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Investigation of the global protein content from healthy human tears

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Abstract

Considering absence of invasiveness and side effects, tears emerge as a particularly attractive fluid for biomarker discovery and therefore for daily clinical use. However, to date, this fluid remains poorly studied in healthy condition. Here, we present an updated in-depth characterisation of the human healthy tear protein composition using proteomics approach. Both eyes of eight healthy controls were collected using the Schirmer's strip method. After liquid digestion and off-gel electrophoresis fractionation, three independent proteomics analyses were performed. Resulting files were searched against the uniprot_swissprot database (2017_05_10) using Thermo Proteome Discoverer (version 2.2) and a false discovery rate of 1% was selected. Globally, 1351 proteins were identified with 2 unique peptides. More specifically, 39% of the lacrimal proteins were enzymes, with high numbers of dehydrogenases, phosphatases, kinases and ligases. Immunoglobulins, serpins and 14-3-3 domains proteins emerged also as abundant lacrimal proteins. Pathway analyses highlighted among others the glycolysis and the coagulation and complement cascades. Our study therefore complements the existing data on healthy tears proteome. Nevertheless, extensive studies for deeply and definitively characterise this promising fluid are required in the near future in order to be able to routinely use this fluid in clinics. A better understanding of its protein content will probably open new avenues in the biomarker discovery and clinical practice in the near future.

Keywords: tears; proteome; Off-Gel Electrophoresis; Gas-Phase Fractionation; integrative pathways.

49

50 # 1. Introduction

51 Surprisingly, until now, tears and their clinical relevance have been relatively poorly
52 studied. This is particularly illustrated by searching the keywords “Eye tears and
53 biomarkers” in Pubmed. Only 268 items were found during the two last decades
54 (between 1998/01 and 2018/01). In the same period, 264’224 publications containing
55 the words “blood” and “biomarkers” were published. With non-invasive, easy and
56 rapidly collected samples, tear-based approaches open up however new routes for
57 diagnostic methods and for deepening understanding of both ocular and systemic
58 diseases. Differences in the tear protein pattern of patients suffering from diabetic
59 retinopathy (1), Graves’ Orbitopathy (2), dry eye disease (3), multiple sclerosis (4, 5)
60 and even breast cancer (6) or renal failure (7) have already been highlighted. In
61 addition, it is also well known that tears can be an infectious fluid as Herpes simplex
62 virus (8), hepatitis B virus (9), human immunodeficiency virus (10) or parasites (11-
63 13) are detectable. However, despite this clinical interest, tears remain an exotic fluid
64 in research as well in clinics.
65 At this stage, in order to be as performing as possible, the use of tears for biomarker
66 discovery and clinics requires well knowing and understanding their global protein
67 content, mainly in healthy subjects. To the best of our knowledge, to date, only three
68 major studies (14-16) deeply investigated the proteome of human healthy tears. It is
69 not easy to understand or explain why tears, unlike other biological fluids, have yet to
70 arouse real clinical interest. The relatively low volumes available for collection
71 probably contributed significantly to the low numbers of ‘omics’ studies reported so
72 far. In this context, using mass spectrometry-based approaches, our study proposes

an updated and extended list of lacrimal proteins which could be used as additional reference list for clinical applications in the field of the biomarker research.

2. Material and methods

2.1. Patients.

Healthy subjects (N=8; experiment 1: 2 women aged 59 and 61 years; experiment 2: 1 woman, 2 men, aged 21, 24 and 60 years respectively; experiment 3: 1 woman, 2 men aged 26, 24 and 26 years respectively) were included in this study. In accordance with the Declaration of Helsinki, written informed consents were obtained from these subjects. The cantonal ethics committee for research on human beings has approved the patient's informed consent form and the use of biological material (approbation N°516/12, Dec 2012).

2.2. Sample collection.

Tears were collected using Schirmer-Plus® paper strip (Biotech Vision Care PVT LTD, Gujarat, India). To avoid any discomfort to patients, collection was restricted to a maximum of 5 minutes. No external stimulation was done to collect the basal fluid. Topical anaesthesia should be avoided because it reduces tear production. For each patient, tear samples were taken from both eyes. Persons collecting tears wore gloves in order to avoid any contamination. Care was taken to avoid damage to the conjunctive surface and local eye irritation. External factors such as harsh lighting, background noise and extreme room temperature, all known to affect the content of

samples, were strictly supervised in order to ensure satisfactory reproducibility. The strip was then inserted in a tube on ice and centrifuged at 7840 g for 7 min at 4°C without any additional buffer, as described elsewhere (17-19). After centrifugation, tear samples were immediately stored at -80°C until analysis. In order to verify the absence of cellular contamination in our samples, we performed a haematoxylin and eosin (H&E) stain in order to color the potential cells. The conclusion was that the Schirmer's method did not induce cell contamination in our samples.

2.3. Total protein assay.

The protein concentration of the pooled tear sample (from both eyes of each subject) was determined by performing a Bradford Assay according to manufacturer's recommendations (Protein assay Dye reagent concentrate, Bio-Rad, Hercules, US-CA). The absorbance was measured at 595 nm with a spectrophotometer (Ultrospec 2100 Pro, Amersham Biosciences) and the protein concentrations of the three pools were determined using a bovine serum albumin (BSA) calibration curve. They were estimated at 12.9 µg/µl, 11.06 µg/µl and 9.4 µg/µl for the experiments 1, 2 and 3 respectively.

2.4. Reduction, alkylation and digestion.

Three independent proteomics experiments were done. Experiment 1 was performed with a pool of two healthy subjects, experiments 2 and 3 were done using two different pools of three healthy subjects (both eyes of the subjects in all three experiments). 60 µg of proteins were used for all experiments. They were dried under speed-vacuum; then urea (33 µl of 6M; Merck, Darmstadt, Deutschland) diluted in Triethylammonium bicarbonate buffer (0.1M; TEAB, Sigma-Aldrich, Saint Louis, US-

121 MO) and tris-(2-carboxyethyl)phosphine (2 µl of 50 mM; TCEP, Sigma-Aldrich, Saint
122 Louis, US-MO) were added in each tube. After incubation at 37°C during 1 hour,
123 iodoacetamid (1 µl of 400 mM; Sigma-Aldrich, Saint Louis, US-MO) was added and
124 tubes were incubated 30 minutes of in the dark. TEAB (67 µl of 0.1M) was added,
125 then a liquid trypsin digestion (1:20 ratio, 1 microgram of enzyme to 20 micrograms of
126 protein, porcine origin, Promega Corporation, Madison, US-WI) was done overnight
127 at 37°C.
128
129 **2.5. Off-gel electrophoresis (OGE).**
130 Before OGE, samples were dried under speed-vacuum then purified by using
131 Macrospin columns (Harvard Apparatus, Holliston, US-MA) according to
132 manufacturer's recommendations. Tubes were dried under speed-vacuum and A
133 3100 OFFGEL Fractionator (Agilent technologies, Santa Clara, US-CA) was
134 performed over night to separate the sample. Guidelines available in Agilent
135 datasheet were followed, using a 13cm IPG strip (Immobiline DryStrip pH 3-10, 13cm
136 GE Healthcare, Little Chalfont, UK) and 12 OGE wells (20, 21). After fractionation,
137 microspin columns (Harvard Apparatus, Holliston, US-MA) were used according to
138 the manufacturer's recommendations and the 12 fractions of each experiment were
139 dried under speed-vacuum. Peptide concentration of the fractions was theoretically
140 approximated, considering that 1/12 of the pooled sample was found in each fraction
141 after OGE.
142
143 **2.6. Mass spectrometry (MS) analyses.**
144 The fractions resulting from OGE were dissolved in 94.9% H2O / 5% Acetonitrile
145 (ACN) / 0.1% Formic acid (FA). For the three experiments, 1µg of fraction was

146 injected in GPF4 mode (22), meaning that each fraction of each experiment was
147 injected four times (GPF1, GPF2, GPF3 and GPF4). They were analysed by tandem
148 MS (Liquid Chromatography-MS/MS) using a Linear Trap Quadrupole (LTQ) Orbitrap
149 Velos Pro (ThermoFisher instruments, San Jose, US-CA) coupled to a nanoflow high
150 pressure liquid chromatography (HPLC, NanoAcquity system from Waters, Milford,
151 US-MA). Peptides were trapped on a home-made 5 µm 200 Å Magic C18 AQ
152 (Michrom) 0.1 × 20 mm pre-column and separated on a commercial 0.075 x 150 mm
153 Nikkyo (Nikkyo Technology, Tokyo, JPN) analytical nanocolumn (C18, 5 µm, 100 Å).
154 More precisely, trapping was done during 15 minutes with a flow rate of 3 µl/min
155 using a gradient of H2O/FA 99.9%/0.1% (solvent A) and CH3CN/FA 99.9%/0.1%
156 (solvent B), where 95% of solvent A were mixed with 5% of solvent B. Then the
157 analytical separation was run for 85 min with a flow rate of 220 nl/min as follows: 0–1
158 min 95% A and 5% B, 1-55 min 65% A and 35% B, 55-65 min 20% A and 80% B, 65-
159 67 min 20% A and 80% B, 67-69 min 95% A and 5% B and 69-85 min 95% A and
160 5% B. min. For MS survey scans, the OT resolution was set to 60000 and the ion
161 population was set to 5 × 105 with an m/z window from 400 to 2000. Five precursor
162 ions were selected for collision-induced dissociation (CID) in the LTQ. The
163 normalised collision energies were set to 35% for CID. The different m/z windows for
164 the gas-phase fractions were set as following: 400-520 for GPF1, 515-690 for GPF2,
165 685-979 for GPF3 and 974-2000 for GPF4. Peak lists and resulting files, combined
166 from the different experiments, were searched against the uniprot_sprot
167 (2017_05_10) database using Thermo Proteome Discoverer (version 2.2.0388;
168 Thermo Fisher Scientific, San Jose, US-CA). Oxidised methionine was set as
169 variable amino acid modifications and carbamidomethylation of cysteines were set as
170 fixed modification. Trypsin was selected as the enzyme, with one potential missed

171 cleavage. The precursor mass tolerance and the fragment mass tolerance were 10
172 ppm and 0.6 Da respectively. A false discovery rate (FDR) of 1% was selected at
173 peptide and protein levels and only the Master proteins were kept. The list of
174 identified protein was generated containing proteins matching with two different and
175 unique peptide sequences. The datasets supporting the conclusions of this article are
176 available in the ProteomeXchange Consortium repository via the PRIDE database
177 (submission identifier: PXD008702). The reviewer account details are the following:
178 username: reviewer05998@ebi.ac.uk; password: ycE67P81.

179

180 **2.7. Process and pathway analyses.**

181 Database for Annotation, Visualization and Integrated Discovery (DAVID), a web-
182 accessible program, was used to perform the Gene Ontology (GO) Analysis
183 classification of the identified proteins (23). Some subcategories were grouped in
184 order to reduce the numbers of GO categories and simplify the pie charts.
185 Search Tool for the Retrieval of Interacting Genes/Proteins /STRING) database was
186 used to classify the proteins into protein families (24).
187 Kyoto Encyclopedia of Genes and Genomes (KEGG) was also used to study and
188 visualise the pathways in which the proteins were involved (25).
189 A manual merge of the protein lists obtained with the three experiments was done
190 using Venny (http://bioinfogp.cnb.csic.es/tools/venny/index.html).
191 Top 10, 20, 50, 100 and 200 of the most abundant proteins for the three experiments
192 were obtained by classifying the proteins according to their Peptide Spectrum
193 Matches (PSM). PSM of a protein corresponds to the number of identified peptide
194 spectra matched for this protein. The same classification was done to obtain the top-
195 100 and top-200 of the less abundant proteins of the different experiments.

196

197 # 3. Results

198 In order to obtain a robust in-depth characterisation, three independent proteomics
199 experiments were performed on three different pools of healthy tears. Individual and
200 general lists of proteins were then generated, analysed and compared to the
201 literature. The study design is summarised in the graphical abstract. In addition,
202 comparisons between the lacrimal protein content with other ocular fluids will be
203 proposed.

204

205 ## 3.1. Characterisation of the human tear proteome

206 By combining all the experiments, 1351 proteins with 2 unique peptides were
207 identified. By excluding the keratins, the original list was finally reduced to 1337
208 proteins with 2 unique peptides (874, 837 and 1143 proteins for experiment 1, 2, and
209 3 respectively; global and detailed lists in S1 dataset).
210 Interestingly, 45.5% (608 proteins) were in common between the three different
211 experiments. For further analyses, only list of proteins without keratins was
212 considered.
213 Top-10 of the most abundant proteins for each experiment was highly stable with 7
214 proteins always present whatever the experiment observed (Table 1). As expected,
215 the rank one protein was the lactotransferrin, one of the major proteins of the tears.
216 The other six common proteins between our three experiments were lipocalin-1,
217 serum albumin, lysozyme C, immunoglobulin heavy constant alpha 1,
218 immunoglobulin kappa constant and polymeric immunoglobulin receptor. By

219 expanding the comparison to the top-20, top-50 or top-100, the percentages of
220 proteins in common were still relatively high (64%, 45.1% and 52.42%, respectively).
221 At the opposite, top-100 and top-200 of the less abundant proteins presented only
222 0.1% and 1.6% of proteins in common between the three experiments.

223 Moreover, the PSM corresponding to the 7 proteins in common between the three
224 top-10 represented between 27.7% (experiment 3) and 36.7% (experiment 1) of the
225 total PSM detected.

226

227 Table 1: Top 10 proteins of the three experiments.

AC	Protein names	PSMs	Pep
P02788	**Lactotransferrin**	**3074**	**60**
P31025	*Lipocalin-1*	*1252*	*14*
P61626	*Lysozyme C*	*1109*	*12*
P02768	*Serum albumin*	*1021*	*54*
P01876	*Immunoglobulin heavy constant alpha 1*	*857*	*13*
P0DOX2	Immunoglobulin alpha-2 heavy chain	546	8
P01833	*Polymeric immunoglobulin receptor*	*541*	*28*
P01877	Immunoglobulin heavy constant alpha 2	521	2
P12273	Prolactin-inducible protein	505	9
P01834	*Immunoglobulin kappa constant*	*389*	*2*

228

AC	Protein names	PSMs	Pep
P02788	**Lactotransferrin**	**1570**	**60**
P61626	*Lysozyme C*	*906*	*13*
P02768	*Serum albumin*	*751*	*57*
P31025	*Lipocalin-1*	*649*	*12*
P01876	*Immunoglobulin heavy constant alpha 1*	*558*	*14*
P12273	Prolactin-inducible protein	487	12
P01833	*Polymeric immunoglobulin receptor*	*385*	*33*
P01834	*Immunoglobulin kappa constant*	*344*	*2*
P25311	Zinc-alpha-2-glycoprotein	268	21
P0DOX7	Immunoglobulin kappa light chain	244	3

229

AC	Protein names	PSMs	Pep
P02788	**Lactotransferrin**	**1913**	**54**
P02768	*Serum albumin*	*1358*	*52*
P61626	*Lysozyme C*	*1179*	*10*
P01876	*Immunoglobulin heavy constant alpha 1*	*677*	*11*
P31025	*Lipocalin-1*	*586*	*10*
P01834	*Immunoglobulin kappa constant*	*432*	*2*
P01833	*Polymeric immunoglobulin receptor*	*353*	*27*
P0DOX2	Immunoglobulin alpha-2 heavy chain	336	8
P25311	Zinc-alpha-2-glycoprotein	305	20

P01024	Complement C3	288	75

230

231 From top to bottom: experiment 1,2 and 3. Bold: same position in the three lists.
232 Italic: present in the three lists. AC: accession number on UniProt; PSMs: Peptide
233 Spectrum Matches for each protein; Pep: number of unique peptides for each protein.

234

235 3.2. Biological analyses of the tear proteome

236 Using the functional annotation-clustering tool from DAVID, the 1337 proteins were
237 classified according to their molecular functions, cellular components and biological
238 processes (Figs 1a, b and c respectively). The cadherin-binding involved in cell-cell
239 adhesion (62%, with for instance some eukaryotic translation elongation factors and
240 several capping actin proteins) and the enzyme activity (16%, containing a lot of
241 alcohol dehydrogenases and aldo-keto reductase) were the two main subcategories
242 emerging from GO molecular functions. For cellular components, half of the proteins
243 were linked to the cell-cell adherens junction (56%), an observation that was
244 confirmed by the biological process classification (cell-cell adhesion 46%).

245

246 Around 39% (518 proteins) of the 1337 proteins appeared to be part of the enzyme
247 protein classes (detailed lists are available as S2 dataset). By classifying these
248 enzymes using UniProt, the three major subclasses were hydrolases (41%, 212
249 proteins), transferases (27%, 138 proteins) and oxidoreductases (15%, 80 proteins)
250 (Fig 2). In the hydrolases category, we highlighted for example the phospholipase A2
251 (26, 15 and 15 PSMs in the three experiments) but also the lactotransferrin and the
252 lysozyme C, which were present in the three top 10 proteins. Several mitogen-

253 activated protein kinases (mitogen-activated protein kinase 1, 3, 13, 14) were found
254 in the transferases category and the oxidoreductases contained a lot of
255 dehydrogenases such as 4 alcohol dehydrogenases (alcohol dehydrogenase, alcohol
256 dehydrogenase 1B, alcohol dehydrogenase 1C, alcohol dehydrogenase class 4,
257 alcohol dehydrogenase class 3) and retinal dehydrogenase 1.

258

259 After noticing the large amount of enzymes in our proteins, we were interested by
260 studying the other major protein families. Using STRING, which is a biological
261 database for protein-protein interactions, it appeared that serpins (10 proteins) and
262 14-3-3 domain (7 proteins) were among the top five protein families.

263 Furthermore, KEGG analyses highlighted two notable biological pathways, the
264 glycolysis/gluconeogenesis (31 proteins, p-Value = $4.6E^{-14}$, with phosphoglycerate
265 kinase (EC 2.7.2.3) and the ATP-dependent 6-phosphofructokinase (EC 2.7.1.11))
266 (Fig 3) and the coagulation and complement cascades (38 proteins, p-Value = $1.7E^{-20}$,
267 among other the complement factor H, the complement factor 3 and the CD59
268 glycoprotein) (Fig 4).

269

270 3.3. Consistency with the existing knowledge

271 To date, only three major publications focused on healthy tear proteome and
272 reported 491, 1543 and 1526 identified proteins respectively (14-16). Summary of
273 these studies compared to our data were described in Table 2. In order to compare
274 them with our study, the same stringent identification criteria that we used were
275 applied: identification of proteins with at least 2 unique peptides, only reviewed

276 proteins and exclusion of keratins. Subsequent new lists of identified proteins were
277 generated for each study, greatly reducing their lists at 478, 1026 and 662 proteins.
278 By merging all the information (the 3 published studies and our study), 1620 proteins
279 were identified in tears (Fig 5). Only 197 proteins (11.2%; S3 dataset) were in
280 common across all 4 studies. Among them, a mean of 55% were found in the top-200
281 of the most abundant proteins of our experiments (53.6%, 53.3% and 55.3% in the
282 experiment 1, 2 and 3 respectively). At the opposite, between 1% and 7.1% of these
283 197 proteins were found in the 200 less abundant proteins of the experiments (4.6%,
284 7.1% and 1% in the experiment 1, 2 and 3 respectively). Furthermore, 425 proteins
285 (24.1%) were described for the first time in our study (S4 dataset), including some
286 translation initiation factors (eukaryotic translation initiation factors 2A and subunit 2,
287 factor 3 subunits A, B, C, D, E, F, M, factors 4 gamma 1 and 2, factor 5), ribosomal
288 proteins (60S ribosomal protein L14, L17, L18a, L22, L23, L24, L3, L30, L32, L9, 40S
289 ribosomal protein S2, S21, S4, S5, S7 and ribosomal protein S6 kinase alpha-1,
290 alpha-3) and mitogen-activated protein kinases 1, 13 and 14. An important point is
291 that around 40% of these 425 proteins were identified at least in two out of our three
292 experiments (S4 dataset), reinforcing our results.
293
294
295 Table 2: Comparison of the four tears proteome studies.

	De Souza et al.	**Zhou et al.**	**Aass et al.**	**Dor et al.**
Collection	1 healthy patient	4 healthy patients	3 healthy patients	8 healthy patients
	Microcapillary tube	Schirmer's strip	Schirmer's strip	Schirmer's strip
Protein quantity (µg)	NM (13 µl)	400	NM	180
Pool	/	Yes	Yes	Yes

Digestion	In-gel digestion Trypsin	In-solution Trypsin	In-solution Lys-C+ trypsin	In-solution Trypsin
Fractionation	SDS-PAGE	Offline SCX	Offline SCX	Off-Gel Electrophoresis
Injection MS buffer	NM	2% ACN	NM	5% ACN
Mass spectrometer	LTQ-Orbitrap + LTQ-FT	Triple TOF 5600	LTQ-Orbitrap	LTQ-Orbitrap
Identification conditions	FDR <0.1%, 2 unique peptides (Orbitrap), 1 peptide (FT)	FDR <1%, 1 peptide	FDR <1%, unspecified number of peptide	FDR <1%, 1 peptide
Maximum identification†	491‡	1543	1526	1351‡
Stringent identification*	478	1026	662	1337

296

297 NM: not mentioned; Lys-C: proteinase lys-C; FT: Fourier transform; †: with 1 peptide
298 and non-reviewed proteins; ‡: with 2 peptides; *: with 2 unique peptides, without
299 keratins and reviewed proteins.

300

301 3.4. The tear proteome and the other ocular fluids

302 The tear proteome generated in our study was then compared to the proteome of
303 vitreous and aqueous humors (VH and AH respectively), particularly well-investigated
304 by Murphy et al. (26, 27). Interestingly, 291 (21.8%) and 197 (14.7%) tear proteins
305 were also found in VH and AH respectively (% of proteins we found in tears; with two
306 peptides, only reviewed and without keratins; S5 and S6 datasets). Proteins found in

307 common were represented by clusterin but also proteins involved in the glycolysis
308 (among others the lactate dehydrogenases A and B chains and the enolase 1) and
309 complement and coagulation pathways (6 proteins of the serpin family and 16
310 complement factors). Several cathepsins (B, D, L1 and Z) were also commonly found
311 in both tears, VH and AH.

312

313 # 4. Discussion

314 Located on eye surface, tears play a key role in the correct function and health status
315 of the eye. Tears provide oxygen and nutriments to the ocular surface cells and
316 improve the optical properties of the eye (lubricating eye surfaces). Furthermore, tear
317 fluid protects the eye's epithelium thanks to antibacterial properties and by flushing
318 contaminants from the ocular surface. But beside their crucial roles for vision, tear
319 composition has been proposed to also reflect general health state and subsequent
320 local or systemic disturbances (1-7). Tears can therefore be used as a diagnostic
321 tool, either for detecting external agents (virus, parasites, bacteria) (8-13) or for
322 measuring changes in response to infection and more generally to pathological
323 situation. Consequently, the ability to measure modifications in human tear content
324 offers promising opportunities for screening not only ocular but also systemic
325 diseases and for discovering potentially new biomarkers for these clinical situations.
326 Nevertheless, despite this promising clinical value, tears remain largely under-
327 investigated and proteomics field is not an exception. However, we strongly believe
328 that having a complete and robust overview of its composition in healthy condition is
329 a mandatory step before starting using it in clinical practice. Three major studies
330 investigated so far the healthy tear proteome. Even if these studies are technically

331 and biologically relevant, this is, in our point of view, probably not enough to
332 definitively close the question of the complex composition of this fluid in healthy
333 condition.

334

335 We propose therefore here a new exploratory proteomics study that will complement
336 existing data. Notably, 1337 tear proteins were identified in tears of healthy subjects.
337 As expected, lactoferroxin, involved in the anti-inflammatory and antimicrobial
338 processes (28), and lipocalin-1, also known for its antimicrobial activity (29). were
339 among the most abundant proteins. This highlighted the importance of tears to
340 maintain a very clean environment for the eyes. Moreover, even if some differences
341 in term of abundance (PSM number) have been observed, the presence of these 5
342 first proteins (lactotransferrin, lipocalin-1, lysozyme C, serum albumin,
343 immunoglobulin heavy constant alpha 1) were largely conserved in all experiments.
344 The principal tear proteins are directly secreted by lacrimal glands (lactoferrin,
345 epidermal growth factor (EGF), tear lipocalin, secretory immunoglobulin A (sIgA) or
346 by lysosomes (lysozyme, which was found in the three experiments in the top 10
347 more abundant proteins). However, some serum proteins such as albumin,
348 transferrin, IgG and IgM were also found in the tear fluid probably as a result of
349 passive transport from the blood and are suggestive of serum leakage. It is also
350 known that cell infiltrating conjunctiva (T cells, B cells among others) secrete Igs and
351 cytokines in various conditions (30). Finally, local contribution of the neighbouring
352 cells releasing proteins in tears cannot be excluded.

353

354 This hypothesis could explain why a huge variety of intracellular proteins were
355 detectable in healthy tears. The detection of 10 serpins and 7 known isoforms of 14-

356 3-3 protein families (14-3-3 protein beta/alpha, epsilon, eta, gamma, sigma, theta,
357 zeta) in the lacrimal content was also very interesting. Both families have been
358 extensively studied for various cellular functions.14-3-3 family has been shown as a
359 potential marker for ocular hypertension induced by glaucoma (31). Several serpins
360 such as serpinA1 (serpin peptidase inhibitor clade A member 1) or serpinB1
361 (plasminogen activator inhibitor-1) were proposed as targets for diagnosis but also
362 therapy in cancer (32-34). Tracing these proteins in tears in response to a treatment
363 or to predict patient outcome could be clinically relevant and open new way to
364 manage cancer patients. Until now, no information related to the eye can be found
365 concerning these protein families, but we may suggest that it is related to the weak
366 overall investigation of tears.
367
368 Two key cellular processes, glycolysis and complement and coagulation cascades
369 were also reported in our proteomics exploration of healthy tears. Alterations of
370 these pathways have been already shown to be involved in some eye diseases such
371 as Age-Related Macular Degeneration (AMD) or diabetic retinopathy (35, 36).
372 Yokosako et al.(35) described the urinary levels of lactate and pyruvate as
373 deregulated IN AMD patients. In the present study, we were able to identify the
374 lactate dehydrogenase and the pyruvate kinase, which may play a role in the
375 deregulation observed by Yokosako et al.(35). As the AMD is an ocular disease, it
376 could be relevant to investigate if the levels of these two proteins are also
377 deregulated in tears. Karamichos et al.(37) also highlighted the
378 glycolysis/gluconeogenesis as part of the most significantly affected pathways in
379 case of keratoconus (KC), which is a non-inflammatory corneal disease. They noticed
380 the 1,3 diphosphoglycerate and the 3-phosphoglycerate as significantly up-regulated

381 in patients suffering from KC compared to healthy controls. In our study, we identified
382 the glyceraldehyde-3- phosphate dehydrogenase and the phosphoglycerate kinase
383 which may have an influence on the metabolites studied by Karamichos et al.(37). De
384 Souza et al.(15) also reported in their tear study a high number of hydrolases
385 involved in the glycolysis. Since the glycolysis takes place within the cells, the source
386 of these proteins might be damaged cells. These cells could be epithelial cells
387 covering the eyes but we cannot exclude that lacrimal cells released them. The fact
388 that these proteins were deregulated in a disease context emphasises their role or
389 importance. Regarding the complement and coagulation pathways, proteins that are
390 involved in play a major role in the protection of the ocular surface (38, 39) but also in
391 several diseases (36). As one of the tear main functions is protection and lubrication
392 of the eye, it is completely relevant to find some proteins related to these processes.
393 Detecting in patient tears involved at different levels in these pathways is clinically
394 relevant and offer new therapeutic strategies.
395

396 Even if we used three different pools of healthy subjects, we observed around 45.5%
397 of overlapping between our experiments, which is quiet good considering the
398 potential individual variations. Technical bias could also have been induced during
399 trypsin digestion (40), off-gel fractionation and mass spectrometry injections (41).
400 Moreover, the differences between our experiments were mainly found on the less
401 abundant proteins, meaning identified with few numbers of unique peptides. It could
402 explain why these proteins were found in some experiments but not in all three.
403 Our study revealed for the first time 425 specific proteins in tears of healthy subjects,
404 when compared with 3 other studies (14-16). If we exclude these 425 specific
405 proteins from our three lists of top-100 and three lists of top-200 less abundant

406 proteins, it does not greatly change the low percentages of shared proteins which
407 were previously found (2% instead of 0.1% between the three top-100 and 5.7%
408 instead of 1.6% between the three top-200).
409 Only three studies investigated the global proteome of the tears in healthy subjects.
410 The number of shared proteins (197) between the 4 studies was quite low. Around
411 55% of these 197 proteins were present in the top 200 more abundant proteins in our
412 experiments. Therefore, the less abundant proteins (between 1% and 7.1% of the
413 197 proteins were found in the 200 less abundant proteins of our experiments) are
414 probably the main cause of variability between these studies. This result is in
415 correlation with the low number of shared proteins between the three top-100 and
416 top-200 less abundant proteins of our own experiments. It is then important to
417 enhance the identification of these low abundant proteins, as they are more likely
418 than abundant proteins to contain potential biomarkers for diseases.
419
420 We specifically identified some ribosomal proteins. They are part of small guanosine
421 trisphosphatases (GTPases) (42). Depending on the binding to guanosine
422 trisphosphate (GTP) or guanosine diphosphate (GDP), Rab proteins (Ras-related in
423 brain) are active or inactive, respectively (43). They are involved in the membrane
424 traffic and vesicles formation and transport. Several mitogen-activated protein
425 kinases which are in charge of the signal transduction by the phosphorylation of
426 serine and threonine protein residues were also found (44). They could be released
427 after apoptosis of the cells in contact with tears. Why did we find these proteins
428 specifically in our study and how could we explain the low number of proteins in
429 common (192) between the studies? The use of different technical workflows (Table
430 2) could answer these questions.

Firstly, the method of sample collection could impact tear composition (17). De Souza et al.(15) used microcapillary glass tubes whereas the other studies sampled tears with Schirmer's strip. Schirmer method is reported to be more comfortable and pleasant for patients, easier to handle and can not wound neither cornea, nor conjunctiva. Capillary tube required more technical expertise from the person collecting tears. The good angle and position of the capillary on the ocular surface are not trivial, which frequently need capillary tube repositioning. More risks to seriously damage the conjunctiva and/or the cornea are associated to this technique. Finally, collection with a capillary tube takes more time since it must be stopped when the patient moves or blinks. Even if this technique is suspected to limit cell contamination from conjunctival or epithelial cells compared to Schirmer collection, this cannot be completely excluded. Posa et al. (17) concluded that both methods were suitable for protein analyses. However, they reported that all subjects experienced the Schirmer strip much more pleasant compared to the capillary tube. Our personal data also support the conclusion of Posa et al. (17). Therefore, regarding the balances between various advantages and few limitations, we chose the Schirmer test. Another parameter is the Schirmer extraction method. Whereas we only centrifuged the Schirmer's strip to collect tears, Zhou et al.(16) used extraction buffer comprising 100 mM of ammonium bicarbonate and protease inhibitor. Aass et al. (14) worked with different buffers containing NH4HCO3, NaCl, a surfactant, or a combination of the three, the consequences on recovered proteins following these extraction methods were not investigated yet.

Another different technical point could be the off-gel electrophoresis fractionation (based on the isoelectric point of the peptides) while Aass et al.(14) and Zhou et

456 al.(16) used strong cation exchange (SCX) chromatography. It has been proven that
457 both methods were suitable to separate a complex sample and enhance the number
458 of identified proteins (45, 46). A few studies compared these two methods (47, 48).
459 The off-gel electrophoresis provides the isoelectric point of the peptides, a useful
460 information, and shows a high resolution, a high sample loading and a flexibility
461 concerning the choice of the pH gradient (20, 45, 49). This allows to focus on a
462 specific pH range, to better separate the samples in this pH gradient. The SCX
463 method is described to be a faster method (20), but evidences about enhancing the
464 number of identified proteins are contradictory (47, 49, 50). AS expected, the
465 methods lead to different protein profiles (49, 51). We also would like to mention that
466 we did not measure peptide concentration of the fractions after OG. Regarding the
467 difficulty in quantifying peptides (52, 53) and the low quantity of material per fraction,
468 we estimated the concentration by considering a homogeneous quantity of peptides
469 in each fraction.

470 Peptide length and tryptic missed cleavage sites could also explain the differences
471 between the 4 studies that we observed. While we used LTQ-Orbitrap like Aass et
472 al.(14), Zhou et al.(16) performed their analyses with a TripleTOF 5600. On another
473 hand, Ass et al.(14) supplemented the trypsin with Lys-C enzyme. This combination
474 of two proteases was described to enhance the efficiency of the digestion, and
475 influences the identified proteins (54).

476 Like Zhou et al.(16) and Aass et al.(14), we pooled different collected samples, while
477 De Souza et al.(15) analysed one single subject. Pooling different samples permitted
478 to reach a sufficient quantity and work with an average sample in term of peptide
479 content (55) in the analysed sample. However, pooling loses the sample identity and

480 the inter-variability information. In our case, we were interested in the global tear
481 proteome so we chose to pool samples.
482
483 In our pie charts, we did not obtain the same category names as Zhou and al. (16),
484 probably because of a different software version. Nevertheless, the main categories
485 seemed similar for each GO classification, which was completely relevant with the
486 fact that almost 50% (776) of proteins were found in common in their study and ours.
487 This observation strengthened our results but also emphasised the need to analyse
488 more samples, to consider the potential effects of physiological parameters (age,
489 gender, circadian rhythm and potential drugs) and to standardise the proteomics
490 workflow.
491
492 So far, VH and AH were not very much studied. VH is a transparent and gelatinous
493 substance situated between the lens and the retina (56). Its global proteome,
494 meaning in healthy subjects, was described in only two main studies (26, 57). This
495 fluid should contain some information about the physiological condition of the retina.
496 AH is situated at the anterior and posterior chambers of the eye and plays a crucial
497 role in cleaning the lens and the cornea, but also permitting the distribution of
498 nutriments and drugs to several ocular structures (58). We showed that clusterin was
499 common to tears, AH and VH. This protein was recently demonstrated in VH as a
500 potential biomarker of the AMD (59) and suspected to be a marker of Alzheimer
501 disease in plasma (60). Cathepsin D, identified in the three different ocular fluids, was
502 recently proposed as biomarker candidate in AH for neovascular AMD also (61).
503 Finding in tears some proteins also present in VH and AH and related to certain

diseases is very promising. Indeed collecting VH and AH requires surgery, which is an invasive process. Using tears to find the same information could be a real advantage for both clinicians and patients. Moreover, it could allow easier and better identification of proteins of interest in certain pathologies.

The comparisons between tears, AH and VH have some limitations. Indeed, two studies of Murphy (26, 27) were done with AH and VH of patients with cataract, because the need to a surgery does not permit to collect complete healthy persons. We do not know how the cataract status could affect the results obtained in these studies, and so the comparisons that we made. However, we are aware that these remarks are somewhat speculative and that more experiments will be needed to support our hypothesis.

5. Conclusion

To conclude, the merge of all tear protein lists has enabled the identification of 1620 proteins (with stringent conditions), suggesting that there is probably room for improvement in tear protein identification. Indeed, the fact that the combination of different studies greatly enhances our knowledge about this fluid clearly demonstrates that additional experiments will be required for the establishment of a reliable proteome for the tears. Through this study, we first complemented the knowledge on tear proteome but we also proposed a global overview of their great potential for clinical research and biomarker discovery.

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530 Figure captions

531

532 **Graphical Abstract. Summary of the experimental workflow followed in this**
533 **study.** GPF: gas-phase fractionation mode; LTQ: linear trap quadrupole; IPG:
534 immobilized pH gradient. DAVID: Database for Annotation, Visualization and
535 Integrated Discovery; STRING: Search Tool for the Retrieval of Interacting
536 Genes/Proteins; KEGG: Kyoto Encyclopedia of Genes and Genomes.

537

538 **Fig 1. Pie charts of molecular function (a), cellular component (b) and**
539 **biological process (c) obtained with the 1337 proteins using DAVID software.**
540 Some subcategories were grouped to reduce the number of GO categories

541

542 **Fig 2. Classification of the 518 enzymes identified in our study.** Data were
543 obtained using the “enzymes classes” available on UniProt. Details of the content of
544 each category could be found in S2 dataset.

545

546 **Fig 3. Glycolysis/Gluconeogenesis pathway obtained using KEGG database**
547 **(25).** Number of genes that are involved in is higher than the number of bold boxes

548 on the figures due to the isoforms of the proteins. It means that one bold box can
549 encompass several proteins. 5.4.2.2: phosphoglucomutase 1, 2; 5.3.1.9: glucose-6-
550 phosphate isomerase; 5.1.3.3: galactose mutarotase; 3.1.3.11: fructose-
551 bisphosphatase 1; 2.7.1.11: phosphofructokinase muscle, platelet, liver types;
552 4.1.2.13: aldolase fructose-bisphosphate C, A; 5.3.1.1: triosephosphate isomerase 1;
553 1.2.1.12: glyceraldehyde-3-phosphate dehydrogenase; 2.7.2.3: phosphoglycerate
554 kinase 1; 5.4.2.11: phosphoglycerate mutase 1; enolase 1; 2.7.1.40: pyruvate kinase
555 (muscle); 1.1.1.27: lactate dehydrogenase A, B; 6.2.1.1: acyl-CoA synthetase short-
556 chain family member 2; 1.2.1.3: aldehyde dehydrogenase 9 family member A1, 7
557 family member A1; 1.2.1.5: aldehyde dehydrogenase 1 family member A3, 3 family
558 member A1; 1.1.1.1: Alcohol dehydrogenase 1B, 1C, 5, 7; 1.1.1.2: aldo-keto
559 reductase family 1 member A1.

560

561 **Fig 4. Coagulation and complement cascades obtained using KEGG database**
562 **(25).** Number of genes that are involved in is higher than the number of bold boxes
563 on the figures due to the isoforms of the proteins. It means that one bold box can
564 encompass several proteins. AT3: serpin family C member 1; A1AT: serpin family A
565 member 1; A2M: alpha-2-macroglobulin; CD59: CD59 molecule; CPB2:
566 carboxypeptidase B2; CR3: fibrinogen gamma chain; CR4: fibrinogen gamma chain;
567 C1INH: serpin family G member; C1qrs: complement C1q B chain, C1q C chain, C1r,
568 C1s; C2: complement factor 2; C3: complement C3; C4: complement C4A; C5:
569 complement C5; C6, 7, 8, 9: complement C6, C7, C8 alpha chain, C8 beta chain, C9;
570 DAF: CD55 molecule; FB: complement factor B; FD: complement factor D; FH:
571 complement factor H; FI: complement factor I; Fibrinogen: fibrinogen gamma chain,
572 beta chain, alpha chain; F2: coagulation factor 2 (thrombin); F5: coagulation factor 5;

573 F12: coagulation factor 12; F13: coagulation factor 13 B chain; HCII: serpin family D
574 member 1; kallikrein: kallikrein B1; kininogen: kininogen 1; PCI: serpin family A
575 member 5; PLG: plasminogen; α2AP: serpin family F member 2.

576

577 **Fig 5. Comparison between the studies of De Souza et al.(15), Zhou et al.(16),**
578 **Aass et al.(14) and us**. The following criteria were applied to the four protein lists:
579 only reviewed proteins, 2 unique peptides and exclusion of keratins. For each
580 category, percentage represents the ratio between the proteins contain in this
581 category and the total number of proteins. Major information is the proteins in
582 common to the four studies (197; 11.2%) and our specific proteins (425; 24.1%).

583

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724

725

726 Supporting information

727 S1 dataset: Proteins identified in experiment 3 (2 peptides, no keratins)

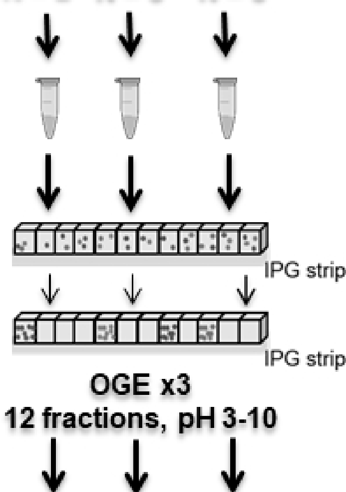
728 S2 dataset: details of the six enzyme categories

729 S3 dataset: Common proteins between the four studies

730 S4 dataset: Proteins specifically identified in this study

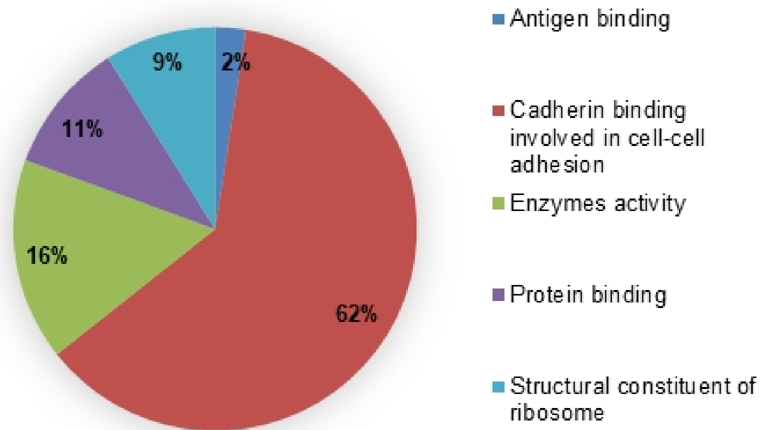
731 S5 dataset: Proteins in common between tears and vitreous humor

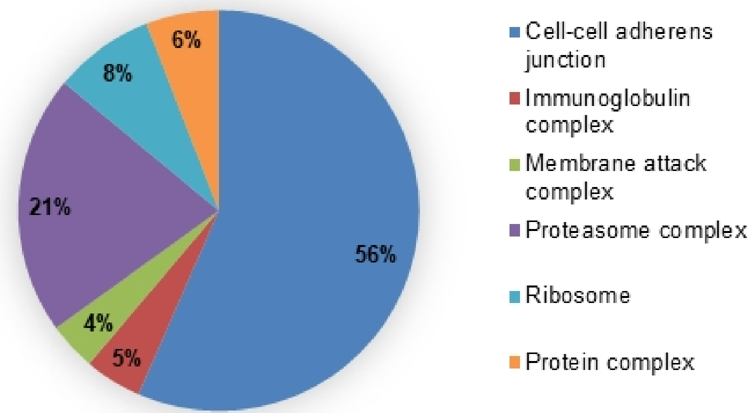
732 S6 dataset: Proteins in common between tears and aqueous humor

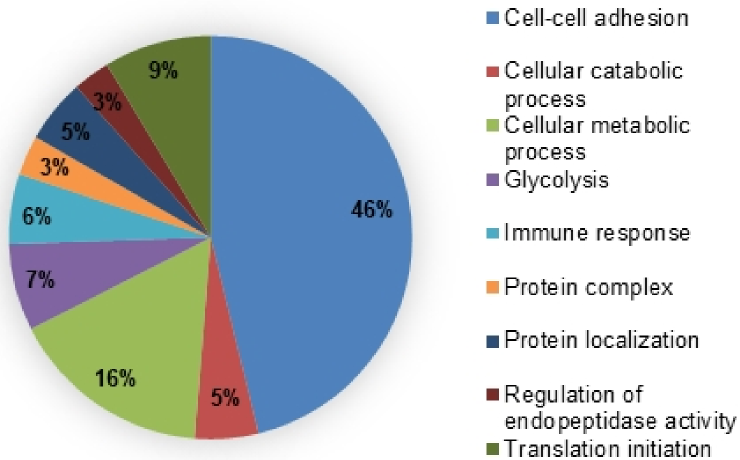


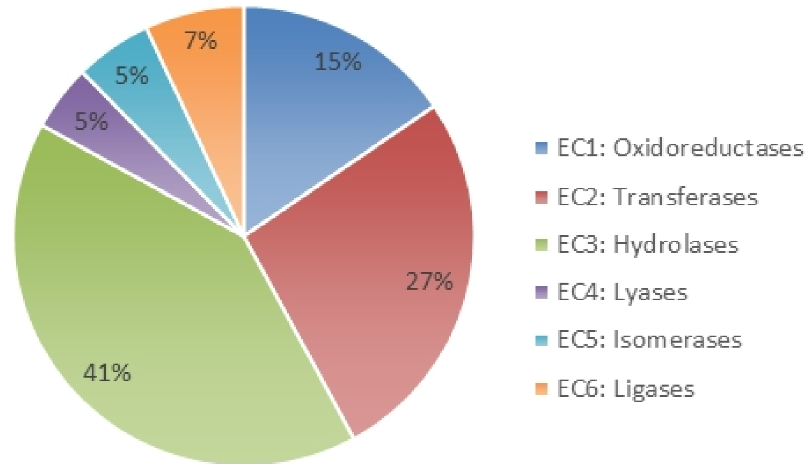
3 different pools of healthy subjects
N = 2
N = 3
N = 3
In-solution trypsin digestion
Experiments 1, 2 and 3
Fractionation of the sample
with Off-Gel Electrophoresis
(OGE)
IPG strip
IPG strip
OGE x3
12 fractions, pH 3-10
Mass spectrometry analysis
(ESI-LTQ Orbitrap Velos)
GPF4 mode injections
MS data processing
using Proteome
Discoverer 2.2
List of identified proteins of the 12 fractions for each experiment
3 individual lists
Final list of proteins (merge of the 3
individual lists)
Bioinformatics analyses

DAVID
STRING
KEGG


Molecular function
Antigen binding
Cadherin binding involved in cell-cell adhesion
Enzymes activity
Protein binding
Structural constituent of ribosome
2%
62%
16%
11%
9%
(a)


Cellular component
Cell-cell adherens junction
Immunoglobulin complex
Membrane attack complex
Proteasome complex
Ribosome
Protein complex
56%
5%
4%
21%
8%
6%
(b)


Biological process
Cell-cell adhesion
Cellular catabolic process
Cellular metabolic process
Glycolysis
Immune response
Protein complex
Protein localization
Regulation of endopeptidase activity
Translation initiation
46%
5%
16%
7%
6%
3%
5%
3%
9%
(c)


Enzyme classes
15%
27%
41%
5%
5%
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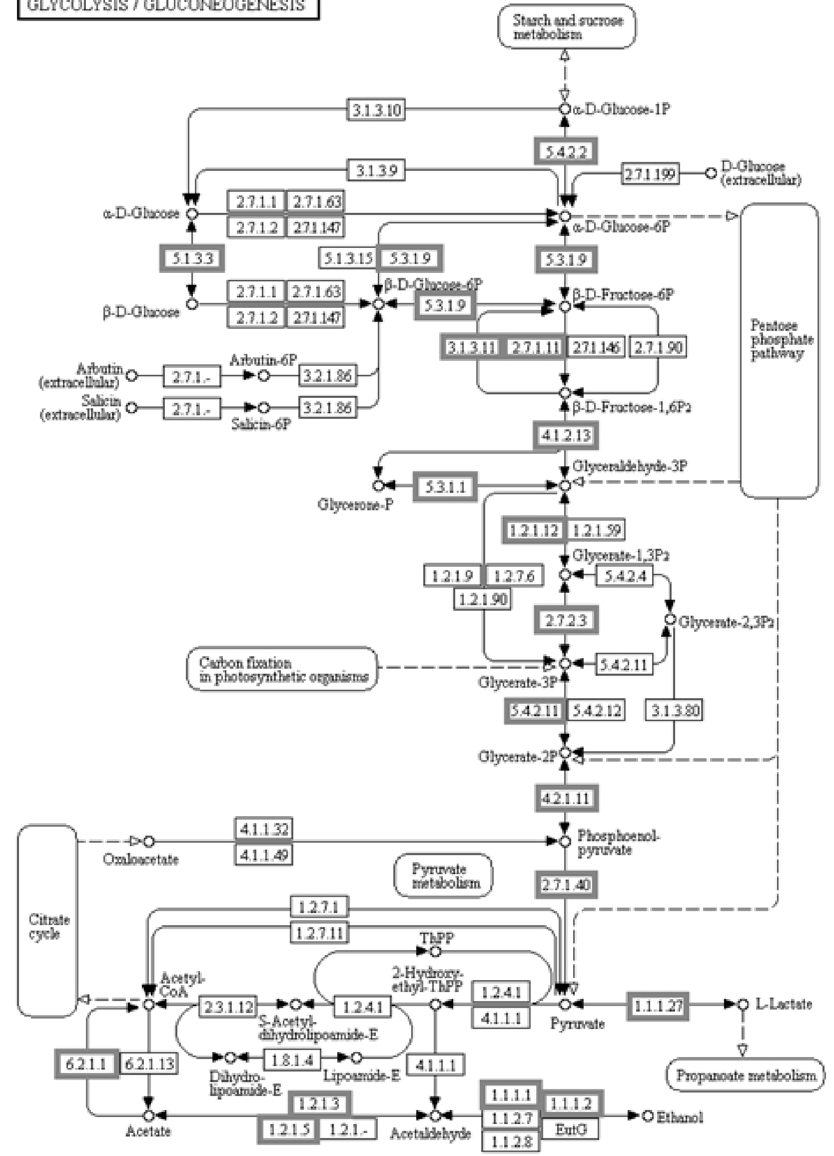
EC1: Oxidoreductases
EC2: Transferases
EC3: Hydrolases
EC4: Lyases
EC5: Isomerases
EC6: Ligases



GLYCOLYSIS / GLUCONEOGENESIS
Starch and sucrose metabolism
α-D-Glucose-1P
3.1.3.10
5.4.2.2
3.1.3.9
2.7.1.199
D-Glucose (extracellular)
α-D-Glucose
2.7.1.1
2.7.1.63
2.7.1.2
2.7.1.147
α-D-Glucose-6P
5.1.3.3
5.1.3.15
5.3.1.9
β-D-Glucose
β-D-Glucose-6P
β-D-Fructose-6P
Pentose phosphate pathway
3.1.3.11
2.7.1.11
2.7.1.146
2.7.1.90
Arbutin (extracellular)
2.7.1.-
Arbutin-6P
3.2.1.86
Salicin (extracellular)
Salicin-6P
β-D-Fructose-1,6P2
4.1.2.13
Glyceraldehyde-3P
5.3.1.1
Glycerone-P
1.2.1.12
1.2.1.59
Glycerate-1,3P2
1.2.1.9
1.2.7.6
5.4.2.4
1.2.1.90
2.7.2.3
Glycerate-2,3P2
Carbon fixation in photosynthetic organisms
5.4.2.11
Glycerate-3P
5.4.2.12
3.1.3.80
Glycerate-2P
4.2.1.11
4.1.1.32
4.1.1.49
Oxaloacetate
Phosphoenol-pyruvate
Citrate cycle
Pyruvate metabolism
2.7.1.40
1.2.7.1
1.2.7.11
ThPP
Acetyl-CoA
2.3.1.12
S-Acetyl-dihydrolipoamide-E
1.2.4.1
2-Hydroxy-ethyl-ThPP
4.1.1.1
Pyruvate
1.1.1.27
L-Lactate
6.2.1.1
6.2.1.13
1.8.1.4
Dihydro-lipoamide-E
Lipoamide-E
Propanoate metabolism
1.2.1.3
1.2.1.5
1.2.1.-
1.1.1.1
1.1.1.2
1.1.2.7
EutG
1.1.2.8
Ethanol
Acetate
Acetaldehyde
00010 12/26/16
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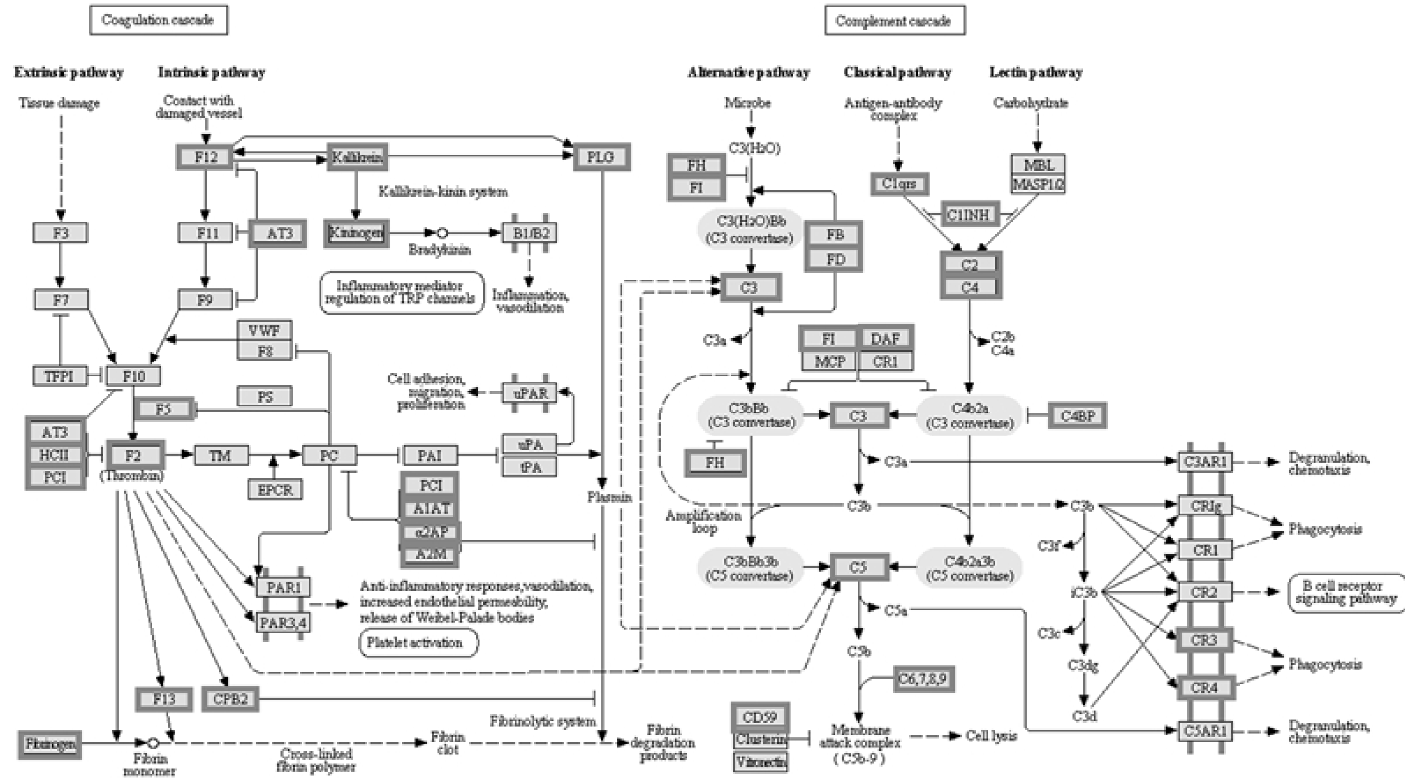
COAGULATION AND COMPLEMENT CASCADES
Coagulation cascade
Complement cascade
Extrinsic pathway
Intrinsic pathway
Alternative pathway
Classical pathway
Lectin pathway
Tissue damage
Contact with damaged vessel
Microbe
Antigen-antibody complex
Carbohydrate
F12
Kallikrein
PLG
Kallikrein-kinin system
Kininogen
Bradykinin
B1/B2
Inflammatory mediator regulation of TRP channels
Inflammation, vasodilation
F3
F11
AT3
F7
F9
VWF
F8
TFPI
F10
PS
F5
HCII
PCI
F2
(Thrombin)
TM
PC
PAI
uPA
tPA
uPAR
Cell adhesion, migration, proliferation
EPCR
A1AT
α2AP
A2M
Plasmin
PAR1
PAR3,4
Anti-inflammatory responses,vasodilation, increased endothelial permeability, release of Weibel-Palade bodies
Platelet activation
F13
CPB2
Fibrinolytic system
Fibrinogen
Fibrin monomer
Cross-linked fibrin polymer
Fibrin clot
Fibrin degradation products
C3(H2O)
FH
FI
C3(H2O)Bb
(C3 convertase)
FB
FD
C1qrs
MBL
MASP1/2
C1INH
C2
C4
C3
C3a
C2b
C4a
DAF
MCP
CR1
C3bBb
C4b2a
C4BP
Amplification loop
C3b
C3AR1
Degranulation, chemotaxis
CRIg
Phagocytosis
C3f
iC3b
CR2
B cell receptor signaling pathway
C3bBb3b
(C5 convertase)
C5
C4b2a3b
C5a
C3c
CR3
C5b
C6,7,8,9
C3dg
CR4
C3d
CD59
Clusterin
Vitronectin
Membrane attack complex
(C5b-9)
Cell lysis
C5AR1
04610 2/10/16
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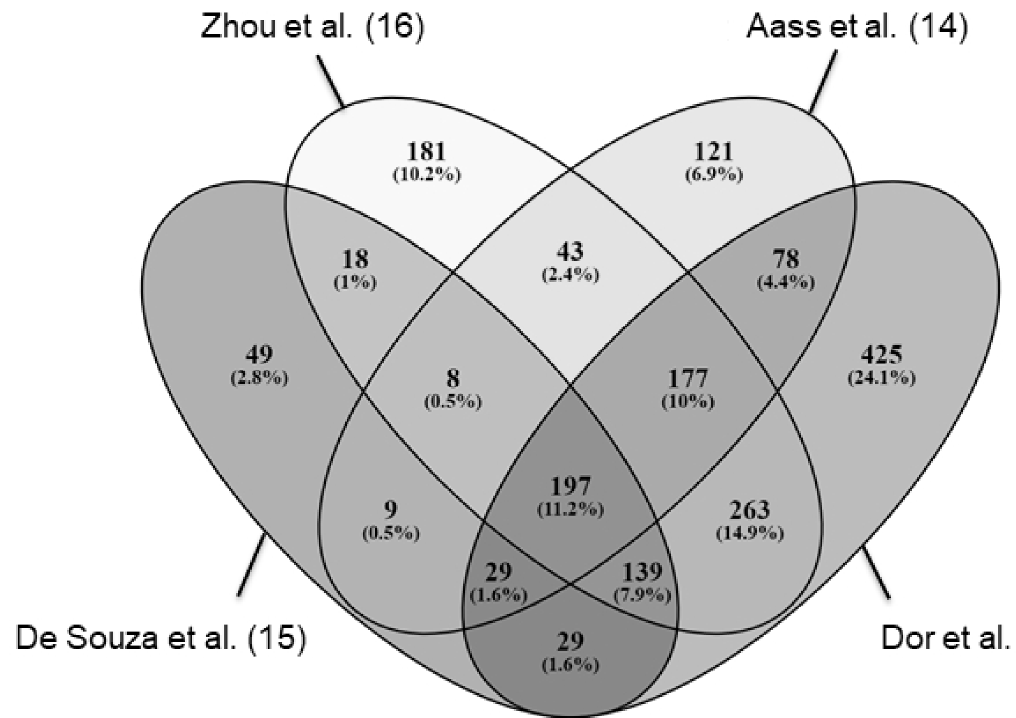
Zhou et al. (16)
Aass et al. (14)
De Souza et al. (15)
Dor et al.
181
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43
(2.4%)
78
(4.4%)
49
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8
(0.5%)
177
(10%)
425
(24.1%)
197
(11.2%)
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