Lifebrain

D3.5. DBS assays for relevant biomarkers for data enrichment

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Executive summary

The aim of D3.5. was to develop, test and validate a set of DBS assays for relevant biomarkers for data enrichment in use in Lifebrain. The initial list of candidate biomarkers was based on discussions before and during the project. The list was quite comprehensive and involved multiple analytical techniques. As expected, some limitations were discovered during method development and validation. Thus, biomarkers possible to measure from DBS evolved during this phase. An updated list of biomarkers has been produced along with method performance data. Some validation is still ongoing; method adjustment may be necessary, and we will probably be able to measure a few more of the markers before the start of the Lifebrain sample analysis.

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List of acronyms/ abbreviations

Lifebrain	Healthy minds from 0 to 100 years: Optimizing the use of European brain imaging cohorts
AC	Administrative Coordinator
CA	Consortium Agreement
CRP	C-reactive protein
DBS	Dried Blood Spots
EB	Executive Board
EC	European Commission
EU	European Union
GA	Grant Agreement
GC-MS	Gas liquid Chromatography – Mass Spectrometry
HR-TOF	High Resolution - Time of Flight
ICP-MS	Inductively coupled plasma mass spectrometry
LB	Lifebrain
LCBC	Centre for Lifespan Changes for Brain and Cognition
M	Month
MMA	Methyl Malonic Acid
MS	Mass Spectrometry
SME	Small and medium-size enterprises
UiO	University of Oslo
UPLC	Ultra Performance Liquid Chromatography
WP	Work Package

1. Introduction

1.1. Deliverable description

Task 3.5. Develop and validate novel Dried Blood Spots (DBS) assays for relevant biomarkers. Lead: Vitas (M1-M24)

DBS is a modern and less invasive alternative to classical venous puncture for collecting blood samples. A minute amount of capillary blood is collected from the fingertip and dried on a specialty filter paper. The dried sample can be collected at home, sent by regular mail and stored at ambient temperature. Vitas will develop and validate new additional biomarkers on DBS tailored for the Lifebrain project. Candidates for new biomarkers are specific proteins like IGF-1, APOE4, BDNF, leptin, Adiponectin, C-peptide, GLP-1, Aβ38, Aβ40, Aβ42, α-Synuclein, amyloid precursor protein (APP), DJ-1/PARK7 and Tau. Also, from Vitas comprehensive UPLC-HR-TOF-MS lipidomics platform, a panel of lipid markers with potential to predicts preclinical transition to clinical stages of Alzheimer's will be selected and developed for DBS. From a similar metallomics platform run on ICP-MS relevant metals like alumina, lead, copper, iron, and zinc will be adapted to DBS and validated. These novel DBS assays will then be added to already established assays like vitamins, diabetes markers like HbA1c, cholesterol and fatty acids.

1.2. Objectives of the deliverable

Develop and validate new biomarkers on DBS tailored for the Lifebrain project

1.3. Collaboration among partners

The list of biomarkers was discussed prior to and during the writing of the application. Then the first draft was presented and discussed at the Lifebrain kick-off meeting in Brussels January 2017. Continuous discussions between relevant partners and the coordinator have taken place via e-mail, Slack, telephone and meetings up to the consortium meeting in Barcelona in November 2017 where the final list was discussed and settled.

1.4. Inputs received from the Lifebrain General Assembly

During the Lifebrain consortium meeting in Barcelona all partners agreed on the list of biomarkers; these analyses should form the basis for method development by Vitas in WP3. The biomarkers are presented in Table 1. It was decided on this meeting to exclude ApoE4 because this will be evaluated in the genetic analyses.

Table 1. Biomarkers to be developed in Lifebrain

Assay A	Assay D	Assay E	Assay K
	Acetic acid	IFN-g	DAG 36:3/36:2 (sleep)
25OHvitaminD_3	Propionic Acid	IL-1b	Sphingolipids (Ceramides)
Testosterone	Butyric Acid	IL-1ra	Phospholipids
Cortisol	Isobutyric Acid	IL-6	Cholesterol esters
Cortisone	Valeric Acid	IL-8	Triglycerides
Estradiol	Proline	IL-10	
Pregnenolone	Asparigine	IL-17	Assay L
17-OH-pregnenolone	Aspartic acid	MCP-1	Mercury (Hg)
Dehydroepiandrosterone	Glutamic acid	TNF-a	Lead (Pb)
Estrone	Phenylalanine	Assay F	Cadmium (Cd)
24OH-Cholesterol	Glutamin	hsCRP	Arsenic (As)
	Tyrosine	Assay G	Chromium (Cr)
Assay B	Tryptophan	mBDNF	Nickel(Ni)
Fatty acids & cholesterol	Kynuurenine	Assay H	Magnesium (Mg)
Assay C	Alanine	ProBDNF	Selenium (Se)
HbA1c	Glycine	Assay J	Copper (Cu)
	a-aminobutyric acid	IGF-1	Lithium (Li)
	Valine		
	Leucine		
	Isoleucine		
	Threonine		
	Serine		
	B12		
	Homocysteine		

2. Biomarker assays – method development and validation

2.1. Biomarkers to be tested and validated

Biomarkers to be tested and validated are presented in Table 1.

During testing and validation, it became clear that some of the suggested biomarkers could not be measured when DBS should be used as sample matrix for various reasons as discussed in detail below.

Assay A. Steroids

AM-434

The goal for this panel is to include 25OH-vitamin D_3, testosterone, cortisol, cortisone, estradiol, pregnenolone, 17OH-pregnenlone, dehydroepiandrosterone, estrone and 24OH-cholesterol. 17OH-pregnenlone could not be assayed at endogenous levels in DBS because of interference from the DBS paper. Moreover, it was not possible to achieve sufficiently low detection limits for estradiol and estrone in DBS because of very low concentrations and the sample amount from DBS is very low. 24OH-cholesterol showed interference with other substances with the same molecular weight.

Thus, 25OH-vitaminD_3, testosterone, cortisol, cortisone, pregnenolone and dehydroepiandrosterone will be in the final DBS panel to be used on the Lifebrain DBS samples analyzed in WP2. These steroid molecules have been suggested as important factors in the promotion or prevention of development of neurodegenerative diseases.

Assay B. Fatty acids and total cholesterol

AM-289

The goal for this panel is to include the following 11 fatty acids: C12:0, C14:0, C15:0, C16:0, C16:1n7, C17:0, C18:0, C18:1,t6-11, C18:1,c9, C18:1,c11, C18:2,n-6, C20:0, C18:3,n-6, C18:3,n-3, C20:1,n-9, C20:2,n-6, C22:0/C20:3,n-6, C20:4,n-6, C20:5,n-3, C24:0, C22:5,n-3, C22:6,n-3 in addition to total cholesterol. All these 12 lipid biomarkers will be included in the final DBS panel to be used on the Lifebrain DBS samples analyzed in WP2.

Assay C. HbA1c

AM-381

This biomarker performed very well and will be included in the final DBS biomarker panel. Vitas is one of few laboratories with a validated method for measuring this marker of glycated haemoglobin, and it is the preferred marker for detection of insulin resistance and diabetes (WHO). It is solid evidence for increased risk of developing dementia in the presence of diabetes mellitus (Lancet 2016).

Assay D. Short chain fatty acids (SCFA) and free amino acids, MMA and homocysteine

AM-xxx and AM-373

Initially, the intention was that SCFA and FFA should be included in one single method. During development it was discovered that this was not possible and that SCFA will be developed as a separate method. These are then denoted:

Assay D1. SCFA

AM-xxx

These short chain fatty acids are of special interest concerning the effect of microbiota producing in particular buturic acid, which may be of importance for developing neurodegenerative diseases (Dinan &Cryan, J. Physiol. 2017,595,489-503). The SCFA has proven to be difficult to develop methods for DBS samples. For several of the SCFA there is disturbing background even when using the new GC-MS technology. The volatility of SCFA, the low sample volumes and the presence of very similar substances in the blank paper used for DBS collection cause problems. SCFA need more work before it can be included in the Lifebrain DBS panel.

Assay D2. FAA, MMA and homocysteine

AM-373

These methods have been developed to include 16 different free amino acids in addition to methyl malonic acid (MMA) a secondary marker for vitamin B_{12} status as well as homocysteine. All parameters are quantified in one run using GC-MS. Stable isotopes are used as internal standards. Kynurenine was not compatible with this panel and will not be included. The 18 other biomarkers will be analysed for the Lifebrain DBS samples in WP2. Blood concentrations of certain amino acids like cysteine and branched chain amino acids (leucine, isoleucine and valine) are markedly associated with chronic diseases like diabetes and certain forms of cardiovascular and cancer types.

Assay E. Cytokines

AM-454

The cytokines to be included are IFNα, IL-1β, IL-1RA, IL-6, IL-8, IL-10, IL-17, MCP11 and TFNα. All these proteins are of interest in relation to inflammatory processes that may be of significant importance for development of diseases or conditions in the central nervous system.

Because this is a custom multiplex assay where all markers are assayed simultaneously and some of these markers are only detectable in people with certain conditions, all markers will be included in the final assay. The method development has been aimed at achieving the lowest possible detections limits and best possible performance in DBS.

Assay F. CRP

AM-438

This biomarker is developed on a single plex ELISA and performed very well and will be included in the final DBS biomarker panel. CRP is an acute phase protein synthesized by the liver in response to general/ systemic inflammation.

Assay G/H. BDNF

AM-446

BDNF is produced in the brain as well as in skeletal muscle and released during physical activity, and it seems to be important for brain function (Mathhews et al. Diabetologia 2009, 52, 1409-18, bathina &Das. Arch.Med.Sci. 2015,11,1164-78). Two subtypes of BDNF were tested using single plex ELISA; proBDNF and mature BDNF (mBDNF). There was a rapid and extensive conversion of proBDNF to mBDNF and that mBDNF makes up the majority of the total BDNF. So, the loss of proBDNF affects the concentration of proBDNF to such an extent that proBDNF cannot be included. The change in mBDNF is still very small and will be included in the final DBS panel to be used on the Lifebrain DBS samples analysed in WP2.

Assay J. IGF-1/2

AM-248

Based on literature studies IGF-1 was selected because it seems to be related to many chronic conditions and diseases (Fagerberg et al. Mol cell proteomics 2014,13,397-406). This assay was developed on a single plex ELISA. The method development for IGF-1 om DBS was successful and this marker will be included in the final panel.

Assay K. Lipidomics

AM-406

Lipidomics represent a highly sophisticated and potentially powerful omics technology that rarely is performed on samples from DBS. Vitas has dedicated a substantial amount of time developing this. E.g. a set of novel biomarkers from diacylglycerols, believed to be unique biomarkers of sleep deprivation, have been tested and validated. In addition, several hundred lipids from all major lipid classes will be included in the final Lifebrain panel. We have observed that free fatty acids (FFA) increase substantially when DBS are stored. The reason for this is that even a minor hydrolysis in each of the thousands of other lipid classes will produce the same FFAs. For the same reason lyso-lipids, containing only one fatty acid moiety will be somewhat less stable than all others.

Assay L. Metals and minerals

AM-372

Mercury, lead, cadmium, arsenic, chromium, nickel, magnesium, selenium, copper and lithium were intended to be included in the panel. Several of these are present in very low concentrations in the DBS samples. The low volume of blood was anticipated to be an additional challenge. Copper seem to be difficult to include due to especially high background in the lot of DBS paper used in the LB kits. Nickel will be difficult because of high contamination from the sample introduction system. All other elements will be screened in the LB DBS samples.

2.2. Final biomarker panel

As a result of the method development and the findings from testing and validating the assays, and updated table of biomarkers in shown in Table 2.

Table 2. Markers included in the final panel

Assay A	Assay D1	Assay E	Assay K
	Need more work		
25OHvitD$_3$	Assay D2	IFN-g	DAG 36:3/36:2 (sleep)
Testosterone		IL-1b	Sphingolipids (Ceramides)
Cortisol	Proline	IL-1ra	Phospholipids
Cortisone	Asparagine	IL-6	Cholesterol esters
Dehydro-Epiandrosterone	Aspartic acid	IL-8	Triglycerides
	Glutamic acid	IL-10	
Assay B	Phenylalanine	IL-17	Assay L
Fatty acids & cholesterol	Glutamine	MCP-1	
	Tyrosine	TNF-a	Mercury (Hg)
Assay C	Tryptophan		Lead (Pb)
HbA1c	Alanine		Cadmium (Cd)
	Glycine	Assay F	Arsenic (As)
	a-aminobutyric acid	hsCRP	Chromium (Cr)
	Valine	Assay G	Nickel (Ni) (!)
	Leucine	mBDNF	Magnesium (Mg)
	Isoleucine	Assay J	Selenium (Se)
	Threonine	IGF-1	Lithium (Li)
	Serine		
	MMA(B12)		
	Homocysteine		

All methods have separate AM numbers (analytical method) and standard operating procedures. These are listed in Table 3 below together with contributors.

Table 3. Method overview

Analyte	AM-nr	Revision nr	Author	Contributors
FA + cholesterol	AM-289	04	MFV	JI/TB/DM/TEG
Steroids + 25-OH vit D	AM-434	00	AS	AMH/TEG
HbA1c	AM-381	01	SEK	JI/ND/PAD/TEG
hs-CRP	AM-438	00	KB	SEK/MFV/PAD/TEG
Cytokines (9-plex)	AM-454	00	ES	SEK/DM/JI/PAD/TEG
Lipidomics	AM-406	00	TBJ	ND/JI/TEG
MMA, HCY, amino acids	AM-373	02	DM	AS/TEG
mature-BDNF	AM-446	00	DM	SEK/MFV/JI/PAD/TEG
IGF-1	AM-248	02	KB	SEK/MFV/PAD/TEG
Metals & minerals	AM-372	03	PAD	AA/TEG

2.3. Main validation results

The validation of the methods is ongoing. For some methods the validation is completed and for some it is close to completion. Validation features like long term stability and precision will take some time to complete. As samples from the Lifebrain partners seem to be somewhat delayed, this should not be an issue. As samples arrive, they will be assayed with completed methods first and then as new methods are finalized these will be applied to the samples. The current status of method validation and the main results are shown in Tables 4-9 in Appendix 1. More detailed validation tables and all raw data are kept in the archives of Vitas.

3. Conclusions

In conclusion the method development has been successful, and most biomarkers have been possible to measure on DBS. The portfolio of DBS biomarkers available to the Lifebrain project and for Vitas to commercialise in other projects, have thus been extensively broadened during this part of the Lifebrain project.

Appendix 1 – Main validation results

Table 4 – Single biomarkers

Assay	Range DBS	Normal range (serum/whole blood	LOD DBS	LOQ DBS	Unit	Repeatability % LOQ/High QC2	Intermediate-Precision % ULOQ/high QC	Accuracy %	Stability (days)
1. Cholesterol	0.5-10	2.9-7.8	0,0003	0,001	mmol/L	6.0-6.9	9,3	95	21
4. HbA1c	4.0-14.0	4.0-14	3,6	3,9	NGSP%	4.8(n=6)	7.2(n=40)	96	15
5. hsCRP	0.08-40	0.1-100	0,03	0,08	mg/L		8.9 (n=22)	119**	ongoing
12. mBDNF	7.8-500	8-46*	0.55 (n=42)	1.64 (n=42)	ng/mL	6.6 (n=10)	14 (n=38)	na	15
13. IGF-1	10.1-640	10-600	3,4	10,1	ng/mL	6.7 (n=8)		na	ongoing
na = not applicable									
* https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4675070/									
** European Reference Material ERM®-DA474/IFCC, Human serum.									

Table 5 – Steroids

Assay	Range DBS	LOQ DBS	Unit	Repeatability % QC	Intermediate-Precision % QC 2	Accuracy %	Stability (days)
Cortisone	5-500	0,6205	ng/mL	15,2	15,61585909	99	21
Cortisol (Hydrocortisone)	1-500	4,98	ng/mL	10,7	11,40208592	96,6	21
Testosterone	0.86-80	0,86526	ng/mL	15,7	17,23233583	100,4	21
Dehydroepiandrosterone	2-500	1,9974	ng/mL	37,4	34,43911603	97,9	21
Pregnenolone	1-500	1,05	ng/mL	13,4	26,33050128	102,7	no*
Vitamin D (25-hydroxy Vitamin D3)	5-500	5	ng/mL	10,0	27,33388606	95,2	21
*not stable under current conditions, more work needed							

Table 6 - FAA, MMA and homocysteine

Assay	Range DBS	Normal range (serum/whole blood	LOQ DBS	Unit	Repeatability % LOQ/low QC	Intermediate-Precision % LOQ/low QC2	Accuracy %	Stability (days)
MMA	0.4-1.016	0.1-3	0,4	µM	21 %	13 %	-	15
Alanine	69.7-696.5	N.A	69	µM	1 %	2 %	105	15
Glycine	60.1-600.5	N.A	60	µM	2 %	2 %	113	15
alfa-Aminobutyric acid	5-50	N.A	5	µM	2 %	2 %	-	15
Valine	50-500	N.A	50	µM	2 %	2 %	107	15
Leucine	30-299.6	N.A	30	µM	2 %	2 %	110	15
Isoleucine	20.8-207.5	N.A	20	µM	2 %	2 %	109	15
Threonine	49.9-499.4	N.A	50	µM	2 %	3 %	107	15
Serine	50.5-505.3	N.A	50	µM	3 %	8 %	115	15
Proline	50-500.5	N.A	50	µM	2 %	4 %	113	15
Asparigine	19.8-198.1	N.A	19	µM	2 %	2 %	97	15
Aspartic acid	28.1-281.4	N.A	28	µM	2 %	3 %	109	15
Glutamic Acid	49.9-499.4	N.A	49	µM	4 %	4 %	98	15
Phenylalanine	15.1-151.5	N.A	15	µM	3 %	2 %	109	15
Glutamine	100-1000.1	N.A	100	µM	1 %	1 %	101	15
Homocysteine	5-29.0	5-20.0	5	µM	7 %	7 %	-	15
Tyrosine	20-199.7	N.A	20	µM	2 %	3 %	115	15
Tryptophan	10.1-100.9	N.A	10	µM	3 %	2 %	-	15

Table 7 - Lipidomics

Assay	Range DBS	LOQ DBS	Unit	Repeatability % LOQ/High QC2 (4)	Intermediate-Precision % ULOQ/high QC (4)	Accuracy %	Stability (days)
Diacylglycerophosphocholines	0.5-800 µM	0,5	µM	<2%	<10%	70%-95% (3)	14
Diacylglycerophosphoethanolamines	2-800 µM	2	µM	<8%	<15%	70%-100% (3)	14
Diacylglycerophosphoserines	5-50 µM (1)	5	µM	<10%	<15%	85%-115% (3)	14
Diacylglycerophosphoinositols	(2)	(2)	µM	<5%	<15%	70%-100% (3)	14
Sphingomyelins	0.5-800 µM	0,5	µM	<7%	<15%	70%-100% (3)	14
Ceramides	0.2-50 µM (1)	0,2	µM	<7%	<25%	85%-105% (3)	14
Diacylglycerol	0.5-800 µM	0,5	µM	<8%	<25%	105% - 120% (3)	14
Triacylglycerol	1-600 µM	1	µM	<7%	<25%	55%-70% (3)	10
Cholesterol esters	(7)	(7)	µM	<5%	<18%	55%-70% (3)	14
Lysophosphocholines	1-50 µM (1)	1	µM	<15%	<15%	50%-75% (3)	2 (6)
Free fatty acids	(5)	(5)	µM	(5)	(5)	na	not stable

na = not applicable - Information on these complex assays are given in dedicated tables

Range of the DBS assay is defined by LOQ and ULOQ . Include unit

Accuracy is estimated by measuring reference material or spiked samples where no reference materiale is available.

Precision is estimated from QC values and is such a between-day precision, list number of total replicates.

Stability - list number of days analyte(s) is stable at room temperature (25°C). Analyte is considered stable if recovery is within 15% of nominal value. If correction factors are used, recovery is calculated on corrected value)

Multiplex/omics parameters are listed in a separate table: Tabeller for ytelsesparametre for omics (X:/EU prosjekter/Lifebrain)

Table 8 – Cytokines

Assay	Range DBS	Normal range (serum/whole blood	LOQ DBS	Unit	Repeatability % LOQ/low QC	Repeatability % LOQ/High QC2	Accuracy %	Stability (days)
IFN-γ	4.25 - 17420	na	4,25	pg/ml	3,4	8,2	86	ongoing
IL-10	0.95 - 3900	0.55 - 315	0,95	pg/ml	3,1	7,5	85	ongoing
IL-17A	5.86 - 24000	na	5,86	pg/ml	11,7	9,1	100	ongoing
IL-1RA	0.99 - 4050	na	0,99	pg/ml	15,1	10,7	86	ongoing
IL-1β	0.95 - 3900	0 - 39	0,95	pg/ml	2,9	4,1	89	ongoing
IL-6	0.52 - 2120	0.38 - 1.07	0,52	pg/ml	8,8	11,8	88	ongoing
IL-8	0.56 - 2300	na	0,56	pg/ml	5,0	6,7	-	ongoing
MCP-1	1.60 - 6550	11.8 - 17.6	1,60	pg/ml	9,8	12,9	92	ongoing
TNF-α	0.83 - 3420	2.08 - 672	0,83	pg/ml	6,4	6,9	73	ongoing

Table 9 - Metals & Minerals

Assay	Range DBS	Normal range whole blood	LOQ DBS	Unit	Repeatability % LOQ/low QC	Repeatability % LOQ/High QC2	Intermediate-Precision % ULOQ/high QC	Accuracy %	Stability (days
Magnesium	4-60 µg/ml	1.9 - 2.7 mEq/L	4	µg/ml	4.3 % (14.4 µg/l)	ongoing	12,2	ongoing	stable
Selenium	0.015 - 1.0 µg/ml	0.023-0.190 µg/ml	0,015	µg/ml	11.5% (0.060 µg/ml)	6.7% (0.272 µg/ml)	13	ongoing	stable
Lithium	3-1000	<1 ng/ml	3	ng/ml	ongoing	ongoing	ongoing	ongoing	stable
Nickel	na	0-10 ng/ml	na	ng/ml	na	na	na	na	na
Copper	na	600-1600 ng/ml	na	ng/ml	na	na	na	na	na
Arsenic	8-100	0-12 ng/ml	8	ng/ml	7.5% (14.1 ng/ml)	4.5% (30.4 ng/ml)	ongoing	ongoing	stable
Cadmium	5-100	0-5 ng/ml	5	ng/ml	15.7% (5 ng/ml)	10.0% (12.10 ng/ml)	ongoing	ongoing	stable
Mercury	9-100	0-10 ng/ml	9	ng/ml	26.9% (1.5 ng/ml)	9.2% (37.1 ng/ml)	ongoing	ongoing	ongoing
Lead	10-1000	0-50 ngml	10	ng/ml	17.3 (9.9 ng/ml)	4.2 % (447 ng/ml)	ongoing	ongoing	stable
Chromium	9-100	0-5 ng/ml	9	ng/ml	ongoing	ongoing	ongoing	ongoing	stable