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39 Identification of cytokine and lipoprotein markers for analyses in SHARE Wave 6 dried blood spots

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- ▶ The identification of inflammation markers in the blood can help elucidate cardiovascular and Alzheimer's disease risk factors
 - ▶ We identified ten promising blood-based protein and inflammation markers from SHARE Wave 6 DBS samples that could potentially predict the risk of cardiovascular and Alzheimer's diseases
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39.1 Introduction

In 2015, SHARE introduced dried blood spot (DBS) sampling in its Wave 6 to complement self-reported health and to gain more detailed and objective information on common ageing-related health conditions, such as cardiovascular disease, diabetes, declines in kidney function and cognitive impairment. Blood collected by this minimally invasive method from survey respondents can be reliably analysed for several blood-based protein markers and blood fats.

SHARE attempts to understand the complex relationships among the biology, health behaviour and socioeconomic status in the ageing process. Among the underlying factors that cause ageing-related diseases, inflammatory pathways are considered to play an important role in, for example, atherosclerosis, cardiovascular disease, obesity, diabetes, mild cognitive decline and Alzheimer's disease. Cytokines are small blood-based proteins prominently involved in the inflammatory process. They are signalling molecules between cells and are important in other immune responses, which they modulate as pro- or anti-inflammatory molecules because sometimes inflammation is the body's response to fighting off infections. However, cytokines can become dysregulated and pathological in some forms of inflammation, autoimmune disease, trauma and sepsis.

Cytokines are not routinely analysed in a blood count, but in research they offer the opportunity to more closely investigate low-level chronic inflammation, such as in atherosclerosis, to fine-tune the search for the risk

of cardiovascular disease among the survey respondents beyond blood-fat (such as cholesterol) values and to biologically back up the results of objective cognitive testing in the survey questionnaire. Using multiplex immunoassay analysis, multiple markers (up to ten) can be analysed from very small amounts of blood, such as in DBS. By introducing cytokine analyses from the DBS collected in its Wave 6 survey, SHARE is testing an innovative method to gain a more detailed look at cardiovascular disease and cognitive decline in a population, not a patient sample. Here, we describe the selection of ten protein markers to be determined from SHARE Wave 6 DBS.

39.2 Identifying markers

After searching the literature for the involvement of cytokines and other blood-based analytes in cardiovascular (Mehra et al 2005) and neurodegenerative diseases (Brosseron et al 2014), 31 potentially interesting protein markers involved in ageing-related health conditions were identified for screening (Table 39.1). This list also includes the Apolipoproteins APOe4 and Clusterin (APOJ) which are involved in cholesterol transport to the brain and clearance of damaged cells, respectively. People carrying the *APOe4* gene variant are known to have a higher risk for cardiovascular disease and cognitive decline (Rasmussen et al. 2015).

It was unknown to us, which of the markers we would possibly detect in DBS samples collected under field work conditions during the SHARE survey and at which concentration levels. Our goal was to establish a multiplex immunoassay (see section 39.1) to test the SHARE DBS samples for the occurrence and levels of up to ten of the markers listed in Table 39.1 to use the full potential of our analysis platform (see below).

Therefore, we screened the 31 candidate markers to reveal the ones that could be measured simultaneously in the assay. We used excess DBS samples from the field collection from the youngest (60 years or younger) and the oldest (80 years or older) respondents in the SHARE Wave 6 cohort to check for the detectability of a marker and to cover the extremities of analyte concentration levels. Having chosen age as a condition that affects analyte levels, this procedure allowed us to understand the lowest and highest levels of the analytes, in particular, those that change with age to ensure that the method was sensitive to and independent of the age of participants.

Table 39.1: Short description of the 31 cytokine and lipoprotein markers chosen from the literature for screening.

Marker/Analyte	Short description of marker type	Pass/Fail of marker in round		
		1	2	3
1 IL-1β	Pro-inflammatory cytokine; increases inflammation (e.g. to regulate defence against pathogens); excess production may contribute to atherosclerosis	–	–	Out
2 IL-6	Pro-inflammatory; also present in adipose and muscle tissue; capable of crossing blood-brain barrier	–	–	Out
3 IL-8	Signalling molecule; potent promotor for the formation of new blood vessels (angiogenesis)	✓	✓	*Selected
4 IL-10	Anti-inflammatory; involved in down regulation of inflammation	–	–	Out
5 IL-1α	Pro-inflammatory; see IL-1 β	–	Out	
6 IL-13	Pro-inflammatory; mediator of allergic reaction (e.g. asthma)	–	Out	
7 IL-16	Pro-inflammatory, chemokine; cerebral pathologies and vascular dementias	✓	*Selected	
8 IL-18	Pro-inflammatory; see IL-1 β ; correlates positively with MMSE in patients with mild cognitive decline	✓	✓	*Selected
9 IL-12/23 p40	Pro-inflammatory; see IL-1 β	✓	*Selected	
10 TNF-α tumour necrosis factor- α	Pro-inflammatory; see IL-1 β	✓	–	Out

(continued)

Table 39.1 (continued)

Marker/Analyte	Short description of marker type	Pass/Fail of marker in round		
		1	2	3
11 IFN- γ interferon- γ	Pro-inflammatory; see IL-1 β ; also involved in viral inhibition, indication of autoimmune/inflammatory disease	–	Out	
12 MCP-1 monocyte chemo-attractant protein	Pro-inflammatory; induces chemical reactions in nearby cells; levels correlate positively with MMSE in patients with mild cognitive decline	✓	*Selected	
13 VEGF-A vascular epithelial growth factor	Signalling molecule in vascular tissue; potent mediator in angiogenesis	✓	*Selected	
14 VCAM-1 vascular cell-adhesion molecule	Vascular injury marker; atherosclerosis, arthritis	✓	✓	Selected
15 BNP (NT-pro BNP) brain natriuretic peptide	Heart failure; cardiac insufficiency	–	Out	
16 YKL; also called chitinase 3 like 1 (CHI3L1)	Inflammatory processes in cardiovascular disease, coronary artery disease, arthritis, cancer, cerebrospinal pathologies	✓	Selected	

17	BDNF brain-derived neurotrophic factor	Neuronal growth factor; neuroprotective, involved in depression, dementia, mild cognitive decline, Alzheimer's disease; levels increase e.g., with mobility/exercise and social contacts	✓	*Selected
18	NGF nerve growth factor	Growth, survival, differentiation of neurons; survival of insulin-producing cells; immune-system regulation	–	Out
19	EGF epidermal growth factor	Stimulates cell growth and differentiation	✓	*Selected
20	Leptin	Hormone involved in body weight regulation; inhibits hunger	–	Out
21	Clusterin Apo1 lipoprotein	Regulation of inflammation, oxidative stress, apoptosis (cell death), ageing, late onset Alzheimer's disease	✓	*Selected
22	GSK 3β glycogen synthase kinase 3 beta	High levels associated with memory loss; dysregulation observed in pathophysiological processes	(✓)	Out
23	Aβ38	Amyloid β (Aβ); main component of amyloid plaques in Alzheimer's disease	–	Out
24	Aβ40			
25	Aβ42			
26	sAPP-α soluble amyloid precursor protein α	Decreases the generation of Aβ	–	Out
27	APOe4 lipoprotein	Involved in fat metabolism of the body, principal carrier of cholesterol to the brain; involved in atherosclerosis, cognitive impairment, cerebrovascular and Alzheimer's disease	(✓)	*Selected

(continued)

Table 39.1 (continued)

	Marker/Analyte	Short description of marker type	Pass/Fail of marker in round		
			1	2	3
28	Total Tau (τ)	Abundant in neurons and the central nervous system; misfolded forms appear in neurodegenerative disease (tangles in Alzheimer's disease); elevated in cerebrospinal fluid and plasma of dementia patients and mild cognitive decline	Out		
29	Tau (τ) 181				
30	S100b	Calcium-binding protein; altered expression of gene implicated in neurological, neoplastic and other diseases	-	Out	
31	A1M Alpha- 1-macroglobulin	Protective against oxidative stress, radical scavenger; degrades heme	Out		

Note: Source of test kits:

A) Antibodies and reagents for markers 1–21, 23–26 bought from MesoScale Diagnostics; **B)** Kits for cell culture analyses plus recombinant calibrator for markers 22, 28, 29 bought from Signalchem/MyBioSource; tests adjusted by Statens Serum Institut; **C)** Antibodies and recombinant calibrator for markers 27, 30, 31 bought from R&D Systems/Agilent/MyBioSource; tests developed by Statens Serum Institut.

✓: Marker well placed in the middle or higher than standard curve;

(/): samples within detection limit; clear division between positive and negative samples (APOe4); -: in the low part or below standard curve;

*: selected as marker for the final 10-plex analysis platform. MMSE = Mini-mental state examination.

39.2.1 Multiplex immunoassay: The method of choice

For the qualitative and/or quantitative determination of very small amounts of physiologically relevant molecules (such as cytokines) in the limited amount of blood material available from the DBS samples, immunoassays are the method of choice. They take advantage of a matching antibodies' strong specificity for a particular protein. Immunological methods allowing for simultaneous measurements of multiple proteins in the same small sample volume are called multiplex immunoassays.

Current technological platforms – the chemistry and the detection instrument as a unit is called a platform – allow measuring up to ten specific proteins in one reaction in as little as 25 µL of protein extract (e.g. MesoScale Diagnostics). The protein extract of each sample is derived from two small punches, each 3.2 mm in diameter, cut out of the blood-containing filter paper of a DBS (Skogstrand et al. 2005).

To enable immunological multiplex analyses, it is essential to obtain antibodies with high specificity for the protein analytes that are to be measured. In addition, to allow for simultaneous measurements, it is important that the concentration levels of the analytes to be determined are within a similar range in the measured samples. The screening of each of the 31 marker candidates occurred in three rounds (Table 39.1). In the first round, analyses were performed according to the conditions recommended by the antibody manufacturer (e.g. MesoScale Diagnostics). If the analyte was measurable in the DBS samples, the analyses parameters were changed in rounds 2 and 3 to meet the preferred conditions for multiplex analyses. Three to six random samples of SHARE participants ≤ 60 years of age and ≥ 80 years of age were tested for each analyte in each round.

39.2.2 The selected markers

From the screening, 12 final candidates were identified and the ten markers subsequently listed were selected for cytokine testing of the SHARE DBS samples. The other 19 candidates did not meet the technical and methodological requirements. Typically, the marker in the sample material was not sufficiently abundant. To confirm the results the ten selected candidates were QC (quality)-tested at MesoScale Diagnostics before being combined to one final 10-plex analysis platform (Tables 39.1 and 39.2).

- Regarding the pro-inflammatory markers **IL-8**, **IL-16**, **IL-18**, **IL-12/23p40** and **MCP-1**, the elevated levels of one or several of these cytokines will better inform us about the type of inflammation, its degree and the inflamed

Table 39.2: 12 Markers measurable in DBS samples comparing Europeans ≤ 60 years old with those ≥ 80 years old.

Markers having passed selection criteria	General detection range of markers	Detection range in youngest respondents	Detection range in oldest respondents
	≤ 60 years old (n = 5) \pm SD	≥ 80 years old (n = 5) \pm SD	
Concentration in	pg/ml	pg/ml	pg/ml
IL-8*	0.0587–165	3.57 \pm 0.99	7.59 \pm 5.02
IL-12/23 p40*	0.469–928	4.14 \pm 1.90	11.06 \pm 3.18
IL-16*	6.48–2,450	151.4 \pm 52.1	146.9 \pm 35.8
IL-18*	0.197–2,500	86.8 \pm 12.3	89.5 \pm 21.0
VEGF-A*	0.287–315	31.1 \pm 8.53	25.2 \pm 2.69
BDNF*	2.94–10,000	166 \pm 44.4	158 \pm 40.4
MCP-1*	1.62–448	49.8 \pm 10.5	61.1 \pm 5.75
VCAM-1	38.7–56,800	5,623 \pm 866	8,807 \pm 2,179
YKL	27.2–100,000	4,359 \pm 1,394	4,828 \pm 1,015
EGF*	0.317–465	13.3 \pm 8.46	6.50 \pm 1.90
Clusterin ^{a)} *	19.2–200,000	500,000–1,000,000	500,000–1,000,000
APOe4 ^{b)} *			

*Markers selected for the final 10-plex.

a): The high level of Clusterin well above the standard curve was one of the challenges when creating this assay. During QC-testing at MesoScale Diagnostics, Clusterin was artificially lowered to allow the inclusion of this marker in the final 10-plex.

b): APOe4 does not vary with age. The protein is either present or not but its level may vary.

tissue type. The results from these markers may confirm a report of cardiovascular disease by a respondent or show undetected inflammation and/or (onset of) cognitive impairment.

- **VEGF** and **EGF** both signal cell growth, which can be important in healing (e.g. in blood vessels) but may also indicate cancer.
- **BDNF** is a neuronal growth factor. It is neuroprotective, and its level increases e.g., with movement/exercise and social embeddedness and is decreased during cognitive decline. The results from the assay can be connected to reports of SHARE respondents regarding their social networks and exercise behaviour (Salinas 2016).

- Last not least are **APOe4** and **Clusterin (APOJ)**; the e4-variant of the apolipoprotein indicates an elevated risk of cardiovascular disease and cognitive impairment (Rasmussen et al 2015). Clusterin has the general vital function in the organism to clear misfolded proteins or cell debris and seems to act neuroprotective in mild cognitive decline and Alzheimer's disease.

The preparation of the SHARE DBS samples, the screening of the 31 candidate markers and the establishment of the final 10-plex analysis platform was performed at Statens Serum Institut in Copenhagen.

39.3 The next steps

With this analysis tool in hand, ca. 16,000 SHARE samples were prepared for analyses of the selected markers. As previously mentioned, the analysis of cytokines and other markers involved in cardiovascular disease and mild cognitive decline gained from DBS collected in a population survey during fieldwork is new. To our knowledge, whether the DBS values of cytokines gained here can be converted to match values gained from venous blood samples in other studies is unknown. Therefore, a validation experiment that compares the analysis results for these markers from venous blood and matched DBS samples and that also controls for sample exposure during blood collection in a survey will have to follow. SHARE will initiate such a study.

A small control study comparing blood samples (venous and DBS) from patients diagnosed with mild cognitive decline and age-matched controls is ongoing. The results for both studies will be reported once available.

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