Nanoparticle-aided glycovariant assays to bridge biomarker performance and ctDNA results

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ABSTRACT

Numerous immunoassay based cancer biomarkers established in the 1970 and 1980'ies are widely used in clinical routine. Initial expectations of biomarkers such as CEA, CA125, CA19-9, AFP to provide decisive help in the diagnosis of early stage, pre-symptomatic cancers have not been realized. Thus, they are primarily used for monitoring disease progression and occasionally being useful as prognostic indicators. This limitation is due to the marker also being measurable in healthy individuals and frequently at elevated concentrations in common benign conditions. Most conventional tumor markers are glycosylated and interestingly specific alterations of the glycostructure part can often be seen early in the cancerous process. Conventional double monoclonal immunoassays are however blind to such changes as they are based on peptide epitope recognition. Wide selections of carbohydrate recognizing macromolecules, lectins, but also glycan structure recognizing antibodies are potentially useful for detecting such changes. Despite numerous attempts generating proof-of-principle evidence for this, such assays have generally not been successfully introduced into clinical routine. The affinity constants of lectin and glycan specific antibodies for their corresponding carbohydrate structures may be up to several orders too low to provide the detection limits and robustness expected from routine tumor markers. In this review, we describe an approach based on the use of highly fluorescent Eu^{3+}-chelate dyed nanoparticles onto which lectins or glycan specific antibodies are coated to provide the necessary binding strength and signal amplification to provide low detection limits, while maintaining the original glycan-structure specificity. This concept applied to three markers, PSA, CA125 and CA15-3 provide glycoform assays of greatly enhanced cancer specificity using sample volumes similar or lower than corresponding traditional ELISAs. For ovarian cancer, we show that this new approach when applied to ovarian cyst fluid samples provide results similar to the performance obtained with ctDNA determinations of a set of 17 driver mutations and greatly superior compared to corresponding conventional immunoassays. Based on our results, we predict that the nanoparticle-lectin concept will enable a new generation of simple, low-cost biomarker assays of highly improved cancer specificity. Such tools should ideally be evaluated together with determination of ctDNA to establish early detection schemes for cancers e.g. ovarian, pancreas, lung where the detection rate of early stage disease is presently unacceptably low.

1. Introduction

1.1. Present state of conventional diagnostic biomarkers

An ideal biomarker aiming at the primary diagnosis of a cancer likely to become life threatening is defined by several criteria. Although no present immunoassay based cancer biomarker is fully cancer specific, a clinically useful marker should be sufficiently elevated above the levels of healthy, age matched controls and most confounding benign conditions at a disease stage early enough to enable use of curative treatment options. In reality, the diagnostic use of cancer biomarkers mostly occurs as suggested by emerging clinical symptoms, family history or genetic susceptibility. The definitive diagnosis is based on identification of cellular changes in tissue biopsies obtained after variously invasive interventions and microscopic evaluation. Adequately reliable biomarkers for cancers of low prevalence and late appearance of symptoms such as ovarian and pancreas cancers are presently not available to justify their use in the early diagnosis. In the highly

prevalent prostate cancer (PCa), especially in men above 60 years, PSA is elevated early in the disease and a result above a selected cut-off indicating enhanced cancer risk can be seen by magnetic resonance imaging (MRI) and tested with a relatively safely performed routine biopsy procedure. However, besides being elevated in common and age related benign prostatic conditions, a more serious circumstance is that many cases of latent, non-aggressive lesions are found in the biopsy specimen leading to over diagnosis and subsequent overtreatment (Schröder et al., 2012). The risk for over diagnosis is in fact shared by many other cancers.

With few exceptions, presently and commonly available cancer biomarkers are primarily useful for monitoring the course of the disease as it progresses naturally or in response to various therapeutic interventions and sometimes in providing prognostic information (Burtis and Bruns, 2014) In patients with suggestive symptoms or with a family history, a positive biomarker results can be used to initiate a more definitive diagnostic procedure. Unsolicited use of cancer biomarkers in apparently healthy individuals is not recommended unless the intention is to establish a personal baseline value.

However, the search for individual tumor markers or novel biomarker approaches with enhanced early detection potential continues actively with the emergence of various high-throughput or -omics platforms. This includes simultaneous determination of both traditional and novel biomarkers using various multi-marker techniques, such as microarray technologies or capture bead based suspension array platforms (Bernhard et al., 2011; Enroth et al., 2019) with the intention to speed up the discovery process for identifying complementary marker combinations or to establish the diagnosis through more complex pattern recognitions. For the early detection of prostate cancer, several recent approaches have been commercially launched, where several prostate kallikrein sub-forms and some other markers are combined to specifically reduce the over-diagnosis of latent cancers while identifying early lesions with enhanced potential for an aggressive disease path (Dani and Loeb, 2017).

1.2. Addressing the genomic background for precision diagnostics – liquid biopsy of ctDNA

The past 10–15 years have witnessed tremendous increase in the understanding of the genomic molecular background underlying common cancers (Van Hoeck, Tjoonk, Van Boxtel and Cuppen, 2019; Vogelstein et al., 2013) as well as the timelines from initial critical mutations to the continuing cascades eventually leading to late stage and widespread metastatic disease. A central realization is that a relatively small number of "driver mutations" in different combinations are commonly found in many cancers. The timeline from initiation of the malignancy to late stage disease is typically spread over 2-3 decades. Further more important is the realization that seeding of malignant cells to eventually give rise to metastatic disease may in itself be a process stretched over several years. Consequently, determination of driver gene mutations in the circulation has been suggested to provide a window of diagnostic opportunity when the cancer still in many cases is eligible for curative interventions.

The concept of applying liquid biopsy technologies for early diagnosis of cancer through specific and sensitive determination of circulating tumor DNA (ctDNA) among circulating free DNA (cfDNA) in plasma, is a relatively recent approach with considerable promise but also presenting with great challenges (Costa and Schmitt, 2019; Fiala and Diamandis, 2017; Fukushima et al., 2009). Determination of cancer gene mutations either through addressing a pre-selection of a limited number of driver mutations or by applying more global detection concepts by various sequencing techniques are two available approaches. The many intriguing opportunities suggested by various ctDNA determinations for early cancer detection, especially compared to the notoriously poor performance of classical cancer biomarkers have been extensively and critically addressed recently (Fiala and

Diamandis, 2017). It was concluded that currently ctDNA does not have the sensitivity and specificity to be applied to asymptomatic patients.

In a recent publication (Cohen et al., 2018), a novel multi-analyte blood test concept (CancerSEEK) was described and evaluated on a cohort of 1005 patients presenting with eight common cancers (ovarian, liver, stomach, pancreas, esophagus, colorectal, lung or breast) all clinically detected (stages I-III) in comparison with 812 healthy controls. The liquid biopsy part encompassed a selection of 61 amplicons theoretically estimated to enable cancer detection rates ranging from 47 to 82%. With the PCR based sequencing assay - 60% detection was obtained for liver cancer and 100 percent of ovarian cancer. A unique advantage of the ctDNA approach used is the expected very high specificity – in this study >99% was reached relative to healthy controls. However, at the outset, several limitations were acknowledged especially regarding the ability to detect the very early stage cancers and the fact that the same driver mutations are likely to be found in many of the tumors precluding reliable information on the anatomical origin of the tumor. Therefore, in order to obtain additional diagnostic support, eight proteins were selected out of a total of 39 potential protein biomarkers markers tested on a multi-marker platform. The overall sensitivities obtained with the combined liquid biopsy/protein determination approach ranged from 33% (breast) to 98% (ovary) for all cancers and 20% (esophageal) and 100% (liver) for stage I cancers. However, in the case of ovarian cancer only 24 percent of the cases represented early stage I-II cancers. Since healthy controls were used as reference and none of the selected protein biomarkers by themselves are even remotely approved for early detection of any cancer due to their presence in many benign conditions, these figures have to be considered optimistic in a real-life early detection scheme. Notably, of the eight protein biomarkers, CA125, CEA and CA19-9 contributed most to the algorithm – these are all known to be widely found in many cancers although traditionally conceived as preferential markers for ovarian, colorectal and pancreas cancer.

2. A novel immunodiagnostic concept incorporating glycosylation

2.1. Biomarker glycosylation

Changes in the glycosylation of malignant cells has long been recognized as a hallmark of cancer, affecting numerous characteristics of biological behavior, cell signaling and communication, tumor cell dissociation and invasion, cell-matrix interactions, metastasis (Fuster and Esko, 2005; Hakomori, 1996, 2002). The molecular changes mainly include loss of or excessive expression of certain forms, increased expression of incomplete or truncated forms and is thought to be non-random with a high degree of tissue specificity. A large number of altered glycosylated epitopes are classified as tumor-associated carbohydrate antigens. Among these, the aberrant expression of Tn and sialyl-Tn antigens, L-fucose and terminal *N*-acetylglucosamine (GlcNAc) have been widely detected in different cancers. The large majority of conventional tumor markers are in fact glycosylated starting from single carbohydrate structures of PSA and AFP to hundreds of glycan structures in large molecular weight mucins such as MUC16 (CA125) and MUC1 (CA15-3) (Chen et al., 2013; Gilgunn et al., 2013; Peracaula et al., 2003; Saldova et al., 2013; Sato et al., 1993; Korekane et al., 2012). The notion that assays specifically recognizing the cancerous forms of the biomarker could conceivably provide a valuable vehicle for early detection of the cancer has been widely recognized. However, with very few exceptions conventional biomarker immunoassays are based on the use of antibodies generated towards peptide epitopes as these enable robust high sensitivity measurement suitable for laboratory routines. In the cases where antibodies to more cancer specific glycan structures are used (e.g. CA19-9) the assays may not be well characterised as several protein carriers may express the same glycan structure (Barnett et al., 2017; Yue et al., 2009, 2011, 2012).

Lectins are carbohydrate structure recognizing molecules widely

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found in plants, animals and mushrooms. The enormous diversity offered in specificities and the easy access to lectins has made them very attractive for employment as binders recognizing more cancer specific structures of common biomarkers. For instance, fucosylation of alpha-fetoprotein (AFP), which reacts with the lectin *Lens culinaris* agglutinin, has been shown to discriminate AFP produced by liver cancer from that originating from non-malignant liver (Taketa et al., 1993). Automated assay for measuring glycovariant of AFP (AFP-L3) has been developed and introduced in clinical use (Li et al., 2001). However, whereas the dissociation constants (K_d) of antibodies for conventional immunoassays are typically in the range of 1–10 nM, those for lectins are generally several orders of magnitude lower, 1–10 μM and even in the mM range (Cummings RD, Darvill AG, Etzler ME, 2017; Heinonen et al., 1999; Hirabayashi, 2008; Syed et al., 2016). Even if some lectins have successfully been used for proof of principle assays with improved cancer specificity, this fact has effectively prevented lectins to become successfully used for routine cancer biomarker assays with simplicity comparable to conventional immunoassays (Llop et al., 2016). This is easily comprehended by reference to immunoassay theory and the central importance of reagent affinity on central immunoassay characteristics.

Glycosylation-based biomarkers are presently considered one of the most promising areas for biomarker discovery with numerous attempts reported to harness the inherent cancer specificity of biomarker glycovariants using different technological approaches and platforms. To arrive at robust and sensitive glycovariant assays of equal simplicity as state-of-art immunoassays, the special features of lectins and glycan reactive antibodies – especially low affinity and glycostructures being shared by a multitude of proteins - have to be efficiently addressed (Kirwan, Utratna, O'Dwyer, Joshi and Kilcoyne, 2015; Narimatsu, 2014).

2.2. Nanoparticles to address affinity limitations of glycan specific reagents

An attractive cure to the insufficient affinity of lectins (as well as glycan specific antibodies of low affinity) is the use of large fluorescent nanoparticles onto which a number of lectins can be immobilized to create a reporter of exquisite reactivity (the bioavidity effect) while preserving the original specificity. The approximately 30000 fluorescent europium (Eu) chelates contained within each of the polystyrene particles furthermore provide an enormous signal amplification in comparison to the direct coupling (5–10/molecule) of the fluorescent reporter to an individual lectin or antibody molecule. In 2001, using this approach a supersensitive, antibody-assisted assay for free PSA was reported showing an up to 70-fold improvement over a conventional double monoclonal sandwich assay (Soukka et al., 2001). While this example used a high affinity antibody (6×10^9 l/mol) and still mostly relied on kinetically enhanced monovalent binding recognition, in the case of low affinity binders such as lectins, the positive affect of the nanoparticle concept combined with multivalent binding (retarded dissociation) is expected to provide even more decisive relative improvement. The study furthermore predicted that the nanoparticle based non-competitive assays would be particularly effective for large multi-epitope (such as multi-glycan) targets. Although the nanoparticle concept using antibodies would in principle be compatible with one-step incubations the preferred mode is a two-step assay with solid-phase analyte capture reaction prior to nanoparticle conjugate incubation. With lectins and glycan specific antibodies immobilized on nanoparticles, a two-step protocol is mandatory to circumvent the interaction of nanoparticle conjugates with the excess of other glycated proteins in crude samples.

3. Novel nanoparticle based constructs for establishing sensitive glycovariant assays

In order to test the nanoparticle concept employing specific lectins

with potentially enhanced cancer specificity for detection of conventional tumor markers first captured with antibodies reactive with protein epitopes, we here report results from three conventional used tumor markers PSA, CA125 and CA15-3.

3.1. Prostate specific antigen and prostate cancer

We initially sought to test the lectin nanoparticle approach using *Aleuria aurantia* (AAL) a fucose-specific lectin, with affinity towards glycoproteins having core fucα1-6/3GlcNAc. AAL as well as other fucose-binding lectins, have been used to study the alterations in PSA fucosylation in PCa and benign prostatic hyperplasia, however with variable and conflicting results (Fujita et al., 2016; Fukushima et al., 2009; Kekki et al., 2017; Ohyama et al., 2004; Peracaula et al., 2008). From a large selection of monoclonal antibodies reactive with different epitopes on either free or ACT-complexed PSA we identified two antibodies (Fab fragments of free PSA specific 5A10 and total PSA specific H50) with good signal formation and low backgrounds with AAL coated on 98 nm Eu-chelate dyed nanoparticles (dl < 5 ng/ml) using PSA derived from the LNCaP cancer cell-line. In comparison, Eu-chelates used for direct labelling of AAL resulted in a calibration curve with more than 10 times lower signals, exemplifying the bioavidity effect inherent in the use of nanoparticles (Fig. 1a). Interestingly, in comparison the conventional seminal plasma derived PSA standard was virtually unreactive with both AAL Eu-nanoparticle based assays (Fig. 1b). Further testing with patient derived material, tissue lysates and urine samples indicated promising results in improved cancer detection. Lysates made from biopsies obtained from identified cancer foci showed highly significant increases (mean difference 3-fold, p = 0.005) in signals compared to biopsies taken from control areas away from the cancer foci. Conventional PSA determination (free or total PSA) showed no difference between the two lysate groups (Fig. 2). As reported (Kekki et al., 2017) free and total PSA assays could not discriminate between urine samples from young healthy controls and prostatic disease patients with either benign or cancerous lesions. The proportion of the AAL-nanoparticle assay result relative to PSA significantly separated the PCa group (Gleason 7–9) from the benign group. However, applying the AAL-nanoparticle assay to serum or plasma samples has not been successful, with highly variable recoveries of the LNCaP PSA standard and high backgrounds in these sample matrices. Clinical evaluation with a large panel of urine samples (multi IMPROD, ClinicalTrials.gov Identifier NCT02241122) is presently ongoing.

Screening for additional cancer vs non cancer discriminating lectins has recently been performed from the extended lectin nanoparticle library of paragraph 4. Five lectins of different glycosylation specificities; mannose-binding lectin (MBL), *Trichosanthes japonica* agglutinin II (TJA-II), wheat germ agglutinin (WGA), *Wisteria floribunda* lectin (WFL) and macrophage galactose-type lectin (MGL) (binding specificity to mannose, fucose, terminal *N*-acetylglucosamine, *N*-acetylgalactosamine, respectively) show initial promise also for serum and plasma matrices.

The fact that PSA has only a single *N*-oligosaccharide chain (Asn 45) has some critical implications on the performance of a lectin-nanoparticle assay. At low concentrations, PSA bound to the solid phase capture antibody is scattered over the whole capture surface of the microtiter well resulting in a suboptimal bioavidity effect of the lectin-nanoparticle (Fig. 3a). Significant signal enhancement (5-120-fold Fig. 3 b) can be obtained by creating a very dense surface of site-specifically oriented capture Fab fragments to a reduced area (2–4 mm spots) on the bottom of the well as described by us previously (Savukoski et al., 2014). Such an approach is likely to be of significant advantage with targets of small molecular proteins with single or few carbohydrate moieties. As a conclusion from these observations and considerations, we predict that the bioavidity effect of large molecular weight molecules with simultaneous presence of several peptide epitopes on tandem repeat units and large numbers of glycans would be
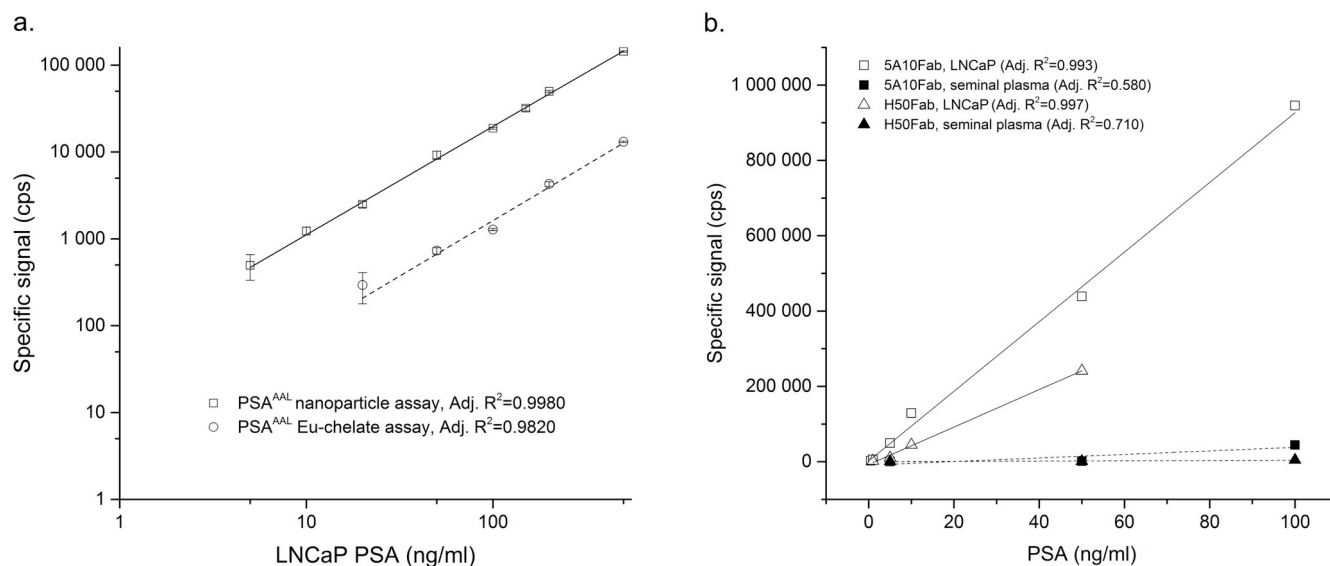
Fig. 1. AAL based PSA assays **A**) Calibrator curves for LNCaP PSA with nanoparticle-based lectin-immunoassay (solid line), and Eu-chelate labelled lectin-immunoassay (dashed line). **B**) LNCaP PSA calibration curves (open symbols) and seminal plasma PSA (closed symbols) for AAL nanoparticle-based assays using Fab fragments of total PSA specific H50 (box symbols), and free PSA specific 5A10 (triangle symbols).
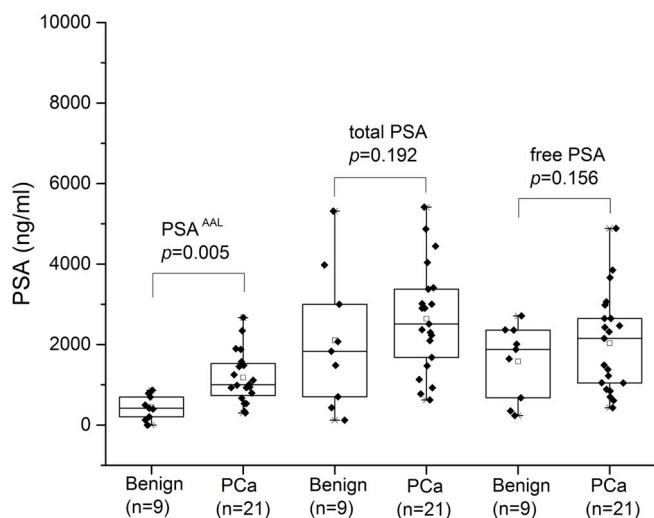

Fig. 2. PSA concentrations measured by PSA AAL nanoparticles-based assays, and conventional total and free PSA assays in benign and cancerous prostate tissue lysates. Cancer foci were graded Gleason 2–5 with percentage of cancer cells between 20 and 80 %. A statistically significant ($p = 0.005$) difference was seen only with the PSA AAL nanoparticle assay. The 10/25/50/75/90th percentiles are marked in the figures.

ideally suited for the lectin-nanoparticle concept (Fig. 3 c).

3.2. CA125 and ovarian cancer

CA125 is a large transmembrane mucin-like molecule (MUC16) with abundant *N*- and *O*-glycans (249 and 3700 potential glycosylation sites, respectively) comprising 28% of its molecular mass (Saldova et al., 2013; Wong et al., 2003; Yin and Lloyd, 2001). Circulating CA125 by immunoassay is based on antibodies against epitopes present on up to 68 tandem repeats and is presently perceived as the best prognostic biomarker of ovarian cancer, although CA125 is found as well in other common cancers (Burtis and Bruns, 2014; Haglund, 1986; Haridas et al., 2011). It is well established that aberrantly glycosylated forms are found in tumors and the circulation of cancer patients, a diversity which cannot be recognized by the conventional immunoassays (Ideo

et al., 2015; Ricardo et al., 2015). Furthermore, CA125 by immunoassay is detectable in healthy males and females and in elevated form during pregnancy, normal menstrual cycle and in many benign inflammatory conditions such as endometriosis, liver cirrhosis etc., a fact that seriously diminish the utility of CA125 for the early detection of ovarian cancer (Akita et al., 2012; Chen et al., 2013). In pre-diagnostic samples, increases of CA125 by immunoassay are seen even up to 2 and 3 years before the clinical presentation (Jacobs et al., 2016) but such increases are not diagnostically useful with the low prevalence of ovarian cancer demanding very high clinical specificity to minimize the risk of false positives.

CA125, with its large molecular weight (some forms up to 2000kD) and numerous peptide epitopes in tandem repeat units and abundant cancer modified glycan structures, is an ideal analytical target for the lectin-nanoparticle concept described above. We have recently described two Eu-nanoparticle based assay constructs for glycovariants of CA125. The C-type lectin receptor, MGL was identified from 16 different lectins to provide preferential measurement of ovarian cancer cell line (OVCAR3) secreted CA125 over CA125 from placental homogenate or liver cirrhosis derived ascites (Fig. 4) (Gidwani et al., 2016). More recently antibodies reactive with the sialylated form of the truncated Tn carbohydrate (STn) immobilized on the nanoparticles resulted in a similarly improved assay, disclosing variable reactivity towards CA125 preparations from different sources (Fig. 5b) (Gidwani et al. JALM, in press). It is of interest to note that assays based on direct labelling of the STn antibody or MGL lectin with Eu-chelates did not produce useful standard curves whereas conjugation of STn antibody or MGL lectin onto Eu-nanoparticles resulted in highly improved signals (Fig. 5c and d). The STn-mAb exclusively recognizes Sialyl-Tn antigen, whereas MGL recognizes terminal *N*-acetylgalactosamine (GalNAc) residues, including sialylated and nonsialylated Tn antigen (van Kooyk et al., 2015). Both glycovariants of CA125 (CA125 MGL and CA125 STn) when applied to marginally elevated (35–200 U/ml) epithelial ovarian cancer (EOC) samples in comparison to samples from endometriosis patients showed a highly significant discrimination relative to the conventional CA125 immunoassay (Fig. 6). In a small cohort, the combination of the two nanoparticle aided assays showed some evidence of complementarity. We noticed excessive false signals from healthy serum when using whole mAb as a capture with our CA125STn assay. The nonspecificity sustained even after adding various blockers commonly employed for the reduction of heterophilic immunoassay
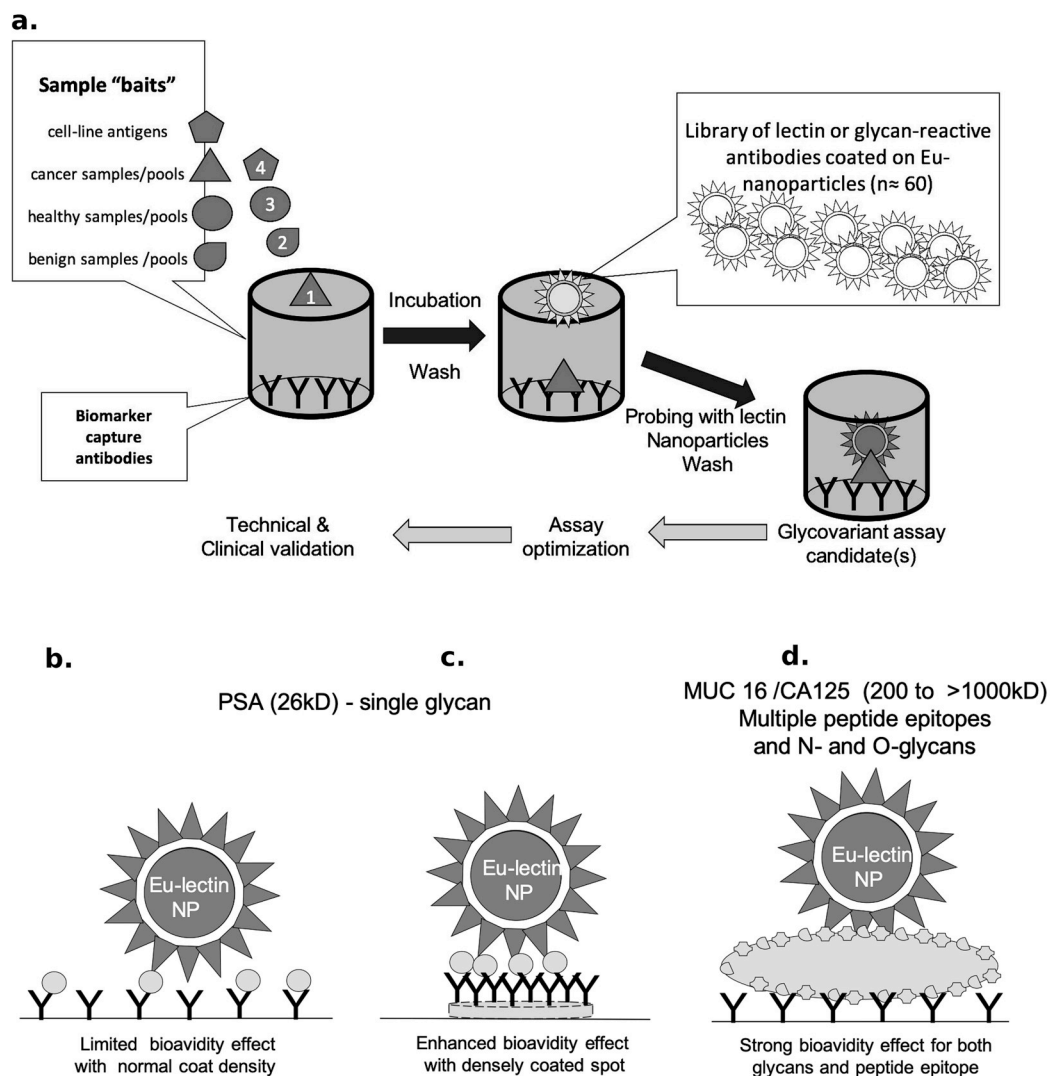
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Fig. 3. Schematic illustration of the nanoparticle aided bioavidity effect with the single glycan PSA target (left and middle) vs a multiepitope/multiglycan target (CA125/MUC16). The weak bioavidity affect with PSA at low concentrations can be strengthened by a more dense antibody "spot" coating.

interferences. In an alternate strategy to eliminate nonspecific interaction, F(ab')2 fragments obtained through enzymatic digestion of the capture antibody. The approach dramatically reduced the cross-reactivity of control sera without affecting signals from EOC sera and OvCa-CA125 standard (Gidwani et al., 2019).

In a recent more extensive evaluation of the diagnostic performance of these exploratory versions of the MGL and STn nanoparticle assays using pre-diagnostic samples from a cohort of 549 pelvic mass patients diagnosed with EOC, benign ovarian tumors or endometriosis, it was concluded that both glycovariants significantly reduced the false positivity rate of the conventional CA125 (Salminen et al. In press). Among the 288 postmenopausal patients (204 EOC, 84 benign) the STn assay increased the sensitivity from 73.5% with the conventional assay to 84.8% (p = 0.0009). As was expected the real advantage is to be seen in the group (63 EOC and 35 benign) with marginally elevated CA125 (35–200 IU/L) where, at 90 percent specificity, the STn assay detected 79.4 percent compared to 25.4 percent with the conventional CA125 assay.

With the Eu-nanoparticle based glycovariant concept with highly reduced biomarker elevations associated with benign conditions the substantially lowered cut-off limits are of special interest in situations seeking to detect an emerging condition prior to the clinical debut. In the evaluations made so far, samples were obtained based on clinical suspicion (pelvic mass) of a possible malignancy. In ovarian cancer, diagnosing the malignancy prior to clinical signs is highly desirable given the poor prognosis following the detection based on clinical suspicion. The repeated use of CA125 determination to establish a person's own baseline in combination with imaging by transvaginal ultrasound (TVU) has been extensively tested as a screening vehicle and has shown some promise (Jacobs et al., 2016). However, the poor specificity of the conventional CA125 test remains a serious obstacle for early detection efforts (Simmons et al., 2019).

We expect that the nanoparticle aided glycovariant concepts described may substantially improve the prospect for early diagnostics and screening of ovarian cancer. However, with the drastically reduced signals from confounding benign conditions, it is conceivable that signals from non-specific sources need to be addressed as they can mask small true increases of the biomarker. With the nanoparticle concept, the likelihood for such heterophilic interferences may actually be increased by the very same bioavidity mechanism strengthening the detection of the targeted biomarker. This calls for careful optimization with use of conventional antibody-based blockers, use of fragmented antibodies as well as careful optimization of buffer conditions and assay protocols to remove any artefactual signals that may obscure the true biomarker related clinical cut-off of the assay. With such optimized assays, the monitoring of pre-diagnostic samples obtained prior to the
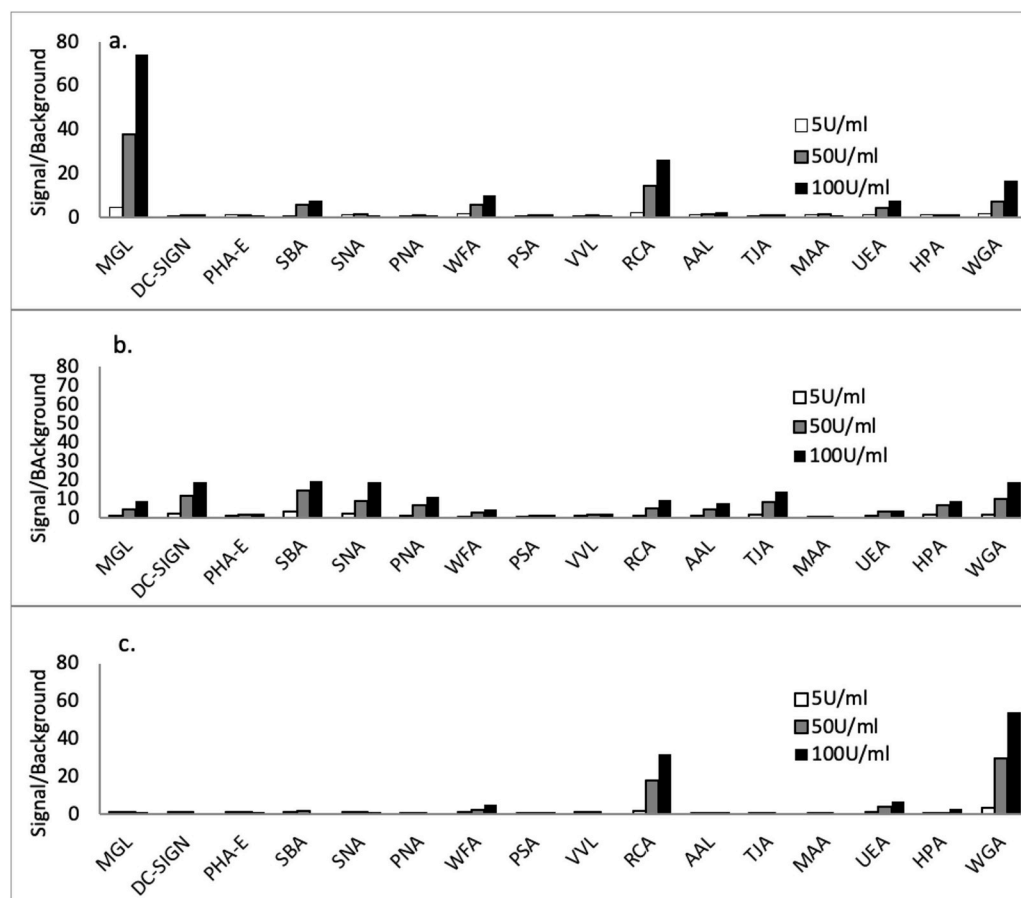
Fig. 4. Binding nanoparticles separately coated with 16 different lectins to CA125 preimmobilized from a) primary OvCa cell line OVCAR-3 (OvCa-CA125), b) placental homogenate (Pla-CA125), and c) liver cirrhosis-derived ascites fluid (LC-CA125). The *X*-axis shows different lectin-NPs used while *Y*-axis shows the signal to background ratios for three CA125 concentrations.

emergence of the clinical disease will become a realistic and prioritized task.

As mentioned previously the use of liquid biopsy methods for the specific detection of driver mutations appears to be highly attractive, primarily due to the claimed excellent specificity, whereas the sensitivity of the technique despite using large sample volumes is likely not sufficient for the early stages of cancer. In a recent study Wang et al. determined a set of driver mutations from 72 ovarian cyst fluids (54 cancers, 18 benign). The AUC for separating cancers from healthy controls was an impressive 0.935. In another five cyst fluids the amount of DNA was not sufficient to perform the mutational analysis (Wang et al., 2016). From the same samples we performed three glycovariant assays (CA125 STn, CA125 MGL and CA15-3 Tn) as well as conventional CA125 and CA15-3 ELISAs. The very high concentrations obtained with conventional CA125 showed poor discrimination of cancers from non-cancers (AUC 0.706) whereas all the tested glycovariant isoforms were found at substantially lower concentrations yet provided significantly improved separations (AUCs of 0.862 and 0.974, for both CA125-glycovariants and in combination with CA15-3 Tn respectively) (Fig. 7). Ongoing studies aim to expand these tests to 325 cyst fluids for which parallel serum samples are available.

These results illustrate the combined technical and diagnostic advantage of the glycan and nanoparticle based technology. The simple and direct protocol applied to minute volumes of crude biological fluids can provide accurate assessment of more cancer associated forms of conventional tumor markers to achieve similar or superior discrimination compared to the more complicated and sample consuming ctDNA determinations of scientifically well established, cancer specific driver mutations. To compare and position these two technological approaches in the diagnosis of early stages of cancer calls for evaluations using extensive cohorts of longitudinally collected samples obtained prior to the clinically evident disease.

3.3. CA15-3 and breast cancer

Mucin 1 (MUC1, also known as cancer antigen 15–3 and 27–29 i.e. CA15-3 and CA27-29) is a transmembrane protein that consists of 1255 amino acids in its canonical sequence and has a heavily glycosylated extracellular domain. The molecular weight of the amino acid backbone is 122 kDa and the apparent molecular weight varies greatly depending on its glycosylation. Alternative splicing, exon skipping, and intron retention leads to MUC1 having a large number of isoforms (Zhang et al., 2013). MUC1 has variable number tandem repeats (VNTR) of amino acids that are characteristic of the mucin family and, in MUC1's case, carry five potential O-glycosylation sites, which are glycosylated to a varying degree depending on the tissue of origin. MUC1 is both *N*- and *O*-glycosylated, and the N-glycosylation is mostly high mannose, hybrid, or acidic complex type. The glycosylation of MUC1 has been observed to change greatly during malignant transformation (Brockhausen et al., 1995; Ghosh et al., 2013) and the changes in glycosylation have been linked to the early phases of oncogenesis (Guo et al., 2010).

MUC1 from normal cells has extensive core 2 O-glycosylation, but the O-glycosylation of breast cancer cells is core 1 type and has increased sialylation, which leads to truncated structures. MUC1 can be differentially expressed in other cancers, and for example in colon cancer cells MUC1 has an overexpression of sialylated Lewis X and sialylated Lewis A antigens. Aberrantly glycosylated MUC1 is expressed in most epithelial cancers, not only breast and colon cancer (Nath and Mukherjee, 2014). MUC1 is shed into the bloodstream in amounts that are easily detectable by immunoassays, which makes it an interesting target for diagnostics.

Both CA15-3 and CA27.29 assays measure MUC1, the main difference of the assays being the antibodies they utilize (Burtis et al., 2012). The CA15-3 assay should not be used in primary diagnosis as the
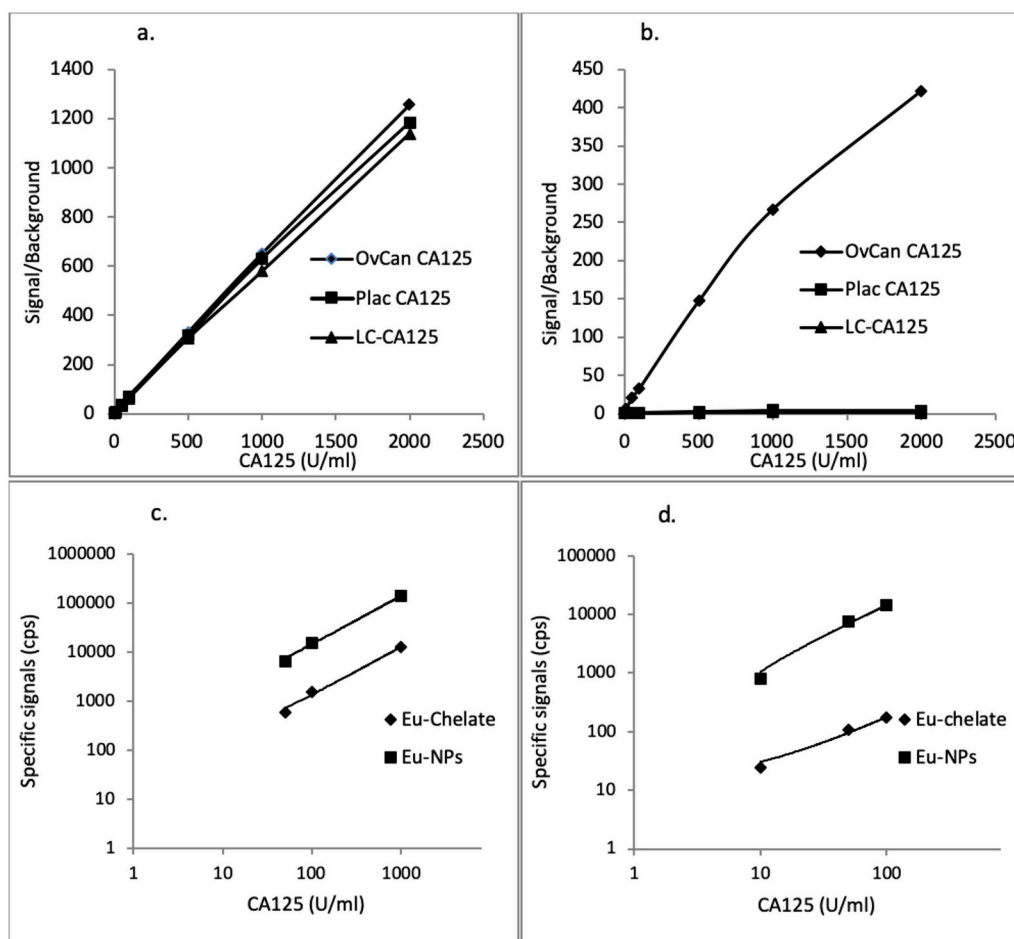
Fig. 5. a) In Conventional CA125 Immunoassay both capture and tracer mAbs detect protein epitopes of CA125 and do not discriminate between CA125 of different tissue origin (OvCan = Ovarian cancer OVCAR-3 cell line, Plac = placental and LC = liver cirrhosis), b) In CA125-STn-nanoparticle assay OvCan-CA125 results in markedly higher signal to-background ratio as compared to those observed for CA125 from non-malignant origins. OvCan-CA125 calibration curves shown for c) STn-mAb and d) MGL-lectin based assays using the nanoparticle vs Eu-chelate approach.

incidence of its elevation in breast cancer patients is low and it can be elevated in benign breast and liver diseases (Burtis et al., 2012). The CA27-29 assay seems slightly better for initial diagnosis than the CA15-3 assay (Gion et al., 1999), but still cannot be recommended for anything else than monitoring therapy and disease recurrence. Recently, the glycovariants of MUC1 have received increased interest in the research fields concerning both diagnostics and therapeutics (Choi et al., 2018; Taylor-Papadimitriou et al., 2018). The use of newly discovered glycovariants is expected to improve upon the conventional assays and some glycovariants have already displayed potential in our initial experiments on metastatic breast cancer patients (Terävä et al., 2019).

In our initial experiment to find glycovariants of MUC1, we screened 28 lectin nanoparticles that we had in our "library" at the time. For the immobilization of MUC1, we used monoclonal anti-MUC1 antibodies Ma552 and Ma695, which are specific for the MUC1 core protein, and MUC1 oligosaccharides, respectively. From the screening, we identified two lectins that had outstanding affinity for MUC1 glycans; macrophage galactose-type lectin (MGL), and wheat germ agglutinin (WGA). (Terävä et al., 2019).

The glycovariants detected by MGL and WGA were measured from 54 metastatic breast cancer patients at the start of treatment and from 20 healthy, age and sex matched, individuals. The samples were collected by Tampere University Hospital (TAYS). The receiver operating characteristics (ROC) curves were generated for the MGL and WGA assays, as well as for the conventional CA15-3 assay (Fig. 8a) and the differences in ROC areas under the curve (AUC) were evaluated with

pairwise comparisons to the conventional CA15-3 assay. The difference between the WGA assay and conventional assay AUC was significant ($\alpha = 0.05$) but the difference between MGL assay and conventional assay AUCs was not. Thus, it was concluded, that in the cohort evaluated, the WGA assay was superior in discriminating the controls from cases.

CA125, as discussed in the paragraph 3.2, is elevated in many other cancers besides ovarian cancer. We therefore wanted to test the two CA125 glycovariants along with the conventional CA125 using the aforementioned metastatic breast cancer samples (n = 54) and benign samples (n = 20), and compare them to our CA15-3 glycovariants. Interestingly we found that the conventional CA125 assay was discriminating the controls from cases statistically significantly ($\alpha = 0.05$) better (AUC = 0.884) than either glycovariant assays, the AUCs being 0.572, and 0.561 for CA125-STn, and CA125-MGL, respectively (Fig. 8b). These results are in line with the widely held notion that glycosylation is tissue specific. Consequently, with future plans to evaluate CA125 in other cancers, e.g. lung, and pancreatic cancer, it is imperative to start by screening a large variety of different glycan specific Eu-nanoparticles.

4. Discussion – implementation of the nanolec glycovariant concept for early detection of cancer

From the examples shown, the use of highly fluorescent Eu3+-chelate dyed nanoparticles as a vehicle for lectins or glycan specific
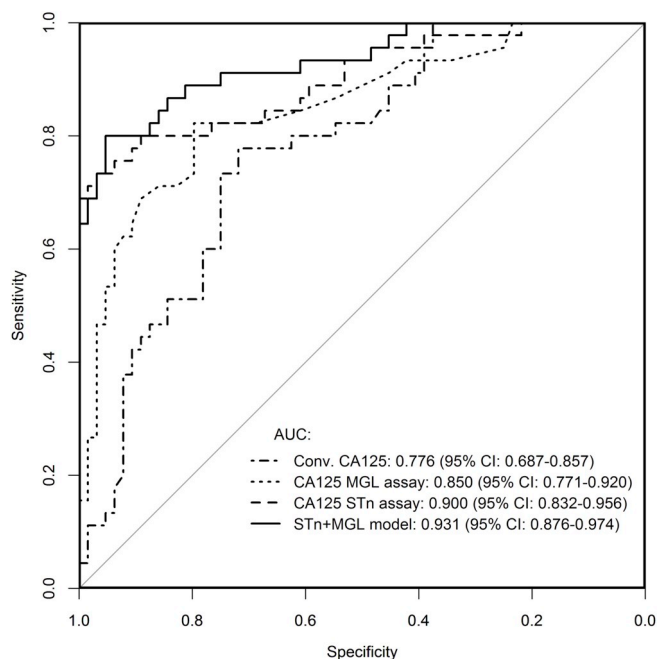
Fig. 6. ROC plot for serum CA125 for the diagnosis of ovarian cancer was performed in marginally elevated CA125 (35–200 U/ml) serum samples from ovarian cancer (n = 45) and endometriosis (n = 41) patients. Highest area under curve (AUC) was 0.90 for CA125-STn nanoparticle, 0.850 for CA125 MGL and least 0.78 for conventional CA125 immunoassay. The perpendicular line (solid black) showing 90% specificity, where the sensitivity of increases from 42% with conventional CA125 ELISAs to 80% with CA125 nanoparticle based assays.
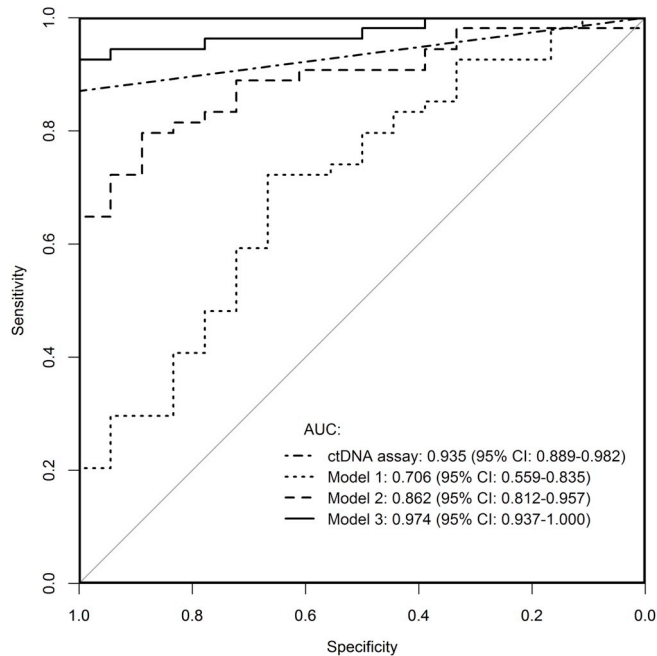

Fig. 7. Receiver operating characteristics (ROC) plots displaying the AUC of different assays in 72 preoperative ovarian cyst fluids (54 cancers, 18 benign) using a combination of conventional CA125 and CA15-3 IA (Model 1 = 0.706), a combination of STn and MGL glycovariants of CA125 (Model 2; AUC = 0.862), and a combination CA125 and CA15-3 glycovariants (Model 3; AUC = 0.974) in comparison ctDNA determination alone (AUC = 93.5) as reported in Wang et al. (2016) The areas under the curve are denoted with the 95% confidence interval (95% CI).

antibodies with insufficient binding affinities, it is possible to construct efficient analytical tools for cancer associated glycovariants of commonly used tumor biomarkers. Despite the extensive and detailed understanding of the potential diagnostic utility in harnessing the glycosylation variation of conventional biomarkers to obtain improved specificity and sensitivity, very little beyond suggestive proof-of-principle results have been brought into clinical use. Here we argue that the bioavidity effect and the amplification potential of the nanoparticles can be a crucial factor for construction of robust, highly sensitive glycovariant applications.

The advantages of this Nanolec technology is particularly evident for large molecular weight tumor marker such as members of the mucin family, offering both numerous peptide epitopes of the tandem repeat segments as well as huge numbers of a *N*- and O-glycosylation sites. However with the example of PSA – a one chain protein, with a single glycosylation unit – we have shown the Nanolec approach to be equally applicable with relatively simple additional technical measures. Many extensively used cancer biomarkers are of similar single protein/limited glycosylation type and constitute attractive targets for construction of lectin assisted glycovariant assays.

The two-fold characteristics of the nanoparticles – bioavidity and signal amplification - can provide biomarker applications of exquisite analytical sensitivity with required samples volumes of just a few microliters as compared to 25–50 µl used in traditional ELISA assays and the use of 5–10 ml blood for liquid biopsy methods.

With the apparent possibility of ultrasensitive assays of improved specificity seen as decisively decreased signals from healthy controls and benign conditions approaching the analytical detection limit of the assay, such novel tumor marker applications will have to be carefully optimized for background control and avoidance of sources of positive heterophilic interferences and other artefactual mechanisms affecting the test signal. This is in clear contrast to many conventional tumor markers such as CA125 and CA15-3, where the commonly occurring non-cancerous isoforms contribute to a high cut-off value, against which tiny interferences are of relative insignificant importance. These considerations are of particular value when considering opportunities for applying these tools to pre-diagnostic samples with intentions for early detection or population screening. Luckily, these problems and corresponding technical solutions have been extensively covered in the immunodiagnostic literature (Tate and Ward, 2004).

The overall approach of the Nanolec concept both for the initial identification – biodiscovery – of novel glycoforms and the further technical and clinical validation of new biomarker candidates is illustrated in Fig. 9. Very central is the establishment of a nanoparticle library presently with about 60 different lectins (plant, mammalian and recombinant prokaryotic lectins) or glycan specific antibodies coated on 100 nm Eu3 + -chelate dyed nanoparticles. For each conventional biomarker for which glycovariant assays are being sought, it is essential to have access to a selection of well characterised "baits" representing real targets of cancerous, benign and healthy control samples as well as cell-line produced antigens eventually providing material for glycovariant assay standardization. In a first incubation step these preparations are captured to available capture antibodies, preferentially antibodies used in conventional, well established ELISA-type immunoassays. Following the first immobilization step and a wash to remove the unbound or loosely bound sample matrix components, the immobilized material is then probed with each of the lectin or glycan antibody labelled nanoparticles. From this nanoparticle-lectin screening phase several initially promising candidates (low background combined with good cancer vs benign discrimination) are selected for further optimization and stepwise extension of testing with individual clinical samples.

As was seen with the CA125 STn and MGL glycovariants, selected and optimized for improved detection ovarian cancer derived CA125, and then applied to breast cancer specimens, which they detected poorly, glycovariants are expected to have increased specificity for the
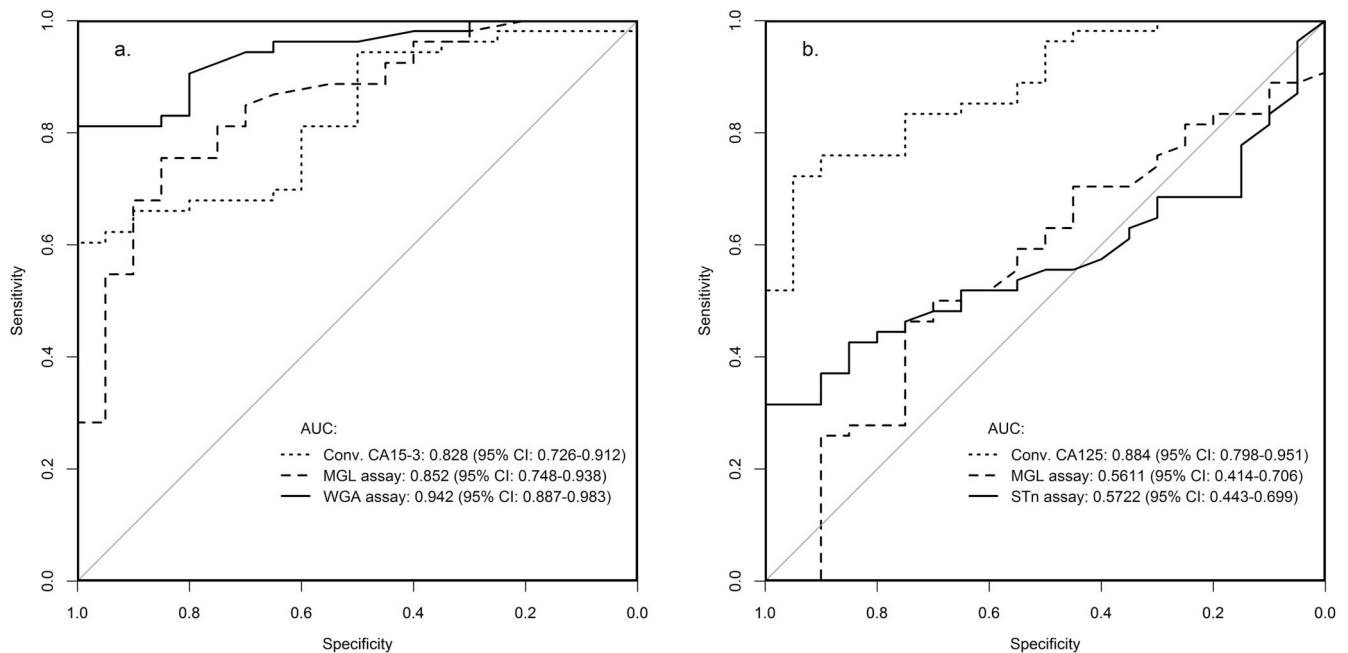
Fig. 8. Tampere University Hospital (TAYS) cohort receiver operating characteristics (ROC). a.) ROC of the glycovariant and conventional CA15-3 assays in TAYS metastatic breast cancer samples. b.) ROC of the glycovariant and conventional CA125 assays in TAYS metastatic breast cancer samples. The areas under the curve are denoted with the 95% confidence interval (95% CI) in parentheses in both (a.) and (b.).

intended target. This is in line with widely accepted notion that the glycosylation machinery is largely tissue specific. From numerous unpublished investigations using the same basic target (e.g. CA125, CEA or CA15-3) with "bait" preparations representing different cancer, different lectins are being selected.

These are still early days of this nanoparticle based technology for identifying cancer or tissue specific glycovariants of common cancer biomarkers. With the available proof-of-principle results we believe that new generations of biomarkers are possible to generate with decisively improved cancer specificity opening up the possibility for their use in the primary diagnostics of several common cancers. Of especially interest are the cancers (ovarian, pancreas, lung), which with presently available diagnostic tools are all too often detected at a stage where the chances for successful curative therapy is low.

The liquid biopsy approach for determination of circulating mutated tumor DNA has lately been given much attention, rightfully due to its

apparently excellent specificity. Presently, the limitations of the various ctDNA technologies lie in the difficulty to detect the very early lesions and the inadequate information about the source (tissue, cancer type) of commonly shared driver mutations. The specified glycosylated isoforms of CA125 will further strengthen the sensitivity of the test as an early indicator of disease. Combined with liquid biopsies i.e. from the cervix and uterus brushings for ctDNA analysis, we will not only get closer to the very origin of the cancer we might also detect precancerous and early stage lesions (Wang et al., 2018). Cohen et al., described a set of traditional tumor marker proteins that was able to substantially improve the early detection performance of the ctDNA analyses. With improved differential diagnostic capacity vs benign conditions as well as better separation of different cancers using glycosylation-based isoforms of the same biomarker target, the next generation of conventional tumor markers as suggested with the Nanolec concept may be an attractive way forward.
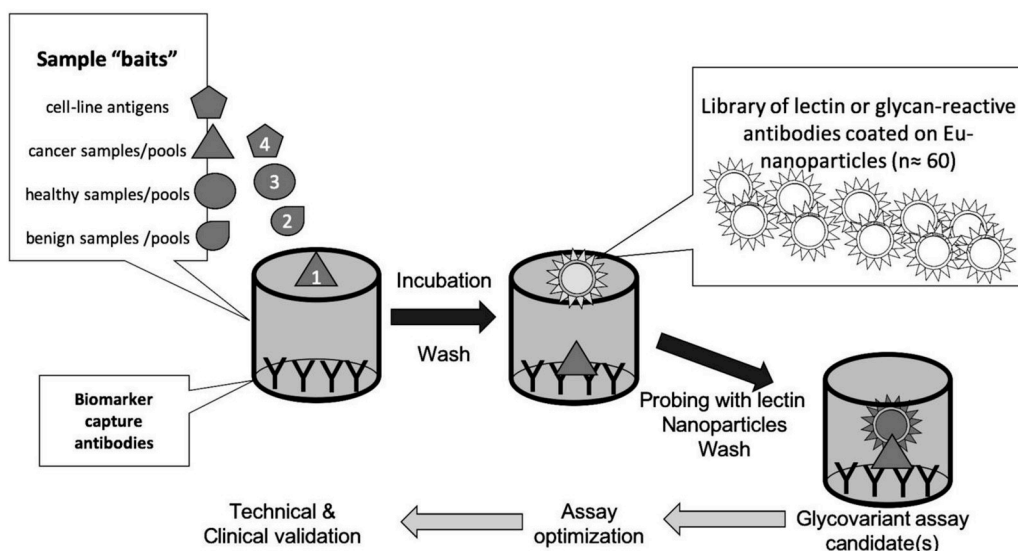

Fig. 9. Schematic overall presentation of the Nanolec glycovariant concept. Different sample preparations (pools/ individual blood samples from benign or cancerous conditions) are used as "baits" by first binding to coated peptide epitope specific conventional antibodies. Following a stringent wash, the immobilized antigens are individually probed by different glycan specific reagents immobilized on nanoparticles (nanoparticle library N ≈ 60). Selection of promising glycovariant candidates are based on analytical performance (assay background, analytical detection limits) and separation of clinical groups (healthy controls, benign and cancerous groups).

Acknowledgments

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