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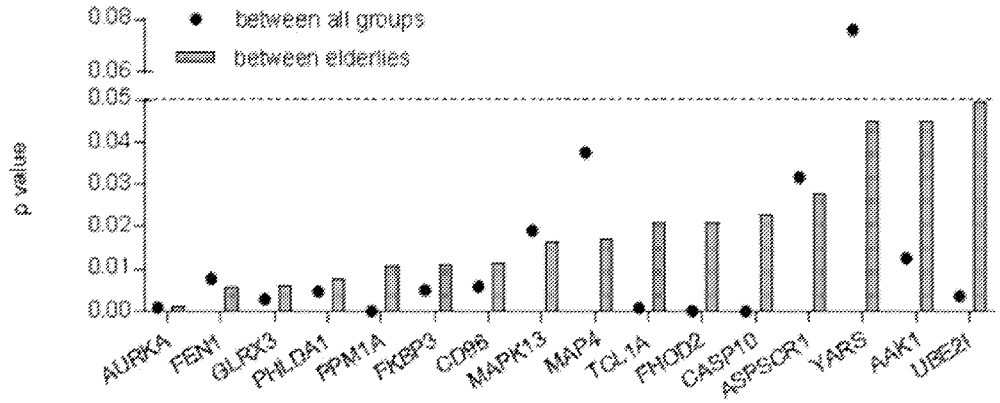
(54) Title: IDENTIFICATION OF HEALTH STATUS IN THE ELDERLY USING IMMUNOLOGICAL BIOMARKERS

Figure 3A



(57) Abstract: A method for determining the health status of an elderly individual by testing the sample extracted from the individual for the presence of biomarkers, the bio markers being autoantibodies to antigens comprising MAPK13, CD96, FKBP3, PPM1A, PHLDA1, GLRX3, FEN1 and AURKA, wherein the antigens may further comprise one or more of UBE2I, AAK1, YARS, ASPSCR1, CASP10, FHOD2, TCL1A and MAP4, wherein PHLDA1 and CD96 correspond to healthy, AURKA, FEN1, CASP10 and AAK1 correspond to intermediate health, and UBE2I, YARS, ASPSCR1, FHOD2, TCL1A, MAP4, MAPK13, FKBP3, PPM1 A and GLRX3 correspond to unhealthy.

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[Continued on next page]

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IDENTIFICATION OF HEALTH STATUS IN THE ELDERLY USING IMMUNOLOGICAL BIOMARKERS.

Field of Invention

The invention relates to the detection of immunological biomarkers, particularly autoantibodies, to determine the health status and/or aging trajectory in the elderly.

Background

Despite technological advances in the area of proteomics research, there are only a handful of biomarkers that have entered the clinic, and 90% of the biomarkers are protein biomarkers [1]. Autoantibody biomarkers as described herein are autoantibodies to antigens, autoantibodies being antibodies which are produced by an individual which are directed against one or more of the individual's own proteins ('self' antigens). Some of the main reasons for failure of biomarkers [2] to make it into clinical practice are:

1) Low sensitivity and specificity
2) Low prognostic/predictive value
3) Not important for clinical decision making
4) Original claims fail validation (false discoveries)

The management of care of elderly individuals depends less on age than on the effect of their comorbidity history (past and present) on their current health status [3]. These comorbidities impose a certain stress on the immune system which has been challenged over the years to deal with infections, cancer or chronic inflammatory diseases [4].

An aim of the invention is therefore to provide an improved panel of autoantibody biomarkers for assessing the health status of elderly individuals.

Summary of Invention

In one aspect of the invention, there is provided a method for determining the health of an individual from a sample extracted from that individual, comprising the steps of:

(i) testing the sample for the presence of biomarkers specific for health;
(ii) determining whether the subject is healthy, is of intermediate health, or is unhealthy, based on the detection of said biomarkers;

characterised in that the biomarkers are autoantibodies to antigens comprising AURKA, FEN1, GLRX3, PHLDA1, PPM1A, FKBP3, CD96 and MAPK13.

In one embodiment the individual is elderly, typically at least 60 years old.

Advantageously the autoantibody biomarkers can be used in the characterization (or diagnosis) of the health status of an elderly individual (Healthy, Intermediate and Unhealthy) by measuring the distribution of plasma-antibody levels. Furthermore a subset of these autoantibody biomarkers, particularly those associated with Healthy and Intermediate, may have a protective role against non-communicable disease.

In one embodiment the sample is tested using a panel of antigens that correspond to the autoantibody biomarkers. Typically, the antigens are biotinylated proteins. Advantageously the biotinylation ensures that the antigens are folded in their correct form to ensure accuracy of detection by the autoantibody biomarkers.

In one embodiment the antigens may include one or more from the group comprising of UBE2I, AAK1, YARS, ASPSCR1, CASP10, FHOD2, TCL1A and MAP4.

It should be noted that not all antigens generate an autoantibody response and it is not possible to predict *a priori* which antigens will do so in a given cohort – of more than 1500 antigens tested, only autoantibodies against the 16 antigens described above are suitable as biomarkers to identify health and aging status.

In one embodiment each biotinylated protein is formed from a Biotin Carboxyl Carrier Protein (BCCP) folding marker which is fused in-frame with the protein.

In a further embodiment the biotinylated proteins are bound to a streptavidin-coated substrate.

Advantageously full-length proteins are expressed as fusions to the BCCP folding marker which itself becomes biotinylated *in vivo* when the fusion partner is correctly folded. By comparison misfolded fusion partners cause the BCCP to remain in the ‘apo’ (i.e. non-

biotinylated) form such that it cannot attach to a streptavidin substrate. Thus, only correctly folded fusion proteins become attached to the streptavidin substrate via the biotin moiety appended to the BCCP tag.

In one embodiment the substrate comprises a glass slide, biochip, strip, slide, bead, microtitre plate well, surface plasmon resonance support, microfluidic device, thin film polymer base layer, hydrogel-forming polymer base layer, or any other device or technology suitable for detection of antibody-antigen binding.

In one embodiment the substrate is exposed to a sample extracted from a person, such that autoantibody biomarkers from the sample may bind to the antigens.

Typically, the sample comprises any or any combination of exosomes, blood, serum, plasma, urine, saliva, amniotic fluid, cerebrospinal fluid, breast milk, semen or bile.

In one embodiment following exposure to the sample, the substrate is exposed to a fluorescently-tagged secondary antibody to allow the amount of any autoantibodies from the sample bound to the antigens on the panel to be determined. Typically, the secondary antibody is anti-human IgG, but it will be appreciated that other secondary antibodies could be used, such as anti-IgM, anti-IgG1, anti-IgG2, anti-IgG3, anti-IgG4 or anti-IgA.

In one embodiment the healthiness of the individual corresponds to the relative or absolute amount of autoantibodies from the sample specifically binding to the antigens.

In one embodiment the method is performed *in vitro*.

In one embodiment the method comprises detecting upregulation/downregulation of one or more biomarkers.

In a further aspect of the invention, there is provided a method for manufacturing a kit for determining the health of an elderly individual from a sample extracted from that individual, comprising the steps of:

for each antigen in a panel, cloning a biotin carboxyl carrier protein folding marker in-frame with a gene encoding the said antigen and expressing the resulting biotinylated antigen;

binding the biotinylated antigens to addressable locations on one or more streptavidin-coated substrates, thereby forming an antigen array;

such that the amount of autoantibodies from the sample binding to the antigens on the panel can be determined by exposing the substrate to the sample and measuring the response;

characterised in that the antigens comprise AURKA, FEN1, GLRX3, PHLDA1, PPM1A, FKBP3, CD96 and MAPK13.

In one embodiment the antigens may include one or more from the group comprising of UBE2I, AAK1, YARS, ASPSCR1, CASP10, FHOD2, TCL1A and MAP4.

In a further aspect of the invention there is provided a method for determining the health of an elderly individual by exposing a composition comprising a panel of antigens as herein described to a sample extracted from that individual, and determining the level of autoantibodies from the sample binding to the antigens.

In a yet further aspect of the invention there is provided a method for determining the health of an elderly individual by exposing a composition comprising a panel of antigens as herein described to a sample extracted from that individual *in vitro*, and determining the level of autoantibodies from the sample binding to the antigens.

In further aspect of the invention, there is provided a composition comprising a panel of antigens for determining the health of an elderly individual, characterised in that the antigens comprise AURKA, FEN1, GLRX3, PHLDA1, PPM1A, FKBP3, CD96 and MAPK13.

In one embodiment the antigens may include one or more from the group comprising of UBE2I, AAK1, YARS, ASPSCR1, CASP10, FHOD2, TCL1A and MAP4.

In one embodiment the antigens are biotinylated proteins

In one embodiment the amount of one or more autoantibody biomarkers binding *in vitro* to the antigens in a sample from a patient can be measured to determine the health status of the patient.

In yet further aspect of the invention, there is provided a composition comprising a panel of autoantibody biomarkers for determining the health status of an elderly patient;

wherein the level of one or more autoantibody biomarkers are measured in a sample from the patient;

characterised in that the one or more autoantibody biomarkers are selected from autoantibodies specific for one or more of the following antigens: AURKA, FEN1, GLRX3, PHLDA1, PPM1A, FKBP3, CD96 and MAPK13.

Brief Description of Drawings

It will be convenient to further describe the present invention with respect to the accompanying drawings that illustrate possible arrangements of the invention. Other arrangements of the invention are possible, and consequently the particularity of the accompanying drawings is not to be understood as superseding the generality of the preceding description of the invention.

Figure 1 illustrates the structure of the *E. coli* Biotin Carboxyl Carrier Protein domain.

Figure 2 illustrates the pPRO9 plasmid used as a vector.

Figure 3 illustrates proteins associated with cell-cycle and cell-death as (A) a chart; (B) linked pathways.

Figure 4 illustrates a clustering analysis: (A) Representation of clusters defined within the elderlies by the 16 antigens by tSNE clustering analysis [5]; (B) Expression density of antibodies for each target protein; (C) Autoantibodies specific to each of the health status groups;.

Figure 5 illustrates the cohort selection: (A) Schematic describing the workflow used to select and categorize elderly individuals in the study; (B) Distribution of elderly and young individuals according to age and gender; Statistical analysis performed with Kruskal-Wallis test with Dunn's correction; (C) Range of clinical variables used for the categorization of elderly individuals; (D) Characteristic of elderly individuals selected in the study for the 6 determining clinical parameters. .

Detailed Description

Materials and Methods

Gene synthesis and cloning. The pPRO9 plasmid (see Figure 2 below) was constructed by standard techniques and consists of a c-myc tag and BCCP protein domain, preceded by a multi-cloning site. A synthetic gene insert was assembled from synthetic oligonucleotides and/or PCR products. The fragment was cloned into pPRO9 using SpeI and NcoI cloning sites. The plasmid DNA was purified from transformed bacteria and concentration determined by UV spectroscopy. The final construct was verified by sequencing. The sequence congruence within the used restriction sites was 100%. 5µg of the plasmid preparation was lyophilized for storage.

The recombinant baculoviruses are generated via co-transfection of a bacmid carrying the strong viral polyhedrin promoter together with a transfer vector carrying the coding sequences of protein of interest, into the Sf9 cell line which is a clonal isolate derived from the parental *Spodoptera frugiperda* cell line IPLB-Sf-21-AE. Homologous recombination initiated by the viral system causes the transfected cells to show signs of viral cytopathic effect (CPE) within few days of culture incubation. The most common CPE observed was the significantly enlargement of average cell size, a consequences of viral progeny propagation. These baculoviruses known as P0 were then released into the culture medium, and viral amplification were done to generate a higher titre of P1 viruses.

Protein Expression. Expressions were carried out in 24 well blocks using 3ml cultures containing $6x10^6$ Sf9 cells per well. High titre, low passage, viral stocks of recombinant baculovirus (>10^7 pfu/ml) were used to infect sf9 insect cells. The infected cells were

then cultured for 72 hours to allow them to produce the recombinant protein of interest. The cells were washed with PBS, resuspended in buffer, and were frozen in aliquots at -80°C ready for lysis as required. Depending on the transfer vector construct and the nature of the protein itself, recombinant protein lysate can be pelleted either from the cultured cell or the cultured medium. Positive recombinant proteins were then analyzed via SDS-PAGE and Western blot against Streptavidin-HRP antibody. In total, 1557 human antigens were cloned and expressed using this methodology.

Array fabrication. HS (hydrogel-streptavidin) slides were purchased from Schott and used to print the biotinylated proteins. A total of 9 nanoliters of crude protein lysate was printed on a HS slide in quadruplicate using non-contact piezo printing technology. Print buffer that have a pH between 7.0 and 7.5 were used. The slides were dried by centrifugation (200 x g for 5 min) before starting the washing and blocking. The printed arrays were blocked with solutions containing BSA or casein (concentration: 0.1 mg/ml) in a phosphate buffer. The pH was adjusted to be between 7.0 and 7.5 and cold solutions were used (4 °C - 20 °C). Slides were not allowed to dry between washes and were protected from light. In total, each resultant 'Immunome array' comprised 1557 antigens, each printed in quadruplicate.

Experimental Procedure. Each critical experimental step of running the Immunome array required a second trained person to thoroughly check, precisely record and cross-check all steps in the protocol, in order to reduce operator bias. Samples were picked, randomised and assigned to assay racks accordingly. These samples were then stored at -20°C until the experimental setup was complete.

1. Study cohort

The study cohort was divided into 2 age groups: young control individuals (YC) and the elderly individuals. The YC group (n=60) composed of male (n=34) and female (n=26) individuals of Chinese ethnicity from 18 to 27 years of age. They are clinically healthy with no reported comorbidities nor active medical treatments. The selection of elderly individuals was performed within elderly individuals of Chinese ethnicity of 60 years of age and beyond. This initial selection increases the analytical power and outcome of this study by removing an ethnicity bias.

Further selection of elderly individuals into health classes (Healthy, Intermediate and Unhealthy) was based on the combination of 6 clinical parameters (Figure 5A):

- Commorb5: Variable reflecting the total number of comorbidities excluding eye problems
- NADL: Total number of disabilities affecting Activities of Daily Living of the elderly individual [7]
- Wo_sf1: Parameter measuring the general quality of life.
- Frailty: A clinical syndrome where the elderly individual is progressively highly vulnerable to internal and external stressors. It is a multidimensional variable taking into account the physical strength and cognitive abilities [8]
- MMSEtot: Total score of the Mini-Mental State Examination. This is indicative of the cognitive capabilities of the elderly individual [9].
- GDStot: Total score of the Geriatric Depression Scale. This is a self-reported assessment used to identify the depression in the elderly [10].

The characterization of the health status of the elderly individuals takes into accounts the 6 parameters previously described and resulted in the selection of the following groups (Figure 5A):

- 115 Healthy elderlies,
- 111 elderlies with Intermediate health status,
- 114 Unhealthy elderlies.

There are no significant variations of age between the health groups although a gender difference can be observed as more females are present in each health group (Figure 5B, Figure 5D). In Figure 5D, bold numbers determine the grade of the individuals for the specific category and score. Numbers between brackets correspond to number of individuals with specific traits. ND: Not determined. Ave: Average age of all elderly individuals for each health groups [6].

Overall the repartition of the individuals showed that unhealthy elderly individuals present an accumulation of comorbidities, an increased frailty status and cognitive decline associated with higher depressive status and an increased quality of life (Figure 5C, Figure 5D).

2. Serum/Plasma Dilution

Samples were then placed in a shaking incubator set at +20°C to allow thawing for 30 minutes. When completely thawed, each sample was vortexed vigorously three times at full speed and spun down for 3 minutes at 13,000 g using a microcentrifuge. 22.5 µL of the sample was pipetted into 4.5 mL of Serum Assay Buffer (SAB) containing 0.1% v/v Triton, 0.1% w/v BSA, 10% v/v PBS (20°C) and vortexed to mix three times. The tube was tilted during aspiration to ensure that the sera was sampled from below the lipid layer at the top but does not touch the bottom of the tube in case of presence of any sediment. This Serum/Plasma dilution process was carried out in a class II Biological Safety Cabinet. Batch records were marked accordingly to ensure that the correct samples were added to the correct tubes.

3. Biomarker Assay

The array was removed from the storage buffer using forceps, placed in the slide box and rack containing 200 mL of cold SAB (4°C) and shaken on shaker at 50 rpm, for 5 minutes. When the slides have completed washing, the slide was placed, array side up, in a slide hybridization chamber with individual sera which had been diluted earlier. All slides were scanned using the barcode scanner into the relevant batch record and incubated in a refrigerated shaker at 50 rpm for 2 hours at 20°C.

4. Array Washing After Serum Binding

The protein array slide was then rinsed twice in individual “Pap jars” with 30 mL SAB, followed by 200 mL of SAB buffer in the slide staining box for 20 minutes on the shaker at 50 rpm at room temperature. All slides were transferred sequentially and in the same orientation.

5. Incubation with Cy3-anti IgG

Binding of autoantibodies to the arrayed antigens on replica Immunome arrays was detected by incubation with Cy3-rabbit anti-human IgG. Arrays were immersed in hybridization solution containing a mixture of Cy3- rabbit anti-human IgG solution diluted 1000-fold in SAB buffer for 2 hours at 50 rpm in 20°C.

6. Washing After Incubation with Cy3-anti IgG

After incubation, the slide was dipped in 200 mL of SAB buffer, 3 times for 5 minutes at 50 rpm at room temperature. Excess buffer was removed by immersing the slide in 200 mL of pure water for a few minutes. Slides were then dried for 2 min at 240g at room temperature. Slides were then stored at room temperature until scanning (preferably the same day). Hybridization signals were measured with a microarray laser scanner (Agilent Scanner) at 10μm resolution. Fluorescence intensities were detected according to the manufacturer's instructions, whereby each spot is plotted using Agilent Feature Extraction software.

Spot segmentation Semi-automatic QC process was carried out in order to produce a viable result. The output from the microarray scanner is a raw .tiff format image file. Extraction and quantification of each spot on the array were performed using the GenePix Pro 7 software (Molecular Devices). A GAL (GenePix Array List) file for the array was generated to aid with image analysis. GenePix Pro 7 allows for automatic spot gridding and alignment of each spot on the array for data extraction. Following data extraction, a GenePix Results (.GPR) file was generated for each slide which contains numerical information for each spot; Protein ID, protein name, foreground intensities, background intensities etc.

Bioinformatics analysis.

1. Image Analysis: Raw Data Extraction

The aim of an image analysis is to evaluate the amount of autoantibody present in the serum sample by measuring the median intensities of all the pixels within each probed spot. A raw .tiff format image file is generated for each slide, i.e. each sample. Automatic extraction and quantification of each spot on the array are performed using the GenePix Pro 7 software (*Molecular Devices*) which outputs the statistics for each probed spot on the array. This includes the mean and median of the pixel intensities within a spot along with its local background. A GAL (GenePix Array List) file for the array is generated to aid with image analysis. This file contains the information of all probed spots and their positions on the array. Following data extraction, a GenePix Results (.GPR) file is generated for each slide which contains the information for each spot; Protein ID, protein

name, foreground intensities, background intensities etc. In the data sheet generated from the experiment, both foreground and background intensities of each spot are represented in relative fluorescence units (RFUs).

2. Data Handling and Pre-processing

For each slide, proteins and control probes are spotted in quadruplicate - 4 arrays on each slide. The following steps were performed to verify the quality of the protein array data before proceeding with data analysis:

Step 1:

Calculate net intensities for each spot by subtracting background signal intensities from the foreground signal intensities of each spot. For each spot, the background signal intensity was calculated using a circular region with three times the diameter of the spot, centered on the spot.

Step 2:

Remove replica spots with RFU ≤ 0.

Step3:

No saturated pixels should be visible within the spots across array which may exceed scanner's reading capacity (maximum RFU for our scanner is 65536 RFU). Therefore, spot/s that show saturation in > 20% of the pixels were removed if it occurs in ≤ 2 replica/s. If saturated spots occur in 3 or more replicas of that protein or probe, these proteins/probes will be flagged as "SAT" and excluded from the downstream analyses.

Step 4:

Zero net intensities if only 1 replica spot remaining.

Step 5:

Calculating percentage of coefficient of variant (CV%) of to determine the variations between the replica spots on each slide.

$$CV\% = \frac{S.D.}{Mean} \times 100\% \qquad \textbf{Equation 1}$$

Flag a set of replica spots with only 2 or less replica/s remaining and CV% > 20% as "High CV". The mean RFU of these replica spots (i.e. proteins) will be excluded from the downstream analysis.

For proteins/controls with a CV% > 20% and with 3 or more replica spots remaining, the replica spots which result in this high CV% value were filtered out. This was done by calculating the standard deviation between the median value of the net intensities and individual net intensities for each set of replica spots. The spot with the highest standard deviation was removed. CV% values were re-calculated and the process repeated.

Step 6:

Calculating the mean of the net intensities for the remaining replica spots.

Step 7:

Composite normalisation of data using both quantile-based and total intensity-based modules. This method assumes that different samples share a common underlying distribution of their control probes while considering the potential existence of flagged spots within them. The Immunome array uses Cy3-labelled biotinylated BSA (Cy3-BSA) replicates as the positive control spots across slides. Hence it is considered as a housekeeping probe for normalisation of signal intensities for any given study.

The quantile module adopts the algorithm described by Bolstad et al., 2003 [11]. This reorganisation enables the detection and handling of outliers or flagged spots in any of the Cy3BSA control probes. A total intensity-based module was then implemented to obtain a scaling factor for each sample. This method assumes that post-normalisation, the positive controls should have a common total intensity value across all samples. This composite method aims to normalise the protein array data from variations in their measurements whilst preserving the targeted biological activity across samples. The steps are as follows:

Quantile-Based Normalisation of all cy3BSA across all samples

($i = spot\ number\ and\ j = sample\ number$)

1. Load all Cy3-BSA across all samples, j, into an i X j matrix X
2. Sort spot intensities in each column j of X to get X_{sort}
3. Take the mean across each row i of X_{sort} to get $< X_i >$

Intensity-Based Normalisation

1. Calculate sum of the mean across each row i , $\sum < Xi >$
2. For each sample, k, calculate the sum of all Cy3-BSA controls, $\sum Xk$
3. For each sample, k,

$$\text{Scaling factor (k)} = \frac{\sum < \text{Xi} >}{\sum \text{Xk}} \qquad \textbf{Equation 2}$$

Data Analysis

The fluorescence signals from the 1557 autoantibody measurements were logarithmically transformed to ensure normality prior to any parametric analysis. One way ANOVA was carried on each of the 1557 autoantibody measurements against i) between all groups (healthy elderlies, elderlies with intermediate health status, unhealthy elderlies and the young controls) and ii) between the elderlies (healthy, intermediate and unhealthy) to identify autoantibodies which were significantly different in at least one of the groups compared to the rest (Table 1). An initial P-value threshold of 0.05 was used to indicate significance. Autoantibody biomarkers towards 16 antigens were identified in this manner: YARS [12], UBE2I [13], TCL1A [14], PPM1A [15], PHLDA1 [16], GLRX3 [17], FHOD2 [18], FEN1 [19], CASP10 [20], MAPK13 [21], MAP4 [22], FKBP3 [23], CD96 [24], AURKA [25], ASPSCR1 [26] and AAK [27], shown in figure 3A. Amongst these 16 antigens, AURKA, FEN1, GLRX3, PHLDA1, PPM1A, FKBP3, CD96 and MAPK13 were found to have P-values of < 0.02 in both analyses (between all groups and between elderlies).

Table 1

Biomarker	P-Values	
	between all groups	between elderlies
AURKA	0.000912	1.41E-03
FEN1	0.00772	0.00592
GLRX3	0.00289	0.00633
PHLDA1	0.00476	0.00799
PPM1A	7.61E-05	0.0108
FKBP3	0.00504	0.0114
CD96	0.00593	0.0116
MAPK13	0.0191	0.0165
MAP4	0.0376	0.0172
TCL1A	0.000814	0.0211
FHOD2	1.05E-04	0.0212
CASP10	5.91E-05	0.023
ASPSCR1	0.0317	0.0281
YARS	0.0761	0.045
AAK1	0.0126	0.0453
UBE2I	0.0036	0.0496

Pathway enrichment analysis showed that 4 of the 16 (PHLDA1, AURKA, FEN1 and UBE2I) are involved in Cell Cycle and DNA repair pathways which are altered in the aging process.

Given that each of the 16 individual autoantibodies are weak predictors of the health status on their own, dimension reduction using tSNE was carried out to identify the collective capabilities of the 16 autoantibodies to differentiate the health groups. As seen in the figure 4A, dimension reduction using tSNE show that the 2 tSNE dimensions were able to differentiate the 3 health groups. The specificity of the 16 autoantibodies is shown in figure 4B.

To identify autoantibodies specific to each of the health status, a series of t-tests with Welch correction was used to test each of the health status against the rest for all 16 identified autoantibodies. For each of the autoantibodies, the best t-test result amongst the three health statuses were selected as the autoantibody of choice for that health status. This identified PHLDA1 and CD96 as being specific for the healthy group, AURKA,

FEN1, CASP10 and AAK1 as being specific for the intermediate group and the rest as being specific for the unhealthy group (figure 4C). The healthy and intermediate autoantibodies may have a protective role against non-communicable disease. The mean RFU is shown for each of the health status groups together with the P-values of the t-tests demonstrating the significance of the autoantibody in discriminating the health status group of interest against the rest

The invention utilises the Biotin Carboxyl Carrier Protein (BCCP) folding marker which is cloned in-frame with the gene encoding the protein of interest, as described above and in EP1470229. The structure of the *E. coli* BCCP domain is illustrated in Figure 1, wherein residues 77-156 are drawn (coordinate file 1bdo) showing the N- and C- termini and the single biotin moiety that is attached to lysine 122 *in vivo* by biotin ligase.

BCCP acts not only as a protein folding marker but also as a protein solubility enhancer. BCCP can be fused to either the N- or C-terminal of a protein of interest. Full-length proteins are expressed as fusions to the BCCP folding marker which becomes biotinylated *in vivo*, but only when the protein is correctly folded. Conversely, misfolded proteins drive the misfolding of BCCP such that it is unable to become biotinylated by host biotin ligases. Hence, misfolded proteins are unable to specifically attach to a streptavidin-coated solid support. Therefore, only correctly folded proteins become attached to a solid support via the BCCP tag.

The surface chemistry of the support is designed carefully and may use a three-dimensional thin film polymer base layer (polyethylene glycol; PEG), which retains protein spot morphologies and ensures consistent spot sizes across the array. The PEG layer inhibits non-specific binding, therefore reducing the high background observed using other platforms. The solid support used to immobilize the selected biomarkers is thus designed to resist non-specific macromolecule adsorption and give excellent signal-to-noise ratios and low limits of detection (i.e. improved sensitivity) by minimising non-specific background binding. In addition, the PEG layer also preserves the folded structure and functionality of arrayed proteins and protein complexes post-immobilisation. This is critical for the accurate diagnosis because human serum antibodies are known in general to bind non-specifically to exposed hydrophobic surfaces

on unfolded proteins, thus giving rise to false positives in serological assays on arrays of unfolded proteins, moreover, human autoantibodies typically bind to discontinuous epitopes, so serological assays on arrays of unfolded proteins or mis-folded proteins will also give rise to false negatives in autoantibody binding assays.

As biotinylated proteins bound to a streptavidin-coated surface show negligible dissociation, this interaction therefore provides a superior means for tethering proteins to a planar surface and is ideal for applications such as protein arrays, SPR and bead-based assays. The use of a compact, folded, biotinylated, 80 residue domain BCCP affords two significant advantages over for example the AviTag and intein-based tag. First, the BCCP domain is cross-recognised by eukaryotic biotin ligases enabling it to be biotinylated efficiently in yeast, insect, and mammalian cells without the need to co-express the *E. coli* biotin ligase. Second, the N- and C-termini of BCCP are physically separated from the site of biotinylation by 50Å (as shown in Figure 1), so the BCCP domain can be thought of as a stalk which presents the recombinant proteins away from the solid support surface, thus minimising any deleterious effects due to immobilisation.

The success rate of BCCP folding marker mediated expression of even the most complex proteins is in excess of 98%. The technology can therefore be applied in a highly parallelised pipeline resulting in high-throughput, highly consistent production of functionally validated proteins.

The addition of BCCP permits the monitoring of fusion protein folding by measuring the extent of *in vivo* biotinylation. This can be measured by standard blotting procedures, using SDS-PAGE or *in situ* colony lysis and transfer of samples to a membrane, followed by detection of biotinylated proteins using a streptavidin conjugate such as streptavidin-horseradish peroxidase. Additionally, the fact that the BCCP domain is biotinylated *in vivo* is particularly useful when multiplexing protein purification for fabrication of protein arrays since the proteins can be simultaneously purified from cellular lysates and immobilised in a single step via the high affinity and specificity exhibited by a streptavidin surface.

Figure 3 illustrates discriminating proteins in the elderly, characterized by processes of cell-cycle and cell-death. (A) p-values related to the 16 protein-targets discriminating the various elderly health statuses. Only readouts of serum/plasma antibody to the YARS protein do not also discriminate between the elderlies and YC individuals ($p>0.05$) (B) 5 protein readouts were tightly associated with regards to pathways linked to cell-cycle (PHLDA1, AURKA, FEN1), DNA repair (UBE2I, FEN1) and translation (YARS) by enrichment pathway analysis (String).

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Table 2

Protein Name	UniprotID	Description
AAK1	Q2M2I8	HUMAN AP2-associated protein kinase 1

Nucleotide Sequence **(Seq ID No. 1)***:*
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GN=ASPSCR1 PE=1 SV=1

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AURKA O14965 HUMAN Aurora kinase A

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CASP10 Q92851 HUMAN Caspase-10

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CD96 P40200 HUMAN T-cell surface protein tactile

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GCTTACTAAAGAATGTATTTCCCAAAGCAAATATCACATGGTTTATAGATGGAAGTTTTCTTCATGATGAAAAAGA
AGGAATATATATTACTAATGAAGAGAGAAAAGGCAAAGATGGATTTTTGGAACTGAAGTCTGTTTTAACAAGGGT
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ACTGAGCTAT

Protein Sequence **(Seq ID No. 21)**:
>sp|P40200|TACT_HUMAN T-cell surface protein tactile OS=Homo sapiens OX=9606 GN=CD96 PE=1 SV=2

MEKKWKYCAVYYIIQIHFVKGVWEKTVNTEENVYATLGSDVNLTCQTQTVGFFVQMQWSKVTNKIDLIAVYHPQYGF
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GIYITNEERKGKDGFLELKSVLTRVHSNKPAQSDNLTIWCMALSPVPGNKVWNISSEKITFLLGSEISSTDPPLSVTES
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FEN1 P39748 HUMAN Flap endonuclease 1

Nucleotide Sequence **(Seq ID No. 6)**:
>P000413_SIG_SIG1-2_FEN1_2237_Homo sapiens flap structure-specific endonuclease 1_BC000323.2_AAH00323.1_P39748_53567_0_1143_0_1140

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GGGGATGTGCTGCAGAATGAGGAGGGTGAGACCACCAGCCACCTGATGGGCATGTTCTACCGCACCATTCGC
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Protein Sequence **(Seq ID No. 22)**:
>sp|P39748|FEN1_HUMAN Flap endonuclease 1 OS=Homo sapiens OX=9606 GN=FEN1 PE=1 SV=1

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YCESIRGIGPKRAVDLIQKHKSIEEIVRRLDPNKYPVPENWLHKEAHQLFLEPEVLDPESVELKWSEPNEEELIKFMCG
EKQFSEERIRSGVKRLSKSRQGSTQGRLDDFFKVTGSLSSAKRKEPEPKGSTKKKAKTGAAGKFKRGK

FKBP3 Q00688 HUMAN Peptidyl-prolyl cis-trans isomerase FKBP3

Nucleotide Sequence **(Seq ID No. 7)***:*
>P001211_CAG_CAGp1_FKBP3_2287_Homo sapiens FK506 binding protein 3
25kDa_BC016288.1_AAH16288.1_Q00688_0_0_675_0_672

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TCTGAAGAGACCCTGGATGAGGGTCCACCAAAATATACTAAATCTGTTCTGAAAAAGGGAGATAAAACCAACTTT
CCCAAAAAGGGAGATGTTGTTCACTGCTGGTATACAGGAACACTACAAGATGGGACTGTTTTTGATACTAATATT
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Protein Sequence **(Seq ID No. 23)***:*
>sp|Q00688|FKBP3_HUMAN Peptidyl-prolyl cis-trans isomerase FKBP3 OS=Homo sapiens OX=9606 GN=FKBP3
PE=1 SV=1

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SKVSEQVKNVKLNEDKPKETKSEETLDEGPPKYTKSVLKKGDKTNFPKKGDVVHCWYTGTLQDGTVFDTNIQTSAK
KKKNAKPLSFKVGVGKVIRGWDEALLTMSKGEKARLEIEPEWAYGKKGQPDAKIPPNAKLTFEVELVDID

FMNL2 Q96PY5 HUMAN Formin-like protein 2

Nucleotide Sequence **(Seq ID No. 8)***:*
>P000661_TRN_TRNp1_FHOD2_114793_Homo sapiens formin homology 2 domain containing
2_BC036492.2_AAH36492.1_Q96PY5_0_0_537_0_534

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GCAGAAGAGGAAAATGAGCTGAGGAAAAAGCAGGAACAAGCTCTCATGGAAAAACTCCTAGAGCAAGAAGCTC
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Protein Sequence **(Seq ID No. 24)***:*
>sp|Q96PY5|FMNL2_HUMAN Formin-like protein 2 OS=Homo sapiens OX=9606 GN=FMNL2 PE=1 SV=3

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KQTLLHYISNVVKEKYHQVSLFYNELHYVEKAAAVSLENVLLDVKELQRGMDLTKREYTMHDHNTLLKEFILNNEGKL
KKLQDDAKIAQDAFDDVVKYFGENPKTTPPSVFFPVFVRFVKAYKQAEEENELRKKQEQALMEKLLEQEALMEQQD
PKSPSHKSKRQQQELIAELRRRQVKDNRHVYEGKDGAIEDIITVLKTVPFTARTAKRGSRFFCEPVLTEEYHY

GLRX3 O76003 HUMAN Glutaredoxin-3

Nucleotide Sequence **(Seq ID No. 9)**:
>P000071_KIN96_KIN_TXNL2_10539_Homo sapiens thioredoxin-like clone MGC:12349_BC005289_AAH05289_O76003_48409.07_0_1008_0_1005

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Protein Sequence **(Seq ID No. 25)**:
>sp|O76003|GLRX3_HUMAN Glutaredoxin-3 OS=Homo sapiens OX=9606 GN=GLRX3 PE=1 SV=2

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MAP3K13 O43283 HUMAN Mitogen-activated protein kinase kinase kinase 13

Nucleotide Sequence **(Seq ID No. 10)**:
>P001569_Q106_Q106p2_MAP3K13_9175_Homo sapiens MAP3K13 mitogen-activated protein kinase kinase kinase 13_NM_004721_0_0_0_0_0_0_0

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Protein Sequence **(Seq ID No. 26)**:
>sp|O43283|M3K13_HUMAN Mitogen-activated protein kinase kinase kinase 13 OS=Homo sapiens OX=9606 GN=MAP3K13 PE=1 SV=1

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MEYCAHGQLYEVLRAGRKITPRLLVDWSTGIASGMNYLHLHKIIHRDLKSPNVLVTHTDAVKISDFGTSKELSDKSTK
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MQTKRPDLLRSEGIPTTEVAPTASPLSGSPKMSTSSSKSRYRSKPRHRRGNSRGSHSDFAAILKNQPAQENSPHPT
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MAP4 P27816 HUMAN Microtubule-associated protein 4

Nucleotide Sequence **(Seq ID No. 11)**:
>P000490_SIG_SIG1-3_MAP4_4134_Homo sapiens MAP4 microtubule-associated protein 4_BC008715.2_AAH08715.1_P27816_113843.4_0_2940_0_2937

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Protein Sequence **(Seq ID No. 27)***:*
>sp|P27816|MAP4_HUMAN Microtubule-associated protein 4 OS=Homo sapiens OX=9606 GN=MAP4 PE=1 SV=3

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KPTLLANGGHGVEGSDTTGSPTEFLEEKMAYQEYPNSQNWPEDTNFCFQPEQVVDPIQTDPFKMYHDDDLADLVF
PSSATADTSIFAGQNDPLKDSYGMSPCNTAVVPQGWSVEALNSPHSESFVSPEAVAEPPQPTAVPLELAKEIEMASE
ERPPAQALEIMMGLKTTDMAPSKETEMALAKDMALATKTEVALAKDMESPTKLDVTLAKDMQPSMESDMALVKDME
LPTEKEVALVKDVRWPTETDVSSAKNVVLPTETEVAPAKDVTLLKETERASPIKMDLAPSKDMGPPKENKKETERAS
PIKMDLAPSKDMGPPKENKIVPAKDLVLLSEIEVAQANDIISSTEISSAEKVALSSETEVALARDMTLPPETNVILTKDKA
LPLEAEVAPVKDMAQLPETEIAPAKDVAPSTVKEVGLLKDMSPLSETEMALGKDVTPPPETEVVLIKNVCLPPEMEVA
LTEDQVPALKTEAPLAKDGVLTLANNVTPAKDVPPLSETEATPVPIKDMEIAQTQKGISEDSHLESLQDVGQSAAPTF
MISPETVTGTGKKCSLPAEEDSVLEKLGERKPCNSQPSELSSETSGIARPEEGRPVVSGTGNDITTPPNKELPPSPEK
KTKPLATTQPAKTSTSKAKTQPTSLPKQPAPTTIGGLNKKPMSLASGLVPAAPPKRPAVASARPSILPSKDVKPKPIAD
AKAPEKRASPSKPASAPASRSGSKSTQTVAKTTTAAAVASTGPSSRSPSTLLPKKPTAIKTEGKPAEVKKMTAKSVP
ADLSRPKSTSTSSMKKTTTLSGTAPAAGVVPSRVKATPMPSRPSTTPFIDKKPTSAKPSSTTPRLSRLATNTSAPDLK
NVRSKVGSTENIKHQPGGGRAKVEKKTEAAATTRKPESNAVTKTAGPIASAQKQPAGKVQIVSKKVSYSHIQSKCGS
KDNIKHVPGGGNVQIQNKKVDISKVSSKCGSKANIKHKPGGGDVKIESQKLNFKEKAQAKVGSLDNVGHLPAGGAVK
TEGGGSEAPLCPGPPAGEEPAISEAAPEAGAPTSASGLNGHPTLSGGGDQREAQTLDSQIQETSI

PHLDA1 Q8WV24 HUMAN Pleckstrin homology-like domain family A member 1

Nucleotide Sequence **(Seq ID No. 12)***:*
>P002080_Q305_Q305p3_PHLDA1_22822_Homo sapiens pleckstrin homology-like domain family A member 1_BC018929.2_AAH18929.3_Q8WV24_0_0_780_0_777

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GCTCTGGAAGAAAAAGTGTTGCATCCTCACCGAGGAAGGGCTGCTGCTTATCCCGCCCAAGCAGCTGCAACAC
CAGCAGCAGCAGCAACAGCAGCAGCAGCAGCAGCAACAACAGCCCGGGCAGGGGCCGGCCGAGCCGTCCCA
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GACCGTGGACTGTGTGGAGCGCAAGGGCAAGTACATGTACTTCACTGTGGTGATGGCAGAGGGCAAGGAGAT
CGACTTTCGGTGCCCGCAAGACCAGGGCTGGAACGCCGAGATCACGCTGCAGATGGTGCAGTACAAGAATCG
TCAGGCCATCCTGGCGGTCAAATCCACGCGGCAGAAGCAGCAGCACCTGGTCCAGCAGCAGCCCCCCTCGCA
GCCGCAGCCGCAGCCGCAGCTCCAGCCCCAACCCCAGCCTCAGCCTCAGCCGCAACCCCAGCCCCAATCACA
ACCCCAGCCTCAGCCCCAACCCAAGCCTCAGCCCCAGCAGCTCCACCCGTATCCGCATCCACATCCACATCCA
CACTCTCATCCTCACTCGCACCCACACCCTCACCCGCACCCGCATCCGCACCAAATACCGCACCCACACCCAC
AGCCGCACTCGCAGCCGCACGGGCACCGGCTTCTCCGCAGCACCTCCAACTCTGCC

Protein Sequence **(Seq ID No. 28)***:*
>sp|Q8WV24|PHLA1_HUMAN Pleckstrin homology-like domain family A member 1 OS=Homo sapiens OX=9606 GN=PHLDA1 PE=1 SV=4

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TRLSLCRDPEPPPPLCLLRVSLLCALRAGGRGSRWGEDGARLLLLPPARAAGNGEAEPSGGPSYAGRMLESSGCK
ALKEGVLEKRSDGLLQLWKKKCCILTEEGLLLIPPKQLQHQQQQQQQQQQQQQPGQGPAEPSQPSGPAVASLE
PPVKLKELHFSNMKTVDCVERKGKYMYFTVVMAEGKEIDFRCPQDQGWNAEITLQMVQYKNRQAILAVKSTRQKQQ
HLVQQQPPSQPQPQPQLQPQPQPQPQPQPQPQSQPQPQPQPKPQPQQLHPYPHPHPHPHSHPHSHPHPHPHPH
PHQIPHPHPQPHSQPHGHRLLRSTSNSA

PPM1A P35813 HUMAN Protein phosphatase 1A

Nucleotide Sequence **(Seq ID No. 13)***:*
>P000364_SIG_SIG1-1_PPM1A_5494_Homo sapiens protein phosphatase 1A (formerly 2C) magnesium-dependent alpha isoform tr_BC026691.1_AAH26691.1_P35813_53422.23_0_1149_0_1146

ATGGGAGCATTTTTAGACAAGCCAAAGATGGAAAAGCATAATGCCCAGGGGCAGGGTAATGGGTTGCGATATG
GGCTAAGCAGCATGCAAGGCTGGCGTGTTGAAATGGAGGATGCACATACGGCTGTGATCGGTTTGCCAAGTGG
ACTTGAATCGTGGTCATTCTTTGCTGTGTATGATGGGCATGCTGGTTCTCAGGTTGCCAAATACTGCTGTGAGC
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CGAATTCAGAATGCAGGTGGCTCTGTAATGATTCAGCGTGTGAATGGCTCTCTGGCTGTATCGAGGGCCCTTG
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GACACCTGTTTGTATAAGGGAAGTCGAGACAACATGAGTGTGATTTTGATCTGTTTTCCAAATGCACCCAAAGTA
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Protein Sequence **(Seq ID No. 29)***:*
>sp|P35813|PPM1A_HUMAN Protein phosphatase 1A OS=Homo sapiens OX=9606 GN=PPM1A PE=1 SV=1

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HLLDHITNNQDFKGSAGAPSVENVKNGIRTGFLEIDEHMRVMSEKKHGADRSGSTAVGVLISPQHTYFINCGDSRGL
LCRNRKVHFFTQDHKPSNPLEKERIQNAGGSVMIQRVNGSLAVSRALGDFDYKCVHGKGPTEQLVSPEPEVHDIER
SEEDDQFIILACDGIWDVMGNEELCDFVRSRLEVTDDLEKVCNEVVDTCLYKGSRDNMSVILICFPNAPKVSPEAVKK
EAELDKYLECRVEEIIKKQGEGVPDLVHVMRTLASENIPSLPPGGELASKRNVIEAVYNRLNPYKNDDTDSTSTDDM
W

TCL1A P56279 HUMAN T-cell leukemia/lymphoma protein 1A

Nucleotide Sequence **(Seq ID No. 14)***:*
>P000179_CAN_CAN1-1_TCL1A_8115_Homo sapiens T-cell leukemia/lymphoma 1A_BC005831.2_AAH05831.1_P56279_0_0_345_0_342

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CCTATCATGTGGCAGCTCTACCCTGATGGACGATACCGATCCTCAGACTCCAGTTTCTGGCGCTTAGTGTACCA
CATCAAGATTGACGGCGTGGAGGACATGCTTCTCGAGCTGCTGCCAGATGAC

Protein Sequence **(Seq ID No. 30)***:*
>sp|P56279|TCL1A_HUMAN T-cell leukemia/lymphoma protein 1A OS=Homo sapiens OX=9606 GN=TCL1A PE=1 SV=1

MAECPTLGEAVTDHPDRLWAWEKFVYLDEKQHAWLPLTIEIKDRLQLRVLLRREDVVLGRPMTPTQIGPSLLPIMWQ
LYPDGRYRSSDSSFWRLVYHIKIDGVEDMLLELLPDD

UBE2I P63279 HUMAN SUMO-conjugating enzyme UBC9

Nucleotide Sequence **(Seq ID No. 15)***:*
>P001344_CAG_CAGp1_UBE2I_7329_Homo sapiens ubiquitin-conjugating enzyme E2I (UBC9 homolog yeast) transcript variant 1_BC000427.2_AAH00427.1_P50550_0_0_477_0_474

ATGTCGGGGATCGCCCTCAGCAGACTCGCCCAGGAGAGGAAAGCATGGAGGAAAGACCACCCATTTGGTTTC
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Protein Sequence **(Seq ID No. 31)***:*
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MSGIALSRLAQERKAWRKDHPFGFVAVPTKNPDGTMNLMNWECAIPGKKGTPWEGGLFKLRMLFKDDYPSSPPKC
KFEPPLFHPNVYPSGTVCLSILEEDKDWRPAITIKQILLGIQELLNEPNIQDPAQAEAYTIYCQNRVEYEKRVRAQAKKF
APS

YARS P54577 HUMAN Tyrosine--tRNA ligase, cytoplasmic

Nucleotide Sequence **(Seq ID No. 16)***:*
>P001370_CAG_CAGp2_YARS_8565_Homo sapiens tyrosyl-tRNA synthetase_BC004151.2_AAH04151.1_P54577_0_0_1587_0_1584

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CAGGCTGGTAGTGGTGCTGTGCAACCTGAAACCCCAGAAGATGAGAGGAGTCGAGTCCCAAGGCATGCTTCTG
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GCTCCATTTCCTGTAAATCGCTGAAAGGGGGGAACATTAGCC

Protein Sequence **(Seq ID No. 32)**:
>sp|P54577|SYYC_HUMAN Tyrosine--tRNA ligase, cytoplasmic OS=Homo sapiens OX=9606 GN=YARS PE=1 SV=4

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PLLSGLLYPGLQALDEEYLKVDAQFGGIDQRKIFTFAEKYLPALGYSKRVHLMNPMVPGLTGSKMSSSEEESKIDLLD
RKEDVKKKLKKAFCEPGNVENNGVLSFIKHVLFPLKSEFVILRDEKWGGNKTYTAYVDLEKDFAAEVVHPGDLKNSV
EVALNKLLDPIREKFNTPALKKLASAAYPDPSKQKPMAKGPAKNSEPEEVIPSRLDIRVGKIITVEKHPDADSLYVEKID
VGEAEPRTVVSGLVQFVPKEELQDRLVVVLCNLKPQKMRGVESQGMLLCASIEGINRQVEPLDPPAGSAPGEHVFV
KGYEKGQPDEELKPKKKVFEKLQADFKISEECIAQWKQTNFMTKLGSISCKSLKGGNIS

Claims

1. A method for determining the health of an individual from a sample extracted from that individual, comprising the steps of:

   (i) testing the sample for the presence of biomarkers specific for health;

   (ii) determining whether the subject is healthy, is of intermediate health, or is unhealthy, based on the detection of said biomarkers;

   characterised in that the biomarkers are autoantibodies to antigens comprising AURKA, FEN1, GLRX3, PHLDA1, PPM1A, FKBP3, CD96 and MAPK13.

2. The method according to claim 1 wherein the antigens further comprise one or more of UBE2I, AAK1, YARS, ASPSCR1, CASP10, FHOD2, TCL1A and MAP4.

3. The method according to claim 1 or 2 wherein the antigens are exposed to a sample extracted from a person, such that autoantibody biomarkers from the sample may bind to the antigens.

4. The method according to claim 3 wherein the antigens are subsequently exposed to a fluorescently-tagged secondary antibody to allow the amount of any autoantibodies from the sample bound to the antigens to be determined.

5. The method according to claim 4 wherein the health status of the person corresponds to the relative or absolute amount of autoantibodies from the sample specifically binding to the antigens.

6. The method according to any preceding claim wherein the sample comprises any or any combination of exosomes, blood, serum, plasma, urine, saliva, amniotic fluid, cerebrospinal fluid, breast milk, semen or bile.

7. The method according to any preceding claim wherein the steps are performed *in vitro*.

8. The method according to any preceding claim wherein the method comprises detecting upregulation/downregulation of one or more biomarkers.

9. The method according to any preceding claim wherein PHLDA1 and CD96 correspond to healthy, AURKA, FEN1, CASP10 and AAK1 correspond to intermediate health, and UBE2I, YARS, ASPSCR1, FHOD2, TCL1A, MAP4, MAPK13, FKBP3, PPM1A and GLRX3 correspond to unhealthy.

10. The method according to any preceding claim wherein the individual is elderly.

11. The method according to any preceding claim wherein the antigens are biotinylated proteins.

12. The method according to claim 11 wherein each biotinylated protein is formed from a Biotin Carboxyl Carrier Protein folding marker which is fused in-frame with a protein.

13. The method according to claim 11 or 12 wherein the biotinylated proteins are bound to a streptavidin-coated substrate.

14. The method according to claim 13 wherein the substrate comprises a hydrogel-forming polymer base layer.

15. A method for manufacturing a kit for determining the health of an elderly individual from a sample extracted from that individual, comprising the steps of:

for each antigen in a panel, cloning a biotin carboxyl carrier protein folding marker in-frame with a gene encoding the said antigen and expressing the resulting biotinylated antigen;

binding the biotinylated antigens to addressable locations on one or more streptavidin-coated substrates, thereby forming an antigen array;

such that the amount of autoantibodies from the sample binding to the antigens on the panel can be determined by exposing the substrate to the sample and measuring the response;

characterised in that the antigens comprise UBE2I, AAK1, YARS, ASPSCR1, CASP10, FHOD2, TCL1A, MAP4, MAPK13, CD96, FKBP3, PPM1A, PHLDA1, GLRX3, FEN1 and AURKA.

16. A composition comprising a panel of antigens for determining the health of an elderly individual, characterised in that the antigens comprise MAPK13, CD96, FKBP3, PPM1A, PHLDA1, GLRX3, FEN1 and AURKA, and optionally

comprising one or more of UBE2I, AAK1, YARS, ASPSCR1, CASP10, FHOD2, TCL1A, and MAP4.

17. A composition according to claim 16 wherein the antigens are biotinylated proteins.

18. A composition according to any of claims 16-17 wherein the amount of one or more autoantibody biomarkers binding *in vitro* to the antigens in a sample from a patient can be measured to determine the health status of the patient.

19. A composition comprising a panel of autoantibody biomarkers for determining the health status of an elderly patient:

wherein the level of one or more autoantibody biomarkers are measured in a sample from the patient;

characterised in that the one or more autoantibody biomarkers are selected from autoantibodies specific for one or more of the following antigens: MAPK13, CD96, FKBP3, PPM1A, PHLDA1, GLRX3, FEN1 and AURKA;

and optionally comprising one of more of the following antigens: UBE2I, AAK1, YARS, ASPSCR1, CASP10, FHOD2, TCL1A, and MAP4.

Figure 1

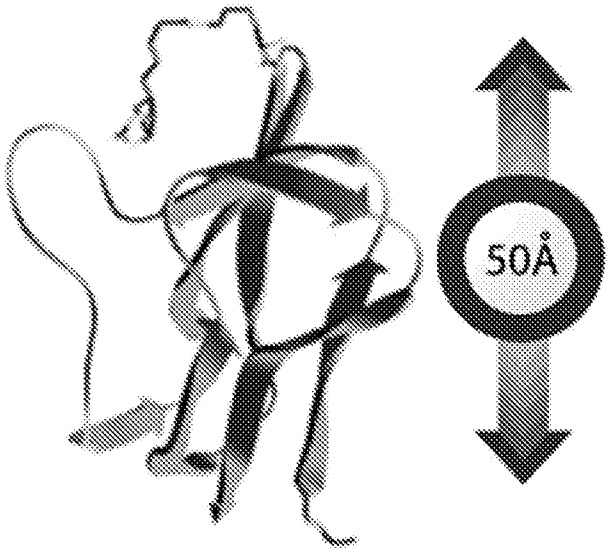


Figure 2

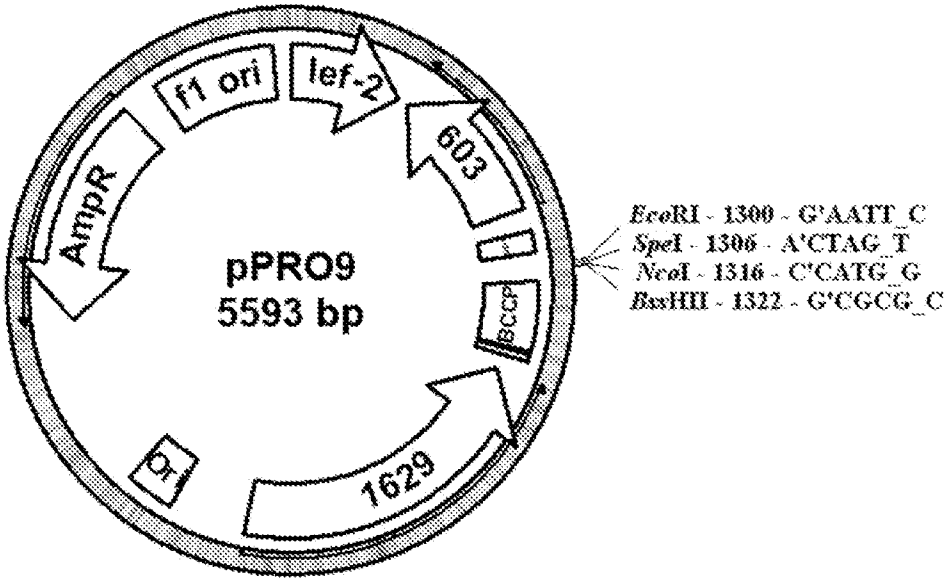


Figure 3A

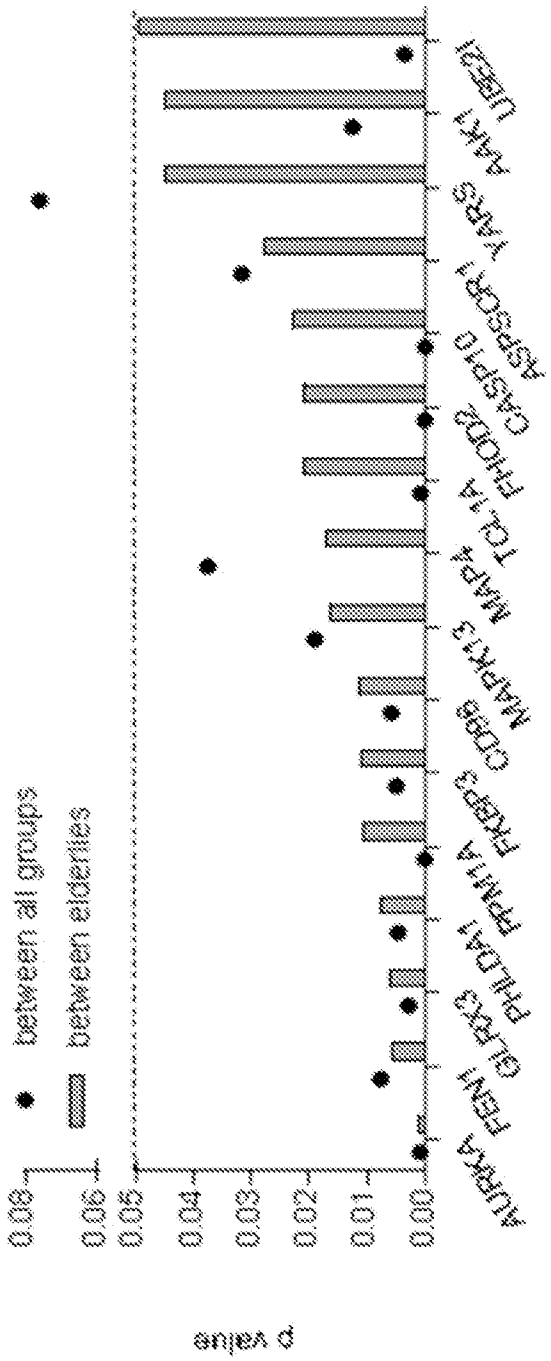


Figure 3B

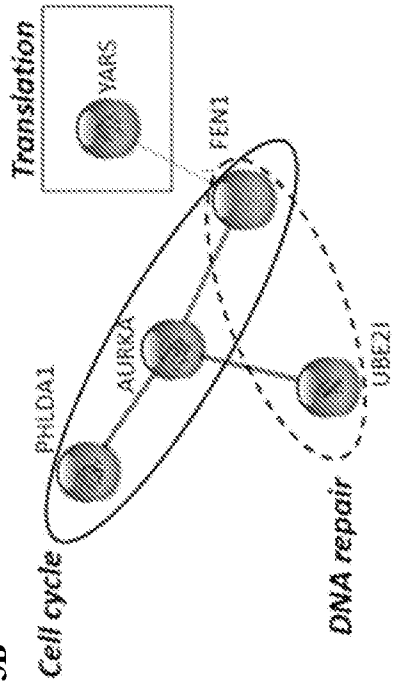


Figure 4B

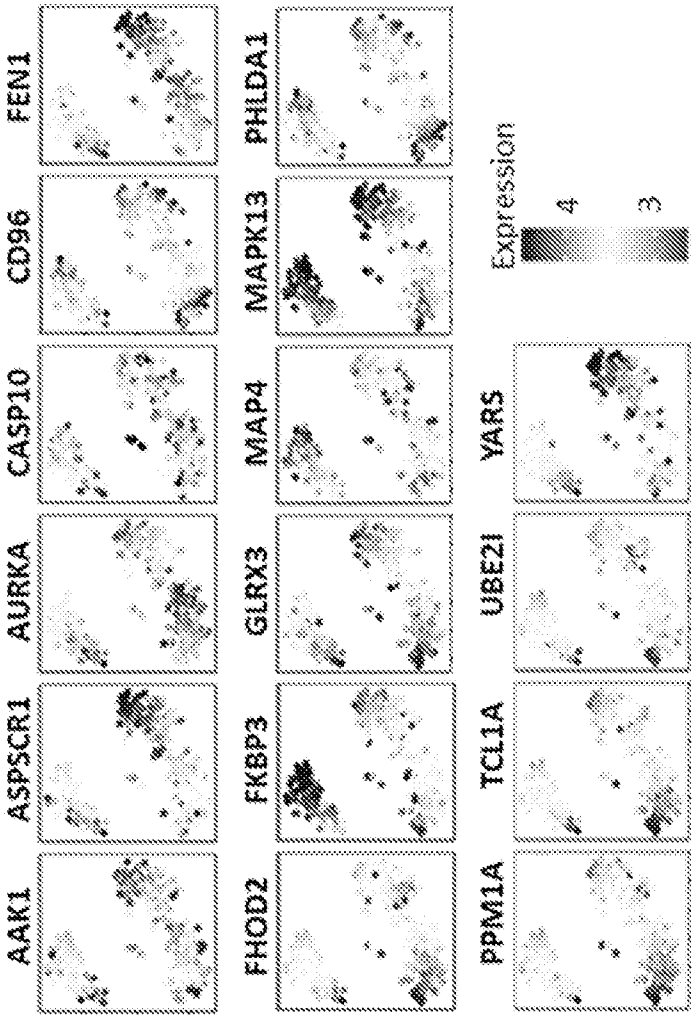
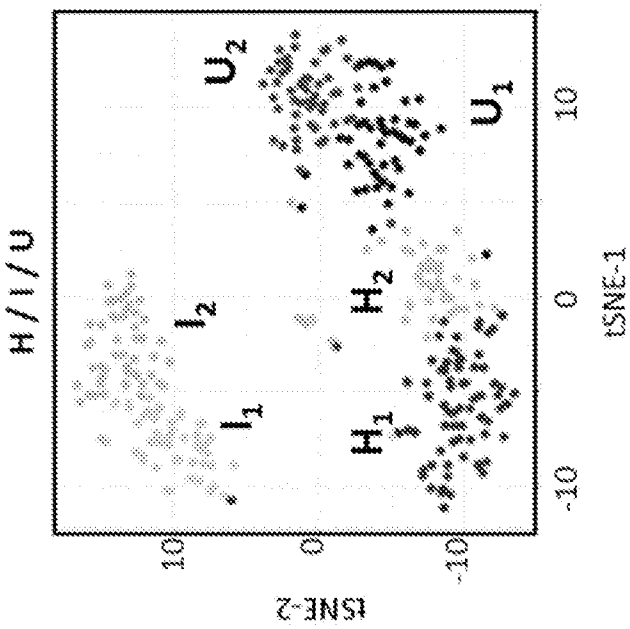
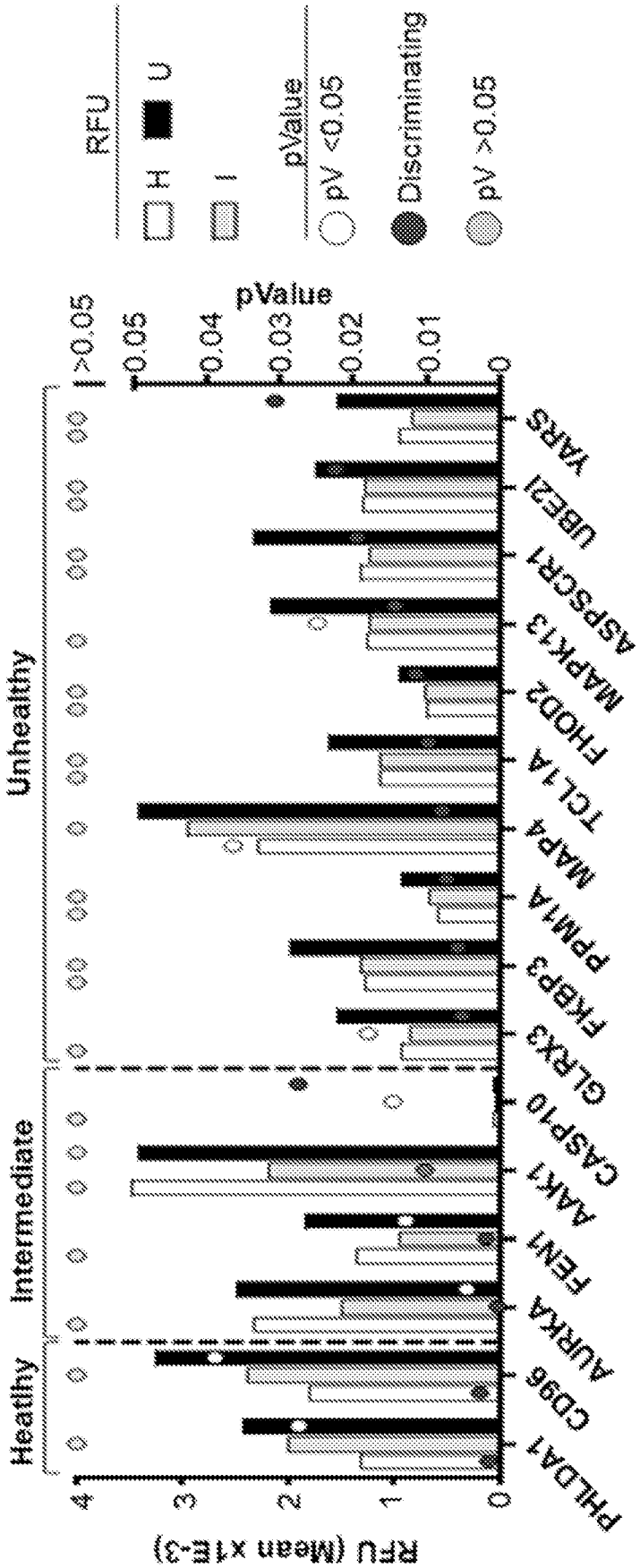


Figure 4A




RFU
H
U
I
pValue
pV <0.05
Discriminating
pV >0.05
pValue
>0.05
0.05
0.04
0.03
0.02
0.01
0
Heathy
Intermediate
Unhealthy
RFU (Mean x1E-3)
4
3
2
1
0
PHLDA1
CD96
AURKA
FEN1
AAK1
CASP10
GLRX3
FKBP3
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MAP4
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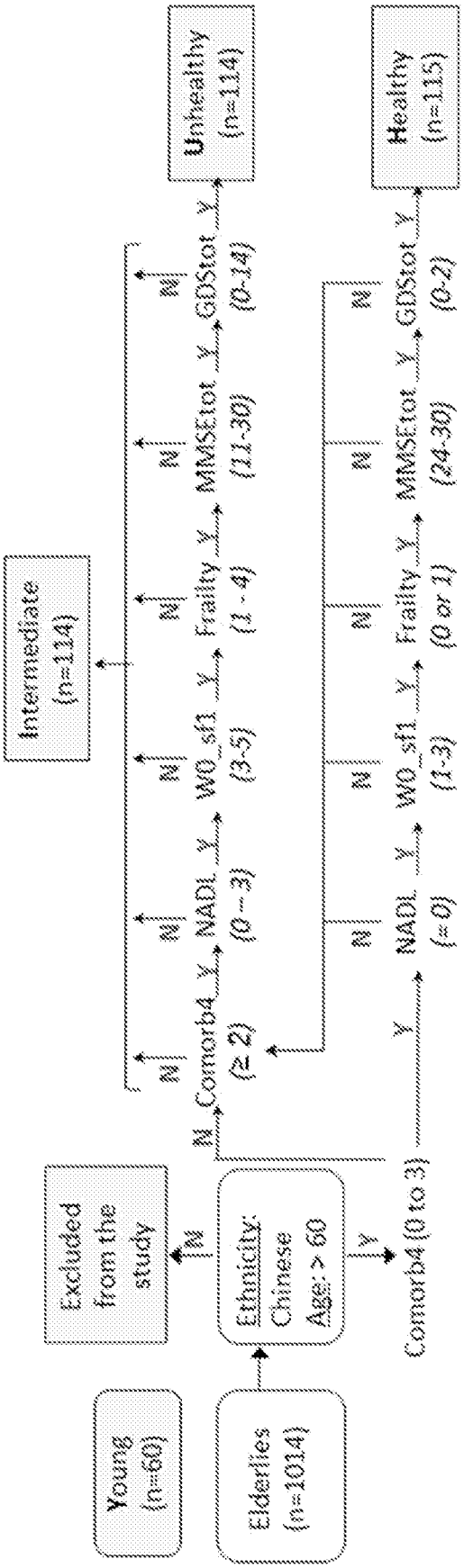
FHOD2
MAPK13
ASPSCR1
UBE2I
YARS

Figure 4C

Figure 5A


Young
(n=60)
Elderlies
(n=1014)
Excluded
from the
study
N
Ethnicity:
Chinese
Age: > 60
Y
Comorb4 (0 to 3)
N
Comorb4_Y
(≥ 2)
Y
NADL_Y
(= 0)
N
NADL_Y
(0 – 3)
N
W0_sf1_Y
(1-3)
N
W0_sf1_Y
(3-5)
N
Frailty_Y
(0 or 1)
N
Frailty_Y
(1 - 4)
N
MMSEtot_Y
(24-30)
N
MMSEtot_Y
(11-30)
N
GDStot_Y
(0-2)
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Intermediate
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Healthy
(n=115)
Unhealthy
(n=114)

Figure 5C

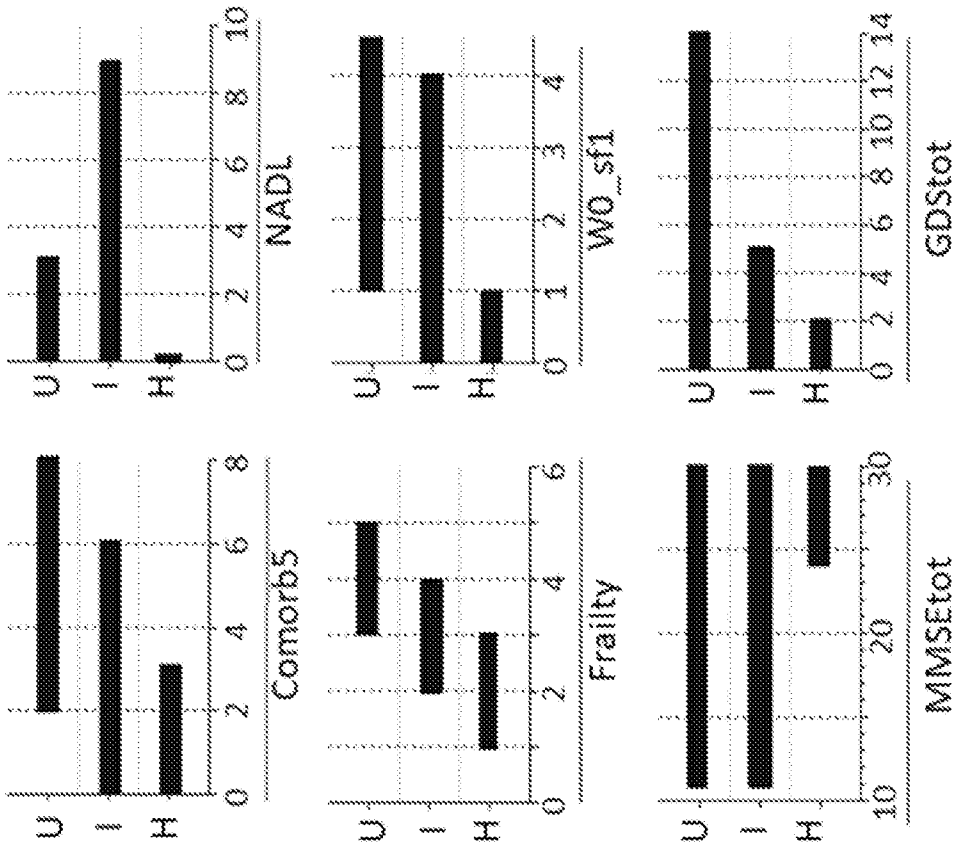

Figure 5B

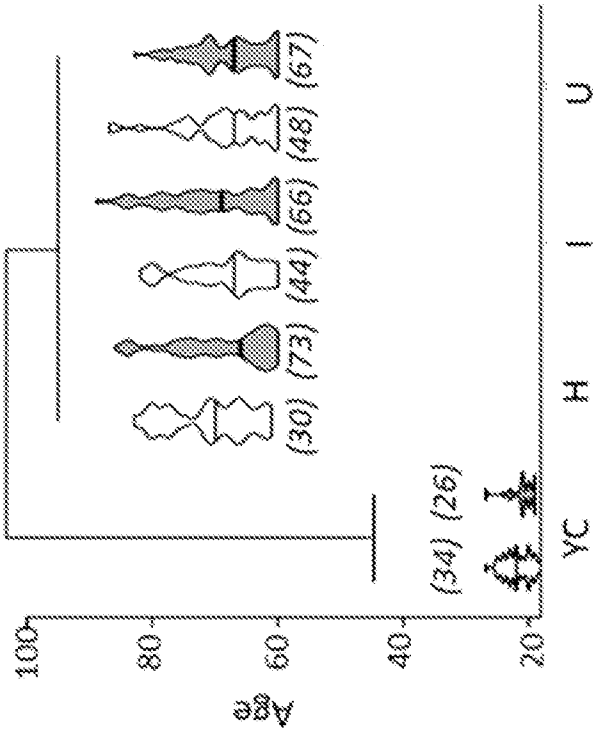

Figure 5D

	Comorb4	NADL	w0_sf1	Frailty	MMSEtot	GDStot
Healthy *(114)* Male *(42)* Female *(72)* Age: 60-82 Ave: 66.44	**0-3**	**0**	**1-3**	**0 -1**	**24-30**	**0-2**
	0 *(34)* **1** *(48)* **2** *(28)* **3** *(3)* Diabetes - 0 *(114)* HBP – 0/1 *(55/59)* Chol_2gp – 0/1 *(73/41)*	**0** *(114)*	**1** *(4)* **2** *(27)* **3** *(83)*	**0** *(74)* **1** *(40)*	**27-30** *(99)* **24-26** *(15)*	**0** *(88)* **1** *(21)* **2** *(5)*
Intermediate *(109)* Male *(49)* Female *(60)* Age: 60-89 Ave: 69.39	**0-7**	**0-2, 9, ND**	**2-4, ND**	**0-4, ND**	**11-30, ND**	**0-5, ND**
	0 *(6)* **1** *(24)* **2** *(36)* **3** *(16)* **4** *(12)* **5** *(5)* **6** *(7)*	**0** *(105)* **1** *(1)* **2** *(1)* **9** *(1)* **ND** *(1)*	**2** *(23)* **3** *(56)* **4** *(23)* **ND** *(7)*	**0** *(40)* **1** *(23)* **2** *(15)* **3** *(19)* **4** *(3)* **ND** *(9)*	**27-30** *(63)* **25-26** *(7)* **≤ 23** *(38)* **ND** *(1)*	**0** *(60)* **1** *(28)* **2** *(11)* **3** *(7)* **4** *(2)* **5** *(1)* **ND** *(1)*
Unhealthy *(113)* Male *(42)* Female *(71)* Age: 60-86 Ave: 70.09	**2-8**	**0-3**	**3-5**	**1-5**	**11-30**	**0-14**
	2 *(9)* **3** *(42)* **4** *(34)* **5** *(17)* **6** *(8)* **7** *(2)* **8** *(1)*	**0** *(109)* **1** *(2)* **2** *(1)* **3** *(1)*	**3** *(65)* **4** *(45)* **5** *(3)*	**1** *(59)* **2** *(33)* **3** *(17)* **4** *(3)* **5** *(1)*	**27-30** *(91)* **24-26** *(13)* **≤ 23** *(9)*	**0-4** *(108)* **≥ 5** *(5)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SG2020/050540

A. CLASSIFICATION OF SUBJECT MATTER

G01N 33/68 (2006.01) G01N 33/53 (2006.01) G01N 33/49 (2006.01)

According to International Patent Classification (IPC)

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

FAMPAT, BIOSIS, EMBASE, MEDLINE and internet; autoantibodies, elderly, AURKA, FEN1, GLRX3, PHLDA1, PPM1A, FKBP3, CD96, MAPK13, and similar terms

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	AKINTOLA A.A. ET AL., A simple and versatile method for frequent 24 h blood sample collection in healthy older adults. *MethodsX*, 26 December 2014, Vol. 2, pages 33–38 [Retrieved on 2020-11-24] <DOI: 10.1016/J.MEX.2014.12.003> Method details	19
X	MARANGON K. ET AL., Diet, antioxidant status, and smoking habits in French men. *Am J Clin Nutr*, 01 February 1998, Vol. 67, No. 2, pages 231–239 [Retrieved on 2020-11-24] <DOI: 10.1093/AJCN/67.2.231> Subjects and Methods	19
X	ANDERSEN-RANBERG K. ET AL., High prevalence of autoantibodies among Danish centenarians. *Clin Exp Immunol*, October 2004, Vol. 138, No. 1, pages 158-163 [Retrieved on 2020-11-24] <DOI: 10.1111/J.1365-2249.2004.02575.X> Materials and Methods, Results, Fig. 1	19

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"D" document cited by the applicant in the international application

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24/11/2020 *(day/month/year)*	*25/11/2020* *(day/month/year)*

Name and mailing address of the ISA/SG	Authorized officer
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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NJEMINI R. ET AL., The prevalence of autoantibodies in an elderly sub-Saharan African population. *Clin Exp Immunol*, January 2002, Vol. 127, No. 1, pages 99-106 [Retrieved on 2020-11-24] <DOI: 10.1046/J.1365-2249.2002.01713.X> Participants and Methods, Results	19

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SG2020/050540

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☐ forming part of the international application as filed:

      ☐ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

      ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

   Although a sequence listing has been filed or furnished, it was not used for the purposes of this search.

   Since only one version or copy of a sequence listing has been filed or furnished, the statements under item 2 are not required.