Implementation of Proteomics in Clinical Trials

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ABSTRACT

The application of protein (or peptide) biomarkers in clinical studies is a dynamic, ever-growing field. The introduction of clinical proteomics/ peptidomics, such as mass spectrometry- based assays and multiplexed antibody- based protein arrays, has reshaped the landscape of biomarker identification and validation, allowing the discovery of novel biomarkers at an unprecedented rate and reliability. To reflect the current status with respect to implementation of protein/peptide biomarkers, an investigation of the most recent (last 6 years) clinical studies from clinicaltrials.gov is presented. Forty-two clinical trials involving the direct use of protein or peptide biomarkers in patient stratification, and/or disease diagnosis and prognosis is highlighted. Most of the clinical trials that include proteomics/ protein assays are aiming towards implementation of non-invasive diagnostic tools for early detection, while many of the clinical trials are targeting to correlate the protein abundance with the risk of a disease event. Less in number are the studies in which the protein biomarkers are applied to stratify the patients for intervention. All the above areas of application are considered of great importance for improving disease management, in an era where implementation towards precision medicine is the desired outcome of proteomics biomarker research.

INTRODUCTION

By definition, a biomarker is an indicator of a pathophysiological state. This includes not only disease diagnosis, but also other areas in disease management, such as assessing disease onset and progression, monitoring or even predicting the patients' response and susceptibility to a certain treatment. Proteins, representing the end-product of the so-called "Central dogma of life", are major players in all biochemical reactions in the body, hence of great biomarker potential. The surge in proteomics techniques in the last decades vitalized both research in protein biomarkers and their applicability. Advancements in mass spectrometry-based approaches [1, 2] have allowed the simultaneous identification of thousands of proteins/peptides in a biological sample, and empowered their relative quantification and differential expression analysis based on label-free (e.g. peak intensity, spectral counting) [3] or label-based approaches, such as SILAC (stable isotope labeling of amino acids in cell culture), ITRAQ (isobaric tag for relative and absolute quantification), and ICAT labeling (isotope-coded affinity tag) [4]. In addition, high-throughput micro-array-based proteomics technologies, such as antibody or reverse-phase protein arrays have also expedited the discovery and validation of novel protein biomarkers. [2, 5] To evaluate the state of the art in protein/peptide/proteomics biomarker implementation, in this review, we searched for and investigated clinical trials involving the direct application of protein biomarkers in patient stratification, disease diagnosis and prognosis during the last six years.

METHODS

A comprehensive, unbiased search strategy was implemented to retrieve clinical studies from clinicaltrials.gov. Our search criterion allowed selection of studies registered after 01/01/2013, excluding trials that had been withdrawn or are of unknown status. Six keywords (protein marker, proteomics, proteome, peptide marker, peptidomics, peptidome) were employed for the search sequentially, which collectively yielded a total of 2749 studies. A further keyword check, selecting for studies that contain "protein" or "proteomics" in their title or outcome measurement resulted in a list of 700 studies. Further manual inspection of these trials excluded studies being not directly relevant to protein use as biomarkers (for example, studies on the nutritional value of milk protein), and an additional of studies not revealing the protein identity or aiming at discovering new biomarkers. Collectively, these resulted in a final list of 42 trials involving the direct use of protein/peptide biomarkers in stratification, diagnosis or prognosis, as the focus of this review. The search was performed on 02/11/2018. A schematic illustration of our search strategy is demonstrated in **Figure 1**.

PROTEIN BIOMARKERS IN CLINICAL TRIALS

A. Biomarkers as Stratification Tools

Protein biomarkers have been applied to stratify patients according to their probability to respond to a certain therapy and therefore, guide intervention. There are altogether three trials which use protein biomarkers as stratification tools. These biomarkers

target different type of diseases, including cardiovascular disease, kidney dysfunction, cancer and inflammation (**Table 1**).

Kidney Dysfunction

In the ongoing Phase II/III PRIORITY study, a classifier based on urinary peptides was used to stratify 1777 patients with type 2 diabetes and normoalbuminuria, so that those at a higher risk of developing nephropathy can be randomized to spironolactone treatment (NCT02040441).[6] The classifier (CKD273) consists of 273 urinary peptides measured by Capillary Electrophoresis (CE) coupled with Mass Spectrometry (CE-MS), has demonstrated the capability to detect diabetic nephropathy at a very early stage. [7] CKD273 is composed predominantly of a diverse class of collagen fragments, as well as fragments derived from abundant blood proteins, inflammatory proteins, fibrinogen, apolipoproteins, and alpha-1 antitrypsin. [8]

Cancers

The BIORISE trial targets to validate a panel of five blood proteins, AK2 (adenylate kinase 2), IDH2 (isocitrate dehydrogenase 2), ANX1 (annexin 1), APEX1 (DNA-apurinic or apyrimidinic site lyase) and HSC70 (heat shock cognate 71kDa) for their predictive value for radiation-induced toxicity in 500 breast cancer patients (NCT03252717). Based on the expression profiles of the five proteins, breast cancer patients can be stratified so that those predicted to suffer less from radiation-induced toxicity can be recommended for radiotherapy. These five proteins were obtained

from differential analysis using quantitative tandem mass spectrometry, and they are currently under the patent application. [9]

Inflammatory diseases

In the trial entitled "Duration of Antibiotic Therapy in Critically Ill Patients: CRP-guided Therapy Versus Best Practice", serum concentration of CRP (C-reactive protein), which is a classical inflammatory molecule, is adopted as a stratification tool suggesting antibiotic suspension (in case of low CRP levels <35mg/L) or in contrast maintaining the antibiotic treatment and triggering careful medical evaluation for persistent infection (in case of high CRP levels) in ICU (Intensive Care Unit) patients with an infection. This experimental arm (employing CRP for stratification) will be compared to the 'no intervention' arm following the currently 'best practice' guidelines for such ICU patients (NCT02987790).

B. Biomarkers in Diagnosis

According to the aforementioned selection criteria, 24 clinical trials were selected as being directly associated with the use of biomarkers in diagnostics. Among them, some studies aimed at validating the novel biomarkers for early disease detection in comparison to regular practice, others make use of the well-characterized biomarkers to measure the primary outcome. Information on the clinical trials that correspond to disease diagnosis is summarized in **Table 2**.

Brain Injury and Neurological Disorders

S100B, a member of the S100 protein family, is the golden standard for detecting and assessing neuronal damage in a non-invasive manner. [10] As a result, S100B is almost omnipresent in detecting brain damage induced by trauma or disease. Interestingly, several studies have employed the S100 protein assays as diagnostic tests to indicate brain injury with some of those also targeting implementation of the assay in the clinical guidelines and are presented below.

In the study "Investigation of cerebrospinal fluid (CSF) and Further Tissue Samples for Biomarkers Indicating Spinal Ischemia and Organ Failure in Patients with Thoracoabdominal Aortic Aneurysm (TAAA)" (NCT03093857), the purpose was to evaluate S100B alongside several other serum and urinary markers, including neuropeptides P and Y, NFL (neurofilament triplet protein) and GFAp (glial fibrillary protein) as diagnostic biomarkers to detect early stage spinal ischemia. The clinical trial is currently in recruiting phase, with an initial estimation of 100 TAAA patients to be enrolled. Baseline and post-surgical measurements are under investigation to assess the diagnostic ability of the above markers.

Furthermore, protein biomarkers were evaluated in clinical trials in order to be considered as part of standardized management of hospitalization of brain trauma in two independent studies. In an effort to include the S100B protein assay in the clinical guidelines for traumatic brain injury, in PROS100B (NCT02819778) involving 4000 pediatric patients, the elevated levels of serum S100B were regarded as an indicator of brain lesion. The above trial was based on a previously published prospective study[11] and designed to validate the routine use of S100B, thus obviating the need of

prescribed cranial computed tomography (CCT) to pediatric patients, given that plenty of research links childhood CCT exposure to increased cancer risk. [12] Moreover, it was also suggested that S100B as an alternative to CCT may reduce time spent in pediatric emergency room and hospitalization.

In another study, entitled "Serum S100B Protein Assay in Mild Head Injury" (NCT03345602), S100B protein assay was applied to exclude the presence of brain lesion within three hours after a light head trauma in 400 participants suffering from head injury. The study aims at investigating if S100B could minimize radiation exposure and cost while achieving the same diagnostics value in comparison to CCT. Likewise, S100B is used as an exclusion marker in the "Using the S100B Protein for Emergency Headache Management Care" (NCT03490500). In the above trial including 250 subjects who demonstrated severe headache, the risk of subarachnoid and intracranial haemorrhage can be excluded according to the level of serum S100B; the conclusion made by S100B will be compared to and confirmed by the brain CCT scan.

Moreover, the serum protein levels of S100 have been also evaluated as a marker of brain damage and are therefore applied as indicators of reaching the primary outcome. In the study entitled "Multi-Modal Brain Monitoring and Cardiac Surgery", three brain monitoring devices were compared, i.e. NIRS (Nearinfrared Spectroscopy), TCD (Transcranial Doppler) and BIS (Bispectral index) for detecting brain damage in 1200 post-cardiac surgery patients (NCT02916069). In this clinical trial, the serum level of protein S100 was used as a marker of brain injury and as such, indicator of

the primary outcome. In a similar study aiming at the comparison of different anesthetic methods for pre-eclamptic parturient, the outcome was assessed by serum protein levels of S100B and NSE (neuron specific enolase). Both proteins act as neuronal damage indicators and were considered as the primary evidence of possible brain damage in 50 mothers and their newborns after anesthesia (NCT03551223). Venous blood and umbilical blood were retrieved by the mother and the baby respectively, for the biomarker measurement and neural damage assessment.

Infectious and Autoimmune Diseases

In the context of the clinical trial entitled "Early Diagnostic of Sepsis and Potential Impact on Antibiotic Management Based on Serial PSP measured Using the Abioscope", the alterations in serum PSP concentration were evaluated as a marker for early diagnosis of sepsis in 300 ICU patients (NCT03474809).

In another trial focusing on autoimmune disease entitled "Serum/Urinary MCP-1 Level as a Marker for Lupus Nephritis" (NCT03164720), serum/urinary MCP-1 (monocyte chemoattractant protein 1) assay was implemented in order to differentiate the high-risk subpopulation of systemic lupus erythematosus nephritis (SLE nephritis) patients in a pool of 60 SLE patients, so that the former can be proffered with prompt treatment. The implementation of a non-invasive protein marker to guide intervention can reduce unnecessary renal biopsies.

Rather than using a single biomarker, multiple biomarkers from different biofluids were examined for the purpose of higher specificity and sensitivity in diagnosis. For example, in the trial, entitled "alpha-Defensin and Synovial Proteins to Improve

Detection of Pediatric Septic Arthritis" (NCT03704766), a series of serum (CRP, procalcitonin, D-dimer) and synovial (CRP, alpha-defensin, leukocyte esterase, neutrophil elastase) biomarkers were employed to improve detection of pediatric septic arthritis in 42 subjects. The above clinical trial aims at evaluating a panel of biomarkers from serum and synovial fluid in order to rapidly detect bacterial joint infection. Implementation of such a diagnostic test is expected to overcome synovial fluid cultures, which are currently the "golden" standard to detect infection and take several days for the result to be delivered.

In the "Diagnosing of Acute Tuberculosis" trial (NCT03667742), four proteins from blood and sweat, namely ESAT-6 (early secretory antigenic target 6), CFP-10 (culture filtrate protein 10), C1q (complement component 1q) and CRP are validated as a quick diagnostic test of acute tuberculosis on 90 subjects.

Cancers

Novel markers that detect carcinoma at its nascent, more regional state are always of great importance in clinical applications and of huge interest for cancer research. For instance, the BIGHPANC trial with 80 participants evaluated the correlation between protein expression of ßIG-He, earlier reported to be overexpressed in tumorgenesis[13] , and the onset and severity of pancreatic adenocarcinoma, one of the deadliest subtypes of pancreatic cancer (NCT03472716). The principle behind is that pancreatic carcinoma is often detected when the disease is already at an advanced stage. Therefore, early detection markers will be of high value. In another study with title "Assessment of BMI-1 on Protein and Molecular Levels in Oral Dysplasia and

Squamous Cell Carcinoma" (NCT03345966) with 18 participants, the expression level of BMI-1 (polycomb complex protein) was compared with results from tissue biopsy, in order to assess the diagnostic value of the novel biomarker in squamous cell carcinoma and oral epithelial dysplasia. The primary outcome was the discrimination of different grades of oral squamous cell carcinoma, based on the protein expression of BMI-1.

In a clinical trial concerning HCC (hepatocellular carcinoma), newly emerging biomarkers AFP-L3 (alpha-fetoprotein L3) and PIVKA-II (protein induced by vitamin K absence or antagonist-II) were tested side by side with traditional liver injury and viral biomarkers (alanine aminotransferase, aspartate aminotransferase, glutamyl transferase, antigen against hepatitis C virus and surface antigen against hepatitis C virus) to uncover their diagnostic potential (NCT03460080). The trial entitled "Diagnostic Value of AFP-L3 and PIVKA-II in HCC" involves 200 participants, in which serum samples were evaluated for the tumor markers, and the marker result compared to imaging methods such as MRI (magnetic resonance imaging) and CT (computed tomography).

In the trial entitled "Serum Biomarkers in Diagnosis and Predicting Prognosis of Ovarian Cancers", the potential of four serum proteins TFF3 (trefoil factor 3), sFRP-4 (secreted frizzled related protein 4), Romo1 (reactive oxygen species modulator 1) and NF(nuclear factor)-kB as biomarkers for the diagnosis pre-surgery as well as prediction of disease-free survival following surgery for ovarian carcinoma is evaluated (NCT03112733). The diagnostic power will be assessed by comparison between groups of individuals with ovarian cancer, benign adnexal mass and those

with no sign of ovarian disease. Noticeably, these four proteins (TFF3, sFRP-4, Romo 1 and NF-kB) had been shown to be candidates for diagnosis of small intestinal endocrine tumor, hepatocellular carcinoma, non-small cell lung cancer, and prostate cancer respectively. [14-17] Results of the trial have not been posted yet.

Cardiovascular Diseases

The timely diagnosis is essential for effective clinical management in patients complaining for chest pain. For instance, an algorithm that comprises of four heart-specific proteins (FABP (fatty-acid-binding protein), troponin, phosphokinase and phosphokinase-MB) was used to detect non-ST segment myocardial infarction in a clinical trial that involves 20 participants (NCT03507270). The clinical trial is called "One-hour Diagnostic Algorithm for NSTEMI", aiming at the evaluation of the above diagnostic algorithm for early detection of myocardial infarction, at 4 hours after a suspected onset of non-ST elevation myocardial infarction. In the RAPIDA trial with similar purpose, the incremental diagnostic value of heart-type FABP in acute coronary syndrome with a finger-prick test upon usual care based on a number of 400 participants was suggested (NCT01826994). The goal of the above trial is to implement the protein assay on patients with any new-onset chest complaint (lasting for not more than 24 hours) to guide further examinations by a cardiologist.

The importance of biomarkers can be demonstrated in stroke management too. The Treat-NASPP study utilized two acute stroke markers, GFAP (glial fibrillary acidic protein) and RBP4 (retinal binding protein 4), to distinguish the more fatal intracerebral hemorrhage from ischemic attack (NCT03158259). In the above trial

including 400 participants, the objective is to investigate the above diagnostic markers for pre-hospital diagnosis to guide intravenous thrombolytic treatment of an ischemic stroke.

Moreover, diagnostics based on previously identified proteins based on the use of proteomics technologies is also observed. The SpecTRA study aims at validating the proteomics biomarkers discovered by multiplexed and targeted mass spectrometry, in combination with decision-making software in distinguishing ischemic stroke from the mimic. In one SpecTRA study with identifier NCT03070067, the abundance of 16 proteomic biomarkers (apolipoprotein B100, FABP3, L-selectin, atrionatriuretic peptide receptor 1, insulin-like growth factor binding protein 3, F5 (coagulation factor V), F9, F10, adiponutrin, von Willebrand factor, thrombospondin 1, prolactin, paraoxonase 3, epidermal growth factor receptor, vascular endothelial growth factor, henopexin, myeloblastin, plasma serine protease inhibitor, heparin cofactor II, hyaluronan-binding protein) was first determined by mass spectrometry in patients' sample and the results were analyzed with a decision-making software in 450 patients. The 16 proteins make up a diagnostic and prognostic panel of ACVS (acute cerebrovascular syndrome), and they are currently under patent review with the application number 15/925629. [18]

Another SpecTRA study with identifier NCT0305099 utilized the same strategy to verify 141 proteomic biomarkers in 1150 participants. The 141 proteins are integrated from a previous study in the cohort of ACVS patients. [19] In the study entitled "Pilot Study of Cardiac MR in Patients with Muscular Dystrophy", the serum concentration of the heart-specific marker, ST2, was used to detect heart dysfunction in 100 patients

with MD (muscular dystrophy) (NCT02921321). In this trial, ST2 acts as a reference to the new imaging technology, as an effort to assess the effectiveness of cardiac MRI in the detection of cardiomyopathy, which is a severe consequence of MD.

Other Complications

In the GESPACE trial that involves 75 participants, the serum level of CRP acts as an early diagnostic indicator of anastomotic fistula induced by surgical resection of colorectal cancer (NCT03097276). This involved assessment of the serum protein concentration of CRP, compared with CT scan.

Moreover, in the study entitled “Study of Glycogen Storage Disease”, CRP, creatine kinase, prealbumin, microalbumin and hemoglobin A1C combined to form a detection panel of glycogen storage disease (NCT02057731). The aim of the trial is to assess the diagnostic potential of the above markers in relation to the glycogen storage disease, while evaluation of the primary outcome was performed by measuring the serum cholesterol level.

C. Biomarkers in Prognosis

Prognosis of disease is of paramount importance, as it enables prevention and early intervention. There are 18 trials that apply protein biomarkers in disease prognosis (summarized in **Table 3).**

Cardiovascular Disease and Heart Failure

Risk markers for CVD that correspond to a lipoprotein profile or proteins involved in inflammation, hemostasis and coagulation are frequently used in clinical trials. In the vast majority of cases, their application targets to evaluate if a certain treatment, exercise program or dietary supplementation lowers the risk of CVD.

The primary aim of the DBS study is to compare the measurements between conventional phlebotomy and dried blood spot testing using ELISA (enzyme-linked immunosorbent assay) or mass spectrometry in the determination of change in cardiovascular risk factors after statin treatment (NCT02402803). In the study, the abundance of ApoB (Apolipoprotein B) and ApoA-I (Apolipoprotien A-I) in finger-prick DBS (dried blood spot) will be defined based on the two different methods (ELISA and MS) to evaluate the change in cardiovascular risk following the initiation of statin therapy. The trial has been actively recruiting at the time of the search (11/2018).

In addition, the levels of lipoproteins, CRP, TNF(tumor necrosis factor)-alpha, IL (interleukin)-6 as well as vascular adhesion molecules VCAM (vascular cell adhesion molecule) and ICAM(intracellular adhesion molecule)-4 were measured in 46 participants in a study that evaluates if intake of walnuts has an influence on arterial stiffness, central blood pressure, lipoproteins and other cardiovascular risk factors (NCT02210767). The trial completion date was April 2018, nevertheless no results have been posted yet. A very similar study evaluating the impact on CVD risk of cashew nut consumption in 40 participants has also been conducted (NCT02628171). Changes in lipoproteins and apolipoproteins, CRP, TNF-alpha, IL-6, PCSK9

(Proprotein convertase subtilisin/kexin type 9), endothelin-1, serum amyloid A, as well as fibrinogen and coagulation factor VII were monitored. The study was completed in March 2016, yet no results have been posted in the clinicaltrials.gov website. Likewise, protein biomarkers were used to assess the effect of intake of dietary fatty acid in the trial with identifier NCT02145936. A series of blood proteins were evaluated towards that end including: lipoproteins, CRP, TNF-alpha, IL-1, IL-6, MCP (monocyte chemoattractant)-1, slCAM(soluble forms of intracellular adhesion protein)-1, sVCAM(soluble vascular cell adhesion molecule)-1, sE-selctin, sP-selectin, LpPLA2 (lipoprotein associated phospholipase A2) as well as desaturase and prothrombin.

The study with identifier NCT03262714 investigates if dancing can improve cardiovascular risk factors (as reflected in serum CRP and TNF-alpha levels, among others) in advanced age women. In a Phase II trial with identifier NCT02062190, CRP and six cytokines (IL (interleukin)-12p70, IL-6, IL-10, IL-1b, IL-8 and TNF-α) were used to examine possible cardiovascular benefits after Resveratrol supplementation in 19 schizophrenia patients. The study was completed in May 2015 yet no results have been posted on the website. In the Phase III D-COR study that aims at assessing the cardiovascular effect of vitamin D supplementation, ApoA-I and ApoB were used as CVD risk markers and were evaluated in 411 participants (NCT02750293).

Peptide prognostic markers are also implemented as predictors of the outcome of heart transplantation. In the uPROPHET trial, the urinary peptidome of 352 recipients of allogenic heart transplant is profiled by high-dimensional classifiers, such as HF1

and HF2. [20] This guides therapy by selecting the most suitable treatment option that can be assigned to these patients with the ultimate goal of maximizing graft survival. HF1 and HF2 were developed from case-control studies involving patients with diastolic left ventricular dysfunction and -matched controls. They contain 85 and 671 urinary peptides respectively; these peptides include a myriad of collagen fragments. [21, 22] The study was completed at end of February 2018, yet the results have not been posted on the website yet.

Kidney Dysfunction

In the trial "Prognostic Biomarkers For Acute Kidney Injury In Liver Cirrhosis" urine and serum levels of KIM-1, as well as urinary l-FABP and protein/creatinine ratio were measured in 52 liver cirrhosis patients, to evaluate their prognostic value for acute kidney injury occurrence or worsening. (NCT03156426). The trial was completed in November 2017, yet no results have been posted yet. Similarly, urinary IGFBP7 (insulin-like growth factor binding protein 7) and TIMP-2 (tissue inhibitor of metalloproteinases 2) were evaluated as predictors of acute kidney damage after major surgery in the Navigate AKI trial (n=240 participants; NCT02114138). The expected study completion date is in December 2019.

Cancers

In the trial entitled "Serum Biomarkers in Diagnosis and Predicting Prognosis of Ovarian Cancers", the potential of four serum proteins TFF3 (trefoil factor 3), sFRP-4 (secreted frizzled related protein 4), Romo1 (reactive oxygen species modulator 1)

and NF(nuclear factor)-kB as prognostic biomarkers for prediction of overall and disease-free survival in ovarian cancer patients will be evaluated in 180 subjects (NCT03112733).

Brain Injury and Neurological Disorders

The SeizS100B trial targets to evaluate the prognostic value of serum levels of the protein S100B for seizure recurrence in patients with epilepsy. [23] The trial involving 75 participants is expected to be completed in December 2019 (NCT02424123).

In the ICON-TBI study, the main objective is to investigate if IL-1ß is predictive of the neuro-radiological evolution in traumatic brain injury in a cohort of 82 patients (NCT03659006). At the same time, the presence of serum ß-amyloid and Aß1-42, T-tau, P-tau181P in CSF (cerebrospinal fluid) are also monitored to investigate their potential associations to pathological phenotypes as estimated by multimodal MRI. The study is expected to be completed in 07/2020.

Inflammation and Autoimmune Diseases

In the trial "Prognostic Value of Three New Biomarkers of Multiple Sclerosis in Patients with Radiologically Isolated Syndrome (T-RIS)" (NCT03357887), the prognostic value of TNF receptor, secreted glycoprotein and two anonymous proteins in cerebrospinal fluid and serum is addressed. The trial targets to recruit 100 participants and even though it was initially foreseen to be completed by December 2018, its latest update (March 2018) states that recruitment has not been initiated yet.

In the study “Impact of Periodontal Disease on Outcome in Diabetes”, bone turnover markers, bone-specific alkaline phosphatase, as well as markers of systemic inflammation, hCRP (high-sensitivity CRP) and TNF-alpha, are investigated as predicative elements of the severity of periodontal disease in 24 diabetes patients (NCT02289066).

Other Complications

Pre-eclampsia is a disorder during pregnancy that if left unattended, can lead to dreadful outcomes for both mother and her infant. In the study named “Pancreatic Stone Protein (PSP) in Pregnant Woman”(NCT02247297), 486 pregnant women were recruited to study the prognostic value of PSP, previously associated with gastrointestinal pathologies to pre-eclampsia and in general inflammatory complications.

The EDMOCS trial investigates the prognostic value of CRP and procalcitonin (PCT) for post-surgical anastomotic leaks in 170 subjects undergoing ovarian cancer surgery with intestinal resection (NCT03131492). In the trial, serum levels of CRP and PCT are measured based on earlier studies supporting the prognostic value of these proteins following colorectal cancer surgery. [24-28]

In a phase IV trial (NCT02788474), the rate of change of CRP, collagen 1 and collagen 3 levels in baseline and week 12, are evaluated as predictors of ECM turnover in 347 patients with IPF (idiopathic pulmonary fibrosis). These marker measurements are an integral part of the evaluation of the effect imposed by the new

drug ninetedanib in IPF patients. The trial was completed in June 2018, with no results posted yet.

DISCUSSION

In this review, a systematic search was conducted to provide an overview of the clinical trials that involve peptide/ protein markers. The aim was to critically assess the contribution of proteomics in the field. The search strategy that we employed initially retrieved 2794 trials, which were gradually filtered based on the use of specific keywords and manual inspection of trials where proteins were used for disease diagnosis, prognosis, and prediction of treatment response or patient stratification for treatment selection. This strategy, relying at least to a good extent on a systematic search based on keywords, has apparent limitations as pertinent trials may have been possibly omitted. In a hypothetical scenario, a trial that uses procalcitonin to predict sepsis risk would have been excluded as it did not include the term “protein” in its title and biomarker measurement. However, through the critical investigation of 700 clinical trials, an informative overview of the current status of the field can be provided. Noticeably, a relatively large proportions of the 700 trials represented studies aiming at the discovery of biomarkers. In these trials, samples from patients and controls were profiled and analyzed so that biomarkers that correspond to stratification, diagnosis, prognosis can be identified. Although these trials were not included in the review as the focus was placed on studies which actually apply protein/ peptide biomarkers, they reflect the popularity of proteomics in biomarker discovery research.

According to the investigation criteria considering only trials over the last six years, we shortlisted 42 clinical trials that were further thoroughly examined. Most of them (n=22) include use of proteins as diagnostic markers, followed by 18 trials involving disease prognosis biomarkers and three studies using or validating proteins as stratification biomarkers to guide intervention. One probable reason of such under-representation of stratification studies in number can be that they are more demanding compared to diagnosis or prognosis studies in nature. Stratification trials require not only knowledge of the selection of biomarker(s) from previous studies, but also the clinical intervention according to results of stratification had to be well-defined. A schematic representation of the distribution of these trials is presented in the pie chart in **Figure 2A**.

As also shown (**Figure 2B**) most of the biomarkers are investigated in the context of cardiovascular disease (n=14), followed by brain injury/neurological disorder (n=8), inflammation/Autoimmune disease (n=7), cancer (n=5), and kidney dysfunction (n=3). **Figure 2C** illustrates the number of protein quantification assays used in the clinical trials. There are nine and six trials that employ the use of immunoassays and mass spectrometry respectively for protein quantification, and 28 trials which do not specify which kind of protein measurement assay they adopted. Unfortunately, no information about the sensitivity or specificity of biomarkers in the trials could be retrieved from the website or literature search. Considering the long time course of a clinical study, in particular for the prognosis trials, it is stipulated that some trials are still in progress while the others have not published the results yet. Owing to the same reason, no information about the cost-efficiency of biomarker(s) in use in each trial is

yet available. Nevertheless, we were able to acquire the comparison of cost-effectiveness of a few novel biomarkers versus standard care in previous studies with similar purposes, which may be considered as a fair reference to the readers. For instance, Westwood and colleagues concluded from 16 studies, that high-sensitivity cardiac troponin assay is cost-effective for early exclusion of acute myocardial infarction in a health-economic analysis.[29] With a decision-analytic Markov model, Petrovic et al. suggested that urinary liver-type FABP yields both higher effectiveness and lower cost than NGAL (neutrophil gelatinase-associated lipocalin) and serum cystatin C in diagnosing acute kidney injury in pediatric cardiac surgery.[30] (PMID: 26110039) Using a similar model, Critselis and colleagues reached the conclusion that an annual CKD273 classifier-based approach is more effectively, albeit more costly, in screening chronic kidney disease progression in comparison with urinary albumin excretion test.[31] (PMID: 29106632)

When examining the clinical trials, it is difficult to ignore the fact that most of the trials (n=38) are marked with "Not Applicable" for their clinical phases. Therefore, it is admitted that a sensible estimation about how close these studies from clinical implementation are hard to make, as some of these studies are observational or retrospective such that "phase" is not applicable. It is suggested that number of participants might offer some evidence for such estimation as it is clear that larger the sample size, the more statistically confident a trial is. Nevertheless, it ought not to be the only criterion to judge whether a trial is more "qualified" than the other, because there are other circumstances that shall be taken into consideration. For example, it is harder to recruit as many participants who suffer from a rare type of cancer as

hypertension; the quality comparison between such clinical trials is therefore not feasible. In addition, fewer participants could be a consequence of more stringent exclusion criteria, which can be extremely common in case-match and epidemiological studies. In these scenario, the quality of study is not compromised albeit smaller sample size. All in all, it is not easy to comment on the quality of individual trial regarding the fact that a lot of information of these ongoing trials is not available. To avoid subjectiveness, the **supplementary tables 1a, 1b, 1c** were constructed so that each trial can be bridged with the measurement of biomarkers as well as the original use of biomarkers in human study for the same clinical condition(s), in the hope that they will provide useful information to the readers.

The advancements of the proteomics technology have not only sparked the growth of protein biomarker discovery, but also reached technological advancements that allow clinical application. As a consequence, there are more and more clinical trials that target the validation or use of these novel biomarkers in a clinical setting. Nevertheless, the use of mass spectrometry as a measurement platform is still not that common when comparing to classical biochemical assays, such as ELISA (enzyme-linked immunosorbent assay) or immunohistochemistry. Although the limitation of mass spectrometry in absolute quantification is sometimes raised as a drawback, but with considerable effort spent on the development of more precise methods in labeling, control set-up and data acquisition, there has been a dramatic increment in the resolution and reliability of mass spectrometry. In addition, when perusing the 42 trials, it is observed that the combinatorial use of multiple biomarkers in the form of stratification/diagnostic/prognostic panels is gradually gaining popularity over the

evaluations based on single/ few biomarkers. For example, the stratification classifier CKD273 in PRIORITY [6] and the prognostic classifiers HF1 and HF2 [21, 22] in uPROPHET consist of 273, 85 and 671 urinary peptides respectively, while the diagnostic panel in SpecTRA [19] utilizes 16 and 141 proteomic biomarkers respectively.

While the growth of protein biomarkers is supported by the development of proteomics techniques, precision medicine clearly depends on the implementation of stratification and prognosis biomarkers assessing patient's risk of disease and/or anticipated response to treatment. [32] Although the partition in **Figure 2A** suggests that the diagnostic biomarkers are still dominating, stratification and prognostic biomarkers are gradually gathering momentum through their use in ever increasing numbers of clinical trials, promising to have a significant impact on disease management. Particularly the stratification markers have the potential of largely facilitating and improving the trials' results hence ultimately reducing associated costs and facilitating drug discovery by pre-selecting for patients that are highly likely to be benefited from the specific intervention. To further advance the field, posting of the trial results is required to guide trial design in the future, as well as data and sample sharing to allow for direct data comparability and cross-correlations. Even though easier said than done, certainly such an approach is feasible and worth-pursuing to rapidly validate biomarkers in disease management.

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Conflict of interest statement

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Figure s

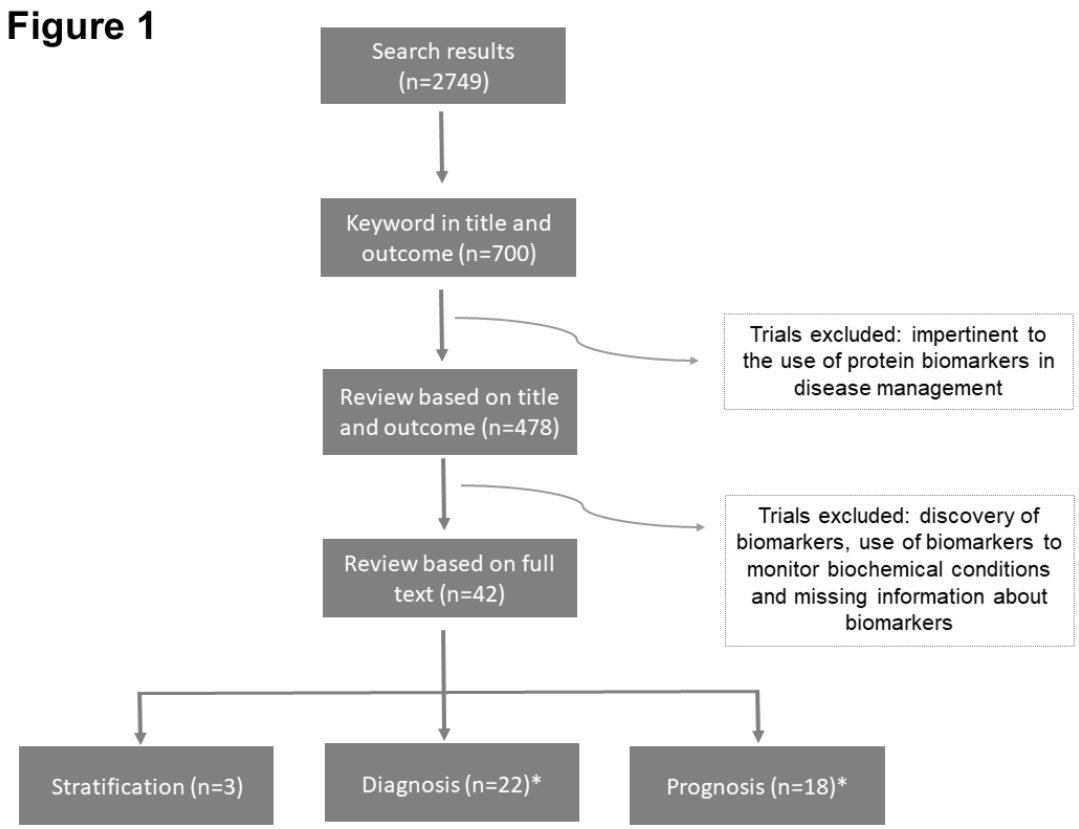

Figure 1. Schematic illustration of how 42 unique clinical trials were shortlisted from 2749 search results from clinicaltrails.gov. *The trial NCT03112733 is categorized both as "Diagnosis" and "Prognosis" due to its dual purpose

Figure 2A

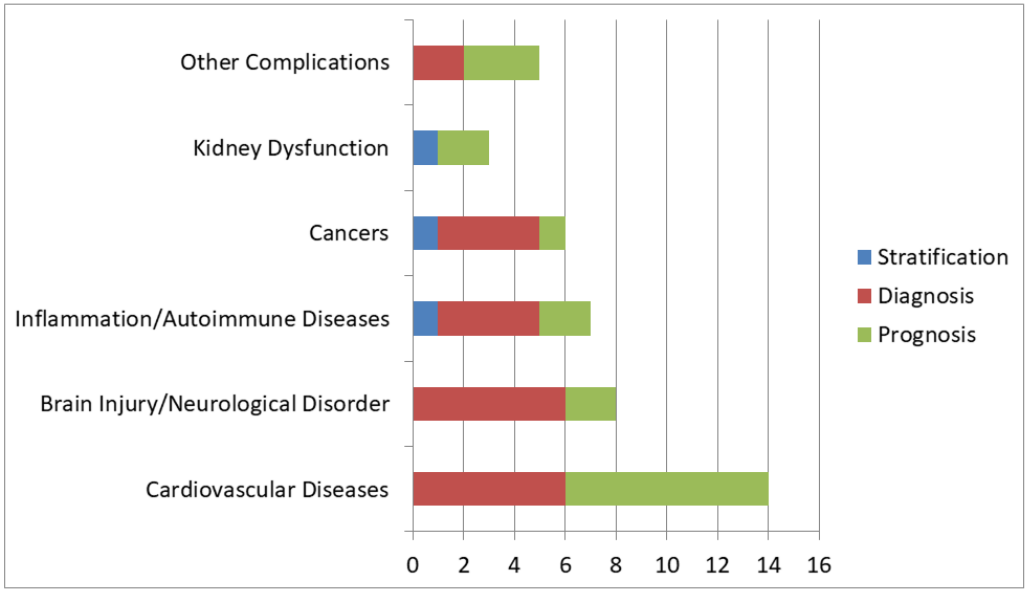

Figure 2B

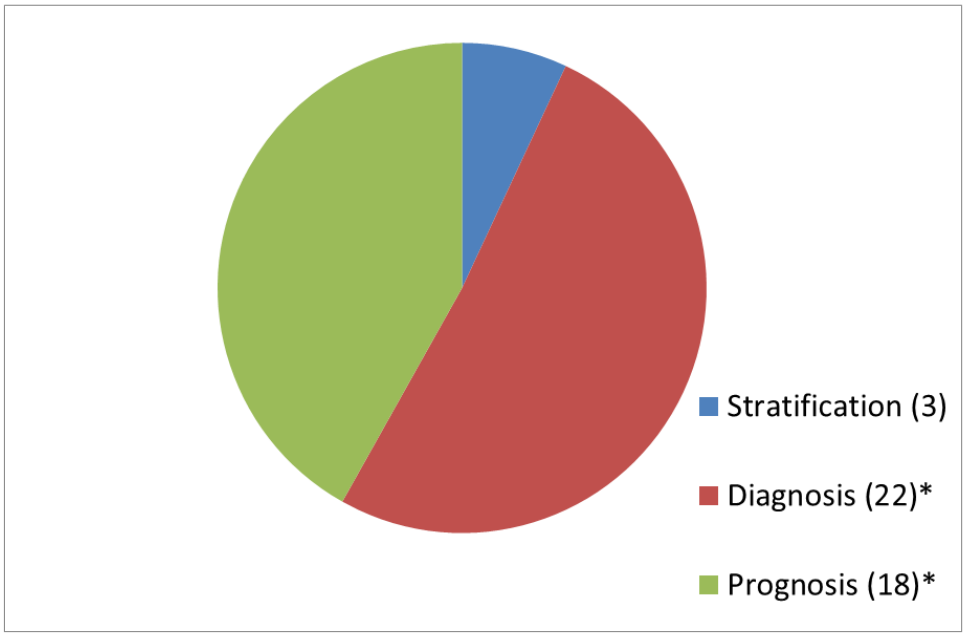

Figure 2C

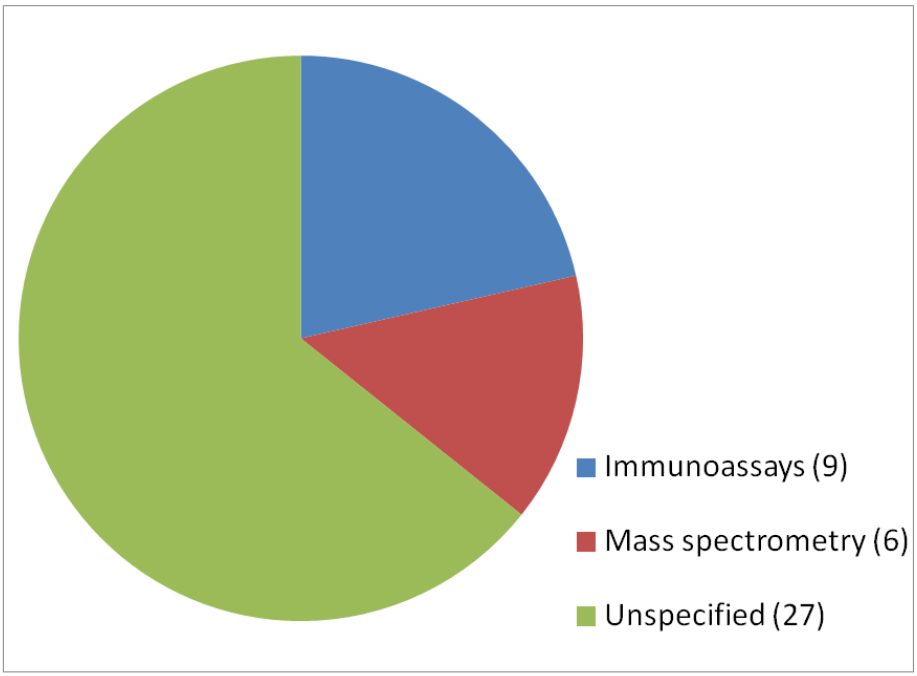

Figure 2. Overview on the application of protein biomarkers in clinical trials. **A)** Distribution of protein biomarkers in stratification, diagnosis and prognosis, **B)** Distribution of biomarkers for stratification, diagnosis and prognosis in different disease catalogs. **B)** Distribution of protein quantification assays. *The trial NCT03112733 is categorized both as “Diagnosis” and “Prognosis”.

Table 1. Stratification Biomarkers

Identifier	*Title*	*Biomarker(s)*	*Condition(s)*	*Participants*	*Phase*
NCT02987790	Duration of Antibiotic Therapy in Critically Ill Patients: CRP-guided Therapy Versus Best Practice	Serum CRP	Sepsis	135	NA
NCT03252717	Predictive Role of New Biomarkers for Hypersensitive Patients to Radiation in Breast Cancer (BIORISE)	Blood AK2, IDH2, ANX1, APEX1 and HSC70	Breast cancer	500	NA
NCT02040441	Proteomic Prediction and RAAS Inhibition Prevention Of Early Diabetic nephRopathy In Type 2 Diabetic Patients With Normalbuminuria (PRIORITY)	Urinary peptide CKD273	Diabetic Nephropathy Diabetic Retinopathy	1777	II/III

Abbreviations: *CRP: C-reactive protein; CVD: cardiovascular disease; LDL-C: low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol; Apo: apolipoprotein; AK2: adenylate kinase 2; IDH2: isocitrate dehydrogenase 2; ANX1: annexin 1; APEX1: DNA-(apurinic or apyrimidinic site) lyase; HSC70: heat shock cognate 71 kDa.*

Table 2. Diagnostic Biomarkers

Identifier	*Title*	*Biomarker(s)*	*Condition(s)*	*Participants*	*Phase*
		BRAIN INJURY AND NEUROLOGICAL DISORDERS			
NCT02916069	Multi Modal Brain Monitoring and Cardiac Surgery	Serum S100	Neural damage after cardiac surgery	1200	NA
NCT03551223	Neural Damage and Anesthetic Treatment in the Preeclamptic Parturient; a Prospective Observational Study	Serum S100B and NSE	Neural damage after anaesthesia	50	NA
NCT03093857	Investigation of the Cerebrospinal Fluid and Further Tissue Samples for Biomarker Indicating Spinal Ischemia and Organ Failure in Patients With TAAA	Neuropeptide P, neuropeptide Y, NFL, S100B, GFAp in serum or urine	Spinal ischemia	100	NA
NCT02819778	Study Assessing Evaluation of the Interest of Serum S100B Protein Determination in the Management of Pediatric mTBI (PROS100B)	Serum S100B	Pediatric mTBI	4000	NA
NCT03345602	Serum S100B Protein Assay in Mild Head Injury	Serum S100B	Head injury	400	NA
NCT03490500	Using the S100B Protein for Emergency Headache Management Care	Serum S100B	Headache	250	NA
		INFLAMMATION AND AUTOIMMUNE DISEASES			
NCT03474809	Early Diagnostic of Sepsis and Potential Impact on Antibiotic Management Based on Serial PSP Measured Using the AbioScope.	PSP in blood	Sepsis	300	NA
NCT03704766	Alpha-Defensin and Synovial Proteins to Improve Detection of Pediatric Septic Arthritis	Serum CRP, PCT, and D-dimer; Synovial CRP, alpha-defensin, leukocyte esterase, neutrophil elastase	Septic arthritis	42	NA

NCT03164720	Serum/Urinary Monocyte Chemoattractant Protein-1 Level as a Marker for Lupus Nephritis	Serum and urinary MCP1	Systemic lupus	60	NA
NCT03667742	Diagnosing of Acute Tuberculosis	CRP, ESAT-6, CFP-10 and C1q in blood or sweat	Tuberculosis	90	NA
		CANCERS			
NCT03472716	The ßIG-H3 Protein: a New Marker in PANCreatic Adenocarcinoma (BIGHPANC)	ßIG-H3 in blood	Pancreatic adenocarcinoma	80	NA
NCT03345966	Assessment of BMI-1 on Protein and Molecular Levels in Oral Dysplasia and Squamous Cell Carcinoma: A Diagnostic Study	BMI-1 in blood	Oral squamous cell carcinoma	18	NA
NCT03460080	Diagnostic Value of AFP-L3 and PIVKA-II in HCC	Serum AFP-L3, PIVKA-II, ALT, AST, glutamyl transferase, gamma-glutamyl transferase, HbsAg and anti-HCV	Hepatocellular carcinoma	200	NA
NCT03112733	Serum Biomarkers in Diagnosis and Predicting Prognosis of Ovarian Cancers	Serum TFF3, sFRP-4, Romo1 and NF-κB	Ovarian cancer	180	NA
		CARDIOVASCULAR DISEASES			
NCT03507270	One-hour Diagnostic Algorithm for NSTEMI	FABP, troponin, CPK, CPK-MB in blood	Non-ST Segment Elevation Myocardial Infarction	20	NA
NCT03158259	Prehospital Advanced Diagnostics and Treatment of Acute Stroke (Treat-NASPP)	GFAP and RBP4 in blood	Ischemic (Brain) stroke, hemorrhagic Stroke	400	NA
NCT01826994	Incremental Value of Point of Care H-FABP Testing in Primary Care Patients Suspected of Acute Coronary Syndrome (RAPIDA)	H-FABP in blood	Acute coronary syndrome,	303	NA

			angina pectoris (unstable/stable), thoracic diseases		
NCT03050099	SpecTRA; An Observational Study of the Verification of Protein Biomarkers in Transient Ischemic Attack	141 pre-selected proteomic biomarkers in blood	Transient ischemic attack	560	NA
NCT03070067	SpecTRA; A Study of the Validation of Protein Biomarkers in Transient Ischemic Attack	ApoB100, FABP, SELL, ANPR-1, IGFBP3, F5, F9, F10, ADPN, vWF, THBS1, PRL, EGFP, VEFG, HPX, MBT, Serpin A5, HCII, HABP2 in blood	Transient ischemic attack	1150	NA
NCT02921321	Pilot Study of Cardiac MR in Patients With Muscular Dystrophy	Serum ST2	Heart dysfunction in muscular dystrophy	100	NA
		OTHER COMPLICATIONS			
NCT03097276	Management of Patients With High C-reactive Protein After Scheduled Resection of Colorectal Cancer (GESPACE)	Serum CRP	Anastomotic fistula	75	NA
NCT02057731	Study of Glycogen Storage Disease Expression in Carriers	Creatine kinase, prealbumin, microalbumin, hemoglobin A1C and CRP in blood, urine and saliva	Glycogen storage disease	114	NA

Abbreviations: *NSE: neuron specific enolase; NFL: neurofilament triplet protein, GFAp: glial fibrillary protein; TAAA: Thoracoabdominal Aortic Aneurysm; mTBI: mild traumatic brain injury; CCT: cranial computed tomography; PSP: pancreatic stone protein; PCT: procalcitonin; MCP1: Monocyte chemoattractant protein-1; BMI-1: polycomb complex protein; ESAT-6: early secretory antigenic target 6; CFP-10: culture filtrate protein 10; C1q: complement component 1q; CRP: C-reactive protein; AFP-L3: alpha-fetoprotein-L3; PIVKA-II: protein induced by vitamin K absence or antagonist-II, ALT: alanine aminotransferase; AST: aspartate aminotransferase; TFF3: trefoil factor 3; sFRP-4: secreted frizzled related protein 4; Romo1: reactive oxygen species modulator 1; NF-κB: nuclear factor-κB; FABP: fatty acid binding protein; CPK: creatine phosphokinase; GFAP: Glial fibrillary acidic protein; RBP4: retinal binding protein 4; h-FABP: heart-type fatty acid binding protein; MR: magnetic resonance; ApoB100: apolipoprotein B100; FABP3: fatty acid binding protein 3, SELL: L-selectin; ANPR-1: atrionatriuretic peptide receptor 1; IGFBP3: insulin-like growth factor binding protein 3; F5: coagulation factor V; ADPN: adiponutrin; vWF: von Willebrand factor; THBS1: thrombospondin 1, PRL: prolactin, paraoxonase 3; EGFP: epidermal growth factor receptor; VEFG: vascular endothelial growth factor; HPX: henopexin; MBT: myeloblastin; Serpin A5: plasma serine protease inhibitor; HCII: heparin cofactor II, HABP2: hyaluronan-binding protein hCRP: high-sensitivity C-reactive protein; ECM: extracellular matrix.*

Table 3. Prognostic Biomarkers

Identifier	*Title*	*Biomarker(s)*	*Condition(s)*	*Participants*	*Phase*
		CARDIOVASCULAR DISEASES			
NCT02402803	Dried Blood Spot- Statin Pilot Study (DBS)	Apo B and ApoA-I in fingerprick dried blood sample and plasma	CVD	20	NA
NCT02210767	Effects of Walnuts on Central Blood Pressure, Arterial Stiffness Indices, Lipoproteins, and Other CVD Risk Factors	Proteins in blood include: 1) Lipoproteins; 2) Inflammation: CRP, IL-6, TNF-α; 3) Vascular adhesion: VCAM, ICAM4	CVD	46	NA
NCT03262714	Effects of Dancing on Cardiovascular and Functional Risk Factors in Older Women	Serum CRP, TNF-α	CVD	30	NA
NCT02145936	Effect of Dietary Fatty Acids on CVD Risk Indicators and Inflammation	Proteins in blood include: 1) Lipoproteins, LpPLA2; 2) Inflammation: IL-6, TNF-α, MCP-1, CRP; 2) Vascular adhesion molecules: slCAM-1, sVCAM-1, sE-selctin, sP-selectin; 3) Desaturase; 4) Prothrombin	Dyslipidemia, CVD	20	NA
NCT02628171	Impact of Cashew Nuts in the Human Diet: Measured Energy Value and Effects on Cardiovascular Disease Risk Factors	Proteins in blood include: 1) Lipoproteins; apolipoproteins, PCSK9; 2) Inflammation: IL-6, TNF-α**,** CRP, serum amyloid A; 3)Vascular health: adhesion molecules and endothelin-1; 4)Hemostasis: fibrinogen and factor VII	CVD	40	NA
NCT02750293	The Effect of Vitamin D Supplementation on Cardiovascular Risk Factors (D-COR)	1)Apolipoprotein A1 and B in blood; 2) Proteomic profile from biopsy sample examined by mass spectrometry	CVD	411	III
NCT02062190	Resveratrol, Cardiovascular Risk Markers And	Serum CRP and cytokines (IL-12p70, IL-6, IL-10, IL-	CVD	19	II

	Cognitive Performance In Patients With Schizophrenia	1ß, IL-8 and TNF-α)			
NCT03152422	Urinary Proteomics in Predicting Heart Transplantation Outcomes (uPROPHET)	HF1 and HF2 urinary peptide classifiers	Heart transplant	352	NA
		BRAIN INJURY AND NEUROLOGICAL DISORDERS			
NCT02424123	Is Protein S100B a Predictor of First-to-chronic Seizure Conversion in Adults? (SeizS100B)	Serum S100B	Epilepsy, seizure	75	NA
NCT03659006	Identification of Predictive Neuroinflammatory Biomarkers of Neuro-radiological Evolution in Severe TBI (ICON-TBI)	IL-1ß in serum and CSF	TBI	82	NA
		KIDNEY DYSFUNCTION			
NCT03156426	Prognostic Biomarkers For Acute Kidney Injury In Liver Cirrhosis	KIM-1 in plasma and urine; urinary l-FABP and protein/creatinine ratio	Acute kidney injury in liver cirrhoses	52	NA
NCT02114138	Network Analysis of Urinary Molecular Signature Complements Clinical Data to Predict Postoperative Acute Kidney Injury (Navigate AKI)	Urinary IGFBP7 and TIMP-2	Acute kidney injury	240	NA
		INFLAMMATION AND AUTOIMMUNE DISEASE			
NCT03357887	Prognostic Value of Three New Biomarkers of Multiple Sclerosis in Patients With Radiologically Isolated Syndrome (T-RIS)	Tumor necrosis factor receptor, glycoprotein, protein X and protein Y in serum and CSF	Multiple sclerosis	100	NA
NCT02289066	Impact of Periodontal Disease on Outcomes in Diabetes	TNF-α, hCRP, bone-specific alkaline phosphatase in blood	Periodontal disease in diabetes[i]	250	NA

		CANCERS			
NCT03112733	Serum Biomarkers in Diagnosis and Predicting Prognosis of Ovarian Cancers	Serum TFF3, sFRP-4, Romo1 and NF-κB	Ovarian cancer	180	NA
		OTHER COMPLICATIONS			
NCT02247297	PSP in Pregnant Women	Serum PSP	Pre-eclampsia	486	NA
NCT03131492	Early Dehiscence Markers in Ovarian Cancer Surgery (EDMOCS)	CRP and PCT in blood	Intestinal anastomotic leak after ovarian cancer surgery	170	NA
NCT02788474	Effect of Nintedanib on Biomarkers of Extracellular Matrix Turnover in Patients With Idiopathic Pulmonary Fibrosis and Limited Forced Vital Capacity Impairment	hCRP, Collagen 1, Collagen 3 in blood	ECM turnover in idiopathic pulmonary fibrosis	347	IV

Abbreviations: CRP: C-reactive protein; IL-6: interleukine-6, TNF-alpha: tumor necrosis factor-alpha; ICAM4: intracellular adhension molecule 4; MCP-1: monocyte chemoattractant protein 1; slCAM-1: soluble forms of intercellular adhesion protein-1; sVCAM-1: soluble vascular cell adhesion protein 1; LpPLA2: lipoprotein associated phospholipase A2; PCSK9: Proprotein convertase subtilisin/kexin type 9; KIM-1: kidney injury molecule 1; l-FABP: liver type fatty acid binding protein; IGFBP7: insulin-like growth factor-binding protein 7; TIMP-2: tissue inhibitor of metalloproteinases-2; CSF: cerebrospinal fluid; TFF3: levels of trefoil factor 3; sFRP-4: secreted frizzled related protein 4; Romo1: reactive oxygen species modulator 1; NF-κB: nuclear factor-κB; PSP: pancreatic stone protein; PCT: procalcitonin; ECM: extracellular matrix.