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Prevalence of high affinity naturally occurring IgG2 antibodies against human chorionic gonadotropin and its subunits in patients with ovarian cyst

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ABSTRACT

Naturally occurring antibodies to tumour antigens are gaining interest as clinically important cancer biomarkers for early diagnosis, prognosis and for the development of anti-cancer therapeutics. The glycoprotein αβ heterodimer hormone human chorionic gonadotropin (hCG) and its β subunit (hCGβ) are produced by various cancers, and their increased serum levels correlate with poor prognosis. We have previously reported that patients with benign ovarian cysts, but not the malignant tumours, were characterized by augmented serum levels of naturally-occurring IgG antibodies to hCG and hCGβ. Here we further characterise these antibodies in patients with ovarian cysts.

IgG and IgM antibody binding to whole hCG, hCGβ, hCGα, hCGβ C-terminal peptide (hCGβCTP), and the hCGβ core fragment (hCGβCF) were measured in the sera from 36 patients with ovarian cysts and 12 healthy non-pregnant women using a standard ELISA. IgG subclass usage and affinity was also determined together with cross-binding to whole hCG and its subunits of four selected commercial monoclonal antibodies generated against ovarian cyst mucins.

Our results showed that 91.7% of the sera tested contained elevated IgG, but not IgM antibodies to one or several antigens, with an overwhelming prevalence of high affinity IgG2 indicating their binding to carbohydrate epitopes and possibly ovarian cyst mucins. Anti-mucin commercial antibody ab212418 (Abcam) produced against Gal1-3GalNAc, exhibited strong cross-binding to hCGαβ, hCGβ, hCGα and hCGβCTP. The protective anti-cancer potential of these antibodies will be further investigated and could lead to the development of novel treatment strategies for ovarian cancer.

1. Introduction

Human chorionic gonadotropin (hCG) is a member of the glycoprotein hormone family, together with luteinizing hormone (LH), follicle stimulating hormone (FSH) and thyroid stimulating hormone (TSH). These hormones are heterodimers consisting of a family-shared α-chain non-covalently associated with a hormone-specific β-chain (hCGβ) (Cole, 2017). hCG is produced by various cells such as cytotrophoblast cells and villous syncytiotrophoblasts during pregnancy as well as by placental, trophoblast-derived and germ-cell derived tumour cells during tumorigenesis (Cole, 2017; Iles et al., 2010). Different types of cells produce different isoforms of hCG which share a common amino acid backbone, but vary in carbohydrate side chain structure (Cole, 2012; Stenman et al., 2006). In pregnancy, hCG is produced by the trophectoderm of the pre-implantation embryo within a few days of fertilization. Its binding to the joint hCG/LH receptor is essential for the production of progesterone and estrogen by the *corpus luteum* to ensure its maintenance through the duration of pregnancy (Fishel et al., 1984), and for the suppression of maternal macrophage attack on fetal and placental tissues (Cole, 2017). Hyperglycosylated hCG is produced by

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cytotrophoblast cells of the blastocyst and acts through the transforming growth factor β (TGFβ) receptor promoting production of collagenases and metalloproteinases required for blastocyst invasion and implantation in the uterus (Sasaki et al., 2008; Staun-Ram & Shalev, 2005). The hCG isoform with sulfated oligosaccharides is produced by pituitary gonadotropic cells during the menstrual cycle (Birken et al., 1996; Odell & Griffin, 1987).

Importantly, hCG is a well established biomarker for placental, trophoblast-derived and germ-cell derived tumours. The trophoblastic carcinomas produce hyperglycosylated whole hCGαβ, whilst placental and germ-cell derived cancers, mostly release hyperglycosylated hCGβ free subunits (Cole, 2012; Iles et al., 2010). The tumour cells not only secrete hCG, but it is also expressed on their surface as an α/β dimer or the hCGβ-chain only (Acevedo et al., 1995). It is believed that hCG plays a role as an autocrine growth factor for tumour cells (Acevedo et al., 1992; Sheaff et al., 1996) and may act at different levels to facilitate cancer progression: (a) as a transforming growth factor; (b) as an immunosuppressive agent; (c) as an inducer of metastasis and (d) as an angiogenic factor (Acevedo et al., 1992; Acevedo et al., 1995; Cole, 2012; Sheaff et al., 1996). Bioneutralisation of hCGβ in carcinomas therefore represents a desirable approach for targeted anti-cancer therapy. Neutralization of soluble hCG with a monoclonal antibody, naturally occurring or vaccine induced antibody, may abrogate hCG-mediated tumour growth, angiogenesis, and immune escape (Geissler et al., 1997; Porakishvili et al., 2002; Triozzi et al., 1994; Yu et al., 2007).

Recently we have demonstrated, that it is possible to develop a potential cancer vaccine, with high immunogenicity, which selectively targets hCG and its β subunit (Kvirkvelia et al., 2018). We have also reported that naturally occurring anti-hCG antibodies can be used as a predictive biomarker for non-malignant ovarian cysts and hypothesized that they might play a protective role against them becoming malignant (Chikadze et al., 2010).

Ovarian cysts are often asymptomatic fluid-filled sacs which can affect women of any age and vary widely in etiology, from physiological, to complex benign, and finally to neoplastic (Jaroslava, 2019; Sutton, 1886). Therefore, there is still an obvious need for new biomarkers that would serve as precise diagnostic and/or prognostic indicators. We have previously shown that the majority of patients with an ovarian cyst, but not those with ovarian carcinoma, had significantly elevated levels of naturally occurring serum antibodies of IgG isotype against both, hCG and hCGβ, whilst patients with ovarian carcinoma expressed non-appreciable levels of these antibodies (Chikadze et al., 2010).

In order to better understand the predictive role of these antibodies, in this study we further characterise them by measuring the binding of antibodies in the sera from ovarian cyst patients to hCG and its subunits - hCGβ, hCGα, hCGβ C-terminal peptide (hCGβCTP), and hCGβ core fragment (hCGβCF), their subclass usage and their binding affinity. In addition, we have investigated the cross-binding ability of some monoclonal antibodies produced against ovarian cyst mucins to hCG and its subunits.

2. Materials and methods

2.1. Patients and healthy control individuals

Serum samples from 36 clinically diagnosed ovarian cyst patients aged 22–61 were collected in preservative-free test-tubes at the "IQ clinic" in Tbilisi, Georgia (care of Dr Maia Janelidze). All patients were newly diagnosed and untreated at the time, and were not pregnant. The patients and healthy, non-pregnant control females (n = 12), were enrolled onto the study following informed consent by the collaborating group of clinicians observing full anonymity and ethical permission granted by the ethics committee of National Center for Disease Control (NCDC) and Public Health of Georgia (registration number – IRB000021).

2.2. Sera collection

10 ml of peripheral blood were collected in anticoagulant-free test tubes following standard procedures. Samples were left to clot at room temperature for 30 min and then centrifuged at 2500 rpm for 15 min. Separated sera were stored in 0.5 ml aliquots at −20 °C for no longer than 2 months.

2.3. Antibody titres

For the assessment of the titres of naturally-occurring autoantibodies of various IgG isotypes to hCGαβ, hCGβ, hCGα, hCGβ carboxy-terminal peptide (hCGβCTP) and hCGβ core fragment (hCGβCF), a standard enzyme linked immunosorbent assay (ELISA) method was used as we have previously described (Chikadze et al., 2010; Kvirkvelia et al., 2018; Porakishvili et al., 2002). Briefly, Nunc MaxisorpC 96-well flat-bottomed microtitre plates were coated with 50 μl of hCGαβ (Sigma, USA), hCGβ (Sigma, USA), hCGα (Fitzgerald, USA), hCGβCTP (Sigma, USA) or hCGβCF (National Institute for Biological Standards and Control (NIBSC), UK) at the concentration 1 μg/ml in 0.05 M carbonate-bicarbonate buffer (CBB, pH9.6; Sigma, USA.). The plates were incubated overnight at 4 °C. Blocking was performed using Pierce™ Protein-Free Blocking Buffer (Thermo Fisher Scientific, USA). Sera were serially diluted 1:25–1:6400 in 5 % bovine serum albumin (BSA, Thermo Fisher Scientific, USA) in phosphate-buffered saline (PBS, Thermo Fisher Scientific, USA). 50 μl of each dilution was added to corresponding wells in duplicates. For the detection, goat anti-human IgG horse-radish peroxidase (HRP)-conjugated antibody (Sigma, USA) and the substrate 3,3′,5,5′-Tetramethylbenzidine (TMB) (Thermo Fisher Scientific, USA) were used. The plates were read at the optical density (OD) of 450 nm in a spectrophotometer Selecta (Spain). The 50 % and 75 % titres were calculated as the dilution of serum corresponding to 50 % or 75 % of the plateau respectively, and the end-point titre as the highest but one dilution giving an OD above the control.

2.4. Antibody relative affinity

To evaluate the binding avidity of sera to all tested antigens the 12 sera were selected on the basis of the highest detected levels of IgG. The chaotropic agent ammonium thiocyanate (ATC) elution was used as previously described (Goldblatt et al., 1993; Macdonald et al., 1988; Porakishvili et al., 2002) Briefly, sera dilutions which correspond to 75 % of the plateau binding as defined above were added to antigen-coated microtitre plates, the plates were incubated for 2 h at 37 °C, washed three times in PBS-Tween (0.05 %) and 100 μl of ATC (Sigma, USA) in PBS was added for 15 min at RT. The chaotropic agent ATC dissociates antibody–antigen binding in a molarity-dependent manner and was used at 0.0625–4 M. Control wells were incubated with PBS without ATC. Following 3 washes with PBS, the secondary HRP- conjugated antibody (Sigma, USA) was added and OD readings performed as above.

The amount of IgG in control (ATC-free) wells, was taken as 100 % and those with different concentrations of ATC, were expressed as proportions of the total IgG. 50 % inhibitory concentration [I_{50}] of ATC was used as a measure of avidity: $I_{50} < 0.5$ M was considered to be a low binding avidity, 0.5 M^{-1} M as an intermediate and I_{50} more than 1 M as a high binding avidity.

2.5. IgG subclasses

For the identification of IgG-subclasses, Nunc Maxisorp C 96-well flat-bottomed microtiter plates were coated with the different proteins as described above. Following the application of an optimal serum dilution, defined above as the 50 % titre, rabbit HRP conjugated antibody (Sigma, USA) to human IgG subclasses (IgG1, IgG2, IgG3 and IgG4) was added at a concentration 1 μg/ml and the rest of the essay was performed as above.

2.6. Cross binding of monoclonal antibodies raised against ovarian cyst mucins to hCG and hCGβ

Four commercially available and purchased murine monoclonal antibodies generated against mucins isolated from ovarian cyst fluid were used in order to assess the cross-binding ability to hCGαβ, hCGβ, hCGα, hCGβCTP and hCGβCF:

1. ab212418 -(Abcam, USA), which preferably reacts with carbohydrate determinants of chain A and H type 3 (Gal1-3GalNAc-R) and 4 (Gal1-3GalNAc-R), but not with type 1 and 2 chain structures. It is not reactive with immuno-dominant A trisaccharide.
2. SPM522 - to Lewis A blood group antigen (Novus Biologicals, USA) recognizing a carbohydrate determinant of Gal 1–3(Fuc 1–4) GlcNAc.
3. ab3968 - (Abcam, USA) reacts with Lewis B blood group antigen, a carbohydrate determinant carried on both glycolipids and glycoproteins, detected on the surface of red blood cells, certain epithelial cells, and in secretions of certain individuals. The exact binding domain is unknown.

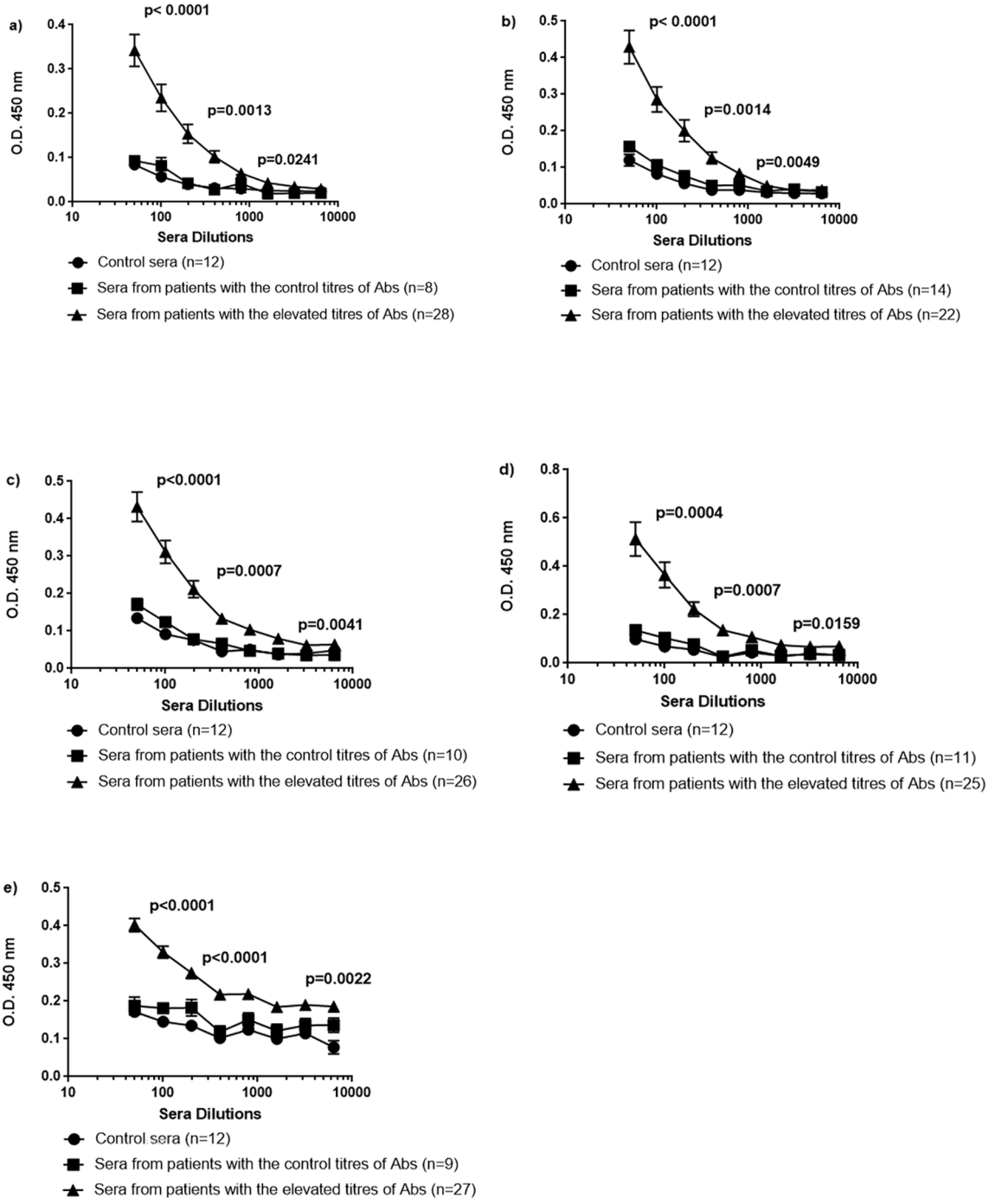

Fig. 1. IgG titration of blood sera from female patients aged 22–61 diagnosed with ovarian cyst (n = 36) and healthy age and gender-matched controls (n = 12) was performed using an enzyme-linked immunosorbent assay (ELISA). Binding of the sera to the following antigens were tested: a) hCGαβ, b) hCGβ, c)hCGα, d)hCGβ C-terminal peptide (hCGβCTP)and e)hCGβ core fragment (hCGβCF). The cut-off was calculated as a mean of optical density (OD) ± 2 × Standard Deviation (SD) determined for the control sera OD.

4. SPM297 - (Novus Biologicals, USA), recognizes the core fragment of mucin 5AC, the exact carbohydrate determinant is unknown.

The rest of the assay was performed as above. Nunc Maxisorp C 96-well flat-bottomed microtitre plates coated with hCGαβ, hCGβ, hCGα, hCGβCTP or hCGβCF. Pierce™ Protein-Free Blocking Buffer (Thermo Fisher Scientific, USA) was used for blocking. Antibodies were serially diluted 2 μg/ml – 62.5 ng/ml in PBS. Goat anti-mouse IgG1 peroxidase-conjugated antibody (Sigma, USA) was used for the detection and 3,3′,5,5′-Tetramethylbenzidine (TMB) (Sigma,USA) as the substrate. The plates were read at A450 nm in a spectrophotometer as above.

2.7. Statistical analysis

Unpaired two-tailed Student's *t* test was used to determine statistical significance. P values of <0.05 were considered significant. Data were analyzed using GraphPad Prism 7 and Ms. Exel 2016 software.

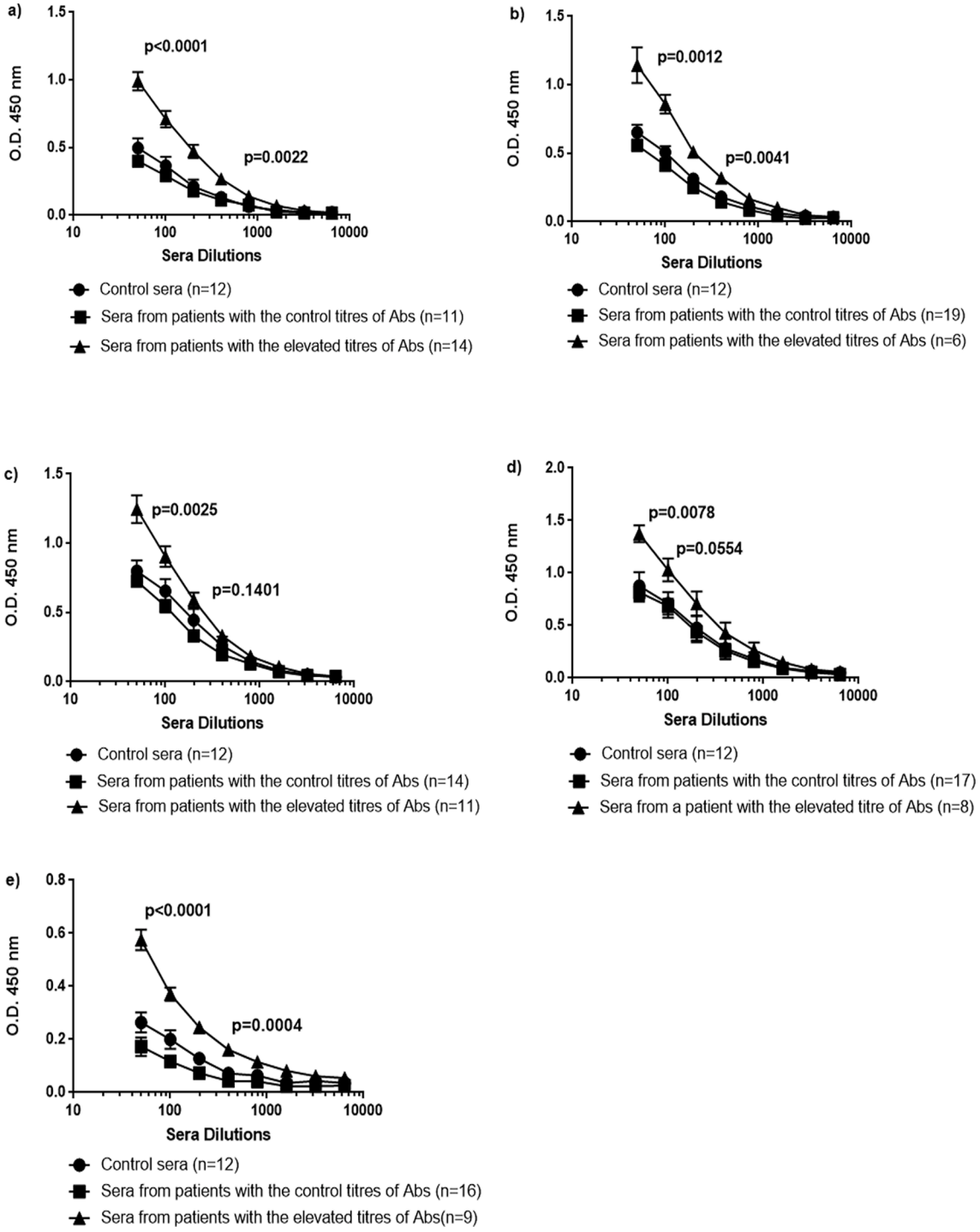

Fig. 2. IgM titration of sera from female patients aged 22–61 diagnosed with an ovarian cyst (n = 25) and healthy age and gender -matched controls (n = 12) was performed using enzyme-linked immunosorbent assay (ELISA). Binding of the sera to the following antigens were tested: a) hCGαβ, b) hCGβ, c)hCGα, d)hCGβ C-terminal peptide (hCGβCTP)and e)hCGβ core fragment (hCGβCF). The cut-off was determined as a mean of optical density (OD) ± 2 × Standard Deviation (SD) determined for the control sera OD.

3. Results

3.1. Elevated levels of naturally-occurring IgG antibodies to hCGαβ, hCGβ, hCGα, hCGβCTP and hCGβCF in patients with ovarian cysts

Sera from 36 ovarian cyst patients and 12 clinically healthy volunteers were titrated against the following antigens: hCGαβ, hCGβ, hCGα, hCGβ CTP and hCGβ CF (Fig. 1.) For each protein the results were separated into two groups: sera with normal (within the same range as for controls) and elevated titres. The cut-off was determined as a mean of OD plus 2 × Standard Deviation (SD) of the control sera ODs.

Sera from 33 out of 36 (91.7 %) patients with ovarian cysts contained significantly higher levels of IgG antibodies binding to the tested antigens, compared to normal controls, using the cut-off described in the material and methods. Elevated levels of naturally-occurring IgG antibodies to hCGαβ heterodimer were detected in 84.8 % (28 of 33) patients (p,<0.0001), to hCGβ in 66.67 % (22 of 33) patients ($p = 0.0001$), to hCGα in 84.8 % (28 of 33) patients ($p < 0.0001$), to hCGβCTP in 75.76 % (25 of 33) of patients ($p = 0.0004$), to hCGβCF in 90.9 % (30 out of 33) of patients ($p < 0.0001$). Binding to all tested antigens was seen in seventeen out of 33 patients sera (51.5 %), 4 patient sera (12 %) bound to four, another 4 (12 %) - to three, 6 sera (18 %) to two tested antigens, and two sera (6 %) to one tested antigen only - hCGβ or hCGα (Fig. 1).

Interestingly, measurement of the levels of IgM antibodies with binding capacity to the same antigens revealed that all 12 control sera contained elevated levels of these antibodies (Fig. 2). Furthermore, in 56 % of the patients' sera (14 of 25) the levels of the IgM antibodies to all tested antigens were similar in range to those of control individuals, and the cut-off value was established based on control sera titration as described in the material and methods. There were three sera with elevated levels of IgM antibodies to all five antigens above the cut off, two - to four tested antigens, two - to 3 antigens (hCGαβ, hCGβ, hCGα)– two - to 2 antigens (hCGαβ and hCGβ CF) and two – to only hCGαβ.

Therefore, it is the elevated levels of IgG antibodies, rather than IgM antibodies to hCG and its subunits that delineates ovarian cyst patients from clinically healthy individuals.

3.2. Binding avidity of naturally-occurring IgG antibodies to hCGαβ, hCGβ,hCGα, hCGβCTP and hCGβCF differs according to the antigens used

In order to better characterise the detected antibodies we further evaluated the binding avidity of the selected sera to all tested antigens and we have established that these antibodies expressed a range of avidity to the tested antigens: high to hCGβCF (I_{50} greater than 4 M ATC), intermediate to hCGαβ, hCGβ and hCGα (I_{50} 0.125 M ATC) and low to hCGβCTP (I_{50} 0.25 M ATC) (Fig. 3). We next determined the distribution of IgG subclasses amongst these antibodies.

3.3. IgG2 subclass is prevalent amongst anti-hCG naturally occurring antibodies in patients with ovarian cysts

The distribution of the detected naturally-occurring IgG isotypes, was determined in 12 selected sera with high levels of IgG autoantibodies to any of the following antigens: hCGαβ, hCGβ, hCGα, hCGβCTP and hCGβCF, using ELISA as described in the materials and methods.

The results demonstrated an overwhelming prevalence of the IgG2 subclass in the pool of naturally occurring antibodies to all tested antigens (Fig. 4). Low titres of autoantibodies of the IgG3 isotype to hCGαβ were detected in 7 out of the 12 sera ($OD = 0.1256 \pm 0.0301$, $p < 0.0001$), to hCGβ in 2 sera ($OD = 0.11225 \pm 0.00375$, $p = 0.1419$) to hCGβCTP in 7 sera ($OD = 0.235429 \pm 0.137386$, $p < 0.0001$) and to hCGβCF in 4 sera ($OD = 0.123125 \pm 0.01291$, $p < 0.0001$). anti-hCGα autoantibodies of the IgG3 isotype were not detected. Interestingly, a range of the titres of autoantibodies of the IgG4 isotype were found in some sera, especially those which bind to hCGβCF, although the average values did not differ from those of IgG3.

Importantly, no appreciable levels of IgG1 isotype antibodies to any of the tested hCG subunits were detected in the sera of patients with ovarian cysts.

The prevalence of the IgG2 isotype suggested that these naturally occurring antibodies might be directed to the carbohydrate chain of the hCG and its subunits and in non-pregnant cancer free females being generated in response to the ovarian cyst mucins.

3.4. Commercial monoclonal antibodies against the range of ovarian cyst mucins cross-bind to the hCG whole hormone or hCGβ

To test this hypothesis, we have assessed the cross-binding to hCG and its subunits of four monoclonal antibodies generated against ovarian cyst mucin(s). The following commercially available monoclonal antibodies (mAb) were used: ab212418, SPM522, ab3968 and SPM297. The data showed that one of them - ab212418 - which reacts to the carbohydrate determinant - Gal1-3GalNAc-R, cross-binds to hCGαβ, hCGβ, hCGα and hCGβCTP (Fig. 5). The respective optical densities (OD) reflecting the binding at 2 μg/ml concentration of the antibody were as follows: to whole hCG – 0.54733 ± 0.038, to hCGβ – 0.687333 ±

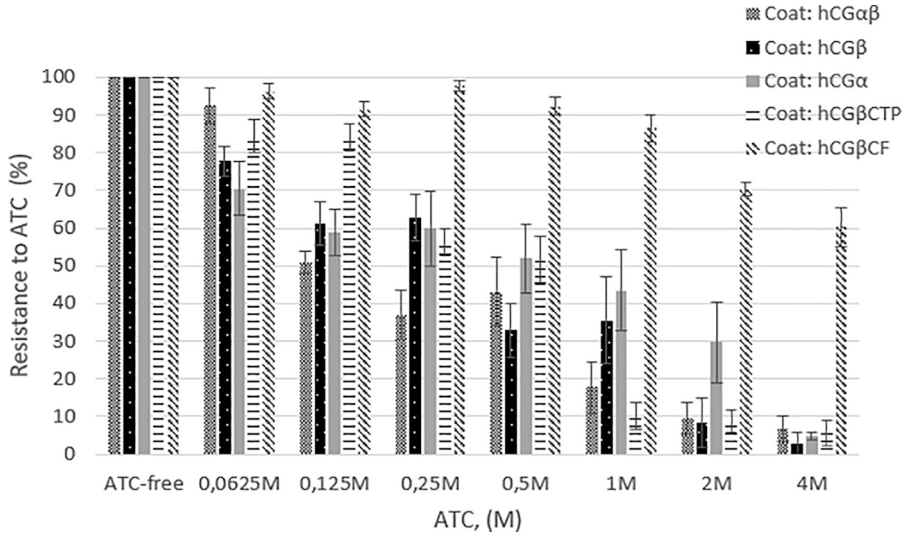

Fig. 3. The binding avidity of the IgG antibodies of sera from patients with an ovarian cyst (n = 12) to tested antigens. The binding of an antibody and its corresponding antigen was disrupted by ammonium thiocyanate solution of different molarity (from 0.0625 M to 4 M). The graphs show the mean O.D. ± SE.

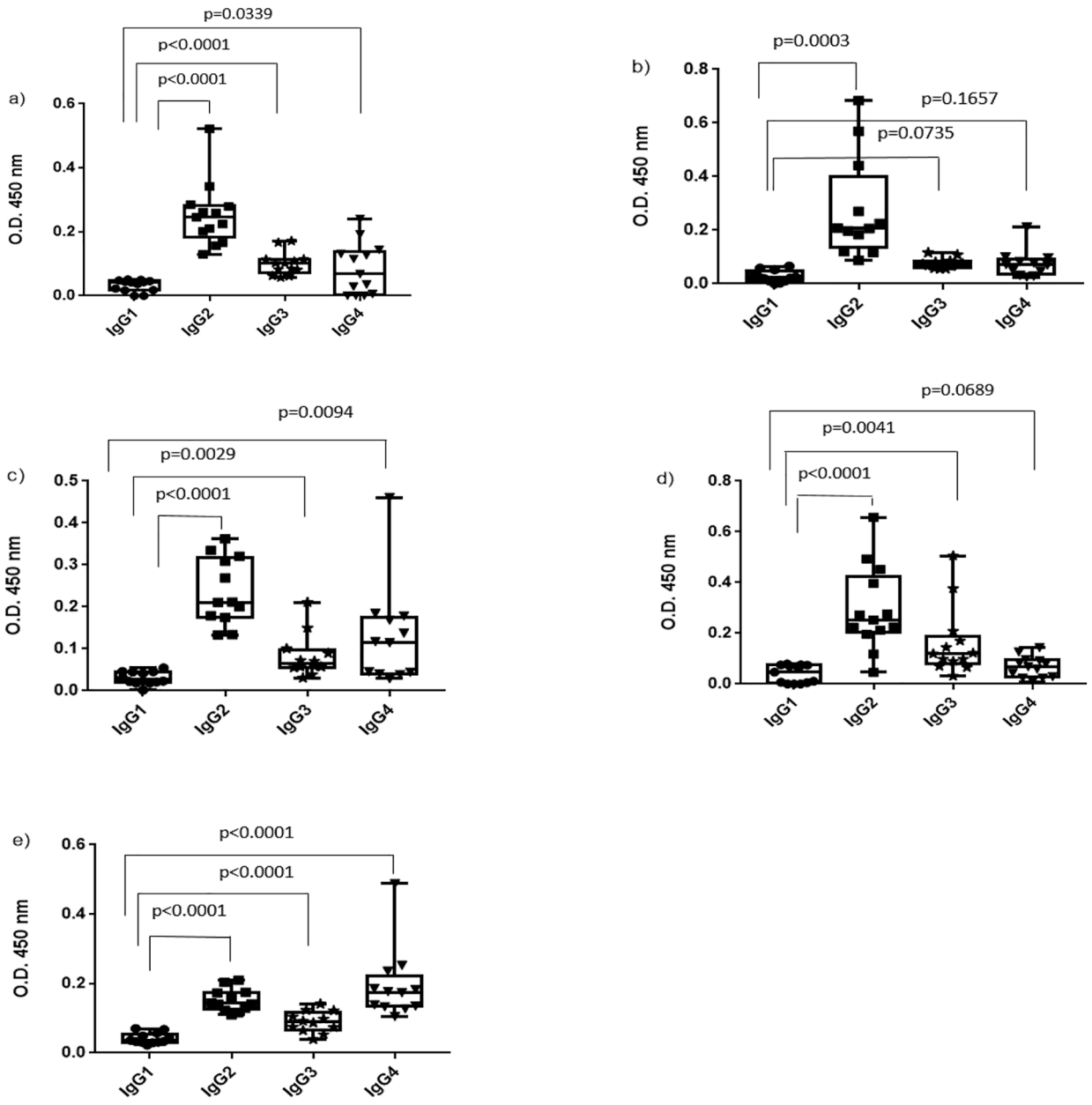

Fig. 4. Distribution of naturally-occurring IgG Isotypes to a) hCGαβ, b) hCGβ, c) hCGα, d)hCGβCTP, e) hCGβCF in patients with an ovarian cyst (n = 12).The results are shown using box-and-whisker diagrams where the middle line of the box represents the median or middle number. The x in the box represents the mean. The median divides the data set into a bottom half and a top half. The bottom line of the box represents the median of the bottom half or 1st quartile. The top line of the box represents the median of the top half or 3rd quartile. The whiskers extend from the ends of the box to the minimum value and maximum value.

0.1262, to hCGα 0.453333 ± 0.0136 and to hCGβCTP 0.362333 ± 0.0906 (control OD was subtracted in all cases). Interestingly, this antibody did not bind to hCGβCF, which contains only one glycosylation site at Asn-78 (*N*-glycan). In addition, there was very poor cross-binding of SPM522 to hCGβ and hCGβCF with respective ODs reflecting the binding at 2 μg/ml concentration – 0.19 ± 0.1325 and 0.1 ± 0.014 (Fig. 5). Neither ab3968 nor SPM297 monoclonal antibodies showed appreciable cross-binding.

4. Discussion

Naturally-occurring antibodies to different antigens have been associated with various human diseases including autoimmune diseases (Haller-Kikkatalo et al., 2012; Rieder et al., 2011), neurologic diseases (Wootla et al., 2015), various malignances (Schwartz-Albiez, 2012) and etc. Studies have focused mainly on the assessment of their role in accurate and early diagnosis and/or further prognoses (Chapman et al., 2007; Lu et al., 2008).

We report here for the first time, elevated levels of naturally-occurring IgG antibodies that bind to hCG whole hormone and its subunits in 91.7 % of the sera from non-functional ovarian cyst patients. These antibodies from different serum samples are characterised by a range of titres and binding avidity to hCGαβ and/or its subunits in various combinations.

Most importantly, there was a consistent overwhelming prevalence of IgG2 subclass in the antibodies to all tested antigens strongly indicating their binding to carbohydrate epitopes within hCG and its components. Indeed, IgG2 is second to IgG1 as the most prevalent antibody isotype in human serum and represents the bulk of the reactivity to many glycans (Vidarsson et al., 2014). Accordingly, hCG is a highly

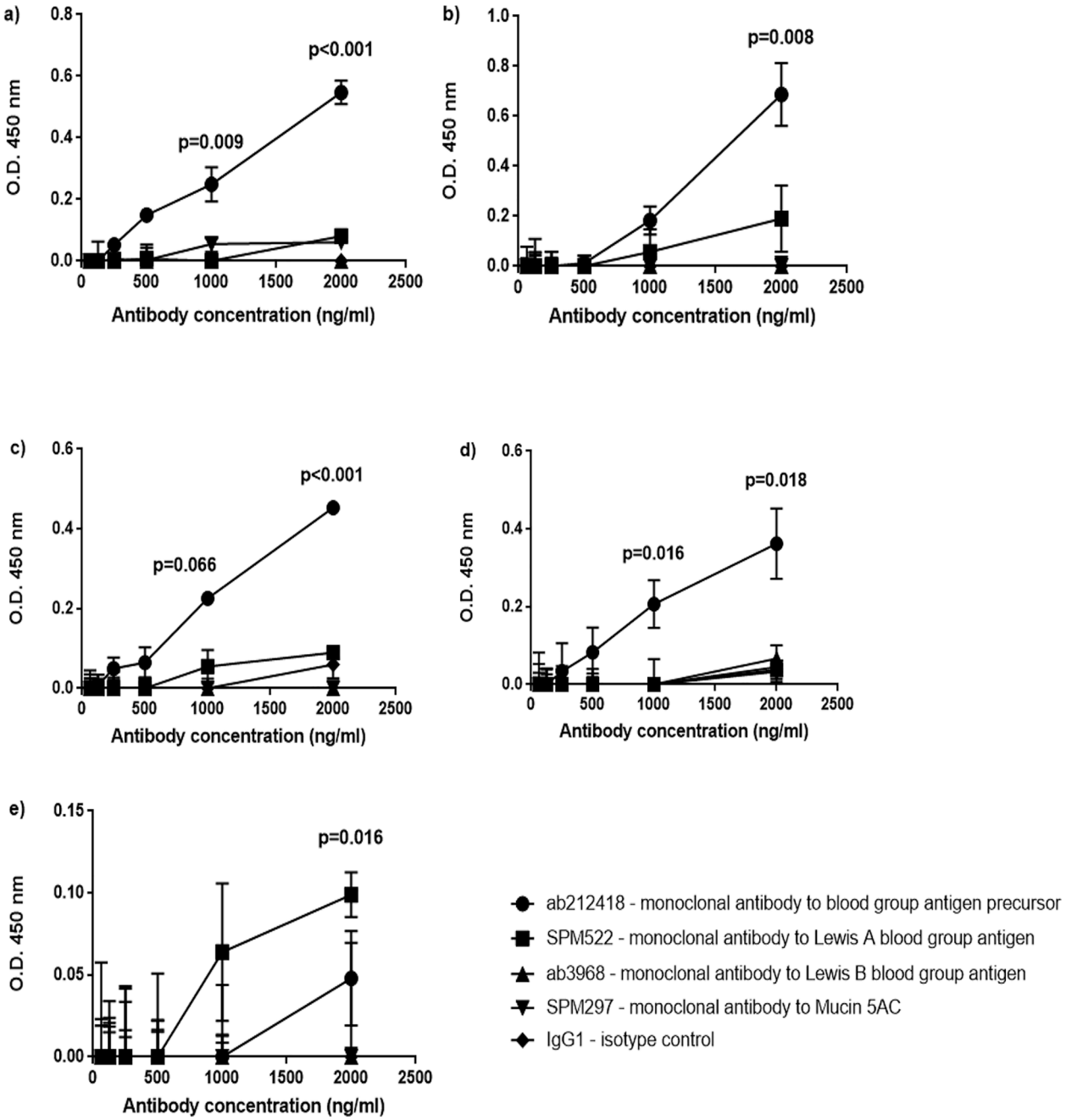

Fig. 5. Binding of different concentrations of commercial monoclonal antibodies: ab212418 (Abcam), SPM522 (Novus Biologicals), ab3968 (Abcam), SPM297 (Novus Biologicals) and IgG1 (Life technologies) as a isotope control to a) hCG, b) hCGβ, c) hCGα, d) hCGβCTP, e) hCGβCF assessed by ELISA. Antibodies were serially diluted 2 μg/ml – 62.5 ng/ml in PBS and added to plates coated with tested antigens in triplicates. The graphs show the mean absorbance ± SD indicated as bars through each data point.

glycosylated glycoprotein with more than 30 % carbohydrate by mass that is composed of two non-covalently associated glycosylated subunits (Fournier, 2016). The α subunit shared by all members of glycoprotein hormone family contains 92 amino acids and bears two *N*-glycosylation sites at Asn-52 and Asn-78 (Bellisario et al., 1973; Kobata & Takeuchi, 1999). In addition, the free form of the α subunit contains one O-glycosylation in position Thr-43 (Cole, 1987). The β subunit (hormone-specific) contains 145 amino acids and bears two *N*-glycosylation sites at Asn-13 and Asn-30 in its core region and in four sites of O-glycosylation at Ser 121, 127, 132 and 138 in its carboxy-terminal peptide (Ibeto et al., 2020; Kobata & Takeuchi, 1999). The mechanisms of the generation of these high avidity anti-carbohydrate antibodies in non-pregnant women need to be established.

Here we hypothesize that these IgG2 class antibodies are generated against the glycans present in ovarian cysts which cross-bind to side sugar chains of hCG and/or its subunits. It is well known, that ovarian cyst fluids constitute one of the richest sources of mucins termed "blood group substances" (Morgan & van Heyningen, 1944; Toll et al., 2006; Wu, 1988). The carbohydrate chains of human ovarian cyst mucins are extremely heterogeneous with respect to both size and structure (Yu et al., 2009) and we propose that hCG and some mucin(s) in ovarian cyst fluid share carbohydrate determinant(s). This probably explains the heterogeneous binding of sera from different patients. Since IgG2 antibodies are generated following the T cell-dependent activation of B cells the carbohydrate moiety of ovarian cyst mucin(s) can be recognized by glycan-specific B cells, that receive necessary co-stimulation from the T

cells recognizing the same glycopeptidic antigens (Kappler & Hennet, 2020). Generated antibodies hence could bind to other glycoproteins bearing the carbohydrates with the similar moieties.

To confirm this 'sharing' hypothesis, we assessed the binding of four commercial monoclonal antibodies generated against different mucins isolated from ovarian cyst fluid, to hCG and its subunits. Our data demonstrated that monoclonal antibody - ab212418, which preferably binds to determinants of chain A and H type 3 (Gal1-3GalNAc-R) and 4 (Gal1-3GalNAc-R), but not to type 1 and 2 chain structures strongly cross-reacted with four out of five tested antigens - hCGαβ, hCGβ, hCGα, hCGβ CTP (Fig. 5). GalNAc-linked glycans, often referred to as mucin-type glycans, are O-linked oligosaccharides (Cahoreau et al., 2015), and the Galβ1-3GalNAc is the predominant O-glycan structure of standard pregnancy urine hCG according to Birken et al. (2003). We therefore propose that the common epitope to which ab212418 antibody binds on hCGαβ, hCGβ, hCGα, hCGβCTP molecules is formed by O-linked glycans. In fact, hCGβCTP bears four sites of O-glycosylation, and free hCGα used in our experiments contains one such site (Cole, 1987). hCGβCF to which ab212418 antibody does not bind contains only one site at Asn-78 for *N*-linked glycosylation (Kobata & Takeuchi, 1999), which might be conformationally hidden or insufficient for a high avidity binding. Monoclonal antibody SPM522 weakly cross-binds to hCGβ and hCGβCF, indicating that *N*-glycosylation sites within the hCGβ might contain Gal 1–3(Fuc 1–4) GlcNAc determinant (s).

That the naturally-occurring IgG2 antibodies bind not only to hCG and its subunits bearing the O- glycosylation sites, but to hCGβCF (peptide with *N*-glycans only) as well, indicates that the pool of detected antibodies is polyclonal. Moreover, the binding avidity to hCGβCF is the highest. It is possible that the binding avidity to the same epitope(s) within the hCGβCF on hCGβ and on the whole hCG are influenced by the tertiary and quaternary structures of these proteins respectively.

The levels of four classes of IgG (IgG1, igG2, IgG3, IgG4) change with age. The major changes are observed during childhood, puberty (Oxelius, 1979) and in elderly people (>75 years) (Khan et al., 2021), but there are not any significant differences in concentrations of IgG subclasses between young and middle-aged adults (Rasmussen et al., 2021). Our findings are consistent with these observations. We didn't find any age-related differences (data not shown) while analyzing our results, indicating that the prevalence of IgG2 antibodies in patients with ovarian cyst is not affected by age.

Our data demonstrates that carbohydrate branches on hCG and its subunits can react with some anti-carbohydrate IgG2 antibodies. Samples from a smaller subgroup of patients (44 %) contained IgM class of antibodies binding to various subunits of hCG above the control levels, and these could have derived from oligospecific B1 cells (Haji-Ghassemi et al., 2015).

It is well known that anti-carbohydrate antibodies are part of anti-tumor immune responses (Schwartz-Albiez, 2012) and, together with other naturally-occurring antibodies, they are gaining much scientific interest as potential clinically useful cancer biomarkers for early diagnosis, prognosis and informing development of novel therapeutics for poorly curable cancers (Yadav et al., 2019). Li et al. (2008) reported diagnostic potential of a panel of 13 autoantibodies against tumor associated antigens (TAAs) for the early detection of ovarian cancer with 62.5 % of sensitivity and 85.4 % of specificity (Li et al., 2008). Mucin 1 (MUC1) autoantibodies were shown to have prognostic value in predicting survival in ovarian cancer patients (Richards et al., 1998). Several monoclonal antibodies are showing promising results when they were incorporated into treatment regimens against ovarian cancer (Tse et al., 2014). Here we propose anti – carbohydrate antibody detection as a novel biomarker for early diagnosis of and/or predictor for therapy of ovarian cancers. The hypothesis would require further confirmation using a larger cohort of patients, both with ovarian cyst and ovarian carcinoma. Future studies should also include identification of a carbohydrate determinant which generates cross-binding antibodies to both tumour-associated mucins and hCG which would assess the importance of these antibodies in the ovarian cancer prevention leading to anti-tumour vaccine development.

In conclusion, our findings confirm the importance of further studies on these naturally-occurring anti-carbohydrate antibodies in patients with ovarian cysts that could lead to the development of novel tumour prevention, its stratification and early detection strategies.

5. Ethics approval and consent to participate

The present study was approved by the ethics committee of National Center for Disease Control and Public Health of Georgia (registration number – IRB000021). Written informed consent was obtained from the patients and healthy controls prior to their participation in the research.

CRediT authorship contribution statement

N. Chikadze: Conceptualization, Methodology, Investigation, Original draft preparation. **M. Tevzadze:** Investigation, Formal analysis. **M. Janelidze:** Investigation. **P. Lydyard:** Writing – review & editing. **N. Porakishvili:** Conceptualization, Methodology, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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