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# A protein corona-enabled blood test for early cancer detection

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Pancreatic cancer is a very aggressive malignancy that is often diagnosed in advanced stage with the implication that long-term survivors are extremely rare. Thus, developing new methods for pancreatic cancer early detection is an urgent task of current research. To date, nanotechnology offers unprecedented opportunities for cancer therapeutics and diagnosis. The aim of this study was the development of a new pancreatic cancer diagnostic technology based on the exploitation of the nano-bio-interactions between nanoparticles and blood samples. Here blood samples from 10 pancreatic cancer patients and 5 patients without malignancy were let to interact with designed lipid nanoparticles leading to formation of a hard "protein corona" at the nanoparticle surface. After isolation, the protein patterns were characterized by sodium dodecyl sulphate polyAcrylamide gel electrophoresis (SDS PAGE). We found that the protein corona of pancreatic cancer patients was much more enriched than that of healthy individuals. Statistical analysis of SDS-PAGE results allowed us to discriminate between healthy and pancreatic cancer patients with a total discriminate correctness rate of 93%.

## Introduction

Despite recent advances in molecular diagnostics, noninvasive screening tests for early stage tumor detection are almost nonexistent for most cancer types. Furthermore, no validated screening tools are available for several neoplasms; among these, pancreatic cancer is a very lethal tumor with high mortality within a year after diagnosis and a median survival of less than six months [1]. Five-years survival rates of 15-20% are reported when radical surgical resection is performed. Unfortunately, at moment of diagnosis, surgery is feasible only in 20% of patients. Surgical approach is precluded to 80% of patients, due to the presence of local invasion of vascular structures or for distant metastases. Avoiding delayed diagnosis is demanding. However, asymptomatic nature, absence of non-invasive imaging diagnostic tools, lack of any sensitive and specific biomarker make pancreatic cancer early diagnosis challenging. Moreover, tumor complex biology and its impenetrable architecture represent a hard basis to develop an effective treatment in advanced stages in order to improve outcomes [2, 3]. Pancreatic intraepithelial neoplasia (PanIN) lesions, several intraductal papillary mucinous neoplasms (IPMN) and mucinous cystic neoplasms (MCN) have been identified as pancreatic cancer precursors [4], but, their diagnosis is often challenging for both radiologists and endoscopists. Moreover, chronic pancreatitis can lead to pancreatic neoplasms and cyto-histological analysis can be unsatisfying [5]. To date, a non-biopsy-based-test detecting serum carbohydrate antigen 19-9 (CA19-9) is routinely used. However, this approach fails to be specific and is poorly sensitive. Therefore, it becomes a priority to develop more specific and sensitive diagnostic tests. In this field, recently Melo et al. detected and isolated pancreatic cancer-cell-derived exosomes circulating in blood and, through mass spectrometry analysis, identified a cell surface proteoglycan, glipican-1 ($GPC1^+$), specifically enriched on cancer-cell-derived exosomes [6]. $GPC1^+$ exosomes were detected in serum of pancreatic cancer patients allowing to distinguish healthy subjects and patients with benign pancreatic disease from those with early-and late-stage pancreatic cancer. More recently, Xie et al. identified two long non-coding RNAs (lncRNAs) in pancreatic cancer patients' saliva. They found that salivary levels of HOTAIR and PVT1 lncRNAs were significantly higher in pancreatic cancer patients compared to healthy ones [7]. Using quantitative PCR (qPCR), they demonstrated that both salivary lncRNAs discriminated pancreatic cancer patients from healthy controls and benign pancreatic lesions patients with sensibility ranging from 60 to 97%. However, despite being promising, the above-mentioned methods depend on numerous and laborious steps (i.e. exosome isolation,

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Electronic Supplementary Information (ESI) available: **Table S1.** Inclusion citeria in healty and pancreatic cancer group. **Table S2.** Nanoparticle versus blood test results. **Table S3.** Measured concentrations of clinically relevant proteins in histologically proven pancreatic cancer patients and healthy patients. **Tables S4-S6.** Demographic and clinical characteristics of pancreatic cancer patients and healthy patients. See DOI: 10.1039/x0xx00000x

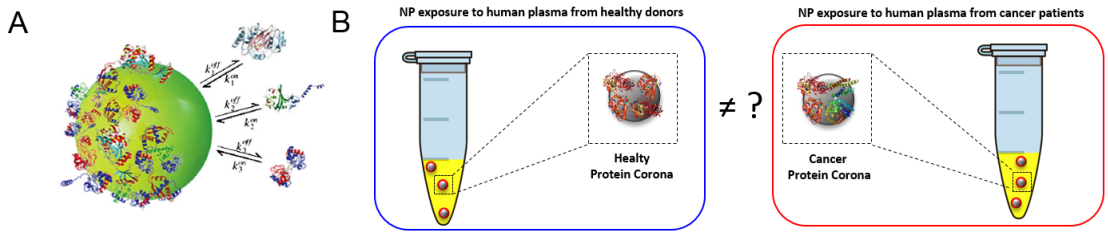


**Figure 1.** (A) When nanoparticles come into contact with physiological environments (e.g. blood, interstitial fluids etc.) they are immediately surrounded by proteins forming a long-standing coating referred to as protein corona. (B) Following exposure to blood samples, the protein corona works as a "nano-accumulator" and allows detecting alterations in circulating proteins as those occurring in cancer. Are the protein coronas formed upon interaction with the blood of pancreatic cancer patients and healthy volunteers equal to each other? This is a key question towards bringing the protein corona from preclinical studies to the clinic.

identification of cell surface proteoglycan using mass spectroscopy or immunogold-TEM and qPCR). Nonetheless, they are extremely expensive and time consuming thus being not appropriate for early cancer detection. As a consequence, the development of fast, cheap, user-friendly and non-invasive tools for pancreas cancer early diagnosis is needed. Recent advance in nanotechnology has brought many innovative approaches and solutions to biomedical research. Due to the peculiar properties that arise when a material is reduced to the nanoscale (1-100 nm), nanomaterials in general and nanoparticles (NPs) in particular are being utilized in almost limitless applications. When NPs are dispersed in a biological fluid, they encounter an immediate and dramatic challenge of their "synthetic identity" [8], as a consequence of the establishment of chemical–physical interactions between the NP surface and medium components. The result is the development of a new interface onto NPs surface, the so-called "bio–nano interface", composed by biomolecules such as proteins, sugars and lipids. This biomolecular shell is dynamic by nature and, given the protein enrichment, it is usually referred to as protein corona (PC) [9]. Comparably to the atmosphere of the Sun, named the *solar corona*, proteins are supposed to establish a dynamic layer around NPs; hence, the phenomenon was nicknamed "protein corona" [9, 10]. It is made of two components, the 'hard' corona (HC) and the 'soft' corona (SC). HC is made of proteins strongly adsorbed to the bare NP surface, while SC is composed of proteins with an affinity for the HC itself and in rapid exchange with the surrounding environment. The long-standing HC is the interface "seen" and processed by living systems, thus it influences various biological responses, such as pharmacological and toxicological effects [11, 12]. Nowadays, it is well recognized that formation and composition of HC depends on several factors [13-21]: i) surface properties of NPs (i.e. size, shape, curvature, surface chemistry and surface charge); ii) characteristics of biological media including protein concentration, protein source, and temperature; iii) incubation time. Because the protein pattern in cancer patients' blood differs from that of healthy donors, the molecular composition of HC formed around NPs could change between cancer and non-cancer patients blood. These alterations are often small and difficult to be detected by conventional blood analyses. On the other side, the HC acts as a "nano-concentrator" of those serum proteins with affinity for the NP surface. The most relevant implication is that thoroughly characterization of HC 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 alteration in circulating level of proteins could not be diagnosed if investigated by blood tests. In this work, we explored the feasibility of developing a novel technology for pancreatic cancer detection based on the recently reported differences in the HC formed onto lipid NPs after exposure to pancreatic cancer and non-cancer blood serum samples [22] (Figure 1). To this end, we employed a liposomal formulation made of a lipid composition made of HSPC (hydrogenated soy phosphatidylcholine),
Cholesterol (Chol) and DSPG (1,2-distearoyl-*sn*-glycero-3-[phospho-rac-(1-glycerol)]) that is the basis of the liposomal amphotericin B. Notably, we found that the HCs varied between pancreatic cancer and healthy human blood. On the other side, common blood tests did not allow discriminating blood samples. Our results show that emerging HC-based technologies could open new horizons towards pancreatic cancer early diagnosis.

## Results and Discussion

Following 1-hour exposure to HP of healthy volunteers, HCs were detached from NP surface and analyzed by 1D SDS/PAGE. We observed that: (i) four major protein bands located at ≈ 110 kDa, 90 kDa, 75 kDa and 37 kDa are in common for various healthy cases (not reported for space consideration); (ii) the intensity of the same protein band from different healthy cases varies. Collectively, these findings indicate that the HCs formed in the HP of healthy subjects are similar to each other. The observed changes in protein band intensities likely arise from plasma alterations occurring among healthy people due to several factors such as genetic background, life style and geographical origin. It is known that circulating levels of proteins can be altered by clinical manifestations. Anyway, no protein biomarker has been clinically validated to diagnose pancreatic cancer so far. Carcinoembryonic antigen (CEA) has been proposed as potential biomarker of pancreatic cancer, but the protein is not produced by all pancreatic tumours and has lower

sensitivity than CA19-9. On the other side, the HC of NPs could work as a "nano-concentrator" of those serum proteins with affinity for the bare NP surface. Some of them could be simultaneously altered by cancer not only in concentration, but also in their structure and function. Slight protein changes (i.e. not detectable by routinely blood tests) could have a deep impact on both nanoparticle-protein and protein-protein interactions within HC. As a result of such global change, exposure of NPs to HP from pancreatic cancer patients could lead to modified HCs. Present exploration was therefore aimed at establishing whether exposure of NPs to HP samples from healthy *versus* cancer patients may result in significant modifications of the HC. 1D SDS/PAGE was therefore used to analyse the protein patterns of the HC of pancreatic cancer patients.

By visual inspection, the same four protein bands identified for healthy individuals (≈ 110 kDa, 90 kDa, 75 kDa and 37 kDa) dominated the protein patterns of pancreatic cancer patients. A densitometry analysis of electrophoretic gels was performed for quantitative comparison. As clearly shows, the protein bands are much more intense in the HC of pancreatic cancer patients than in that of healthy volunteers. As the band intensity does correlate with protein binding, results of Figure 2 suggest that the HC of pancreatic cancer patients is more enriched than that of control subjects.

However, the great variability between both groups makes band intensity alone not enough to discriminate between cancer patients and healthy subjects. To properly evaluate changes in the HC, PCA was performed. As Figure 3A clearly shows, pancreatic cancer patients and healthy ones were strongly separated in the PC1-PC2 plane. This result clearly demonstrates that the PCA performed using the intensities of four selected protein bands is a powerful tool to discriminate between the two groups. To provide a quantitative estimation of group separation, LDA on PCs was accomplished. In Figure 3B we report the PC centroids of the two groups and the linear discriminant function that splits the PCs Cartesian plane in two prediction regions. Notably, statistical analysis on the intensity of the four major bands found in coronas' protein patterns identified correctly 9 of 10 cancer patients and 5 of 5 healthy subjects. The test with 90% specificity and 100% sensitivity in distinguishing healthy control from cancer case reached an overall correctness of 93% (Table S2). To compare the prediction ability of the particle-enabled technology with that feasible by means of routinely blood test, PCA and LDA were replicated using the 24 haematological values as input parameters (Table S3). As panels C and D of Figure 3 clearly show, samples centroids were almost super-imposed in the PCs Cartesian plane. Our findings imply that statistical analysis applied to the results of blood tests does not allow to accurately distinguishing cancer vs. non-cancer patients.

We underline that conclusions of our study are far from being general due to the small number of samples. Moving from descriptive to predictive modelling will require larger series of experiments. However, our findings represent the proof of concept that the protein corona technology is a promising tool for the early diagnosis of pathological conditions where alterations of circulating proteins occur. Implications could be extremely relevant in pancreatic cancer, wherein early diagnosis is the only change of access to treatment with curative intent. In the next future, nanotechnological solutions based on the exploitation of the protein corona will open new horizons for clinical diagnosis.

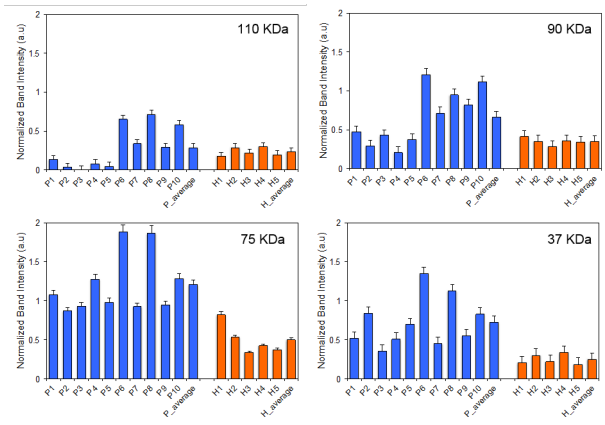


**Figure 2.** Major proteins bands (110 kDa, 90 kDa, 75 kDa and 37 kDa) identified in the protein coronas of lipid nanoparticles after exposure to human plasma of pancreatic cancer (P, blue bars) and healthy (H, orange bars) patients. Results are the average of three independent measurements standard deviation.

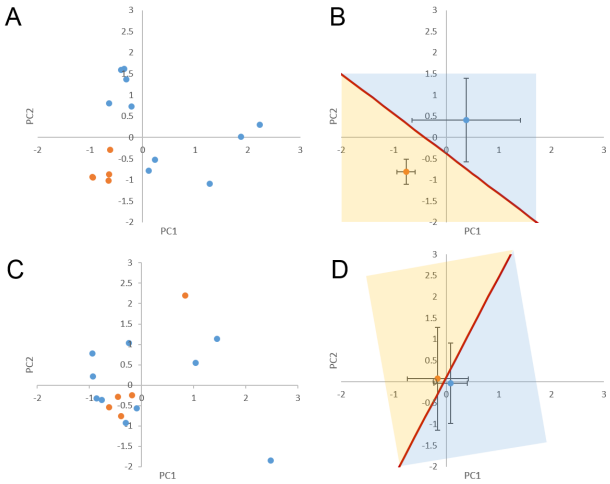


**Figure 3.** (A) Principal components (PC1, PC2) calculated from the set of the four protein bands reported in Figure 2. Blue and orange circles refer to the pancreatic cancer and healthy patients respectively. (B) Centroids of the distributions of panel A. The straight line separates the healthy volunteers group from pancreatic cancer patients group according to the results of the linear discriminant analysis (LDA). (C) Principal components (PC1, PC2) calculated using the set of 24 hematological values reported in Table S3 as input parameters. (D) Centroids of the two distributions reported in panel C are almost superimposed.

## Materials and Methods

**Demographic characteristics and clinical laboratory tests**

Blood samples from 10 consecutive histologically proven pancreatic adenocarcinoma (median age 72 years) and from a group of 5 patients (median age 66 years) affected by surgical benign diseases that met the criteria reported in Table S1 have been collected. In the present manuscript, the last group of patients are considered 'healthy" as well as their group. "Healthy" mean that they were not affected by malignancies and represent the control group. Data regarding medical history and clinical-instrumental work-up have been assessed for each patient; particularly, total protein, serum albumin and serum protein electrophoresis have been considered in both groups. Total protein and albumin were dosed on Dimension Vista (Siemens) system. Obtained values are expressed in g/dl for both albumin and total protein. Electrophoresis has been performed on Capillarys Sebia electrophoresis system for half an hour (zonal electrophoresis in liquid phase). It is a semi-quantitative analysis and values of various protein fractions α1 (e.g. anti-trypsin), α2 globulin (e.g. α2-macroglobulin, aptoglobine, ceruloplasmine), β1 globulin (e.g. transferrin), β2 globulin (e.g. β 2-microglobulin), γ globulin (e.g. immunoglobulin)) are expressed as percentage intervals. For patients in pancreatic cancer group, CEA and Ca 19.9 have been evaluated too. Blood collection and plasma preparation and storage have been performed according to methods already described [23]. According to their clinical stage, pancreatic cancer patients underwent surgery, neoadjuvant chemo-radiotherapy or chemotherapy. For surgical resected patients, pathological staging data have been collected. Clinical and pathological stages are reported according to TNM staging system [24]. The Ethical Committee of University "Campus Bio-Medico di Roma" approved the present study. Tables S4-S6 show demographic and clinical characteristics of our series.

**Liposomes preparation**

Hydrogenated soy phosphatidylcholine (HSPC), 1,2-distearoyl-sn-glycero-3-[Phospho-rac-(1-glycerol)] (Sodium Salt) (DSPG) were purchased from Avanti Polar Lipids (Alabaster, AL) Cholesterol (Chol) were purchased from Sigma-Aldrich (Milan, Italy). Ambisome-like liposomes were synthesized according to standard protocols at the molar ratios HSPC:DSPG:Chol (2.5:1:1.2) [25]. Lipid films were hydrated with phosphate saline buffer (PBS) 10 mmol/l (pH= 7.4) to a final lipid concentration 1 mg/mL. The obtained liposome suspension was extruded 20 times through a 0.05 µm polycarbonate carbonate filter with the Avanti Mini-Extruder (Avanti Polar Lipids, Alabaster, AL). Liposomes were small in size (particle diameter ≈ 130 ± 5 nm) and negatively charged (surface charge ≈ - 30 ± 3 mV). Measurements of size and surface charge were performed at room temperature on a Zetasizer Nano ZS90 (Malvern, UK) equipped with a 5 mW HeNe laser (wavelength λ= 632.8 nm) and a digital logarithmic correlator. More detailed information can be found elsewhere [26].

**Liposome-protein complexes**

Liposome-protein complexes were prepared by incubating liposomes with HP (1:1 v/v) at 37 °C for 1-hour. Experimental conditions (i.e. plasma concentration, temperature and incubation time) were chosen according to previous investigations [26-29] After incubation, liposome-protein complexes were isolated by centrifugation for 15 min at 14,000 rpm. Then pellets were washed three times with PBS to remove unbound proteins obtaining the "hard corona."

**1D-SDS-PAGE experiments**

The hard corona-coated NPs were re-suspended in 20 µl of Laemmli Loading buffer 1X (10 µl βME/1 ml Laemmli Loading buffer) and boiled for 5 min at 100 °C. Identical volumes (10 µl) of each sample were loaded on a gradient polyacrylamide gel (4-20% Criterion TGX precast gels, Bio-Rad) and run at 100 V for about 150 minutes. Gels were washed in double-distilled water ($ddH_2O$) and fixed over night for 12 hours in a de-staining solution (MeOH 50% in $ddH_2O$) with gentle agitation at room temperature, with at least one solution change. In order to determine differences in corona composition, we stained the proteins with highly sensitive silver-ammonia solution for 30 minutes. Then, gels were rinsed 3 times in ddH2O, 40 seconds each rinse, then incubated with acidic developer solution from 3 to 5 minutes. Once all the protein bands started to clearly appear, developer solution was removed and stop solution (acetic acid 10%, methanol 45% in $ddH_2O$) was added. Pictures of gels were captured by KODAK Digital DC120 .

**Statistical analysis**

Principal component analysis (PCA) is a classical, well-established method frequently used in physical as well as in social and biological sciences [30]. Its aim is to project a multidimensional phenomenon onto a reduced set of orthogonal axes (principal component scores or eigenfunctions) with minimal loss of information [31]. The new coordinates are linear combinations of the original ones: they are orthogonal by construction, each representing an independent aspect of the data set. Moreover, the principal components are endowed with the property of explaining the system variability in a hierarchical way: this implies that we can save the meaningful (signal-like) part of the information retained by the first principal components and discard the noise, which is supposed to concentrate on the minor components. The set of original variables obtained under each explored condition is thus linearly transformed by the PCA into a new set of variables (called PC1, PC2, etc.) endowed with the property of being orthogonal to each other and ordered by the decreasing amount of explained variance (information)

in the data. We took extensive advantage of that property to include within some of principal components a major fraction of the information present in the hematological values describing the patients' condition. By means of this analytical technique, we compared two different, even if correlated, datasets. One dataset was composed by 24 different hematological parameters. The second dataset was composed of 4 variables, i.e. the intensity of four major bands identified in the patterns of nanoparticle-corona by 1D-SDS-PAGE and located at 110 kDa, 90 kDa, 75 kDa and 37 kDa. Thus, we clustered and classified the patients by means of Linear Discriminant Analysis (LDA) performed on the principal components [32]. LDA is the method of election to determine whether meaningful differences exist between the groups and to identify the discriminating power of each variable [32]. The technique constructs a set of linear functions of predictors, known as discriminant functions, such that L = b1 x1 + b2 x2 + … + bn xn + c, where the bi are discriminant coefficients the xi are the input variables or predictors and c is a constant. L is a class index that usually takes the values of 1 and –1 respectively for the two classes to be discriminated, so that a positive value of L points to class A and a negative value to class B. We performed LDA on the two principal components of the two considered datasets.

## Conclusions

Extensive clinical investigations are necessary to validate the clinical applications of the new protein corona-based test, and rigorously evaluate the factors that may influence its specificity and sensitivity. This would further validate the potential of protein corona for early detection of pancreatic cancer thus leading novel opportunities in terms of treatment and outcomes.

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