Proteomic investigation on bio-corona of Au, Ag and Fe nanoparticles for the discovery of triple negative breast cancer serum protein biomarkers

Maríadel Pilar Chantada-Vázquez^a, Antonio Castro López^b, María García Vence^c, Sergio Vázquez-Estévez^d, Benigno Acea-Nebril^e, David G. Calatayud^f, Teresa Jardiel^f, Susana B. Bravo^c, Cristina Núñez^a

^a Research Unit, Hospital Universitario Lucus Augusti (HULA), Servizo Galego de Saúde (SERGAS), 27002 Lugo, Spain

^b Breast Unit, Hospital Universitario Lucus Augusti (HULA), Servizo Galego de Saúde (SERGAS), 27002 Lugo, Spain

^c Proteomic Unit, Instituto de Investigaciones Sanitarias-IDIS, Complejo Hospitalario Universitario de Santiago de Compostela (CHUS), 15706 Santiago de Compostela, Spain

^d Oncology Division, Hospital Universitario Lucus Augusti (HULA), Servizo Galego de Saúde (SERGAS), 27002 Lugo, Spain

^e Department of Surgery, Breast Unit, Complejo Hospitalario Universitario A Coruña (CHUAC), SERGAS, A Coruña, Spain

^f Department of Electroceramics, Instituto de Cerámica y Vidrio-CSIC, Kelsen 5, Campus de Cantoblanco, 28049 Madrid, Spain

Highlights

- Interaction of AuNPs, AgNPs and MNPs with human serum formed a protein corona.
- Methods DDA for the qualitative analysis of the protein coronas.
- SWATH-MS for the quantitative analysis of the protein coronas.
- The analysis of the protein coronas allowed the identification of novel biomarkers of TNBC.
- Role of different protein biomarkers in the diagnosis of TNBC.

Abstract

Nowadays, there are no targeted therapeutic modalities for triple negative breast cancer (TNBC). This disease is associated with poor prognosis and worst clinical outcome because of the aggressive nature of the tumor, delayed diagnosis, and non-specific symptoms in the early stages. Therefore, identification of novel specific TNBC serum biomarkers for screening and therapeutic purposes remains an urgent clinical requirement.

New user-friendly and cheap methods for biomarker identification are needed, and nanotechnology offers new opportunities. When dispersed in blood, nanoparticles (NPs) are covered by a protein shell termed “protein corona” (PC). While alterations in protein patterns are challenging to detect by conventional blood analyses, PC acts as a “nano-concentrator” of serum proteins with affinity for NPs’ surface. So, the characterization of PC could allow the detection of otherwise undetectable changes in protein concentration at an early stage of the disease or after chemotherapy or surgery.

To explore this research idea, serum samples from 8 triple negative breast cancer (TNBC) patients and 8 patients without malignancy were allowed to interact with gold nanoparticles (AuNPs: 10.02 ± 0.91 nm), silver nanoparticles (AgNPs: 9.73 ± 1.70 nm) and magnetic nanoparticles (MNPs: 9.30 ± 0.67 nm). Here, in order to identify biomarker candidates in serum of TNBC patients, these nanomaterials were combined with electrophoretic separation (SDS-PAGE) to performed qualitative and quantitative comparisons of the serum proteomes of TNBC patients ($n = 8$) and healthy controls ($n = 8$) by liquid chromatography tandem-mass spectrometry (LC-MS/MS) analysis. The results were validated through a sequential window acquisition of all theoretical mass spectra (SWATH) analysis, performed in total serum samples (patients and controls) using this approach as a multiple reaction monitoring (MRM) analysis.

Significance

It is well known that several proteins presented in human serum are important biomarkers for the diagnosis or prognosis of different diseases, as triple negative breast cancer (TNBC). Determining how nanomaterials as gold nanoparticles (AuNPs: 10.02 ± 0.91 nm), silver nanoparticles (AgNPs: 9.73 ± 1.70 nm) and magnetic nanoparticles (MNPs: 9.30 ± 0.67 nm) interact with human serum will assist not only in understanding their effects on the biological system (biocompatibility and toxicity), but also to obtain information for developing novel nanomaterials with high specificity and selectivity towards proteins with an important biological function (prognostic and diagnostic protein biomarkers).

1. Introduction

Breast cancer (BC) is the most frequently diagnosed cancer and the leading cause of cancer death in women worldwide, accounting for 23% of total new cancer cases [1].

Mainly, triple negative breast cancer (TNBC) is a heterogeneous disease that is characterized by a lack of estrogen receptor/progesterone receptor (ER/PgR) expression

and absence of human epidermal growth factor receptor 2 (HER2) overexpression or amplification. This subgroup accounts for 12–15% of all types of breast cancer and exhibits a distinct molecular profile, clinical behavior, and response to therapy [2]. Notably, triple negative tumors are usually high grade and exhibit increased aggressiveness, poor prognosis, and worst clinical outcome [3]. Because hormonal (tamoxifen) and HER2-directed (trastuzumab) therapies are not effective, TNBC patients are managed with standard chemotherapy; however, a high rate of local and systemic relapse is frequently associated with treatment. Unfortunately, no useful biomarkers neither targeted therapeutic modalities exist for this breast cancer subtype [4].

It is well known that proteins secreted from tumor tissues have a higher likelihood of reaching the systemic circulation and may, therefore, serve as potential biomarkers for early detection [5]. Serum proteomics is a valuable tool that can facilitate comprehensive and systematic elucidation of the serum proteome under both healthy and disease conditions as well as identification of serum protein markers used for disease diagnosis and prognosis, particularly for identifying breast cancer-specific markers [5].

Current proteomic technologies that promote large-scale sample screening and facilitate the identification of proteins associated with disease and treatment are developing rapidly [6]. Mass spectrometry (MS), a powerful proteomics tool, has evolved to a high-throughput level, allowing rapid and accurate analysis of several thousand proteins in a single study [7]. Several studies have addressed the possibility of applying MS proteome analysis to diagnostics of TNBC, revealing protein patterns specific for patients with TNBC at either early or late clinical stages [8]. The peptide markers identified with differentiating patterns include glycolytic enzymes (as for example MDH2, PGK1, TKT, Aldolase1), cytokeratins (CK7, 8, 9, 14, 17, 19), further structure proteins (vimentin, fibronectin, L-plastin), for NME1-NME2, lactoferrin, and members of the Annexin family, among others [9].

SWATH-MS is an emerging technique that combines deep proteome coverage capabilities with quantitative consistency and accuracy [10]. Mainly, SWATH-MS analysis offers several advantages, including high reproducibility and reliability of quantitative information, in discovery proteomics [11]. Furthermore, SWATH-MS methods can be interchanged to MRM approaches focused on the validated biomarkers. Therefore, SWATH-MS is an important tool not only for the biomarker discovery but also for the development of preliminary validation studies [12].

However, currently available proteomic tests detect only a tiny fraction of potential biomarkers due to their deficient concentration in biofluids, in addition to the ‘swamping’ effect, caused by non-specific highly abundant molecules. The issue of signal-to-noise exceeds the current capability of proteomic analysis and therefore limits the diagnostic information that can be obtained [13].

To overcome these challenges, several approaches have been developed, comprising depletion of high-abundance proteins that mask less abundant proteins [14,15], chromatographic or electrophoretic sample prefractionation, to decrease complexity before mass spectrometric analyses [16,17], and direct isolation of preferred proteins [18]. Nevertheless, none of the methods can provide a standard solution to biomarker discovery

or can give a reproducible diagnostic platform for establishing biomarker guides. In this way, one promising way taken is the use of nanoscale materials [19].

Nanotechnology-based platforms hold great promise in addressing the above fundamental and technical issues of biomarker discovery to overcome persistent deficiencies of conventional methods. Currently, it is well known that the surfaces of nanoparticles (NPs) are rapidly covered by different types of biomolecules when they contact biological media called protein corona (PC) [20].

The protein composition and content in the corona depend on several parameters, including: i) physicochemical properties of the NPs (i.e. composition, size, shape, curvature, surface chemistry and surface charge, hydrophobicity/hydrophilicity) [21,22]; ii) characteristics of biological media (i.e. protein source, and temperature) [23,24]; iii) incubation time [25].

Notably, the composition of the protein corona varies among healthy individuals, as well as among patients with various diseases/medical conditions. Thus, the same NPs may have different protein coronas in different individuals. These alterations are often small and challenging to be detected by conventional blood analyses.

On the other side, the protein corona can act as a “nano-concentrator” [26] of those serum proteins with affinity for the NP surface. Therefore, characterization of protein corona could allow detecting minor changes in protein concentration at the very early stages of disease development or even after chemotherapy or surgery (i.e., when an alteration in circulating level of proteins could be undetectable by blood tests).

Keeping in mind that each disease is characterized by different plasma/serum proteomes, inducing the formation of different PCs on the same nanomaterial, M. Mahmoudi, et al. introduced the novel concept of “personalized protein corona” (PPC) [27]. More specifically, depending on the type, period and severity of the disease (which determines the serum/plasma alterations), each patient may have a personalized protein corona.

In the present study, gold nanoparticles (AuNPs: 10.02 ± 0.91 nm), silver nanoparticles (AgNPs: 9.73 ± 1.70 nm) and magnetic nanoparticles (MNPs: 9.30 ± 0.67 nm) were used to pre-concentrate and separate proteins from sera samples of eight patients with TNBC as well as from eight healthy people. For protein biomarkers identification and quantification, the proteome map changes between both groups were detected using a proteomic approach based on electrophoretic separation (SDS-PAGE) and mass spectrometry (nLC-MS/MS).

2. Materials and methods

2.1. Chemicals and reagents

All reagents and solvents used were HPLC-grade or higher. Sodium citrate tribasic dihydrate, tannic acid, silver nitrate, ammonium hydroxide,

iron(III) chloride hexahydrate and iron(II) sulfate heptahydrate, sodium borohydride (NaBH_4), trypsin, trifluoroacetic acid, DL-Dithiothreitol (DTT), Iodoacetamide (IAA),

acrylamide/bis-acrylamide 30% solution (37.5:1), Glycerol 86–88%, Tris-base, Coomassie Brilliant Blue R250 (CBB), sodium carbonate, and the Sigma Marker wide range 6.5–200 kDa were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium dodecyl sulfate (SDS) and formaldehyde were purchased from Panreac (Barcelona, Spain). β -mercaptoethanol was purchased from Merck (Hohen-Brunn, Germany), and bromophenol-blue was purchased from Riedel-de Haen (Seelze, Germany). Hydrogen tetrachloroaurate (III) hydrate (99.9%-Au) (49%Au) at 10% w/v was purchased from Strem Chemicals (Newburyport, MA, USA). Ammonium bicarbonate (ambic) and formic acid were purchased from Fluka (Steinheim, Germany).

2.2. Instrumentation

Microscopic characterizations of AuNPs, AgNPs, and MNPs were performed by transmission electron microscopy (TEM) using a Jeol JEM 1011 microscope. Samples for TEM were prepared by pipetting a drop of the colloidal dispersion onto an ultrathin carbon-coated copper grid and allowing the solvent to evaporate. AuNPs, AgNPs and MNPs ζ -potentials were measured at 25°C before and after protein corona formation using a Malvern Zetasizer Nano ZS instrument. For ζ -potential measurements samples were diluted in 1 mL milli-Q water and placed in Zetasizer disposable cuvettes. A minimum of 3 measurements per sample were made.

Power Pac Basic power supply from Bio-Rad (CA, USA) was used for sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) protein separation. Protein quantification was accomplished by measuring the absorbance at 280 nm with the use of a Qubit™ 4 Quantitation Starter Kit from Thermo Fisher Scientific. Gel image acquisition was carried out with a UVP PhotoDoc-It™ Imaging System from Analytik Jena.

2.3. Synthesis of inorganic nanoparticles

2.3.1. Synthesis of citrate-gold nanoparticles (10.02 ± 0.91 nm)

Gold nanoparticles (AuNPs) were synthesized by the citrate reduction method in aqueous solution [28]. Briefly, 60 ml of sodium citrate tribasic solution (0.075% w/v) was heated to 100 °C, and then gold was added as 54 μ L of 10% w/v of hydrogen tetrachloroaurate (III) hydrate solution. The reaction mixture was kept under reflux until a deep red color was detected. The solution of nanoparticles is chilled at room temperature and stored at 4 °C for a maximum of one month.

2.3.2. Synthesis of citrate-silver nanoparticles (9.73 ± 1.70 nm)

Silver nanoparticles (AgNPs) were synthesized by the citrate reduction method in aqueous solution by the method reported by V. Puentes et al. [29]. A 100 mL volume of an aqueous solution containing sodium citrate (SC) (5 mM) and tannic acid (TA) (0.025 mM) was prepared and heated with a heating mantle in a three-neck round-bottomed flask for 15 min under vigorous stirring. A condenser was used to prevent the evaporation of the solvent. After boiling had commenced, 1 mL of AgNO₃ (25 mM) was injected into this solution. The solution became bright yellow immediately. Resultant Ag NPs were purified by centrifugation at 18,000 $\times g$ to remove the excess of TA and further redispersed in Milli-Q-water before sample characterization.

2.3.3. Synthesis of Fe_3O_4 magnetic nanoparticles (9.30 ± 0.67 nm)

The synthesis of magnetic nanoparticles was performed according to a previously described procedure [30]. Briefly, 6 mL of concentrated ammonium hydroxide and 4 mL of water saturated with N_2 were mixed in a round-bottom flask under an inert atmosphere. Subsequently, in another vessel, 1 g of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ and 0.25–0.5 g of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ were dissolved in 10 mL of water saturated with N_2 . After mixing both solutions, the system was kept under constant stirring for 80 min at 80 °C. The final reaction product was washed three times with deionized water and magnetically separated for 24 h at room temperature.

2.4. Sample resources

Venous blood samples were obtained from eight females with triple negative breast cancer (TNBC) and eight disease-free individuals with the use of *VACUETTE® Serum Clot Activator Tubes* (10 mL). The collected blood samples were allowed to clot for 15 min and then centrifuged for 5 min at 4°C and 1,800×g. Sera were transferred into clean plastic tubes (1 mL) and immediately frozen at -80°C at Research Unit, Hospital Universitario Lucus Augusti (HULA). Clinical features of TNBC tumors, including tumor size, histology, receptor status, clinical stage, and nodal status, are summarized in Table 1.

Table 1. Clinical features of triple negative breast cancer tumors.

Characteristics		Number
Patients		8
Age (years)	< 40	1
	40–70	4
	> 70	3
Tumor size (cm)	< 2	3
	2–5	4
	> 5	1
Histological types	Ductal invasive carcinoma	8
Receptor status	Triple negative	8
Clinical stage	I	1
	II	4
	III	3
Nodal status	N0	7
	N1	1

2.5. Sample preparation

2.5.1. Optimization of protein corona formation in serum using magnetic nanoparticles

A series of serum sample aliquots were used for checking the effects of sample pH and temperature on the high-abundance proteins depletion using DTT, MNP/protein ratios, and pH of the medium on the washing steps.

2.5.1.1. Depletion of multiple high abundant proteins

Human serum aliquots (x8) were filtered with Miller-GP® Filter Unit (Millipore) with a size of 0.22 µm. Four aliquots of human serum (30 µL) were depleted with dithiothreitol (DTT) according to the protocol described by Warder et al. [31,32]. Briefly, fresh DTT 500 mM (3.3 µL) in milli-Q water was mixed with 30 µL of human serum and vortex quickly. Samples were then incubated at room temperature until a viscous white precipitate persisted (60 min), followed by centrifugation at $18,840 \times g$ for 20 min. Supernatants were transferred to a clean tube before the protein alkylation and nanoparticles (NPs) fractionation.

To evaluate the effects of sample pH and temperature on the high-abundance proteins depletion with DTT, four aliquots of human serum (30 µL) were depleted with dithiothreitol (DTT) following a modification of the protocol previously published by Arruda et al. [33]. Four aliquots of 30 µL of human serum were mixed fresh DTT 500 mM (3.3 µL) in ambic (12.5 mmol L^{-1}) and vortexed. Samples were incubated for 60 min at 37°C, centrifuged at $13,000 \times g$ for 40 min to separate supernatants to be alkylated and fractionated with nanoparticles (NPs).

2.5.1.2. NPs protein alkylation and fractionation

After protein depletion, the reduced SH-groups were alkylated with iodoacetic acid (IAA) for 45 min at room temperature and protected from light. Volumes of serum reduced and alkylated, were diluted to a final volume of 100 µL in Tris-HCl (0.1 mol L^{-1} , pH 5.5), and mixed with MNPs (5 µg) to obtain the following MNP/protein ratios: 1:1, 1:2, 1:4, 1:10. Then, all NPs-serum solutions were incubated at 25 °C with shaking (300 rpm) in a thermostatic bath for 30 min and then pellets were harvested by centrifugation at $20,186 \times g$ for 30 min. To evaluate the effects of sample pH on the stabilization of the protein corona in the washing steps, a fraction of pellets were washed (x3) with 50 µL of Tris-HCl (0.1 mol L^{-1} , pH 5.5) and another fraction with 50 µL of milli-Q water (x3). In both cases, pellets were harvested again by centrifugation at $20,186 \times g$ for 30 min to remove unbound proteins.

2.5.2. Incubation of nanoparticles with serum samples

Serum aliquots (x2) belonging to the eight disease-free individuals and eight triple negative breast cancer patients were depleted with dithiothreitol (DTT) following the method of Warder et al. [33,34]. After that, the reduced SH-groups were alkylated with iodoacetic acid (IAA) at room temperature (45 min in the dark).

After protein reduction and alkylation, serum aliquots ($\times 2$) belonging to the eight disease-free individuals and eight triple negative breast cancer patients were incubated with AuNPs (10.02 ± 0.91 nm) and AgNPs (9.73 ± 1.70 nm) (4 aliquots per individual, 2 with each nanoparticle type) following the method described by C. Núñez et al. [34]. Briefly, 75 μ L of AuNPs (10.02 ± 0.91 nm) and 75 μ L of AgNPs (9.73 ± 1.70 nm) were added to each different serum aliquots ($\times 2$) belonging to the eight disease-free individuals and eight triple negative breast cancer patients (4 aliquots per individual, 2 with each nanoparticle type), followed by the addition of 40 μ L of citrate/citric acid buffer to a final pH of 5.8. Then, all NPs-serum solutions were incubated at 37 °C with shaking in a thermostatic bath for 30 min. Pellets were harvested by centrifugation at $18,840 \times g$ for 30 min. In all cases, pellets containing proteins bound to nanoparticles were washed three times with 25 μ L citrate/citric acid buffer and harvested again by centrifugation at $18,840 \times g$ for 30 min to remove unbound proteins.

In the particular case of magnetic nanoparticles, each different reduced and alkylated serum aliquots ($\times 2$) from disease-free individuals ($n = 8$) and negative breast cancer patients ($n = 8$) were incubated (shaking at 300 rpm, 25 °C, 30 min) with 5 μ L of MNPs (9.30 ± 0.67 nm) after the addition of 87 μ L of Tris-HCl (0.1 mol L^{-1} , pH 5.5).

After centrifuging ($15,000 \times g$, 30 min), pellets were separated and washed ($\times 3$) with 50 μ L of Tris-HCl (0.1 mol L^{-1} , pH 5.5) and centrifuged again ($20,186 \times g$, 30 min).

2.5.3. Gel electrophoresis

After that, pellets were reconstituted in 10 μ L of a buffer with 0.2 M Tris-HCl, 2% w/v SDS and 20% v/v glycerol. This 10 μ L was mixed with 4 μ L of SDS-PAGE loading buffer (10% w/v SDS, Tris-Base 40 mM, pH 6.8, 50% v/v glycerol, 0.1% v/v bromophenol blue, 10% v/v β -mercaptoethanol) in a final volume of 20 μ L. Then, all samples were denatured by heating at 100 °C for 5 min and loaded into a 10% acrylamide/bis-acrylamide, stacking gel/12.5% acrylamide/bis-acrylamide running gel, of 1 mm thickness, and separated at 180 V (constant voltage) for 120 min. After electrophoresis, the gel was fixed for 30 minutes with 40% (v/v) ethanol and 10% (v/v) acetic acid and then stained overnight with Colloidal Coomassie Blue [35]. Gels were rinsed with distilled water and a 0.5 M sodium chloride solution until a clear background was observed. Gel imaging was carried out with a UVP PhotoDoc-ItTM Imaging System.

2.5.4. In-gel protein digestion

Protein bands were excised manually and transferred to 2.5-mL Lo-Bind tubes, and then washed twice with water and with 50% (v/v) acetonitrile/25 mM ammonium bicarbonate (ambic) until the blue color disappeared.

Before the trypsin digestion, gel spots were washed with 25 mM ambic and dehydrated with acetonitrile. Then, 30 μ L of trypsin ($20 \text{ ng } \mu\text{L}^{-1}$ in 12.5 mM ambic/2% (v/v) acetonitrile) was added to the gel spots and incubated for 60 min at 0 °C.

After this time, gel spots were inspected, trypsin solution not absorbed into the gel was removed, and the gels were covered with 100 μ L of 12.5 mM ambic. Samples were incubated for 12 h at 37 °C. Then 50 μ L of 5% (v/v) formic acid was added, and the

supernatant was transferred to a new Lo-Bind tube and the peptides were further extracted from the gel twice with 50% (v/v) acetonitrile/0.1% (v/v) trifluoroacetic acid (TFA) ($\times 3$) and acetonitrile (ACN) ($\times 1$). Samples were dried-down and stored at -20°C [36].

2.6. Protein identification by mass spectrometry (LC-MS/MS) and data analysis

Digested peptides of each sample were separated using Reverse Phase Chromatography. The gradient was developed using a micro liquid chromatography system (Eksigent Technologies nanoLC 400, SCIEX) coupled to high-speed Triple TOF 6600 mass spectrometer (SCIEX) with a microflow source. The analytical column used was a silica-based reversed phase column Chrom XP C18 150×0.30 mm, 3 mm particle size and 120 Å pore size (Eksigent, SCIEX). The trap column was a YMC-TRIART C18 (YMC Technologies, Teknokroma with a 3 mm particle size and 120 Å pore size, switched on-line with the analytical column. The loading pump delivered a solution of 0.1% formic acid in water at 10 $\mu\text{L}/\text{min}$. The micro-pump provided a flow-rate of 5 $\mu\text{L}/\text{min}$ and was operated under gradient elution conditions, using 0.1% formic acid in water as mobile phase A, and 0.1% formic acid in acetonitrile as mobile phase B. Peptides were separated using a 25 minutes gradient ranging from 2% to 90% mobile phase B (mobile phase A: 2% acetonitrile, 0.1% formic acid; mobile phase B: 100% acetonitrile, 0.1% formic acid). The injection volume was 4 μL .

Data acquisition was carried out in a TripleTOF 6600 System (SCIEX, Foster City, CA) using a Data dependent workflow. Source and interface conditions were as follows: ion spray voltage floating (ISVF) 5500 V, curtain gas (CUR) 25, collision energy (CE) 10 and ion source gas 1 (GS1) 25. The instrument was operated with Analyst TF 1.7.1 software (SCIEX, USA). Switching criteria were set to ions greater than mass to charge ratio (m/z) 350 and smaller than m/z 1400 with a charge state of 2–5, mass tolerance 250 ppm and an abundance threshold of more than 200 counts (cps). Former target ions were excluded for 15s. The instrument was automatically calibrated every 4 hours using as external calibrant tryptic peptides from PepcalMix (Sciex).

After MS/MS analysis, data files were processed using ProteinPilot™ 5.0.1 software from Sciex, which uses the algorithm Paragon™ for database search and Progroup™ for data grouping. Data were searched using a Human-specific UniProt database. False discovery rate was performed using a non-linear fitting method displaying only those results that reported a 1% Global false discovery rate or better [37,38].

2.7. Protein quantification by SWATH (Sequential Window Acquisition of all Theoretical Mass Spectra)

2.7.1. Creation of the spectral library

To construct the MS/MS spectral libraries, the peptide solutions were analyzed by a shotgun data-dependent acquisition (DDA) approach by micro-LC-MS/MS. To get a good representation of the peptides and proteins present in all samples, pooled vials of samples from each group (control and triple negative breast cancer patients) were prepared using equal mixtures of the original samples. 4 μL (4 mg) of each pool was separated into a micro-LC system Eksperit nLC425 (Eksigent, Dublin, CA, USA) using a column Chrom XP C18 150×0.30 mm, 3 mm particle size and 120 Å pore size (Eksigent,

Sciex) at a flow rate of 5 μ L/min. Water and ACN, both containing 0.1% formic acid, were used as solvents A and B, respectively. The gradient run consisted of 5–95% B for 30 min, 5 min at 90% B and finally 5 min at 5% B for column equilibration, for a total run time of 40 min. When the peptides eluted, they were directly injected into a hybrid quadrupole-TOF mass spectrometer Triple TOF 6600 (Sciex, Redwood City, CA, USA) operated with a data-dependent acquisition system in positive ion mode. A Micro source (Sciex) was used for the interface between microLC and MS, with an application of 2600 V voltage. The acquisition mode consisted of a 250 ms survey MS scan from 400 to 1250 m/z followed by an MS/MS scan from 100 to 1500 m/z (25 ms acquisition time) of the top 65 precursor ions from the survey scan, for a total cycle time of 2.8 s. The fragmented precursors were then added to a dynamic exclusion list for 15 s; any singly charged ions were excluded from the MS/MS analysis.

The peptide and protein identifications were performed using Protein Pilot software (version 5.0.1, Sciex) with a Data were searched using a Human-specific UniProt database, specifying iodoacetamide as Cys alkylation. The false discovery rate (FDR) was set to 1 for both peptides and proteins. The MS/MS spectra of the identified peptides were then used to generate the spectral library for SWATH peak extraction using the add-in for PeakView Software (version 2.2, Sciex) MS/MSALL with SWATH Acquisition MicroApp (version 2.0, Sciex). Peptides with a confidence score above 99% (as obtained from Protein Pilot database search) were included in the spectral library).

2.7.2. Relative quantification by SWATH acquisition

SWATH-MS (Sequential Window Acquisition of all Theoretical Mass Spectra) acquisition was performed on a TripleTOF® 6600 LC-MS/MS system (Sciex). Samples from control and triple negative breast cancer patients were analyzed using data-independent acquisition (DIA) method (30 total samples). Each sample (4 μ L (from a mg/ml solution) was analyzed using the LC-MS equipment and LC gradient described above for building the spectral library but instead using the SWATH-MS acquisition method. The method consisted of repeating a cycle that consisted of the acquisition of 65 TOF MS/MS scans (400–1500 m/z, high sensitivity mode, 50 ms acquisition time) of overlapping sequential precursor isolation windows of variable width (1 m/z overlap) covering the 400–1250 m/z mass range with a previous TOF MS scan (400–1500 m/z, 50 ms acquisition time) for each cycle. The total cycle time was 6.3 s. For each sample set, the width of the 100 variable windows was optimized according to the ion density found in the DDA runs using a SWATH variable window calculator worksheet from Sciex.

2.7.3. Data analysis

The targeted data extraction of the fragment ion chromatogram traces from the SWATH runs was performed by PeakView (version 2.2) using the SWATH Acquisition MicroApp (version 2.0). This application processed the data using the spectral library created from the shotgun data. Up to ten peptides per protein and seven fragments per peptide were selected, based on signal intensity; any shared and modified peptides were excluded from the processing. Five-minute windows and 30 ppm widths were used to extract the ion chromatograms; SWATH quantitation was attempted for all proteins in the ion library that were identified by ProteinPilot with an FDR below 1%.

The retention times from the peptides that were selected for each protein were realigned in each run according to the iRT peptides spiked in each sample and eluted along the whole-time axis. The extracted ion chromatograms were then generated for each selected fragment ion; the peak areas for the peptides were obtained by summing the peak areas from the corresponding fragment ions. PeakView computed an FDR and a score for each assigned peptide according to the chromatographic and spectral components; only peptides with an FDR below 1% were used for protein quantitation. Protein quantitation was calculated by adding the peak areas of the corresponding peptides.

The integrated peak areas (processed, mrkvw files from PeakView) were directly exported to the MarkerView software (Sciex) for relative quantitative analysis. The export will generate three files containing quantitative information about individual ions, the summed intensity of different ions for a particular peptide, and the summed intensity of different peptides for a specific protein. MarkerView has been used for the analysis of SWATH-MS data reported in other proteomics studies [39,40] because of its data-independent method of quantitation. MarkerView uses processing algorithms that accurately find chromatographic and spectral peaks direct from the raw SWATH data. Data alignment by MarkerView compensates for minor variations in both mass and retention time values, ensuring that identical compounds in different samples are accurately compared to one another.

To control for possible uneven sample loss across the different samples during the sample preparation process, we performed a global normalization based on the total sum of all the peak areas extracted from all the peptides and transitions across the replicates of each sample. Unsupervised multivariate statistical analysis using principal component analysis (PCA) was performed to compare the data across the samples. The average MS peak area of each protein was derived from the biological replicates of the SWATH-MS of each sample followed by Student's *t*-test analysis using the MarkerView software for comparison among the samples based on the averaged area sums of all the transitions derived for each protein. The *t*-test will indicate how well each variable distinguishes the two groups, reported as a *p*-value. To set of differentially expressed proteins (*p*-value <0.05) with a 1.5 fold in- or decrease was selected.

Functional analysis was performed by FunRich open access software (Functional Enrichment analysis tool) for functional enrichment and interaction network analysis (<http://funrich.org/index.html>).

2.8. TNBC biomarkers validation

A SWATH-MS analysis was performed using the same conditions described in [Section 2.7](#). In the validation phase, total serum samples from control and triple negative breast cancer patients previously depleted with DTT were used.

To perform the biomarker validation, the SWATH library was performed using not only serum pools from incubation with the different nanoparticles but also pools of total serum samples. Therefore, we improve our library and perform better protein quantification to achieve biomarker validation.

3. Results

Following the synthetic methods described by R. López-Cortés [30], V. Puentes et al. [31] and F. Schüth et al. [32], AuNPs (10.02 ± 0.91 nm), AgNPs (9.73 ± 1.70 nm) and MNPs (9.30 ± 0.67 nm) were successfully obtained, respectively.

The sizes and ζ -potential of AuNPs (10.02 ± 0.91 nm), AgNPs (9.73 ± 1.70 nm) and MNPs (9.30 ± 0.67 nm) were examined before and after their incubation with two pools of human blood serum from healthy individuals and triple negative breast cancer patients, following the conditions described in [Section 2.5.2](#).

TEM and ζ -potential measurements after the incubation of AuNPs (10.02 ± 0.91 nm), AgNPs (9.73 ± 1.70 nm) and MNPs (9.30 ± 0.67 nm) with serum demonstrated that, in all cases, the size did not change significantly and the surface charge remained negative (see Figs. S1–S10). Upon serum incubation, the mean particle surface charge of the AuNPs (10.02 ± 0.91 nm) increased (became less negative) from -37.0 mV to -29.7 mV, and the same measurement for the MNPs (9.30 ± 0.67 nm) increased from -30.5 mV to -29.3 mV. However, upon serum incubation the mean particle surface charge of the AgNPs (9.73 ± 1.70 nm) decreased (became more negative) from -27.4 mV to -30.0 mV. These results are in agreement with previous studies suggesting that negatively charged NPs do not exclusively interact with positively charged proteins, as electrostatic interactions are not the only driving force behind NP-corona interactions [41,42]. Interestingly, for AuNPs (10.02 ± 0.91 nm) and MNPs (9.30 ± 0.67 nm), where the ζ -potential was shifted toward less negative values, it could be suggested preferential interaction with positively charged proteins. The presence of negatively charged proteins can be explained by a sequential model of protein binding, in which positively charged proteins initially bind the NP, followed by negatively charged ones [45,46].

3.1. Optimization of parameters for the protein corona formation in serum using MNPs

As mentioned above, a great number of variables could influence the efficiency of protein adsorption on the MNPs surface [43]. For this reason, three parameters were evaluated: (i) the effects of sample pH and temperature on the depletion of high-abundance proteins presented in serum using DTT, (ii) MNP/protein ratios; (iii) and pH of the medium on the washing steps. For this study, shaking and incubation temperature were previously defined as 300 rpm and 25°C , respectively.

In order to evaluate the effects of sample pH and temperature on the depletion of high-abundance proteins, four human serum samples ($\times 2$) ($30\ \mu\text{L}$) were depleted with fresh DTT 500 mM ($3.3\ \mu\text{L}$) in milli-Q H_2O for 60 min at room temperature (protocol of Warder et al. [33,34]), and four human serum samples ($\times 2$) ($30\ \mu\text{L}$) were depleted with fresh DTT 500 mM ($3.3\ \mu\text{L}$) in ambic ($12.5\ \text{mmol L}^{-1}$) for 60 min at 37°C (modification of protocol described by Arruda [35]). In both cases, after the incubation and centrifugation, supernatants were transferred to a clean tube before the protein alkylation and nanoparticles (NPs) fractionation. Depletion with fresh DTT 500 mM in milli-Q for 60 min at room temperature showed more reproducible results (see Fig. S4).

Protein concentration is another critical parameter that may affect the capacity and kinetics of protein adsorption. To investigate the influence of the MNP/protein ratio on the formation of the protein corona, volumes of serum reduced and alkylated ($\times 2$) were mixed with MNPs (9.30 ± 0.67 nm), at MNP/protein ratios of 1:1, 1:2, 1:4, and 1:10 (see [Section 2.5.1](#)). Maintaining the amount of adsorbent (*i.e.*, MNPs) constant and increasing the protein concentration, would be expected to lead to a decrease of available adsorption sites, reducing the efficiency of protein removal [\[44\]](#). As a compromise between MNPs and protein corona formation, the 1:2 ratio (MNP/protein) was then selected for future experiments (see Fig. S5).

The pH value is an essential parameter because it influences the charge state of proteins, therefore influencing their interaction with MNPs [\[45\]](#). To evaluate the effects of sample pH on the stabilization of the protein corona in the washing steps, a fraction of pellets were washed three times with 50 μ L Tris-HCl (0.1 mol L^{-1} , pH 5.5) and another fraction was washed three times with milli-Q water. The first one was selected as the preferred method, because the washes with milli-Q water promoted the destabilization of the protein corona formed around the MNPs, due to the modifications of the pH (data not shown).

3.2. Serum fraction preparation and protein corona purification (patients vs. controls)

Serum aliquots ($\times 2$) belonging to the eight disease-free individuals and serum samples from eight triple negative breast cancer patients were depleted with dithiothreitol (DTT) according to the protocol described by Warder et al. [\[33,34\]](#). After that, the reduced SH-groups were alkylated with iodoacetic acid (IAA) for 45 min at room temperature and protected from light.

After protein reduction and alkylation, serum aliquots ($\times 2$) belonging to the eight disease-free individuals and eight triple negative breast cancer patients were incubated with AuNPs (10.02 ± 0.91 nm), AgNPs (9.73 ± 1.70 nm) and MNPs (9.30 ± 0.67 nm) (6 aliquots per individual, 2 with each nanoparticle type) and further processed as described in Section 2.5.2.

Two protein fractions were thus obtained in each case, one in the supernatant and the second one attached to the surface of each nanoparticles types (protein corona). Then, protein fractions (AuNPs-protein corona, AgNPs-protein corona, MNPs-protein corona) were separately loaded onto a 1D-SDS-PAGE. Proteins were separated and, after staining, gel bands were excised and submitted to the sample incubation described in [Section 2.5.4](#). The resulting peptides were then analyzed by mass spectrometry (LC-MS/MS) for protein identification.

[Fig. 1](#) shows the 1D gels for the protein corona formed around the three different NPs (AuNPs-protein corona, AgNPs-protein corona, MNPs-protein corona) visible after Coomassie staining. As may be seen, it is quickly noted that there is a difference in the intensity of the bands on the gel profiles for the healthy controls (from C1 to C8) and the patients (from P1 to P8) for each type of nanoparticle. However, no conclusion can be drawn unless the proteins are identified.

Fig. 1. 1D-SDS-PAGE of protein coronas formed around 10.02 ± 0.91 nm gold nanoparticles (AuNPs), 9.73 ± 1.70 nm silver nanoparticles (AgNPs) and 9.30 ± 0.67 nm magnetic nanoparticles (MNPs) after their incubation with serum aliquots (x2) belonging to the eight disease-free individuals (C1–C8) and eight triple negative breast cancer patients (P1–P8). On the left, it marks the lane with Mw protein standards.

As Fig. 2 shows, 192, 161 and 142 proteins were commonly detected in the protein corona formed around AuNPs, AgNPs, and MNPs, after their incubation with serum samples of the eight triple negative breast cancer patients and eight healthy controls, respectively.

Fig. 2. Quantitative Venn diagrams showing the number of identified proteins found in the protein corona of 10.02 ± 0.91 nm gold nanoparticles (AuNPs), 9.73 ± 1.70 nm silver nanoparticles (AgNPs) and 9.30 ± 0.67 nm magnetic nanoparticles (MNPs) after their incubation with serum from eight triple negative breast cancer patients and eight healthy controls.

In the particular case of all serum samples from healthy controls, 285, 292 and 206 were identified on the surface of AuNPs (10.02 ± 0.91 nm), AgNPs (9.73 ± 1.70 nm) and MNPs (9.30 ± 0.67 nm), respectively. From them, 149 proteins were commonly detected in the protein corona of all three different types of NPs. However, 202 different proteins were found on the three distinct NPs surface: 71 different proteins on the AuNPs, 85 on the AgNPs, and 46 individual proteins on the MNPs (see Table S1).

Furthermore, 231, 206 and 203 were found in the surface of AuNPs (10.02 ± 0.91 nm), AgNPs (9.73 ± 1.70 nm) and MNPs (9.30 ± 0.67 nm) after their incubation with all serum aliquots ($\times 2$) from eight triple negative breast cancer patients (6 protein samples per individual: 2 incubated with AuNPs, 2 with AgNPs and 2 with MNPs) (see Table S2). A total of 138 proteins were commonly found in the protein corona of all three different types of NPs. However, 142 different proteins were found in the three different NPs surface: 56 different proteins on the AuNPs, 33 on the AgNPs and 53 individual proteins on the MNPs.

Fractionation of the proteome using AuNPs, AgNPs, and MNPs allows for the identification of 39 (see Table 2), 45 (see Table 3) and 61 (see Table 4) protein biomarkers in the pellet of all patient samples, respectively (see Fig. 3). Remarkably, these proteins were not identified in the healthy control. These potential biomarkers came from different cellular components; most of them from the nucleus and cytoplasm (see Fig. 4). Protein biomarkers also showed different functionality and are constituted by proteins implicated in the immune response, followed by proteins with an enzymatic function, structural, transporter, inflammatory, signal transduction, and with antibiotic/antibacterial properties.

Table 2. Selection of identified single-detected corona proteins bound to the 10.02 ± 0.91 nm AuNPs after 30 min incubation and subsequent washing.

Protein name	UniProt name	Entry name	Gene	Mass (kDa)	Function
Actin, cytoplasmic 2	ACTG_HUMAN	P63261	ACTG1	41.7	Cell mobility
Atypical kinase COQ8B, mitochondrial	COQ8B_HUMAN	Q96D53	COQ8B	60.1	Involved in the biosynthesis of coenzyme Q
Beta-ureidopropionase	BUP1_HUMAN	Q9UBR1	UPB1	43.1	Catalytic activity
Brefeldin A-inhibited guanine nucleotide- exchange protein 3	BIG3_HUMAN	Q5TH69	ARFGEF3	240.6	Participates in the regulation of systemic glucose homeostasis
85/88 kDa calcium- independent phospholipase A2	PLPL9_HUMAN	O60733	PLA2G6	89.9	Catalytic activity
Cilia- and flagella- associated protein 100	CP100_HUMAN	Q494V2	CFAP100	71.1	Plays a role in ciliary/flagellar motility by regulating the assembly and the activity of axonemal inner dynein arm

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Protein name	UniProt name	Entry name	Gene	Mass (kDa)	Function
Coagulation factor XI	FA11_HUMAN	P03951	F11	70.1	Catalytic activity
Contactin-associated protein-like 2	CNTP2_HUMAN	Q9UHC6	CNTNAP2	148.2	Plays a role in the formation of functional distinct domains critical for saltatory conduction of nerve impulses in myelinated nerve fibers
DNA topoisomerase 1	TOP1_HUMAN	P11387	TOP1	90.7	Catalytic activity
Dynein assembly factor 1, axonemal	DAAF1_HUMAN	Q8NEP3	DNAAF1	80.0	Plays a role in cytoplasmic preassembly of dynein arms
E3 ubiquitin-protein ligase SHPRH	SHPRH_HUMAN	Q149N8	SHPRH	193.1	Enzyme involved in DNA repair
Estrogen sulfotransferase	ST1E1_HUMAN	P49888	SULT1E1	35.1	Catalytic activity. May play a role in the regulation of estrogen receptor activity by metabolizing free estradiol
[F-actin]-monooxygenase MICAL3	MICA3_HUMAN	Q7RTP6	MICAL3	224.3	Catalytic activity
Glial fibrillary acidic protein	GFAP_HUMAN	P14136	GFAP	49.9	Cell-specific marker during the development of the central nervous system
Histone-lysine N-methyltransferase 2D	KMT2D_HUMAN	O14686	KMT2D	593.4	Catalytic activity. Acts as a coactivator for estrogen receptor by being recruited by ESR1, thereby activating transcription
Immunoglobulin lambda variable 3-27	LV327_HUMAN	P01718	IGLV3-27	12.2	Immune response

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Protein name	UniProt name	Entry name	Gene	Mass (kDa)	Function
Inositol hexakisphosphate kinase 2	IP6K2_HUMAN	Q9UHH9	IP6K2	49.2	Catalytic activity
Inositol 1,4,5-trisphosphate receptor type 3	ITPR3_HUMAN	Q14573	ITPR3	304.1	Receptor for inositol 1,4,5-trisphosphate
Intraflagellar transport protein 74 homolog	IFT74_HUMAN	Q96LB3	IFT74	69.2	Transporter activity
Janus kinase and microtubule-interacting protein 1	JKIP1_HUMAN	Q96N16	JAKMIP1	73.2	Plays a role in the microtubule-dependent transport of the GABA-B receptor
Lebercilin	LCA5_HUMAN	Q86VQ0	LCA5	80.5	Transporter activity
MaFF-interacting protein	MAFIP_HUMAN	Q8WZ33	MAFIP	13.9	Inhibits cell growth and colony-forming efficiency
Matrix metalloproteinase-9	MMP9_HUMAN	P14780	MMP9	78.5	Catalytic activity
Microtubule-associated protein 2	MTAP2_HUMAN	P11137	MAP2	199.5	Stabilizes the microtubules against depolymerization
Mucolipin-3	MCLN3_HUMAN	Q8TDD5	MCOLN3	64.2	Pays a role in the regulation of membrane trafficking events
Multidrug resistance protein 1	MDR1_HUMAN	P08183	ABCB1	141.5	Responsible for decreased drug accumulation in multidrug-resistant cells
Nebulette	NEBL_HUMAN	O76041	NEBL	116.4	Plays an important role in the assembly of the Z-disk
Nucleoporin 188 homolog	NUP188_HUMAN	Q5SRE5	NUP188	196.1	May function as a component of the nuclear pore complex (NPC)

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Protein name	UniProt name	Entry name	Gene	Mass (kDa)	Function
PC membrane recruitment protein 2	AMER2_HUMAN	Q8N7J2	AMER2	69.5	Negative regulator of the canonical Wnt signaling pathway involved in neuroectodermal patterning
Protein ELYS	ELYS_HUMAN	Q8WYP5	AHCTF1	252.5	Required for the assembly of a functional nuclear pore complex (NPC) on the surface of chromosomes
Protein phosphatase 1 regulatory subunit 26	PPR26_HUMAN	Q5T8A7	PPP1R26	127.3	Inhibits phosphatase activity of protein phosphatase 1 (PP1) complexes. May positively regulate cell proliferation.
Protein ZGRF1	ZGRF1_HUMAN	Q86YA3	ZGRF1	236.6	Zinc ion binding that inhibits factors Xa and XIa of the coagulation cascade
Putative transmembrane protein encoded by LINC00477	CL067_HUMAN	Q96M19	LINC00477	18.2	Product of a dubious CDS prediction. May be a non-coding RNA
Serine protease 33	PRS33_HUMAN	PRSS33	PRSS33	29.8	Catalytic activity
Serine-protein kinase ATM	ATM_HUMAN	Q13315	ATM	350.7	Activates checkpoint signaling upon double strand breaks (DSBs), apoptosis and genotoxic stresses
Serum amyloid A-1 protein	SAA1_HUMAN	P0DJ18	SAA1	13.5	Inflammatory response
Sodium-dependent noradrenaline transporter	SC6A2_HUMAN	P23975	SLC6A2	69.3	Transporter activity

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Protein name	UniProt name	Entry name	Gene	Mass (kDa)	Function
Suppressor of tumorigenicity protein	7 ST7_HUMAN	Q9NRC1	ST7	67.2	Acts as a tumor suppressor
Vasorin	VASN_HUMAN	Q6EMK4	VASN	71.7	May act as an inhibitor of TGF-beta signaling

The accession number, gene name, species (Human), molecular weight (kDa) and protein function were reported.

Table 3. Selection of identified single-detected corona proteins bound to the 9.73 ± 1.70 nm AgNPs after 30 min incubation and subsequent washing.

Protein name	UniProt name	Entry name	Gene	Mass (kDa)	Function
Amyloid-beta A4 precursor protein-binding member 2	A APBA2_HUMAN	Q99767	APBA2	82.5	Function in synaptic vesicle exocytosis by binding to STXBP1, an essential component of the synaptic vesicle exocytotic machinery
Ankyrin repeat domain-containing protein 20B	AN20B_HUMAN	Q5CZ79	ANKRD20A8P	93.9	–
Ankyrin repeat and sterile alpha motif domain-containing protein 1B	ANS1B_HUMAN	Q7Z6G8	ANKS1B	138.1	Plays a role as a modulator of APP processing
Bone morphogenetic protein 10	BMP10_HUMAN	O95393	BMP10	48.0	Inhibits endothelial cell migration and growth. May reduce cell migration and cell matrix adhesion in breast cancer cell lines.
Bromodomain adjacent to zinc finger domain protein 2A	BAZ2A_HUMAN	Q9UIF9	BAZ2A	211.2	Essential component of the NoRC (nucleolar remodeling complex) complex

Protein name	UniProt name	Entry name	Gene	Mass (kDa)	Function
					that mediates silencing of a fraction of rDNA
Caspase recruitment domain-containing protein 11	CAR11_HUMAN	Q9BXL7	CARD11	133.3	Involved in the costimulatory signal essential for T-cell receptor (TCR)-mediated T-cell activation. Also activates the TORC1 signaling pathway
Complement factor H-related protein 3	FHR3_HUMAN	Q02985	CFHR3	37.3	Involved in complement regulation
Complement factor H-related protein 4	FHR4_HUMAN	Q92496	CFHR4	65.3	Plays a role in lipid metabolism
Cyclin-dependent kinase 2	CDK2_HUMAN	P24941	CDK2	33.9	Catalytic activity
Dual specificity protein phosphatase 9	DUS9_HUMAN	Q99956	DUSP9	41.9	Inactivates MAP kinases
Dystonin	DYST_HUMAN	Q03001	DST	860.6	Acts as an integrator of intermediate filaments, actin and microtubule cytoskeleton networks
EGF-containing fibulin-like extracellular matrix protein 1	FBLN3_HUMAN	Q12805	EFEMP1	54.6	Binds EGFR, the EGF receptor, inducing EGFR autophosphorylation and the activation of downstream signaling pathways. May play a role in cell adhesion and migration
GREB1-like protein	GRB1L_HUMAN	Q9C091	GREB1L	214.3	Plays a major role in early metanephros

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Protein name	UniProt name	Entry name	Gene	Mass (kDa)	Function
					and genital development
Hemoglobin subunit gamma-2	HBG2_HUMAN	P69892	HBG2	16.1	Gamma chains make up the fetal hemoglobin F, in combination with alpha chains
Histone-lysine methyltransferase 2A	N-KMT2A_HUMAN	Q03164	KMT2A	431.8	Catalytic activity
Immunoglobulin heavy variable 3-23	HV323_HUMAN	P01764	IGHV3-23	12.6	Immune response
Importin-4	IPO4_HUMAN	Q8TEX9	IPO4	118.7	Functions in nuclear protein import as nuclear transport receptor
Keratin, type I cytoskeletal 9	K1C9_HUMAN	P35527	KRT9	62.1	Structural
LINE-1 type transposase domain-containing protein 1	L1TD1_HUMAN	Q5T7N2	L1TD1	98.8	Single-stranded RNA binding
Lysosomal-trafficking regulator	LYST_HUMAN	Q99698	LYST	429.1	Required for sorting endosomal resident proteins into late multivesicular endosomes
Matrix metalloproteinase-9	MMP9_HUMAN	P14780	MMP9	78.5	Catalytic activity
Microtubule-associated protein 1A	MAP1A_HUMAN	P78559	MAP1A	305.5	Structural protein involved in the filamentous cross-bridging between microtubules and other skeletal elements
MORC family CW-type zinc finger protein 1	MORC1_HUMAN	Q86VD1	MORC1	112.9	Zinc ion binding.

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Protein name	UniProt name	Entry name	Gene	Mass (kDa)	Function
Nuclear distribution protein nudE-like 1	NDEL1_HUMAN	Q9GZM8	NDEL1	38.4	Required for organization of the cellular microtubule array and microtubule anchoring at the centrosome
Polymeric immunoglobulin receptor	PIGR_HUMAN	P01833	PIGR	83.3	This receptor binds polymeric IgA and IgM at the basolateral surface of epithelial cells
Polypeptide N-acetylgalactosaminyltransferase 13	GLT13_HUMAN	Q8IUC8	GALNT13	64.0	Catalytic activity
Probable guanine nucleotide exchange factor MCF2L2	MF2L2_HUMAN	Q86YR7	MCF2L2	126.9	Functions as a guanine nucleotide exchange factor
Pro-epidermal growth factor	EGF_HUMAN	P01133	EGF	133.9	Stimulates the growth of various epidermal and epithelial tissues in vivo and in vitro
Properdin	PROP_HUMAN	P27918	CFP	51.3	A positive regulator of the alternate pathway of complement
Protein MMS22-like	MMS22_HUMAN	Q6ZRQ5	MMS22L	142.3	Maintain genome integrity during DNA replication
Protein phosphatase homolog PTC7	PPTC7_HUMAN	Q8NI37	PPTC7	32.6	Catalytic activity
Protein Shroom3	SHRM3_HUMAN	Q8TF72	SHROOM3	216.8	Controls cell shape changes in the neuroepithelium during neural tube closure

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Protein name	UniProt name	Entry name	Gene	Mass (kDa)	Function
Protein ZGRF1	ZGRF1_HUMAN	Q86YA3	ZGRF1	236.6	Zinc ion binding that inhibits factors Xa and XIa of the coagulation cascade
Putative solute carrier organic anion transporter family member 1B7	SO1B7_HUMAN	G3V0H7	SLCO1B7	71.2	May encode a non-functional truncated protein
Ras-interacting protein 1	RAIN_HUMAN	Q5U651	RASIP1	103.4	Acts as a critical and vascular-specific regulator of GTPase signaling, cell architecture, and adhesion
Ribosomal protein S6 kinase alpha-1	KS6A1_HUMAN	Q15418	RPS6KA1	82.7	Catalytic activity
Short coiled-coil protein	SCOC_HUMAN	Q9UIL1	SCOC	18.0	Positive regulator of amino acid starvation-induced autophagy
Structural maintenance of chromosomes protein 6	SMC6_HUMAN	Q96SB8	SMC6	126.3	Structural
Synaptotagmin-5	SYT5_HUMAN	O00445	SYT5	42.9	May be involved in Ca ²⁺ -dependent exocytosis of secretory vesicles
Tudor domain-containing protein 1	TDRD1_HUMAN	Q9BXT4	TDRD1	132.0	Acts via the piRNA metabolic process, which mediates the repression of transposable elements during meiosis
Tyrosine--tRNA cytoplasmic ligase,	SYYC_HUMAN	P54577	YARS	59.1	Catalytic activity

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Protein name	UniProt name	Entry name	Gene	Mass (kDa)	Function
Vigilin	VIGLN_HUMAN	Q00341	HDLBP	141.4	Protect cells from over-accumulation of cholesterol
von Willebrand factor	VWF_HUMAN	P04275	VWF	220	Plays a major role in blood coagulation
Wee1-like protein kinase	WEE1_HUMAN	P30291	WEE1	71.6	Acts as a negative regulator of entry into mitosis
Zinc finger protein 114	ZN114_HUMAN	Q8NC26	ZN114	47.7	May be involved in transcriptional regulation.

The accession number, gene name, species (Human), molecular weight (kDa) and protein function were reported.

Table 4. Selection of identified single-detected corona proteins bound to the 9.30 ± 0.67 nm MNPs after 30 min incubation and subsequent washing.

Protein name	UniProt name	Entry name	Gene	Mass (kDa)	Function
ATP-binding cassette sub-family B member 5	ABCB5_HUMAN	Q2M3G0	ABCB5	138.6	Transporter activity
Bile salt-activated lipase	CEL_HUMAN	P19835	CEL	79.3	Catalytic activity
Cathelicidin antimicrobial peptide	CAMP_HUMAN	P49913	CAMP	19.3	Antibacterial activity
Ceramide synthase 4	CERS4_HUMAN	Q9HA82	CERS4	46.4	May be either a bona fide (dihydro)ceramide synthase or a modulator of its activity
C-reactive protein	CRP_HUMAN	P02741	CRP	25.0	Displays several functions associated with host defense

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Protein name	UniProt name	Entry name	Gene	Mass (kDa)	Function
C-type lectin domain family 4 member F	CLC4F_HUMAN	Q8N1N0	CLEC4F	65.5	Receptor with an affinity for galactose and fucose. Could be involved in endocytosis
Cystatin-F	CYTF_HUMAN	O76096	CST7	16.4	May play a role in immune regulation through inhibition of a unique target in the hematopoietic system
DDB1- and CUL4-associated factor 15	DCA15_HUMAN	Q66K64	DCAF15	66.5	May be involved in ubiquitination and degradation through a DDB1-CUL4 E3 protein-ubiquitin ligase
DNA topoisomerase binding protein 1	2- TOPBP1_HUMAN	Q92547	TOPBP1	170.7	Binds double-stranded DNA breaks and nicks as well as single-stranded DNA
Dynein heavy chain 10, axonemal	DYH10_HUMAN	Q8IVF4	DNAH10	514.8	Presents ATPase activity
Ellis-van Creveld syndrome protein	EVC_HUMAN	P57679	EVC	111.9	Involved in endochondral growth and skeletal development
Fanconi anemia group B protein	FANCB_HUMAN	Q8NB91	FANCB	97.7	DNA repair protein required for FANCD2 ubiquitination

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Protein name	UniProt name	Entry name	Gene	Mass (kDa)	Function
F-box only protein 42	FBX42_HUMAN	Q6P3S6	FBXO42	77.8	Specifically recognizes p53/TP53, promoting its ubiquitination and degradation
Galectin-3-binding protein	LG3BP_HUMAN	Q08380	LGALS3BP	65.3	Stimulate host defense against viruses and tumor cells
HEAT repeat-containing protein 4	HEAT4_HUMAN	Q86WZ0	HEATR4	117.2	–
Immunoglobulin variable 6-1	heavy HV601_HUMAN	A0A0B4J1U7	IGHV6-1	13.5	Immune response
Immunoglobulin variable 2-24	kappa KV224_HUMAN	A0A0C4DH68	IGKV2-24	13.1	Immune response
Immunoglobulin variable 3-27	lambda LV327_HUMAN	P01718	IGLV3-27	12.2	Immune response
Kallistatin	KAIN_HUMAN	P29622	SERPINA4	48.5	Enzyme regulator inhibitor
Keratin, type I cytoskeletal 14	K1C14_HUMAN	P02533	KRT14	51.6	Structural
Keratin, type II cuticular Hb1	KRT81_HUMAN	Q14533	KRT81	54.9	Structural
Lebercilin	LCA5_HUMAN	Q86VQ0	LCA5	80.5	Transporter activity
LINE-1 type transposase domain-containing protein 1	L1TD1_HUMAN	Q5T7N2	L1TD1	98.8	Single-stranded RNA binding
Lipopolysaccharide-binding protein	LBP_HUMAN	P18428	LBP	53.4	Immune response
LRP chaperone MESD	MESD_HUMAN	Q14696	MESD	26.1	Assisting the folding of beta-propeller/EGF

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Protein name		UniProt name	Entry name	Gene	Mass (kDa)	Function
						modules within the family of low-density lipoprotein receptors (LDLRs)
MANSC domain-containing protein 1		MANS1_HUMAN	Q9H8J5	MANSC1	46.8	–
Meckelin		MKS3_HUMAN	Q5HYA8	TMEM67	111.7	Required for ciliary structure and function
Mitochondrial oxoglutarate/malate carrier protein	2-	M2OM_HUMAN	Q02978	SLC25A11	34.1	Catalytic and transporter activities
NAD(P)H dehydrogenase [quinone] 1		NQO1_HUMAN	P15559	NQO1	30.9	Catalytic activity
N-acetyllactosaminide beta-1,3-N-acetylglucosaminyltransferase 2		B3GN2_HUMAN	Q9NY97	B3GNT2	46.0	Catalytic activity
Nck-associated protein 1		NCKP1_HUMAN	Q9Y2A7	NCKAP1	128.8	As component of the WAVE1 complex, required for BDNF-NTRK2 endocytic trafficking and signaling from early endosomes
Nesprin-1		SYNE1_HUMAN	Q8NF91	SYNE1	1011.1	Forms a linking network between organelles and the actin cytoskeleton to maintain the subcellular

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Protein name	UniProt name	Entry name	Gene	Mass (kDa)	Function
					spatial organization
Nuclear receptor coactivator 6	NCOA6_HUMAN	Q14686	NCOA6	219.1	Involved in the coactivation of the NF-kappa-B pathway
PAN2-PAN3 deadenylation complex subunit PAN3	PAN3_HUMAN	Q58A45	PAN3	95.6	Regulatory subunit of the poly(A)-nuclease (PAN) deadenylation complex
Pentatricopeptide repeat-containing protein 1, mitochondrial	PTCD1_HUMAN	O75127	PTCD1	78.8	Mitochondrial protein implicated in negative regulation of leucine tRNA levels, as well as negative regulation of mitochondria-encoded proteins and COX activity
Phosphatidylcholine translocator ABCB4	MDR3_HUMAN	P21439	ABCB4	141.5	Acts as a positive regulator of biliary lipid secretion
1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase gamma-2	PLCG2_HUMAN	P16885	PLCG2	147.8	Catalytic activity
Phospholipase D1	PLD1_HUMAN	Q13393	PLD1	124.2	Catalytic activity
Polypeptide N-acetylgalactosaminyltransferase 3	GALT3_HUMAN	Q14435	GALNT3	72.6	Catalytic activity

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Protein name	UniProt name	Entry name	Gene	Mass (kDa)	Function
Protein salvador homolog 1	SAV1_HUMAN	Q9H4B6	SAV1	44.6	Regulator of STK3/MST2 and STK4/MST1 in the Hippo signaling pathway which plays a pivotal role in organ size control and tumor suppression by restricting proliferation and promoting apoptosis
Protein S100-A7	S10A7_HUMAN	P31151	S100A7	11.5	–
Protein S100-A8	S10A8_HUMAN	P05109	S100A8	10.8	Plays a prominent role in the regulation of inflammatory processes and immune response
Protein S100-A9	S10A9_HUMAN	P06702	S100A9	13.2	Plays a prominent role in the regulation of inflammatory processes and immune response
Protein Z-dependent protease inhibitor	ZPI_HUMAN	Q9UK55	SERPINA10	50.7	Enzyme regulator activity
Protein ZGRF1	ZGRF1_HUMAN	Q86YA3	ZGRF1	236.6	Zinc ion binding that inhibits factors Xa and XIa of the coagulation cascade

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Protein name	UniProt name	Entry name	Gene	Mass (kDa)	Function
Protocadherin-12	PCD12_HUMAN	Q9NPG4	PCDH12	128.9	Cellular adhesion molecule that plays an important role as a regulator of cell migration, probably via increasing cell-cell adhesion
Roundabout homolog 4	ROBO4_HUMAN	Q8WZ75	ROBO4	107.4	Mediates the inhibition of primary endothelial cell migration by Slit proteins
Serum amyloid A-2 protein	SAA2_HUMAN	P0DJ19	SAA2	13.5	Inflammatory response
Short coiled-coil protein	SCOC_HUMAN	Q9UIL1	SCOC	18.0	Positive regulator of amino acid starvation-induced autophagy
Structural maintenance of chromosomes protein 6	SMC6_HUMAN	Q96SB8	SMC6	126.3	Structural
Supervillin	SVIL_HUMAN	O95425	SVIL	247.7	Structural
Syncoilin	SYNC1_HUMAN	Q9H7C4	SYNC	55.3	Plays a supportive role in the efficient coupling of mechanical stress between the myofibril and fiber exterior
TBC1 domain family member 9	TBCD9_HUMAN	Q6ZT07	TBC1D9	143.2	Acts as a GTPase-activating

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Protein name	UniProt name	Entry name	Gene	Mass (kDa)	Function
Transportin-1	TNPO1_HUMAN	Q92973	TNPO1	102.3	protein for Rab family protein(s) Transporter activity
Tudor domain-containing protein 5	TDRD5_HUMAN	Q8NAT2	TDRD5	109.7	Acts via the piRNA metabolic process, which mediates the repression of transposable elements during meiosis
Ubiquitin-like modifier-activating enzyme 1	UBA1_HUMAN	P22314	UBA1	117.8	Catalytic activity
E3 ubiquitin-protein ligase MSL2	MSL2_HUMAN	Q9HCI7	MSL2	62.5	Component of histone acetyltransferase complex
Villin-like protein	VILL_HUMAN	O15195	VILL	95.9	Tumor suppressor
Vitamin K-dependent protein C	PROC_HUMAN	P04070	PROC	52.1	Catalytic activity
Zinc finger RNA-binding protein	ZFR_HUMAN	Q96KR1	ZFR	117.0	Involved in the nucleocytoplasmic shuttling of STAU2
Zinc finger protein 99	ZNF99_HUMAN	A8MX4	ZNF99	100.8	May be involved in transcriptional regulation

The accession number, gene name, species (Human), molecular weight (kDa) and protein function were reported.

Fig. 3. Quantitative Venn diagrams showing the number of identified protein biomarkers found on the surface of the three different nanoparticles (color: black) and commonly found on the surface of AuNPs (10.02 ± 0.91), AgNPs (9.73 ± 1.70 nm) and MNPs (9.30 ± 0.67 nm) (color: grey).

Fig. 4. Localization of the 39, 45 and 61 protein biomarkers found in the surface of AuNPs (10.02 ± 0.91), AgNPs (9.73 ± 1.70 nm) and MNPs (9.30 ± 0.67 nm), respectively, in the different cellular components.

Importantly, the GRF-type zinc finger domain-containing protein 1 (protein ZGRF1) was identified in the protein corona of AuNPs, AgNPs and MNPs after their incubation with the serum samples of all triple negative breast cancer patients. Zinc-finger proteins (ZNFs) are one of the most abundant groups of proteins and have a wide range of molecular functions. Given the wide variety of zinc-finger domains, ZNFs can interact with DNA, RNA, PAR (poly-ADP-ribose) and other proteins. Thus, ZNFs are involved in the regulation of several cellular processes. ZNFs are implicated in transcriptional regulation, ubiquitin-mediated protein degradation, signal transduction, actin targeting, DNA repair, cell migration, and numerous other processes [46]. Notably, overexpression of similar zinc finger proteins has been shown to promote cell growth and metastasis in laryngeal squamous cell carcinoma, glioma, non-small cell lung cancer, gastric cancer, oral squamous cell carcinoma, gallbladder cancer, and breast cancer [47], and also in triple negative breast cancers [48].

Matrix metalloproteinase-9 (MMP9) was identified in the protein corona of AuNPs and AgNPs after their incubation with the serum samples of all triple negative breast cancer patients. Members of the matrix metalloproteinase (MMP) family have been identified as poor prognosis markers for breast cancer patients and as drivers of many facets of the tumor phenotype in experimental models [49]. Studies of the pathological processes involved in tumor progression and metastasis revealed matrix metalloproteinases (MMPs) as prominent molecules engaged in shaping the tumor microenvironment and driving cancer progression and metastasis [50,51]. Mainly, MMP9 was investigated as a potential tumor marker in breast cancer [52]. MMP-9 is one of 70 genes in the Rosetta poor prognosis signature for breast cancer patients [53], the basis for the clinically implemented Mammaprint prognostic assay (Agendia Inc., Irvine, CA). MMP-9 was also highly expressed in node-positive tumors and the preoperative blood serum of patients, but MMP-9 activity was appreciably inhibited in blood serum samples collected after surgery.

Lebercilin and Immunoglobulin lambda variable 3-27 (LV327) were identified in the protein corona of AuNPs and MNPs after their incubation with the serum samples of all triple negative breast cancer patients. While the protein expression of lebercilin was observed in several tissue cancers like colorectal cancer, breast cancer, prostate cancer, lung cancer (The Human Protein Atlas, <https://www.proteinatlas.org/ENSG00000157578-LCA5L/pathology>), immunoglobulin free light chains as LV327 are biomarkers of poor prognosis in basal-like breast cancer and are potential targets in tumor-associated inflammation [54].

LINE-1 type transposase domain-containing protein 1 (LITD1), structural maintenance of chromosomes protein 6 (SMC6) and short coiled-coil protein (SCOC) were identified in the protein corona of AgNPs and MNPs after their incubation with the serum samples of all triple negative breast cancer patients.

LITD1 is an RNA-binding protein that involved with self-renewal of undifferentiated human embryonic stem cells and cancer cell proliferation [55].

The structural maintenance of chromosomes (SMC) proteins are essential for successful chromosome transmission during replication and segregation of the genome in all organisms. SMC proteins function together with other proteins in a range of chromosomal

transactions, including chromosome condensation, sister-chromatid cohesion, recombination, DNA repair, and epigenetic silencing of gene expression. Notably, the protein expression of SMC6 was observed in different tissues as colorectal cancer, breast cancer, prostate cancer, lung cancer, and liver cancer (The Human Protein Atlas, <https://www.proteinatlas.org/ENSG00000163029-SMC6/pathology>). In humans, SCOC is required for autophagosome formation during amino acid starvation [56]; however, this relation with cancer is unknown until the moment.

3.3. Proteomic alterations in triple negative breast cancer serum revealed by SWATH-MS analysis

Label-free SWATH experiments were carried out on a Triple TOF 6600 mass spectrometer (SCIEX). After a comparison between the different groups of samples (controls and TNBC patients), it was observed a variation in the number of statistically significant proteins in the protein corona formed around the three different NPs: 10.02 ± 0.91 nm gold nanoparticles (AuNPs), 9.73 ± 1.70 nm silver nanoparticles (AgNPs) and 9.30 ± 0.67 nm magnetic nanoparticles (MNPs) (see [Table 5](#)).

Table 5. Number of proteins identified by LC-MS/MS by SWATH analysis.

SWATH-MS

Library	180
Samples	
TNBC patients vs. controls (AuNPs)	48 (p -value ≤ 0.05)
TNBC patients vs. controls (AgNPs)	140 (p -value ≤ 0.05)
TNBC patients vs. controls (MNPs)	57 (p -value ≤ 0.05)

After the analysis of the protein corona formed around AuNPs (10.02 ± 0.91 nm), a total of 177 non-redundant proteins were quantified, out of which 48 were found to be differentially regulated. 14 proteins had elevated expression, while 34 proteins showed down-regulation (see [Table 6](#)).

Table 6. Significant proteins (p -value < 0.05) in comparisons between triple negative breast cancer and controls after the analysis of the protein corona of AuNPs (10.02 ± 0.91 nm).

Protein	UniProt ID	p -value	Fold change
TNBC patients vs. controls			
Complement component C6	CO6	0.021032066	3.249007224 ↑ TNBC
Vitamin D-binding protein	VTDB	0.000044522	1.663281358 ↑ TNBC
Complement component C9	CO9	0.000172961	1.658309079 ↑ TNBC

Protein	UniProt ID	p-value	Fold change	
Complement C4-A	CO4A	0.003482887	1.525866959	↑ TNBC
Complement C3	CO3	0.000115208	1.451520626	↑ TNBC
Plasminogen	PLMN	0.005392898	1.329388215	↑ TNBC
Vitronectin	VTNC	0.004766057	1.282037959	↑ TNBC
Apolipoprotein L1	APOL1	0.022535013	1.251913563	↑ TNBC
Afamin	AFAM	0.020811026	1.248070740	↑ TNBC
Complement factor H	CFAH	0.020861177	1.204471284	↑ TNBC
Complement C5	CO5	0.016073885	1.185058578	↑ TNBC
Serum albumin	ALBU	0.004803944	1.172992995	↑ TNBC
Kininogen-1	KNG1	0.024754757	1.167133709	↑ TNBC
Galectin-3-binding protein	LG3BP	0.030403083	1.088223023	↑ TNBC
Glutathione peroxidase 3	GPX3	0.01510168	7.16326536	↑ CONTROL
Immunoglobulin heavy variable 5-51	HV551	0.02782821	4.90904368	↑ CONTROL
Immunoglobulin constant mu	heavy IGHM	0.04111454	4.42849357	↑ CONTROL
Apolipoprotein C-I	APOC1	0.00460538	4.09989381	↑ CONTROL
Immunoglobulin variable 3-9	lambda LV39	0.0394842	3.35634858	↑ CONTROL
Apolipoprotein D	APOD	0.04050732	3.22034685	↑ CONTROL
Immunoglobulin constant alpha 1	heavy IGHA1	0.00960489	3.19128417	↑ CONTROL
Immunoglobulin kappa variable 2-29	KV229	0.00334596	2.48673552	↑ CONTROL

Protein		UniProt ID	p-value	Fold change	
Immunoglobulin constant alpha 2	heavy	IGHA2	0.02785381	2.43044159	↑ CONTROL
Inter-alpha-trypsin heavy chain H4	inhibitor	ITIH4	0.02521756	2.41781135	↑ CONTROL
Immunoglobulin variable 4-69	lambda	LV469	0.00833282	2.37374475	↑ CONTROL
Apolipoprotein(a) sapiens	OS=Homo	APOA	0.03681681	2.12362227	↑ CONTROL
Pigment epithelium-derived factor		PEDF	0.00858685	1.94114234	↑ CONTROL
Alpha-2-macroglobulin		A2MG	0.00477503	1.9190963	↑ CONTROL
Hemoglobin subunit beta		HBB	0.00500101	1.90068671	↑ CONTROL
CD5 antigen-like		CD5L	0.0397185	1.7759815	↑ CONTROL
Serum amyloid A-4 protein		SAA4	0.04115058	1.77144695	↑ CONTROL
Tetranectin		TETN	0.01611908	1.7097494	↑ CONTROL
Apolipoprotein A-II		APOA2	0.01467561	1.67488772	↑ CONTROL
Inter-alpha-trypsin heavy chain H2	inhibitor	ITIH2	0.00096553	1.61898708	↑ CONTROL
Carboxypeptidase B2		CBPB2	0.00728968	1.46619249	↑ CONTROL
Transthyretin		TTHY	0.04987991	1.45277849	↑ CONTROL
Complement subcomponent-like protein	C1r	C1RL	0.03887632	1.43784936	↑ CONTROL

Protein		UniProt ID	p-value	Fold change	
Inter-alpha-trypsin inhibitor heavy chain H1	inhibitor	ITIH1	0.00031251	1.41502216	↑ CONTROL
Serum paraoxonase/arylesterase 1		PON1	0.00142352	1.40629048	↑ CONTROL
Apolipoprotein F		APOF	0.03956903	1.39106396	↑ CONTROL
Apolipoprotein M		APOM	0.00064927	1.36179665	↑ CONTROL
Apolipoprotein A-I		APOA1	0.02341567	1.34721575	↑ CONTROL
Serotransferrin		TRFE	0.0020036	1.31516423	↑ CONTROL
Selenoprotein P		SEPP1	0.00426885	1.30905254	↑ CONTROL
Mannan-binding lectin serine protease 1	lectin serine	MASP1	0.03643268	1.29206286	↑ CONTROL
Carboxypeptidase N subunit 2		CPN2	0.03700623	1.28514087	↑ CONTROL
Protein AMBP		AMBP	0.0084045	1.22749917	↑ CONTROL
Plasma protease C1 inhibitor		IC1	0.04106727	1.2095684	↑ CONTROL

In the case of the protein corona formed around AgNPs (9.73 ± 1.70 nm), a total of 176 non-redundant proteins were quantified, out of which 140 were found to be differentially regulated. 64 proteins had elevated expression, while 76 proteins showed down-regulation (see [Table 7](#)).

Table 7. Significant proteins (p -value < 0.05) in comparisons between triple negative breast cancer and controls after the analysis of the protein corona of AgNPs (9.73 ± 1.70 nm).

		UniProt ID	p -value	Fold change
TNBC patients vs. controls	C-reactive protein	CRP	0.00550613	4.84567669 ↑ TNBC
	Histidine-rich glycoprotein	HRG	3.22E-09	3.93436379 ↑ TNBC
	Complement component C9	CO9	6.28E-08	3.56825414 ↑ TNBC
	Complement C3	CO3	1.95E-08	3.03698879 ↑ TNBC
	Complement factor B	CFAB	4.13E-09	2.83840576 ↑ TNBC
	Immunoglobulin kappa variable 2-24	KV224	0.00067261	2.77717871 ↑ TNBC
	Ficolin-3	FCN3	7.79E-08	2.73769215 ↑ TNBC
	Immunoglobulin kappa variable 1-13	KV113	0.00613408	2.71041552 ↑ TNBC
	IgGFC-binding protein	FCGBP	0.00010163	2.70272053 ↑ TNBC
	Phosphatidylinositol-glycan-specific phospholipase D PE=1 SV=3	PHLD	0.00305474	2.66489178 ↑ TNBC
	Coagulation factor XIII B chain	F13B	2.66E-07	2.60635841 ↑ TNBC
	Fetuin-B	FETUB	1.50E-06	2.55082102 ↑ TNBC
	Apolipoprotein L1	APOL1	2.53E-10	2.52932257 ↑ TNBC
	Serum albumin	ALBU	4.46E-09	2.51568102 ↑ TNBC
	Insulin-like growth factor-binding protein complex acid labile subunit	ALS	4.46E-09	2.49955167 ↑ TNBC
	Inter-alpha-trypsin inhibitor heavy chain H3	ITIH3	7.30E-07	2.38250842 ↑ TNBC
	Complement factor I	CFAI	1.02E-07	2.3647495 ↑ TNBC
	Complement factor H	CFAH	4.83E-09	2.35326116 ↑ TNBC
	Vitronectin	VTNC	1.40E-09	2.31946741 ↑ TNBC

Protein	UniProt ID	p-value	Fold change
C4b-binding protein alpha chain	C4BPA	0.00011973	2.28632913 ↑ TNBC
Hyaluronan-binding protein 2	HABP2	3.29E-07	2.28034134 ↑ TNBC
Plasminogen	PLMN	7.79E-05	2.23378207 ↑ TNBC
Keratin type II cytoskeletal 1	K2C1	0.00022394	2.20416961 ↑ TNBC
Kininogen-1	KNG1	1.18E-08	2.19276786 ↑ TNBC
Fibronectin	FINC	3.36E-08	2.08858855 ↑ TNBC
Complement C5	CO5	2.42E-08	2.01278029 ↑ TNBC
Complement C1q subcomponent subunit C	HUMAN	4.55E-05	2.01080564 ↑ TNBC
Apolipoprotein A-IV	APOA4	4.61E-06	2.00364799 ↑ TNBC
Keratin type I cytoskeletal 10	K1C10	0.00259975	1.97893282 ↑ TNBC
Tetranectin	TETN	4.94E-05	1.95250374 ↑ TNBC
Clusterin	CLUS	1.82E-06	1.9173471 ↑ TNBC
Complement component gamma chain C8	CO8G	6.29E-06	1.89453705 ↑ TNBC
Serotransferrin	TRFE	4.33E-05	1.8939175 ↑ TNBC
Complement C4-B	CO4B	0.03028632	1.89103397 ↑ TNBC
Complement C1q subcomponent subunit B	C1QB	0.00018878	1.88786101 ↑ TNBC
Plasma kallikrein	KLKB1	2.85E-06	1.88182482 ↑ TNBC
Galectin-3-binding protein	LG3BP	1.76E-09	1.85166575 ↑ TNBC
Alpha-2-HS-glycoprotein	FETUA	1.70E-06	1.82851516 ↑ TNBC
Complement C1q subcomponent subunit A	C1QA	0.00028866	1.80691958 ↑ TNBC
Prothrombin	THRB	0.0030656	1.68707742 ↑ TNBC
Retinol-binding protein 4	RET4	5.98E-06	1.65111784 ↑ TNBC

Protein	UniProt ID	p-value	Fold change	
Carboxypeptidase N catalytic chain	CBPN	0.00562324	1.62654965	↑ TNBC
Complement component C8 beta chain	CO8B	0.00298689	1.58131021	↑ TNBC
Sex hormone-binding globulin	SHBG	0.04298875	1.5528487	↑ TNBC
Serum amyloid P-component	SAMP	0.00436056	1.55023657	↑ TNBC
Zinc-alpha-2-glycoprotein	ZA2G	0.00133379	1.54906716	↑ TNBC
Beta-2-glycoprotein 1	APOH	0.00192644	1.53648273	↑ TNBC
Vitamin K-dependent protein S	PROS	1.47E-06	1.52146296	↑ TNBC
Complement C4-A	CO4A	0.00378789	1.50875703	↑ TNBC
Mannan-binding lectin serine protease 1	MASP1	0.00057947	1.49286664	↑ TNBC
Complement C2	CO2	1.04E-05	1.48927332	↑ TNBC
Coagulation factor X	FA10	0.00022691	1.47652463	↑ TNBC
Complement C1r subcomponent	C1R	0.03355753	1.46634915	↑ TNBC
Alpha-1-acid glycoprotein 2	A1AG2	0.04347799	1.46608046	↑ TNBC
Antithrombin-III	ANT3	0.00109057	1.45858581	↑ TNBC
Afamin	AFAM	0.02956547	1.42380083	↑ TNBC
Vitamin D-binding protein	VTDB	0.00689951	1.40641874	↑ TNBC
Hemopexin	HEMO	0.00014568	1.38633651	↑ TNBC
Carboxypeptidase N subunit 2	CPN2	0.03697862	1.36334001	↑ TNBC
Protein AMBP	AMBP	0.0013811	1.32566307	↑ TNBC
Apolipoprotein E	APOE	0.00068645	1.28506343	↑ TNBC
Attractin	ATRN	0.0072892	1.27421822	↑ TNBC
N-acetylmuramoyl-L-alanine amidase	PGRP2	0.00138778	1.23991258	↑ TNBC

Protein	UniProt ID	p-value	Fold change	
Apolipoprotein M	APOM	0.01360078	1.23954254	↑ TNBC
Immunoglobulin heavy variable 3-15	HV315	4.18E-06	75.3967905	↑ CONTROL
Immunoglobulin heavy constant gamma 1	IGHG1	1.34E-07	42.3987588	↑ CONTROL
Alpha-1-antichymotrypsin	AACT	6.14E-10	40.3625379	↑ CONTROL
Immunoglobulin heavy variable 3-73	HV373	6.06E-07	37.7464233	↑ CONTROL
Immunoglobulin heavy constant gamma 3	IGHG3	3.68E-06	36.0478431	↑ CONTROL
Ceruloplasmin	CERU	2.42E-09	33.0108942	↑ CONTROL
Immunoglobulin heavy constant gamma 2	IGHG2	1.72E-06	32.3391318	↑ CONTROL
Alpha-1-antitrypsin	A1AT	1.14E-10	28.6542019	↑ CONTROL
Immunoglobulin heavy constant mu	IGHM	7.89E-05	26.3705332	↑ CONTROL
Corticosteroid-binding globulin	CBG	1.19E-09	25.9868345	↑ CONTROL
Immunoglobulin heavy constant gamma 4	IGHG4	0.00011456	21.9488461	↑ CONTROL
Cholinesterase	CHLE	1.48E-08	21.7678062	↑ CONTROL
Immunoglobulin kappa variable 4-1	KV401	2.21E-07	20.676772	↑ CONTROL
Immunoglobulin heavy variable 4-59	HV459	4.18E-08	19.791237	↑ CONTROL
Immunoglobulin heavy variable 3-30-5	HV335	1.61E-07	18.8319962	↑ CONTROL

Protein	UniProt ID	p-value	Fold change	
Immunoglobulin lambda variable 1-47	LV147	6.19E-05	18.715722	↑ CONTROL
Immunoglobulin lambda variable 8-61	LV861	2.74E-08	18.5700233	↑ CONTROL
Immunoglobulin kappa variable 6-21	KV621	0.0062062	16.6110906	↑ CONTROL
Immunoglobulin heavy variable 3-49	HV349	2.43E-08	16.3703203	↑ CONTROL
Immunoglobulin kappa constant	IGKC	2.49E-06	15.6449675	↑ CONTROL
Immunoglobulin lambda constant 3	IGLC3	1.61E-05	15.2226153	↑ CONTROL
MMS19 nucleotide excision repair protein homolog	MMS19	0.01743218	15.0005868	↑ CONTROL
Immunoglobulin kappa variable 3-20	KV320	5.98E-09	14.9565997	↑ CONTROL
Immunoglobulin kappa variable 3-15	KV315	4.41E-09	13.7845573	↑ CONTROL
Immunoglobulin lambda-like polypeptide 1	IGLL1	9.67E-08	11.9419924	↑ CONTROL
Immunoglobulin lambda variable 3-9	LV39	4.22E-07	11.8083139	↑ CONTROL
Immunoglobulin heavy variable 5-51	HV551	4.24E-07	11.4284904	↑ CONTROL
Immunoglobulin kappa variable 3-11	KV311	5.08E-09	11.3796551	↑ CONTROL
Immunoglobulin kappa variable 1-33	KV133	7.64E-07	11.2884931	↑ CONTROL
Immunoglobulin heavy constant alpha 2	IGHA2	6.08E-05	10.9418774	↑ CONTROL

Protein		UniProt ID	p-value	Fold change	
Immunoglobulin lambda-like polypeptide 5 V=2		IGLL5	9.57E-05	10.7682004	↑ CONTROL
Angiotensinogen		ANGT	3.42E-11	8.97430276	↑ CONTROL
Pigment epithelium-derived factor		PEDF	8.81E-10	8.87937962	↑ CONTROL
Immunoglobulin lambda variable 7-43		LV743	3.62E-06	8.19552518	↑ CONTROL
Hemoglobin subunit alpha		HBA	0.0003338	7.79643124	↑ CONTROL
Immunoglobulin heavy constant alpha 1		IGHA1	1.48E-07	7.66824876	↑ CONTROL
Immunoglobulin lambda variable 3-25		LV325	1.49E-06	7.44370609	↑ CONTROL
Immunoglobulin heavy variable 4-28		HV428	1.38E-06	7.26533223	↑ CONTROL
Immunoglobulin kappa variable 1D-12		KVD12	1.17E-09	7.18559345	↑ CONTROL
Heparin cofactor 2		HEP2	2.44E-07	7.13401112	↑ CONTROL
Inter-alpha-trypsin inhibitor heavy chain H4		ITIH4	3.63E-08	6.70198308	↑ CONTROL
Immunoglobulin lambda variable 9-49		LV949	0.00017391	6.43567288	↑ CONTROL
Alpha-1B-glycoprotein		A1BG	1.70E-09	6.00033312	↑ CONTROL
Apolipoprotein A-I		APOA1	1.65E-09	5.16689397	↑ CONTROL
Immunoglobulin lambda variable 3-19		LV319	1.92E-08	5.05592895	↑ CONTROL

Protein	UniProt ID	p-value	Fold change	
Immunoglobulin kappa variable 2-29	KV229	2.59E-07	5.02231162	↑ CONTROL
Immunoglobulin heavy variable 6-1	HV601	2.19E-05	4.85408469	↑ CONTROL
Immunoglobulin heavy variable 1-46	HV146	9.86E-06	4.84652628	↑ CONTROL
Immunoglobulin heavy variable 3-23	HV323	1.25E-05	4.75302522	↑ CONTROL
Cholesteryl ester transfer protein	CETP	1.22E-05	4.64058921	↑ CONTROL
Apolipoprotein A-II	APOA2	2.16E-08	4.62885891	↑ CONTROL
Thyroxine-binding globulin	THBG	1.80E-08	4.45994071	↑ CONTROL
Protein Z-dependent protease inhibitor	ZPI	4.44E-05	4.43951224	↑ CONTROL
Serum amyloid A-4 protein	SAA4	1.38E-07	4.39608833	↑ CONTROL
Immunoglobulin heavy variable 1-69	HV169	0.00058138	4.33088897	↑ CONTROL
Immunoglobulin kappa variable 1-9	KV109	3.99E-06	4.20727924	↑ CONTROL
Gelsolin	GELS	7.03E-09	4.09546999	↑ CONTROL
CD44 antigen	CD44	0.00233936	4.0837648	↑ CONTROL
Apolipoprotein C-I	APOC1	0.00038806	3.66742486	↑ CONTROL
Alpha-2-macroglobulin	A2MG	3.58E-09	3.65331919	↑ CONTROL

Protein	UniProt ID	p-value	Fold change	
Apolipoprotein D	APOD	3.95E-08	3.53083248	↑ CONTROL
Kallistatin	KAIN	4.78E-10	3.48621242	↑ CONTROL
Immunoglobulin kappa variable 1-5	KV105	8.42E-05	3.22054945	↑ CONTROL
Leucine-rich alpha-2-glycoprotein	A2GL	1.29E-08	3.00855446	↑ CONTROL
Hemoglobin subunit beta	HBB	8.64E-05	2.890312	↑ CONTROL
Coiled-coil domain-containing protein 8	CCDC8	6.16E-07	2.88193259	↑ CONTROL
Immunoglobulin kappa variable 2D-28	KVD28	0.00104876	2.6282284	↑ CONTROL
Immunoglobulin kappa variable 6D-21	KVD21	0.01695916	2.61227937	↑ CONTROL
Immunoglobulin lambda variable 4-69	LV469	8.14E-05	2.35130225	↑ CONTROL
Biotinidase	BTD	0.00622085	2.19095666	↑ CONTROL
Plasma serine protease inhibitor	IPSP	3.04E-05	2.1835559	↑ CONTROL
Carboxypeptidase B2	CBPB2	8.55E-05	1.8115321	↑ CONTROL
Immunoglobulin heavy variable 2-26	HV226	0.00977698	1.69947422	↑ CONTROL
Apolipoprotein B-100	APOB	2.35E-05	1.66025805	↑ CONTROL
Alpha-2-antiplasmin	A2AP	0.00014843	1.60147275	↑ CONTROL

Protein	UniProt ID	p-value	Fold change	
Apolipoprotein C-III	APOC3	0.02144075	1.39259865	↑ CONTROL

In the protein corona formed around the MNPs (9.30 ± 0.67 nm), a total of 176 non-redundant proteins were quantified, out of which 57 were found to be differentially regulated. 45 proteins had elevated expression, while 12 proteins showed down-regulation (see [Table 8](#)).

Table 8. Significant proteins (p -value < 0.05) in comparisons between triple negative breast cancer and controls after the analysis of the protein corona of MNPs (9.30 ± 0.67 nm).

Protein	UniProt ID	p-value	Fold change	
C-reactive protein	CRP	0.00169149	3.40849571	↑ TNBC
Lipopolysaccharide-binding protein	LBP	0.00837847	2.93851762	↑ TNBC
Immunoglobulin kappa variable 2-24	KV224	0.00583062	2.27733934	↑ TNBC
Serum amyloid P-component	SAMP	0.00010388	2.20872021	↑ TNBC
Keratin. type II cytoskeletal epidermal	K22E	0.01682722	2.10615702	↑ TNBC
Complement C4-B	CO4B	0.04180946	1.9800469	↑ TNBC
Complement component C9	CO9	5.75E-06	1.90848624	↑ TNBC
IgGfC-binding protein	FCGBP	0.01481938	1.86546782	↑ TNBC
Protein Z-dependent protease inhibitor	ZPI	0.00252121	1.78800298	↑ TNBC
Complement C1q subcomponent subunit B	C1QB	0.00199072	1.78457812	↑ TNBC
Phosphatidylinositol-glycan-specific phospholipase D	PHLD	0.00435099	1.73844701	↑ TNBC
Prothrombin	THRB	0.00750791	1.72802617	↑ TNBC
Complement C4-A	CO4A	0.00510952	1.67059345	↑ TNBC

TNBC
patients
vs. control

Protein	UniProt ID	p-value	Fold change	
Alpha-1-acid glycoprotein 1	A1AG1	0.02552784	1.64048407	↑ TNBC
Apolipoprotein C-III	APOC3	0.03298589	1.61371634	↑ TNBC
Keratin. type II cytoskeletal 1	K2C1	0.01154011	1.60905471	↑ TNBC
Sex hormone-binding globulin	SHBG	0.02523265	1.59168855	↑ TNBC
Inter-alpha-trypsin inhibitor heavy chain H3	ITIH3	0.00066933	1.55061894	↑ TNBC
Complement C3	CO3	6.41E-06	1.53090904	↑ TNBC
Keratin. type I cytoskeletal 10	K1C10	0.0384444	1.52567319	↑ TNBC
C4b-binding protein alpha chain	C4BPA	0.00027605	1.51193215	↑ TNBC
Complement factor H	CFAH	2.79E-05	1.46546396	↑ TNBC
Plasma serine protease inhibitor	IPSP	0.00973255	1.4623027	↑ TNBC
Histidine-rich glycoprotein	HRG	0.01164428	1.4616738	↑ TNBC
Complement factor B	CFAB	0.00061903	1.45925143	↑ TNBC
Pregnancy zone protein	PZP	0.00155229	1.44791482	↑ TNBC
Complement C1q subcomponent subunit A	C1QA	0.02763702	1.4062295	↑ TNBC
Coagulation factor V	FA5	0.01312931	1.40131004	↑ TNBC
Complement factor I	CFAI	7.52E-05	1.40080904	↑ TNBC
Complement C2	CO2	0.00061386	1.33762187	↑ TNBC
Hemopexin	HEMO	3.66E-05	1.29733733	↑ TNBC
Coagulation factor XIII B chain	F13B	0.01256843	1.28491873	↑ TNBC
Complement C1q subcomponent subunit C	C1QC	0.03780247	1.26491165	↑ TNBC
Kininogen-1	KNG1	0.00429848	1.26291248	↑ TNBC
Beta-2-glycoprotein 1	APOH	0.04746608	1.24963502	↑ TNBC
Apolipoprotein L1	APOL1	0.00237944	1.23613552	↑ TNBC

Protein	UniProt ID	p-value	Fold change	
Antithrombin-III	ANT3	0.01869897	1.22888805	↑ TNBC
Protein AMBP	AMBP	0.01434176	1.22857819	↑ TNBC
Vitronectin	VTNC	0.01666796	1.21690616	↑ TNBC
Inter-alpha-trypsin inhibitor heavy chain H1	ITIH1	0.01847152	1.20880937	↑ TNBC
Insulin-like growth factor-binding protein complex acid labile subunit	ALS	0.03555036	1.20445695	↑ TNBC
Hyaluronan-binding protein 2	HABP2	0.01352977	1.18411135	↑ TNBC
Clusterin	CLUS	0.04346683	1.1756767	↑ TNBC
Alpha-2-HS-glycoprotein	FETUA	0.02891723	1.16730605	↑ TNBC
Galectin-3-binding protein	LG3BP	0.00292722	1.116735	↑ TNBC
Apolipoprotein C-I	APOC1	0.02222358	2.50213212	↑ CONTROL
Immunoglobulin heavy constant mu	IGHM	0.00022527	2.032717896	↑ CONTROL
Immunoglobulin lambda variable 8-61	LV861	0.00063688	2.023228572	↑ CONTROL
Immunoglobulin lambda variable 3-19	LV319	0.0082843	1.73294072	↑ CONTROL
CD5 antigen-like	CD5L	0.02066785	1.731935965	↑ CONTROL
Immunoglobulin lambda variable 3-9	LV39	0.02497423	1.576244295	↑ CONTROL
Apolipoprotein F	APOF	0.02131359	1.556435198	↑ CONTROL
Immunoglobulin kappa variable 6-21	KV621	0.00608326	1.473240834	↑ CONTROL
Immunoglobulin heavy variable 3-23	HV323	0.02960462	1.38352045	↑ CONTROL

Protein	UniProt ID	p-value	Fold change	
Immunoglobulin heavy variable 4-28	HV428	0.02327515	1.304707447	↑ CONTROL
Plasma kallikrein	KLKB1	0.04204343	1.23839695	↑ CONTROL
Apolipoprotein M	APOM	0.01063485	1.232467766	↑ CONTROL

A SWATH library was developed. To this aim, the ProteinPilot software (AB Sciex; version 4.0) was used where the proteins were identified with minimum of 2 peptides along with a confidence score above 99% and FDR below 1% as threshold criteria. Therefore, a spectral library containing 180 proteins found in the nanoparticle surfaces after serum incubation was employed. In this study, it was shown that this strategy provided a more comprehensive and reproducible coverage of the proteins that can join the different nanoparticle surfaces.

We fixed the cut off to considerate a deregulated protein at ≥ 1.5 for up-regulation and ≤ 0.67 for down-regulation. Only proteins with a p -value ≤ 0.05 were selected.

In the analysis of the protein corona formed around the three nanoparticles (AuNPs, AgNPs, and MNPs), eight common proteins showed to be statistically significant and appeared quantitatively increased (up-regulated) in triple negative breast cancer patients *versus* controls (healthy people) (Fig. 5). These proteins are complement component C9 (CO9), complement C4-A (CO4A), complement C3 (CO3), vitronectin (VTNC), apolipoprotein L1 (APOL1), complement factor H (CFAH), kininogen-1 (KNG1), galectin-3-binding protein (LG3BP). However, three common proteins appeared quantitatively decreased (down-regulated) in triple negative breast cancer patients *versus* controls (healthy people) (Fig. 6). These proteins are immunoglobulin heavy constant mu (IGHM), immunoglobulin lambda variable 3-9 (LV39) and apolipoprotein C-I (APOC1).

Fig. 5. Quantitative Venn diagrams showing the number of up-regulated proteins found in the protein corona of 10.02 ± 0.91 nm gold nanoparticles (AuNPs), 9.73 ± 1.70 nm silver nanoparticles (AgNPs) and 9.30 ± 0.67 nm magnetic nanoparticles (MNPs) after their incubation with serum from eight triple negative breast cancer patients and eight healthy controls.

Fig. 6. Quantitative Venn diagrams showing the number of down-regulated proteins found in the protein corona of 10.02 ± 0.91 nm gold nanoparticles (AuNPs), 9.73 ± 1.70 nm silver nanoparticles (AgNPs) and 9.30 ± 0.67 nm magnetic nanoparticles (MNPs) after their incubation with serum from eight triple negative breast cancer patients and eight healthy controls.

After the analysis of the protein corona formed around AuNPs (10.02 ± 0.91 nm), AgNPs (9.73 ± 1.70 nm) and MNPs (9.30 ± 0.67 nm), the principal components analysis (PCA) clearly revealed that the samples of the triple negative breast cancer patients and healthy people were separated in the PC1 axis, which explains 47.7, 80.9 and 79.6% of the variance between the samples, respectively (Figs. S13–S15).

In all cases (AuNPs, AgNPs, MNPs), the separation between the groups of samples is visible between the group of healthy people and the group of triple negative breast cancer.

3.4. TNBC biomarker validation

Mass spectrometry-based validation assays were performed in a different cohort of total serum patients samples ($n=8$) and controls ($n=8$) which were run in triplicate using a TripleTOF® 6600 LC-MS/MS system (Sciex). A correct protein validation was performed. To this aim, the library was improved after the addition of DDA data acquired from total serum pools (controls and patient), obtaining a total of 205 identified proteins. After comparing the results obtained by this analysis, with previous analysis performed on serum samples after the incubation with the different nanoparticles, fascinating results were observed.

Graphically, these variations can be observed through charts such as the volcano plot. Volcano plots (see Fig. 7) of the global quantification of proteins between healthy and triple negative breast cancer patients with A: AuNPs (10.02 ± 0.91 nm), B: AgNPs (9.73 ± 1.70 nm) and C: MNPs (9.30 ± 0.67 nm) were generated by plotting the log 2-fold changes for the identified proteins against their corresponding adjusted p-value.

Fig. 7. Volcano plots of the SWATH analysis of proteins between healthy and triple negative breast cancer patients with AuNPs (10.02 ± 0.91 nm), AgNPs (9.73 ± 1.70 nm) and MNPs (9.30 ± 0.67 nm). X-axis shows $\log_2$ -fold change and Y-axis the statistical significance through $-\log_{10}$ -pvalue. The dot lines represent the cut off (p value ≤ 0.05). Proteins biomarkers are those that were common and statistical significant in all the nanoparticles.

Validation assays verified and confirmed that transthyretin (TTHY) was statistically significant and appeared quantitatively decreased (down-regulated) in triple negative breast cancer patients when compared with controls (healthy people). This result was observed after the analysis of the protein corona formed around AuNPs (10.02 ± 0.91 nm). A similar observation was recently reported, where it was found that transthyretin (TTHY) was predominantly (68.75%) down-regulated ($n = 33/48$) in the sera of breast cancer patients [57].

Furthermore, validation assays verified and confirmed that complement component C8 gamma chain (CO8G), ficolin-3 (FCN3), retinol-binding protein 4 (RET4), fibronectin (FINC), fetuin-B (FETUB) and apolipoprotein A-IV (APOA4), were up-regulated in triple negative breast cancer when compared with controls. However, apolipoprotein C-III (APOC3), immunoglobulin kappa variable 2D-28 (KVD28), immunoglobulin kappa variable 1-5 (KV105), immunoglobulin kappa variable 4-1 (KV401), immunoglobulin kappa variable 1D-12 (KVD12), immunoglobulin heavy variable 1-46 (HV146), immunoglobulin heavy variable 3-30-5 (HV335), immunoglobulin heavy constant gamma 2 (IGHG2), immunoglobulin heavy constant gamma 3 (IGHG3), and immunoglobulin heavy constant gamma 4 (IGHG4) were down-regulated in triple negative breast cancer when compared with controls. These results were observed after the analysis of the protein corona formed around AgNPs (9.73 ± 1.70 nm).

Recently, similar studies found that elevated serum levels of retinol binding protein 4 (RBP4) [58] and fibronectin (FINC) [59] were associated with breast cancer risk and they could be useful markers for predicting poor prognosis in breast cancer patients.

In the particular case of apolipoprotein A-IV (APOA4), comparative proteomic profiling of immunodepleted plasma of healthy and of BC individuals revealed that this protein was also up-regulated in the plasma of the BC individuals. Furthermore, this protein was

found to be involved in the pathogenesis of cancer and played an important role in the regulation of metastasis [60].

Validation assays verified and confirmed that pregnancy zone protein (PZP), coagulation factor V (FA5), protein Z-dependent protease inhibitor (ZPI), alpha-1-acid glycoprotein 1 (A1AG1) and inter-alpha-trypsin inhibitor heavy chain H1 (ITIH1), were statistically significant and appeared quantitatively up-regulated in triple negative breast cancer patients when compared with controls (healthy people). This result was observed after the analysis of the protein corona formed around MNPs (9.30 ± 0.67 nm).

Mainly, the pregnancy zone protein (PZP) was found to be implicated the pathogenesis of breast cancer [61].

It is well known that cancer is associated with hypercoagulability, and circumstantial evidence suggests that tumor-expressed coagulation factors actively support cancer pathogenesis and progression. Notably, single nucleotide polymorphisms (SNPs) in F5, encoding coagulation factor V (FA5), have been found associated with breast cancer. In 2018, M. Tinholt et al. [62] found that FA5 expression was higher in breast tumors compared to normal tissue. Importantly, FA5 expression was significantly increased in patients with tumors of aggressive nature (hormone receptor negative-, triple negative-, HER2 enriched-, and basal-like tumors). These authors also suggested that tumor-expressed FA5 could be a possible marker of aggressive breast cancer, and it could emerge as a promising tumor suppressor candidate.

Finally, protein Z-dependent protease inhibitor (ZPI) were identified by immunochemistry in breast cancer cells, whereas they were absent from normal breast tissue [63], and alpha-1-acid glycoprotein (AGP) was also found to be a potential biomarker for breast cancer in 'at risk' individuals, particularly, TNBC patients [64].

4. Discussion

In this study, we provided by the first time the application of a combined proteomic approach as DDA and SWATH-MS to develop the characterization and quantification of triple negative breast cancer proteins after bio-corona nanoparticle protein pre-concentration.

Gold nanoparticles (AuNPs: 10.02 ± 0.91 nm), silver nanoparticles (AgNPs: 9.73 ± 1.70 nm) and magnetic nanoparticles (MNPs: 9.30 ± 0.67 nm) were assessed in biomarker discovery as a tool for the pre-concentration and separation of proteins from complex proteomes. To this end, sera from eight healthy individuals were compared with sera from eight patients diagnosed with triple negative breast cancer. The application of these nanomaterials, combined with mass spectrometry, has allowed the identification of seven potential biomarkers for the diagnostic and control of TNBC progression: GRF-type zinc finger domain-containing protein 1 (protein ZGRF1), Matrix metalloproteinase-9 (MMP9), Lebercilin and Immunoglobulin lambda variable 3-27 (LV327) and LINE-1 type transposase domain-containing protein 1 (LITD1), structural maintenance of chromosomes protein 6 (SMC6) and short coiled-coil protein (SCOC).

After performing over these samples, a SWATH analysis to quantify the protein changes, the separation between the group of healthy people and the group of triple negative breast cancer patients was observed. Moreover, a lot of deregulated proteins among both groups were observed. However, these proteins are not among the altered proteins find by the qualitative DDA assay. The proteomic methods used in this study are complementary and allow improving characterization studies. The fact that they are complementary and not necessarily identify the same proteins is due to the search methods DDA (qualitative), and IDA (SWATH-quantitative) analysis is different.

So, in the analysis of the protein corona formed around the three nanoparticles (AuNPs, AgNPs, and MNPs), eight common proteins showed to be statistically significant and appeared quantitatively increased (up-regulated) in triple negative breast cancer patients versus controls (healthy people). These proteins are complement component C9 (CO9), complement C4-A (CO4A), complement C3 (CO3), vitronectin (VTNC), apolipoprotein L1 (APOL1), complement factor H (CFAH), kininogen-1 (KNG1), galectin-3-binding protein (LG3BP). And three common proteins appeared quantitatively decreased (down-regulated) in triple negative breast cancer patients versus controls (healthy people). These proteins are immunoglobulin heavy constant mu (IGHM), immunoglobulin lambda variable 3-9 (LV39), and apolipoprotein C-I (APOC1).

Moreover, a lot of deregulated proteins not common to all samples incubated with the different nanoparticles were found. Therefore, protein corona formed around AuNPs in breast cancer patients showed 14 proteins up-regulated and 34 proteins down-regulated, in comparison with the protein corona formed around AuNPs in healthy people. In the case of the protein corona formed around AgNPs, 64 proteins were found to be up-regulated, and 76 proteins down-regulated. Finally, in the protein corona formed around the MNPs, 45 proteins had elevated expression, while 12 proteins showed down-regulation. From this point of view, AgNPs seem to be more suitable for a clinical translational purpose, because the analysis of the protein corona formed around these systems allows better differentiation between both groups of study: healthy and diseased individuals.

All these proteins can be considered potential triple negative breast cancer biomarker candidates; however, these proteins were found when serum samples were concentrated using a bio-corona nanoparticle. Thus, it was thought that the best validation could be finding these proteins in total serum samples. To this aim, it was performed a new SWATH-MS analysis improving the library, and it allowed us to find several validated proteins.

When it was compared total serum SWATH with concentrated proteins in AgNPs, we found again complement component C8 (**CO8G**), ficolin-3 (**FCN3**), retinol-binding protein 4 (**RET4**), fibronectin (**FINC**), fetuin-B (FETUB) and apolipoprotein A-IV (**APOA4**), up-regulated; and immunoglobulin heavy constant gamma 4 (**IGHG4**), immunoglobulin kappa variable 2D-28 (**KVD28**), immunoglobulin kappa variable 1-5 (**KV105**), apolipoprotein C-III (**APOC3**), immunoglobulin heavy variable 1-46 (**HV146**), immunoglobulin heavy constant gamma 2 (**IGHG2**), immunoglobulin heavy constant gamma 3 (**IGHG3**), immunoglobulin kappa variable 4-1 (**KV401**), immunoglobulin kappa variable 1D-12 (**KVD12**) and immunoglobulin heavy variable 3-30-5 (**HV335**), down-regulated. It is consistent with data from previous studies, and these

proteins can be considered validated. In the comparison between total serum SWATH and MNPs only found 5 up-regulated proteins pregnancy zone protein (**PZP**), coagulation factor V (**FA5**), protein Z-dependent protease inhibitor (**ZPI**), alpha-1-acid glycoprotein 1 (**A1AG1**) and inter-alpha-trypsin inhibitor heavy chain H1 (**ITIH1**). And finally, in the comparison between total serum SWATH and AuNPs, only one down-regulated protein was validated: transthyretin (**TTHY**).

This study shows that serum proteomics is a valuable tool that can facilitate comprehensive and systematic identification of the serum proteome under both healthy and disease conditions. Thus, serum proteomics could be used for disease diagnosis and prognosis. In our case, we found several breast cancer-specific markers that can be used in the diagnosis. However, due to we found a lot of differentiated proteins, it is necessary complementary assays to reduce the number of protein biomarkers.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

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Supplementary material

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