e	3 Ketoacyl coA thiolase	41.67/8.4 7	iTRAQ	-	-	-	AALDAAGLKPDQVDSVNIGQ VLVLSSTDGAFLLPR LACAGELGLDINKLNL NGAQDILVGAH TAGTASGI ASGSRITG	Involved in biosynthetic pathways such as poly beta-hydroxybutyrate synthesis or steroid biogenesis		this work
Ensangp00000010689 ^e	cell division Kinase	74.82/9.3 3	iTRAQ	-	-	-	SLDLLDSMLVLDP PGSEDLSGEEDIGSPLLPSNRD TIQNLTPSG REIKILRQ AGINMM GGAGAPAG	Protein phosphorylation	cytoplasmic	this work
Ensangp00000020132 ^e	Low density lipoprotein receptor	179.24/6. 29	iTRAQ	-	-	-	DGTERVLIQSQNL GSQRVELITK IVTAEIQAPDG SPDDAPADHVCACPQGLMLL K	Lipid metabolism	membranar	this work

Ensangp00000020384 ^e)	Low density lipoprotein receptor	17.3/5.04	LC MS/MS	-	-	-	GRTN BISRAGICDGK	Lipid metabolism	membranar	this work P [50]
Ensangp00000022875 ^e)	Lysozyme precursor	15.33/8.56	LC MS/MS iTRAQ	-	-	-	NGSTDYGFQINNK YWBDSGYGSNDCK NLLNDDITDDIK KLPNVSSCF	Immunity related Antibacterial enzyme	secreted	P [12], P [50]
Ensangp00000011006 ^e)	Malate dehydrogenase	35.37/6.95	2-DE-MS		96	PSD	DDLFTNTNASIVR	Participates in the citric acid cycle	cytoplasmic	this work
Ensangp00000020184 ^d) ^e)	Malate dehydrogenase	35.27/9.52	LC MS/MS	-	-	-	ANTFVGEAAGVDPQK	Metabolic enzymes which catalyse the last step in anaerobic glycolysis	mitochondrial	P [12]
Ensangp00000017682 ^d) ^e)	Maltase	67.21/5.87	1-DE-MS 2-DE-MS LC MS/MS iTRAQ	27%	6-8, 12	(17-43)	AMPSGAIANWVLGNHDNSR DQPETYDMVHQWR ELNVAAQLAAPR GITQTIDYLK	Sugar digestion Converts sucrose in nectar to glucose and fructose	secreted	P [12], T [9]
Ensangp00000015067	Mitochondrial carrier		2-DE-MS	16		186				
Ensangp00000004215 ^e)	Mitogen activated kinase kinase kinase	159.64/10.04	LC MS/MS	-	-	-	NIATYYGAFIK	Protein kinase ATP binding	cytoplasmic	this work
Ensangp00000003748 ^e)	Myosin	121.89/10.23	iTRAQ	-	-	-		Contractile protein	cytoplasmic	this work P [52]
Ensangp00000003978 ^e)	N acylneuraminatase cytidyltransferase	21.1/5.67	LC MS/MS	-	-	-	HLTLARILLGME	Forms CMP-NeuAc, the nucleotide sugar donor used by sialyltransferases (modification may be important in pathogenesis)	cytoplasmic	this work

Ensangp00000021120 ^e)	NADPH dependent carbonyl reductase	27.05/7.7 3	LC MS/MS	-	-	-	MDFTGKVVLTIGASSGIGAST AK	Carbohydrate metabolism	cytoplasmic	this work
Ensangp0000002613 ^e)	Nucleolar RNA associated protein	117.56/7.05	iTRAQ	-	-	-	LSSETIDELEK	Appears to be associated with ribosome biogenesis	cytoplasmic	this work
Ensangp00000011253 ^d) ^e)	Nucleoside diphosphate kinase	19.01/8.4 6	1-DE-MS iTRAQ	32%	-	-	GDLCVQVGR	Maintenance of cellular pool of nucleoside triphosphates	cytoplasmic and plasma membrane	this work T [9]
Ensangp00000012716 ^{*,d,e})	Putative 5' nucleotidase precursor	63.47/7.0 1	1-DE-MS 2-DE-MS LC MS/MS iTRAQ	20%	10, 13- 33, 38- 51, 54, 57, 60, 64, 65, 77-82, 85-87, 130- 134, 140- 141, 144- 145	(15-30) PSD	APFPLTLIHINDLHAR DQIYVVVVPSYLADGKDGFA MK ECIAGIAR GLAPYLAELEK LGTQVIGTTEVFLDRESCR LSGADLWSAIDHSFTLDDEFR MKIPTVVANLEK NVNIIIVVLSHCGLDGDK QLAEEAGDLIDVIVGAHSHSLL LNK YDTIEGDYPLVVKK VVIENTNGTCSWDLDSQR NPIEKGDITNGLAIEAAPYGSS VDMIK	Anti-platelet	secreted	P [12]
Ensangp00000020778	Peptidyl prolyl cis trans isomerase	18.29/8.9 7	LC MS/MS iTRAQ	-	-	-	FFDMTVDNQPLGR IVIELRPDVVVK HVVFGSVVEGMDVVR	Accelerates protein folding	cytoplasmic	this work
Ensangp00000028058 ^d) ^e)	Peroxidase precursor	24.99/8.2 3	1-DE-MS LC MS/MS iTRAQ	16%	-	-	AFAGAININDHMFNPTVLER CFAIPVRPDDPVLASAGGIQCLD LVR LLPAEYGDGVYVPR SNITPELTILHVAFLR TTLVNMQFGQLVAHDMGLR WEDFVELR	Vasodilator	secreted	P [12], P [50]
Ensangp00000020634 ^e)	Peroxisomal targeting signal 2 receptor	36.41/6.2 2	iTRAQ	-	-	-	VSGSGDGSVQLWNT FTTNR TNLAS SVQLWNTNLASN	Family of protein implicated in a variety of	?	this work

							SQFYGLAGGGT	functions ranging from signal transduction and transcription regulation to cell cycle control and apoptosis	?	?	this work
Ensangp00000024749 ^e)	Pheromone/general odorant binding protein OBP56	27.15/5.5 2	iTRAQ	-	-	-	SASEVQDDKCK	?	?	this work	
Ensangp00000013993 ^e)	Phosphatidylethanolamine-binding protein	24.17/6.6 7	iTRAQ	-	-	-	YVFLVYK	Proteinase inhibitor	?	this work P [52]	
Ensangp00000012460 ^d)	Phosphoglycerate kinase	43.84/7.5 4	2-DE-MS	-	109	27	-	Glycolysis	cytoplasmic	this work	
Ensangp00000015800 ^d) ,e)	Phosphoglycerate mutase	28.7/6.8	LC MS/MS	-	148	25	YGEEQVLIWR	Involved in energy pathways	cytoplasmic	this work	
Ensangp00000020531 ^e)	Precursor	200.9/4.5	iTRAQ	-	-	-	ERTGEIMLLQR AGTIVGNVSALDEDVGPNG TRDARLDRDTNPESYAI GTIFVNSTLNINYAAVI VERQLDYEE VSGVLDLDRFTVEMQERLANAN LELS	Cadherin	membranar	this work	
Ensangp00000012492 ^d)	Precursor	28.47/5.0 4	1-DE-MS	22%	-	-	-	EGF-like domain	?	this work	
Ensangp00000013568 ^d)	Precursor	41.83/5.4	2-DE-MS	-	75	-	-	Aspartic protease A1	secreted	this work	
Ensangp00000016366 ^d)	Precursor	45.95/9.4 3	2-DE-MS	-	142	23	-	Glucose-methanol-choline oxidoreductase	cytoplasmic	this work	
Ensangp00000019046 ^d)	Precursor	12.39/8.7 5	LC MS/MS	-	-	-	ANDRAMVK	EGF-like domain	?	this work	
Ensangp00000029324 ^e)	Precursor	25.94/4.8	LC MS/MS	-	-	-	TLTFVLKPTK	Alpha 2	intracellular	this work	

Ensangp00000031578 ^e	Precursor	58.96/9.68	iTRAQ	-	-	-	DMPNITLLNLDGNQLSR NLLQNLDLALFVAMPQLLNLN ASSPV ANNLT SAPIA PVTGR PNITLLN VSAPIGL NKITTFNIT LAQFLDQAAAVAK	macroglobulin domain Leucine rich repeat Putatively involved in protein-protein interaction	?	this work
Ensangp00000020734 ^d	Pterin 4 alpha carbinol amine dehydratase	21.20/10.23	LC MS/MS	-	-	-	LAQFLDQAAAVAK	Transcriptional activator/pterin dehydratase	?	this work
Ensangp00000027538 ^c	Retrovirus related pol polyprotein	9.51/11.28	2-DE-MS	-	181, 183	PSD	-	?	nuclear	this work
Ensangp00000021077 ^d ,e)	Ribonuclease	14.41/8.04	LC MS/MS iTRAQ	-	-	-	ALAPYNQAIIVADR	Inhibits protein synthesis by cleavage of mRNA	?	this work
Ensangp00000006850 ^e	DNA directed RNA polymerase	68.25/8.18	iTRAQ	-	-	-	LSYISALGMMTR	Transcription	nuclear	this work
Ensangp00000019607 ^e	Ryanodine receptor 1	577.53/5.18	LC MS/MS	-	-	-	YFDMFLKLLK	Ca ²⁺ release channels involved in secretory pathways ?	membranar	this work
Ensangp00000020530 ^e	Serine protease precursor	25.2/4.57	iTRAQ	-	-	-	NGQNDIALLQLDRK VITSAQCTTDEGNGIPSVVRLG GTK SVLFAVL LIWDSVV ALLQLDRKIIIN TTDEGNGIPSVVR LAAETDILHEVVNEGISR	Involved in immunity or in coagulation cascade	secreted	this work
Ensangp00000016680	Serpin 9	46.36/7	iTRAQ	-	-	-	LAAETDILHEVVNEGISR	Serine protease inhibitor Involved in immunity	secreted	T [11]

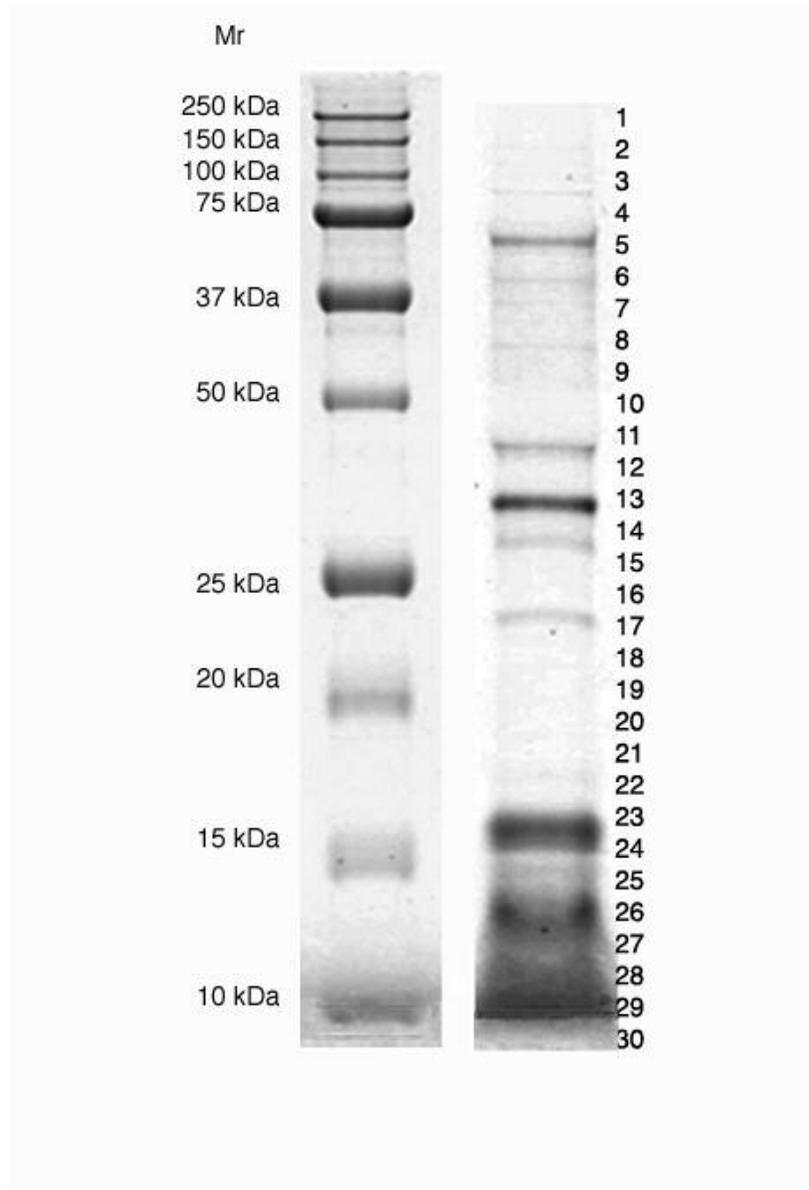
Ensangp00000017327	putative Salivary protein SG1B	46.6/7.37	iTRAQ	-	-	-	DYESYLGAMFAADAFHVVEY AD GK	?	secreted	P [12]
Ensangp00000032098 ^e)d	Salivary D3 protein		iTRAQ	-	-	-	AAAGPAPDPSSQFCQQLDDA QR	Saglin	?	P [12]
Ensangp00000027418 ^d)*	Salivary gland 1-like 3 protein	44.51/6.0 4	1-DE-MS	30%	-	-	-	?	secreted	P [12]
Ensangp00000009988 ^e)	SG3	20.01/4.3	iTRAQ				ATGPLFLPHFGQGPR RGQQ LIFLAA SVERNPA ATIAVASAAT ASPTTAEA QQRQQVQR DVEGLLKAEVALK	Mucin	secreted	T [51]
Ensangp00000008103 ^e)	Stromal interaction molecule precursor	54.49/6.3 6	LC MS/MS	-	-	-		Role in RNA binding	membranar	this work
Ensangp00000009009 ^e)	Fact complex subunit facilitates chromatin transcription	71.65/6.2 8	iTRAQ	-	-	-	RPLSAYMLWLNSAR	Recombination signal sequence recognition T160	nuclear	this work
Ensangp00000016164 ^e)	Superoxyde dismutase	15.67/5.4 5	iTRAQ	-	-	-	SLVVHADPDDLGVGGHELK	Metalloprotein that prevents damage by oxygen-mediated free radicals	intracellular	this work
Ensangp00000018041 ^d)	Toll precursor	16.69/4.5 1	2-DE-MS	-	152	17	-	Toll IA Involved in signal transduction pathways in response to pathogens	plasma membrane	this work P [50]

Ensangp00000028309 ^e)	Trans enoyl COA isomerase mitochondrial precursor	30.18/7.13	LC MS/MS	-	-	-	ALEQAVAFNLR	Fatty acid metabolism	mitochondrial	this work
Ensangp00000021085 ^e)	Translationally controlled tumor protein TCTP	19.54/4.42	iTRAQ	-	-	-	LVDDVMYEVYGK	Histamine-releasing factor	?	T [9], T[11]
Ensangp00000017522 ^e)	Trio protein	43.78/6.4	iTRAQ	-	-	-	SMYDLIGQLVQSSK	?	secreted	P [12]
Ensangp00000018152 ^d ,e)	Triosephosphate isomerase	22.52/5.09	1-DE-MS LC MS/MS	30%	-	-	AIFGETDELIAEK DWSNVVIAAYEPVWAIGTGK SLLPETIGVAAQNCYK DLGLGWVILGHSER	Central enzyme in the glycolytic pathway Plays an important role in several metabolic pathways Proteolytic enzyme	cytoplasmic	this work P [52]
Ensangp00000025045 ^e)	Trypsin precursor	28.65/6.49	iTRAQ				QIGIVSWGDTQCVGT RGGSTL NETDLTVR RLALTAGH NGNFVPNL PAPARATGRIV	Proteolytic enzyme	secreted	this work
Ensangp00000008105 ^e)	E3 Ubiquitin ligase	201.24/6.46	iTRAQ				GLAMADLRLEK QQLCIKP NPDN SEHRNHK GTYHSVN TQASQQQAPL LRDGSRVMMMG SPIILLDDIFDK	Involved in protein degradation pathway	cytoplasmic	this work
Ensangp00000000334 ^c ,e)	Unknown	39.57/7.29	LC MS/MS	-	-	-		ATP/GTP-binding site motif A (P-loop)	intracellular	this work
Ensangp00000012072 ^d)	Unknown	29.2/4.4	2-DE-MS	-	135	20	DSTLIMQLLR	14-3-3 protein. Family of conserved regulatory molecules	cytoplasmic	P [12]

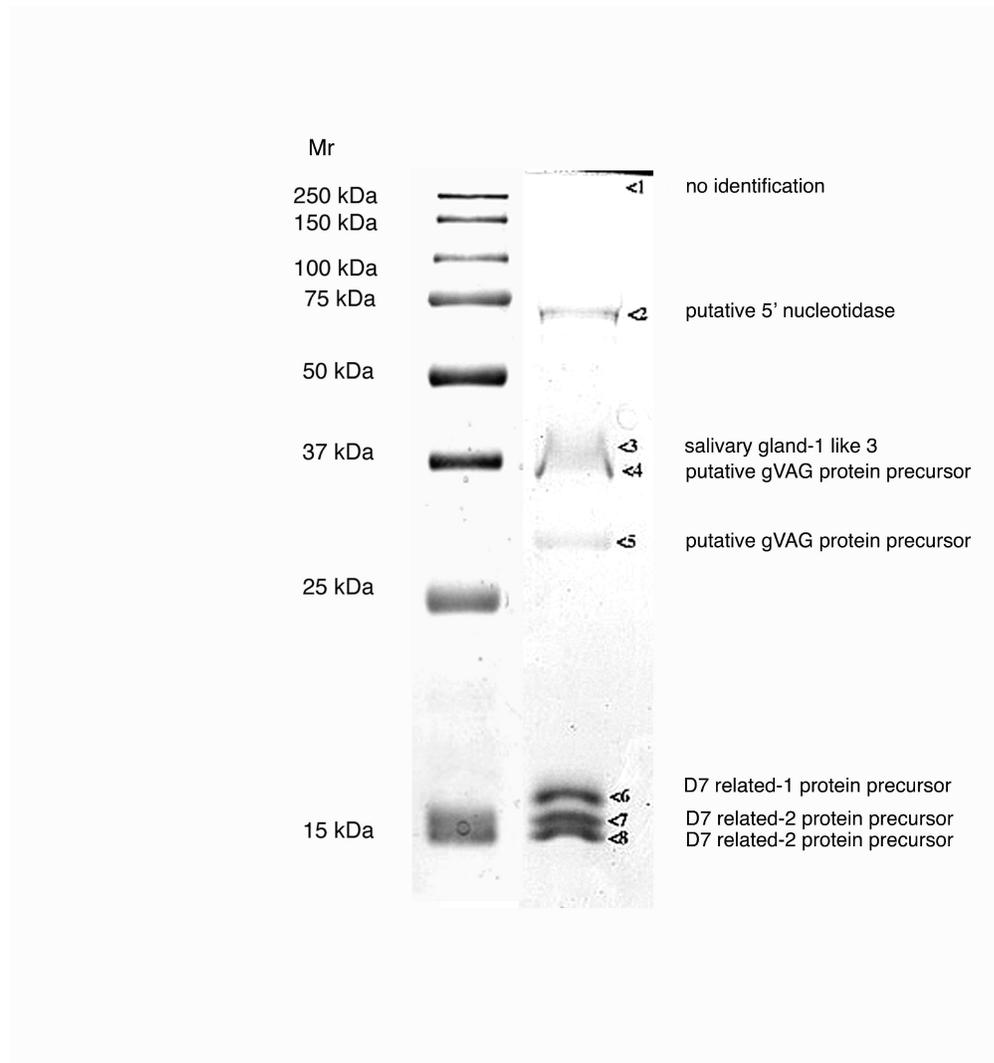
Ensangp00000012822 ^e)	Unknown	74.9/7.88	iTRAQ				DVQASHISRLGTSSIVSYTP TLRNGTPQASNSI YCTLRNGT NVSMC PDTIDSD	that bind a multitude of functionally diverse signaling proteins Immunoglobulin-like domain Involved in cell adhesion	membrane	this work
Ensangp00000015472 ^c) ^{e)}	Unknown	15.64/10.38	1-DE-MS	20%	-	-	-	InterPro Zn-finger, C2H2 type nucleic acid-binding protein	nuclear ?	this work P [50], T [9]
Ensangp00000016832 ^e)	Unknown	19.42/4.88	iTRAQ				QQAAAAAETTSQAAGTLMDH AK	Anti-freeze protein	?	this work
Ensangp00000017135 ^e)	Unknown	85.43/8.64	LC MS/MS	-	-	-	IKCGLLLEGVR	?	?	this work
Ensangp00000019537 ^e)	Unknown	90.81/7.41	iTRAQ	-	-	-		?	?	this work
Ensangp00000019887 ^d)	Unknown	70.9/5.1	2-DE-MS	-	9	18	-	Heat-shock 70 domain May be involved in response to stress	cytoplasmic and organelles	P [12]
Ensangp00000028177 ^e)	Unknown	36.81/10.03	iTRAQ	-	-	-	LGIGSSSINGSGAVVRK	Basic helix-loop-helix dimerisation region		this work
Ensangp00000028294 ^d)	Unknown	15.18/4.57	LC MS/MS	-	-	-	GSTINLTBAVK	Immunoglobulin-like domain Involved in cell adhesion	membrane ?	this work
Ensangp00000029447 ^e)	Unknown	20.35/6.24	iTRAQ	-	-	-	EQQLALDVR	?	secreted	this work
Ensangp00000012893 ^{e)})	Unknown	72.74/4.92	iTRAQ	-	-	-	ELEDIVQPIIAK	Hsp70 and tropomyosin domains	ER ?	this work

Ensangp00000011593 ^e)	Wilm's tumor 1 associating WT1 associated pr splicing regulator female lethal 2-D homolog	33.55/4.7 8	LC MS/MS	-	-	-	FTPDSNTGKR	Potential role in transcriptiona l regulation Involves in alternative splicing regulation	nuclear	this work
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^{a)} When several spots corresponded to the same protein, the percentage range of the sequence coverage is indicated in parenthesis. ^{b)} Subcellular localization is inferred from sequence or structure similarity with orthologous proteins. ^{c)} Identification was performed using Ensembl database v35 of november 2005. ^{d)} proteins identified from salivary gland extracts of young blood-fed females. ^{e)} Proteins identified from salivary gland extracts of old blood-fed females. ^{f)} Proteins allowing a correction of incorrect genome annotation (the part of the sequence in bold is that described in Ensembl v43. PSD : post source decay. Shaded lines : Proteins identified for the first time by a proteomic approach. * means that the proteins were also identified in saliva. References are underlined when they correspond to proteins identified in human saliva.



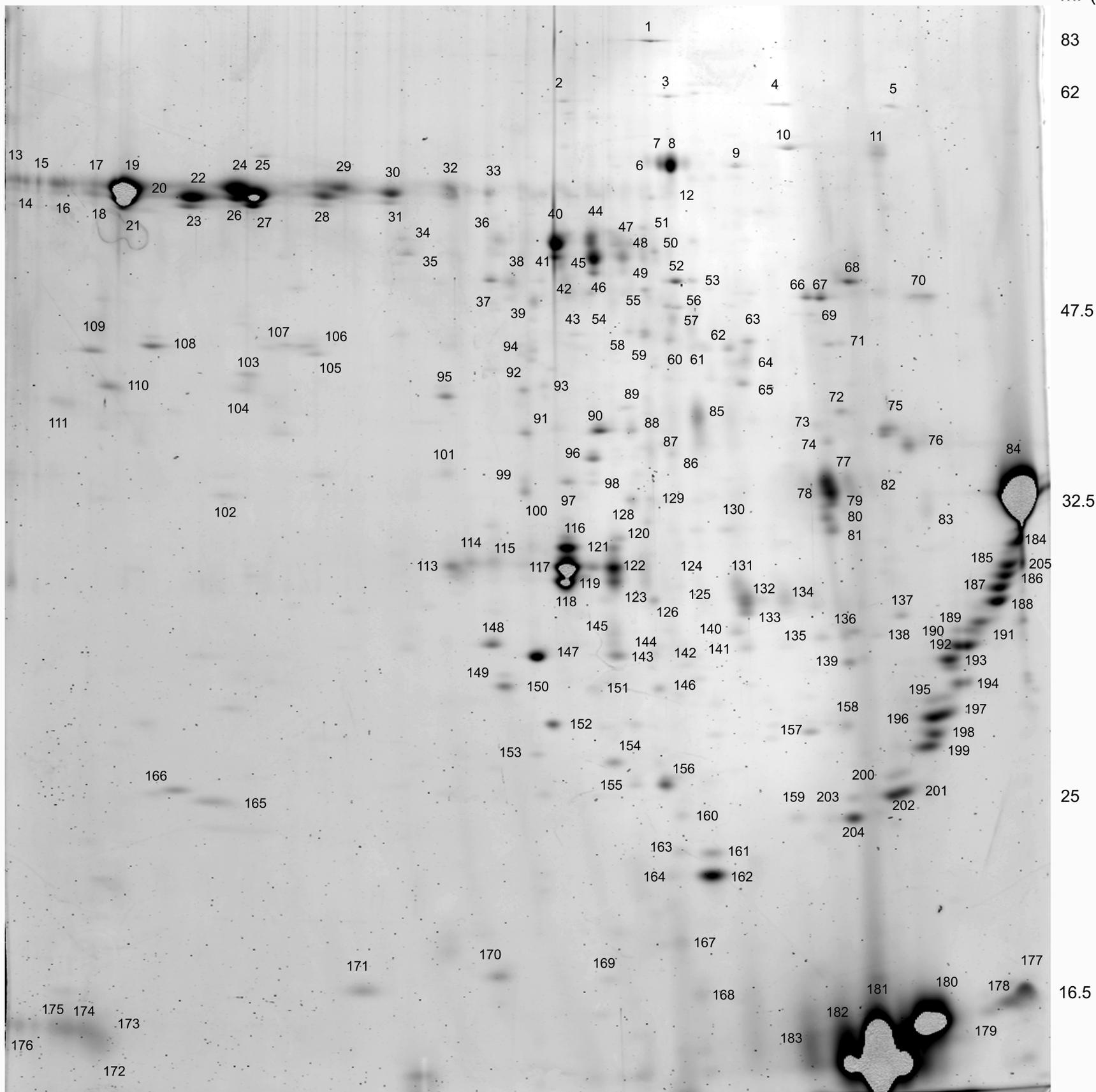
Salivary components were separated by a 12% NU-PAGE Bis-Tris gel under denaturing and reducing conditions. Molecular mass markers are shown on the left. After Coomassie staining, the gel was cut into millimeter slices as indicated by the numbers on the right side of the figure. The plugs obtained were analyzed by mass spectrometry as described in the Methods section.



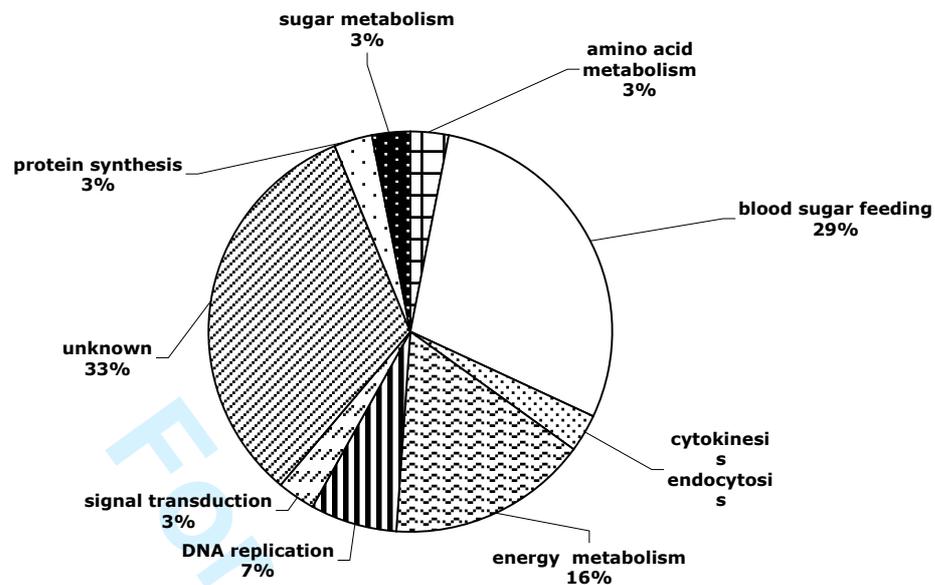
Saliva was collected from 7200 females using artificial feeders. After lyophilisation, saliva components were re-suspended in water and aliquots were analyzed by SDS-PAGE. Following silver nitrate staining, the numbered protein bands were analyzed by mass spectrometry

pl 8

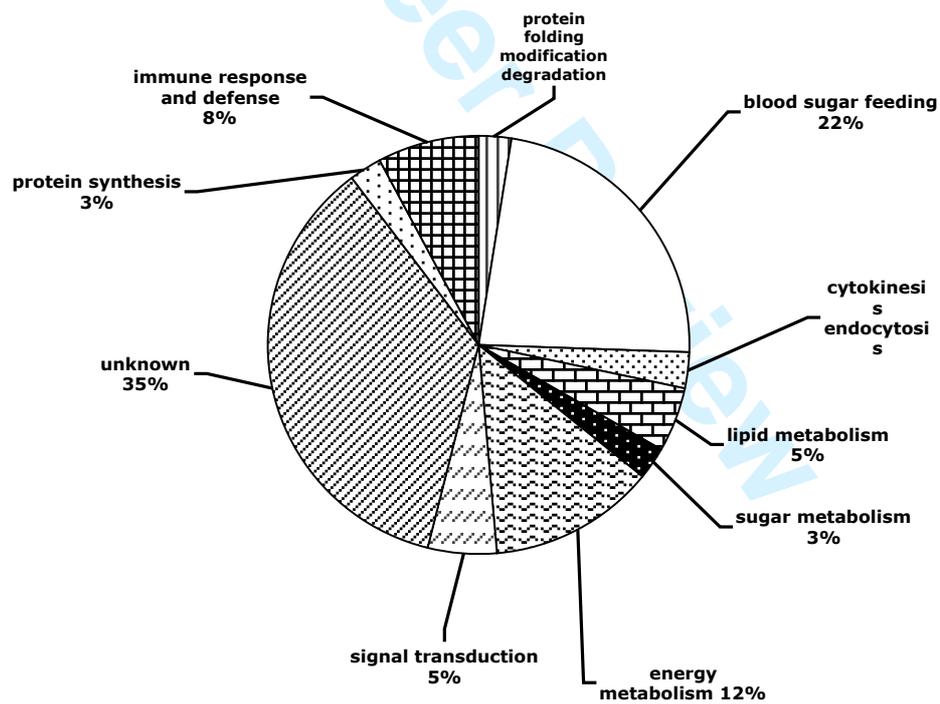
4 Mr (kDa)



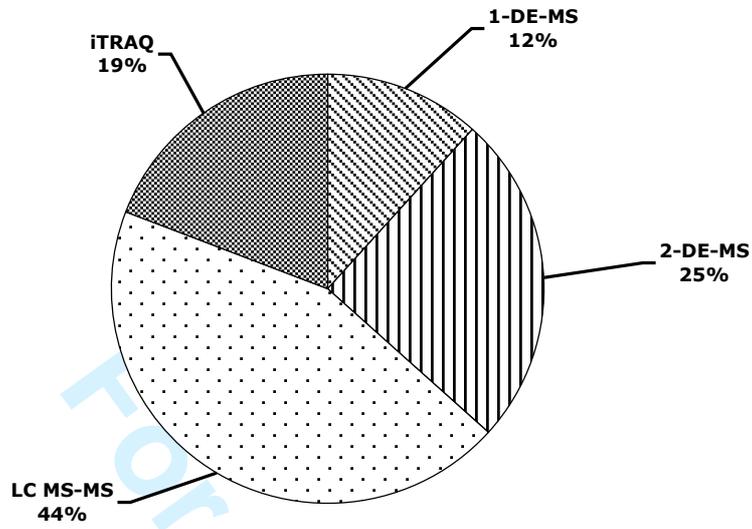
A



B



A



B

