# Gamma-glutamyltransferase-associated glycoprotein patterns in human seminal plasma of normozoospermic men: a new aspect of biomarker heterogeneity

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**Background.** Gamma-glutamyltransferase (GGT) is a well-known laboratory biomarker. In spite of high concentration and the possible biomedical importance of estimating GGT in human seminal plasma (hSP), it has not been widely explored in reproductive physiology. This study aimed to complement existing data on its diversity, previously obtained on seminal extracellular vesicles, by analyzing matched soluble fraction of hSP. The GGT-associated patterns of selected glycoproteins were analyzed in order to establish an adjunct referent parameter for differentiation between known high molecular mass forms of GGT. Getting insight into distinct GGT-associated glycoprotein patterns should contribute to define them together as possible multimarkers.
**Methods.** GGT forms in soluble, membrane-free-fraction isolated form hSP of normozoospermic men were analyzed using gel filtration and lectin blotting using WGA (wheat germ agglutinin) and Con A (concanavalin A).
**Results.** Widely distributed GGT (with two to three partially resolved peaks), which may correspond to high molecular mass aggregates, were detected. GGT-associated patterns of selected glycoproteins (at position of big, medium, and small-GGT) all comprised high molecular mass WGA-reactive smears, but differed in the presence of Con A-reactive glycans, as well as mucin-associated antigens CA19-9 and CA125.
**Conclusions.** GGT contributes to several molecular patterns that differ between the soluble and extracellular vesicle fractions of hSP. Their glycobiochemical heterogeneity is due to difference in the presence of distinct sialylated and mannosylated glycans. Moreover, GGT-associated glycoprotein patterns differentiate between high molecular mass forms of GGT in the soluble fraction of hSP. They hold promise as possible targets for increasing biomarker potential of GGT.

**Key words:** human seminal plasma, gamma-glutamyltransferase, sialylated glycans, CA19-9, CA125

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## INTRODUCTION

Gamma-glutamyltransferase (GGT, EC 2.3.2.2) belongs to a family of highly conserved enzymes associated with the membranes of almost all cells, especially those of secretory/absorptive epithelial tissues[1,2]. It is a heterodimer consisting of a large heavily glycosylated subunit (47-55 kDa) that anchors it to the membrane and a small catalytic subunit (22-25 kDa) (ref.[3,4]). GGT subunits' molecular masses were found to vary due to differences in glycosylation in source tissue, but they are also influenced by isolation methods used for the release of the enzyme from the membrane[5-8]. In addition, higher molecular mass single chain enzyme (120–150 kDa) as well as structurally unrelated antigens (85 kDa – more than 95 kDa) are suggested to represent highly glycosylated non-processed GGT precursors or distinct large subunit itself [9-11].

GGT activity has been implicated in a variety of diseases, and it is a well-known laboratory biomarker[12-14]. Aiming at increasing the specificity and sensitivity of the GGT test, the chromatographic method for separation of GGT forms of distinct molecular mass was established[15]. It pointed out four main GGT forms: big GGT (b-GGT) of 2000 kDa, medium GGT (m-GGT) of 1000 kDa, small GGT (s-GGT) of 250 kDa and free GGT (f-GGT) of 70 kDa[15,16]. The formation and nature of these forms are not fully understood[16]. It is proposed that b-GGT represents an enzyme associated with different membrane structures including extracellular vesicles, but that there is also the possibility of existence of other forms of b-GGT as a result of enzyme involvement in macromolecular protein complexes[17]. In general, aggregation or association of GGT with lipid moieties or (glyco) proteins resulting in its different masses and charges could be responsible for high molecular mass GGT and its pronounced heterogeneity. Elevation of GGT concentration in serum found in different pathologies is associated with alterations in specific fractional GGT pattern which specifically indicated changes in the ratio of b-GGT and s-GGT to f-GGT[13,18-20].

Human seminal plasma (hSP) is a rich source of GGT, but it is not thoroughly investigated and there are no data on its fractional pattern. In contrast to serum GGT, which is a liver enzyme, seminal GGT is assumed to be of prostatic origin[21]. In this regard, seminal GGT could indicate

the secretory status of prostate gland, which is very important in regulation of fertility[22]. The data on associations of GGT and male reproductive health are rare. It was shown that activity of seminal GGT correlates with sperm concentration, but not motility, volume and age[23]. Additionally, it was found to be lower in ejaculates with abnormal liquefaction, and in ejaculates of infertile men with or without leukocytospemia[24,25]. Regarding serum GGT, the available data also indicated a relation to semen quality, that is, its concentration was negatively related to sperm number[26].

Taking advantage of high concentration and the possible biomedical importance of estimation of GGT in hSP, this study was aimed at complementing existing data on its diversity, by analyzing soluble hSP as an unexplored source[27]. The definition of GGT forms in hSP was performed in the membrane-free fraction that match to distinct membranous fraction associated with previously characterised prostasomes, abundant seminal extracellular vesicles[28,29]. GGT is a membrane-bound protein, and its presence in biological fluids could be the result of different processes, suggesting the importance of detailed considerations of analytical perspective of the soluble proteome, in general. Establishment of the pattern of GGT forms in normal hSP is a starting point necessary for monitoring of changes apropos different aspects of reproductive health.

At present, the GGT forms are annotated according to their molecular mass only[15,16]. Aiming to resolve the presumed heterogeneity of high molecular mass GGT forms, underlying patterns of associated glycoproteins were also analyzed. Thus, in response to the general need for the introduction of additional assays to complement the determination of the activity or concentration of different analytes[30], reflection on broad dynamic distribution/redistribution of a particulate molecule of its chemical diversity was considered as an adjunct target. This concept of mapping GGT in the context of specific soluble molecular assemblies has not been applied so far, since GGT is commonly determined in unfractionated body fluids. This represents a new way to take into account the structural properties of a particular molecule to potentially deliver new analytical protocols in the field of biomarkers.

## MATERIALS AND METHODS

### Material

Bovine serum albumin (BSA) and Sepharose 2B were from Sigma (St. Louis, Missouri, USA). Biotinylated plant lectins: Con A (Concanavalin A), WGA (wheat germ agglutinin), and the Elite Vectastain ABC kit were from Vector Laboratories (Burlinghame, California, USA). Sephadex G 200 was from Pharmacia AB (Uppsala, Sweden). SDS-PAGE molecular mass standards (P7717) were from New England Biolabs (Ipswich, Massachusetts, USA). Nitrocellulose membranes, Pierce ECL Western Blotting Substrate were from Thermo Fisher Scientific (Waltham, Massachusetts, USA). The gamma-glutamyltransferase (GGT) colorimetric assay kit was from Bioanalytica (Madrid, Spain). CA19-9 antigen and CA125-antigen were determined using ELSA CA19-9 and ELSA II CA125 assay form CisBio, Biointernational (Codolet, France), respectively.

### Human semen samples

This study was performed on archived leftover, anonymized samples of human semen taken for routine analysis and, since existing human samples were used, it is not considered research on human subjects. It was approved by the institutional ethics committee (No: 02–1462/2) according to the guidelines which conform to the Declaration of Helsinki (as revised in 2013). Sperm parameters were assessed according to the recommended criteria of the World Health Organization (released in 2021), concerning numbers, morphology and motility.

### Separation of soluble and prostasomal fractions from hSP

Two pools (10 samples each) of hSP of normozoospermic men (n) were used. The results of semen analysis were: sperm concentration of 31–117 x $10^6$ sperm/mL (pool 1) and 45–89 x $10^6$ sperm/mL (pool 2); liquefaction over 30 min (in both pools); volume of 3–5 mL (in both pools); progressive motility of 42–73% (pool 1) and 39–68% (pool 2); abnormal morphology of 0–2% (in both pools); leukocyte concentration of 0–0.1 x $10^6$ leukocytes/mL (pool 1) and 0–0.3 x $10^6$ leukocytes/mL (pool 2).

Pooled hSP was first centrifuged at 17000 × *g* for 30 min and the obtained supernatant was then centrifuged at 100000 × *g* for 2 h (Ti 50.2 rotor, k factor=157.7) using an Optima L-90K ultracentrifuge (Beckman Coulter, Indianapolis, CA, USA). The resulting supernatant (S100-N) representing the soluble fraction of hSP was used for downstream analysis. The prostasomes (P100-N) were purified from the matching pellet (100000 × *g*) according to a protocol described previously[31]. They were used for column calibration as such (native) or after treatment with 1% Triton X-100, as previously described[29].

### Precipitation of soluble hSP using polyethylene glycol (PEG)

The precipitation of soluble hSP was performed using polyethylene glycol (PEG) as previously described for the isolation of immunoglobulins and lipids with slight modifications[32,33]. The GGT activity in the precipitate was measured using the GGT colorimetric assay kit according to the manufacturer's instructions on Biosystems A25 (Barcelona, Spain) as described previously[28,29].

### Gel filtration

S100-N (1 mL) was loaded on a Sephadex G-200 (bed volume 35 mL) and/or Sepharose 2B column (bed volume 25 mL) equilibrated and eluted with 0.03 M TRIS-HCl, pH 7.6, containing 0.13 M NaCl. Fractions (1mL) were collected. The elution of GGT, CA19-9 antigen and CA125 antigen was monitored in corresponding fractions, as described above. Both columns were calibrated using native prostasomes, P100-N (100 000 × *g* pellet) which elute at the position of b-GGT and GGT released from detergent-treated P100-N which elute approximately at

the position of f-GGT. The fractional GGT area was calculated using a PeakFit programme (Ver 4.12), (Inpixon HQ, California, USA) to resolve overlapping peaks.

**SDS-PAGE and lectin-blotting**

Gel filtration-separated fractions were resolved on 10% separating gel with 4% stacking gel under denaturing and reducing conditions[34]. The gel was calibrated with SDS-PAGE molecular weight standards (broad range).

The samples were transferred to a nitrocellulose membrane by semi-dry blotting using a Bio-Rad Laboratories Trans-blot SD (Hercules, California, USA). The conditions were: transfer buffer, 0.025 M TRIS containing 0.192 M glycine and 20% methanol, pH 8.3 under a constant current of 1.2 mA/cm$^2$ for 1 hour. The membrane was blocked with 1% bovine serum albumin (BSA) in 0.05 M phosphate buffer saline (PBS), pH 7.2, overnight at 4 °C, and then incubated with biotinylated plant lectin Con A and WGA (0.2 µg/mL in 0.05 M PBS, pH 7.2) for one hour at room temperature. After washing six times in 0.05 M PBS, pH 7.2, avidin/biotinylated horseradish peroxidase (HRPO) from a Vectastain Elite ABC kit (prepared according to the manufacturer's instructions) was added and incubated for 30 minutes at room temperature. The membrane was then rinsed again six times in 0.05 M PBS, pH 7.2, and proteins were visualized using Pierce ECL substrate solution, according to the manufacturer's instructions.

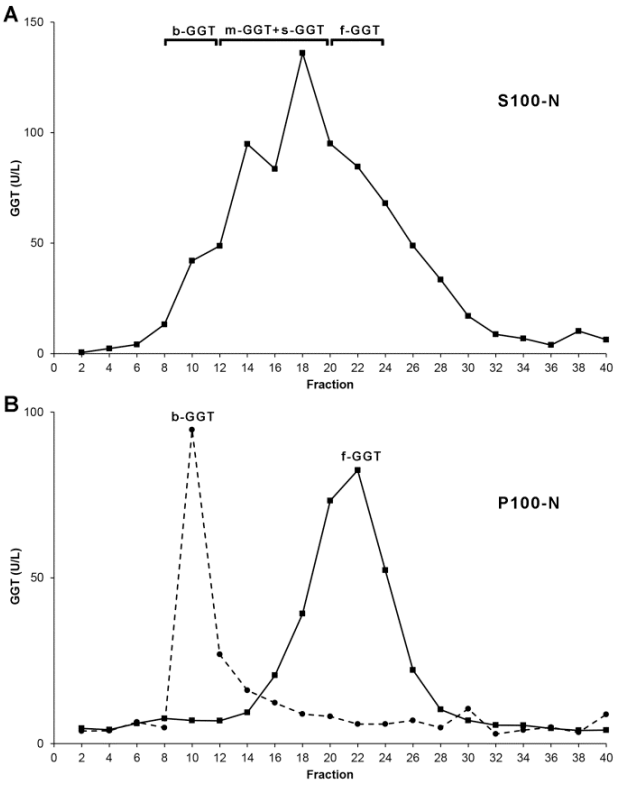


**Fig. 1.** Size-based separation of gamma-glutamyltransferase from soluble hSP fraction.

A. S100-N (100 000 × *g* supernatant) from human seminal plasma (hSP) was separated on Sepharose 2B Fractions of 1 mL were collected. Elution of GGT (gamma-glutamyltransferase) was monitored by measuring enzyme activity (U/L). The expected elution positions of the GGT forms were marked as: b-GGT, big GGT: fractions 8–12; m-GGT, medium GGT and s-GGT, small GGT: fractions 12–20 and f-GGT, free GGT: fractions 20–24.

B. The column was calibrated using native prostasomes, P100-N (100 000 × *g* pellet) eluting at the the position of b-GGT and GGT released from detergent-treated P100-N eluting approximately at the position of f-GGT.

## RESULTS

**Distribution and basic structural properties of GGT from the soluble hSP fraction**

Differential centrifugation of pooled hSP separated GGT activity across both S100-N, soluble (membrane-free) fraction (100 000 x *g* supernatant) and P100-N, extracellular vesicles (EVs) *i.e.*, prostasomes fraction (100 000 x *g* pellet). The GGT concentration in S100-N (of two examined pools) was 2941 U/L and 3082 U/L and GGT concentration in the P100-N were 739 U/L and 2158 U/L.

To resolve if GGT activity in S100-N is due to its association with remnants of prostasomes or its complexes with immunoglobulins, it was subjected to precipitation with PEG. The results obtained indicated that GGT was not associated with these molecules, since 2.5–3.6% of total GGT activity was precipitated.

Initial insight into GGT forms in S100-N was obtained using gel filtration on Sephadex G-200 chosen to include and separate free enzyme (app. 70 kDa) and exclude (concentrate) higher molecular mass forms (> 200 kDa). The elution profiles obtained showed that the major GGT peak was mostly eluted in the void volume, whereas a minor one entered the column at its tailing edge (data not shown).

**Size-based separation of GGT forms from soluble hSP fraction**

Based on the GGT elution profile on Sephadex G-200, further separation of high molecular mass forms was attempted on Sepharose 2B, having a more appropriate fractionation range. Widely distributed enzyme (with two to three partially resolved peaks depending on the pool used) eluting mainly after b-GGT was observed (Fig. 1). It suggested high molecular mass aggregates/complexes that may be principally related to the positions of m-GGT and s-GGT. Specifically, the share of eluted GGT calculated according to the total peak area for two pools of S100-N was: at the position of b-GGT (11.5 and 13.8%), m-GGT and s-GGT (56 and 65.1%) and f-GGT (23.4 and 30.2%).

**GGT-associated patterns of sialylated and mannosylated glycoproteins from soluble hSP fraction**

When selected fractions resolved by gel filtration of soluble hSP were subjected to electrophoresis and lectin-blotted, distinctive underlying glycoprotein patterns associated with GGT forms of different molecular mass were observed (Fig. 2). In general, WGA-reactivity was

distributed across high molecular masses regions and it overlapped distribution of GGT activity. It was associated with constitutively present (in all examined pools of S100-N) high molecular mass smear in stacking gel and at the border of stacking and separating gel (Fig. 2A). It differed strikingly from that of prostasomes where reactivity was low (Fig. 2C) and could occasionally be associated with distinct lower molecular mass Con A-reactive proteins.

Regarding Con A-reactivity in the region that overlaps GGT activity, Con A bound constitutively present 150 kDa and 200 kDa glycoprotein bands (Fig 2B), and occasionally present 66 kDa and 50 kDa glycoprotein bands. In the low molecular mass region, overlapping the tailing edge of the peak of GGT activity, Con A-reactivity was predominant and associated with numerous bands entering the gel (Fig. 2B, fractions 18–24). They had a ladder appearance suggesting considerable aggregation that significantly influenced variation in the presence and distribution of particular Con A-reactive bands between the examined pools of S100-N. However, two differently abundant 50 kDa and 45 kDa Con A-reactive bands were constitutively detected (in all examined pools of S100-N).

As for prostasome fraction, a cluster of abundant Con A-reactive bands at the position of b-GGT was clearly present (Fig. 2D, fractions 8-10). It was, obviously, concentrated upon pelleting hSP, that is, it was completely depleted from S100-N.

Together, high molecular mass GGT forms can be annotated with high molecular mass WGA-reactive glycans. They were clearly different from particulate high molecular mass b-GGT which can be annotated with low molecular mass Con A-reactive glycoproteins.

Based on the electrophoretic appearance of high molecular mass WGA-reactive smears being reminiscent of mucins, their presence was checked by determination of soluble mucin 1-associated CA19-9 antigen and mucin 16-associated CA125 antigen in the gel filtration resolved fractions (Fig. 3). The elution profile of the CA19-9 antigen revealed that it only partially overlapped b-GGT and mainly m-GGT. As for the CA125 antigen, it was low abundant to easily follow but two broad peaks at positions overlapping b-GGT and s-GGT could be deduced.

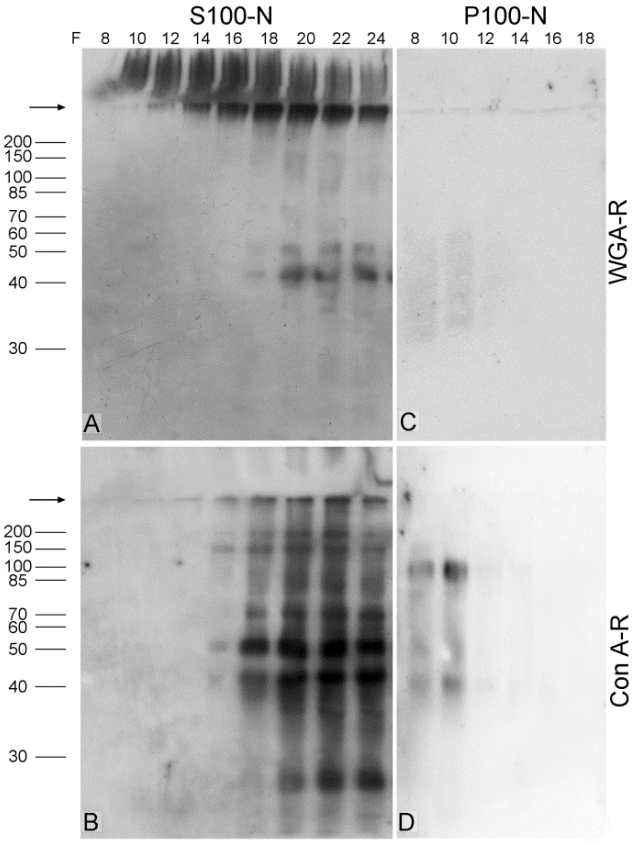


**Fig. 2.** Gamma-glutamyltransferase-associated glycoprotein patterns of soluble hSP.
Representative gel filtration (Fig. 1A) resolved fractions of S100-N (A, B) and P100-N (C, D) were subjected to 10% SDS-PAGE, transferred onto a membrane and subjected to lectin-blot using WGA (A, C) and Con A (B, D). The numbers indicate the position of the molecular mass standards (kDa). The arrow indicates the border of stacking and separating gels. F-fraction. S100-N, soluble fraction of human seminal plasma (hSP); P100-N (100 000 × *g* pellet). WGA-R: wheat germ agglutinin-reactive; Con A-R: concanavalin A-reactive.

## DISCUSSION

Membrane-associated molecules, comprising many known biomarkers among them GGT, may be assessed in both particulate/membrane fraction and the soluble fraction. As for hSP, its composition is affected by proteolytic processes, and there may be different mechanisms responsible for the decomposition of GGT-containing membranes or the release of GGT. Depending on the mode of release, hydrophilic species (with no membrane domain) and hydrophobic species prone to autoaggregation or complexes with lipid moieties could be present as high molecular mass forms[5,35]. Moreover, different tissues which shape hSP composition can secrete structurally different GGT molecules[36-38].

It was previously reported that f-GGT is most abundant in soluble fractions of serum of healthy adults, following s-GGT and minor m-GGT and b-GGT (ref.[39]). In general, f-GGT could only be recovered from ultracentrifugation of human serum/sputum/bile supernatants[10,16,39]. The results obtained here indicated that GGT at the positions of m-GGT and s-GGT represented the main part of the enzyme activity in hSP. A broad elution pattern of high molecular mass GGT was observed, which may suggest that most of the released enzyme is not devoid of the hydrophobic domain. Since it is responsible for interaction between GGT molecules themselves or with different carriers, this may result in different ratio of particular GGT forms observed in hSP (ref.[5,35]). Compared to other sources/biological fluids, the possibility of m-GGT and s-GGT of different origins might also exist. This could be in agreement with data suggesting heterogeneity of b-GGT entities, as sources for generation of m-GGT and s-GGT by their progressive modification[16]. However, the underlying mechanism is not known[16].

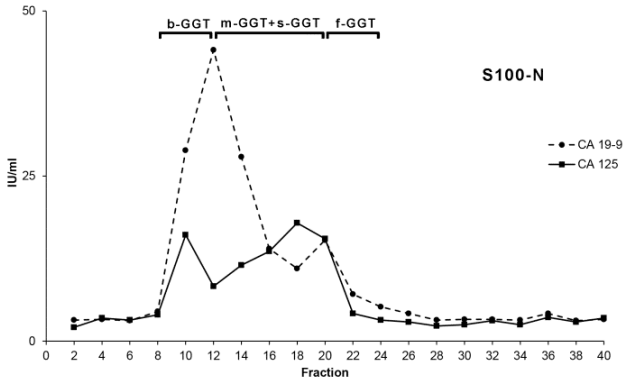


**Fig. 3.** Size-based separation of mucins-related antigens in soluble hSP.
S100-N (100 000 × *g* supernatant) from human seminal plasma (hSP) was separated on Sepharose 2B. Fractions of 1 mL were collected. Elution of CA19-9 and CA125 were monitored by measuring their concentration (IU/L). The expected elution position of the gamma-glutamyltransferase (GGT) forms was marked as: b-GGT, big GGT: fractions 8–10; m-GGT, medium GGT and s-GGT, small GGT: fractions 12–20 and f-GGT, free GGT: fractions 22–24.

Thus, it was previously proposed that a difference between b-GGT in particulate/membrane fraction and b-GGT in the soluble fraction could be due to different origin/composition (secretory vesicles *vs*. specific granules) or subsequent modification in some pathophysiological conditions such as inflammation[10,40,41]. The results described here suggested that b-GGT in soluble fraction (100 000 x *g* supernatant) and b-GGT associated with prostasomes (100 000 x *g* pellet) are different *i.e.,* that there is heterogeneity of this form in unfractionated hSP. These observations point to the general importance of resolving the issue of difference in b-GGT between the particulate fraction and the soluble fraction. Regarding human serum as an analyte, depletion of microvesicles and isolation of the true exosomal fraction (pandan to prostasomal fraction) or overlapping soluble complexes of the same size should be advantageous for following specific changes regarding GGT or any selected marker[42]. In general, separation of b-GGT associated with exosomes is preferable when considering the application of exosomes as a liquid biopsy in clinical diagnosis[43,44]. Moreover, this is necessary when considering GGT-associated molecular patterns as an adjunct parameter for differentiation between GGT forms or as a distinct parameter for multimarker analysis.

In this study, the underlying patterns of GGT-associated sialylated and mannosylated glycoproteins were also analyzed in order to annotate them with size-resolved GGT forms. This choice was based on existing data that showed their association with prostasomes *i.e.* b-GGT itself[29]. Specifically, data on molecular pattern of prostasomes from normozoospermic man indicated GGT/Con A-reactive glycans as common constituents. However, compared to the soluble subproteome, contributing Con A-reactive bands were strikingly different (with respect to molecular masses) and much more abundant, whereas high molecular mass WGA-reactive glycans were low abundant[28]. The results obtained on soluble hSP indicated that GGT-associated patterns of selected soluble glycoproteins (at position of big- , medium- and small-GGT) all comprised high molecular mass WGA-reactive smears, but differing in the presence of Con A-reactive glycans (only partially at position of s-GGT), as well as mucin-associated antigens CA19-9 (predominantly at position of m-GGT) and CA125 (at position of b-GGT and s-GGT).

In general, GGT distribution/concentration was inversely correlated with that of CA19-9 antigen. Thus, the results obtained might point out GGT as part of mucins interactome previously shown to comprise glutathione (GSH) and GSH-related enzymes[45]. GGT plays key roles in glutathione (GSH) homeostasis by breaking down extracellular GSH and also initiates the metabolism of glutathione S-conjugates as a product of anti-oxidative processes[46]. The meaning of putative GGT-mucins complexes is to be resolve, in light of finding that interaction of glutathione S-transferase with mucins affects their rheological properties. It is suggested that this may play a functional modulatory role, in terms of inhibiting or enhancing the activity of associated enzymes[45]. So far, in an unrelated examination, possible use of the ratio of CA19-9 to total serum GGT is suggested for the prediction of recurrence and long-term prognosis in patients with pancreatic head carcinoma and cholangiocarcinoma[47,48].

Taken together, the results obtained allowed a new perception of the heterogeneity of existing GGT forms and suggested different molecular patterns as possible targets for evaluating their biomarker potential. To accomplish this goal, in general, combined detection of GGT activity and distinct immunoreactivity of different user-selected classes of molecules in specified proteomes should be used as a reference. This poses a challenge with respect to the technical design of the assay, since molecular activity must be preserved during the capture and detection procedures. Biomarker discovery is a multistep process, and this study initiates the discovery phase, as the first phase that precedes the validation of differences in glycobiochemical properties of GGT-associated molecular patterns, which may be advantageous over analysis of unfractionated GGT.

## CONCLUSION

GGT in unfractionated hSP (soluble membrane-free fraction and extracellular vesicles fraction) is presented in several molecular assemblies. Related GGT-associated molecular patterns were established by describing the main contributing glycoproteins of the soluble hSP proteome of normozoospermic man, as an adjunct parameter in addition to the molecular mass currently used to differentiate between previously established GGT forms. Principally, the distribution and compositions of these assemblies in terms of one particular contributing / associated molecule could be due to its structure, molecules

relevant for its traffic or interacting ligands / partners. In this way, the recognitive properties (as new determinant) of a heterogeneous analyte such as GGT can be approached by their mapping in the context of native proteome and should be taken into consideration concerning the changes in enzyme concentration occurring under different pathological conditions. Getting insight into GGT and distinct GGT-associated glycoprotein patterns and their disparity from referent one, merits consideration in terms of improving its biomarker potential and might point to possible role in reproductive physiology.

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**Author contributions:** MJ: designed and coordinated the study; TJ, JDL, SG: contributed to the study design; TJ, JDL, SG, NM: performed the experiments; TJ, JDL, SG, NM, LjH, MJ: contributed to the interpretation and in writing the manuscript. All authors reviewed and edited the manuscript and approved the final version of the manuscript.

**Conflict of interest statement:** The authors state that there are no conflicts of interest with respect to the publication of this article.

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