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Serum Protein Profiling of Patients at Risk to Develop Gastric Disease Based on a DSC Test

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Simple Summary

Early gastric cancer can be difficult to detect, because of the lack of specific initial symptoms. We analysed at protein level serum of patients at risk for gastric cancer or advanced atrophy, according to our DSC test developed in a medium-risk population (test score 2, S2). An increase in beta-2-microglobulin and a decrease in pregnancy zone protein resulted hallmarks in serum S2. These findings may be translationally significant in helping to guide clinicians in developing a screening strategy.

Abstract

Background: At present, the gold standard for gastric cancer (GC) confirmation relies mostly on histopathology, an invasive procedure. Noninvasive detection methods using serum for large-scale screening maybe useful for the early diagnosis of GC. *Helicobacter pylori* (HP) infection and chronic atrophic gastritis are major GC risk factors. We recently developed a noninvasive test called DSC test—, based on patient's age, sex, their serum PGI and PGII, anti-HP immunoglobulin (IgG), and gastrin G17 levels –predicting GC risk as low (score 0, S0) or high (score 2, S2). The comparative investigation at serum protein level of the two different patient groups detected by our DCS test (S0 and S2) may undoubtedly help to identify gastric disease-dependent proteins, resulting from bacterial infection or gastric mucosa inflammation, as well as get better insight the molecular scenario associated to pre-cancerous conditions. **Methods:** Mass spectrometry-based protein analysis of tryptically digested proteins was performed, followed by univariate statistical analysis for the different DSC groups from two cohort of patients (exploratory and validation). Significantly differentially abundant proteins differing more than 1.5-fold between groups were selected and validated, and their putative role(s) in gastritis and GC discussed. **Results:** We present data of comparative protein analysis of sera from patients at high risk to develop gastric cancer or advanced atrophy, depending on their DSC score. In particular, we used untargeted liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based proteomics as semiquantitative method to profile proteins specifically associated with score 2 in sera of 80 patients. In both the exploratory and the validation cohorts, four proteins (beta-2-microglobulin, EGF-containing fibulin-like extracellular matrix protein 1, complement factor D, and Cystatin-C) were more abundant, while two (sex hormone-binding globulin and pregnancy zone protein) were less abundant in sera of S2 individuals ($|\text{fold change}| \geq 0.6$, $p < 0.05$, *t*-test). The higher presence of beta-2-microglobulin (B2M) and the lower content of pregnancy zone protein (PZP) in S2 sera were validated by immunoblotting. **Conclusions:** This study identified a proteomic signature differentially associated to sera of patients with a different risk to develop GC/advanced atrophy according to our DSC test. The protein marker panels

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presented in this work will contribute to improve GC diagnostics, once they have been transferred from a research result to a practical tool.

Keywords: biomarker; gastric cancer; gastritis; mass spectrometry; proteomics; screening; beta-2-microglobulin

1. Introduction

Gastric cancer (GC) is a highly aggressive and complex malignancy representing a global health problem: it is the fifth most commonly diagnosed cancer and the fifth leading cause of cancer-related death worldwide in 2022 [1].

GC is classified into two main histological subtypes, with distinct characteristics: diffuse (poorly cohesive cells, including signet ring cells and linitis plastic) and intestinal (glandular growth pattern), this last representing the large majority of GCs worldwide[2,3]. Diffuse GC is largely driven by mutations in pathways regulating cell-extracellular matrix interactions, while intestinal GC typically arises from chronic atrophic gastritis, primarily triggered by *Helicobacter pylori* (HP), a class I carcinogen, through a succession of a well-delineated sequential series of precancerous steps, known as the "Correa cascade", the gastric advanced atrophy predisposing to intestinal metaplasia, dysplasia and, finally, adenocarcinoma [4,5]. While HP and premalignant lesions are known risk factors, most affected individuals do not develop gastric cancer. Therefore, clear guidelines on who to screen, when to start, and which methods to use are still lacking.

The etiology of GC is notably multifactorial, its development arising from an interplay of several risk factors: several modifiable (HP, obesity, diet, smoking and alcohol consumption) and nonmodifiable (gene susceptibility, age, family history and male gender) factors may interact and impact GC risk/progression, thus suggesting potential preventive strategies [6].

At present, primary (HP test-and-treat) and secondary (upper gastrointestinal endoscopy and histology) preventive strategies for GC are available. In particular, the gold standard for screening asymptomatic individuals or surveillance of high-risk subgroups for GC is upper endoscopy with biopsy, despite its invasive nature, which detects early-stage cancer or premalignant gastric lesions (atrophy, dysplasia or intestinal metaplasia) [7]. In this context, the endoscopic management of gastric precancerous lesions has been recently updated in the MAPS II guidelines [8].

In Western countries, GC is frequently diagnosed at an advanced stage as incurable/metastatic disease with a poor prognosis, so that its early diagnosis becomes crucial in the improvement of therapy efficacy and long-term patient survival [9]. In recent years, evidence has strongly suggested that despite the availability of effective diagnostic tools for GC, there is no 'one-size-fits-all' screening method. Strategies must therefore be adapted to regional contexts, tailoring approaches for high-risk populations versus those in lower-risk areas [10–13]. At present, national population-wide screening programs for GC have been implemented in only few East Asian countries, at high prevalence of GC [14]. While in Western Europe, at low prevalence of GC, routine endoscopic screening for GC is currently not suitable for large-scale screening protocols, due to the low incidence of GC and the high cost of the procedure [15]. Most Western nations, including Italy, recommend a personalized or risk-stratified screening strategy, in which endoscopy is reserved exclusively for high-risk subjects [13]. In this context, current efforts to identify high-risk populations include several rigorous testing protocols. The clinical utility of serum-based markers like CEA, CA 19-9, and CA 72-4 remains limited in screening and early diagnosis in GC, as they do not meet the required thresholds for sensitivity and specificity [14]. However, serology may have an important role in GC prevention, in particular with the so-called GastroPanel® test and its simultaneous detection of pepsinogen type I and II (PGI and PGII), PGI/PGII ratio, gastrin-17 (G17) and anti-HP IgG antibodies. This test has been originally conceived to diagnose diffuse atrophic gastritis [16]. However, since the 1990s, these markers have been also utilized to identify patients at high risk for GC (characterized by reduced PGI and PGI/PGII

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values). Although their sensitivity remains low, they help identify candidates who may require an upper gastrointestinal endoscopy [17–20].

Recently, we have developed a model—called DSC test—, based on patient's age, sex, their serum PGI and PGII, anti-HP immunoglobulin (IgG), and G17 levels, to early individuate patients at higher risk of developing GC in a medium-risk GC Italian area [21] . The DSC model grouped patients based on GC risk classes into negative (score 0), neutral, and positive (score 2) results, with an overall 74.66% accuracy rate and a good specificity (around 75%) for GC diagnosis. In this work, we employed liquid chromatography mass spectrometry to identify the serum proteome signature in individuals with a positive DSC test, which may help us to get better insight the molecular phenotypes of our DSC-selected subjects at risk for GC and, indirectly, improve the diagnostic utility of our test.

2. Results

2.1. Demographic Characteristics and Hematological Parameters

Table 1 shows the characteristics of patients in the discovery (n=20) and validation (n=80) cohorts. None of the laboratory-tested parameters (HP IgG, PGI, PGII, PGI/PGII, G-17) differed significantly between the two cohorts. The cohorts were also comparable in gender distribution (~30% male). However, a statistically significant difference was observed in age distribution: individuals aged 50–70 years were more prevalent in the discovery cohort (40%), whereas subjects aged <50 years were more frequent in the validation cohort (51%) than in the discovery one (25%) (p-test = 0.045).

Table 1. Demographic and serological data in the discovery and validation cohorts.

	Discovery cohort	**Validation cohort**	
	n=20	n=80	p-value
Class age <50y (%)	5 (25)	41 (51)	0.045
50-70y (%)	8 (40)	14 (18)	0.039
>70y (%)	7 (35)	25 (31)	0.792
Gender, male (%)	7 (35)	26 (33)	1
Helicobacter pylori IgG > 30 EIU (%)	3 (16)	5 (6)	0.178
PGI ng/ml, median (IQR)	93 (53-126)	90 (75-103)	0.73
PGII ng/ml, median (IQR)	11 (10-16)	11 (8-15)	0.476
PGR, median (IQR)	8 (6-12)	10 (8-13)	0.134
G-17 pmol/l, median (IQR)	5 (3-10)	4 (3-10)	0.683

EIU, Enzyme Immune Units; PG I, pepsinogen I; PG II, pepsinogen II; PGR, pepsinogen I to pepsinogen II ratio; G-17, gastrin-17.

2.2. Plasma Protein Profiling

In Part I of the study (discovery phase), serum samples from patients S0 and S2 were analyzed using label-free LC-MS/MS. Based on ($|FC(log2)|\geq 0.6$, p <0.05, t-test), a total of 14 differentially abundant proteins were identified (Table 2; Supplementary Table 1; Figure 1c). In particular, quantitative comparisons between the S2 and S0 groups identified 7 proteins that were significantly more abundant in the serum of patients at high-risk for gastric disease, and 7 proteins that were more abundant in the serum of individuals of low-risk (t-test, p-value <0.05).

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Table 2. Differences in the abundance of proteins in the serum of individuals by DSC score (S2 *vs.* S0) in the discovery cohort (p <0.05).

Acc.	Description	Gene Symbol	Score Sequest HT:	FC (log2): (S2) / (S0)	FC p-value: (S2) / (S0)	FC adj. p-value: (S2) / (S0)	Ab. S0	Ab. S2	Ab. CV [%]: S0	Ab. CV [%]: S2
More abundant in S2 (n = 7)										
P06331	Immunoglobulin heavy variable 4-34	IGHV4-34	361.90	1.4	0.0398	0.4079	55.9	144.1	71.50	62.02
Q12805	EGF-containing fibulin-like extracellular matrix protein 1°	EFEMP1	25.97	1.2	0.0023	0.1314	59.5	140.5	56.23	56.98
P61769	Beta-2-microglobulin°	B2M	115.81	1.2	5.05E-05	0.0068	61.4	138.6	37.54	17.51
P00746	Complement factor D°	CFD	70.48	1.1	4.58E-05	0.0068	64.7	135.3	37.40	25.69
P01034	Cystatin-C°	CST3	8.60	1.0	0.0351	0.4079	68.2	131.8	58.68	38.43
A0A075B6S5	Immunoglobulin kappa variable 1-27	IGKV1-27	117.64	0.9	0.0100	0.2692	70.7	129.3	51.34	43.63
P1842860709	Lipopolysaccharide-binding protein	LBP	157.77	0.8	0.0194	0.3275	72.9	127.1	33.98	39.33
More abundant in S0 (n = 7)										
Q9UGM5	Fetuin-B	FETUB	148.12	-0.6	0.0024	0.1314	119.3	80.7	29.85	24.86
P03951	Coagulation factor XI	F11	12.58	-0.7	0.0023	0.1314	124.3	75.7	22.77	33.13
P27169	Serum paraoxonase/arylesterase 1	PON1	1020.78	-0.7	0.0048	0.2000	125.1	74.9	25.30	39.52
P35858	Insulin-like growth factor-binding protein complex acid labile subunit	IGFALS	564.07	-0.8	3.87E-05	0.0067	127.7	72.3	20.08	27.34
A5A3E0	POTE ankyrin domain family member F	POTEF	17.73	-0.9	0.0361	0.4078	128.8	71.2	32.03	40.91
P04278	Sex hormone-binding globulin°	SHBG	309.79	-1.1	0.0092	0.2619	137.7	62.3	69.30	58.49
P20742	Pregnancy zone protein°	PZP	2448.02	-2.2	0.0129	0.2739	164.3	35.7	171.08	103.30

difference in protein abundance confirmed in the validation cohort (Table 3); Acc., UniProt Knowledgebase accession number; Ab., abundance; FC, fold change (LFQ intensity ratio between S2 and S0).

In Part II of the study (validation phase), label-free LC-MS/MS analysis identified a total of 21 proteins differing in abundance —based on FC and p-values— between the S2 and S0 groups in our validation cohort (Table 3; Supplementary Table 2; Figure 1d).

Table 3. Differences in the abundance of proteins in the serum of individuals by DSC score (S2 *vs* S0) in the validation cohort (p <0.05).

Acc.	Description	Gene Symbol	Score Sequest HT:	FC (log2): (S2) / (S0)	FC p-value: (S2) / (S0)	FC adj. p-value: (S2) / (S0)	Ab. S0	Ab. S2	Ab. CV [%]: S0	Ab. CV [%]: S2
More abundant in S2 (n = 13)										
P02675	Fibrinogen beta chain	FGB	23.61	1.35	0.00061	0.008814037	56.4	143.6	184.45	71.58
P08519	Apolipoprotein(a)	LPA	2876.20	1.32	0.0400	0.182228738	57.3	142.7	125.62	102.8
P07998	Ribonuclease pancreatic	RNASE1	30.74	0.95	0.0004	0.005736136	68.2	131.8	77.57	62.48
P02679	Fibrinogen gamma chain	FGG	47.87	0.86	0.0038	0.038226774	70.9	129.1	128.83	48.20
P00746	Complement factor D	CFD	421.54	0.84	3.1088E-09	9.80828E-07	71.8	128.2	36.02	34.98
Q9NQ79	Cartilage acidic protein 1	CRTAC1	114.32	0.71	3.27164E-05	0.001056618	76.0	124.0	55.67	45.09

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P61769	Beta-2-microglobulin	B2M	493.31	0.70	1.19552E-08	2.075E-06	76.1	123.9	28.39	34.61
Q12805	EGF-containing fibulin-like extracellular matrix protein 1	EFEMP1	250.78	0.68	1.17896E-06	0.000106275	76.8	123.2	50.14	32.62
P0DJI9	Serum amyloid A-2 protein	SAA2	171.40	0.64	0.0253	0.137709754	78.2	121.8	387.24	117.58
Q92954	Proteoglycan 4	PRG4	231.91	0.63	0.0006	0.009371515	78.4	121.6	57.05	36.88
O75460	Serine/threonine-protein kinase/endoribonuclease IRE1	ERN1	376.58	0.59	0.0009	0.012633807	79.7	120.3	47.32	37.62
Q02985	Complement factor H-related protein 3	CFHR3	746.82	0.58	0.0392	0.180446882	80.2	119.8	63.18	58.96
P01034	Cystatin-C	CST3	288.43	0.58	0.0012	0.014245287	80.3	119.7	37.17	42.23
More abundant in S0 (n = 8)										
P14151	L-selectin	SELL	21.06	-0.55	0.0018	0.020011824	118.7	81.3	39.87	38.02
A0A0G2JS06	Immunoglobulin lambda variable 5-39	IGLV5-39	44.61	-0.62	0.0039	0.039217887	121.2	78.8	59.70	59.55
P01705	Immunoglobulin lambda variable 2-23	IGLV2-23	86.94	-0.64	0.0431	0.191508477	121.8	78.2	52.82	67.65
P04211	Immunoglobulin lambda variable 7-43	IGLV7-43	692.58	-0.81	0.0004	0.006648553	127.2	72.8	46.28	48.86
P01704	Immunoglobulin lambda variable 2-14	IGLV2-14	95.38	-0.84	0.0027	0.029178574	128.3	71.7	61.53	57.76
P04278	Sex hormone-binding globulin°	SHBG	2369.20	-0.92	0.0015	0.018350946	130.8	69.2	105.91	60.86
P20742	Pregnancy zone protein°	PZP	19422	-0.95	0.0010	0.012633807	131.6	68.4	234.45	107.22
P09172	Dopamine beta-hydroxylase	DBH	188.40	-1.28	0.0033	0.034356657	141.8	58.2	69.46	95.01

Acc., UniProt Knowledgebase accession number; Ab., abundance; FC, fold change (LFQ intensity ratio between S2 and S0).

Principal component analysis of the differently-abundant proteins was able to discriminate S2 and S0 groups, albeit with a marked biological variability from patient to patient, in both the discovery and validations cohorts (Figure 1, c and d).

The following six proteins were confirmed as being differentially abundant in S2 versus S0 groups in both discovery and validation cohorts: complement factor D (CFD), beta-2-microglobulin (B2M), EGF-containing fibulin-like extracellular matrix protein 1 (EFEMP1), and cystatin-C (CST3), which were more abundant in S2 groups; sex hormone-binding globulin (SHBG), and pregnancy zone protein (PZP), which were less abundant in S2 individuals.

Differences in the abundance of B2M and PZP were examined by immunoblotting serum proteins from the S0 and S2 groups of the validation cohort (pools of 51 and 29 samples, respectively). The higher level of B2M as well as the lower level of PZP was confirmed in S2 serum compared to S0 (Figure 2).

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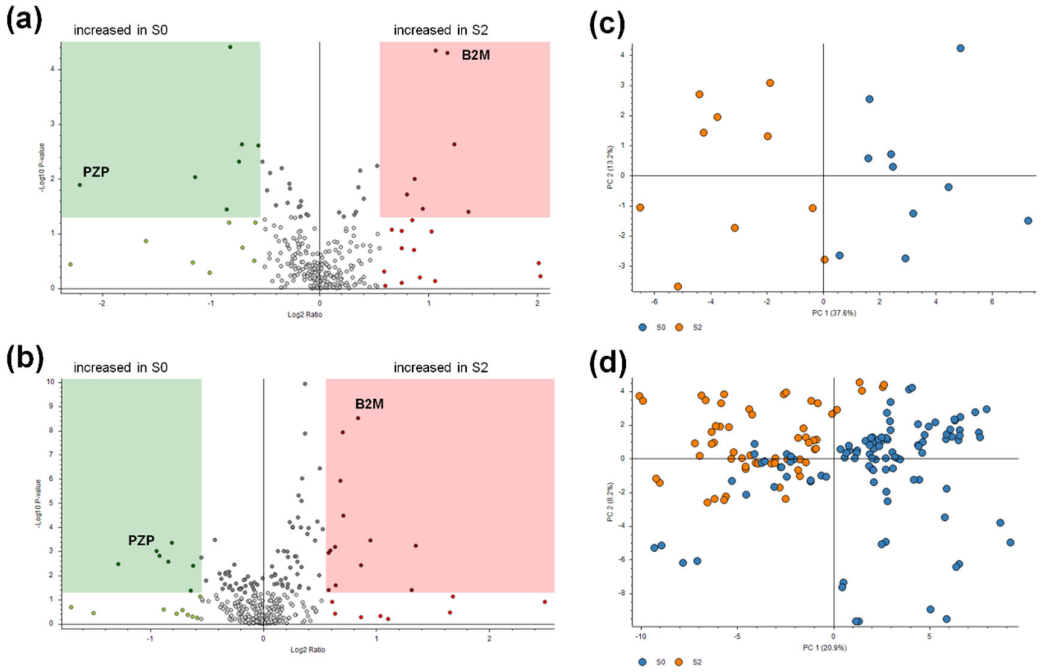

Figure 1. Volcano plot showing proteomic data by DSC score status (S2 *vs*. S0) in the discovery and exploratory cohorts (a, b). Principal component analysis (PCA) of the proteins identified by LC-MS/MS analysis (normalized protein abundances) as being differently expressed by the DSC score groups in the discovery and exploratory cohorts (c, d). In both Volcano plots (a, b), Log2 transformed abundance ratios for each protein are plotted on the *x*-axis. Negative log10 transformed *p*-values are plotted on the *y*-axis. Proteins significantly more abundant in S2 patient sera (red circles) are evidenced by the red area, while those more abundant in S0 patient sera (green squares) are evidenced by the green area (*p*-value≤ 0.05). The proteins with differences that were not statistically significant are shown in grey. Positions of B2M and PZP proteins are evidenced on the graphs. In both PCA (c, d), each circle represents an individual patient's protein profile. In (d) both technical replicates for each patient are shown. B2M, beta-2-microglobulin; PZP, pregnancy zone protein.

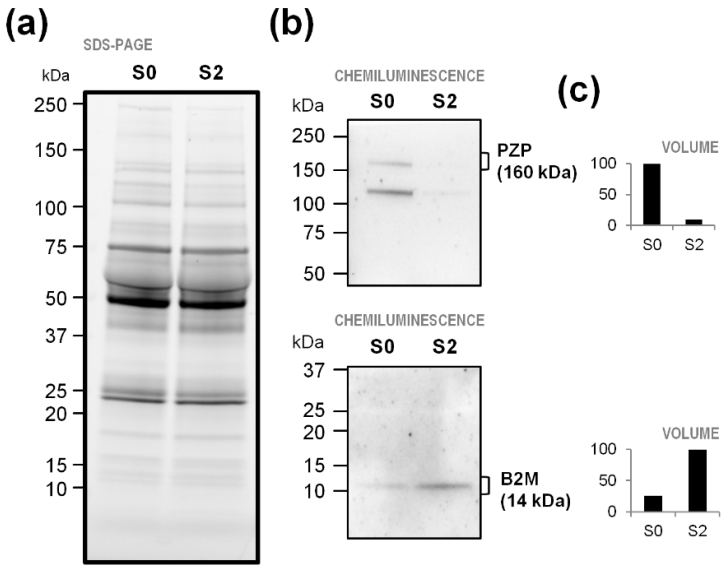

Figure 2. Immunochemical detection of two differentially abundant serum proteins, indicated by gene names on the right. Samples are pools of serum proteins from individuals with DSC scores 0 and 2 of the validation

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cohort. (a) Stain-free gel acquired before transfer as control for equal loading of protein extracts. (b) Cross-reactive bands against PZP or B2M antibodies. (c) Band volume intensities from immunoblotting. B2M, beta-2-microglobulin; PZP, pregnancy zone protein.

A significant correlation was observed between serum relative abundance of B2M and age (r = 0.659,*p* = 0.002), and between PZP levels and sex (r = -0.205, *p*<0.001), respectively (Table 4).

Table 4. Correlation between B2M and PZP levels and serological parameters.

	B2M		**PZP**	
Serological parameter	r	*p*-value	r	*p*-value
Sex	26.000	0.122	5.000	<0.001
Age	0.659	0.002	-0.205	0.387
PGI	0.159	0.502	-0.259	0.271
PGII	0.104	0.671	-0.079	0.748
PGI/PGII	0.116	0.627	-0.277	0.238
G17	0.111	0.643	0.103	0.666
HP IgG	-0.026	0.913	-0.322	0.166

G-17, gastrin-17; HP, *Helicobacter pylori*; PG I, pepsinogen I; PG II, pepsinogen II.

3. Discussion

We recently proposed a noninvasive screening method called DSC test – based on patient's age, sex, serum PGI and PGII, anti-HP IgG, and G17 levels – predicting the risk of developing gastric disease and categorizing patients into low- (score 0, S0) and high-risk (score 2, S2) groups for GC in Italian geographical areas with medium GC risk (Veneto and Friuli-Venezia Giulia) [21]. This test suggested being valuable as a potential screening strategy capable of identifying asymptomatic individuals who require upper gastrointestinal diagnostic endoscopy. Hereby, extending our previous research, we present a study adopting a mass spectrometry-based proteomic approach to identify new candidate markers associated with early-GC risk, intended for incorporation into the DSC test to improve its accuracy.

To date, numerous studies have investigated predictive biomarkers for the early detection of stomach cancer or gastric precancerous at the protein level [23–25]. The identification of circulating biomarkers suitable for population screening and early detection of GC yielded significant advancements both in plasma (5-protein panel, [26]; 13-protein panel, [27]; HLA-G, [28]; miscellaneous, such as [29]; sex hormone-binding globulin [30]) and in serum (ITIH4 [31]; survivin, [32]; cytokines, [33]). However, these markers still lack sufficient diagnostic power, highlighting the urgent need for more sensitive and specific alternatives.

In differently large clinical cohorts of patients, many studies have reported lists of candidate disease-associated proteins, using different technological platforms, among which mass spectrometry-based proteomics, has become a cornerstone of biomedical research, enabling the routine, comprehensive, and high-throughput identification/quantification of proteins in complex biological samples. In particular, the "bottom-up" approach is the dominant method for discovery-driven profiling, bypassing the need for extensive prefractionation by digesting entire proteomes into peptides for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis [34].

Our LC-MS/MS-based label-free quantitative proteomic approach succeeded in identifying quantifiable differences in serum protein levels across S0 and S2 patients, in two independent cohorts (discovery and validation). In sera of S2 patients of both cohorts, five proteins were found to be significantly more abundant (complement factor D, CFD; beta-2-microglobulin, B2M; EGF-containing fibulin-like extracellular matrix protein 1, EFEMP1, and cystatin-C, CST3), while two resulted to decrease in content (sex hormone-binding globulin, SHBG, and pregnancy zone protein, PZP). Among them we focused on B2M and PZP, whose differential content was validated by immunoblotting. In particular B2M and PZP were found to be significantly increase and decrease in relative abundance in serum of S2 patients as compared with S0.

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B2M, a low-molecular weight house-keeping protein (around 12 kDa), is synthesized by nearly all nucleated cells, including lymphocytes. It is non-covalently linked to the α-chain of major histocompatibility complex class I (MHC-I) molecules, the B2M subunit facilitating antigenic peptide presentation to natural killer cells and CD8+ cytotoxic T lymphocytes but also reinforcing MHC-I structural integrity [35,36]. Mainly metabolized in the glomerulus after its dissociation from the MHC-I molecule and excreted by the kidneys, this immune surveillance molecule is a sensitive indicator of proximal tubular dysfunction [37]. In certain diseases, B2M is either cleared less efficiently or produced more rapidly, leading to elevated levels in the blood; at the same time, because it lacks covalent bonds to MHC-I and direct anchoring to cell membrane, B2M can detach as free soluble molecule circulating in the extracellular fluid [38]. Soluble B2M is present in bodily fluids, such as serum, with stable concentrations and at low levels under normal physiological conditions [25], while elevated serum B2M levels have been associated to a variety of malignancies, including certain cancers of the blood and bone marrow (i.e., myeloma [39], Hodgkin disease [40], Burkitt lymphoma [41], chronic lymphocytic leukemia [42], and diffuse large B-cell lymphoma, DLBCL [43]), non-hematological cancers (i.e., oral squamous cell carcinoma [44]; prostate [45]; small cell lung [46]; glioma [47]), in most of which circulating B2M appeared as useful biomarker for clinical diagnosis, follow-up and prognosis.

Our observed higher B2M levels in S2 patients may come from a higher inflammatory status, a growing evidence emerging about the relationship between circulating B2M and systemic inflammation, high cell turnover and immune activation. Interestingly, we found an association between serum B2M abundance and age, in accordance with previous studies reporting increased circulating B2M levels with aging and [50,51]. The higher B2M levels observedin S2 patients at increased risk of GC are also consistent with the DSC screening test, as GC incidence is strongly associated with aging [52]. In contrast, no correlation was observed between serum B2M level and the serological parameters (PGI, PGII, PGR, G-17, and HP IgG). Similarly, in Yeniova et al. (2014) work investigating 120 patients (70.8% HP positive of which 27.5% CagA positive) serum B2M level did not correlate with HP infection severity and intensity according to Sydney classification and CagA status [51]. Comparable serum B2M levels in HP and control groups were also found by Akay et al. (2008), who detected subendothelial B2M in 63.3% cases with HP and none of the HP-negative control group ($p<0.001$), B2M accumulating in the majority of HP-positive patients with active chronic gastritis, whereas any correlation between serum and tissue levels being found [52]. B2M deposition have been also found in the subepithelial layer of gastric biopsies from patients with HP-positive active chronic gastritis, this process being associated with the significant leucocyte infiltration during HP infection and its subsequent release of B2M from their surface [53]. In our study, we did not measure B2M tissue levels and a possible B2M deposition at (inflammatory) gastric level. Therefore, it should be tempting to investigate tissue concentrations of B2M in order to evaluate B2M gastric accumulation and its possible differential concentration depending on stomach atrophy and HP infection.

In several solid cancers (i.e., prostate, breast, lung and renal cancer), *in situ* B2M can exert tumorigenic effects independently of MHC activating epithelial-mesenchymal transition and inducing cancer lethality and bone metastasis by interacting with the hemochromatosis -I, proteins [54]. In B2M can stimulate tumor cell growth/viability and suppress apoptosis by interfering with specific pathways (i.e., cAMP-dependent PAK and PI3K/AKT in prostate and renal carcinomas, respectively [55,56]). In colorectal cancer, low B2M-expression correlated with aggressive behaviour (immune cold environment and high-risk histologic features) and worse disease-specific survival [57].

PZP is an estrogen-dependent highly conserved glycoprotein belonging to the α-2-macroglobulin superfamily, composed by two identical disulfide-bridged subunits of around 160 kDa. It is mainly produced by the liver, expressed in extracellular compartments of several tissues, but also secreted in body fluids, such as serum (revised by Wu [58]). PZP plays fundamental roles as broad spectrum protease activity inhibitor, immune regulator/immunosuppressant and inhibitor of

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misfolded protein aggregates [59,60]. During pregnancy PZP serum levels dramatically increase, but declining rapidly post partum; while in serum of non-pregnant females and healthy males, PZP is a trace protein (<0.01 mg/ml)[61,62]. Circulating (plasma/serum) PZP levels can also vary (increase or decrease) in different diseases, including cancer [58]. For instance, elevated serum PZP levels were identified in uterine leiomyoma, endometrial cancer and some ovarian tumors [63], and lung adenocarcinoma with type 2 diabetes mellitus [64]. Other pathologies investigated in terms of PZP plasma levels variation include metabolic diseases, such as diabetes and hypertensive disorders in pregnancy [65], and conditions involving chronic inflammation (i.e., rheumatoid arthritis, RA [66]; inflammatory bowel disease, IBD [67]).

During the last years, there has been also a particular interest in deciphering the possible role(s) of PZP as regulator of tumor immune microenvironment *in situ*, and thus, indirectly, as candidate immunotherapy target, its role as potential tumor marker being recently revisited [68]. The possible relationship between PZP and GC has been exploited at transcript level by Oshima et al. (2024) in GC tissues and adjacent normal gastric mucosa of 253 patients with pStage II/III GC who underwent curative resection [69]. The authors proposed a clinical utility of PZP as independent predictor of poor OS (hazard ratio=1.984, 95% confidence interval=1.307-3.012, p=0.0013), its expression in GC tissues resulting associated with differentiated histological types, venous invasion, and pathological stage. Previous studies in patients with either hepatocellular carcinoma or lung cancer reported low PZP expression in tissues, where the protein was proposed as potential prognostic indicator and tumor suppressor [70,71].

To our knowledge, at present the possible relationship between gastric atrophy or dysplasia and PZP protein is still to be investigated. When autoimmune, atrophic gastritis is accompanied by increase in lymphocytic infiltrates (predominantly T cells and plasma cells) in the gastric submucosa and lamina propria and production of high levels of different pro-inflammatory cytokines, such as IFN-γ and TNF-α by CD4+ Th1 cells [72,73]. Chronic inflammation in gastric mucosa is also a typical hallmark of HP-induced gastritis [74], which notably presents increasing PGII serum levels [75]. The inhibitory role played by PZP during pregnancy is known to selectively modulate T-cell activation, particularly by suppressing Th1-type immune responses [76,77]. The lower serum PZP levels observed in S2 patients may stem from persistent gastric inflammation and mucosal atrophy, potentially triggered by the autoimmunity or HP infection alongside environmental changes in the gastric milieu.

Serum PZP levels were not significantly associated with any of the serological parameters individually tested, but showed a significant association with sex, being higher in female than in males. This finding is consistent with the DSC screening test, which associated the S2score with male sex, and with epidemiological evidence reporting a higher GC incidence in males [77].

To gain better insight into the potential contribution of PZP to altered local and systemic immune responses, future studies should investigate PZP expression in gastric tissues and correlate its levels with those of pro-inflammatory cytokines. An additional role played by PZP may also be as tissue remodeling as protease inhibitor, this may also the case for some S2 patients at risk for **precancerous lesions** development into intestinal-type gastric cancer.

Therefore, our proteomics analysis revealed increased B2M levels and decreased PZP levels in patients at high risk of GC, supporting the validity of the DSC screening test, in which the S2 score is associated with older age and male sex.

Our study has several limitations. First, the overall sample size of our selected patient cohorts was small and may only partially reflect the high heterogeneity of the serum proteomes. Future analyses should be conducted on larger cohorts, stratified by clinical parameters such as gastric atrophy, intestinal metaplasia, and dysplasia. Another limitation is the inherent complexity of the serum matrix; protein profiles vary considerably based on factors such as age, sex, hormonal status, and metabolism. Therefore, potential confounding factors affecting protein levels must be carefully considered. Finally, a further objective could be the *in situ* investigation of B2M and PZP levels in selected S2 patients with extreme protein abundance (highest *vs*. lowest), to get better insight their

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potential role as candidate markers for pre-cancerous gastric conditions. In line with our primary objective of identifying novel early GC risk markers for the DSC test, further studies will evaluate whether additional proteins identified here (i.e., CFD, EFEMP1, CST3, and SHBG) can be integrated to further improve the test's accuracy.

4. Conclusions

In two independent cohorts classified as S0 and S2 by our DSC screening test, patients at higher GC risk (S2) displayed a distinct serum protein profile compared with those at lower risk (S0). Quantitative label-free LC-MS/MS analysis identified two proteins, B2M and PZP, whose serum abundance differed between S2 and S0, supporting their potential as early biomarkers of GC risk and their association with age and male sex. Both proteins may play a role in the immune response occurring during the early stage of gastric mucosa transformation along the Correa cascade.

5. Materials and Methods

5.1. Patient Selection

The study enrolled consecutive patients undergoing esophagogastroduodenoscopy and gastric biopsy at the Unit of Oncological Gastroenterology of Centro di Riferimento Oncologico di Aviano (CRO) between April 2014 and December 2016. Patients whose sera were analyzed gave written informed consent. The study protocol no. CRO-2024-04 was approved by the Ethical Committee of Friuli Venezia Giulia (CEUR-2024-Os-128/July 16th 2024) and registered at ClinicalTrials.gov (ID NCT07374731). The study was conducted in compliance with the Declaration of Helsinki principles and Good Clinical Practice. Written informed consent was obtained from all participants. Inclusion criteria were: age over 18 years; ability to understand, accept, and sign informed consent for the study, data processing and the collection of a serum aliquot for research purposes; fasted subjects with suspected pre-neoplastic/neoplastic gastric lesion or with a request for pepsinogen testing, for whom endoscopic examination data are available. Exclusion criteria were: pregnant women; patient with co-existing or prior diagnosis (within the last 5 years) of another malignant neoplasm; patient unable to understand, accept, and sign consent for data processing. Based on their DSC score, individuals were grouped into “score 0” (S0) or “score 2” (S2), indicating “low-risk” or “high-risk” for developing severe gastric diseases, including GC [21].

5.2. Study Design

Patients were divided into four groups: (i) an discovery group of score 0 (n = 10); (ii) an discovery group of score 2 (n = 10); (iii) a validation group of score 0 (n = 51); and (iv) a validation group of score 2 (n = 29). Our discovery and validation cohorts consisted of the two discovery and two validation groups, respectively. Label-free LC-MS/MS was used to analyze and compare the two groups (S0 and S2) in the discovery cohort in Part I of the study, and in the validation cohort in Part II. The relative abundance of some selected proteins was found to differ between the two groups in both cohorts.

5.3. Label Free Proteomic Profiling by LC-MS /MS

Proteins were reduced, alkylated, digested, and peptides cleaned up with the EasyPep Mini MS Sample Prep Kit (Thermo Fisher Scientific) according to the manufacturer’s protocol. Concentration of peptides resuspended in 0.1% formic acid was measured with the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Peptide mixtures were analyzed using a Q-Exactive Plus Hybrid Orbitrap mass spectrometer equipped with a UHPLC Vanquish (Thermo Fisher Scientific), as previously reported [22]. Briefly, each tryptic peptide sample (15 µg) was fractionated in a XBridge Peptide BEH C18 column (3.5 µm 2.1 x 150, Waters, Sesto San Giovanni, Milan, Italy) at a flow rate of 200 µL/min using 0.1% formic acid/acetonitrile gradient over a period of 80 min and sprayed onto the mass

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spectrometer using a heated electrospray source probe in positive mode (Thermo Fisher Scientific). The mass spectrometer was run in data-dependent mode with positive polarity at electrospray voltage of 3.52 kV and capillary temperature of 325 °C. Full scan MS spectra (m/z 375-1500) were acquired followed by MS/MS scans on the top 10 intense ions, applying a dynamic exclusion window of 30 seconds. Individual samples of the exploratory cohort were run in single runs, while those from the validation cohort in duplicate. Commercially available peptides from tryptic digested HeLa proteins (Thermo Fisher Scientific) were used as quality control standards. Label-free quantification (LFQ) and database search were done with Proteome Discoverer software (version 2.5.0.400) using the Sequest search engine against the human database (UniProt release 2025_04). Only proteins identified with a false discovery rate (FDR) high (1%), a Sequest Score ≥1, and a t-test p-value <0.05 were considered. The abundance ratio (or fold change, FC) of statistically significant proteins was calculated as the ratio of the average LFQ intensities between the two matched groups, with differential proteins increasing or decreasing in abundance depending on the FC values ≥2 (FC Log2≥1) or ≤2 (FC Log2≤-1) respectively.

5.4. Statistics

Demographic and serological variables in the discovery and validation cohorts were compared using Fisher's exact and Man-Whitney as appropriate. In the discovery cohort, correlation between serum levels of selected differentially abundant proteins (S2 vs. S0) and serological parameters was evaluated by Mann-Whitney U test and Spearman rank's correlation.

5.5. Validation of Candidate Markers by Immunoblotting

Plasma pools (S0 and S2) were treated with 2-D Clean-Up Kit (Cytiva) and centrifuged. The resulting pellets were resuspended in lysis buffer (Thermo Fisher Scientific). Protein concentration was determined with Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Samples were stored at -80 °C until analysis. Protein (30 and 50 µg per pool for pregnancy zone protein PZP and beta-2-microglobulin B2M respectively) was fractionated on 4-20% gradient Criterion TGX Stain-Free gels (Bio-Rad). Gel images were acquired with the Chemidoc system (Bio-Rad) to document equal protein loading among samples. Then, separated proteins were electrotransferred onto nitrocellulose membranes and processed with PURITY™ WB horseradish peroxidase (HRP) Detection System (Vilber, Eberhardzell Germany), according to the manufacturer's protocol. Protein bands were visualized by chemiluminescence using SuperSignal West Femto Substrate Maximum Sensitivity Substrate (Thermo Fisher Scientific) and imaged with ChemiDoc (Bio-Rad). The following primary antibodies were tested: anti-PZP (1:1000; #PA5-110249, Thermo Fisher Scientific), and anti-B2M (1:500; #ab14714, AbCam).

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Supplementary Table S1: Supplementary Table S2.

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