Nutrition and biomarkers in psychiatry

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Research on micronutrient deficiencies in schizophrenia,
the role of the intestine in the hyperserotonemia of autism,
and a method for non-hypothesis driven discovery of biomarkers in urine

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Contents

LIST OF ABBREVIATIONS

1DE	one-dimensional gel electrophoresis
2DE	two-dimensional gel electrophoresis
5-HIAA	5-hydroxyindoleacetic acid
5-HT	5-hydroxytryptamine or serotonin
5-HTT	5-HT transporter
5-HTP	5-hydroxytryptophan
AA	arachidonic acid
ACN	acetonitrile
ADHD	attention deficit hyperactivity disorder
ADI-R	autism diagnostic interview - revised
ADOS	autism diagnostic observation schedule
AKT1	serine-threonine kinase 1
APO-AIV	apolipoprotein A-IV
ASD	autism spectrum disorders
AUC	area under the curve
BD	bipolar affective disorder
BDNF	brain-derived neutrophic factor
BMI	body mass index
BPP	brain proteome project
C_1	carbon-1; methyl
CDCV	common disease common variant
CDD	childhood disintegrative disorder
CMV	cytomegalovirus
CNV	copy number variation
COMT	catechol-O-methyl transferase
CPZ	chlorpromazine
CRMP-2	collapsing response protein 2
CSF	cerebrospinal fluid
CV	coefficient of variation
DALY	disability-adjusted life year
DAO	D-amino-acid oxidase
DAOA	D-amino-acid oxidase activator
DBI	diazepam binding inhibitor
DHA	docosahexaenoic acid
d-IBS	diarrhea predominant irritable bowel syndrome
DISC1	disrupted in schizophrenia 1
DLX5	distal-less homeobox 5 transcription factor
DM	diabetes mellitus
DNMT1	DNA-methyltransferase 1
DRD1	dopamine receptor D1
DRD2	dopamine receptor D2
DSM-IV-TR	diagnostic and statistical manual of mental disorders, 4th edition, text revision
DTNBP1	dysbindin; dystrobrevin binding protein 1
DZ	dizygotic
ECS	electroconvulsive seizures
EFA	essential fatty acid
EIC	extracted ion chromatogram
ELISA	enzyme-linked immunosorbent assay
EN2	engrailed 2
EPA	eicosapentaenoic acid
ESI	electrospray
FA	fatty acid (*in part I*)
FA	formic acid (*in part II*)
FAS	fetal alcohol syndrome
FDA	food and drug administration
FMR1	fragile-X mental retardation 1
fMRI	functional magnetic resonance imaging
GABA	gamma-aminobutyric acid
GAD67	glutamate decarboxylase 67
GI	gastrointestinal
Glo1	glyoxoalase I
GluR7	glutamate receptor 7
GR	glucocorticoid receptor
GRK3	G-protein receptor kinase 3
GRK4	G-protein receptor kinase 4
GSSG	oxidized glutathione
GTP	guanosine triphosphate
Hcy	homocysteine
HDAC	histone deacetylase
HDM	histone demethylase
HDSS	high-dimensionality small-sample-size
HMT	histone methyltransferase
HPLC	high performance liquid chromatography
HUPO	human proteome organization
ICD-10	international statistical classification of diseases and related health problems, 10th revision
IMGSAC	international molecular genetics study of autism consortium
L/M	lactulose-mannitol ratio
LBW	low birth weight
LC	liquid chromatography
LCPUFA	long-chain polyunsaturated fatty acid
LG-ABN	licking, grooming and arched-back nursing
LMR	lifetime morbid risk
LOD	lower limit of detection
LOOCV	leave-one-out cross-validation

MALDI	matrix-assisted laser desorption and ionization
MAO-A	monoamine-oxidase A
MD	Mahalanobis distance
MECP2	methyl-CpG-binding protein 2
MIAME	minimal information about experiments for genomics and transcriptomics
MIAMET	minimal information about experiments for metabolomics
MIAPE	minimal information about experiments for proteomics
MnSOD	manganese superoxide dismutase
MRM	multiple reactions monitoring
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MTHFR	methylene tetrahydrofolate reductase
MUFA	monounsaturated fatty acids
MZ	monozygotic
NBEA	neurobeachin
NF1	neurofibromatosis 1
NGFI-A	nerve growth factor inducible factor A
NLG	neuroligin
NMDA	N-methyl-D-aspartic acid
NOS	not otherwise specified
NPAS3	neuronal PAS domain protein 3
NRG1	neuregulin 1
NSC	nearest shrunken centroid
NTD	neural tube defects
OCD	obsessive-compulsive disorder
PC	prinicipal component
PCA	prinicipal component analysis
PDD	pervasive developmental disorders
PE	phosphatidylethanolamine
PG	prostaglandin
PKU	phenylketonuria
PLA2	phospholipase A_2
PLT	platelet; thrombocyte
PON	paraoxonase
PPAR-a	peroxisome proliferator activated receptor-α
PRKCB	protein kinase C β
PRODH2	proline dehydrogenase 2
PRP	platelet-rich plasma
PTSD	post-traumatic stress disorder
PUFA	polyunsaturated fatty acids
PWS	Prader-Willi Syndrome
Q-TOF	quadrupole time-of-flight
RBC	red blood cell; erythrocyte
RELN	reelin
RIA	radioimmunoassay
RP	reversed-phase
RR	relative risk
RV	reference value
SAFA	saturated fatty acids
SAH	S-adenosyl homocysteine
SAM	S-adenosyl methionine
SAT	differential sugar absorption test
SD	standard deviation
SELDI	surface-enhanced laser desorption and ionization
SES	socioeconomic status
SHANK3	SH3 and multiple ankyrin repeat domains
SID	stable-isotope dilution
SNP	single nucleotide polymorphism
SOX-10	SRY (sex-determining region)-box 10
SREBP	sterol regulatory element binding protein
SUMO	small ubiquitin related modifier proteins
Tbx1	T-box 1
TCP-1	t-complex protein 1
TFA	trifluoroacetic acid
TIC	total ion chromatogram
TOF	time-of-flight
TPH2	tryptophan hydroxylase 2
Tryp	tryptophan
TSC1	tuberous sclerosis 1
TSC2	tuberous sclerosis 2
UBE3A	ubiquitin-protein ligase E3A
UPD	uniparental disomy
UV	ultraviolet-visible
WHO	world health organization
XBP1	X-box binding protein 1
YLD	year lived with disability

Scope, aim and outline of this thesis

This thesis describes the study of markers of nutrition and intestinal motility in mental disorders with a focus on schizophrenia and autism, and the development, evaluation and application of a biomarker discovery method for urine. The aim of the thesis is to investigate the role of long-chain polyunsaturated fatty acids (LCPUFA), B-vitamins and platelet (PLT) serotonin (5-HT) in schizophrenia and autism. The thesis proposes also that biomarker research in psychiatric disease is of great relevance and describes a biomarker discovery method in urine using a non-hypothesis driven '-omics'-like approach. The thesis ends by summarizing its contents and putting biomarker research in psychiatric disease and its implications in a broader perspective.

In the **Introduction** the complex etiology and potential role of non-hypothesis driven biomarker research in psychiatric disease is reviewed, with an accent on schizophrenia and autism. The enormous economic and psychosocial global burden of mental disorders is described as well as their epidemiology, clinical presentation and classification/diagnosis. Hypothesized etiological factors are discussed to create a framework in which biomarkers and the research thereof can be positioned. Furthermore, advances in the field of biomarker research in psychiatry are discussed in the context of epigenetics, proteomics and metabolomics.

The first part (**Part I**) of this thesis describes a study of LCPUFA and B-vitamins in schizophrenia, of PLT 5-HT and intestinal permeability in autism, and of the value of PLT 5-HT as marker of intestinal motility in newborns. The chapters, in which these studies are described, are preceded by a review (**Chapter 1**), which gives an overview of the role of LCPUFA and folate in the etiology and severity of psychiatric diseases such as depression, bipolar disorders, schizophrenia and autism. Pregnancy complications and folate-substrated carbon-1 metabolism are considered and their possible epigenetic effect on the etiology of mental disorders is described. Other nutritional factors, such as LCPUFA, that are important for brain development, physico-chemical properties of membranes, signal transduction and DNA-transcription, and that have been used in supplementation trials, are suggested to be important factors in the origin and severity of schizophrenia. In **Chapter 2** we describe the results from a study concerning the essential fatty acid (EFA) and functional B-vitamin status in patients with schizophrenia. Aberrant EFA-status and increased homocysteine (Hcy; a marker of functional B-vitamin deficiency), have been reported before in subgroups of patients with schizophrenia. We describe the characteristics of large subgroups with marginal to severe deficiencies of LCPUFA and B-vitamins, notably folate and

vitamin B_{12}. Deficiencies proved easily correctable in the most severely deficient patients upon supplementation with ω3 fatty acids and B-vitamins. **Chapter 3** attempts to integrate and link previously reported findings of increased intestinal permeability and increased PLT 5-HT levels in subgroups of children with pervasive developmental disorders (PDD). Platelet 5-HT and intestinal permeability were assessed in children with PDD in Curaçao. Differential urinary excretion of inert sugars after ingestion of a sugar solution was used as marker of intestinal permeability. In **Chapter 4** we examined the potential of PLT 5-HT as marker of intestinal motility. For this we studied whole blood and PLT 5-HT in mothers (normal motility) and their newborns (developing intestinal motility) at birth. The course of PLT 5-HT in relation to changes in feeding mode (i.e. parenteral/enteral) was investigated in a small group of preterm born infants to see whether PLT 5-HT was responsive to changes in intestinal motility. **Chapters 2, 3 and 4** describe hypothesis-driven research in mental disorders. However, the advance of knowledge about mental disorders is slow and it is likely to benefit from complementation by information that is generated through non-hypothesis driven research with state-of-the art techniques that profile proteins (i.e. proteomics) and metabolites (i.e. metabolomics). These '-omics' techniques are likely to deliver a multitude of candidate diagnostic and prognostic markers as well as therapeutic targets, compared to hypothesis driven research.

Part II is thus devoted to the development, evaluation and application of such a non-hypothesis driven method. In **Chapter 5** we describe the comparative analysis of low molecular weight urinary components using LC-MS and subsequent multivariate statistical analysis of the processed LC-MS data. This chapter deals with the development, evaluation and preliminary application of the method to proteinuria in humans. The potential and pitfalls of the method are contemplated upon. **Chapter 6** describes an advanced proof-of-principle of the method through the comparison of urinary profiles from pregnant and non-pregnant females using the improved methodology described in Chapter 5. The methodology is significantly optimized with respect to data processing and multivariate statistical analysis. More focus is put on the selection of discriminatory peaks.

General Introduction

General Introduction

Biomarker research in mental disorders
Linking biomarkers to etiology

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Abstract

Amongst complex diseases, psychiatric disorders are probably the least understood. Unfortunately, at the same time they contribute enormously to psychological, social and economic suffering on a global and individual level. Especially in third-world countries an affected relative is really detrimental for a household. It is remarkable that despite the wide availability of psychiatric services and psychiatrists in Western countries, under-diagnosis and under-treatment are still common. This may be related to currently available diagnostic and treatment modalities that are far from perfect. A largely unknown disease etiology and a diagnostic system that is based on the observation of symptom clusters can be held, at least partially, responsible for this situation. In view of the large number of epidemiological studies, studies of animal models for anxiety, depression and addiction and extensive research into mental disorders, one might expect otherwise. Today most new medication for psychiatric disorders is still being discovered by serendipity in spite of our growing knowledge of molecular and cellular processes involved in neural signaling and neuronal development. In most cases there is no effective medication without harmful side-effects that can cure neurodevelopmental and mental disorders.

Research has, however, enriched our knowledge with the identification of psychosocial and cultural risk factors and early-life events that increase the risk of developing a mental disorder. Genetic analysis has yielded some candidate genes that, depending on the disorder in question, have a rather small to negligible contribution to the overall risk. Notwithstanding all these efforts no single gene has thus far been identified that can explain the complex etiology of a mental disorder except for Rett's syndrome. Altered expression of genes caused by epigenetic deregulation rather than mutations of the DNA sequence may be more relevant for the development of mental disorders. Upcoming unbiased, non-hypothesis driven approaches based on genomics, transcriptomics, proteomics and metabolomics technologies hold promise to increase our comprehension of mental disorders through the (expression-) profiling of hundreds to thousands of genes, gene-transcripts (mRNA), proteins and metabolites, respectively. For the interpretation of the results, powerful bioinformatics approaches are crucial. Integration of these '-omics' results in comprehensive functional correlation networks offer the possibility to study psychiatric diseases in a systems biology approach. By doing so it is expected that new prognostic, diagnostic and therapeutic biological markers or panels of markers will be discovered, because 'childhood-diseases' that have impaired biomarker research in cancer seem to have been dealt with.

New markers or marker panels should have superior sensitivity and specificity and should preferably relate in a causative manner to the organ, tissue, cell or

molecular pathway that is involved in the pathophysiology of the given disease. This may also simplify the present picture of mental disorders that is defined by phenomenological observations into biochemically related classes. In the end it may even be possible to make mental disorders run a less severe course, to prevent or delay the onset, to decrease the impact of environmental risk-factors, to identify highly-susceptible individuals, and to prevent or even cure mental disorders in generations to come.

1. Introduction

The world health organization (WHO) reported in 2001 that 450 million (10%) of all people suffer from mental or neurological disorders or from psychosocial problems such as those related to alcohol and drug abuse [1]. One in 4 people will be affected by a neuropsychiatric disorder at some stage of their life [1]. Men and women are equally affected with some exceptions, such as a higher prevalence of alcohol and substance abuse disorders in men and of unipolar depressive disorder in women [1]. Other examples of neuropsychiatric disorders include bipolar affective disorder (BD), schizophrenia, epilepsy, Alzheimer's and other dementias, post traumatic stress disorder (PTSD), obsessive and compulsive disorder, panic disorder, and primary insomnia. All of these involve cognitive, emotional, behavioral and interpersonal impairments. It is alarming that the largest portion of individuals with neuropsychiatric disorders remains untreated [2]. Next to the enormous suffering of families with affected relatives there is the associated economic burden of mental disorders caused by health care and social service needs, lost employment and reduced productivity (e.g. mental health problems account for 35–45% of absenteeism from work [3]), impact on families and caregivers, levels of crime and public safety, and the negative effect of premature mortality.

The high life-time prevalence puts also a significant burden on primary care, because 24% of all patients attend these facilities because of a mental disorder [4]. Mental disorders thus contribute largely to the global burden of disease and health care costs. Importantly, projections based on figures of the 1990 WHO Global Burden of Disease Study in 1997 by Murray and Lopez [5] note that there will be a 40% increase (from 10.5 to 14.7%) of disability-adjusted life years (DALY; see 'glossary' at the end of the 'References' section) caused by neuropsychiatric disorders from 1990 to 2020. An update of these projections, based on figures of the WHO from 2002, even shows unipolar depressive disorders will be ranked as the second leading cause (5.7%) of DALYs in 2030 [6].

In regions with high-income countries such as Europe, neuropsychiatric disorders account for over 40% of chronic diseases and these disorders are the greatest cause of years lived with disability (YLD). With 19.5% of DALYs, they come in second after cardiovascular disease [3]. In westernized countries unipolar depressive disorder will become the leading cause of illness, while in low-income countries it will rank third [6] on the list of DALY causatives.

Few reports are available regarding the economic impact of mental disorders in developing countries. However, reports from industrialized countries show, for example, that in the U.S.A. 7% of total health care expenditures are spent on mental illness and that the total cost of mental disorders is about 2% (38.4 billion €)

of the U.S. gross domestic product [7]. Europe's mental health care budgets constitute on average 5.8% of total health expenditure with a wide variation between countries (0.1% to 12%) [3]. The cost of mental disorders constitutes a significant proportion of the overall economy and the negative economic consequences of mental illness greatly exceed the cost of treatment. It is thus important to prevent and treat mental illness for these two reasons.

One of the ten recommendations in the world health report of 2001 by the WHO is to support research into biological and psychosocial aspects of mental health in order to increase the understanding of mental disorders and to develop more effective interventions [1]. The European Union adds to this that interventions should also be evaluated for their cost–effectiveness [3]. A recent cost-effectiveness study [8] shows that current treatments, such as first-generation antipsychotic, antidepressant and antianxiolytic drugs combined with psychosocial treatment, are very cost-effective and steps should be undertaken to increase treatment coverage and adherence. However, even if unlimited funding was available and all individuals affected by a psychiatric disease were treated optimally, about 60% of the burden would remain unavertable [9]. The burden of mental illness is especially heavy in developing countries in which poverty, HIV/AIDS, violence, prejudice etc. use up most of the resources. Treating mental illness is often seen as a luxury in these countries [10]. These problems need to be addressed before findings of more applied and fundamental research can be implemented.

Although research focusing at biological aspects of mental health may not find its way into pharmacological interventions directly, it will probably contribute to the understanding of the cause, course and outcome of disease. The use of biomarkers, i.e. molecules that indicate a physiological alteration due to development of disease for preventive, diagnostic, prognostic and therapeutic purposes, is relatively new, but this field of research has gained much interest in the past decades. This article aims to review the role and potential of biomarkers and biomarker research in psychiatric diseases, with the emphasis on schizophrenia and pervasive developmental disorders (PDD; see 'glossary') /autism spectrum disorder (ASD; see 'glossary'). The latter two terms will be interchangeably used throughout this review. Although this review will cover many topics regarding the epidemiology and etiology of mental disorders, it will not be exhaustive in this respect. Many comprehensive and recommendable reviews regarding specific topics discussed in this review have been published and the authors will refer the reader to these reviews for more detailed overviews where appropriate.

2. Epidemiology and economic costs

A more scientific view on the causes and symptoms of psychiatric diseases was adapted after superstition, e.g. demonic possession and witchcraft, had been abandoned. The puritan clergyman Cotton Mather (1663-1728) was one of the first to advance physical explanations for mental illnesses in Renaissance Europe [11], while the Arab physician Rhazes already described definitions, symptoms, and treatments for mental illness in the 10th century [12]. Acceptance that mental illness is caused by an unlucky combination of genetic background and environmental factors made room for epidemiological studies that identified gradients across time and/or space. From these studies underlying risk factors were identified. MEDLINE has cited studies on the epidemiology of depression, bipolar disorder, schizophrenia and developmental disorders since the 1970s.

Proper estimation of incidence and prevalence rates, and detection of trends, have in the past been hampered by different ascertainment strategies across epidemiological studies. With the global implementation and acceptance of classification systems for mental disorders (i.e. Text-Revision of the 4th (IV) edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV-TR) of the American Psychiatric Association [13], and the 10th revision of the International Statistical Classification of Diseases and Related Health Problems (ICD-10) of the WHO [14]) more reliable numbers on prevalence and incidence have become available. The bias of an underestimation of the prevalence of psychiatric diseases in the developing world may nevertheless be present, because many epidemiological studies reported in languages other than English, have not been accounted for in systematic reviews in the past. Up to a decade ago, it was, for example, believed that schizophrenia was randomly distributed across different cultures and regions. However, recent epidemiological data show otherwise [15].

The many evolution-based theories that are founded on the dogmatic belief that the incidence of schizophrenia is invariant across time and place, are currently being challenged. Systematic reviews on incidence and prevalence estimates of schizophrenia show a median (10-90th percentile) incidence of 15.2 (7.7-43.0) per 100,000 persons [16], and a median (10-90th percentile) lifetime prevalence of 4.0 ‰ (1.6-12.1) [17]. It seems that about 7-8 individuals per 1,000 will develop schizophrenia during their lifetime, which is referred to as the lifetime morbid risk (LMR; see 'glossary'). In addition, the incidence estimates showed that males have a 1.4-times higher risk to develop schizophrenia than females and that migrants have a 4.6-times higher risk than native-born individuals. Living in an urban area also increases the risk of developing schizophrenia compared to living in mixed urban/rural sites [16]. Remarkably, there were no differences in prevalence estimates for different gender and urbanicity status [17]. The investigators observed

a 1.8-times higher prevalence in migrants compared to native-born individuals [17]. Developing nations had a lower prevalence of schizophrenia than developed nations, but this finding should be interpreted with caution, because a country's status of development was only based on a single crude economic variable (per capita gross national product). More research into factors that differentially influence the course of illness in men and women around the world, especially from poorer countries, is therefore warranted [17]. Risk factors associated with the incidence of schizophrenia were not fully congruent with those for prevalence. More importantly, these results call for the unraveling of factors that cause a differential course of schizophrenia between risk groups. For example, the fact that high latitude is associated with a higher prevalence of schizophrenia [18] is intriguing.

The economic burden of schizophrenia in Europe [19] and in the U.S. [20] (direct medical, non-medical and indirect costs) associated with psychotic disorders are respectively estimated to 35 billion € and 32.5 billion $ per year, which is primarily due to the early onset in adulthood and the fact that 2/3 of affected individuals have persistent and/or fluctuating symptoms despite optimal treatment [13]. The cost per capita spent on care for patients with schizophrenia are estimated at 1.1 million $ in the U.S. and 2.3 million $ in Canada [21]. Cost-effectiveness studies are necessary to maintain mental health care accessible for large groups of patients.

Despite its lower prevalence compared to psychotic disorders, pervasive developmental disorders (PDD or ASD) are also characterized by a significant burden of disease and economic cost, because of their early onset, lost productivity and required adult care. PDDs have a prevalence rate of about 60 persons per 10,000 [22] and the prevalence of autistic disorder and Asperger's disorder are estimated at about 13/10,000 and 3/10,000 [22], respectively. Childhood disintegrative disorder (CDD) is rarer with a prevalence of about 0.2/10,000 [22]. Rett's disorder seems to have a genetic origin, i.e. mutations of the methyl-CpG-binding protein 2 (MECP2) gene cause the majority of the cases of Rett syndrome (80% of affected females [23]) via an influence on chromatin remodeling [24]. A more recent systematic review of PDD prevalence studies showed slightly lower prevalence estimates of 7.1/10,000 for autism and 20/10,000 for ASD [25]. The 60% of variation in prevalence estimates between studies was attributed to changing diagnostic criteria, age of the sample, urban or rural location of sampling and retrospective or prospective case-assessment [25]. The Center for Disease Control in the U.S. [26] found that 5.2-7.6 of 1,000 children aged 8 years have ASD. This CDC study group also noted a trend for non-Hispanic white children to have slightly higher prevalence estimates than non-Hispanic black children. It is unclear why

prevalence estimates varied by race/ethnicity. Overall, their data also corroborate the notion that more males than females are affected (male-female ratio: 3.4-4.2 / 1) [26]. Interestingly, females (58%) were more prone to be cognitively impaired than males (42%) [26]. There is an apparent increase in incidence of PDD [26;27], but it is disputed whether this trend can be accounted for by other than methodological factors and increased awareness [22;27]. However, environmental risk factors cannot be ruled out. It is important for decision makers in health care to acknowledge this increasing trend. The CDC urges policy makers to improve early identification of ASD. In the future results from developmental epidemiology can contribute to the understanding of psychopathology as well as to the identification of environmental risk factors. Research into gene-environment correlations and interactions give hope that we can reduce the burden of child and adolescent mental illness by devising preventive and therapeutic measures [28].

Up-to-date information about prevalence rates is important because the societal economic costs, including education and treatments for children with ASD, amount to approximately 35 billion $ per year [29]. Total expenditures per 10 000 covered lives associated with ASD increased 142.1% over a 5-year period [30], because of a 20% increase in average health care expenditures from 2000 to 2004 and rising prevalence rates. Ironically, ASD creates a smaller burden on health insurances than other childhood disorders such as mental retardation, because of the relatively lower prevalence.

The lifetime per capita incremental societal cost of autism is $3.2 million [31]. Earlier identification and more proactive treatment will increase the burden of autism on the health care system, so efforts should be made to ensure that access to care for this vulnerable population is not compromised. Overall the utilization and cost of health care are significantly higher for children with PDD compared with children without PDD, underscoring the need to find more appropriate treatment options including biomedical approaches that target the core PDD symptoms [32]. Biomarker research into the causes, traits and treatment options of children with PDD should be stimulated by governmental institutions at least for matters of cost-effectiveness.

3. Diagnosis and classification

The statement that “current (diagnostic) criteria reflect perceived similarity of symptoms and prognoses, which is potentially influenced not only by actual etiological similarity, but also by the cultural and inherent person-perception biases of those perceiving the sufferer, and the categorization demands of legal, medical and research systems” [33] is both frustrating as well as liberating, because it might explain why many findings are only relevant for subgroups of patients with a

certain psychiatric diagnosis. It also stresses the need for other than categorical ways to characterize the different symptom dimensions of mental disorder, e.g. by endophenotypes [34]. Endophenotypes (see 'glossary') may provide an alternative approach to observation-based classification systems of heterogeneous disorders. An example of an endophenotype is the inability of schizophrenia patients and their unaffected family members to avoid looking at a visual cue that they have been told to ignore (antisaccade eye movement task). By definition only, endophenotypes differ from biological markers by the fact that the latter lack a (known) genetic basis. However, the question is to what extent and for what means the difference between endophenotypes and biological markers should be upheld.

3.1. Schizophrenia

Schizophrenia [35] is a disabling mental disorder that is caused by disruptions in thought processes. It is characterized by psychosis, apathy, social withdrawal, and cognitive impairment [13]. The positive symptoms of schizophrenia, including false beliefs (delusions) and perceptual experiences not shared by others (hallucinations), and bizarre behavior, are most prominent during a psychotic episode, whereas its negative symptoms, including blunted affect, apathy (loss of interest and motivation), anhedonia (inability to experience pleasure from normal activities) and alogia (diminished speech content), are more persistent [36]. These negative symptoms and deficits in cognition, including problems in attention and concentration, psychomotor speed, learning and memory, and executive functions are strongly associated with impaired psychosocial functioning [37;38], which is present in the prodromal phase of a psychosis [39]. The result is that most patients with schizophrenia have impaired functioning at work or school, in parenting, personal care, independent living, interpersonal relationships and leisure time even before their first psychotic episode [39]. Definitive diagnosis is usually assigned during hospital admission for a psychosis and a follow-up of at least 6 months is generally required [13].

Assignment of schizophrenia is done using either the DSM-IV-TR [13] or ICD-10 [14] criteria. These classification systems objectively define symptoms and characteristic impairments of schizophrenia in a similar way and the reliability of diagnosis between the two systems is high [40], even though a narrower definition of the disorder is used in the DSM-IV-TR. Temporal diagnostic consistency seems moderate (70%) with the highest variability immediately after onset of the disorder in outpatient and emergency settings [41]. However, this is far better than for most mental disorders, e.g. the temporal consistency of specific personality disorder is estimated at only 30% [41]. There is growing support for the view that these disorders should not be seen as discrete 'disease entities' but rather as dimensions of

continuous variations [42]. While the onset of schizophrenia occurs in the 2nd or 3rd decade of life, subtle abnormalities of cognition, social interaction, motor function and physical morphology are frequently observed in individuals who later develop schizophrenia [43], which is suggestive of a developmental vulnerability. The question arises whether there is a gold standard for the assignment of a mental disorder and so whether current treatment recommendations and interventions are always appropriate. The holy grail of biological psychiatry, a lab test for mental disorders [44], remains difficult to attain, because it relies on widely used classification systems of which the short-term consistency seems poor to at most moderate [41]. Multidisciplinary biomarker research may hold the promise of improving classification and follow-up of therapeutic efficacy.

3.2. Autism

Autism and the other PDDs were first described in 1943 by Kanner [45]. Since then, its criteria and ways of assignment have changed considerably. The term ASD is often used to refer to autistic disorder, PDD-NOS and Asperger's disorder, while the term PDD additionally comprises Rett's disorder and CDD [13]. Pervasive developmental disorders are characterized by severe and pervasive qualitative impairment in several areas of development: reciprocal social interaction skills, communication skills, or the presence of a restricted, stereotyped, repetitive repertoire of behavior, interests and activities [13;14]. Children with PDD also suffer frequently from mental retardation (30-70%), seizures (25%), hyperactivity and other behavioral problems [46].

A failure to develop joint attention, including a child's ability to share interests, pleasurable experiences or requests by using gestures or verbal communication in combination with eye contact, is one of the earliest indicators of autism [47]. Impairments are usually evident from about 18 months of age [48]. Early identification of children with PDD is important because early intervention is more effective in children with autism than in children with other developmental disabilities [49] and it helps minimizing the impact on the family. Having a PDD leads to major difficulties in daily living, school and work performance and most families are confronted with extraordinary demands on their time, energy and financial resources.

In addition to general medical evaluation, children are evaluated for the presence/absence of a PDD (categorical diagnosis), different dimensions of PDD (e.g. intellectual functioning, language, behavior) and the individual's most disruptive symptoms, which then become the focus of treatment [46]. Assignment of diagnosis usually involves a 2-level approach, with additional, more specific screening for autism of children that fail routine developmental screening [46;48].

Information from parents, teachers and pediatric clinicians working in primary care settings about the developmental profile in conjunction with standardized instruments is used to assign the clinical diagnosis, since DSM-IV TR criteria, which are considered the gold standard for ASD diagnosis, leave too much latitude for clinical judgment [48]. Autism-specific diagnostic tools are the Autism Diagnostic Interview (ADI-R, [50]) and the Autism Diagnostic Observation Schedule (ADOS, [51]). The ADI-R is a standardized investigator-based semi-structured interview that is applicable from about 18 months into adulthood. It aims to provide data on the behavior of a child or young adult to differentiate between autism and other developmental disorders [50]. It is, however, not suitable for children with a mental age below 2 years [52]. The ADOS is a semi-structured observational instrument based on DSM-IV criteria, which is used to differentiate between autism, PDD-NOS and other developmental disorders by assessment of social interaction, communication, play and imaginative use of materials [51]. The ADOS is highly sensitive in classifying children with ASD in their 2nd and 3rd year of life but its specificity is poor [52]. Revision of ADOS algorithms is ongoing to improve the diagnostic validity [53].

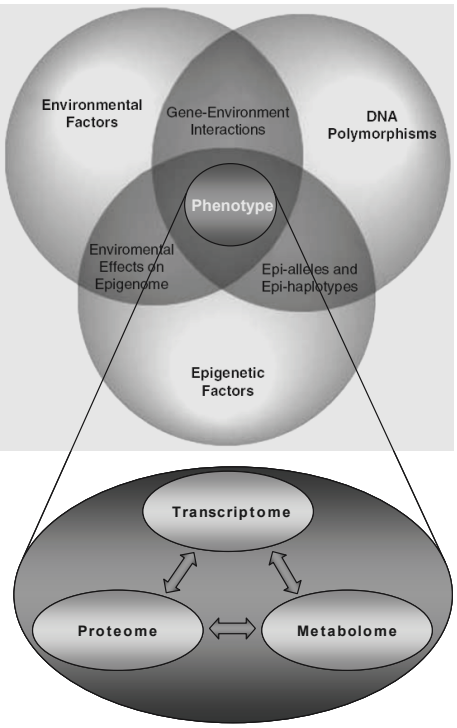
Biomarkers such as proteins, metabolites and cells of the immune system are being tested for their potential in early diagnosis of PDD. However, definitions of PDD and the ways of assigning them have changed throughout time making any correlation with biochemical markers difficult. Because PDD represents a spectrum of disorders with impairments in different areas that range from very mild sub clinical to severe clinical forms, it is inherent to PDD that their presence or absence in an individual can never be confirmed with 100% certainty. The question whether a diagnostic biomarker for PDD is feasible should not deal with the question whether symptom-based diagnosis is better than biomarker based diagnosis (i.e. is the biomarker the consequence of an established diagnostic system?), but merely with the fact whether a biomarker can identify those at risk of developing an impairment, which may be a part of a disorder named 'PDD', and whether these individuals are responsive to available interventions.

4. Etiology of schizophrenia and autism

The etiology of many psychiatric disorders is unknown. To determine which biomarkers (for prevention, diagnosis and treatment) are necessary, it is first important to consider what is known about the etiology. For example, current biomarker research will be less fruitful in establishing the biological causes of increased risk of schizophrenia in migrants, because the causes are likely to be very heterogeneous. Biomarker research should rather aim to identify and characterize groups or individuals with (epi-) genomes, transcriptomes, proteomes or

metabolomes that confer susceptibility to develop a mental disorder or that are responsive to available therapies. Integration of evolutionary medicine (see 'glossary'), epidemiology and life sciences is necessary to identify those individuals carrying susceptible '-omes'. For this we believe it is better to describe mental disorders as arbitrarily defined sets of symptoms that may have a hierarchical or dimensional (e.g. dimensions of positive, negative or general symptoms of schizophrenia) structure rather than as discrete categories [54]. We suggest that an extended model of Mills and Petronis [55] (**Figure 1**) best represents the components of modern complex disease and mental disorders, in particular.

Figure 1. ***Model of disease/phenotype.***
Adapted from [55]. Biomarker research and systems biology focus at molecular changes that determine the phenotype in response to the interplay of environmental factors, DNA polymorphisms and epigenetic factors. The etiology of disease, including that of mental disorders, can be found either in our hardware, i.e. DNA and highly heritable epigenetic imprints, or our software, i.e. the interplay between '-omes' and environmental and epigenetic factors, or both.



This section focuses on environmental risk factors, genetic susceptibility genes and epigenetic regulation in mental disorders to find the many, presumably overlapping, causes of mental disorders. **Table 1** shows a summary of the different risk factors putatively involved in the etiology of schizophrenia, and **Table 2** summarizes the risk factors implicated in the etiology of PDD.

Table 1. *Risk factors for schizophrenia.*

Psychological, Social and Cultural factors	*References*
Migration: migrants vs. native born (RR 2.9), 1st generation migrants (RR 2.7), 2nd generation (RR 4.5), dark-skinned migrants (RR 4.8)	[17;57]
Urbanicity: urban birth and urban childhood	[16;64]
Socioeconomic stress: socioeconomic deprivation at birth, social defeat, discrimination, acculturative stress	[35;64;74]
Childhood trauma: stressful events alter HPA axis and CRF system	[69;71]
Early life events	
Advanced paternal age: >35 years	[79]
Prenatal and perinatal risk factors (RR ±2.0): fetal growth retardation, fetal perinatal hypoxia, perinatal infections, perinatal stress, Rhesus incompatibility, and pre-pregnancy high and late-pregnancy low maternal BMI	[90;93;97;98]
Season of birth	[84;85]
Folate deficiency: periconception folate status, high 3rd trimester maternal homocysteine (Hcy) levels	[63;101;102]
Vitamin D deficiency	[61;62]
Genetics (RR 1.5-2.0)	
NRG1, DTNBP1, DAOA (G72), Tbx1, COMT, PRODH2, NPAS3, GRK4, DISC1,	[142-144]
Epigenetics	
RELN, GAD_{67}, MB-COMT	[153;155]

4.1. Psychological, Social and Cultural Risk Factors

4.1.1. Schizophrenia

Several factors such as migration, urbanicity and environmental stress have been associated with increased risk for schizophrenia [35] and other mental disorders. These risk factors seem to be strongly intertwined, because the many suggested causatives show considerable overlap and mutual modes of action. Unification of risk factors for different mental disorders may prove valuable in establishing common causes of closely-related mental disorders.

Table 2. *Risk factors for pervasive developmental disorders.*

Psychological, Social and Cultural factors	*References*
Migration: being born to mothers born outside Europe (adj. RR 1.4)	[76]
Urbanicity: high degree of urbanization of birthplace (adj. RR >1.6)	[76]
Early life events	
Advanced paternal and maternal age	[77]
Prenatal and perinatal complications (RR 1.5-2.5): low birth weight, short duration of gestation, obstetric complications associated with intrapartum hypoxia, Rhesus incompatibility	[77;94]
Prenatal and perinatal exposure to toxic substances: environmental pollutants, alcohol, tobacco, substances of abuse, medication	[125;126;128]
Genetics	
TSC1, TSC2, NF1, MECP2, SHANK3, SLC6A4, NBEA, PRKCB-I and -II, EN2, MET, *de novo* CNV	[146-149; 167;168]
Epigenetics	
BDNF, DLX5, UBE3A, GABR, RELN, FMR1	[146;152;153]

Migration does not seem to be generally associated with an increased risk for mood disorders [56] but this is not the case for schizophrenia. There is an increased risk of schizophrenia in migrants, notably 2nd generation (relative risk; RR 4.5) and dark-skinned migrants (RR 4.8), which is attributed to psychosocial factors including discrimination, social defeat and acculturative stress (i.e. stress caused by the psychological and social counter-part of cultural diffusion and admixture) as well as to biological factors such as folate and vitamin D deficiency [57]. The idea that experiences of psychosocial adversity by, possibly also genetically susceptible, individuals belonging to an ethnic minority increase their risk of developing schizophrenia fits findings of increased risk in white migrants [58] and non-black minority groups [59]. Furthermore, the high prevalence of PTSD (10%), major depression (5%) and general anxiety (4%) in refugees [60] emphasize the role of psychosocial and cultural factors. However, the vitamin D hypothesis of schizophrenia [61] is also attractive, because it explains many epidemiological issues of schizophrenia [62], for example, why 1st generation dark-skinned migrants and their offspring (2nd generation) are at increased risk. Next to vitamin D, qualitative and quantitative changes in the diet of carbon-1 (C_1) substrates (e.g. folic acid) in combination with polymorphisms in genes related to C_1-metabolism (e.g. the methylene tetrahydrofolate reductase (MTHFR) gene) are suspect of aberrant epigenetic control of DNA transcription in pregnancy resulting in increased

susceptibility for mental disorders [63]. Overall, one can conclude that although factors of psychosocial and cultural nature are important in the etiology of mental disorders, it seems that these risk factors are less easy targets for intervention and prevention than factors of dietary nature. E.g. nutritional fortification is more easily instituted than accomplishing a change in behavior in those that discriminate.

Urban birth or urban childhood compared to rural locations is suggested to be associated with an increased risk for schizophrenia. It explains around 30% of all schizophrenia incidence, thus being a major environmental risk factor [64]. Exposures to infectious agents, low prenatal vitamin D and folate levels [62], toxins associated with pollution and stress (social isolation) have been mentioned, although the real underlying cause remains to be elucidated [65]. Cognitive social capital, aspects of the degree of mutual trust, bonding and safety in neighborhoods are suggested to be important during the rearing of children and each of them modulates the risk of schizophrenia [64]. Selective migration to urban areas of individuals with proneness to schizophrenia has been explained by various factors related to poverty, the availability of services and easier access to cheap accommodation [17]. Remarkably, urbanicity as risk factor seems specific for the psychotic symptoms of schizophrenia and bipolar disorder, because affective illness of bipolar disorder was not shown to be associated with urbanicity [66;67]. While Williams et al. [25] found an increased prevalence rate of PDD in urban areas compared to rural/mixed areas; they attributed this finding to different diagnostic practices between locations. In addition, major depression was more prevalent in urban than rural areas if controlled for confounding factors like age, immigration status, race, working status and marital status [68]. Taken together, urbanicity is associated with an increased risk for at least some mental disorders. The biochemical background against which urbanicity acts in conjunction with other genetic and environmental risk factors will be difficult to draw.

Traumatic events, independent of migration and urbanicity, have been implicated in the etiology of schizophrenia by some [69], but not by others [70]. The relation between childhood trauma and depression, anxiety and panic disorders, PTSD, drug abuse and suicide attempts is much less debated [71]. A neurobiological explanation is sought in persistent, long-term effects of stressful events during a critical period of development on respectively the activity and sensitivity of the corticotropin-releasing factor (CRF) system as well as the hypothalamic-pituitary-adrenal (HPA) axis [71]. These systems are important in the mediation of mood and anxiety symptoms. For example, the CRF system shows increased activity in patients with depressive symptoms [72], while depressed abused children compared to controls or to depressed non-abused children showed increased corticotropin (ACTH) excretion post CRF administration [73]. Altogether,

childhood trauma may be one of the many environmental factors lowering the threshold to develop a mental disorder in those individuals that are (epi-) genetically more susceptible.

4.1.2. Pervasive Developmental Disorders

Risk factors of psychosocial and cultural nature for PDD are less easily identified, because the etiological role of these factors may prove less distinct. Kanner emphasized in 1943 the unusually high educational background and professional achievements of parents [45] of children with autism but the association between autism and socioeconomic class could not be confirmed [22]. In contrast to PDD, socioeconomic deprivation at birth at the level of the individual and community is associated with increased risk of schizophrenia [74] and another recent study implied that socioeconomic status partially explains the observed higher risk of schizophrenia in African Americans compared to white Americans [75]. A recent study in Denmark [76], investigating familial risk factors for autism, identified having an affected sibling (adjusted RR 22.3), maternal history of psychiatric disorder (adj. RR 2.0), a high degree of urbanization of birthplace (adj. RR > 1.6) and being born to mothers born outside Europe (adj. RR 1.4). The latter factor, i.e. maternal migration, is explained by missed immunizations in the new country of residence and/or selective migration of people with a genetic vulnerability to autism [77]. We believe that similar factors underlying the association between migration and urbanicity and schizophrenia, also apply to PDD, because the etiology of several psychiatric disorders might not be so different.

Summarizing, the available literature supports an important role of psychosocial and cultural risk factors in the development of mental disorders. It seems that many of the psychosocial and/or environmental factors, such as urban location, can be termed “mental stress factors”, which challenge the human brain/organism more than it was meant to deal with. Susceptible individuals probably are unable to cope, or cope differently, with this stress and are experienced by others as ‘crazy’. Obviously, identification of key risk factors is important, because it allows us to develop selective or universal preventive measures and targeted interventions. However, the complexity and interaction of the underlying causes may thwart a clear definition of these targets.

4.2. Early-life events

There is reasonable evidence to believe that the origin of many disorders, including that of mental disorders, can be found *in utero*. In analogy to the Barker hypothesis [78], which links fetal malnutrition via abnormal metabolic programming to

cardiovascular disease in later life, perinatal factors disrupting brain development *in utero* may confer increased vulnerability to develop a mental disorder. Suboptimal neurodevelopment may only be partially reparable during post-natal life. In this section we will discuss several early-life events that may be deleterious for cognitive and mental functioning in later life.

Advanced parental age has been implicated in many mental disorders. In autism, advanced paternal and maternal age, were consistently shown to be associated with an increased risk of having a child with ASD [77] and similar results were obtained from a meta-analysis of the effects of paternal age for schizophrenia risk [79]. At the time of conception, the fertilized egg has undergone a maximum of 23 replications, whereas for sperm cells this number varies non-linearly with age from 35 to 840 at the age of 15 and 50 years, respectively [80], with each replication carrying a chance of mutation in the germ-line. Interestingly, paternal and also maternal age independently have an effect on offspring IQ-scores, with increasing maternal age affecting fetal neurodevelopment through age-related alterations in the *in-utero* environment [81]. Paternal *de novo* mutations and/or altered genetic imprinting [82] and maternal nucleotide repeat instability [83] have been proposed as mechanisms.

The season of birth effect on mental disorders has been most consistently shown for schizophrenia [84;85]. Winter/early spring birth in the Northern hemisphere and births 3-4 months after rain season in northern Brazil [86] are associated with an increased risk of schizophrenia. However, winter/spring births are also associated with superior outcomes with respect to physical and cognitive development in the general population, suggesting an impact of season of birth on developmental trajectories [87]. Remarkably, the season of birth effect was not observed in neurodevelopmental disorders such as ASD, hyperkinetic disorder, Tourette syndrome and obsessive-compulsive disorder (OCD), although in this study such an effect could not be ruled out [88]. Significant seasonal effects on dietary intake of micro- and macronutrients, including fat, carbohydrate, vitamin C and D, and B-vitamins during pregnancy has been proposed as explanation for the season of birth effect on cardiovascular diseases and mental disorders [89]. In addition, McGrath et al. [87] suggested differential exposure to the complex of interacting downstream consequences of biometeorological variables (temperature, rainfall, UV-radiation etc.), including those that have an impact on health status, energy expenditure, disease exposure, vitamin D status etc. at critical periods of development *in utero*, to be responsible for the counterintuitive result of the association of winter/spring births with both better physical and cognitive outcomes in the general population and with an increased risk for schizophrenia.

Prenatal and perinatal complications are frequently associated with increased risk for mental disorders, including autism (RR 1.5-2.5) [77], schizophrenia (RR ±2.0) [90] and eating disorders [91]. For bipolar disorder, however, no robust evidence for such an association is present [92]. Low birth weight (LBW), short duration of gestation and obstetric complications associated with intrapartum hypoxia were most consistently found to increase the risk of autism [77]. Fetal growth retardation, fetal perinatal hypoxia and other prenatal risk factors such as infections, medication, stress, nutritional deficiency and Rhesus incompatibility, were found to increase the risk of schizophrenia [93], while growth restriction and newborn hypoxia were found to be risk factors for autism [77]. Smoking, the use of contraceptives at time of conception and Rhesus incompatibility during pregnancy were also found to be associated with an increased risk for autism [94]. There seems to be a consistent association with adverse events during pregnancy, at delivery and during the neonatal phase with the development of at least some mental disorders in early or later life. Perhaps these adverse events are early expressions of the presence of a risk factor, like a susceptible (epi-)genotype that predisposes to the development of obstetrical complications in the mother and mental disorders in the offspring.

Likely, low weight at birth (LBW) (<2500 g) and fetal growth restriction can be considered to be indicators of a heterogeneous set of adverse intrauterine effects. However, both LBW as well as fetal growth restriction are associated with preterm birth. Black race, maternal thinness (BMI<20), a history of a prior preterm birth, a short cervical length and a positive test result for cervical or vaginal fluid fetal fibronectin were identified as strong predictors of preterm birth in the U.S. [95]. Also, intrauterine bacterial infections are thought to be responsible for 85% of spontaneous preterm births [96], especially malaria and HIV in the developing countries. Interestingly, high maternal BMI seems to protect against two risk factors for growth restriction which are maternal smoking, and maternal stress, but interventions directed at increasing birth weight seemed only effective in thin women [96]. Nevertheless, both late-pregnancy low [97] and pre-pregnancy high maternal BMI [98] have been linked to increased risk for schizophrenia in the offspring. Low maternal BMI may mediate its effects by similar mechanisms that are implicated in a two-fold higher risk of having offspring with schizophrenia in women that experience starvation during early pregnancy [99;100]. Low periconception folate status [101] and, conceivably associated, high 3rd trimester maternal homocysteine (Hcy) levels [102] have been implicated as key nutritional mediators of the adverse effects of famine during early pregnancy. The many possible effects of folate and homocysteine on schizophrenia are discussed in detail in [63]. However, elevated 3rd trimester Hcy levels have been suggested to cause

subtle damage to placental vasculature thus compromising oxygen delivery to the fetus [102]. It is likely that besides many factors that adversely affect the fetus' growth, psychosocial factors are important for risk-increasing obstetric events themselves. The quest for a single risk factor that accounts for the effects of LBW and growth restriction on the development of mental disorders should thus be discouraged.

Prolonged or acute oxygen deprivation to the fetus may be a major risk factor for neuropsychological and neuropsychiatric disturbances. Cerebral hypoxia-ischemia harms fetal brain development considerably, but timing and severity determine the outcome in terms of the severity of the damage and the regions of the brain affected [103]. Energy depletion and subsequent generation of reactive-oxygen species are primarily responsible for hypoxia-associated neuronal cell death [103]. The origins of fetal hypoxia are heterogeneous and may include, in addition to overt fetal distress, maternal hypertension (e.g. caused by pre-eclampsia), gestational diabetes mellitus (DM), hemolytic diseases (e.g. Rhesus incompatibility), cord encircling of the neck and prolonged labor [77;104]. The suggestion that genetic vulnerability for schizophrenia [105], autism and other mental disorders increases obstetric sub optimality, is intriguing and warrants further research [77;106]. This relation between genotype and obstetrics would mean that obstetric events are early indicators of susceptibility genes present in individuals at risk of developing a mental disorder.

Several reports have suggested that prenatal exposure to bacterial and viral infections contributes to the etiology of schizophrenia (reviewed in [107]), autism [108;109] and mental retardation [110]. Prenatal infections with polio, rubella, influenza and toxoplasmosis have been associated with schizophrenia. In contrast to the 5-10% increase in risk of schizophrenia through second trimester exposure to the poliovirus [111], prenatal exposure to rubella, a well-known central nervous system teratogen, is associated with a 10-20 fold increase in risk of schizophrenia [112] and with an increased risk of autism [108]. First trimester exposure to influenza confers a 7-fold risk, while early to mid gestation exposure was associated with a 3-fold risk of developing schizophrenia [113]. A 2.5-fold increase of risk for schizophrenia was noted to be associated with elevated maternal IgG antibodies against *Toxoplasma gondii*, a ubiquitous intracellular parasite [114]. Interestingly, a recent report by Cetinkaya et al. [115] found that two third of patients with schizophrenia had elevated serum levels of anti *Toxoplasma gondii* antibodies compared to 20-25% of depressed patients and controls. Second trimester increases of maternal cytokine levels, including interleukin 8 (IL-8), are also significantly associated with pregnancies giving rise to schizophrenia cases [116]. It is important to note that the results from studies of prenatal infection and schizophrenia might

have public health implications, because there are many possible preventive strategies for bacterial and viral infections.

For autism, exposure of the fetus to the cytomegalovirus (CMV) in the 3rd trimester has been implicated in the etiology of some children with autistic-like behavior [109]. Other congenital infections have been implicated in autism, but evidence is less convincing for measles, mumps, varicella and intrauterine human parvovirus [117]. Congenital rubella [118] and herpes simplex [119] seem more consistently associated with autism, mental retardation and behavioral pathology. Prenatal infection with CMV [110] or *Toxoplasma gondii* [120] is also suggested to increase the risk of mental retardation. Altogether, prenatal bacterial or viral infections seem to disrupt normal neurodevelopment with neuropsychiatric consequences in later life, suggesting that the downstream consequence (e.g. cytokine release) of the inflammatory process rather than the inflammatory agent itself (e.g. lipopolysaccharides [121]) is responsible for abnormal neurodevelopment.

A possible explanation for the mechanism by which maternal infection affects embryogenesis is through hypoxia, hyperthermia, malnutrition, and the effect of elevated levels of circulating cytokines on gene expression in the brain. Fatemi et al. [122] showed altered regulation of a subset of genes in brains of mouse offspring exposed to prenatal infection. This potentially leads to permanent changes in brain structure and function. Cytokines, probably of maternal, placental and fetal origin, might suppress expression of genes resulting in subsequent reduced protein availability [123]. Reelin [124], for example, is an important glycoprotein involved in guidance of neuronal and glial cells during embryogenesis, and reduced expression of the reelin-gene through mutation or promoter hypermethylation has been shown to result in cognitive deficits that are similar to those that are often observed in autism and schizophrenia. It is hypothesized that disrupted neurodevelopment, rather than neurodegeneration, is associated with prenatal infection and central to the etiopathogenesis and disease process of schizophrenia and autism [125-127].

Environmental exposure to toxic substances in fetal life such as alcohol, substances of abuse, heavy-metals (lead, methylmercury, arsenic), dry-cleaning agents (tetrachloroethylene), toluene, PCBs, certain classes of medication, and many other industrial chemicals are implicated in the etiology of, mainly, neurodevelopmental disorders [128]. Exposure of the embryo to ethanol is associated with increased risk of the Fetal Alcohol Syndrome (FAS) and its less severe variants known as Fetal Alcohol Effects (FAE). Although congenital anomalies that are usually observed in children with FAS are often not seen in children with autism, considerable overlap in behavioral pathology suggests a

common factor. Therefore, the complex cognitive, behavioral, and physical symptomatology in children with FAS [129] may frequently be misdiagnosed as PDD, for psychological and political reasons that will not be discussed in this review. Prenatal cocaine exposure (PCE) has, however, not been linked with congenital anomalies or medical complications, but it has been found to be an independent risk factor for behavioral problems at school age [130]. Remarkable, this study [130] showed that the combined effects of prenatal and postnatal exposure to tobacco and alcohol on childhood behavior were found to be greater than that of PCE. Use of thalidomide, misoprostol and valproic acid during the 1st trimester, notably during the first 8 weeks of embryogenesis, is associated with autism [126;131], while the use of analgesics during the 2nd trimester [132] and of diuretics for treatment of maternal hypertension in the 3rd trimester [133] are thought to play a role in the etiology schizophrenia. The neurodevelopmental and neurotoxic effects of some industrial chemicals are diverse and a universal mode of action will be hard to find. Disturbance of thyroid function and subsequent harm to the embryo and fetus has been proposed [134]. The different effects, ranging from minor anomalies to malformations, resulting from short-term (1-3 weeks) exposure to the aforementioned exogenous substances during the first trimester, suggest that each of these agents strikes during a critical phase ("vulnerability window") during the embryogenic period, with physical and neuropsychiatric consequences at birth and thereafter. Unfortunately, such insults are often permanent, because there is little potential for later repair. It is imaginable that presence of a susceptible genotype, i.e. a maternal and/or fetal genotype less able to detoxify, can moderate the effects of environmental toxicity. Studies (in animals) into the teratogenic effects of toxic agents may thus benefit from complementary biomarker-related research.

Other life events that are implicated in the etiology of mental disorders are brain trauma and substance abuse. For autism, e.g., a case-series study of patients with PDD in Tanzania with severe malaria and subsequent recovery during the first years of life showed an immediate onset of autistic-like behavior in some patients, which had entirely normal development before their malaria-infection [135]. Tourette syndrome and tics have also been associated with insults to the basal ganglia, such as head trauma, encephalitis and other causes [136]. While substance abuse, e.g. amphetamine (dopamine D_2-receptor), ketamine (N-methyl-D-aspartic acid [NMDA] receptor), phencyclidine (PCP), LSD, heroin, cocaine, can merge into psychosis-like symptoms, the diagnosis of schizophrenia is generally not established in this group of patients. However, substance abusers tend to be overrepresented among patients with mental disorders, advancing a common vulnerability [137].

It is also rewarding to investigate potential links between somatic diseases and psychiatric symptoms to obtain better insight into cause-symptom relations. For example, adults with celiac disease have a high prevalence of depressive symptoms [138]. It seems that these patients are at increased risk for non-affective psychosis [139] and are more likely to have ADHD-like symptomatology [140]. Primary hyperparathyroidism (PHPT), characterized by thirst, fractures, osteoporosis, urolithiasis, is also associated with psychosomatic symptoms that are usually encountered in obsessive-compulsive disorders, depression, anxiety, and paranoia [141]. The occurrence of psychiatric symptoms in somatic disease, and parallels between mental disorders and somatic disorders, may provide new insights into disease-mechanisms and yield valuable ideas regarding new therapies.

In summary, early life events ranging from those that affect the quality of germ-line cells to those that disrupt neurological and cognitive function later in life, and all adverse events in between, have been implicated in the etiology of mental disorders. On one hand this large number of identified risk factors emphasizes the speed of science, but the fact that only few risk factors have been linked to a proven mechanism shows that there is still a large gap in our knowledge on the other hand. Integrative approaches, studies using extensively-characterized population cohorts, longitudinally acquired bio-repositories and life-long follow-up, performed on different continents are only one part of the answer. Intervention/prevention-based medicine might be the other part. Of course, this research can be supported and complemented by biomarker research, elucidating disease-mechanisms, prognostic and diagnostic phenomena and supporting tailored interventions.

4.3. Genetics and epigenetics of mental disorders

There has been a great focus on the genetic and, recently, epigenetic basis of mental disorders. Evidence for a genetic contribution in the etiology of schizophrenia [142-144], bipolar disorder and major depression [143;145], and autism [146-149] has been comprehensively reviewed. The concept of a strongly genetic contribution in the etiology of mental disorders is fuelled by a body of studies in twins and affected families. A major problem with genetic research is the assumption that mental disorders have a predominantly genetic basis, which underestimates the contribution of environmental factors. The assumption appears incorrect because of large effect-sizes of some environmental factors, e.g. 10-20 fold higher risk of schizophrenia associated with prenatal rubella exposure. In addition, the value of conclusions from twin-studies is questioned because these studies of MZ or DZ twin reared-together or reared-apart, may not measure anything more than environmental influences, error, and bias [150]. Interestingly, during their lifetime

twins increasingly differ in their epigenotype, resulting in different phenotypes and different susceptibility for diseases like mental disorders [151]. The role of epigenetic mechanisms in psychiatric disorders is also increasingly recognized [146;152-155].

To understand the mechanisms by which predisposing genes interact with the environment to result in the precipitation of a mental disorder, we will review literature regarding the role of genetics and epigenetics in the etiology of mental disorders. This section, however, will not exhaustively discuss all chromosomes, loci, alleles, and genes etc. that have ever been implied in schizophrenia, bipolar disorder, major depression and autism. Instead, some current achievements and failures as well as difficulties and new approaches in genetic analysis of psychiatric disorders will be reviewed. In addition, their value in diagnostics and treatment will be discussed.

4.3.1. Genetics of mental disorders

It seems a paradox that susceptibility alleles that confer an increased risk for developing a mental disorder are still among us, notably when mental disorders are considered as common, harmful and heritable. Some believe this to be the result of a polygenic mutation-selection balance [156], i.e. that our complex genotype, which is required for our underlying human behavioral phenotype is inevitably prone to suffer to some extent from mutations. This idea is supported by the notion that mental disorders do not inherit by Mendel's law. The majority of the scientific community is in favor of a multi-loci (polygenic) mode of inheritance of mental disorders. Other explanations for the presence of susceptibility genes throughout the millions of years of evolution are based on ancestral neutrality (mismatch between ancestral and current environments) and on balance-selection (adaptive benefits). The different models are commented by Keller and Miller [156], and it might be important to view the genetic etiology of schizophrenia and autism and other mental disorders against this background.

Two approaches are being adopted in the search for mental disorder genes: linkage studies and association studies. Linkage studies aim to select candidate gene loci by genome-wide or local gene mapping in families and by defining the tendency for alleles closely spaced on the same chromosome and coinciding with the phenotype of interest to be transmitted from one generation to another as an intact unit through meiosis. The next step is generally to examine candidate genes by means of association studies. Most molecular genetic studies on mental disorders follow the common disease common variant (CDCV) model, which implies the study of relatively common polymorphism that confer susceptibility to common

diseases like heart disease. However, by doing so rare causative variants may be overlooked.

Unfortunately, unclear diagnostic boundaries, multiloci-type inheritance, epistasis (gene-gene interactions), clinical and etiologic heterogeneity and confounding gene-environment and environment-gene interactions have impeded linkage and association studies considerably leading to many inconclusive and non-replicable findings [142]. More stringent criteria for publication of results from association studies are under way [157] to avoid future statements like "to date, no causative gene or genetic risk factor has been identified for bipolar disorder or depression" [145] and "no gene has been proven to not be an autism disease gene" [158].

While the genetic causes of mental disorders have not been uncovered yet, parallels should also be sought in the ways the etiology and pathogenesis of neurodegenerative disorders, such as Alzheimer's disease, Parkinson's disease and Huntington's disease are investigated. It is important (i) to understand rare familial variants, (ii) to identify (interacting) causative genes to define pathogenic pathways and therapeutic targets, (iii) to investigate commonalities among different disorders, (iv) to identify mutations that increase the risk of developing a disease but that themselves are not causally related, and (v) to consider the neurotoxicity of RNAs and proteins (e.g. in Huntington's disease [159]) deriving from genetic changes such as point or frameshift mutations or deletions [144]. For schizophrenia, which is increasingly viewed as a subtle disorder of neurodevelopment, clues to the etiology and pathogenesis may be provided by severe disorders of cortical development like lissencephalies [144]. For example, an established cause of lissencephaly is mutation of the RELN-gene, which codes for reelin. Reelin has a key role in cortical neuronal migration [124] and reduced expression of RELN in post-mortem brains of individuals with schizophrenia and bipolar disorder has been reported [160]. Moreover, neuropathological investigations of schizophrenia point more to a neurodevelopmental abnormality than a neurodegenerative disease [161]. Most genes that have been implicated in mental disorders have a key role in neurodevelopment and/or neurotransmission.

4.3.1.1. Genetics of schizophrenia [142-144]

The 10-times higher LMR in relatives of patients with schizophrenia [162], the higher concordance rates in monozygotic (MZ, ~50%) than dizygotic (DZ, ~17%) twins [163] have led to an estimate of the heritability of tendency to develop schizophrenia of about 80%. Gene-environment interactions certainly contribute to the overall risk, emphasizing the idea that schizophrenia may be genetically

mediated but not determined [142]. Interestingly, the relative risks for schizophrenia at the loci identified so far are rather low (range 1.5-2.0) indicating modest effect sizes [144].

Regions 1q, 5q, 6p, 6q, 8p, 10p, 13q, 15q and 22q have been confirmed in more than one linkage study, and association replication has been observed for neuregulin 1 (NRG1) and dystrobrevin binding protein I (dysbindin; DTNBP1). Other genes that have, though not consistently, been associated with schizophrenia are D-amino acid oxidase [DAO] activator (DAOA; formerly called G72) and genes located on chromosome 22, catechol-O-methyl transferase (COMT) and proline dehydrogenase 2 (PRODH2).

Neuregulin 1 was identified by fine mapping of chromosome 8p and is believed to code for multiple protein products that are implicated in neuronal differentiation and migration as well as in expression and activation of neurotransmitter receptors like the NMDA-receptor. Polymorphisms leading to alternative splice variants of NRG1 may lead to proteins with enhanced or reduced function. Dysbindin was shown to be associated with schizophrenia through chromosome 6p linkage [142]. Its pre- and postsynaptic presence, its suggested role in glutaminergic neurotransmission, and its reduced expression at both RNA and protein levels in certain brain regions of schizophrenia patients have fostered the search for dysbindin polymorphisms. Dysbindin seems to interact with serine-threonine kinase 1 (AKT1), a protein that is involved in growth-factor induced neuronal survival. The AKT1 gene has recently been shown to be associated with schizophrenia [164]. D-amino acid oxidase is activated by DAOA which oxidizes D-serine, a coagonist at the "glycine site" of the NMDA receptor. The potential therapeutic efficacy of D-serine and reports of reduced blood and cerebrospinal fluid (CSF) D-serine levels in individuals with schizophrenia warrant further investigation of the DAOA-system. Most replication studies have confirmed a role of DAOA in schizophrenia.

Twenty to 30% of patients with the chromosomal microdeletion syndrome (velo-cardio-facial syndrome; a deletion in chromosome 22q11 that encompasses appr. 27 genes) have schizophrenia or another major psychiatric disorder with psychosis-like symptoms. Among the deleted genes is T-box 1 (Tbx1; transcription factor involved in regulation of otocyst development), which is expressed in the microvasculature of the brain. The COMT gene is also coded on chromosome 22q11 and has received more attention, because the enzyme participates in the clearance of catecholamines, including dopamine, from the synapses. Replacement of methionine by valine at codon 108 (soluble form; S-COMT) or codon 158 (membrane-bound form; MB-COMT) has been suggested to decrease COMT-activity, thus resulting in prolonged elevated levels of dopamine in critical central

synaptic clefts. However, reduced COMT-activity appears to be more strongly associated with cognitive function than with schizophrenia. Altered PRODH2 activity influencing the availability of glutamate, has also been linked to deletions in the 22q11 chromosome region. The only two reports considering chromosomal translocations in individuals with schizophrenia deal with neuronal PAS domain protein 3 (NPAS3) and G-protein receptor kinase 4 (GRK4), which code for a transcriptional regulatory factor and glutamate kainate receptor, respectively.

DISC1 (disrupted in schizophrenia 1), is emerging as the best supported candidate gene for schizophrenia. It has been identified via a balanced (1:11) chromosomal translocation, segregating with schizophrenia, bipolar disorder and other mental illnesses. Affected individuals have either schizophrenia or an affective disorder. Also, endophenotypical reduced P300 amplitude and latency linked to schizophrenia, was associated with translocation in both unaffected as well as affected carriers. On a structural/functional level, DISC1 haplotypes are associated with alterations in hippocampal function, fMRI signals, working memory and cognition. Although it is presently unclear whether haploinsufficiency or dominant-negative interactions mediate DISC1 loss-of-function, elucidation of normal DISC1 function is critical for understanding DISC1-related diseases. DISC1 is distributed throughout neurons, nuclei, mitochondria and neurites and its function is linked to neural development via neuronal migration, neurite outgrowth and neuronal maturation, and to neural functioning via modulation of cytoskeleton function, synaptic transmission and plasticity. DISC1 protein interacts with many other proteins to form complexes that are vital for proper neurodevelopment and neural functioning.

The interactivity of DISC1 is suggestive of the idea that multiple subtle dysfunctional genes and/or gene-products can lead to endophenotypes associated with several mental disorders such as schizophrenia, bipolar disorder and depression. Also, the interactivity-requirement supports the notion that the functionality of a protein complex can be adversely affected if a single protein constituent is reduced in quality or quantity. For example, DISC1 interacts with phosphodiesterase 4B to regulate neuronal cAMP cell signaling. Thus, if such a protein-complex is involved in critical processes of brain development, brain functioning and behavior, it can have differential down-stream consequences. Other processes, of which the functionality is subject to genetic variation, may increase or repair the damage, thus adding to the importance of epistasis in mental disorders. Taken together, genes associated with schizophrenia probably have different etiologic and pathogenic effects, each affecting particular neurobiological processes to different extents, in turn causing specific phenotypes.

4.3.1.2. Genetics of bipolar disorder and unipolar depression [143;145]

Familial aggregation is higher for bipolar disorder than for unipolar depression as shown by higher rates of sibling recurrence risk (5-10% vs. 2.5-3.5%) and MZ-twin concordance (45-70% vs. 40-50%). The heritability estimate for bipolar disorder (80-90%) is higher than for unipolar depression (33-42%), supporting a stronger environmental contribution in unipolar depression [143]. The genetic contribution to the etiology of bipolar disorder resembles that of schizophrenia, suggesting a common genetic background. Results from family and twin linkage studies show convergent or overlapping clinical features and susceptibility genes (DAOA, DISC1, NRG1 and COMT) for schizophrenia, schizoaffective disorder and bipolar disorder [165].

Linkage studies in bipolar disorder resulted in suspected loci at 2p, 4p, 4q, 6q, 8q, 9p, 10q, 12q, 11p, 13q, 14q, 16p, 16q, 18q, 21q, 22q and Xq [145]. Unfortunately, results from these linkage studies and subsequent association studies of specific gene loci have not provided unambiguously identified susceptibility genes for bipolar disorder or depression. Functional genetic studies in bipolar disorder have focused on neurotransmission cascades that are also implicated in schizophrenia: the monoamine pathway (dopamine, serotonin [5-HT] and noradrenaline), intracellular signaling systems, the GABA (γ-aminobutyric acid)ergic system, proto-cadherin and genes encoding for other targets of mood-stabilizers such as lithium. Although effect-sizes were modest (RR < 2), functional polymorphisms for monoamine-oxidase-A (MAO-A), COMT and the 5-HT transporter (5-HTT) genes have been implicated. However, these positive results still await replication in independent samples of sufficient size.

For DAOA (13q34), which is also implicated in schizophrenia, no pathologically relevant variant has been identified despite independent confirmation of its variation influencing susceptibility to bipolar disorder. In view of these findings the DAO containing region 12q23, which is implicated in both bipolar disorder as well as unipolar disorder, warrants more thorough study. Another interesting candidate gene is brain-derived neutrophic factor (BDNF). BDNF (putatively located on 11p13), a neurotrophin, has a role in promoting and modifying growth, development and survival of neuronal populations and, in the mature nervous system, it is involved in activity-dependent neuronal plasticity. The common functional Val66Met polymorphism of BDNF is thought to be associated with susceptibility to a specific aspect of the clinical bipolar phenotype, rather than influencing susceptibility to bipolar disorder as a whole. G-protein receptor kinase 3 (GRK3) and XBP1 (encodes a transcription factor that regulates MHC class II genes) (both 22q) have also been associated with bipolar disorder through

circumstantial evidence coming from a rodent model of mania and an effect of the functional polymorphism on mood stabilizer action, respectively. Many other genes have been implicated in bipolar disorder and of these, association of DAOA is most robust [145]. Some studies have increased the meaningfulness of the association by being able to relate gene polymorphisms to persecutory delusion or psychosis, but these findings could not be replicated.

Compared to bipolar disorder, unipolar depression is more heterogeneous and there is evidence for a large environmental component in its etiology. Large sample sizes and correction for confounding environmental factors are thus required. It is not surprising that few consistent positive findings of genetic studies concerning depression have been published. The 12q22-23 region is the only region that has been linked to unipolar depression, and also to anxiety traits. Gender-specificity in linkage signals has been observed, but awaits replication. Functional polymorphisms of 5-HTT, tryptophan hydroxylase 2 (TPH2) and BDNF have often been studied in combination with environmental factors to explain the onset of depression. Most interesting is the interaction between polymorph 5-HTT and occurrence of life events in early childhood. A rare loss-of-function mutation in TPH2, a brain-specific enzyme involved in 5-HT synthesis, was found in approximately 10% of patients with major depression, but this finding was not replicated in four subsequent studies. Despite initial positive findings, many subsequent studies have not been able to associate 5-HTT, BDNF and COMT with depression, possibly due to poor study design or false-positive initial findings.

More robust strategies identifying genetic risk factors or causative genes for mood disorders are required. In addition, researchers may consider experimenting with psychiatric nosologies other than DSM-IV and ICD-10, because the Kraepelin dichotomy that distinguishes schizophrenia and bipolar affective disorder as distinct entities with separate underlying disease processes and treatments seems outdated [165]. Further, the idea of convergent or overlapping susceptibility genes leading to a spectrum of disorders ranging from bipolar disorder to schizophrenia supports the view of Keller and Miller of a polygenetic mutation-selection model for mental disorders [156].

4.3.1.3. Genetics of pervasive developmental disorders [146-149]

Concordance of the narrow phenotype of ASD in monozygotic twins is 82-92%, while this is only 1-10% for dizygotic twins. Sibling recurrence risk is estimated at 2-3% and heritability is estimated to be >90%. Social and non-social autistic traits seem highly, but independently, genetically determined in ASD. Sub-threshold autistic traits are also more frequently present in siblings and parents of individuals with ASD. Together, these findings have incited researchers to believe that autism

is one of the most genetically determined neuropsychiatric disorders. However, the finding of concordance rates below 100% points to a weak but definitely significant influence of environmental factors on the ASD phenotype and the value of results coming from twin-studies should be reconsidered [150]. Gene-environment interactions, the high degree of genetic heterogeneity, the polygenic or oligenic mode of inheritance, and significant epistasis have complicated the search for autism-genes significantly.

Cytogenetic, linkage, candidate gene association, and recently also *de novo* copy number variation (CNV) studies are used to identify ASD susceptibility genes. The former kind has yielded valuable information through the study of 'syndromic' autism. About 10-15% of autism is syndromic, which means that the autism is secondary to a genetic disorder such as the chromosomal rearrangement syndromes Angelman and Prader-Willi (PWS), fragile X syndrome, tuberous sclerosis and neurofibromatosis, or the result of exposure to teratogenic agents. For the majority of autism cases (85-90%) the genetic origin is unknown. However, the cases in which autism is syndromic are especially relevant with regard to prevention of ASD by genetic counseling. Moreover, the syndromic cases present an opportunity for pinpointing the underlying genetic abnormalities and for investigating parallels with non-syndromic cases.

The rate of cytogenetic abnormalities in autistic disorder is estimated to be 3-5%. Duplication or inversion of the chromosomal 15q11-13 region, e.g., has a prevalence rate of about 1% in ASD and this region is also related to other developmental and behavioral syndromes. Deletions of the maternal or paternal chromosome 15q11-13 region are associated with the Angelman syndrome and PWS, respectively. Several genetic mechanisms have been implicated in Angelman, such as interstitial deletion of a maternal chromosome (70-75%), mutation of the ubiquitin-protein ligase E3A (UBE3A) gene or mutation in the imprinting center (20%), abnormal methylation (3-5%), and paternal uniparental disomy (UPD) in combination with the lack of a maternal copy (2-3%). Angelman syndrome has many characteristics in common with ASD, including moderate to severe mental retardation, absence of language development and motor stereotypies. For PWS similar mechanisms have been proposed such as interstitial deletion of the maternal chromosome (70-80%), maternal UPD in combination with the lack of a paternal copy (20-30%), and mutation in the imprinting center (1-2%). PWS subjects with UPD tend to have autistic-like impairment in social interaction.

The other syndromic cases are associated with single-gene mutations of the following genes: fragile-X mental retardation 1 (FMR1), tuberous sclerosis 1 (TSC1), tuberous sclerosis 2 (TSC2), neurofibromatosis 1 (NF1) and MECP2. More rare but treatable syndromes associated with ASD are phenylketonuria (PKU) and the

Smith-Lemli-Opitz syndrome. PKU as a cause of ASD has become rare in countries with an established neonatal screening program. Individuals with the Smith-Lemli-Opitz syndrome, which is associated with increased serum 7-dehydrocholesterol and syndactyly (webbing of fingers or toes) of toes 2 and 3, can be treated with a high-cholesterol diet. The X-linked genes MECP2 and FMR1 are involved in autism secondary to the Rett and the fragile X syndrome, respectively. MECP2 is a methylated-DNA binding protein that regulates gene expression through chromatin remodeling. MECP2-activity is thought to be important for synapse maintenance and remodeling because of the regressive nature of Rett. FMR1-gene silencing by promoter hypermethylation and subsequent reduced translation of FMR protein, which is involved in mRNA transport, results in fragile X syndrome (2-5% of individuals with ASD), a common cause of mild to moderate mental retardation in boys. The FMR protein function is modulated by GTPase activity which is crucial for control of cytoskeletal dynamics. Next to GTPase activating proteins, guanosine exchange factors are involved in ASD and mental retardation.

Mutations in tumor-suppressor genes (TSC1 and TSC2) related to tuberous sclerosis may be responsible for the localization of tubers to the temporal cortex generally observed in ASD. Tuberous sclerosis is an autosomal-dominant neurocutaneous disorder, which is 100-times more prevalent in ASD. Neurofibromatosis type 1 is a disease in which the growth properties of neural-crest derived cells are affected. It is caused by mutation in NF1 (encodes neurofibromin which is a tumor suppressor protein) and is associated with features such as toe syndactyly, cutaneous malformations and mental retardation. Despite fine-mapping of the X-chromosome, which is driven by the marked sex-difference in ASD, most linkage study outcomes are negative. However, linkage studies have shown 7q, 16p and 17q to be linked to ASD in male only pairs. Studies on maternally and paternally imprinted loci are, to date, inconclusive.

Unfortunately, chromosomal abnormality studies have not led to the identification of a common autism risk allele yet. Combining results from studies to rare non-syndromic chromosomal abnormalities in families of ASD subjects with those from candidate genes that reside in suspected chromosomal regions, have indicated a role for UBE3A and GABA-A receptor (GABR) β3, both mapped to 15q11-13, and the neuroligin (NLGN) gene family. The five NLGN-genes are X-linked and encode cell adhesion molecules localized at glutaminergic synapses or GABAergic synapses. They are thought to play a crucial role in synapse formation and their association with scaffolding proteins seemingly regulates the glutamate-GABA balance, possibly explaining the high prevalence of epilepsy in ASD. The above findings exemplify that identification of rare variants may have significant

value and investigations of phenomena of phenotypic overlap is of fundamental importance.

At the synapse, appropriate connectivity between cytoskeleton and membrane proteins is mediated by scaffolding proteins, such as encoded by the SH3 and multiple ankyrin repeat domains (SHANK3) gene, which are crucial for dendritic morphology. SHANK3 is also a binding partner for neuroligins, which is suggestive for a role of SHANK3 in the NLGN-pathway of autism. This pathway connects actin cytoskeleton to the postsynaptic scaffold at glutaminergic synapses. Variants of genes encoding neurotransmitter receptors and transporters might also confer susceptibility to or modulate ASD-associated behavior.

The most studied gene is the SLC6A4-gene, which encodes 5-HTT. However, SLC6A4 gene variants seem to have small effects on blood 5-HT levels in ASD. Functional polymorphism of 5-HTT have also been implicated in stereotyped behavior. The second most genotyped neurotransmission-genes are those of the GABA-receptor cluster. Significant epistasis of GABR α4 – GABR β1 has been reported. This is especially interesting, because of the involvement of the GABA-ergic systems in seizures in ASD. Glutaminergic receptor genes are also likely to be relevant in ASD pathogenesis, because their products are, next to neurotransmission processes, also involved in synapse maintenance and plasticity, and they play a pivotal role as neurohypophyseal hormone receptors in animal models of social interaction.

Second-messenger proteins such as neurobeachin (NBEA), an anchoring protein able to recruit protein kinase A, and protein kinase C β (PRKCB) I and II, are implicated in the differentiation of antigen-presenting dendritic cells whose dysfunction could contribute to altered immune responsiveness in ASD. These proteins are also involved in Ca^{2+} signaling, and perturbation of their synthesis could translate into altered synaptogenesis. Remarkably, while there is no putative genetic explanation, reduced programmed cell death leading to increased cell numbers and maintenance of misplaced cells are described consistently in neuropathological studies of ASD.

Secreted molecules, such as the reelin-protein (RELN), have a pivotal role in neuronal migration and prenatal development of neural connections. This has been confirmed in reeler-mice, which lack reelin and display cytoarchitectonic alterations in numerous brain regions. Post-mortem brain analysis of autistic individuals shows impaired reelin signaling and reduced blood reelin levels have been found in individuals with autism and first-degree relatives. RELN-variants that cause decreased reelin expression are suggested to confer vulnerability to ASD. However, the genetic heterogeneity and reported inconsistencies suggest interpretation of RELN-variants within a framework of a region-specific gene-

environment interaction model. For example, a gene-environment interaction is thought to occur between RELN-variants and exposure to organophosphates. The latter inhibit RELN-associated proteolytic activity on matrix proteins, which is essential for neuronal migration. This assumption is further strengthened by the finding that variants in the paraoxonase (PON) gene, which encodes the organophosphate deactivating enzyme paraoxonase, are linked with RELN-variants in ASD. The laminin β1 (LAMB1)-gene, encoding the β1-chain of laminin, is another interesting candidate. Laminin is an important glycoprotein promoting neuronal migration and neurite outgrowth in the developing nervous system. A knock-out mouse model of the engrailed 2 (EN2) gene (7q) lacking the EN2 homeobox transcription factor resulted in a hypoplastic cerebellum and decrease in the number of Purkinje cells. Neuropathology reminiscent to that of the EN2 knock-out mouse was observed in post-mortem brain studies of individuals with ASD. Moreover, reduced expression of EN2-gene products has been associated with PDD. Reduced expression of the MET gene has recently been implicated in autism [166]. MET receptor tyrosine kinase mediated signaling is involved in neocortical and cerebellar growth and maturation, immune function, and gastrointestinal repair, possibly explaining some of the comorbidity observed in ASD.

It can be concluded that individual genes have been implicated by means of their positional (through association or linkage) and/or functional (through their involvement in neurodevelopment and/or neurotransmission) properties. Findings of reduced programmed cell death and/or increased cell proliferation, altered cell migration with disrupted cortical and subcortical cytoarchitectonics, abnormal cell differentiation with reduced neuronal size, and altered synaptogenesis have been proposed to explain the unbalanced local versus long-distance and inhibitory versus excitatory connectivity possibly underlying altered social information processing in autism. The presented evidence implicates three pathways in ASD pathogenesis: (i) cell migration, (ii) glutamate-GABA equilibrium, (iii) synapse formation and maintenance, as well as dendritic morphology [146]. Genes that are implicated in these pathways encode proteins that can be divided according to their involvement in (i) chromatin remodeling (e.g. MECP2) and regulation of transcription (e.g. FMR1), (ii) actin cytoskeleton dynamics (e.g. TSC1, TSC2 and NF1), (iii) synaptic scaffolding (e.g. SHANK3), (iv) neurotransmission (e.g. SLC6A4), (v) second-messenger systems (e.g. NBEA), (vi) apoptosis, (vii) cell adhesion (NLGN), and (viii) paracrine cell-cell communication (e.g. RELN).

Recently, through the development of high-resolution genome analysis techniques to identify *de novo* genomic deletions and duplications of tens to thousands of kilobases (i.e. copy number variation; CNV), the number of cases with traceable

underlying genetic causes of autism has been raised to 10-20% [167;168] and this number may even grow to 30-40%. *De novo* CNV encompasses at least 12% of the human genome and hundreds of genes [169]. Copy number variation can result in the loss of copy, gain of copy and disruption of a dosage-sensitive gene all with effects at the protein level [170]. Copy number variation might contribute to the interindividual genetic variability even more than single-nucleotide polymorphism (SNP). The existence of a higher rate of CNV and also *de novo* CNV in ASD compared to controls suggests that genetic causes of autism have high heritability but mutations are not inherited, which is explained by the *de novo* aspect of CNV [171]. This new understanding explains a part of the puzzling data from twin studies in autism.

The potential existence of less penetrant CNV that has smaller effects but also contribute to autism is interesting. Copy number variation does not seem to increase with age in contrast to point mutations associated with increased paternal age. Beaudet [171] proposes a mixed epigenetic and genetic *de novo* and inherited model for autism (**Figure 2**), in which individual patients have a genetic (mutation) or epigenetic (epimutation) cause of autism and these components could be inherited in some cases and could be acquired *de novo* in others [172].

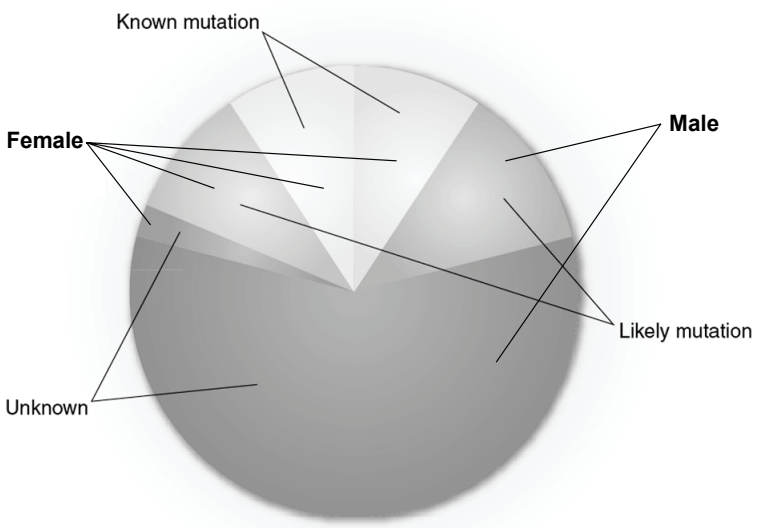


Figure 2.
From [171]. The causes of autism according to genetic contribution and sex.

In summary, results from (epi-)genetic and neuropathological studies in ASD and related behavioral and developmental disorders provide a putative role for genes involved in brain development and all aspects of neurotransmission that are determinants of behavior. These genes should be prioritized for future genetic research. Nevertheless, the impact of the environment should not be neglected as well as the modest effect sizes of individual genes and poor consistency of most implicated genes. Future studies should conform to guidelines for genetic analysis of complex disease [157].

4.3.1.4. Outlook and discussion

Additional research strategies for the identification of mental disorder susceptibility genes have been proposed: (i) use of mouse-models for study of gene-environment interactions, (ii) the study of genetics in a pedigree of a mental disorder accompanied by a specific somatic syndrome, (iii) study of mitochondrial DNA in cases where mental disorders are more frequently observed in maternal relatives of children with mitochondrial diseases or metabolic diseases compared with their paternal relatives, (iv) use of microarrays to study the expression of a large set of genes hypothesized or known to be involved in neurodevelopment, neuroplasticity, neurotransmission, and remodeling or migration of neurons. It is probably also beneficial to focus on single large families to avoid genetic heterogeneity, to take into account geographic origin, or to genotype isolated populations. Another important question is whether to use tissue, e.g. of the brain or other neuronal tissue, or lymphocytes for genetic analysis. The latter is thought to be less representative for gene expression in the brain [145]. However, genotype characteristics are inevitably related to psychiatric phenotypes classified by the DMS-IV and ICD10 criteria, spurring the subject of psychiatric nosology. Phenotype definition is generally difficult in mental disorders, because no sensitive and specific tests are available to distinguish different disease entities.

Another interesting notion is the significant publication bias, which has made the genetic research community to call for a platform for negative results. Interestingly, among the 166 putative associations with bipolar disorder studied at least three times, only six associations were replicated in more than 75% of the studies, which can be explained by small sample-size, population stratification, phenotype definition, genetic heterogeneity, low relative risk, multiple testing, genotyping error, selection bias especially for the control group, and many other factors [173]. Publication bias has likely compromised genetic meta-analyses and published positive findings may prove to be oversimplifications after more detailed analysis. For example, re-analysis of a polymorphic region of the 5-HTT-allele found it to be consisting of at least 14 alleles instead of the previously assumed 2

alleles. This finding is important, because different alleles have functional differences. Guidelines are in preparation to prevent the aforementioned problems and to increase the validity of future reported results.

Evenly important, the unequivocal identification of susceptibility genes caused by the advancement in genotyping raise ethical and psychological concerns regarding the availability of information and services for those under consideration for genetic testing. Age at testing, consent procedures, and post-testing implications for the individual require careful consideration of researchers and policy-makers. However, identification of causative genes provides an opportunity for genetic counseling, and via increased insight in disease mechanisms it may result in the development of effective therapies. For example, careful genetic assessment of children with ASD can be used to determine whether the autism is of syndromic or idiopathic nature. This will help to inform parents with regard to the recurrence risk (e.g. 2-8% for idiopathic autism) of the disorder and it will help them with the psychological coping with the impact of the disorder.

If future research discover genes and their specific pathogenic mutations that can be unambiguously linked to certain mental disorders or associated traits, the feasibility of the following increases: (i) rational drug design, (ii) characterization of genotype-phenotype relations, (iii) identification of environmental risk factors interacting with specific genes, (iv) realistic research into prevention focusing at identifying high-risk individuals [142], and (v) improved psychiatric nosology. It is important that future studies on the 'genes of mental disorder' account for epigenetic regulation of gene expression via interaction with the environment, because gene-mutations alone are not expected to explain the origin of the majority of mental disorders.

4.3.2. Epigenetics

Some evidence for the involvement of epigenetics in several mental disorders has already been presented in the previous section: altered epigenetic control of COMT, RELN, and glutamate decarboxylase (GAD_{67}) in schizophrenia and bipolar disorder, mutations in MECP2 and UBE3A in autism, and of the glucocorticoid receptor (GR) in anxiety and depression [174]. Interaction of our genome with the environment may be mediated through epigenetic modifications of our genotype. The epigenetically modified genome is referred to as the epigenome. Epigenetics refers to heritable, but reversible regulation of various genomic functions, mediated principally through changes in DNA methylation and chromatin structure [175] and non-coding RNA (ncRNA) [176]. The extent and consistency with which epigenetic modifications are transferred from one generation to another during

meiosis and from cell to cell during mitosis (for cell differentiation) are, however, still subject of debate.

A key issue of epigenetic modifications is their lasting effect on gene expression by either up- or down-regulation of gene expression. While the DNA sequence of an organism defines the primary structure of the proteins, epigenetic mechanisms control the quantity, location and timing of gene expression [55]. Epigenetic processes are thus essential for normal cellular development and differentiation, and are thought to be mitotically stable. Parent-of-origin specific effects, also referred to as genomic imprinting, are presumed to be under epigenetic control. Genomic imprinting is the differential expression of genetic material at either a chromosome or allelic level depending on whether the genetic material has been transmitted from the paternal or maternal side [55]. Two metastable epigenetic processes are transgenerational inheritance of phenotype (meiosis) and the interaction of the genome with the environment (fine tuning of phenotype).

Epigenetic control is exerted through cytosine methylation at CpG dinucleotides, the post-translational modification of histones by means of acetylation or methylation, ubiquitylation or small ubiquitin related modifier proteins-ylation (SUMOylation), phoshorylation and ADP-ribosylation, and transcriptional silencing and alterations of DNA-methylation by ncRNA [153]. CpG methylation occurs through DNA methyltransferase catalyzed transfer of a methyl-group (CH_3 or C_1 group) from S-adenosyl methionine (SAM) to cytosine residues. Histones may also be de-methylated or methylated by enzymes that abstract (e.g. demethylases) or transfer (e.g. methyltransferases) a methyl group. Other chromatin remodeling systems that have been implicated in epigenetic changes are nucleosome sliding (mediated by ATP-dependent chromatin remodeling proteins) and histone substitution (exchange of histones from nucleosome with external histones) [153].

Typically many genes show an inverse correlation between the degree of methylation and the level of expression [177]. Methylated CpG sites (2-5% of all DNA bases) are overrepresented in CpG islands of the promoter regulatory region of many genes. Methylation disrupts binding of transcription factors and attracts methyl-binding proteins that are associated with gene silencing and chromatin compaction. The X-linked gene encoding MECP2, e.g., is mutated in Rett's syndrome resulting in decreased MECP2 activity. Decreased MECP2 activity leads to transcriptional de-repression of specific promoters thereby decreasing the expression of genes encoding proteins which are crucial for brain development and plasticity [146]. There is a clear interaction between CpG methylation and histone modification. For example, methylated CpG islands normally recruit active MECP2, which in turn recruits co-repressors such as histone methyltransferase (HMT) and

histone deacetylase (HDAC) complexes. HDAC deacetylates histones thereby changing the chromatin structure from an activated, open state, which allows gene transcription (euchromatin), to an inactivated, condensed state, which does not allow gene transcription (heterochromatin). However, permissive and repressed intermediate chromatin states have also been suggested [153]. Chromatin remodeling has a high temporal and spatial resolution with regard to modulating gene expression by permitting or inhibiting access of the transcriptional machinery to specific promoter regions [153]. The diversity of histone modifications and their different spatial effects on chromatin structure enables the definition of a specific epigenetic state of gene activation or silencing also referred to as the "histone code hypothesis" [178].

Many researcher have adopted an epigenetic perspective in mental disorder research for the fact that it might explain a number of the observed phenomena [55;153]: (i) discordance of MZ twins, (ii) contribution of the environment, (iii) high heritability but slow progress in identifying risk genes, (iv) gene-environment interactions, (v) frequently observed unequal sex-ratios, (vi) parent-of-origin effects, (vii) gradual onset, chronic and remitting course over a lifetime, and (viii) necessity for chronic administration of psychiatric medication to mediate effects. Improved insight into these phenomena through the study of epigenetic processes will contribute to the development of new diagnostic methods and treatments.

Another important aspect of epigenetic traits is their potential heritability. It is not completely clear how epigenetic traits are transferred during gametogenesis and meiosis, or how they are maintained during mitosis. The answer to this question seems to be nuanced. In somatic cells the epigenetic profile is transferred from maternal to daughter chromatids during mitosis [55]. However, there seems to be considerable infidelity in the maintenance of methylation patterns in mammalian cells and *de novo* methylation events are fairly common during mitosis [179]. Especially unmethylated regions outside the promoter regions are unreliably inherited [180]. Metastability of the epigenetic profile during mitosis may have profound effects. Generally it is also assumed that epigenetic profiles are reset and erased during gametogenesis and early embryogenesis, but evidence is mounting that for at least some genes epigenetic marks (epi-alleles) are transmitted during meiosis and thus transmitted from generation to generation [181]. In addition, histone modifications, e.g. methylation, are potentially inherited through 'template reading' and 'writing' mechanisms or indirectly as a result of gene transcription. The coupling of DNA methylation replication and subsequent gene-silencing to modifications of newly-synthesized histones is of special interest and requires further investigation [182]. Taken together, the finding of only partial erasure of

epigenetic marks during gametogenesis has significant implications for heritability and inheritance research in mental disorders.

Because most neurons do not divide in the adult brain, chromatin modifications and DNA methylation are sustained within individual cells and affect activity, survival, and morphology of neurons and ultimately integrated regulation of complex behavior [153]. It is therefore that most epigenetic modifications associated with neurobiological adaptations are long-lasting. In the section dealing with genetics of schizophrenia, bipolar disorder, depression and PDD some epigenetic 'mutations' have already been linked to the increased risk or occurrence of traits/symptoms associated with these mental disorders. The following sections briefly discuss in more detail the possible role of epigenetics in the etiology and severity of these disorders.

4.3.2.1. Epigenetics of schizophrenia and bipolar disorder

The most widely studied epigenetic alteration in schizophrenia concerns that of genes aberrantly expressed in GABAergic neurons (e.g. RELN-promotor region methylation [183]), causing dysfunction of GABA-mediated neuronal circuitry. Recent studies conducted in post-mortem brains of patients with schizophrenia and bipolar disorder found increased expression of DNA-methyltransferase 1 (DNMT1), and reduced expression of RELN and GAD_{67} in GABAergic neurons in several brain regions of patients with schizophrenia [184;185] but not in patients with bipolar disorder [185] compared to non-psychiatric controls. Another study found 2-3 fold increased levels of SAM, the universal methyl-donor, in brains of patients with schizophrenia and bipolar disorder compared to patients with unipolar disorder and controls [186]. The increase in SAM levels in these patients was associated with DNMT1 overexpression in prefrontal cortex GABAeric neurons [186], which is suggestive for SAM-substrated and DNMT1-mediated RELN- and GAD_{67}-promotor hypermethylation and consecutive by reduced gene expression. S-adenosylmethionine is intimately linked to nutrition through C_1-metabolism [63].

Another candidate gene for epigenetic alteration is COMT. Recently, a 2-fold lower rate of promoter region methylation of the gene encoding for MB-COMT was found to be a major risk factor for schizophrenia and bipolar disorder [187]. The authors also found a corresponding increase in MB-COMT transcripts, an inverse relation with dopamine receptor D_1 (DRD1) and a tendency for the Val allele polymorphism to be associated with hypomethylation. This could cause degradation of dopamine at increased rates through overexpression of MB-COMT and hyperactivity of the COMT-allele together with secondary downregulation of DRD_1 expression. The latter is suggested to reduce RELN-gene expression through dopamine-DRD_1 interaction mediated cAMP response element hypermethylation

of the RELN-promoter region [187]. Treatment of patients with schizophrenia and bipolar disorder with epigenetic drugs such as inhibitors of methylating enzymes (e.g. DNMT1) might reverse promoter hypermethylation of suppressed genes. Noteworthy is the finding of DNMT1-inhibition by doxorubicin and subsequent reactivation of the human RELN and GAD_{67} genes in neuronal cell culture, genes that are down-regulated due to modifications in the epigenome [188]. Interestingly, the concentration of doxorubicin used for DNMT1-inhibition did not induce significant cell death, while it resulted in robust induction of RELN and GAD67 mRNA [188]. However, the cell, gene and promoter specificity and selectivity should be extensively investigated *in vitro* and in animal models before these potentially toxic compounds are tested in human subjects.

4.3.2.2. Epigenetics of depression

The persistence of depression is thought to be mediated by slow but stable adaptations, including those of epigenetic nature [153]. It is interesting that one of the proven therapies of depression, i.e. chronic electroconvulsive seizures (ECS) upregulates BDNF expression through changes in chromatin remodeling, which were distinct from changes in chromatin after acute ECS. BDNF upregulation mediates antidepressant activity in animal models [153]. The notion that altered epigenetic regulation is involved in depression is supported by the fact that chronic social defeat stress in an animal model of depression downregulates expression of BDNF splice variants, which is reversed upon chronic administration of imipramine [189]. The possibility to use specific HDAC, HMT and histone demethylase (HDM) inhibitors to treat depression is raised by some [153].

Interestingly, in rats, offspring of mothers with high licking, grooming and arched-back nursing (LG-ABN), display increased expression of glucocorticoid receptor mRNA and protein, and decreased GR promoter methylation compared to pups of mothers with low LG-ABN (reviewed in [190]). The difference in promoter methylation emerged in the first week of life, persisted into adulthood but could surprisingly be reversed by cross-fostering. Furthermore, pups with high LG-ABN mothers have increased expression of nerve growth factor inducible factor A (NGFI-A), which binds the GR promoter regions and enhances its transcription [191]. The third argument in favor of a role for epigenetic contribution to the etiology of depression comes from proband sex effects.

Skewed X-chromosome inactivation is an epigenetic process which might explain the excess rate of unipolar depression in women and the female MZ twin discordance [192]. It is generally assumed that in women X inactivation is stochastic for each cell lineage and that this is maintained throughout subsequent cell divisions. However, in 5-20% of women without X-linked disorders, there is

constitutional skewing of X-inactivation [193]. Moreover, it seems that as many as 15% of genes are expressed from both X-chromosomes [194]. These genes are obvious candidates for explaining sexual dimorphism in disease prevalence in women [55]. Another explanation for sex effects of depression might be mediated by hormone-specific modification of certain genes. A fourth argument is a parent-of-origin effect, i.e. disease susceptibility is mediated by parental factors in a sex-specific manner, e.g. preferential maternal transmission of a GluR7 gene risk allele to patients with recurrent depression has been reported [195]. Genomic imprinting seems to be the underlying mechanism. Last, the interaction between the genotype and epigenotype may be commonplace, and it may better predict the risk of developing mental disorders such as depression. For example, the T-allele of the C677T polymorphism in the MTHFR gene is implicated in depression [196] and the Val allele of COMT has been associated with promoter hypomethylation [187]. These results suggest it is better to perform comprehensive analyses of both the genotype and epigenotype to evaluate whether some epigenetic changes may be associated with specific polymorphisms. Especially for complex diseases with a large environmental component it is unacceptable to study the contribution of genes without the study of DNA methylation and histone modification.

4.3.2.3. Epigenetics of autism

The causative role of epigenetic mechanisms in autistic-like behavior is best exemplified in fragile-X syndrome in which CGG repeat expansion in the FMR1 gene renders it susceptible for epigenetic silencing and subsequent reduced FMR1 expression, and in Rett's disorder heterozygous females in which the gene encoding a key-modulator in epigenetic control (MECP2) is mutated [152]. MECP2 brings about silencing by binding to methylated promoters and recruiting co-repressors and histone-deacetylases. This alteration of gene-expression by decreased MECP2 activity leads to altered levels of products of the following genes: BDNF ↑, distal-less homeobox 5 transcription factor (DLX5) ↑, UBE3A ↓ and GABR β3 ↓ (reviewed in [146]). Expression of the RELN-gene is modulated by promoter methylation [124]. In addition to altered epigenetic control of single-gene expression, parent-of-origin effects and genomic imprinting have been implicated in PDD [197]. Especially in Angelman syndrome and PWS the effect of genomic imprinting is clear (see 'genetics' sections). The aforementioned gene products are implicated in brain development and plasticity. Next to other chromosomal regions subject to imprinting, imprinting errors of 7q and 15q have been associated with Angelman syndrome, PWS and Turner syndrome (X monosomy) [146;152]. The gender bias in autism may also have epigenetic roots, because it is maintained after exclusion of syndromic ASD cases with X-linked genetic origins. An epistatic mechanism in

which genes involved in neurodevelopment and neural signaling interact with genes encoding for products having endocrine functions has been suggested, but could not be confirmed. One suggestion is that maternally derived X-linked loci are silenced and thus not expressed in males, rendering them more vulnerable to impairments in social and communication skills [198].

Taken together, epigenetic mechanisms definitely play an important role in the etiology of mental disorders and it is thus of the utmost importance to take these effects into account when performing analyses directed at the 'genetic' origin of such disorders. With this in mind the function of ligands for transcription factors, e.g. certain long-chain polyunsaturated fatty acids (LCPUFA), and methyl-donors, e.g. folate, requires closer examination because these compounds explain many risk-factors, and gene-environment and environment-gene interactions that are associated with the development of schizophrenia and autism [63]. Furthermore, it is important to recognize the very exciting possibility that we inherit next to the DNA of our ancestors also their lifestyle. It is most likely that the purported genetic and epigenetic changes are observed as differential expression of proteins and metabolites in tissues and bodyfluids. Profiling of protein expression and metabolite abundance could shed more light on the consequences of these changes.

5. Biomarkers for mental disorders

In the previous paragraphs we have dealt with the socioeconomic burden, epidemiology, diagnosis, classification and the etiology of mental disorders. With regard to the vast amount of research that has been performed to mental disorders it is fair to question what knowledge is still needed with regard to prevention (ranging from pre-pregnancy measures to risk-factor reduction in adolescence), diagnosis (psychiatric nosology based on definitive objective and preferably quantifiable measures with a link to etiology) and treatment (effective treatment adapted to an individual's geno- and phenotype) as well as outcome monitoring (remission and relapse estimation) of mental disorders. The review of literature describing hypothesis-driven research may seem somewhat disappointing with respect to findings of generally applicable biomarkers for these purposes. For example, still no diagnostic blood test is available for any mental disorder and treatment outcome monitoring is usually done by assessing a patient's behavior and socioeconomic functioning. Genomic, transcriptomic, proteomic and metabolomic approaches using an in-depth qualitative and quantitative assessment by expression profiling of the respective '-omes' in health, the target disease and closely related diseases, may generate biomarkers that comply with criteria for diagnostic or therapeutic markers.

A consensus definition of a biomarker, shorthand for biological marker, is "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" [199]. Another definition of a biomarker is "a measurable indicator of a specific biological state, particularly one relevant to the risk of contraction, the presence or stage of disease" [200]. Nowadays, the term 'biomarker' is often used to refer to a molecular biomarker but physical traits or physiological metrics (e.g. results from molecular imaging) should be considered [200].

Biomarkers can be used for the diagnosis, staging, screening and prediction of a disease and for monitoring the effectiveness of and patient compliance with a treatment [201;202]. With respect to mental disorders, there is a great need for biomarkers that enable the development of objective, differential diagnostic systems that enable treatment monitoring. The identification of distinct phenotypical subtypes through the use of biomarkers may complement and even replace current diagnostic criteria. The combined use of biomarkers and endophenytopes for early recognition [203] and classification may be another promising approach. Moreover, the identification of biomarkers and other endophenotypes would likely facilitate the setting up of effective programs for prevention and early detection of mental disorders leading to better treatment outcome and a less severe course of the disorder.

Biomarker research into the causes, traits, states and treatment options of patients with mental disorders should be stimulated by governmental institutions for matters of cost-effectiveness. In addition, the psychiatric community is likely to benefit from an objective measure for the classification and treatment of patients with mental disorders. To achieve this, a multidisciplinary approach and multilevel analysis of the results is essential. Unfortunately, using hypothesis-driven research no single biomarker or panel of biomarkers has been discovered that fulfils the criteria for a diagnostic or surrogate-end-point marker. Other approaches complementing hypothesis-driven research are therefore being pursued integrating results from genomic, transcriptomic, proteomic and metabolomic studies.

5.1. Diagnostic biomarkers for mental disorders

A diagnostic biomarker test for a mental disorder preferably should fulfill the following eight criteria [204]: (i) detect a fundamental feature of disease with high sensitivity and specificity, (ii) be validated in confirmed post-mortem cases, (iii) be standardized with proper bioinformatics and proper statistics, (iv) be specific for the disease compared with related disorders, (v) be reliable in many testing environments/labs, (vi) be preferably non-invasive, (vii) be simple to perform, and (viii) be inexpensive. Early diagnosis of schizophrenia, for example, is important

because a longer duration of untreated psychosis after the first manifestation is associated with a deteriorating prognosis. Early intervention, already in the prodromal phase, in these patients reduces the period of untreated psychosis and sometimes it even prevents or delays the onset of psychosis in high-risk individuals [205]. In addition, early intervention improves social and functional outcomes and is cheaper than standard care models that start treatment once a psychosis is manifest [205]. Early intervention in children with autism is likewise associated with a better prognosis [49]. The current diagnostic systems, i.e. DSM-IV and ICD-10, are neutral with respect to theories of etiology. These systems therefore only provide limited insight into which therapy, either pharmacological or psychosocial, is most effective.

Unfortunately, a number of factors hampers biomarker discovery in mental disorders: the absence of an objective biological 'gold standard', frequently prevalent psychiatric comorbidity, heterogeneity and equifinality (same symptomatic or syndromatic clinical diagnostic entity represents different initial conditions that lead to the same clinical endpoint), quantitative traits or intermediate phenotypes and a presumed polygenic/multifactorial etiology [206]. It is also virtually impossible to study the primary affected organ in humans, except for special cases, where cerebrospinal fluid may be taken. Novel approaches of molecular imaging may also allow a more detailed study of brain disorders in humans. A generally applicable biochemical diagnostic marker should, however, be preferably detectable and quantifiable in either peripheral cells, tissues, or body fluids that are easily accessible to be routinely examined in patients. Another major problem is to determine the proper cut-off level of a biological marker or a marker panel: where does normal interindividual variability end and where does pathology start [207]?

Functional tests, e.g. the dexamethasone suppression test (measures the cortisol- response of the adrenal glands to ACTH) that is used to study the HPA-axis, have also been proposed as useful tools in the diagnosis of mood disorders. Despite the poor sensitivity and specificity of the dexamethasone suppression test for patients with depression in general [206], it is a quite well-established predictor of risk of suicide in depressed patients, along with serum cholesterol [208]. If future research uncovers other pathophysiological mechanisms or affected systems that are specific to certain mental disorders, functional tests may be devised that address hypo- or hyperactivity of these systems. Functional testing may provide an alternative path that also provides valuable information with respect to the proper diagnosis and to the most effective treatment.

5.2. Biomarkers for treatment monitoring of mental disorders

Biomarkers for monitoring treatment and outcome for mental disorders are less easily qualified than those for somatic disorder like diabetes. In the (bio)-pharmaceutical industry biomarkers are qualified according to whether they are "fit-for-purpose" [209]. Exploratory biomarkers are used as research and development tools accompanied by some preliminary clinical evidence. Their main purpose is to support the generation of new hypotheses (e.g. gene expression profiling). Valid exploratory biomarkers have demonstrated, but not yet reproducibly, adequate preclinical sensitivity and specificity and are linked to clinical outcome. These biomarker candidates can assist in clinical decision-making (e.g. adiponectin). Fully validated biomarkers have reproducibly proven their adequacy in terms of sensitivity, specificity and their link to clinical outcomes in multiple prospective clinical studies in human. These biomarkers are also used for dose finding in clinical trials and secondary/tertiary claims (e.g. fasting plasma glucose). If a biomarker can function as a substitute for a clinical endpoint it is designated as a surrogate end point. These biomarkers can be used for registration purposes of new pharmaceuticals (e.g. hemoglobin A1c). However, few biomarkers make it from the exploratory phase to the final status of surrogate end point.

A biomarker that may serve as a surrogate end point is most interesting in psychiatry, since clinical endpoints of mental disorders are diverse and not well-defined. The most important indicators of recovery from a mental disorder are: having paid work fit to the patient's educational background, having normal interpersonal relationships and being devoid of any symptoms that impair daily living. Of course, it will be very hard to link these complex psycho-socioeconomic outcomes to any biochemical marker. It thus seems more feasible at present to use biomarkers for the identification of novel pharmaceutical targets and compounds that share the same mechanism of action. Biomarkers may also be valuable tools to validate animal models for mental disorders and to translate preclinical results into the clinic. The study of efficacy and toxicity of new drugs in animal models with predictive power for human clinical trials is another field of application [204].

5.3. Biomarker discovery

5.3.1. Tissue banks and sample collections

Hypothesis-driven biomarker research in mental disorders focuses at those tissues, organs, cells and pathways which are implicated in the etiology and severity of the disease, because of their obvious relation with symptoms. Though this approach has yielded reasonably effective treatments for some mental disorders, it may overlook more hidden biomarkers because of spatial and temporal constraints. For example,

peripheral cells are easier to obtain than post-mortem brain tissue and a spatial constraint might thus be that the investigation of peripheral tissue or cells (e.g. leukocytes) may not fully resemble molecular and cellular processes in the brain, although this is disputed by some [210]. Analysis of fluid more proximate to the affected organ than blood or urine, such as CSF, is preferable but also more difficult to obtain without a clear medical indication. With respect to the temporal constraint, one may have to conclude that earlier and current research has exposed only those abnormalities secondary to the primary causative process. The presumed neurodevelopmental origin of many mental disorders suggests that biomarker studies should start before clear symptoms of the disease are visible. Disease entities with similar symptomatology but different underlying causes can probably not be differentiated by the study of secondary processes. All together, it may be that the search for true diagnostic markers that can differentiate between psychiatric disorders with different etiologies was unsuccessful because we were just too late and studying the wrong thing.

To circumvent these problems large-scale biobanking projects have been set up that coordinate the collection of whole-blood, serum, plasma, erythrocytes, leukocytes (for DNA) and other biofluids (e.g. urine, CSF) and/or post-mortem brain tissue from affected individuals and controls. For proper biobanking, it is also necessary to collect samples from affected and unaffected relatives. Especially a collection of biofluids and pregnancy tissues from the mother before and during pregnancy of the affected individual is important to define vulnerability windows of intrauterine neurodevelopment and their association with psychiatric disorders in later life. For the father pre-pregnancy DNA might be considered important because of changes in epigenetic markers over time and imprinting effects.

Several post-mortem brain collections (http://www.intbbn.org/) are available for studies of schizophrenia, bipolar disorder and depression: the Stanley Foundation brain collection [211], the Oxford brain bank [212], the Harvard brain collection [213], the BrainNet Europe Consortium [214] and others. The tissue in these banks is generally accompanied by the necessary information. Generally, brain tissue is dissected into several brain regions that are sliced and stored as freezing coupes. These brain slices give an opportunity to study different affected regions resulting in increased homogeneity of the sample. Collaboration between tissue banks and standardized collection of tissues as well as extensive accompanying information is the key to successful exploitation of these banks, because only then will sample size be sufficient to control confounding factors and to draw firm conclusions. For autism research, the Brain and Tissue Bank of the University of Maryland is probably the best established collection [215]. Several other initiatives, notably in the USA, have led to the collaboration of different brain

tissue banks to increase the number of samples for the study of (neuro-) developmental disorders. This is necessary because of the low prevalence and decreased likelihood of early death in children with PDD.

Several population cohorts such as the Northern Finland 1966 Birth Cohort Study, the Danish population-based cohort studies, and the NIMH (National Institute of Mental Health) epidemiological catchment area studies [216] have increased the understanding of mental disorders significantly. Prospective collection of blood specimen, DNA together with epidemiological information of both affected individuals, their (un)affected relatives and controls is to this moment less common practice. If biobanking is to be implemented in these cohort studies, standardization of sample collection, sample storage and sample management is of utmost importance. An example of DNA-biobanking in PDD is the International Molecular Genetics Study of Autism Consortium (IMGSAC) initiative from 1994. IMGSAC has provided many researchers with genetic material and clear diagnostic and anthropometric information of a sufficient number of patients to test their hypotheses. These and other large-scale initiatives provide the international research community with a wealth of high-quality samples and information. New diagnostic and therapeutic biomarkers for mental disorders are likely to come from the analysis of these tissue banks (e.g. the human proteome organization (HUPO) brain proteome project (BPP; http://www.hbpp.org/)) using non-hypothesis driven techniques. These techniques assess in a qualitative, semi-quantitative and/or quantitative fashion DNA (genomics), RNA (transcriptomics), proteins (proteomics) and metabolites (metabolomics). The complementary and integrated analysis of these '-omics' data allows evaluation of complete systems using a systems biology approach [217].

5.3.2. Multi-analyte analysis by '-omics' technologies in biomarker research

As mentioned before, many '-omics' techniques use multi-analyte profiling and subsequent analysis of data using bioinformatics to extract useful information from the large amount of data that are generated by these techniques. Generally this causes the problem that datasets contain a low numbers of samples (10's-100's) and a large numbers of variables (1000's-10000's). This is also referred to as the high-dimensionality small-sample-size (HDSS) problem [218]. The analysis of properly collected and well-characterized samples by sophisticated low-throughput '-omics' techniques and subsequent data analysis by appropriate bioinformatics approaches that control or correct for the HDSS problem, enables the extraction of valuable information from which a diagnostic or therapeutic biomarker (or set of biomarkers) may be derived. Unfortunately the rate of introduction of new

diagnostics coming from these '-omics' techniques has been disappointing until now.

While large investments are being made in this area, the rate of introduction in the clinic of new protein biomarkers is falling, at least for cancer diagnostics [219]. The regulatory part of this problem is tackled by the creation of a consortium consisting of regulatory institutions (e.g. food and drug administration; FDA) and (bio)-pharmaceutical industries. This consortium focuses at the evaluation of cost-effectiveness and the qualification of biomarkers for regulatory decision-making [220]. In addition, researchers have begun to propose coherent and comprehensive processes (pipelines) for biomarker development consisting of: discovery, qualification, verification, research assay optimization, clinical validation and commercialization [200] in accordance to guidelines defining the minimal information about experiments for genomics and transcriptomics (MIAME), proteomics (MIAPE) and metabolomics (MIAMET). These pipelines are designed to provide some directions in facing the challenges associated with biomarker discovery. For proteins in blood, these challenges are related to the complexity and dynamic range of proteins in biofluids, the low relative abundance of many disease-specific biomarkers and the extent of variation between individuals and within a given disease [200].

Genomic and transcriptomic methods profile genes or gene-expression using microarrays of PCR-amplified material. However, gene expression data do not consistently correlate with protein expression and cannot identify post-transcriptional (e.g. alternative splicing) or post-translational modifications, which are major modulators of protein function, and presumably pathogenesis [217]. Proteomics and metabolomics may better reflect the dynamics of states and traits of patients. For proteome and metabolome profiling highly selective, sensitive and in certain cases also specific assays are required and these assays are usually preceded by extensive pre- and/or sub-fractionation methods (restricted-access chromatography, affinity-chromatography, depletion of high-abundant proteins etc.). Compared to the enormous number of articles about cancer proteomics, the input of proteomics and metabolomics in psychiatry research is scarce [204]. Some proteomic and metabolomic approaches have been applied to schizophrenia (proteomics reviewed in [204], [221-227]; metabolomics: [222;228]), bipolar disorder [proteomics [221;229]], depression [proteomics [221;223;230]; metabolomics [231]] and autism [proteomics [232;233]], or related animal models [all proteomics: schizophrenia [234;235]; depression [236;237]; anxiety [238]]. However, most of these studies have only shown changes in high abundant markers that are less likely to be related to the primary disease process. As a consequence

these studies have made limited contributions to the understanding of these disorders.

Routinely applied proteomics techniques are two-dimensional gel electrophoresis for protein analysis (2-DE) or liquid chromatography hyphenated with mass spectrometry (LC-MS). These techniques and their application in proteomics have been extensively reviewed [239-241]. In this review no further attempts will be made to review these techniques with respect to their advantages and drawbacks for biomarker discovery. We will merely describe the workflow (**Figure 3**) and challenges that proteomics faces in finding biomarkers for mental disorders as well as the findings of proteomic and metabolomic studies in this area.

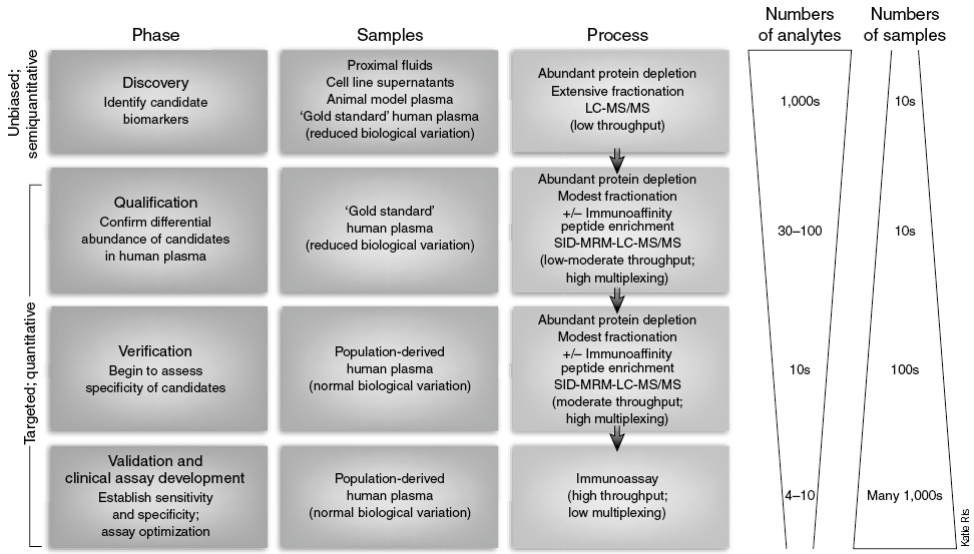


Figure 3. ***Process flow for the development of novel protein biomarker candidates***
From [200]. 'Numbers of analytes' refers to the number of proteins expected to be evaluated as candidate biomarkers in each phase of development. 'Numbers of samples' refers to the sample requirements for each phase. LC-MS/MS, liquid chromatography tandem mass spectrometry; SID, stable isotope dilution; MRM, multiple reaction monitoring.

5.3.3. Biomarker discovery pipeline in mental disorders and challenges

Biomarker discovery starts with the proper selection of patients and controls. Including large groups of patients having different but related mental disorders (symptom domains) according to the DSM-IV or ICD-10 criteria and a control group seems the best approach to find a diagnostic marker or marker panel. However, for the analysis of data an unbiased/unsupervised approach should be

used, since DSM-IV and ICD-10 classifications are not based on etiology. Furthermore, proper matching for age, gender, medication, duration of illness, lifestyle and other factors is of imminent importance to reduce the effect of confounding factors. For '-omics' studies of animal models for psychiatric disorders similar considerations are valid.

The next step in biomarker discovery is the selection of relevant samples, which can be biofluids such as blood or its derivatives (serum, plasma or specific cells), urine, CSF, or tissues (e.g. specific areas of post-mortem brains). Biofluids and their protein constituents are generally used in diagnostics and are thus a logical starting point for biomarker discovery, because of their comprehensiveness and accessibility [200]. Unfortunately, blood contains tens of thousands of proteins, which span ten to eleven orders of magnitude in concentration [200]. Disease-specific markers coming from the brain are likely to be highly diluted in blood and even more diluted in urine, which makes their discovery challenging. Higher concentrations of these markers are more likely to be found proximally to the affected brain in the CSF. A disadvantage of CSF is that it is not readily available, because it can only be acquired by an invasive lumbar puncture in limited volume (mL-scale). Analysis of post-mortem brain tissue is interesting, because it provides direct access to the affected organ, thereby increasing the chance of finding abnormalities that relate directly to the symptomatology. However, post-mortem brain analysis often means analyzing samples of convenience with little control over matching and confounding factors. In addition, brain abnormalities in later life might not be reflective of early processes that are responsible for the onset of, for example, depression or psychosis. Independent of the nature of the sample, samples should be collected in a standardized fashion and should be accompanied by the relevant information in a way that global exchange of material is possible.

Sample pre-treatment and analysis is the next step in the workflow. Often, sample pretreatment is crucial to reach into the lower concentration range of proteins and metabolites. For this, immunodepletion to remove high abundant proteins and affinity-chromatography to enrich proteins with specific post-translational modifications (e.g. glycosylated proteins) or a given functionality are frequently exploited [242]. Another interesting approach is the use of the blood peptidome, which accumulates on high-abundant circulating blood proteins like albumin, thereby serving as concentrators of potential disease-specific peptide markers [243].

Sample analysis is done using relatively low-throughput techniques, because extensive prefractionation must be generally performed. Consequently, one sample results in multiple fractions that need to be analyzed, for example, by LC-MS [242]. This improves sensitivity when hyphenated with hybrid mass spectrometers

exhibiting high mass resolution and mass accuracy, (e.g. linear ion trap/Fourier transform ion cyclotron resonance (LTQ/FT) or LTQ/Orbitrap mass spectrometers [200]). However, such an approach results in many tens of hours analysis time per sample [218]. Each sample may generate data files on the order of hundreds to thousands of Mbytes. There is thus an increasing need for data storage, processing and analysis capacity that should be managed by skilled bioinformaticians with an understanding of analytical chemistry.

Analysis of high-dimensional data from relatively few samples requires extensive knowledge about multivariate statistics and methods to reduce data complexity (binning/meshing), to extract relevant information (noise-reduction/peak-detection [244]) and to render LC-MS data comparable (normalization [245] and alignment of data in m/z and/or time domains [246]). Data processing methods that reduce data complexity by removing redundancy caused by isotopes and charge-states in the raw data have to be integrated in the data-processing workflows [247]. After data processing a matrix is constructed that contains values (intensity/area/volume) of individual peaks (for example, characterized by retention time and mass-to-charge ratio) from different samples (organized in columns). This matrix is subjected to unsupervised or supervised multivariate statistical analysis and visualization [218].

Depending on the goal of the study, multivariate analysis is performed in an unsupervised or supervised manner. In the search for a diagnostic biomarker or biomarker panel that differentiates between bipolar disorder and schizophrenia it is advisable not to use supervised classifying algorithms, because an intermediate phenotype might exist that affects sample classification and that would go undetected by using a supervised classification algorithm. In those cases it is better to use unsupervised principal component analysis (PCA) for class discovery, which in this example might result in the discovery of three classes (e.g. bipolar disorder, intermediate phenotype and schizophrenia) instead of the expected two. On the other hand unsupervised PCA reduces the chance of finding significant differences between classes, which makes it a risky strategy and at the end of the line it may prove an unviable option. The separation of groups of samples can be visualized by the projection of individual samples in a 2D- or 3D-plot consisting of principal components (PC) 1, PC 2 and PC 3. In contrast to diagnostic biomarkers that differentiate between psychiatric disorders, treatment biomarkers and biomarkers distinguishing between controls and affected individuals are more likely to be discovered through the use of supervised classification algorithms. Supervised classification pinpoints to those compounds that best discriminate a group of individuals or individuals before and after treatment. Next to the multivariate analysis of cross-sectional data, multivariate correlation analysis of longitudinal data

of groups of individuals is probably even more sensitive in finding biomarkers, because intra-individual noise is better estimated and corrected for.

The output of the multivariate analysis consists of a number of peaks representing one or more parent compounds. To decide which of the discriminatory peaks should be further studied (for example, after identification by LC-MS/MS), visual inspection, univariate statistical analysis and confirmation in an independent dataset should be performed. Confirmation of the discriminatory properties of identified compounds can be accomplished through spiking experiments or through dedicated analysis schemes (e.g. immunoassays; selective LC-MS/MS approaches).

The last step in the biomarker discovery workflow is the interpretation of the role of the discovered biomarker(s) in disease etiology and its validation in larger groups of patients in comparison with controls. Systems biology may be helpful in relating a given biomarker to disease etiology [217] as may be the study of the literature. Qualification, verification and validation according to FDA-guidelines and the Clinical Laboratory Improvement Amendments law of 1988 guidelines of the biomarker in larger groups should be performed with high-throughput assays such as stable-isotope dilution (SID) multiple-reaction-monitoring (MRM) LC-MS/MS on a triple quadrupole mass spectrometer or with sensitive immunotechniques such as radioimmunoassays (RIA) or enzyme-linked immunosorbent assays (ELISA) [200]. For clinical validation is important to consider the consequences of the chosen (multivariate) cut-off(s). At least sensitivity, specificity and preferably also the negative and positive predictive value should be assessed and compared with other existing assays through receiver operating characteristics curves (ROC). If the marker holds with respect to clinical and analytical validation, it can be implemented in routine care.

5.3.4. Results from proteomic and metabolomic studies in mental disorders

Compared to cancer research, few studies report the use of '-omics' technologies in psychiatry research. In this section we focus on studies that used general proteomics techniques such as 2-DE, LC-MS and MS for the simultaneous analysis of proteins in brain tissue, CSF, plasma and serum as well as on some metabolomics studies that have used special analytical platforms usually GC-FID of derivatizated analytes or ^{1}H-NMR for profiling of glucose, fatty acid and oxidative phosphorylation pathways etc.

Advances of proteomics technologies in schizophrenia research have recently been reviewed [204]. Using 2-DE of the hippocampal proteome of seven patients with schizophrenia and seven controls, 108 differentially expressed proteins were

found. Among these were the diazepam binding inhibitor (DBI) and manganese superoxide dismutase (MnSOD) (both decreased), and the overexpressed collapsing response protein 2 (CRMP-2) and t-complex protein 1 (TCP-1) [248]. The first two proteins are involved in the regulation of GABA-ergic activity and in antioxidant activity, respectively [204]. TCP-1 aides in protein folding and arrangement. CRMP-2 is involved in axon formation. An alteration in turnover of the cytoskeleton through differential post-translational oxidation/nitration was suggested to be ongoing in the brains of patients with schizophrenia [248].

Another study of 89 frontal cortices obtained post-mortem from individuals with schizophrenia (n=24), bipolar disorder (n=23), major psychiatric disorder (n=19) and non-psychiatric controls (n=23) used 2-DE and MS sequencing of proteins [221]. Eight differentially expressed proteins were found: glial fibrillary acidic protein and dihydropyriminidase-related protein 2 were decreased in psychiatric patients, while ubiquinone cytochrome c reductase core protein 1 was only decreased in depression. The authors concluded that because some alterations in proteins were found in patients with different psychiatric diagnoses they may represent features that are common to the different diseases such as non-specific markers of inflammation or a common second messenger pathway [221].

Analysis by LC-MS of trypsin-digested serum proteins from 69 children with autism and 35 typically developing children showed only few differentially expressed peptides that barely reached statistical significance [233]. These peptides could be related to relatively high-abundant proteins such as apo-B100, complement factor H related protein, complement C1q and fibronectin 1, which do not seem to be very specific for autism and may reflect alterations in lipid metabolism and/or the immune system. 2-DE post-mortem brain tissue analysis of individuals with autism revealed a more acidic (polar) form of glyoxoalase I (Glo1) after identification of differentially expressed protein by LC-MS/MS [232]. This more acidic from of Glo1 was traced to a SNP in the Glo1 gene. The gene-product of Glo1 gene was found to have decreased enzyme activity and was later termed as predisposing factor in the etiology of autism [232].

Analysis of CSF from ten patients with schizophrenia and ten controls using 2-DE and MS protein sequencing showed downregulation of apolipoprotein A-IV (apo-AIV) in schizophrenia [249]. Decreased expression of apo-AIV was suggested to lead to reduced satiety signaling thereby increasing the risk of weight gain and insulin-resistance in patients using atypical antipsychotics [204]. MS sequencing of a differentially expressed protein in 2-DE analysis of plasma from treatment-resistant patients with schizophrenia and chlorpromazine-treated rats showed a decrease of plasma apo-AI in patients with schizophrenia and an increase in chlorpromazine-treated rats [227]. This suggests an association of decreased apo-AI

with the pathology of schizophrenia and an association of increased apo-AI with the therapeutic action of chlorpromazine [227]. The 2-DE analysis of CSF from patients with major depression that aimed at finding differences in the CSF-proteome between suicide attempters and non-attempters is another example of how proteomics can be applied to a clinical question [230].

Another frequently used proteomic technique is surface-enhanced laser desorption/ionization (SELDI) time-of-flight (TOF) MS. This technique was applied for the analysis of post-mortem dorsolateral prefrontal cortex from 34 individuals with schizophrenia and 35 controls [250]. Using a combination of 10 peaks the authors were able to diagnose schizophrenia patients with a sensitivity and specificity of around 70% after cross-validation. However, none of the peaks was identified, diagnostic performance was poor and confounding by medication was not controlled for, raising questions about the applicability and usefulness of this SELDI method and study design. SELDI-TOF analysis of CSF from patients with drug-naïve first-onset schizophrenia, depression, OCD and Alzheimer was more successful with a sensitivity of 88% and a specificity of 95% of the discriminatory proteins in an independent test-set [223]. Up-regulation of a VGF-derived peptide and down-regulation of transthyretin, which was co-regulated with a peptide cluster, were found to be discriminatory in this study [223]. Although CSF is generally not collected for the diagnosis of schizophrenia, a CSF-based diagnostic assay seems feasible, and it is of interest to see whether such an assay can also be used for treatment monitoring. Recently, SELDI-TOF MS protein profiling of dorsolateral prefrontal cortex tissue of patients with schizophrenia and bipolar disorder in comparison with controls revealed different groups of proteins involved in cell metabolism, signaling cascades, regulation of gene transcription, protein and RNA chaperoning, and other aspects of cellular homeostasis that could differentiate between the diagnostic clusters or between psychiatric patients and controls by their up- or downregulation [229]. Protein identification was done using matrix-assisted laser desorption-ionization TOF post-source decay MS [229]. Integrated transcriptomic, proteomic and metabolomic analyses of post-mortem prefrontal cortex from 10 patients with schizophrenia and ten matched controls suggested altered mitochondrial energy metabolism and oxidative stress in schizophrenia (in 9/10 patients) [222]. These findings were suggestive for low-grade hypoxia and energy depletion in the prefrontal cortex caused by reduced cerebral blood flow [222]. This study is a fine example of how systems biology can improve the understanding of disease mechanisms and at the same time provides new directions for research.

To improve insight into disease mechanisms and metabolic side-effects of three atypical antipsychotics, a metabolomics platform capable of quantifying 300 lipids

was used to analyze plasma of patients with schizophrenia before and after treatment [228]. Olanzapine and risperidone seemed to affect a broader range of lipid classes than aripiprazole and the former two caused increases of triacylglycerols and decreases of free fatty acid levels in contrast to aripiprazole, which may be related to metabolic side-effects. Interestingly, phosphatidylethanolamine (PE) levels increased after treatment for all drugs, suggesting PE-levels to be associated with therapeutic benefit. Moreover, metabolome profiling may be a promising tool for the identification of therapeutic response markers and non-responders (i.e. pharmacometabolomics), and to assess metabolic side-effects [228]. A metabolomics approach has also been adopted to advance knowledge about late-life depression [231]. Using GC-MS, plasma from depressed, remitted and never-depressed adults was studied. Remitted patients resembled never-depressed adults with respect to their metabolome, and depressed patients had altered levels of several fatty acids, glycerol and GABA, suggesting altered lipid and neurotransmitter metabolism.

The application of proteomics and metabolomics in animal models of mental disorders has been diverse: (i) validation of animal models by studying the underlying pathology (e.g. mechanism by which methamphetamines bring about behavioral sensitization [226]); (ii) investigation of changes caused by the NMDA-receptor antagonist MK-801 (compound with psychotomimetic effects) in the thalamus [235] and cortex [234]; (iii) discovery of anxiety-related proteins in mouse brains [238]; (iv) observation that enduring high levels of circulating cortisol lead to altered cellular morphology and cell death pathways [237]; (v) for monitoring of the effects of medication. The effects of chlorpromazine [225;227] and clozapine [225] treatment on the plasma proteome in rats were studied and a study to the effects of monoamine reuptake inhibitors such as fluoxetine and venlafaxine showed changes within the hippocampal formation, beyond 5-HT/norepinephrenine neurotransmission, which may reflect long-term functional adaptations that are required for antidepressant activity [236].

5.4. Outlook and discussion

'-Omics' technologies provide a new opportunity to study complex diseases such as mental disorders through an integrated approach based on systems biology. These techniques may extend our view beyond present hypotheses. Non-hypothesis driven research is especially needed to solve those clinical questions where hypothesis-driven research has failed up to now. Although biomarkers for diagnostic and treatment purposes should preferably have an etiology-supported basis, their discovery might be through the use of techniques without any etiological assumption on forehand, at least theoretically. The needs of the

psychiatric community in biomarkers that aid in providing directions to the most (cost-) effective treatment with the least side-effects drive this research. A treatment biomarker may even turn out to be responsive to psychosocial therapy. In the end it may be possible to predict the onset of a disorder, to relate alterations in a causative manner to symptoms, and to define markers for recovery, remission and therapeutic response. Of course, all this is not possible without an extensive network that supports biomarker discovery, validation and implementation. Especially in mental disorders of low prevalence, international collaboration is required. The mental disorder biomarker discovery network has to extend from policy-makers that enable the collection of human material in large population cohorts and that provide financial support to researchers, to the end-users that benefit from the outcomes (i.e. the affected individual and his/her family). Technological advances in the field of genomics, transcriptomics, proteomics and metabolomics have been significant and it seems that there are mainly logistic and financial limitations to exploit these techniques in psychiatry research in a manner that is presently done in cancer. Results from cancer research are promising, but a marker discovered by '-omics' approaches has yet to conquer a place in the diagnostic arsenal of a clinical chemistry laboratory.

6. Conclusion

Mental disorders cause enormous psychosocial and socioeconomic suffering to those that are affected and to those living close by (e.g. relatives, friends and neighbors). Future projections about the incidence and prevalence of mental disorders are pessimistic with e.g. predicted increases in unipolar depression. A major problem of most mental disorders is their chronic nature, resulting in impairment of normal living (e.g. in work, relations, family-life etc.) after onset until death. The chronic nature of mental disorders is probably the most important contributor to the large burden-of-disease. For some disorders such as schizophrenia, abnormal behavior may already be present before the disorder is recognized and treated. For autism, children are impaired already from the early start of their life. Significant underdiagnosis and undertreatment in psychiatry is a fact of live, especially in third-world countries. This is mainly due to unequal distribution of psychiatric resources such as medication and the number of psychiatrists per capita. We still lack objective and quantifiable diagnostic measures, such as biological markers. Even in western countries available treatment modalities, either pharmacological or psychosocial, are far from being effective and most patients have residual symptoms for the rest of their lives or experience relapse. More insight into the causative mechanisms and risk factors may enable us to increase the efficacy and reduce the toxicity (e.g. metabolic side effects of

antipsychotics) of existing drugs, or to generate novel pharmacological targets. It is important to account for early-life events and environmental factors as wells as for genetic and epigenetic influences equally when investigating mental disorders. Especially adverse early-life events seem to do considerable harm, thereby significantly increasing the risk of developing a psychiatric disorder. Defining risk factors at different stages of life, ranging from optimal guidance of pregnancies to the prevention of depression in the elderly, through epidemiological research seems fruitful. If it is possible to link these risk factors in a mechanistic way to pathology, we may be able to intervene on a population level or set-up screening programs that track-down high-risk individuals. One possible way by which we can relate risk-factors to mechanistic pathways that cause disease is through the use of well-established '-omics' technologies (genomics, transcriptomics, proteomics and metabolomics). These unbiased (non-hypothesis driven) profiling techniques promise to extend our knowledge through systems biology-based mental disorder research. However, reflection upon the results of proteomics studies in cancer show that the field of biomarker discovery and validation is still in its infancy with regard to the number of biomarkers that have reached the clinic. It seems that, metaphorically speaking, the ripe fruits of knowledge and biomarkers have yet to be harvested from the tree of '-omics' technologies, and these fruits need to be undone of bugs and leaves before they can be eaten. Nevertheless, it is expected that if we combine the results from 'classic' hypothesis-driven experiments with those from unbiased non-hypothesis driven profiling techniques, it will be possible to improve the prospects of people suffering from these devastating disorders.

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Glossary (see also 'List of abbreviations')

abbreviation		*explanation*
ASD	autism spectrum disorders	see 'PDD'
DALY	disability-adjusted life year	a time-based measure that combines, in a single indicator, years of life lost from premature death and years of life lived with a disability
LMR	lifetime morbid risk	theoretical prevalence at any point in life for anyone, regardless of time of assessment
NPV	negative predictive value	proportion of patients with negative test results who are correctly diagnosed
PDD	pervasive developmental disorders	umbrella-term encompassing autistic disorder, Asperger's disorder, Rett's syndrome, childhood disintegrative disorder and PDD-not otherwise specified
PPV	positive predictive value	proportion of patients with positive test results who are correctly diagnosed
	endophenotype	heritable, measurable traits or facets in between genotype and phenotype reflecting neurobiological features underlying a readily apparent disease
	evolutionary medicine	study of present medical conditions in the context of possible discrepancies between current human environments and behaviors and past conditions under which we evolved
	sensitivity	true positive rate: proportion of true positives of all positive (e.g. diseased) cases in the population/sample
	specificity	true negative rate: proportion of true negatives of all negative (e.g. healthy) cases in the population/sample

Part I.

Research on micronutrients in schizophrenia and the role of the intestine in the hyperserotonemia of autism

Chapter 1

Folate and long chain polyunsaturated fatty acids in psychiatric disease

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Abstract

Schizophrenia, autism and depression do not inherit by Mendel's law and the search for a genetic basis seems unsuccessful. Schizophrenia and autism relate to low birth weight and pregnancy complications, which predispose to developmental adaptations by 'programming'. Epigenetics might constitute the basis of programming and depends on folate status and one-carbon metabolism in general. Early folate status of patients with schizophrenia might be compromised as suggested by i) coinciding incidences of schizophrenia and neural tube defects (NTDs) in the Dutch hunger winter, ii) coinciding seasonal fluctuations in birth of patients with schizophrenia and NTDs, and higher schizophrenia incidence in iii) immigrants and iv) methylene tetrahydrofolate reductase 677C→T homozygotes. Recent studies in schizophrenia and autism point at epigenetic silencing of critical genes or chromosomal loci. The long chain polyunsaturated fatty acids (LCPUFA) arachidonic (AA, from meat) and docosahexaenoic (fish) acids are components of brain phospholipids, and modulators of signal transduction and gene expression. Patients with schizophrenia and possibly autism, exhibit abnormal phospholipid metabolism that might cause local AA depletion and impaired eicosanoid-mediated signal transduction. National fish intakes relate inversely with major and postpartum depressions. Five out of 6 randomized controlled trials with eicosapentaenoic acid (fish) have shown positive effects in schizophrenia and 4/6 were favorable in depression and bipolar disorders. We conclude that folate and LCPUFA might be important in both the etiology and severity of at least some psychiatric diseases.

1. Introduction

The stable cross-cultural and cross-racial incidence of schizophrenia, initially noticed by the WHO in 1970, suggests that schizophrenia susceptibility genes have been with us since the origin of *homo sapiens*, some 160,000 years ago. This, together with the lower fecundity of, notably male, schizophrenics raises the question why the disease has survived natural selection [1;2]. Family studies of schizophrenics indicate that schizophrenia is rarely the only psychiatric illness, but that there is a continuum of disorders that is likely to derive from the combination of a small number of susceptibility genes, with intermediate outcomes such as 'schizotypy', depression, bipolar disorders, sociopathy and learning disabilities (including dyslexia). These genes might have been conserved during evolution because they actually code for exceptional creativity and intelligence. There is a long list of famous musicians, writers, philosophers, scientists and inventors with schizophrenic or schizotypal characteristics [2]. Our rapidly changing lifestyle, beginning with the agricultural revolution (commencing some 10,000 years ago), and its acceleration since the industrial revolution (beginning some 200 years ago) might have turned this 'advantageous genotype' into a disadvantage. The WHO predicts psychiatric disease, notably depression, to be ranking in the top of chronic diseases in Western countries in the near future. The present consensus is that the prevalence of autism exhibits an increase that is unlikely to be explained by changes in diagnostic criteria or improvements in case ascertainment. It is e.g. estimated that the prevalence in the USA has shown a >10-fold increase in the past decades, with <3 cases per 10,000 children in the 1970s to >30 per 10,000 in the 1990s [3].

The rapidly increasing incidence, and perhaps severity, of some psychiatric diseases suggests that, analogous to other typically Western diseases such as coronary artery disease, diabetes mellitus type 2 and some cancers (e.g. prostate, breast, colon, lung), we are dealing with a conflict between our contemporary lifestyle and our slowly adapting genome. We review the currently available data in support of the hypothesis that (early) environmental factors, notably those of nutritional nature, play an important role in the etiology and severity of at least some psychiatric diseases. Emphasis is laid on the role of folate and long chain polyunsaturated fatty acids (LCPUFA) in the etiologies of schizophrenia and autism, and the role of dietary folate and LCPUFA in the severity of schizophrenia and depression.

2. Influence of genetics, birth weight and pregnancy complications

Psychiatric diseases, such as schizophrenia (1% of population) and autism (0.1% of children), are among the 'complex' diseases that by definition do not inherit by Mendel's law. They are generally considered to derive from a combination of heritable and environmental factors. Currently, autism holds a respectable list of over 89 candidate genes, provoking the comment that 'as of this date, no gene has been proven to *not* be an autism disease gene' [4]. Also the list of schizophrenia candidate genes is on steady growth, while genes alone cannot explain the 2.7 times higher schizophrenia relative risk of first generation migrants and the 4.5 times higher relative risk of second generation migrants, which notably affect subjects migrating from the developing to developed countries [5].

The higher concordance of monozygotic twins for schizophrenia (about 50%; [6]) and notably autism (60-90%; [7]), as compared with dizygotic twins (schizophrenia: 17%; autism: 0-10%), seem to argue in favor of the importance of genetic factors. Twin studies in support of a genetic background have, however, been seriously criticized because of methodological problems and questionable assumptions [8]. Taking chorionicity into account, it was found that simple monozygotic concordance rates may overestimate schizophrenia heritability, with low birth weight and notably 'programming' probably being of more importance [9;10].

Birth weight has only a small genetic component and reflects mainly the quality of the intrauterine environment [11]. Small and disproportionate babies derive from a dysbalance between fetal nutrient demand and maternoplacental nutrient supply in early and late gestation, respectively, causing what is named the 'thrifty phenotype' [12]. The underlying process of 'programming' stems from a stimulus or an insult at a sensitive or critical period of development with long-term consequences. 'Programming' is a well-known phenomenon in biology. The underlying mechanism contributes to 'developmental plasticity' [13]. Its occurrence is not limited to an adverse environment in intrauterine life that stems from under or malnutrition, but it may also be triggered by infection, season of birth and smoking, or adverse environmental conditions in early infancy. By down-regulation of growth and the induction of other developmental adaptations it is now presumed to affect many tissues, organs and systems, including the central nervous system. Such adaptations may be beneficial for short-term survival but are in the long-term, notably when stimulated by unfavorable postnatal lifestyle, implicated in a number of chronic non communicable diseases at adult age, including schizophrenia [12;14]. A recent study showed that at adolescent age, very low birth weight babies are at risk of developing psychiatric symptoms and reduced

social and academic skills, while term small for gestational age babies have higher risk of emotional, behavioral and attention deficit symptoms [15]. A study of perinatal risk factors for autism among cases, unaffected siblings and controls in W-Australia concluded that we might be dealing with genetic factors that predispose to obstetric complications and that these factors may precipitate to autism by exposure to certain environmental stimuli [16]. Similar perinatal risk factors, including low birth weight, but also parental psychiatric history, were reported in another recent autism case-control study [17]. A meta-analysis of prospective population-based studies revealed that schizophrenia is associated with complications of pregnancy as well [18].

Taken together, the available data suggest that birth weight, pregnancy complications and parental psychiatric history might be important to the development of at least some psychiatric diseases. The plausibility of causality would, however, benefit greatly from the identification of the offending environmental factors and the elucidation of the underlying pathophysiological mechanism(s). Recent developments have shed more light into these issues.

3. Nutritional factors in the etiology and severity of psychiatric disease

Indications in favor of nutritional factors in prenatal life as causative factors in psychiatric disease derive from the two times higher incidence of schizophrenia in the Dutch offspring cohort that was conceived in the last month of the 1944-1945 Dutch hunger-winter [19]. The schizophrenia incidence in this cohort coincided with a 2.5 times higher incidence of neural tube defects (NTDs), which suggests involvement of low folate status. The about two times higher schizophrenia relative risk associated with maternal undernutrition was recently confirmed in a study of the massive 1959-1961 famine in China [20]. Folate involvement is further strengthened by the demonstration of coinciding seasonal fluctuations in birth incidence of patients with NTDs and schizophrenia, with both disorders exhibiting highest conception rates in May-June [21]. A third indication of folate involvement may come from the study of immigrant populations. Immigrants are less likely to use folic acid supplements preconceptionally and in the first trimester [22-25], and they also have higher NTD rates [23]. Both of these seem to relate to the alarmingly higher incidence of schizophrenia in the second-generation offspring (5). A recent meta-study of 2,265 schizophrenia cases and 2,721 controls revealed that the homozygous methylene tetrahydrofolate reductase (MTHFR) 677C→T variant is characterized by a 1.36 (1.07-1.72) higher odds ratio for schizophrenia, as compared with the CC wildtype [26]. MTHFR TT homozygotes are in need of higher folate status for similar MTHFR functioning compared with CT and CC counterparts,

because of the thermolability and reduced activity of the MTHFR 677C→T enzyme [27]. Finally, Moretti et al. [28] reported on a 6-years-old girl with cerebral folate deficiency, developmental delay, psychomotor regression, seizures, mental retardation and autistic features, who after one year of folinic acid supplementation responded favorably with regard to neurological development to exhibit 'classic' autistic features. Other indications in favor of nutritional imperfections in pregnancy and early postnatal nutritional status derive from the association between short birth intervals and schizophrenia in the offspring [29], and the association of schizophrenia with the total number of siblings per household during childhood [30]. Patients with schizophrenia living in 'developing countries' have consistently been found to have a differential advantage in course and outcome of the disease, which is probably on account of environmental factors and notably diet [31]. Schizophrenia runs a more severe course in countries with a relatively high saturated fat intake and low unsaturated fat intake [2;32]. Many studies of patients with schizophrenia have shown low circulating folate and mildly increased homocysteine [33-36] and, occasionally strongly, impaired LCPUFA status, including ω3LCPUFA status [37;38]. Serum folate concentrations in patients with schizophrenia correlate inversely with the severity of negative symptoms [34], while a randomized controlled trial with methylfolate in patients with major depression or schizophrenia improved both clinical and social recovery [39]. The picture emerges that low folate status, or possibly abnormal one-carbon metabolism in general, and low polyunsaturated fatty acid status might be among the offending factors that are involved in both the etiology and the severity of at least some psychiatric diseases.

4. Folate, one-carbon metabolism and epigenetics

'Epigenetics' refers to modifications in gene expression that do not entail a change of DNA sequence. The discipline studies heritable, but potentially reversible, changes in gene expression by DNA methylation and alterations of chromatin structure [40-44]. DNA methylation makes use of S-adenosylmethionine (SAM) as a substrate. SAM is the methyldonor of over 80 methylation reactions known to date, and many micronutrients, including those in the folate cycle, are indirectly involved in its synthesis from the essential amino acid methionine (**Figure**).

SAM-substrated DNA methylation by DNA methyltransferases is predominantly directed at CpG dinucleotides, in which the cytosine is converted to 5-methylcytosine. These CpGs tend to occur in 'islands' that are abundant in promoter regions of genes that are regulated in their expression by methylation. Epigenetic modification of chromatin structure occurs by SAM-substrated methylation of histones and also by their acetylation, phosphorylation and ubiquitylation. Different phenotypic characteristics of somatic cells within a single organism provide a lively example of the biological importance of the resulting 'epigenotype' of which much is based on gene-silencing by DNA methylation, or, alternatively, on gene-activation through methylation of suppressor genes. Most somatic cells are in this manner 'locked' into specific patterns of gene expression, which provides the basis of cell differentiation, and thereby the typical characteristics of e.g. a hepatocyte or neuron. Analogous to the memory contained within a liver cell that it is to remain a liver cell, even after mitosis, it has been suggested that synaptic input or other environmental stimuli lead to epigenetic changes that are at the basis of synaptic plasticity and thereby the formation of long-term memory and adjustment of neural functioning [45].

It has for long been believed that epigenetic modifications that are acquired during the life of an animal are erased during gametogenesis (i.e. meiosis) to restore the totipotency of the fertilized egg, and that these modifications can therefore not be transmitted to the next generation. This proved however incorrect for at least some mammalian alleles (so called metastable epialleles), and it is now clear that through this mechanism phenotype can be inherited by events that are mostly considered stochastic in nature. Transgenerational inheritance of the epigenetic state conserved in meiosis is distinct from parental imprinting and from epigenetic maintenance during mitosis. Following erasure of epigenetic marks during meiosis, parental imprinting entails epigenetic silencing of an allele according to the sex of the animal. It causes 'parent-of-origin specific effects' that derive from monoallelic expression in somatic cells of the offspring and in which the epigenetically inactivated gene may derive either from the mother or father. Mitotic epigenetic maintenance on the other hand refers to the propagation of the epigenetic state during cell division. The fidelity of DNA methylation maintenance in dividing cultured mammalian cells amounts to 97-99.9% per mitosis, whereas the *de novo* methylation amounts to 3-5% per mitosis [43]. The changes in the epigenome following mitosis, driven by (e.g. hormone initiated) developmental programs of cell and tissue differentiation, aging, microenvironment but also stochastic events, may induce further variation in the ultimate phenotypic characteristics. It has e.g. recently been established that with advancing age monozygous twins may exhibit deviant gene activities that trace down to epigenotypic differences [46]. Phenotypic

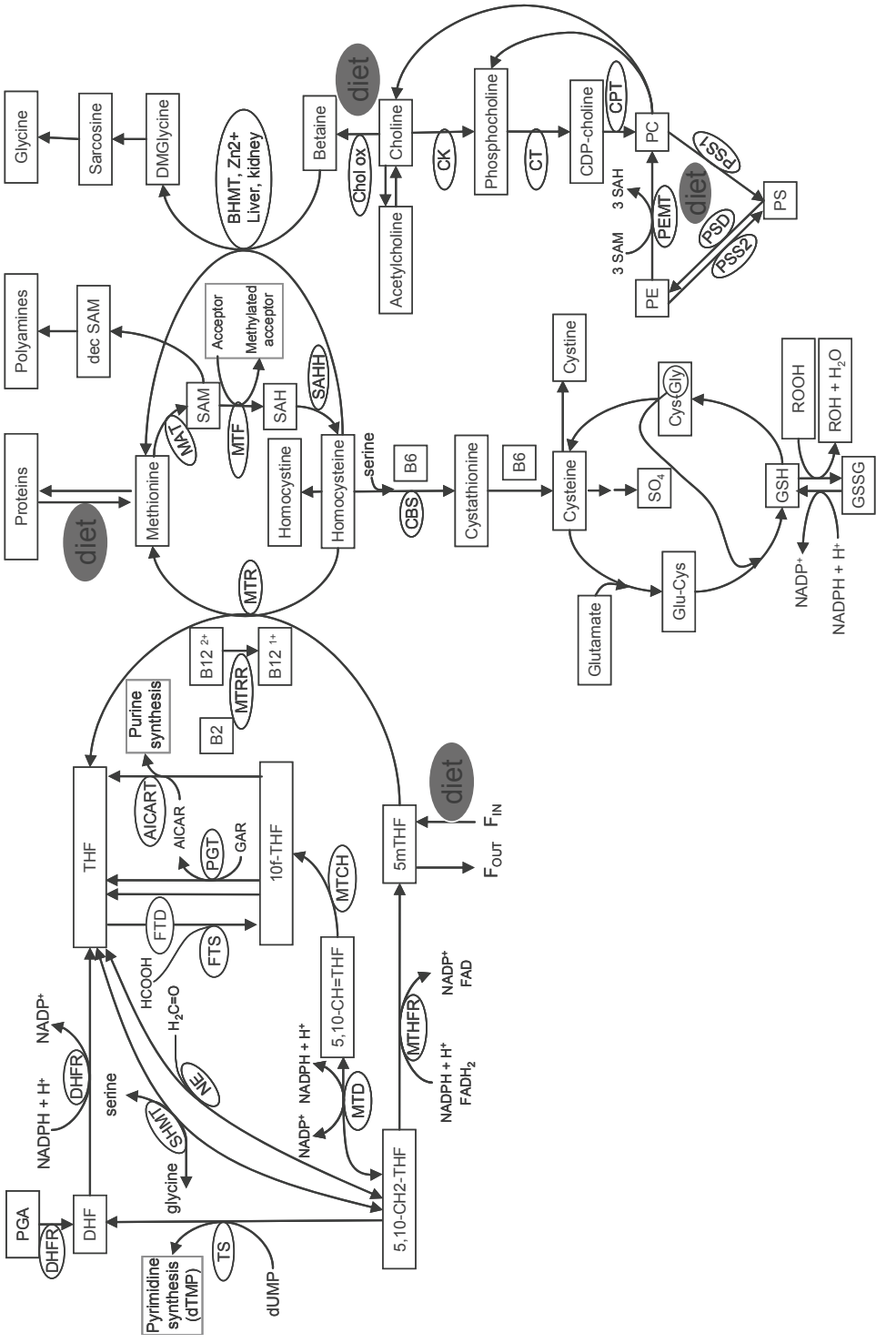
Glycine
Sarcosine
DMGlycine
BHMT, Zn2+ Liver, kidney
Betaine
diet
Choline
Chol ox
Acetylcholine
CK
Phosphocholine
CT
CDP-choline
CPT
PC
PSS1
PS
PSD
PSS2
PE
PEMT
3 SAM
3 SAH
Polyamines
dec SAM
Acceptor
Methylated acceptor
SAM
MAT
MTF
SAH
SAHH
Proteins
diet
Methionine
Homocystine
Homocysteine
serine
B6
CBS
Cystathionine
Cystine
Cysteine
SO4
Cys-Gly
GSH
ROOH
ROH + H2O
GSSG
NADP+
NADPH + H+
Glutamate
Glu-Cys
MTR
B12 2+
B12 1+
MTRR
B2
Purine synthesis
AICART
AICAR
PGT
GAR
THF
10f-THF
FTD
FTS
HCOOH
H2C=O
NE
MTCH
5,10-CH=THF
MTD
NADP+ NADPH + H+
5mTHF
MTHFR
NADP+ FAD
NADPH + H+ FADH2
FOUT FIN
SHMT
serine
glycine
NADPH + H+
DHFR
NADP+
PGA
DHF
TS
dUMP
Pyrimidine synthesis (dTMP)
5,10-CH2-THF

Figure. ***One-carbon metabolism and its immediately surrounding metabolic pathways.***
Indicated are the folate cycle (top, left), the methionine-homocysteine cycle (top, middle), the transsulphuration pathway and its connection with cystathionine/glutathione synthesis (middle, and middle-bottom), the betaine-homocysteine regeneration pathway (top right) and the choline-betaine connection with phospholipid synthesis and phospholipid interconversion (top right, and top middle to bottom). One-carbon metabolism might play an important role in epigenetics, which refers to modification of gene expression that do not entail a change of DNA base sequence. Epigenetics studies heritable, but potentially reversible, changes in gene expression by DNA methylation and/or alteration of chromatin structure. DNA methylation occurs by SAM-substrated methylation of cytosine bases in notably CpG sequences and is catalyzed by DNA methyltransferases. Dysbalances in one-carbon metabolism may cause altered states of DNA methylation and thereby phenotypic changes that in early life are connected with developmental plasticity, and that at later life are associated with complex diseases, including cardiovascular disease, some cancers and psychiatric disease.

- 10f-THF, 10-formyltetrahydrofolate;
- 5,10-CH_THF, 5,10-methenyltetrahydrofolate;
- 5,10-CH2-THF, 5,10-methylenetetrahydrofolate;
- 5mTHF, 5-methyltetrahydrofolate;
- AICAR, aminoamidazolecarboxamide ribotide.
- AICART, aminoimidazolecarboxamide ribotide transformylase;
- B2, vitamin B_2 (flavin adenine dinucleotide)
- B12, vitamin B_{12} (methylcobalamin), $^{1+}$ and $^{2+}$ refer to oxidation state of cobalt atom;
- B6, vitamin B_6;
- BHMT, betaine homocysteine methyltransferase;
- CBS, cystathionine β-synthase;
- CDP, cytidine diphosphate;
- Chol ox, choline oxidase;
- CK, choline kinase;
- CPT, CDP-choline:1,2-diacylglycerol cholinephosphotransferase;
- CT, CTP-phosphocholine cytidylyltransferase;
- Cys, cysteine;
- Cys-Gly, cysteinylglycine;
- decSAM, decarboxylated S-adenosyl methionine;
- DHF, dihydrofolate;
- DHFR, dihydrofolate reductase;
- DMGlycine, dimethylglycine;
- dTMP, thymidine monophosphate;
- dUMP, 2'deoxyuridine monophosphate;
- FAD(H2), oxidized (reduced) flavin adenine dinucleotide (vitamin B_2);
- *F*in and *F*out, the rates at which 5mTHF enters and leaves the cell, respectively;
- FTD, 10-formyltetrahydrofolate dehydrogenase;
- FTS, 10-formyltetrahydrofolate synthase;
- GAR, glycinamide ribotide;
- Glu, glutamine;
- Glu-Cys, glutamylcysteine;
- Gly; glycine;
- GSH, reduced glutathione (Glu-Cys-Gly);
- GSSG, oxidized glutathione;
- MAT, methionineadenosyltransferase;
- MTR, methionine synthase;
- MTCH, 5,10-methylenetetrahydrofolate cyclohydrolase;
- MTD, 5,10-methylenetetrahydrofolate dehydrogenase;
- MTF, methyltransferases (including DNA methyltransferases);
- MTHFR, 5,10-methylenetetrahydrofolate reductase;
- MTRR, methionine synthase reductase;
- NADP(H), oxidized (reduced) nicotinamide adenine dinucleotide phosphate;
- NE, nonenzymatic interconversion of THF and 5,10-CH2-THF;
- PC, phosphatidylcholine;
- PE, phosphatidylethanolamine;
- PEMT, phosphatidylethanolamine N-methyltransferase;
- PGA, pteroyl-L-glutamic acid (folic acid);
- PGT, phosphoribosyl glycinamidetransformylase;
- PS, phosphatidylserine;
- PSD, phosphatidylserine decarboxylase;
- PSS1 and 2, phosphatidylserine synthase;
- ROOH, peroxide;
- SAH, S-adenosylhomocysteine;
- SAHH, S-adenosylhomocysteine hydrolase

adjustment by epigenetic modification, together with long-term adjustment of DNA base-sequence by mutation and short-term adjustment by interaction with the environment through transcription factors, are at the center of our ability to adapt to the 'conditions of existence', which on its turn constitutes the major driving force of evolution. Any change of environment (e.g. current lifestyle) beyond the flexibility of base-sequence, epigenetics or physiological interaction with nuclear transcription factors, puts us at risk of disease development.

Epigenetic deregulated (otherwise perfectly normal) genes, or combinations of these with disease susceptibility genes, are more likely to be at the basis of complex diseases than gene mutations or polymorphisms *per se*. Epigenetic deregulation may notably account for the incomplete penetrance, such as encountered in autism and schizophrenia. Parent-of-origin specific gene regulation by imprinting, and triggers like gender (i.e. hormones) and endocrine rearrangements during life, may unfavorably affect epigenetic status and thereby explain [40;44] the relation of complex diseases with low birth weight and obstetric complications (autism and schizophrenia), gender inequality (male/female=4 in autism), as well as the late onset, the peak periods of onset during life and the fluctuating course of psychosis in schizophrenia [43]. Parent-of-origin imprinting and hormones are well known factors to affect epigenetic status but also nutrition proved intimately involved in epigenetic status and its heritability. The latter was elegantly demonstrated by Waterland and Jirtle [47;48], who studied the influence of 'methylation diets' on phenotype. They supplemented female mice with extra folic acid, vitamin B_{12}, choline and betaine (see Figure) from 2 weeks prior to conception until weaning to show augmented methylation of a retroviral element within the so called 'agouti-gene', which is a gene that determines the color of their coat. The intervention (partially) silenced the agouti-gene by methylation and thereby caused the coat color of the offspring to shift permanently from yellow into the brownish (pseudo-agouti) phenotype, while there was also evidence of transgenerational transmission. Another study emphasized the importance of homocysteine and S-adenosylhomocysteine (SAH). These are products of SAM methylation (Figure) and SAH is a potent inhibitor of methyltransferases. In this study Friso et al. [49;50] showed that genomic DNA methylation correlates directly with folate status and inversely with levels of plasma homocysteine. The study group was a mixed population of patients with and without coronary artery disease, and, consistent with MTHFR activity, the encountered association of global DNA methylation with folate tracked down to lower DNA methylation in MTHFR 677C→T homozygotes with low folate status [49]. Their results suggest that interaction between nutritional status and genetic polymorphism has the potential to modulate gene expression through DNA methylation [49;50]. A study of Ingrosso et al. [51] with

hyperhomocysteinemic patients on hemodialysis revealed global and locus-specific DNA hypomethylation, which was probably mediated by the associated increase of the methyltransferase inhibitor SAH. Importantly, subsequent folic acid supplementation augmented both global and locus-specific DNA-methylation, as derived from the switch from abnormal biallelic expression to normal monoallelic expression for a number of genes with known sensitivity to methylation. The study showed that folate status affects the expression of sex-linked and imprinted genes, which are both characterized by the expression of specific alleles, and that these effects are not limited to early life. A recent study of Lillycrop et al. [52] showed that dietary protein-restriction of pregnant rats, a well known model that reduces fetal growth, causes lower methylation status and activation of the genes for the glucocorticoid receptor and peroxisome proliferator activated receptor-alpha (PPAR-α) in the livers of their offspring. These receptors are important in embryogenesis, and in postnatal blood pressure and metabolic control, and are among the many candidates to be involved in fetal programming. The observed changes proved persistent up to at least 6 days after weaning and could be prevented by fortification of the protein-restricted diet with folic acid. The study showed that methylation of specific genes in specific tissues, as induced by an unbalanced diet, might be at the basis of phenotypic changes in early development [52]. Other causes may be reduced uterine blood flow, maternal postnatal behavior and social interaction [53]. The altered epigenotype might persist throughout the lifespan, passed on to the next generation and one-carbon metabolism might be central from a mechanistic perspective.

There is as yet no solid evidence of epigenetic factors in schizophrenia. The disease has, however, been linked to prenatal deficiencies of folate (see above), vitamin B_6 and vitamin B_{12} [29], which are micronutrients that are either directly or indirectly involved in one-carbon metabolism and thereby in gene expression and repression through methylation (Figure). Petronis et al. [54] conducted a pilot study on the epigenetic status of the 5'-regulatory region of the dopamine D2 receptor gene (DRD2). DRD2 has been listed as a candidate gene for schizophrenia susceptibility and DRD2 antagonism is common to all antipsychotics. They studied two pairs of monozygous twins, one concordant and one discordant for schizophrenia. It appeared that the affected twin from the pair discordant for schizophrenia was epigenetically 'closer' to the affected concordant twins than to his unaffected monozygous co-twin, suggesting that schizophrenic patients have similar epigenetic status of DRD2. Schizophrenia symptomatology is already for some time known to become exacerbated by high doses of methionine (Figure) [55]. Mice receiving prolonged treatment with methionine exhibit behavior patterns that mimic specific phenotypic aspects of schizophrenia and this coincides with

augmented brain contents of SAM, hypermethylation of the reelin promoter and downregulated expression of both reelin and glutamic acid decarboxylase (GAD_{67}). Both reelin and GAD_{67} carry CpG islands in their promoter regions, and the degree of reelin methylation in this region correlates inversely with reelin expression [56;57]. The reelin protein is necessary for neuronal migration, axonal branching, synaptogenesis and cell signaling, while GAD_{67} is one of the two isoenzymes that synthesize the neurotransmitter gamma-aminobutyric acid. Several studies have shown reduced reelin mRNA and protein levels in postmortem brains of patients with schizophrenia, and also in patients with bipolar disorders [58]. Recent studies of postmortem brains of schizophrenic patients and controls revealed reelin gene promoter hypermethylation and downregulation of reelin and GAD67 expression, suggesting an epigenetic basis for their hypoactivity in schizophrenia [58-60]. Also postmortem brains of autistic patients contain low levels of the reelin mRNA and protein, which together with some other anomalies suggest impairment of the reelin signaling pathway in autism as well [61]. Iwamoto et al. [62] reported a tendency towards a highly methylated state of the CpG island of the SOX-10 gene in the brains of patients with schizophrenia. The SOX-10 gene codes for an oligodendrocyte-specific transcription factor and it was found that the percentage methylated allele correlated inversely with relative SOX-10 expression. Catechol-O-methyltransferase (COMT) is a strong candidate in the etiology of schizophrenia. Methylation of the promoter of soluble COMT in the brain of patients with schizophrenia was ruled out as a common cause, but one patient with extreme negative symptoms showed the unique feature of full methylation of the 23rd cytosine [63]. James et al. [64] reported on 20 children with autism and 33 controls in which they studied the plasma concentrations of several metabolites in the methionine transmethylation and transsulfuration pathways (Figure). In autism they found higher SAH, adenosine and oxidized glutathione (GSSG) in conjunction with lower methionine, SAM, SAM/SAH ratio, homocysteine, cystathionine, cysteine, total glutathione and total glutathione/GSSG ratio. This profile is consistent with lower methylation capacity (i.e. lower SAM/SAH ratio) and increased oxidative stress (relatively increased GSSG) and proved correctable by supplementation with folinic acid, betaine and methylcobalamin. A recent study by Lamb et al. [65] identified two discrete loci underlying linkage of autism to chromosome 7 with possible parent-of-origin specific effects and a role of (an) imprinted gene(s). The involvement of *epigenetic* rather than *genetic* variation might explain the lack of causative base-sequence variants so far identified in candidate genes in these regions. It may be concluded that the number of studies on the epigenetic basis of psychiatric disease and the number of investigated patients is as yet small. Multiple genes might be involved, given the possible heterogeneity of

what is presently considered to be single disease entity and given a possible multi-hit etiology that starts in the maternal uterus, or perhaps even prior to conception up to oogenesis in the grandmaternal womb.

5. Long chain polyunsaturated fatty acids and brain development

Low status of long chain polyunsaturated fatty acid (LCPUFA; ≥20 carbon atoms and ≥3 methylene-interrupted cis-double bonds) may play a role as one of the offending factors in both the etiology of psychiatric disease and its severity. LCPUFA are either of the ω6 or ω3 series. Qualitatively and quantitatively important LCPUFA are arachidonic acid (AA, an ω6LCPUFA notably from meat), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (both ω3LCPUFA from fish) [66;67]. They derive from the parent essential fatty acids (EFA) linoleic and alpha-linolenic acids and some of the C_{20} members (i.e. AA, EPA and dihomo-gamma-linolenic acid) are precursors to eicosanoids (prostaglandins, thromboxanes, leukotrienes). LCPUFA are building blocks of membrane phospholipids of all cells, in which they contribute to the physical properties of the membrane and to (synaptic) signal transduction. EFA make up 20% of brain dry weight, including about 6% for AA and 8% for DHA. DHA and AA are determinants of membrane fluidity, which is important for the efficacy of neurotransmitter-receptor interaction and transporters. AA is of special importance as a second messenger in signal transduction [68]. DHA is the major structural lipid of the retinal photoreceptor outer segment membrane, where its fluidity is essential to accommodate the extremely rapid conformational changes of rhodopsine [68]. Both AA and DHA are important to maintain a healthy endothelium of our cardiovascular system [69;70], of which the brain is obviously dependent for adequate nourishment. LCPUFA synthesis from the parent precursors may be subject to 'programming' that affects the vascular endothelium. A high saturated fat diet given to pregnant rats caused reduced AA and DHA and increased linoleic and alpha-linolenic acids in the aorta of their offspring, suggesting poor conversion of precursor EFA to LCPUFA. These abnormalities coincided with vascular dysfunction and persisted to adulthood [71].

LCPUFA are not only important membrane structural elements, but, together with their eicosanoid products, they are also firmly implicated in gene expression. For example, dietary LCPUFA are ligands to peroxisome proliferator activated receptors (PPARs) and suppress the expression of sterol regulatory element binding proteins (SREBPs). These are nuclear transcription factors that can be considered as main switches in the coordinated expression and repression of a variety of (key) enzymes in intermediary metabolism, thermoregulation, energy partitioning,

growth and differentiation, and inflammatory responses [72-75]. 'Nutrigenomics' studies in rats revealed that ω3LCPUFA (i.e. notably EPA and DHA) modulate the expression and repression in brain of a sizeable number of genes that are involved in structure, energy metabolism, neurotransmission, signal transduction and regulation [76;77]. Dietary LCPUFA also influence neurotransmitter physiology. Experiments with rats showed that fish oil supplementation influences several neurochemical and behavioral features of monoaminergic function, causing a 40% higher dopamine content in the frontal cortex, a reduction of monoamineoxidase-B activity, greater binding to DRD2 and 25% lower ambulatory activity as compared to controls [78].

LCPUFA-rich fresh- and salt-water shoreline-based diets are likely to have been at the basis of our larger and more sophisticated brains compared with other primates. A constant dietary LCPUFA supply and notably that of DHA might therefore be important [79-87]. DHA might at least be conditionally essential, since we have limited ability for its synthesis from the parent essential fatty acid alpha-linolenic acid [88-91]. Higher dietary DHA intake may on its turn require higher AA intake to prevent competition between ω3LCPUFA and ω6LCPUFA, while alpha-linolenic acid has an independent role as a precursor to cholesterol synthesis in brain [92]. This lays emphasis on a dietary ω3/ω6 balance [66;67;93;94]; a balance that since the industrial revolution has increasingly become violated in favor of higher intake of ω6 fatty acids (notably linoleic acid), decreasing intake of ω3 fatty acids and increasing intake of saturated and *trans* fatty acids [94]. Deficiency of ω3 fatty acids in primates is, amongst other conditions, associated with psychiatric pathology [95], and with reduced learning, abnormal electroretinograms and visual impairment in humans [96]. AA and DHA status in preterm babies is related to birth weight, head circumference and length at birth [97-100], and both AA and DHA may be protective against the central nervous, visual and auditory damage that is typical for (very) premature babies [101]. Various studies have shown suboptimal neurodevelopment of both preterm and term babies receiving infant formulas without LCPUFA, although many of these effects might be transient [102-108]. It is clear that LCPUFA have important functions in brain and that notably the low ω3LCPUFA status of the contemporary Western diet might put us at risk of suboptimal brain development and functioning.

6. Schizophrenia-phospholipid hypothesis

There are (anecdotic) reports that i) feverish illness in schizophrenics ameliorates their psychiatric symptoms, ii) schizophrenics rarely suffer from rheumatoid arthritis (suggesting a generalized reduced inflammatory response), iii) schizophrenic patients are less capable of producing the typical (prostaglandin-

induced) cutaneous flush that follows nicotinic acid ingestion or topical application, and iv) schizophrenia in developing countries with higher LCPUFA intakes runs a less severe course [2;31;32;109]. Horrobin [2] linked these observations to develop the so called 'phospholipid hypothesis' that states that schizophrenia is a systemic disease with a central theme of impaired AA release and consequently insufficient production of its eicosanoid metabolites to support adequate signal transduction [110]. In other words, we are possibly dealing with a genetically determined generalized 'abnormality' of phospholipid metabolism that might be sensitive to prevention or correction by nutritional factors. These nutritional factors are likely to be LCPUFA, of which the intake has been subject to tremendous decline since the industrial revolution. Lower contemporary intake in Western countries is e.g. suggested by the relatively high AA and DHA status of Tanzanian women who consume an AA and DHA-rich, fresh water fish-based, diet that (in this respect) is likely to be close to our ancient diet [111]. It is possible that the genetic make-up of patients with schizophrenia would in the past not have precipitated to disease and that the LCPUFA-rich diet of our ancestors enabled them to take full evolutionary advantage of the associated intelligence and creativity [2].

Consistent with the increased LCPUFA losses postulated by the phospholipid hypothesis, both patients with schizophrenia [32;112] and autism [113] have increased activity of phospholipase A_2, which releases AA from membrane phospholipids (a process vital to brain cell signaling), while their LCPUFA in erythrocytes appear more sensitive to oxidative stress *in vitro* [113;114]. Brain magnetic resonance spectroscopy studies in schizophrenics showed signs of increased phospholipid turnover, electroretinograms of patients with schizophrenia are abnormal (suggesting low retinal DHA content), and incorporation of AA into phospholipids seems to occur with difficulty [2]. Taken together, these data suggest local AA depletion and insufficient synthesis of AA-derived eicosanoids, which becomes e.g. noticeable by amelioration of psychiatric symptoms by fever-associated eicosanoid release, pain resistance by eicosanoid-shortage at basal conditions, and poor ability to exhibit an eicosanoid-induced flush upon nicotinic acid treatment. Das [115] hypothesized that perinatal supplementation of LCPUFA, especially EPA and DHA, may prevent schizophrenia in the adult. He considers schizophrenia to be a low-grade systemic inflammatory disease with origins in the perinatal period, probably triggered by maternal infection in a genetically susceptible individual that leads to excess production of pro-inflammatory cytokines both in the mother and fetus. The infection compromises LCPUFA status with devastating neurodevelopmental effects that should theoretically be favorably responsive to augmented LCPUFA status.

7. Fish oil, schizophrenia and depression

Low intake of the fish oil fatty acids EPA and DHA is implicated in the high incidence of depression in Western countries. The incidence of depression has increased markedly in recent decades [116] and there is a strong inverse correlation between national dietary fish intakes and rates of major and postpartum depressions [117;118]. Depressive symptoms are more likely to be encountered in infrequent fish consumers and EPA and DHA status is low in depressive patients. There are also close relationships between fish consumption and the incidence of cardiovascular disease and depression, which fuelled the suggestion that depression should be included into the cluster of diseases that are associated with the metabolic syndrome [32]. Data from the UK show that the peak age of schizophrenia onset (i.e. 19-24 years) coincides with the highest intake of burgers (i.e. saturated fat) and full-sugar carbonated drinks and the lowest intake of oily fish [119]. A meta-analysis of dietary patterns in various countries linked the intake of refined sugar and dairy products to a worse 2-year outcome of schizophrenia, while a high national prevalence of depression became predicted from low intake of fish and seafood [120]. These data demonstrate that there are basically no differences between dietary risk factors for poor mental health, cardiovascular disease and some cancers. Five out of 6 double-blind placebo-controlled trials with add-on ω3 fatty acid (notably EPA) supplementation in schizophrenia have so far produced positive results, whereas 4/6 of such trials produced positive effects in depression and bipolar disorders [32;37;121]. In other words, LCPUFA are likely to be involved in the etiology of at least some psychiatric diseases, but also in their presentation in terms of severity at later age.

8. Conclusions

Current research on the etiology of psychiatric disease seems to fall short of the input of nutrition and may be somewhat overdosed with genetics and the traditional search for abnormal neurotransmitter metabolism *per se*. Folate, other one-carbon metabolite micronutrients, and dietary LCPUFA might play important roles in the etiology of at least some psychiatric diseases in their capacity as modulators of gene expression through epigenetic mechanisms (folate), and as brain structural components, precursors of signal-transducing eicosanoids and ligands to nuclear transcription factors (LCPUFA). Low status of micronutrients involved in one-carbon metabolism and low LCPUFA status are also likely to be factors in psychiatric disease severity.

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Chapter 2

Low essential fatty acid and B-vitamin status in a subgroup of patients with schizophrenia and its response to dietary supplementation

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Kemperman RFJ, Veurink M, van der Wal T, Knegtering H, Bruggeman R, Fokkema MR, Kema IP, Korf J, Muskiet FAJ. Low essential fatty acid and B-vitamin status in a subgroup of patients with schizophrenia and its response to dietary supplementation. Prostaglandins Leukot Essent Fatty Acids. 2006 Feb;74(2):75-85. Epub 2005 Dec 27.

Abstract

We assessed essential fatty acid (EFA) and B-vitamin status, together with their determinants, in 61 patients with schizophrenia and established whether those with poor status responded biochemically to the appropriate dietary supplements. As a group, the patients had high erythrocyte saturated fatty acids (FA), monounsaturated FA and low polyunsaturated FA of the ω3 and ω6 series. Patients reporting not to take vitamin supplements had low vitamin B_{12} and high homocysteine. Homocysteine variance proved best explained by folate in both the total group and male patients, and by vitamins B_{12} and B_6 in females. Alcohol consumption and duration of illness are risk factors for low polyunsaturated FA status (<P2.5 of reference range), while male gender and absence of fish consumption predict hyperhomocysteinemia (>P97.5 of reference range). Two patients exhibited biochemical EFA deficiency and 7 showed biochemical signs of ω3/docosahexaenoic acid (DHA) marginality. Four patients exhibited moderate hyperhomocysteinemia with plasma values ranging from 57.5-74.8 μmol/L. None of the 5 patients with either moderate hyperhomocysteinemia, biochemical EFA deficiency, or both, was predicted by their clinicians to have a poor diet. That diet was nevertheless at the basis of these abnormalities became confirmed after supplementing 4 of them with B-vitamins and with soybean and fish oils. We conclude that a subgroup of patients with schizophrenia has biochemical EFA deficiency, ω3/DHA marginality, moderate hyperhomocysteinemia, or combinations. Correction seems indicated in view of the possible relation of poor EFA and B-vitamin status with their psychiatric symptoms, but notably to reduce their high risk of cardiovascular disease.

1. Introduction

Patients with schizophrenia have frequently been shown to have a low status of essential fatty acids (EFA) of both the ω3 and ω6 series. Low erythrocyte (RBC) contents of linoleic acid (LA; 18:2ω6) and the long chain polyunsaturated fatty acids (LCP) arachidonic acid (AA; 20:4ω6) and docosahexaenoic acid (DHA; 22:6ω3) are the most consistent findings [1]. Some results of former studies may have been confounded by LCP losses in the pre-analytical phase, which might be due to the higher sensitivity of the LCP in the RBC of patients with schizophrenia to oxidative stress *in vitro* [2]. Other possible causes for low LCP status in schizophrenia might relate to a reduced rate of LCP incorporation into phospholipids and increased phospholipase-catalyzed loss (the so called 'membrane phospholipid hypothesis' [3]), the interaction with gender and smoking, or low dietary intake [4;5]. Low dietary intake of LCP [6] and low RBC LCP status [7;8], notably AA and DHA, are associated with an increase of psychiatric symptoms that may be at the basis of the well known cross-cultural differences in schizophrenia severity [9;10]. A meta-analysis of 2003 concluded that the use of ω3LCP in schizophrenia is still experimental and that the outcome of large, well designed, studies has to be awaited [11]. At present six out of 7 trials with (add-on) eicosapentaenoic acid (EPA; 20:5ω3) supplements yielded positive results on psychiatric end points [4;12;13].

Patients with schizophrenia also have low functional B-vitamin status which becomes noticeable from above-normal levels of plasma homocysteine [14-18]. Increased homocysteine levels in schizophrenic patients have, however, not consistently been found [19-21]. Homocysteine is a cytotoxic amino acid that is likely to be involved in both neurodegenerative disorders, like Alzheimer's disease [22], and psychiatric disorders, including schizophrenia [23]. The major determinants of increased plasma homocysteine in the general population are low serum folate, vitamin B_{12} and betaine [24-26], which share functions in one-carbon metabolism [27]. Folate status in schizophrenic patients correlates inversely with negative symptoms [19] and in one study supplementation with methylfolate was found to enhance social and clinical recovery [28].

Life expectancy of patients with schizophrenia is 20% shorter compared with the general population. The excess mortality is for 60% attributable to physical illness (circulatory, respiratory, digestive and genitourinary disease) with the remainder on account of suicide (28%) and accidents (12%) [29]. Some newer atypical antipsychotic drugs may have side effects such as weight gain, elevation of serum triglycerides and increased risk of diabetes mellitus type 2. All of these constitute risk of cardiovascular disease in a population segment with little exercise,

poor diet, almost universal smoking, and unhealthy lifestyle in general [13;29]. In other words, patients with psychiatric diseases, especially patients with schizophrenia, may benefit from good nutrition, and not merely with the aim of ameliorating psychiatric end points. Also mild hyperhomocysteinemia [30;31] and low ω3LCP status [32-34] are risk factors for cardiovascular disease events and death. No matter the origin and whether these are features of all patients, both mild hyperhomocysteinemia and low ω3LCP status are correctable by supplementation of folic acid (or a folic acid, vitamin B_{12} and vitamin B_6 combination) and fish oil, which calls for patient-individual dietary counseling and dietary supportive care, if necessary.

Using a cross-sectional study design we determined the frequency of low EFA and B-vitamin status in a representative group of patients with a schizophrenia spectrum disorder. We were also interested to see whether low status of EFA and B-vitamin in patients is predictable by their physicians. Finally, we examined whether those with low statuses improved biochemically after short term supplementation of soybean and fish oils and B-vitamins, including folic acid.

2. Patients and Methods

2.1. Patients, samples and analyses

One hundred patients, classified as having schizophrenia according to the DSM-IV TR [35], were randomly selected from the files of the department of Psychiatry of the University Medical Center of Groningen in The Netherlands. The selected patients were asked to participate through invitation by mail, telephone or during therapeutic sessions with two of us (HK, RB). There were no other inclusion or exclusion criteria. Sixty-one (61%) patients agreed to participate. The study was performed in the summer of 2003. The protocol was in agreement with local ethical standards and the Helsinki declaration of 1964, as revised in 2002.

The participants were physically examined and their anthropometrical data were recorded. Information regarding smoking, alcohol consumption, drug abuse, use of nutritional supplements and fish consumption was obtained with the aid of an assisted questionnaire. Ages at onset of the illness, comorbidity and contemporary medication were taken from the hospital records. Non-fasting blood samples were obtained by venepuncture between 10.00 and 16.00 h for analyses in whole blood, serum, EDTA-plasma and isolated RBC. We analyzed routine clinical chemical and hematological indices in serum and EDTA-blood by standard clinical chemical methods. Serum folate and vitamin B_{12} (cyanocobalamin) were analyzed with a fluoroimmunoassay (Autodelfia, Wallac Oy, Germany), plasma homocysteine with an immunochemical method (ImX, Abbott Laboratories,

Illinois, USA), whole blood vitamin B_6 (pyridoxine) by HPLC [36] and the RBC fatty acid composition (expressed in mol%) by capillary gas chromatography [37]. Addition of the RBC to the antioxidant-fortified transmethylation mixture, following the immediate isolation of RBC by washing, ensured the stability of unsaturated fatty acids in the preanalytical phase [37]. The typical day-to-day coefficients of variation range from 1.1-17.6%, dependent on the abundance of the analyte expressed in mol% [38].

2.2. Supplementation of patients with poor B-vitamin and EFA status

Patients with isolated biochemical EFA deficiency were supplemented with a combination of soybean and fish oils and those with isolated moderate hyperhomocysteinemia were supplemented with B vitamins. For those with combined biochemical EFA deficiency and moderate hyperhomocysteinemia we chose to supplement first with EFA and second with B vitamins. No supplements were to be taken during the day prior to sampling. The patients were asked to continue supplementation beyond the twelfth week until they had their blood samples taken.

Patients with biochemical EFA deficiency (i.e. RBC 20:3ω9>0.46 mol% [38]) were supplemented for 12 weeks with purified fish oil capsules (Triomar; Pronova Biocare, Norway) corresponding with daily dosages of 310 mg EPA and 200 mg DHA. In this period they also received 15 mL soybean oil per day (generic brand from local grocer; Albert Heijn, The Netherlands) corresponding with daily dosages of 1 g ALA and 7 g LA. Soybean oil was added to the hot meal. Folic acid (800 μg) was added to this regimen from weeks 4 to 6 and the B-vitamin combination (800 μg folic acid, 8 mg vitamin B_6, 4 μg vitamin B_{12}) was provided from weeks 6 to 12. Generic brand folic acid and B-vitamin supplements were purchased from a local drugstore (Etos, The Netherlands). Blood samples for analyses were taken at 0, 4, 6 and 12 weeks.

Patients with moderate hyperhomocysteinemia (i.e. homocysteine levels ranging from 31-100 μmol/L [31;39]) were selected for supplementation with 800 μg folic acid per day during 2 weeks. They were subsequently supplemented for 10 weeks with an additional B-vitamin preparation, which resulted in a total daily intake of 800 μg folic acid, 8 mg vitamin B_6 and 4 μg vitamin B_{12}. From 4 to 12 weeks the patients also received the same daily dosages of fish and soybean oils (310 mg EPA, 200 mg DHA and 15 mL soybean oil). Blood samples for the analyses of B-vitamin status parameters and RBC fatty acids were taken at 0, 2, 4 and 12 weeks.

2.3. Data evaluation and statistics

Patient characteristics (i.e. BMI, smoking and use of alcohol) were compared with age and sex matched data of the Dutch general population, as derived from the 2003 Dutch National Food Consumption Survey [40]. Subgroups were defined according to medication type i.e. clozapine, single atypical antipsychotic, multiple antipsychotics and classical antipsychotics. Antipsychotic medication dose was expressed as defined daily dose [41] and chlorpromazine (CPZ) equivalents [42]. Fish fat intake was expressed as 'Q', which equals the product of the number of days of fish consumption per week and a measure for the average fatness of the fish consumed. The average fatness ranged from lean (0.5-5% fat/g flesh) to oily (>5% fat/g flesh), giving rise to a measure of fatness ranging from 1 to 4. Using this index of fish fat intake (Q), the recommended intake of at least two servings of medium-oily fish per week [43] would translate into a Q of 4.

Clinical chemical data were evaluated with the use of reference values as applied in our laboratory. For RBC fatty acids we used reference values (i.e. 2.5 and 97.5 percentiles of healthy omnivorous controls) as previously reported [38]. These cut-off values are independent from the age of 0.2 years and apply for both sexes. RBC 20:3ω9 above 0.46 mol% was defined as biochemical EFA deficiency; an RBC 22:5ω6/AA ratio above 0.068 mol/mol was considered to reflect isolated ω3-deficiency; an RBC 22:5ω6/DHA ratio above 0.22 mol/mol but equal or below 0.48 mol/mol was defined as ω3/DHA marginality; and an RBC 22:5ω6/DHA ratio above 0.48 mol/mol was indicative for ω3/DHA deficiency [38]. For homocysteine we employed the 14.6 μmol/L upper limit of the reference range (i.e. P97.5) as derived from our previous study of healthy controls [44]. In addition we used a cut-off value (P97.5) of 9.3 μmol/L, as derived from data of apparently healthy subjects following supplementation with folic acid and the vitamins B_{12} and B_6 ('vitamin-optimized cut-off value'; [44]). These cut-off values apply for both sexes, but may provide a somewhat conservative estimate for premenopausal female patients. Numbers of patients with scores below and above the local reference values were calculated and expressed as percentages of the total. Stratification for age was employed to investigate the age-dependent increase of homocysteine in young male patients [14;15].

Statistical analyses were performed with the Statistical Product and Service Solutions package version 11.5 (SPSS Inc., Chicago). Differences in characteristics between patients and the Dutch general population were tested by Chi-square tests. Differences between patient and control data were tested at $p<0.05$ using either the Students' t or Mann-Whitney U tests, dependent on the normality of the distributions. P-values were corrected for relevant confounders (i.e. age and gender) using ANOVA or logistic regression, if applicable. Comparison of multiple

subgroups (e.g. based on medication or DSM-IV TR classification) was performed with ANOVA (parametric) after Bonferroni correction or with the Kruskall-Wallis (non-parametric) tests. Bivariate correlations were examined by calculating either the Pearson (linear) or Spearman (non-linear) coefficients. Outcomes were considered significant at $p<0.05$. RBC fatty acids were examined for correlation with B-vitamin status parameters to explore general patterns in nutrition. Predictors of low PUFA (<P2.5 of reference range) and increased homocysteine (>P97.5 of the reference range) in the whole group were examined by multinominal logistic regression in which we tested a selected number of indices that in bivariate comparisons proved most significantly related to PUFA and homocysteine, respectively. Multivariate linear regression was used to explain variance in RBC 20:3ω9 and plasma homocysteine. For this we tested the parameters that exhibited the most significant bivariate correlations.

3. Results

3.1. Patients

Table 1 depicts the patient characteristics. The percentages female and male patients with overweight (BMI>25 kg/m^2) were similar to the age- and gender-matched data of the Dutch general adult population. The percentage obesity (BMI>30 kg/m^2) was higher in female patients (30 vs. 11%; $p<0.001$), but not in male patients. The percentages smoking (55 vs. 30.8%; $p<0.001$), and heavy smoking (31 vs. 8.0%; $p<0.01$) were higher, which was notably on account of heavy smoking in female patients (39 vs. 8%; $p<0.0001$). The percentage patients consuming alcohol was lower (65 vs. 81.7%; $p<0.005$), although the percentage male patients reporting heavy drinking almost reached significance (27 vs. 17.0%; $p= 0.053$). The percentage patients reaching the recommended fish intake ($Q\geq4$) was 21%, while 10% reported not to consume fish at all. Smoking, alcohol and fish consumption did not differ significantly between sexes. The patients reporting intake of vitamins and/or fish oil supplements had better B-vitamin and LCPω3 status, respectively (data not shown). Patients reporting gastrointestinal disturbances (24%) did not differ in their characteristics, RBC fatty acid composition and B-vitamin status parameters, compared with those not having such complaints (not shown).

Table 1. *Patient characteristics.*

Gender *(M / F)*	37 | 24		
Age *(years)*	31.5 (9.2)		
BMI *(kg/m²)* [a]	25.5 (4.1)		
BMI *(<18.5 / 18.5-25 / 25-30 / >30)* [a]	0 | 26 | 18 | 8		
Hospitalized | Community dwelling	14 | 47		
Age at onset of illness *(years)* [b]	23 (16-45)		
Duration of illness *(years)* [b]	5 (1-20)		
Diagnosis according to DSM-IV			
295.3: paranoid type	21/61 (34%)		
295.4: schizophreniform disorder	9/61 (15%)		
298.9: psychotic disorder NOS	8/61 (13%)		
295.9: undifferentiated type	7/61 (12%)		
other	16/61 (26%)		
Medication		**Dose (mg/day)**	**Defined Daily Dose** [c]
olanzapine (N05AH03)	19/61 (31%)	11.8 (5-20)	1.18 (0.5-2)
risperidon (N05AX08)	19/61 (31%)	3.0 (1-8)	0.6 (0.2-1.6)
clozapine (N05AH02)	9/61 (15%)	363 (200-500)	1.21 (0.67-1.67)
other	17/61 (28%)		
no medication	2/61 (3%)		
Average dose		280.5 (78.3)[d]	0.94 (0.26)
	Yes (fraction; %)	**Median (range)**	
Diabetes Mellitus [e]	1/53 (2%)		
Smoking [f]	30/56 (55%)		
Cigarettes/day		20 (1-40)	
heavy smoking [g]	17/56 (30%)		
cigarettes/day in heavy smokers		20 (20-45)	
Recent use of cannabis [b]	12/55 (22%)		
Alcohol [f]	36/56 (64%)		
units/day [h]		0.7 (0.1-23)	
heavy drinking [i]	10/56 (18%)		
units/day in heavy drinking		3.9 (3-23)	
GI disturbances [j]	14/58 (24%)		

Table 1. *Patient characteristics* **(continued).**

	Yes (fraction; %)	Median (range)
Fish intake [j]	52/58 (90%)	
Q [k]		1 (0.04-21)
Q≥4 of those eating fish	11/52 (21%)	
Q of those with Q≥4		8 (4-21)
Use of supplements [e]	5/53 (9%) [l]	

Data are for 61 patients unless otherwise indicated. Data represent numbers, fractions (%), means (SD), or medians (range). [a] n=52, BMI of the patient with diabetes mellitus was 22.3 kg/m²; [b] n=55; [c] defined daily dose calculated with [41]; [d] chlorpromazine equivalents calculated with [41;42]; [e] n=53; [f] n=56; [g] ≥20 cigarettes per day; [h] 1 unit equals 20 mL of pure ethanol; [i] ≥3 alcohol units per day; [j] n=58; [k] Q is an index for fish fat intake, that equals the product of the number of days of fish consumption per week and an arbitrary measure for the average fatness of the fish consumed; [l] vitamins n=4, fish-oil supplements n=2.

3.2. Erythrocyte fatty acids

Table 2 shows the RBC fatty acid composition of patients and controls together with the percentage patients with values below and above the respective reference values. Between (sub)groups comparisons were done with patients reporting no intake of fish-oil supplements. Type or doses of antipsychotic medication were not significantly related to the RBC fatty acid composition. Most remarkable was the higher RBC saturated fatty acids (SAFA) content of the patients ($p<0.001$), with 19 (31%) patients exhibiting levels above the P97.5. Higher SAFA was notably attributable to higher 16:0 ($p<0.001$) and 12 (20%) patients had levels above P97.5. The percentage monounsaturated fatty acids (MUFA) ($p<0.001$), notably 18:1ω9 ($p<0.001$), was also higher, with 15 (25%) and 33 (54%) patients having contents above the P97.5, respectively. Higher RBC SAFA and MUFA coincided with lower polyunsaturated fatty acids (PUFA) ($p<0.001$), with 25 (41%) patients having contents below the P2.5. The lower PUFA status was attributable to different combinations of lower status of ω3 ($p<0.001$; 13% below P2.5), ω6 ($p<0.001$; 13% below P2.5), alpha-linolenic acid (ALA; 18:3ω3) ($p<0.001$; 36% below P2.5), DHA ($p<0.001$; 13% below P2.5), AA ($p<0.05$; 11% below P2.5) and 22:4ω6 ($p<0.001$; 11% below P2.5). Biochemical EFA deficiency (i.e. RBC 20:3ω9>0.46 mol%) was found in one male and one female patient, both 53 years of age of which one reported to not eat fish at all, whereas no such data were available from the other. Isolated biochemical ω3-deficiency, as indicated by increased RBC 22:5ω6/AA ratio, was not observed in our study group.

Biochemical ω3/DHA marginality, as derived from a 22:5ω6/DHA ratio >0.22 but ≤0.48 mol/mol, was observed in 9 (15%) patients, including the 2 with biochemical EFA. We did not observe biochemical ω3/DHA deficiency. Of the remaining 7 with biochemical ω3/DHA marginality 4 reported not to eat fish and no such data were available for one.

Table 2. ***Erythrocyte fatty acid composition of patients with schizophrenia.***

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Data are for 61 patients unless otherwise indicated. Data are presented in mol%. Reference values represent medians (P2.5-P97.5) for controls (n=69). Data for patients represent medians (range). The reference values (i.e. 2.5 and 97.5 percentiles) of the controls were used as cut-off values to evaluate individual patients. The outcomes are expressed as the number (%) of patients with RBC fatty acid content either below the P2.5 (n<P2.5) or above the P97.5 (n>P97.5). P<0.05 was considered significant for between group analyses. Biochemical essential fatty acid deficiency was defined as an RBC 20:3ω9 above 0.46 mol%. Biochemical isolated ω3 deficiency was defined as an RBC 22:5ω6/AA above 0.068 mol/mol. Omega-3/DHA marginality was defined as an RBC 22:5ω6/DHA above 0.22 mol/mol but equal or below 0.48 mol/mol, while ω3/DHA deficiency was indicated by an RBC 22:5ω6/DHA above 0.48 mol/mol [38]. ALA, alpha-linolenic (18:3ω3); EPA, eicosapentaenoic acid (20:5ω3); DHA, docosahexaenoic acid (22:6ω3); LCP, long chain polyunsaturated fatty acid; LA, linoleic acid (18:2ω6); AA, arachidonic acid (20:4ω6); SAFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid. [a] Significance (p) for all patients (n=61) vs. controls; [b] Significance (p) for patients reporting no intake of fish-oil supplements (n=59) vs. controls; [c] Patient (n=1) reporting intake of fish-oil supplements is included; [d] Patients (n=2) reporting intake of fish-oil supplements are included.

	Reference values		*Patients*		*Patients*		*Patients vs. controls*	
	median	(P2.5 - P97.5)	median	(range)	n <P2.5	n>P97.5	p[a]	p[b]
14:0	0.43	(0.30 - 0.54)	0.46	(0.26 - 0.76)	2 (3)	11 (18)	0.103	0.146
16:0	23.77	(22.50 - 25.30)	24.35	(22.91 - 26.83)	0 (0)	12 (20)[c]	<0.001	<0.001
18:0	16.41	(15.18 - 17.78)	16.8	(14.53 - 19.41)	1 (2)	5 (8)[c]	0.001	<0.001
20:0	0.44	(0.36 - 0.53)	0.42	(0.31 - 0.67)	8 (13)	1 (2)	0.419	0.394
22:0	1.85	(1.50 - 2.17)	1.69	(1.32 - 2.15)	4 (7)[c]	0 (0)	<0.001	<0.001
24:0	4.62	(3.88 - 5.19)	4.44	(3.52 - 5.27)	4 (7)	1 (2)	0.129	0.169
26:0	0.23	(0.17 - 0.30)	0.23	(0.16 - 0.33)	3 (5)	2 (3)[c]	0.342	0.436
ALA	0.18	(0.13 - 0.28)	0.14	(0.08 - 0.34)	22 (36)	1 (2)	<0.001	<0.001
EPA	0.44	(0.23 - 0.93)	0.5	(0.16 - 1.98)	2 (3)	2 (3)[d]	0.223	0.501
22:5ω3	1.85	(1.40 - 2.46)	1.87	(1.24 - 2.63)	2 (3)	3 (5)[d]	0.878	0.587
DHA	3.75	(2.29 - 5.45)	3.13	(1.34 - 5.91)	8 (13)	1 (2)[c]	<0.001	<0.001
LCPω3	6.17	(4.39 - 8.28)	5.45	(3.35 - 10.36)	7 (11)	2 (3)[d]	<0.05	<0.005
ω3	6.38	(4.59 - 8.44)	5.69	(3.44 - 10.53)	8 (13)	2 (3)[d]	<0.05	<0.005
LA	10.28	(8.26 - 13.03)	9.79	(8.17 - 12.70)	1 (2)	0 (0)	0.051	0.074
20:2ω6	0.26	(0.18 - 0.42)	0.23	(0.15 - 0.41)	4 (7)	0 (0)	<0.005	<0.05
20:3ω6	1.57	(1.23 - 2.14)	1.78	(1.10 - 3.05)	4 (7)	12 (20)	<0.05	<0.05
AA	13.87	(12.15 - 15.91)	13.42	(9.96 - 16.34)	7 (11)[c]	1 (2)	<0.05	<0.01
22:4ω6	2.77	(1.99 - 3.53)	2.49	(1.46 - 3.28)	7 (11)[c]	0 (0)	<0.001	<0.001
22:5ω6	0.50	(0.35 - 0.70)	0.47	(0.29 - 0.76)	8 (13)[d]	2 (3)	<0.05	0.097
LCPω6	18.73	(16.42 - 21.42)	18.72	(13.79 - 20.29)	5 (8)[c]	0 (0)	0.057	0.136
ω6	29.31	(26.48 - 31.87)	28.62	(23.51 - 31.42)	8 (13)[d]	0 (0)	<0.001	<0.005
18:1ω7	1.76	(1.36 - 2.23)	1.27	(0.93 - 2.65)	42 (69)[c]	1 (2)	<0.001	<0.001
ω7	1.84	(1.40 - 2.31)	1.27	(0.93 - 2.65)	42 (69)[c]	1 (2)	<0.001	<0.001
18:1ω9	10.76	(9.16 - 11.48)	11.61	(9.63 -13.50)	0 (0)	33 (54)[c]	<0.001	<0.001
20:1ω9	0.22	(0.14 - 0.31)	0.22	(0.13 - 0.30)	2 (3)	0 (0)	0.451	0.489
20:3ω9	0.25	(0.13 - 0.42)	0.17	(0.09 - 0.67)	13 (21)	2 (3)	<0.001	<0.001
24:1ω9	3.37	(2.65 - 4.29)	3.75	(2.47 - 4.71)	1 (2)	5 (8)	<0.001	<0.001
ω9	14.48	(13.05 - 15.93)	15.78	(13.72 - 18.52)	0 (0)	27 (44)[c]	<0.001	<0.001
SAFA	47.76	(46.68 - 48.88)	48.57	(46.49 - 53.82)	1 (2)	19 (31)[c]	<0.001	<0.001
MUFA	16.1	(14.44 - 17.54)	16.89	(14.90 - 19.50)	0 (0)	15 (25)[c]	<0.001	<0.001
PUFA	36.13	(34.39 - 37.72)	34.46	(28.62 - 37.24)	25 (41)[c]	0 (0)	<0.001	<0.001
LCPω3+LCPω6	25.19	(23.16 - 27.57)	24.18	(19.57 - 26.86)	12 (20)	0 (0)	<0.001	<0.001
LCPω3/LCPω6	0.32	(0.22 - 0.48)	0.29	(0.18 - 0.75)	4 (7)	3 (5)[d]	0.325	0.062
PUFA/SAFA	0.76	(0.72 - 0.80)	0.71	(0.53 - 0.80)	36 (60)[d]	0 (0)	<0.001	<0.001
ω3+ω6	35.79	(33.83 - 37.74)	34.31	(27.94 - 37.11)	23 (38)	0 (0)	<0.001	<0.001
ω3/ω6	0.21	(0.15 - 0.31)	0.20	(0.11 - 0.45)	6 (10)	2 (3)[d]	0.399	0.096
20:3ω9/20:4ω6	0.02	(0.01 - 0.03)	0.01	(0.01 - 0.06)	11 (18)	3 (5)	0.001	<0.005
22:6ω3/22:5ω3	2.14	(1.14 - 2.97)	1.71	(0.77 - 3.64)	7 (11)	1 (2)	<0.005	<0.005
22:5ω6/20:4ω6	0.04	(0.02 - 0.05)	0.03	(0.02 - 0.06)	0 (0)	1 (2)	0.154	0.201
20:5ω3/22:6ω3	0.12	(0.06 - 0.20)	0.16	(0.07 - 0.33)	0 (0)	18 (30)[d]	<0.001	<0.001
22:5ω6/22:6ω3	0.13	(0.07 - 0.22)	0.16	(0.05 - 0.33)	3 (5)[c]	9 (15)	0.465	0.194
18:2ω6/18:3ω3	56.01	(36.03 - 82.31)	72.72	(28.48 - 142.78)	1 (2)	17 (28)	<0.001	<0.001

BMI correlated positively with RBC 20:0 and 20:3ω6 ($p<0.05$ for both). Duration of illness correlated negatively with LA ($p<0.005$; $r= -0.438$). Smokers had lower RBC 14:0 ($p<0.05$) and higher 24:1ω9 ($p<0.01$). Female smokers had lower 24:0 and 26:0 ($p<0.05$ for both) levels than male smokers. Alcohol users had higher 16:0, 20:0, ω9 (notably 18:1ω9), compared with non-alcohol users, whereas their 26:0, PUFA (notably ω3+ω6) and LCPω3+ω6 were lower ($p<0.05$ for all). The number of alcohol units consumed per day did not correlate with any fatty acid except for 22:5ω3 ($p=0.05$, $r=0.331$). Patients reaching the recommended fish-intake (i.e. $Q\geq4$; 11/52) had higher SAFA (notably 16:0), ω3, LCPω3 (notably EPA) and lower ω6, LCPω6 (notably AA and 22:4ω6) and PUFA/SAFA compared with patients who did not reach the recommended fish intake ($p<0.05$ for all). The index for fish-fat intake (Q) correlated ($p<0.005$ for all) with the ω3/ω6 ratio ($r=0.397$), LCPω3/LCPω6 ratio ($r=0.441$), EPA ($r=0.528$) and LCPω6 ($r= -0.414$).

In a logistic regression model ($p<0.05$) RBC PUFA below the P2.5 of the reference range proved best predicted in the total group by alcohol consumption (Wald $\chi^2 = 5.495$, $p<0.05$; odds ratio = 5.609, 95% CI 1.327 – 23.712) and duration of illness (Wald $\chi^2 = 4.246$, $p<0.05$; odds ratio = 0.822, 95% CI 0.683 – 0.988), when tested for alcohol consumption, duration of illness, age and gender. Linear regression showed the variance in RBC 20:3ω9 to be explained for 52% by ALA ($\beta= -0.298$, $p<0.005$), LA ($\beta= -0.505$, $p<0.001$) and DHA ($\beta= -0.369$, $p<0.001$) in the total group, when we tested for ALA, LA, 22:5ω6, DHA and SAFA. In females 51% of the variance in RBC 20:3ω9 could be explained by LA ($\beta= -0.727$, $p<0.001$), when testing for LA and ALA. In males LA ($\beta= -0.569$, $p<0.001$) and DHA ($\beta= -0.643$, $p<0.001$) explained 49% in RBC 20:3ω9 of the variance, when we tested for ALA, LA and DHA.

3.3. B-vitamins and homocysteine

Table 3 shows the B-vitamin status parameters of the patients, together with the percentage patients exhibiting values below and above the respective reference ranges. Between (sub)group comparisons were done with all patients and with the subgroup reporting no intake of vitamin supplements. B-vitamin status parameters were not related to BMI, DSM-IV classification, medication type and dose, and alcohol consumption. In our data set levels of homocysteine did not differ among the various age groups of male patients.

Table 3. *B-vitamin status parameters of patients with schizophrenia.*

	Reference values	Patients Median	(range)	Patients N<P2.5	n>P97.5	Patients vs. Controls P [a]	P [b]
Folate (nmol/L)	4.0 - 30.0	9	(3.4 - 38)	2 (3)	2 (3) [c]	0.121	0.079
Vitamin B_6 (nmol/L)	35 - 136	68	(26 - 382)	3 (5)	6 (10) [c]	0.587	0.317
Vitamin B_{12} (pmol/L)	170 - 700	211	(77 - 944)	17 (28)	1 (2)	<0.05	<0.001
Homocysteine (μmol/l)	4.7-14.6 [d]	11.6	(5.9 - 74.8)	0 (0)	17 (28)	<0.001	<0.001
	9.3 [e]			13 (21) [f]	48 (79)		

Data are for 61 patients. B-vitamin status parameters were measured in serum (folate, vitamin B_{12}), plasma (homocysteine) and whole blood (vitamin B_6). Data represent medians (range). The numbers (%) of patients with levels either below (n<P2.5) or above (n>P97.5) the reference ranges were assessed. Abnormal homocysteine was also assessed with the use of cut-off values at 9.3 and 14.6 μmol/L, which represent the P97.5 of B-vitamin optimized healthy adults and the P97.5 of these adults before B-vitamin optimization [44], respectively. Raw data for between-group comparisons derived from [44]. P<0.05 was considered significant for between group analyses. [a] Significance (p) for all patients (n=61) vs. controls (n=101); [b] Significance (p) for patients (n=57) vs. controls (n=79) reporting no intake of vitamin supplements; [c] Patients (n=2) reporting vitamin intake are included; [d] P97.5 derived from raw data [44] were used to evaluate individual patients; [e] Vitamin-optimized reference value [44]; [f] Patients (n=4) reporting intake of vitamins are included.

Most remarkable were the lower levels of vitamin B_{12} ($p<0.001$; 28% below P2.5) and the higher levels of homocysteine ($p<0.001$). Twenty-eight percent of patients, versus 2% of controls ($p<0.001$), had homocysteine levels above the P97.5 of the reference range; while 79% had values above the 'vitamin-optimized cut-off value'. A higher percentage of patients than controls had vitamin B_{12} levels below local reference values (28 vs. 3% below 170 pmol/L, $p<0.001$). Four patients, all males, aged 26-53 years, exhibited moderate hyperhomocysteinemia (range 57.5-74.8 μmol/L). None of these reported the intake of supplements. Vitamin B_6 levels were lower ($p<0.05$) in smoking than in non-smoking patients. Schizophrenic female smokers had lower homocysteine ($p<0.005$) than male smokers. Fish fat intake (Q) correlated positively with levels of folate ($p<0.01$, $r=0.371$) and vitamin B_{12} ($p<0.001$, $r=0.548$).

In a logistic regression model ($p<0.05$) a homocysteine above 14.6 μmol/L proved best predicted by male gender (Wald $\chi^2 = 4.774$, $p= 0.029$; odds ratio = 7.693, 95% CI 1.234 – 47.965) and absence of fish consumption (Wald $\chi^2 = 4.224$, $p= 0.039$; odds ratio = 0.1, 95% CI 0.011 – 0.894), when tested for gender, age, absence of fish consumption and smoking. The variance in plasma homocysteine could be explained for 47% by folate ($\beta= -0.611$, $p<0.001$) and ALA ($\beta= -0.317$, $p<0.005$) in the total group, when we tested for folate, vitamin B_{12}, vitamin B_6, fish fat intake, ALA and EPA. In females 45% of the homocysteine variance could be explained by

vitamin B_6 (β= -0.508, $p<0.01$) and vitamin B_{12} (β= -0.348, $p<0.05$), when we tested for folate, vitamin B_{12} and vitamin B_6. In males the homocysteine variance could be explained for 48% by folate (β= -0.701, $p<0.001$), testing for folate, vitamin B_{12} and vitamin B_6.

3.4. Dietary supplementation

None of the 5 patients with either biochemical EFA deficiency, moderate hyperhomocysteinemia or both was expected by their treating physicians to have poor diets. Four of them were available for supplementation with B-vitamins, and soybean and fish oils. The **Figure** shows the courses of their homocysteine and RBC 20:3ω9 during supplementation. The supplementation schemes were different for the 3 patients with hyperhomocysteinemia-only and the one with combined hyperhomocysteinemia and biochemical EFA deficiency. One of the 3 patients with hyperhomocysteinemia-only showed an immediate homocysteine decrease upon administration of the initial daily dosage of 800 μg folic acid, while the remaining 2 showed decreases upon the addition of other B-vitamins, including 8 mg vitamin B_6 and 4 μg vitamin B_{12}. The final homocysteine levels of these 2 patients were below 14.6 μmol/L (P97.5) and only one had a level below the more appropriate vitamin-optimized cut-off value of 9.3 μmol/L. The patient who did not reach a homocysteine below 14.6 μmol/L admitted to his clinician to be incompliant with respect to the intake of the B-vitamin supplement. His relatively low RBC folate level, as compared with the two others (data not shown) also suggested poor compliance, since RBC folate is a parameter of the folate status of the preceding weeks. The patient with combined hyperhomocysteinemia and biochemical EFA deficiency showed an initial increase of plasma homocysteine, which was probably due to our request to discontinue the reported infrequent intake of a multivitamin supplement. Addition of folic acid and other B-vitamins reduced homocysteine to a final level below 14.6 μmol/L but above 9.3 μmol/L.

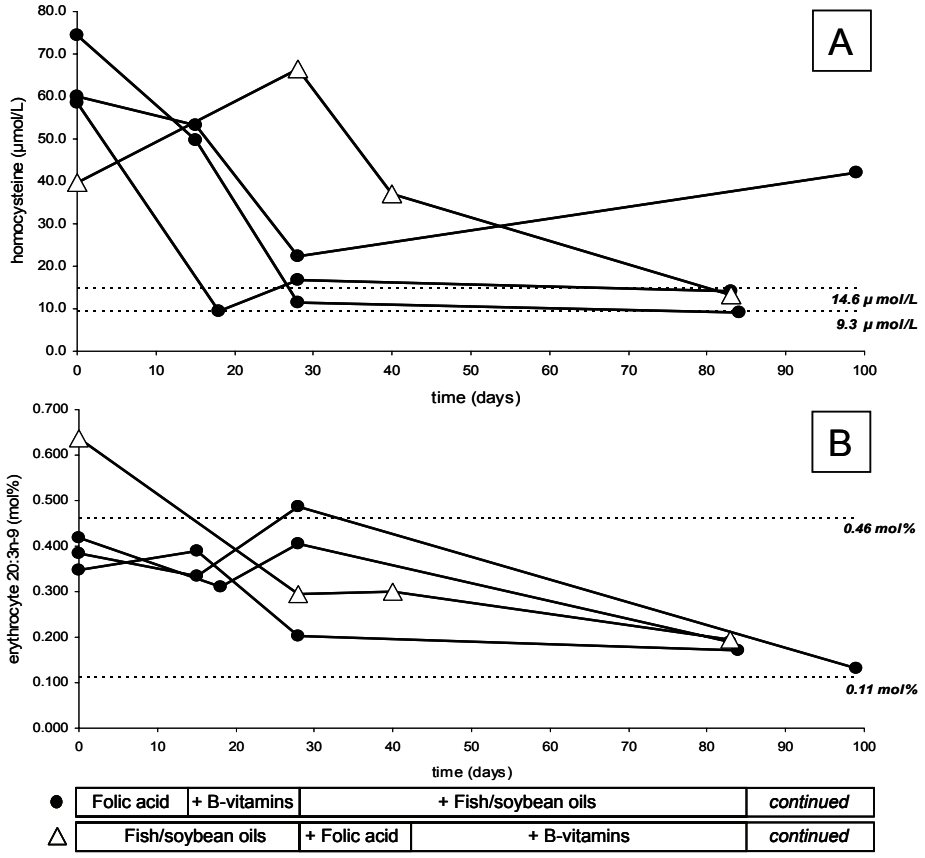

Figure. ***Courses of plasma homocysteine (A) and erythrocyte 20:3ω9 content (B) for 4 patients with schizophrenia during B-vitamin, soybean oil and fish oil supplementation.***

Selection of the 4 patients for supplementation was based on either moderate hyperhomocysteinemia (i.e. 31-100 µmol/L) or biochemical signs of essential fatty acid deficiency (erythrocyte 20:3ω9>0.46 mol%). Supplementation schemes (bottom) were different for the 3 patients with moderate hyperhomocysteinemia (•), and the one with combined hyperhomocysteinemia and essential fatty acid deficiency (△). Daily supplements were: Folic acid (800 µg folic acid), B-vitamins (8 mg vitamin B_6, 4 µg vitamin B_{12} and other B-vitamins), Fish oil (310 mg eicosapentaenoic acid (EPA; 20:5ω3) and 200 mg docosahexaenoic acid (DHA; 22:6ω3), and Soybean oil (15 mL soybean oil). The patient who exhibited an initial homocysteine decrease and a subsequent increase admitted to be incompliant with respect to B-vitamin intake.

Supplementation of the patient with combined hyperhomocysteinemia and biochemical EFA deficiency with soybean and fish oils rapidly reduced RBC 20:3ω9

to levels within the reference range. Also the 3 patients with hyperhomocysteinemia-only exhibited decreases of RBC 20:3ω9, although their changes occurred largely within the reference range. All patients seemed compliant with regard to the intake of the soybean oil and fish oil supplements, since all of them exhibited clear increases in their RBC LA, EPA and DHA (not shown).

4. Discussion

We assessed the frequency of low B-vitamin and EFA status, its determinants and its predictors in a group of patients with schizophrenia. Furthermore we were interested to see whether those with poor status responded biochemically to the appropriate dietary supplements.

Most remarkably we found that, as a group, the patients had high RBC SAFA and MUFA and low PUFA, both from the ω3 and ω6 series. Lower PUFA status seems in contrast to a recent study of Strassnig et al. [45] who reported increased fat, SAFA and PUFA intakes in schizophrenia. Their results were, however, not accompanied by laboratory data showing the actual PUFA status of their patients. RBC PUFA below the P2.5 of the reference range proved best predicted by alcohol consumption and duration of illness. Not unexpectedly, the index of biochemical EFA deficiency (i.e. RBC 20:3ω9) proved best explained by low RBC ALA, LA and DHA. Two patients exhibited biochemical EFA deficiency and 7 showed biochemical signs of ω3/DHA marginality only. Of these nine, at least 5 (i.e. ≥56%) reported not to eat fish at all as compared to 2% in the rest of the study population. The employed index for fish fat intake proved reliable, since there was also a positive relation between the calculated intake of fish oil fats and ω3LCP status. DSM-IV classification, duration of illness, gastrointestinal disturbances, BMI, smoking, alcohol drinking and medication were not related to the indices of EFA (20:3ω9) or B-vitamin (homocysteine) deficiencies.

With respect to B-vitamin status we found low vitamin B_{12} levels (28%) and high homocysteine (28%) in the group of patients not reporting to take vitamin supplements, and lower vitamin B_6 in smoking compared with non-smoking patients. A homocysteine above 14.6 μmol/L proved best predicted by male gender. Homocysteine variance proved best explained by folate and ALA in the total group. The percentage homocysteine variance explained by folate and vitamin B_{12} in our patient group was higher than corresponding data from a recent study in Israel [46]. The influence of ALA was unexpected, but in a subsequent analysis both folate and vitamin B_{12} proved positively related with our index of fish oil intake, suggesting that we were dealing with poor dietary habits in general and not e.g. diminished intake of certain food products. Four patients exhibited moderate hyperhomocysteinemia with plasma values ranging from 57.5-74.8 μmol/L. These

are exceptionally high values that in our experience are only rarely found in routine patient care. One of these patients also had biochemical EFA deficiency, which adds to the contention that some patients are subject to poor dietary habits in general. EFA deficiency [47] and hyperhomocysteinemia with homocysteine levels in Israeli schizophrenic patients up to 80 μmol/L have recently been reported [46]. It should be noted that our study was the first to show a low EFA status as derived from increased 20:3ω9. This fatty acid, also named Mead acid, is a functional parameter of the EFA status [38], and is rarely found to be increased in adults with undisturbed fat absorption consuming typically Western diets.

Importantly, none of the 5 patients with biochemical EFA deficiency, moderately increased homocysteine, or both, was suspected to have a poor diet, when we informed their clinicians of these outcomes. That diet is nevertheless at the basis of their abnormal indices of EFA and B-vitamin status became confirmed by the supplementation study that we performed in 4 of them. This study showed these indices to be easily correctable by supplemental B-vitamins, and soybean and fish oils, with folic acid probably being the most important determinant of hyperhomocysteinemia. However, 3 of the 4 patients did not reach the 9.3 μmol/L upper limit of the plasma homocysteine concentration, that we achieved by supplementation of healthy adults with a combination of folic acid, vitamin B_{12} and vitamin B_6 [38]. Prolonged supplementation or addition of betaine to the supplement might be of aid to reach this treatment goal, but compliance seems to be a more realistic subject of concern.

We conclude that, as a group, patients with schizophrenia have high RBC SAFA and MUFA, low PUFA and increased homocysteine. A subgroup of patients with schizophrenia has biochemical EFA deficiency, ω3/DHA marginality, moderate hyperhomocysteinemia, or combinations. This subgroup is not readily identified by their clinicians, while these conditions proved easily correctable by supplementation. Correction may be indicated in view of the possible relationship of poor EFA and B-vitamin status with their psychiatric symptoms, but also in view of the reduction of their high risk of cardiovascular disease. There is compelling, though not definite, evidence that reduction of homocysteine by B-vitamin supplementation (notably folic acid) and augmentation of ALA and ω3LCP status (by supplementation of soybean and fish oils) decreases risk of cardiovascular disease. Our study does not allow definite conclusions regarding risk factors for low PUFA and increased homocysteine that would allow their easy identification in clinical practice. Alcohol consumption and duration of illness seem to be risk factors for low PUFA status (i.e. <P2.5 of reference range), while male gender and absence of fish consumption seem to predict hyperhomocysteinemia (>P97.5). These factors and their predictive values should be investigated in larger studies. To

this end possible options for the identification of patients with poor diets are monitoring of dietary practices, or the more reliable, but also more costly, laboratory screening. Another possibility is supplementation of all patients without any monitoring or testing. The costs of either of these approaches and the anticipated compliance of intervention must be weighed against the potential clinical benefits with regard to the amelioration of psychiatric symptoms and the reduction of cardiovascular risk.

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Chapter 3

Brief Report: Normal intestinal permeability at elevated platelet serotonin levels in a subgroup of children with pervasive developmental disorders in Curaçao (The Netherlands Antilles)

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Abstract

This study investigated the relationship between platelet (PLT) serotonin (5-HT) and intestinal permeability in children with pervasive developmental disorders (PDD). Differential sugar absorption and PLT 5-HT were determined in 23 children with PDD. PLT 5-HT (2.0-7.1 nmol/10^9 PLT) was elevated in 4/23 patients. None exhibited elevated intestinal permeability (lactulose/mannitol ratio: 0.008-0.035 mol/mol). PLT 5-HT did not correlate with intestinal permeability or GI tract complaints. PLT 5-HT correlated with 24 h urinary 5-hydroxyindoleacetic acid (5-HIAA; p=0.034). Also urinary 5-HIAA and urinary 5-HT were interrelated (p=0.005). A link between hyperserotonemia and increased intestinal permeability remained unsupported. Increased PLT 5-HT in PDD is likely to derive from increased PLT exposure to 5-HT. Longitudinal studies, showing the (in)consistency of abnormal intestinal permeability and PLT 5-HT, may resolve present discrepancies in the literature.

1. Introduction

Autism has been linked to gastrointestinal (GI) disturbances [1]. It is, however, questionable whether GI anomalies in children with autism are specific [2]. An increase of chronic diarrhea, constipation, abdominal bloating and food regurgitation has been found in some studies, but could not be confirmed in more recent studies (reviewed in [2]). Increased GI permeability, as established through the differential sugar absorption test (SAT), was demonstrated in 9/21 (43%) [3] and 19/26 (76%) [4] of patients diagnosed with autism. The SAT measures the integrity of the intestine by the ingestion of two indigestible saccharides that after GI uptake become fully excreted in urine. One of these (usually lactulose) passes the intestinal wall through paracellular transport ('leakage'), while the other (usually mannitol) passes by paracellular and transcellular transport. The urinary lactulose/mannitol ratio is used as a measure of intestinal integrity and permeability [5]. In addition, a recent study found a high prevalence of congenital GI anomalies (adjusted odds ratio 5.1, 95% confidence interval 1.8-14.1), notably pyloric stenosis, in autism, which may be linked to the high rate of GI dysfunction reported by their parents [6].

The implication of the GI tract in autistic pathophysiology warrants more detailed investigation of the gut-brain axis [2]. Especially the role of serotonin (5-hydroxytryptamine; 5-HT), as a messenger within this axis [7], deserves attention. Many different aspects of the 5-HT system in autism have already been studied [8-11]. A recent report [11] on platelet (PLT) 5-HT in PDD showed PLT hyperserotonemia in approximately 36% of patients with autism and in 58% of patients with PDD not otherwise specified (NOS). Using mixture-modeling analysis Mulder et al. [11] derived an empirical cut-off value that enabled dichotomization of patients with PDD into normo- and hyperserotonemic. Extensive behavioral assessments did, however, not show significant correlates with PLT 5-HT or hyperserotonemic status.

A common (developmental) factor, causing both an autistic brain and deregulated 5-HT release from the GI tract, years after birth, may be involved in the etiology of PDD [10]. The primary site of the hyperserotonemia in autism is likely to be located in the GI tract. Serotonin is a biogenic amine that derives from the essential amino acid tryptophan (Tryp) by hydroxylation and subsequent decarboxylation. The GI tract contains about 80% of bodily 5-HT, which is unevenly distributed among the enterochromaffin cells (90-95%) and neurons (5-10%) [12]. The main functions of 5-HT are in smooth muscle contraction, blood pressure regulation, and peripheral and central neurotransmission. Serotonin localized in the basolateral stores of enterochromaffin tissue is released upon

neuronal, chemical or mechanical stimulation. Several 5-HT receptors control GI motility, sensation and secretion [7]. Following its release, 5-HT is removed from the interstitial space by 5-HT selective reuptake transporters [7]. However, part of the 5-HT enters the portal blood and systemic circulation where it is either rapidly taken up and accumulated by PLT, or metabolized by the liver, lung and kidneys into its major metabolite 5-hydroxyindoleacetic acid (5-HIAA) [12]. Platelets store and transport the majority (99%) of circulating 5-HT [13].

Elevated PLT 5-HT levels observed in subgroups of patients with PDD, may be related to increased GI motility. This notion is supported by higher PLT 5-HT in patients with diarrhea predominant irritable bowel syndrome (d-IBS), as compared with healthy controls [12], although this was not consistently found [14]. Patients with d-IBS have augmented GI motility, which is likely to cause increased exposure of their circulating PLT to 5-HT [7;12;14]. Increased PLT 5-HT is also observed in patients with carcinoid tumors [15]. Carcinoid tumors derive from enterochromaffin cells and are characterized by high 5-HT production with diarrhea as a frequent symptom [16]. Consequently, measurement of PLT 5-HT is used as a sensitive marker for the early diagnosis and the subsequent follow-up of patients with carcinoid tumors [15].

The aim of the present study was to investigate whether the subgroup of children with PDD having increased PLT 5-HT levels, is the same as the one exhibiting increased intestinal permeability as established by a SAT.

2. Methods

2.1. Patients

Parents of patients with PDD (n=31) according to the DSM-IV TR [17] were asked for the participation of their affected children via the local patient society and pediatricians. Oral and written informed consent were obtained. Information regarding comorbidity, medication, nutritional supplements and the prevalence of GI related complaints was obtained from medical records and with the aid of assisted questionnaires. The study was performed in Curaçao (The Netherlands Antilles) in the summer of 2004. All collected urine and blood samples were transported in dry ice to the Netherlands for further analyses in the University Medical Center Groningen (UMCG). The study was approved by the Medical Ethical Committee of the St. Elisabeth Hospital in Curaçao.

2.2. Sugar Absorption Test

The SAT was performed according to van Elburg et al. [5]. Shortly, the patients ingested a sugar (lactulose, mannitol and sucrose) containing test fluid after an

overnight fast. All urine voidings during the following 5 h were collected and pooled. Urinary sugars were analyzed by gas chromatography as previously described [18]. A urinary lactulose/mannitol (L/M) ratio above 0.090 was considered to be indicative for abnormal GI integrity/increased intestinal permeability [5].

2.3. Serotonin Assays

For estimation of 5-HT turnover and exposure of PLT to 5-HT, we examined 24 h urinary excretion of 5-HIAA and total 5-HT [15]. For this, parents were asked to abstain their child completely from 5-HT containing foods (e.g. banana, pineapple, kiwi, walnuts) during collection and during the preceding 12 h. The volumes of the urine samples were measured before storing at -20°C. Urinary 5-HIAA and total 5-HT concentrations were determined as previously reported [15]. Urinary 5-HIAA values were evaluated with the use of age-dependent reference values [19].

Non-fasting venous blood (for serum antibodies) and EDTA-anticoagulated blood (all other assays) were collected from children with PDD. EDTA-anticoagulated blood was placed on melting ice. Hematological indices were measured immediately after sampling. Within one hour after collection a 1:1 mixture of K_2EDTA and $Na_2S_2O_5$ was added to PLT-rich plasma (PRP) to prevent oxidation of indoles. Plasma and serum were stored at -80°C. Simultaneous analysis of indoles [Tryp, 5-hydroxytryptophan (5-HTP), 5-HT and 5-HIAA] in PRP was performed as previously described [15]. PLT 5-HT data were compared with both a local cut-off value of 5.4 nmol/10^9 PLT [20] and an empiric cut-off value of 4.55 nmol/10^9 PLT [11]. The local cut-off value represents the 97.5th percentile of a reference group of healthy adults that is employed in our laboratory for the diagnosis of carcinoid tumors. The empirical cut-off value represents the bottom of the valley of the PLT 5-HT bimodal distribution, as exhibited by patients with PDD. This value allows optimal classification into those who are normoserotonemic and hyperserotonemic. Platelet-rich-plasma Tryp data were evaluated with the use of age-dependent reference values [19].

2.4. Exclusion of Celiac Disease

Serum IgA anti-endomysium titers and HLA genotype were assessed to rule-out celiac disease, which is an established cause of increased GI permeability [21].

2.5. Statistics

All data were analyzed using the Statistical Product and Service Solutions package, version 11.5 (SPSS Inc. Chicago). Data were tested for normality using the Shapiro-Wilk W test. Group comparisons (normo- and hyperserotonemic) were performed

with the Mann-Whitney U test (non-parametric). Spearman (non-parametric) tests were used to evaluate correlations at α=0.05, minimizing type-II errors.

3. Results

3.1. Patients

We enrolled the first 24 (77%) of the 31 patients with PDD whose parents agreed to participate. Patient characteristics are reported in **Table 1**. The parents reported 13/23 (57%) of their affected children to have one or more GI symptoms.

Table 1. *Characteristics of patients with pervasive developmental disorders in Curaçao tested for platelet serotonin and intestinal permeability.*

Parameter	
Gender (male/female)	18 (75%) / 6 (25%)
Age (years)	9.9 (±3.9)
DSM-IV TR diagnoses	
- 299.00 (autistic disorder)	8 (33%)
- 299.80 (PDD-NOS)	16 (67%)
Ethnicity	
- Caucasian	8 (33%)
- African-American	13 (54%)
- other	3 (13%)
Comorbidity[a]	
- epilepsy	5 (22%)
- allergy	2 (9%)
- asthma	1 (4%)
- intestinal yeast infection	1 (4%)
Medication for comorbidity	9 (38%)
Nutritional supplements (vitamins/ω3-oils)	9 (38%) / 2 (4%)
Diet (gluten and casein free) [a]	2 (9%)
Physical complaints related to GI tract [a]	13 (57%)
- nausea	0 (0%)
- vomiting	1 (4%)
- diarrhea	4 (17%)
- constipation	4 (17%)
- bloating and gaseousness	8 (35%)

Data represent number (percentage) or mean (±SD) for 24 patients, unless otherwise specified. [a] n=23. PDD-NOS; pervasive developmental disorder – not otherwise specified.

Because of cleanliness problems, no urine samples were obtained from one patient, while from another we only received urine for SAT. Blood sampling from yet another patient was problematic. Consequently, our study comprised urine for SAT

from 23/24 patients, 24 h urine from 22/24 patients and blood samples from 23/24 patients. **Table 2** shows the indices related to 5-HT turnover and intestinal permeability of these patients.

Table 2. *Indices of serotonin (5-HT) turnover and intestinal permeability in patients with pervasive developmental disorders in Curaçao.*

Parameter		Reference values	< RV	≥ RV
PLT 5-HT (nmol/10⁹ PLT) [a]	3.4 (2.0-7.1)	<4.55 [b]		6 (26%)
		<5.4 [c]		4 (17%)
		2-18y: 0-79 [d]	0 (0%)	0 (0%)
Tryp (µmol/L) [a]	50.0 (±10.9)	2y: 35-73 [d]		
		6y: 37-76 [d]	3 (13%)	0 (0%)
		16y: 54-93 [d]		
5-HIAA (µmol/24h) [e]	8.4 (3.9-36.4)	3-8y: 2.1-29.3 [d]		
		9-12y: 5.2-32.9 [d]		
		13-17: 4.7-34.0 [d]	1 (5%)	1 (5%)
		>18y: 5.2-36.6 [d]		
5-HT (nmol/24h) [e]	305 (±92)			
lactulose (mmol/5h) [f]	0.024 (±0.11)			
mannitol (mmol/5h) [f]	1.32 (±0.59)			
L/M ratio (mol/mol) [f]	0.019 (±0.007)	<0.090 [g]	23 (100%)	0 (0%)

Data represent number (percentage), median (range) or mean (±SD). [a] platelet-rich plasma, n=23; [b] empiric cut-off derived from Mulder et al. [11]; [c] upper reference value derived from apparently healthy adults [20]; [d] from reference [19]; [e] 24 h urine, n=22; [f] 5 h urine from sugar absorption test, n=23; [g] from reference [5]. RV; reference value; PLT, platelet; Tryp, tryptophan; 5-HIAA, 5-hydroxyindoleacetic acid; L/M, lactulose/mannitol.

3.2. Sugar Absorption Test

Intestinal permeability, reflected by the L/M ratio [median (range) 0.017 mol/mol (0.008-0.035)], indicated that none of the patients had increased intestinal permeability (i.e. L/M ratio ≥0.090).

3.3. Serotonin Assays

PLT 5-HT [median (range): 3.4 (2.0-7.1) nmol/10⁹ PLT] was elevated in 4 (range: 5.7-7.1 nmol/10⁹ PLT) and 6 [range 4.6-7.1 nmol/10⁹ PLT] patients if compared to the local cut-off value ([20]; <5.4 nmol/10⁹ PLT) or the empirical cut-off value ([11]; <4.55 nmol/10⁹ PLT), respectively. The sole patient exhibiting detectable levels of plasma 5-HIAA (26.0 µmol/L), also exhibited increased PLT 5-HT (6.4 nmol/10⁹

PLT). However, this patient did not exhibit increased urinary total 5-HT or 5-HIAA excretions. Urinary excretion of 5-HIAA was within normal range.

3.4. Exclusion of Celiac Disease

None of the patients were positive for serum IgA anti endomysium and 8/23 (35%) patients had a genotype positive for either HLA-DQ2 (n=5) or HLA-DQ8 (n=3). Based on the results of serology, none of the patients seemed to have celiac disease, although we did not perform further tests to exclude this.

3.5. Statistics

There was no correlation between PLT 5-HT and L/M ratio ($p= 0.663$; $r = -0.098$). Patients exhibiting GI tract complaints did not have higher PLT 5-HT, higher L/M ratios, or higher 5-HIAA and total 5-HT 24 h urinary excretions ($p>0.4$). Platelet 5-HT correlated with 24 h urinary 5-HIAA excretion ($p= 0.034$; $r= 0.465$). Also the 24 h excretion rates of 5-HIAA and total 5-HT showed a positive correlation ($p= 0.005$; $r= 0.580$).

4. Discussion

In this study of children with PDD we did not observe a relation between PLT 5-HT and intestinal permeability, as derived from the urinary L/M ratio. The number of children with PDD exhibiting increased PLT 5-HT was lower compared with reports of others. For instance, the recent study of Mulder et al. [11] showed 23/81 (28%) of Dutch children with PDD to exhibit increased PLT 5-HT, using the same analytical method and a cut-off value of 5.4 nmol/10^9 PLT. Also our data on intestinal permeability contrast with previous reports [3;4] showing that 43-76% of children with autism have increased intestinal permeability, as established by a SAT. A weakness of the current study is its small size and the lack of a local age- and gender matched control group. We have, on the other hand, no indications for deviant reference values for PLT 5-HT or L/M ratios in Curaçao, as compared with The Netherlands. Age and gender do not appear to affect PLT 5-HT [11], but it must be noted that PLT 5-HT reaches highest levels during childhood and gradually decreases during adulthood [22]. Dependent on the cut-off values employed, we nevertheless found 4 and 6 patients with increased PLT 5-HT. Neither of these patients had abnormal intestinal permeability or L/M ratios residing in the upper range of normality. However, the positive correlation between PLT 5-HT levels and 24 h urine 5-HIAA, and also the relation between urinary 5-HIAA and urinary 5-HT, suggest that, also in PDD, exposure of PLT to 5-HT determines PLT 5-HT and its consistently found increase in a subgroup. It is possible that increased PLT 5-HT

and GI permeability are not consistent features of children with PDD in time. Long term, e.g. monthly, monitoring of a well defined patient and control group may shed more light on this potential source of variance as a cause of the conflicting results found by several investigators. Differences in the activity of the 5-HT transporter based on genetic polymorphisms are unlikely, since these seem to have minor effects, if any, on PLT 5-HT levels [23].

In conclusion, the finding of a subgroup of children with PDD exhibiting hyperserotonemia was replicated. None of the children exhibited increased intestinal permeability, while PLT 5-HT was unrelated to both intestinal permeability and GI symptoms. Additional studies are needed to elucidate the etiology of increased PLT 5-HT in PDD and to establish its relation with intestinal pathology, if any.

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Chapter 4

Relation between platelet serotonin and feeding mode in newborns suggests that gut motor activity is a determinant of platelet serotonin content

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Abstract

Background and Aims: The usefulness of platelet serotonin (PLT 5-HT) as a marker of gastrointestinal motility is as yet unclear. We determined whether PLT 5-HT is lower at a condition of relative gut motor activity quiescence (i.e. in newborns at birth) compared with a condition of normal gut motor activity (i.e. in their mothers at birth), and whether in newborns institution and discontinuation of enteral feeding coincide with increases and decreases of PLT 5-HT, respectively. *Design and Measures:* PLT 5-HT was determined in 17 mothers and their 18 healthy full term newborns. Longitudinal PLT 5-HT data and data of feeding modes were available for 5 preterm born infants. *Results:* Newborns exhibited about 2 times lower PLT 5-HT compared with their mothers (medians: 1.5 and 2.9 nmol/10^9 PLT, respectively). Newborn PLT 5-HT related positively with maternal PLT 5-HT and newborn mean PLT volume, and negatively with newborn whole blood tryptophan. In the longitudinally investigated preterm born infants we observed 7 increases and 1 decrease of PLT 5-HT during institution of enteral feeding (in 5 infants), and 2 decreases and 1 increase of PLT 5-HT during parenteral feeding (in 3 infants). *Conclusion:* Lower PLT 5-HT at birth and its change in response to enteral feeding in newborns suggest gut motor activity to be a determinant of PLT 5-HT in early postnatal life.

1. Introduction

Serotonin (5-hydroxytryptamine; 5-HT) is a biogenic amine that derives from the essential amino acid tryptophan (Tryp) by hydroxylation and subsequent decarboxylation. About 80% of the bodily 5-HT is localized in the enterochromaffin cells (90-95%) and neurons (5-10%) [1;2] of the gastrointestinal (GI) tract. Serotonin localized in the basolateral stores of enterochromaffin tissue is released upon neuronal, chemical or mechanical stimulation. Several 5-HT receptors control GI motility, sensation and secretion [1;2]. Following its release 5-HT is removed from the interstitial space by 5-HT selective reuptake transporters [3;4]. Part of the 5-HT also enters the portal blood and systemic circulation where it is either rapidly taken up and accumulated by platelets (PLT), or metabolized by the liver, lung and kidneys into its major metabolite 5-hydroxyindoleacetic acid (5-HIAA) [2;5;6]. Platelets store and transport the majority (99%) of circulating 5-HT [7].

Gastrointestinal motor activity might be reflected by PLT 5-HT. Although not consistently found [8], patients with diarrhea predominant irritable bowel syndrome (d-IBS) have higher PLT 5-HT as compared with healthy controls [2]. The augmented GI motility in these patients with d-IBS is likely to cause increased exposure of their circulating PLT to 5-HT [2]. Increased PLT 5-HT is also observed in patients with carcinoid tumors [9]. Carcinoid tumors derive from enterochromaffin cells and diarrhea is a frequent symptom [10]. Notably those carcinoid tumors originating from the midgut are characterized by high 5-HT production and measurement of PLT 5-HT is used as a sensitive marker for the early diagnosis and the subsequent follow-up of patients with carcinoid tumors [9]. Finally, 36-58% of patients with autism have been reported to exhibit increased PLT 5-HT [11]. Patients with autism have a higher prevalence of GI disturbances, but this observation is controversial [12]. A recent study shows a 5.1 times higher odds of congenital anomalies of the GI tract in children with autism [13].

The usefulness of PLT 5-HT as a marker of GI motility is as yet unclear: increased GI motility may cause increased PLT 5-HT, while decreased motility may relate to low PLT 5-HT. The fetus exhibits little GI motor activity prior to 30 weeks of gestation, but this increases steadily thereafter [14]. After 32 weeks of gestation nonmigrating motor activity patterns lengthen and decrease in occurrence, and migrating motor complexes begin to appear [15]. The use of gut motor patterns to assess gut maturity has thus been suggested [14]. There is substantial evidence that intestinal motor activity in newborns becomes stimulated by enteral nutrition [16]. Better maturation of motor function [17] and increased intestinal trophic responses [18] are observed in enterally fed preterm animals, as compared with parenteral

feeding. Consequently, the rapid doubling of PLT 5-HT levels after birth to near-adult levels during the first postnatal days [19] is likely to be caused by enteral feeding instituted maturation of gut motor activity. The aim of the present study was to contribute to the development of PLT 5-HT as a marker of GI motility by investigating whether PLT 5-HT is lower at an established physiological condition of relative gut motor quiescence (i.e. immediately after birth) and whether it subsequently changes as a function of feeding mode (i.e. enteral or parenteral). Therefore we compared PLT 5-HT of newborns with that of their mothers and monitored the course of PLT 5-HT in a small number of newborns that we were able to study during switches from enteral to parenteral feeding and vice versa.

2. Materials and Methods

2.1. Patients and Samples

For the comparison of PLT 5-HT from newborns and their mothers, venous blood samples of 17 mothers were collected together with venous umbilical cord blood samples of their 18 newborns (i.e. 16 singletons and 1 twin). Both samples were taken at delivery. Pregnant women were eligible to participate if they gave informed consent, had uncomplicated pregnancies, had uncomplicated deliveries at term, and if they did not the following: substances of abuse other than alcohol and tobacco, psychotropic medication and drugs affecting PLT 5-HT levels (e.g. selective 5-HT reuptake inhibitors). The participants were recruited from 28 consecutive expecting mothers visiting the Department of Obstetrics and Gynecology. Only newborns that were appropriate or large for gestational age according to Kloosterman [20] were included in the final evaluation.

For the longitudinal study of neonatal PLT 5-HT we collected venous blood of 20 consecutive preterm born infants (16 singletons and 2 twins) who were admitted to the neonatal intensive care unit (NICU). Additional blood samples (0.5 mL) were collected only when during their stay hematological indices were ordered by the pediatrician. Data on the course of pregnancy and delivery, anthropometrics, medication and diet, GI function (e.g. vomiting, diarrhea, bowel movements etc.) were gathered from the patient records. Only neonates of whom longitudinal samples and clinical data were available were included in the final evaluation. In all cases, enteral feeding consisted of (fortified) breast milk or Similac Special Care (Ross Products Division, Abbott Laboratories, Columbus, OH, USA), whereas parenteral feeding consisted of a 5-10% dextrose solution with amino acids (Vaminolact®); Pharmacia-Upjohn, Sweden), electrolytes, vitamins and trace elements prepared by the hospital pharmacy, and separately Intralipid 20% solution (Kabi-Vitrum, Sweden).

The studies were conducted at the Department of Obstetrics and Gynecology and the Department of Pediatrics of the St. Elisabeth Hospital in Curaçao (Netherlands Antilles). Informed consent was obtained from the mothers or both parents. The study protocols were in accordance with local ethical standards and the Helsinki declaration of 1964, as revised in 2004.

2.2. Analyses

Blood samples were collected in K_2-EDTA, immediately stored in melting ice and divided into two portions. One portion was used for the assay of hematological indices. The second was used for HPLC profiling of indoles in whole blood. A mixture of K_2EDTA and $Na_2S_2O_5$ (1:1) to a final concentration of about 10 g/L was added to prevent oxidation. Samples were subsequently stored at -80 ºC until transportation on dry ice to the University Medical Center in Groningen (The Netherlands). Tryp, 5-hydroxytryptophan (5-HTP) and 5-HT were measured with a single HPLC-fluorometric profiling method, using a previously published method [21]. PLT 5-HT was calculated by dividing the whole blood 5-HT concentration by the PLT count. The outcome was expressed in nmol/10^9 PLT.

2.3. Data evaluation and statistics

All data were analyzed using the Statistical Product and Service Solutions package, version 11.5 (SPSS Inc. Chicago). Data for newborns and their mothers were tested for normality using the Shapiro-Wilk *W* test. Non-Gaussian distributed indices were transformed by ^{e}log transformation. Differences between indices of newborns and their mothers were assessed with the paired Student's t-test with Bonferroni correction, at α=0.0036. Relations between indices of newborns and their mothers were evaluated with Pearson or Spearman correlation, following correction for multiple correlations at α=0.0036. Indices exhibiting correlations with newborn PLT 5-HT or whole blood 5-HT at α=0.05 were further investigated by backward linear regression analysis for their ability to explain the observed variance. The final model was obtained after exclusion of insignificant indices.

A change of PLT 5-HT was considered significant if it exceeded the reference change value (RCV) at a 95% confidence level [22]. The employed RCV was derived from the median intra-individual biological variation as established from PLT 5-HT data of 18 apparently healthy adults [12 males, 6 females; median (range) age 35.0 (20.8-56.6) years], who were monitored during five consecutive days (unpublished observations). Their median intra-individual coefficients of variation (CVi) for PLT 5-HT and whole blood 5-HT amounted to 7.3 and 7.4%, respectively. These figures, together with a 5-HT inter-assay variation (CVa) of ≤2.8% [21], resulted in RCV for PLT 5-HT and whole blood 5-HT of 21.6 and 21.9%,

respectively. Changes of newborn PLT 5-HT exceeding the RCV were related to changes in feeding mode as occurring within the corresponding time intervals.

3. Results

3.1. Newborn and maternal indices and their interrelations

Table 1 shows for both newborns and their mothers some anthropometric data, together with hematological indices, whole blood concentrations of Tryp, 5-HTP and 5-HT, and PLT 5-HT.

Table 1. *Platelet indices, and indoles in whole blood and platelets of newborns and their mothers at delivery*

	Newborns	Mothers	P-value
Anthropometrics			
Gender (Male / Female)	9 / 9[a]	17	
Age	38.9 (37.3-41.9) weeks[b]	33.1 (18.3-39.0) years	
Weight (kg)	3.430 (2.680-4.410)	84.0 (64.0-122.0)[c]	
AGA / LGA	16 / 2		
Platelet indices			
PLT (*10^9/L)	278 (±70)	243 (±54)	0.018[d]
MPV (fL)	8.0 (6.6-11.6)	9.2 (7.2-13.6)	0.532[d]
Indole concentrations			
Tryp (µmol/L)[e]	43.1 (±8.1)	21.7 (±4.0)	< 0.001
5-HTP (nmol/L)[e]	206 (17-301)[f]	17 (17-246)[g]	0.023[h]
5-HT (nmol/L)[e]	388 (240-712)	618 (420-1856)	< 0.001[d]
5-HT (nmol/10^9 PLT)	1.5 (0.8-3.2)	2.9 (1.9-6.4)	< 0.001[d]

Data are medians (range) or means (±SD). P<0.0036 was considered significant. [a] Two girls were twins. AGA, appropriate for gestational age [20]; LGA, large for gestational age [20]; PLT, platelets; MPV, mean platelet volume; Tryp, tryptophan; 5-HTP, 5-hydroxy tryptophan; 5-HT, 5-hydroxytryptamine; M/F male/female. [b] gestational age at birth; [c] n = 16; [d] Student's t-test performed on [e]log transformed data; [e] concentration in whole blood; [f] 5-HTP was below the 17 nmol/L [21] detection limit in 13 mothers; [g] 5-HTP was below the 17 nmol/L [21] detection limit in 8 newborns; [h] Wilcoxon Signed-Rank test.

In all samples 5-HIAA was below the detection limit of 61 nmol/L [21]. Compared with their mothers, newborns had significantly (p<0.0036) higher whole blood Tryp, but lower PLT 5-HT and whole blood 5-HT. The following correlations (Pearson coefficient; p-value) between the corresponding indices of newborns and their mothers were found to be significant (p<0.0036): RBC (r=0.757; p<0.001), Ht (r=0.726; p=0.001) and whole blood 5-HT (r=0.743; p<0.001).

The ^{e}log PLT 5-HT of the newborns was found to be related to: newborn PLT (r= -0.567; p=0.014), newborn ^{e}log MPV (r=0.476; p=0.046), newborn whole blood 5-HT (r=0.686; p=0.002), newborn Tryp (r= -0.479; p=0.044) and maternal ^{e}log PLT 5-HT (r=0.549; p=0.018). Newborn whole blood 5-HT correlated with: newborn ^{e}log PLT 5-HT (r=0.686; p=0.002), maternal ^{e}log whole blood 5-HT (r=0.743; p<0.001) and maternal ^{e}log PLT 5-HT (r=0.741; p<0.001). The ^{e}log PLT 5-HT of the mothers was found to be related to: newborn birth weight (-0.520; p=0.027), newborn whole blood 5-HT (0.741; p<0.001), newborn ^{e}log PLT 5-HT (0.549; p=0.018), and maternal ^{e}log whole blood 5-HT (0.794; p<0.001). The ^{e}log whole blood 5-HT of the mothers correlated with: newborn whole blood 5-HT (0.743; p<0.001), maternal PLT (0.590; p=0.010) and maternal ^{e}log PLT 5-HT (0.794; p<0.001).

Linear regression models for PLT 5-HT and whole blood 5-HT of both newborns and their mothers were constructed by backward stepwise linear regression analysis. Indices that were dependent to either whole blood 5-HT (i.e. PLT 5-HT and PLT) or PLT 5-HT (i.e. whole blood 5-HT and PLT) were excluded. Gestational age (GA) and birth weight were included as additional variables. Newborn ^{e}log PLT 5-HT proved best explained (adjusted R^2=0.799) by (beta, p-value) newborn Tryp (r= -2.010; p=0.002), maternal ^{e}log PLT 5-HT (r=1.035; p=0.016) and newborn ^{e}log MPV (r= 1.750; p=0.018), when testing for newborn GA, newborn birth weight, newborn ^{e}log MPV, newborn whole blood Tryp and maternal ^{e}log PLT 5-HT. Maternal ^{e}log PLT 5-HT proved best explained (adjusted R^2=0.668) by a constant [1.497 (std. error 0.452; p=0.005)] and newborn whole blood 5-HT (0.671; p<0.001) and newborn birth weight (-0.404; p=0.012) as indices, when testing for newborn GA, newborn birth weight, newborn whole blood 5-HT and newborn ^{e}log PLT 5-HT.

3.2. Relation between newborn PLT 5-HT and feeding mode

Of 20 included neonates, longitudinal PLT 5-HT and feeding data were available for only 5, since the majority of uncomplicated patients were most swiftly discharged from the NICU. **Table 2** shows the characteristics of the examined patients at the time of admission. All were preterm. One (no 3) was small for gestational age ([20;23]; birth weight <10th percentile for GA) and all had a low birth weight (<2.500 kg).

Table 2. *Characteristics of the 5 longitudinally studied preterm neonates at the time of admission.*

no	gender	birth weight	GA	corrected GA*
	(M/F)	*(g)*	*(weeks+days)*	*(weeks)*
01	F	1740	32+5	33
02	M	1620	32+6	35
03	F	1560	36+5	37
04	M	1720	30+6	33
05	M	1780	30+6	30

M/F, male/female; GA, gestational age.* Corrected GA is based on the Farr-Mitchell score which uses external characteristics at birth to estimate GA [23].

In this group (**Figure**) we observed 7 increases and 1 decrease of PLT 5-HT following institution of enteral feeding (i.e. in nos 1-5), and 2 decreases and 1 increase following institution of parenteral feeding, i.e. discontinuation of enteral feeding (respectively in nos 1, 4 and 5).

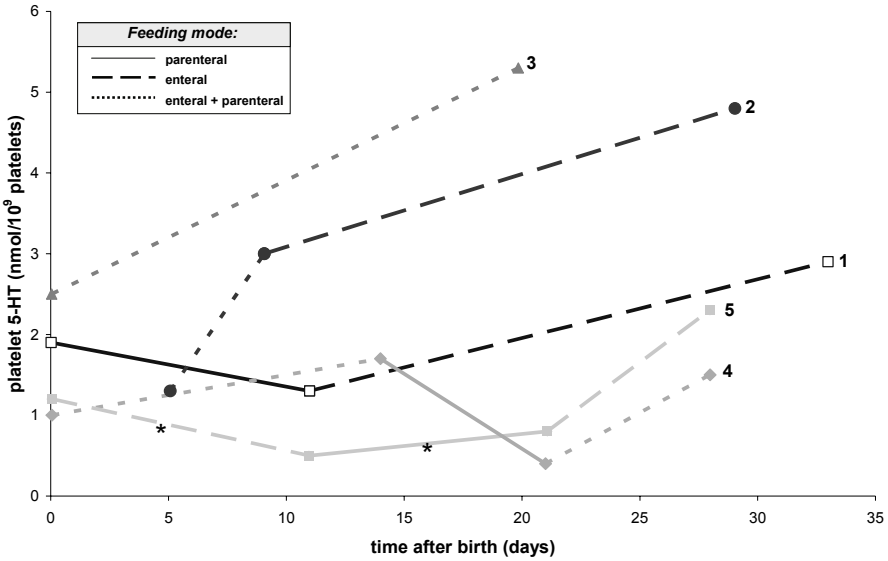

Figure. *Courses of platelet 5-HT content in time in response to changes in feeding mode in 5 preterm born newborns.*

For patient characteristics see **Table 2**. PLT 5-HT is expressed in nmol/10^9 PLT. Feeding mode in indicated periods is shown by filled lines (parenteral), dashed lines (enteral) and dotted lines (enteral plus parenteral). All indicated changes in PLT 5-HT exceeded the reference change value (i.e. 21.6%), which implies that they reflect significance at the $p<0.05$ level. The changes of PLT 5-HT were in line with an increase upon enteral or enteral + parenteral feeding, and a decrease upon parenteral feeding (i.e. discontinuation of enteral feeding), except for 2 observations in patient no 5 (indicated by *).

The 2 observations that were inconsistent with our hypothesis occurred in a single patient (no 5), while in another (no 4) initial institution of enteral feeding and subsequent switches to parenteral and enteral feeding correlated perfectly with the expected course of PLT 5-HT. The courses of whole blood 5-HT in relation to changes in feeding mode (not shown) were similar to those of PLT 5-HT, but showed an even more pronounced pattern consistent with our hypothesis.

4. Discussion

We were interested to see whether PLT 5-HT is lower at the physiological condition of relative gut motor quiescence (i.e. newborns) and whether changes in feeding mode of newborns coincide with changes of PLT 5-HT. For this we compared PLT 5-HT of healthy term newborns (n=18) with that of their mothers (n=17) and linked early postnatal switches from enteral to parenteral feeding and vice versa to changes of PLT 5-HT in 5 preterm born infants. The most important observations were: newborns have about two times lower PLT 5-HT compared with their mothers, postnatal increases of PLT 5-HT coincide with the institution of enteral feeding, and postnatal decreases of PLT 5-HT coincide with the discontinuation of enteral feeding. The most important determinants of newborn PLT 5-HT seem to be newborn whole blood Tryp (suggesting a newborn PLT 5-HT depressing effect), newborn MPV and maternal PLT 5-HT (suggesting a newborn PLT 5-HT augmenting effect). A weakness of the present study is the inevitably low number of longitudinally investigated newborns, the inability to correlate PLT 5HT with a direct measure of GI motility and the use of RCV derived from apparently healthy adults.

We have no explanation for the inverse relation between newborn PLT 5-HT and newborn whole blood Tryp. In contrast to newborns, maternal whole blood Tryp proved not to be an important determinant of maternal PLT 5-HT. Maternal plasma Tryp decreases during pregnancy [24], while higher Tryp in umbilical venous plasma [25],compared with the mothers have previously been noted. The relation between newborn PLT 5-HT and newborn MPV seems conceivable, since large PLT may obviously harbor higher 5-HT contents at similar intracellular 5-HT concentrations, perhaps because of higher numbers of membrane-bound 5-HT transporters and intracellular 5-HT containing dense granules. Also remarkable were detectable levels of whole blood 5-HTP in 4/17 mothers and 11/18 newborns. This intermediate product in 5-HT synthesis is generally not detected in the blood of healthy adults by HPLC or in patients with carcinoid tumors [21], which adds to the notion of altered regulation of 5-HT synthesis in the fetus and/or the pregnant mother. 5-HTP has been detected before in pregnant women and their newborns [26] and several functions of 5-HTP in pregnancy have been suggested.

The about two times lower newborn PLT 5-HT than their mothers and the relation between newborn and maternal PLT 5-HT have been noted before [19;27-29]. Lower newborn PLT 5-HT has been ascribed to higher prenatal 5-HT catabolic activity, such as possibly originating from placental 5-HT degradation [27;29]. This option seems however less plausible, since 5-HT uptake by newborn PLT is considered to be fully functional [19], while at least some patients in the present study indicate that PLT 5-HT may remain below the maternal reference range up to at least 27 days postpartum (**Figure**). Differences between the intensities of newborn and adult GI motility seem more probable. The relation between newborn and maternal PLT 5-HT remains puzzling. Some have suggested heritability of PLT 5-HT as a cause [19]. Other possibilities are mutual influence of GI motility while any transplacental transport of 5-HT remains implausible because of the highly efficient mono- and diamine placental barrier [26;30].

PLT 5-HT half life amounts to 4.2 days and thereby equals approximately PLT half life, at least in adults [21]. This implies that each of the employed blood sampling intervals in our postnatal PLT 5-HT monitoring protocol (Figure) are well above the PLT 5-HT half life. The protocol therefore not only allowed detection of increases of PLT 5-HT deriving from increasing 5-HT exposure, but also detection of losses of PLT 5-HT content due to diminishing exposure of newly secreted PLT to 5-HT. Given the present time intervals we found that postnatal increases and decreases of PLT 5-HT were related to institution and discontinuation of enteral feeding, respectively, although the number of investigated neonates and observations was limited. Replication of our findings in larger groups is necessary, and preferably this study should be embedded in another longitudinal study in which blood is collected from healthy newborns. Our data nevertheless suggest that the mature postnatal PLT 5-HT content will at least partially become established by neurotransmission processes involved in GI motility. One of the investigated preterm infants (no 5, **Figure**) exhibited a pattern inconsistent with this notion. This patient was born at 30 gestational weeks, which is about two weeks prior to the time at which migrating motor complexes in the GI begin to appear [15]. It seems therefore possible that his inconsistent pattern of PLT 5-HT is related to an immature GI tract, and that such a condition may last up to 20 days after birth in preterm born infants.

In conclusion, consistent with relative gut motor quiescence we found that newborn PLT 5-HT is considerably lower as compared with their mothers. Early postnatal PLT 5-HT changes correlate with changes in feeding mode (which affect gut motor activity), such that institution and discontinuation of enteral feeding are linked to a PLT 5-HT increase and decrease, respectively. Further studies are

warranted to establish the link between PLT 5-HT and gut motor activity and the usefulness of PLT 5-HT in the detection and monitoring of GI pathology.

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Part II.

Comparative urine analysis for biomarker discovery

Chapter 5

Comparative urine analysis by liquid chromatography - mass spectrometry and multivariate statistics: method development, evaluation and application to proteinuria

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Abstract

We describe a platform for the comparative profiling of urine using reversed-phase liquid chromatography-mass spectrometry (LC-MS) and multivariate statistical data analysis. Urinary compounds were separated by gradient elution and subsequently detected by electrospray Ion-Trap MS. The lower limit of detection (5.7-21 nmol/L), within-day (2.9-19%) and between-day (4.8-19%) analytical variation of peak areas, linearity (R^2: 0.918-0.999), and standard deviation for retention time (<0.52 min) of the method were assessed by means of addition of seven 3-8 amino acid peptides (0-500 nmol/L). Relating the amount of injected urine to the area under the curve (AUC) of the chromatographic trace at 214 nm better reduced the coefficient of variation (CV) of the AUC of the total ion chromatogram (CV =10.1%) than relating it to creatinine (CV =38.4%). LC-MS data were processed and the common peak matrix was analyzed by principal component analysis (PCA) after supervised classification by the nearest shrunken centroid algorithm. The feasibility of the method to discriminate urine samples of differing compositions was evaluated by (i) addition of seven peptides at nM concentrations to blank urine samples of different origin and by (ii) a study of urine from kidney patients with and without proteinuria. (i) The added peptides were ranked as highly discriminatory peaks despite significant biological variation. (ii) Ninety-two peaks were selected best discriminating proteinuric from non-proteinuric samples, of which 6 were more intense in the majority of the proteinuric samples. Two of these 6 peaks were identified as albumin derived peptides, which is in accordance with the early rise of albumin during glomerular proteinuria. Interestingly, other albumin derived peptides were non-discriminatory indicating preferential proteolysis at some cleavage sites.

1. Introduction

Biofluids such as serum, plasma, whole blood and urine are routinely used for diagnostic purposes and in comparative studies. Urine seems a more suitable biofluid than blood and its derivatives (plasma or serum), because it can be obtained in large quantities by non-invasive sampling. Analytical advantages of urine-analysis are the less complex sample pretreatment due to the much lower protein content, the relatively small size and higher thermodynamic stability of urinary peptides/proteins [1], and the lower complexity and intermolecular interactions compared to proteins in serum [2;3]. The majority of pathological changes in human organs may well be reflected in urine. In this way urine-analysis can aid in disease diagnosis, treatment monitoring and prognosis [4]. Urine, on the other hand, is prone to larger biological variations than the blood compartment, as it samples the metabolic endproducts from the organism destined for excretion. Factors affecting the concentrations of these products in humans, e.g. age and gender [5], can be controlled for by careful matching, whereas this is much harder to do for the influence of factors of cultural and dietary nature [6]. One of the major challenges in biomarker research using urine is thus the large natural variation in the concentration of peptides, proteins and metabolites, which requires careful normalization of the measurements [7].

A frequently used analytical technique to profile urinary compounds for biomarker research is liquid chromatography – mass spectrometry (LC-MS). The advantages of LC-MS are high sensitivity and the feasibility of detecting thermolabile, water soluble compounds without the need for chemical derivatization. Analysis of urine by LC-MS has been applied in metabolomics [8;9], proteomics [4;10-15] and peptidomics™ [16-24] as well as in drug metabolism research. LC-MS is sensitive to matrix effects due to the interference of matrix components with the ionization of analytes (ion suppression) [25]. The use of internal standards and standardization of the injected amount are therefore necessary for quantitative analyses. Comparative profiling using stable isotope labeled standards [26] or dye-labeled proteins as internal standards [27] can overcome these limitations but have proven to be laborious and expensive, making a well-controlled label-free quantitative method as described here of particular interest for clinical studies, where large series of samples have to be processed. A quantitative method for profiling complex biological samples without the need for labeling or spiking with internal standards seems feasible as long as there is linearity of signal versus concentration and a high degree of reproducibility of sample processing and the LC-MS platform [28].

Comparing profiles of urinary compounds, it may be insufficient to normalize the data based on a single compound like creatinine, as this may not be representative of the wide range of molecules with different physico-chemical properties (e.g. molecular weight, pK_a, hydrophobicity). Since the urinary creatinine concentration, which is widely employed to correct for concentration differences and to express the urinary clearance of blood components, is mainly determined by age, gender, muscle mass, kidney function, exercise and diet, it is questionable whether this should be the only standard when it comes to accurate data about renal clearance [5]. To base normalization on a broader molecular basis, we have therefore compared normalization of the injected volume of urine based on a fixed amount of creatinine with a multi-compound normalization strategy based on the area under the curve of the chromatogram at 214 nm (AUC_{214}).

Data processing prior to multivariate statistical analysis is critical for comparing LC-MS data sets that may reach 10^7 to 10^8 data points per analysis or even more, in case high-resolution mass spectrometry is used. Data processing has the goal to correct for unwanted variations in data sets by, for example, correcting shifts in retention time and by discriminating meaningful data points from background, noise and spikes [29-31], thus generating a peak matrix suited for further processing by peak matching, missing peak allocation and finally statistical analysis. Even after data processing there is a need for a further reduction in dimensionality, since the complexity of biological samples like urine generates a great number of significant peaks that generally exceeds the number of analyzed samples with the inherent risk of overfitting the data. One way of further reducing the number of dimensions in the data is by using a regularized linear discriminant classifier method like the nearest shrunken centroid (NSC) algorithm [32]. Visual inspection of patterns in the high-dimensional data space is possible through the use of further dimension-reducing techniques such as principal component analysis (PCA) [33]. For an extensive review and references on data (pre-) processing and multivariate statistical methods we refer to the review of Listgarten and Emili [34].

In the present work, we describe the development of a simple, rapid and robust reversed-phase LC electrospray (ESI) Ion Trap MS platform to reproducibly profile urinary compounds. The platform was evaluated using peptides, which are frequently present at nM concentrations in urine. Dedicated data processing and multivariate statistical approaches were developed and applied to the data to obtain proof-of-principle on several aspects of the method such as: i) the ability to discriminate groups of urine samples based on the absence or presence of standard peptides at approximately 2-10 times the lower limit of detection (LOD; nM range), and ii) the differentiation between the contribution of analytical and biological variation to the final result. Finally the platform was tested in a preliminary study

comparing urine samples from hospitalized patients with and without proteinuria. Using multivariate statistical analysis of LC-MS profiles of urinary compounds is likely to aid in diagnostics, monitoring of disease activity and therapy. It may also be of great value in forming new hypotheses about disease mechanisms and the effect of therapeutic interventions.

2. Experimental

Further detailed information is available under 'Supporting Information'.

2.1. Chemicals

Acetonitrile (ACN) HPLC-S gradient grade), ultra pure water (18.2 MΩ.cm), trifluoroacetic acid (TFA) 99% spectrophotometric grade, formic acid (FA) 98-100% pro analysis were used for reagent preparation. A peptide stock solution [peptide, concentration in mmol/L; VYV, 0.29; YGGFL (leucine enkephalin), 0.20; DRVYIHFP (angiotensin II), 0.10; YPFPGPI (β-casomorphin 7), 0.16; YPFPG (β-casomorphin 5), 0.21; GYPPT (gluten exorphin A5), 0.19; YGGWL (gluten exorphin B5), 0.20] was used for addition experiments and internal standardization.

2.2. Sample Preparation

Urine samples were stored at -20°C, thawed, mixed, acidified with 1% TFA and centrifuged to remove precipitate (5 min at 1500 g and 4°C). The supernatant was diluted 1:1 with 0.2% FA in 10% ACN and stored at 4°C until analysis. The injection order on the LC-MS was randomized. A pooled urine sample was prepared by mixing equal volumes of urine from seven apparently healthy adults (creatinine: 10.6 mmol/L).

Urinary creatinine concentration and total protein content were assayed on an autoanalyzer (MEGA, Merck, Darmstadt, Germany). The study protocol was in agreement with local ethical standards and the Helsinki declaration of 1964, as revised in 2004.

2.3. Reversed Phase HPLC-MS

All LC-MS analyses were performed on an 1100 series capillary HPLC system equipped with a cooled autosampler (4°C), a UV detector (λ=214 nm) and an SL ion trap mass spectrometer (Agilent Technologies). Urine samples were desalted on an Atlantis™ dC_{18} precolumn (2.1 x 20 mm, 3 µm particles and 10 nm pore diameter) using 0.1% FA in 5% ACN at a flow rate of 50 µL/min for 16 min. Urinary compounds were back-flushed from the precolumn onto a thermostated (25 ºC) Atlantis™ dC_{18} analytical column (1.0 x 150 mm, 3 µm particles, 30 nm pores) and

separated in 90 minutes at a flow rate of 50 µL/min during which the percentage of solvent B (0.1% FA in ACN) in solvent A (0.1% FA in ultra pure H_2O) was increased from 5.0 to 43.6% (0.43%/min). During these 90 minutes UV absorption and positive mode MS spectra were acquired. Settings for ESI and mass analysis were as follows: 16.0 psi N_2; drying gas: 6.0 L/min N_2; T: 325 °C; cap. voltage: 3.8 kV; skimmer: 57.5 V; cap. exit: 190.7 V; oct. 1: 4.12 V; oct. 2: 2.49 V; oct. RF: 190.7 V; lens 1: -4.9 V; lens 2: -37.7 V; trap drive: 52.5; scan speed: 5500 m/z s^{-1}; 50 ms accumulation time or 30000 ions; scan range: 100-1500 m/z; Gaussian acquisition filter (width 0.1 m/z) of each scan; rolling average of 5 spectra. Spectra were saved in the centroid mode. Following the gradient, both columns were washed with 85% B for 5 min and equilibrated with 5% B for 10 min prior to the next injection.

Internal standards were injected onto the precolumn prior to injection of the urine samples. Between each injection the injection system was cleaned with 70% ACN and filled again with 0.1% FA in 5% ACN. The amount of internal standard (IS) peptides injected varied per experiment (see section 'Results' for more details).

2.4. Tandem mass spectrometry and database searching

Nano-LC ESI quadrupole time-of-flight (Q-TOF) MS/MS (API QSTAR Pulsar i LC/MS/MS System [Applied Biosystems, MDS Sciex, Framingham, Massachusetts, USA]) was used in the Auto-MS^2 mode (precursor ions >100 counts, 1+ to 4+ ions; spray voltage 2350V; varying collision energies). Using mascot.dll script-processing in Analyst QS 1.1, build 9865 (Applied Biosystems, MDS Sciex) MS-spectra were deconvoluted with respect to charge state and isotopes. The resulting spectra were saved in mascot (Matrix Science, London, UK) generic file format and submitted to an in-house version of the MASCOT [35] search engine (v1.9.05) for UniProt (release 7.7) queries and to a web-based version of the Phenyx [36] search engine (v2.1) for UniProt_Sprot (r. 48.8 of 10-Jan-2006) queries. Enzyme settings were based on chymotrypsin-, trypsin- and caspase-like proteolytic activity of the 26S proteasome in renal cells [37] allowing "half cleavages (Phenyx)" of peptide bonds. Search outcomes were evaluated at a significance level of a probability based Mowse score for MASCOT of ≥61 ($P<0.001$) and at an AC-score of ≥5 for Phenyx. The latter is the sum of the best scores per valid peptide sequence.

The identities of discriminatory peaks, as selected by multivariate statistics, from Ion-Trap and quadrupole-TOF MS data were confirmed by comparing mass spectra and relative retention times using bracketing between added standard peptides. MS/MS data from these discriminatory peaks were processed using MASCOT scripts to obtain a peak list of fragment ions suitable for MS/MS ion search. Prior to subsequent database queries these peak lists were tagged and added to the original MS/MS peak list used for protein identification to evaluate whether

they originated from one of the previously identified proteins. A peak was considered to be identified if the Phenyx z-score ≥4, P≤0.0001 and retention time of the peptide were within 0.05 min and 0.01 amu of the respective discriminatory peak. If the peak/peptide did not reach these criteria, manual conversion of the monoisotopic multiply-charged fragment ions to monoisotopic singly-charged fragment ions was performed prior to another round of searches using MASCOT and Phenyx.

Further detailed information is available under 'Supporting Information'.

2.5. Data processing and analysis

2.5.1. Data analysis for method evaluation

LC-MS chromatographic data were analyzed with Data Analysis software for LC/MSD Trap, version (3.2 build 121) (Bruker Daltoniks, Bremen, Germany). Peak areas and intensities of the spiked peptides described were obtained from the respective smoothed and baseline subtracted extracted ion chromatograms (EIC). One cycle of smoothing with a Gaussian algorithm at a width of 1.8 points preceded baseline subtraction with a flatness of 1. The AUC_{214} was calculated between 30 and 80 min retention time, which corresponds to 14 - 64 min for mass spectrometric data acquisition (MS data acquisition was started 16 min later than the gradient program) during gradient elution.

Univariate statistical analyses were performed with the Statistical Product and Service Solutions package version 11.5 (SPSS Inc., Chicago).

2.5.2. Data (pre-)processing

For processing and multivariate statistical analysis the original Bruker Daltoniks LC-MS data files were converted into ASCII-format with the Bruker Data Analysis software. For further data analysis Matlab (version 7.0.0.19920, Mathworks, Natick, MA, USA) and the PLS toolbox (version 3.5.2, Eigenvector Research Inc., Wenatchee, WA, USA) were used.

Initially the nominal m/z ratios were rounded to the nearest integer to 1 amu bins (instead of the original 0.1 amu in the acquired data) according to Radulovic et al. [38], which is adapted to the accuracy of the ion trap (±0.3 amu). Binning reduced the amount of data by roughly a factor 10 and it also partially corrected for the slight shift in m/z values as a result of trap overfilling occurring during peak elution. For signal filtering and background reduction, data was first smoothed using a moving average filter (3-scan header width, 2 cycles). A modified M-N rule was applied for peak detection in which a pre-defined baseline T (30% of trimmed mean) was multiplied by M (set at 2) to set the threshold (1000 counts), which

should be exceeded by the peak intensity for at least N (set at 5) consecutive observations in the time dimension [38]. If T was lower than 1000 counts, M*1000 was used as a threshold for that bin. For each bin we included the m/z bin value, intensity of the data point with the highest intensity and the mean retention time of the three data points with the highest intensity to generate the peak list.

We used a similar approach as Radulovic et al. [38] to obtain optimal settings for M and N. M (1.5-4) and N (4-8) were applied to two blank LC-MS runs and two LC-MS runs of the pooled urine sample. The settings at which the ratio between the number of peaks (between 30-80 min and broader than 0.3 min at baseline * N) in the sample and in the blank was the highest and at which a minimal number of peaks was extracted from the noise in the blank chromatogram were used.

Time alignment of the chromatograms was not needed, because the median (range) standard deviation of the retention time of the standard peptide peaks was only 0.37 (0.23-0.52) min. One-dimensional peak matching and missing peak allocation in different samples was realized by using a sliding windows technique in which similar m/z bins are evaluated for peaks proximate in time (step size 0.1 min; search window 1.0 min; maximal accepted difference of centroided retention time within a group of matched peaks 0.75 min). Missing peak allocation was performed by extracting the background in given m/z bins at the mean retention time of the other identified peaks. The generated final peak matrix, created from the peak matrices of the individual samples, consisted of a peak(*row*)-sample(*column*)-intensity(*value*) matrix. This final peak matrix was used for multivariate statistical analysis.

All data preprocessing work was done on a personal computer equipped with a +3600 MHz AMD processor and with 4 GB of RAM.

2.5.3. Classification and multivariate statistical methods

To select the most discriminating peaks we applied the NSC classification algorithm [32;39]. NSC regularizes data whereby class-specific centroids are "shrunk" toward the overall (nonclass-specific) centroid, which has the effect of eliminating the influence of the most weakly correlated peaks, thereby reducing the capacity to overfit [34]. This algorithm is used to select peaks that are relevant for the discrimination of the predefined classes in conjunction with permutation tests to validate the classification algorithm [40] using leave-one-out cross-validation (LOOCV) to avoid overfitting due to one outlier. The optimal shrinkage value was the value at which LOOCV showed the lowest classification error. In LOOCV one observation per class is iteratively omitted from the data set that is used to construct the classification model, which is then used to classify the omitted observation as case or control. Variables selected at the highest shrinkage value and lowest

LOOCV error, were employed for construction of the final classification model. The selected peaks were then analyzed and visualized by plotting the first two principal components obtained after PCA [33]. As a measure for class separation the Mahalanobis distance (MD), was calculated [41]. We consider a MD above the cut-off of 6.0, corresponding to a difference of 6 sigma between the mean centroids of the classes [40], as indicative for significant class separation.

3. Results

3.1. Method development and evaluation

3.1.1 Optimization of RP-HPLC

Resolution between the spiked peptides (see Experimental) was optimized by varying gradient steepness and flow rate in a central composite design. The goal was to achieve optimal resolution within a maximal runtime of 90 min and a flow-rate of 50 µL/min, which is adapted to the ESI-source and the column diameter of 1 mm. Optimal resolution was obtained at a gradient steepness of 0.43% solvent B/min. Runs of urine samples of 6 healthy adults without the added peptides assured that there were no detectable peaks in the respective EIC at the retention times of the added peptides. A typical chromatogram of 9.6 µL urine spiked with 0.25-0.73 pmol of standard peptides under these conditions is shown in **Figure 1A**. For two peptides (DRVYIHPF and YPFPGPI) the mono- and di-protonated ions were extracted and combined into one trace, whereas for the other peptides only the mono-protonated ions were observed and thus extracted (**Figure 1B**). **Figure 1C** shows the corresponding chromatographic trace at 214 nm of the in-line UV-detector.

3.1.2. Normalization of injected amount of urine to creatinine or the AUC_{214}

Since ESI is sensitive to matrix effects leading to ion-suppression [25], it is important to standardize the injected amount of urine in comparative analyses. Assuming that matrix effects are proportional to the injected amount, two methods of normalization were investigated: i) to a given amount of creatinine (50 nmol) and ii) to a fixed AUC_{214} value (1.02×10^5 absorbance units [AU]; see below for the calculation). Creatinine was chosen, because of its frequent use in clinical chemistry and the UV-area at 214 nm between 30-80 minutes (AUC_{214}) was chosen, because it normalizes the injected amount based on a large number of compounds, which is likely to be more representative of the overall sample composition.

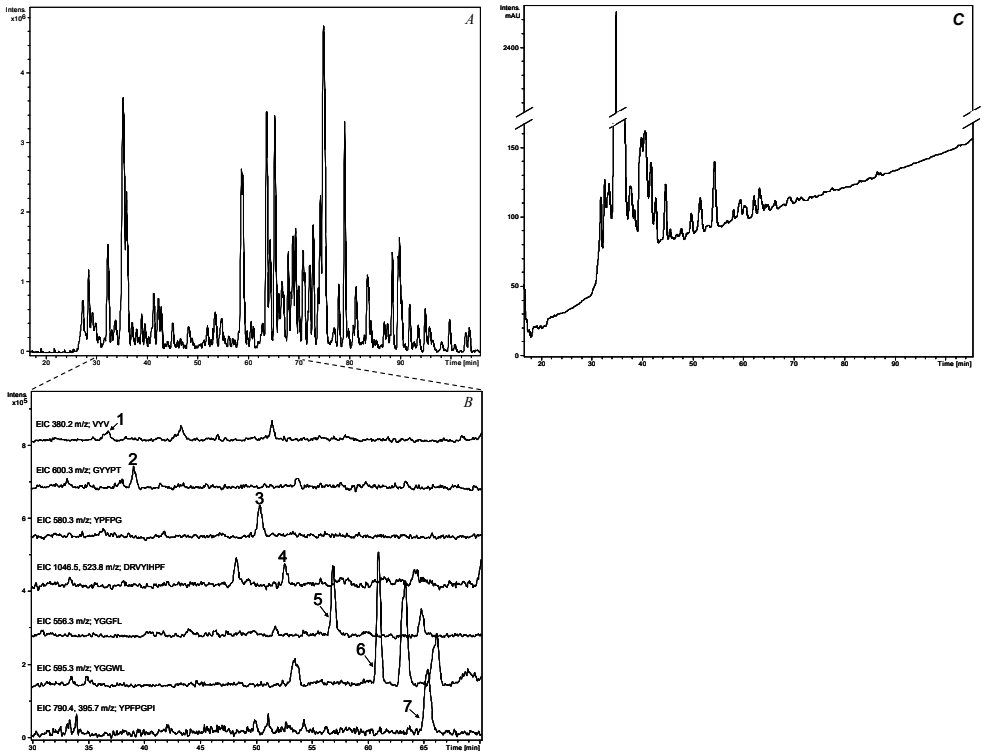


Figure 1. ***Analysis of a pooled urine sample spiked with nM concentrations of internal standard peptides.***

A. Smoothed and baseline subtracted base peak chromatogram of a pooled urine sample (9.6 µL injected, equivalent to 50 nmol creatinine) co-injected with 5 µL of 1:2000 diluted peptide stock solution, after optimization of the resolution by varying gradient steepness in a central composite design.

B. Smoothed and baseline subtracted extracted ion chromatograms (EIC) of the seven peptides used for optimization and internal standardization (same sample as shown in panel A). Peptide peaks correspond to n-times the lower limit of detection (LOD): peptide (n x LOD), 1. VYV (3.9), 2. GYYPT (2.3), 3. YPFPG (4.6), 4. DRVYIHPF (2.2), 5. YGGFL (9.5), 6. YGGWL (3.5) and 7. YPFPGPI (7.3); the absolute values for the LOD can be found in **Table 2**. The calculated LOD for VYV appears to be lower than the LOD that was derived from the signal-to-noise ratio of the peak shown in Figure 1B. Peptide m/z-values and retention times derived from the analysis of diluted stock solutions were used to extract and appoint peaks to the standard peptides spiked into urine.

C. Smoothed and baseline subtracted UV chromatogram (λ=214 nm) of the same urine sample as shown in panel A.

Moreover, normalizing to the AUC_{214} rather than to the AUC of the total ion chromatogram (AUC_{TIC}) avoids confounding effects resulting from ion suppression, since extinction coefficients are characteristic of each compound as long as the linear range of the UV-detector is not exceeded. By starting integration at 30 min we avoided the contribution of very small and very hydrophilic compounds, e.g. small hydrophilic organic acids, to the AUC_{214}.

To investigate which normalization method of the injected amount (injection volume) was preferable, ten different 24-hour urine samples of children (male: female / 5:5; age range 6.8 - 13.6 years) with creatinine levels ranging from 2.0 - 10.6 mmol/L were analyzed. The initial sample volume injected onto the column was equivalent to 50 nmol creatinine and the AUC_{214} between 30 and 80 minutes was a measure of the overall amount of compounds injected. From this area a new injection volume was calculated keeping the 'injected AUC_{214}' constant at $1.02x10^5$ absorbance units, which corresponds to an average amount of 50 nmol creatinine for the ten urine samples. Five µL of the 1:500 diluted peptide stock solution (1.0-2.9 pmol per peptide) was co-injected to evaluate which method of standardization resulted in the most repeatable peak areas for each peptide.

Overall peptide MS-signals showed less variation when the injected volume was normalized to a fixed AUC_{214} value as compared to a constant amount of creatinine (**Table 1**). Similarly, the AUC_{TIC} between 30 and 80 minutes (dead time between UV detector and ion source was approximately 5 sec), which reflects all of the MS-detected urinary compounds, improved with respect to the coefficient of variation (CV) from 38% when normalized to creatinine to 10% when using the AUC_{214} value (**Table 1**). Generally these results indicate that adjusting the injected amount of urine based on the AUC_{214} is preferable to minimize variations in peak area and intensity due to matrix effects, although this seems component dependent. A drawback of using the UV-area is that one compound eluting at 35.0 min retention time determined a large portion (median [range]; 44% [14-62%]) of the total AUC_{214}.

Table 1. ***Peak area and intensity variation of peptides added to urine after normalization of the injected amount to creatinine or UV-area***

	Peptide (sequence)	Creatinine [CV [b] (%)]		AUC_{214} [a] [CV [b] (%)]	
		Area	*Intensity*	*Area*	*Intensity*
1	VYV	30.1	22.2	23.3	20.7
2	GYYPT	19.3	16.2	13.2	11.9
3	YPFPG	19.2	15.1	11.3	11.6
4	DRVYIHPF	13.7	13.3	25.6	23.3
5	YGGFL	21.5	16.3	5.9	6.1
6	YGGWL	11.5	11.1	7.1	9.7
7	YPFPGPI	20.4	20.1	15.4	17.6
	AUC_{TIC} [c]	38.4		10.1	

[a] Area under the curve of the UV-chromatogram (λ=214 nm) between 30 and 80 min retention time;
[b] Coefficient of variation;
[c] Area under the curve of the total ion chromatogram between 30 and 80 min retention time.

3.1.3. Method evaluation

The linearity and lower limit of detection (LOD) of the optimized LC-MS method were determined by duplicate analyses of pooled urine samples spiked with peptides at concentrations of 0 (n=5); 10; 25; 50; 100; 150; 250 and 500 nmol/L (calculated for YGGFL). Calibration curves were based on the respective smoothed and baseline subtracted EIC. Least squares linear regression analysis was employed. The LOD is calculated from the intercept of the y-axis with the upper limit of the 95% confidence interval of the calibration curve [42].

The method exhibited good linearity (all curves $P<0.001$) with R^2 values ranging from 0.918–0.999 for peak area and intensity over the concentration range (data not shown). The LOD ranged from 5.7-21 nmol/L, depending on the peptide (**Table 2**). This corresponds to an injected amount of 54-204 fmol per peptide (**Table 2**). **Figure 1B** shows the EIC of the respective peptides at the calculated 2-10 times their LOD (i.e. 0.25-0.73 pmol injected or 50-145 nmol/L). The calculated LOD for VYV appears to be lower than the LOD that was derived from the signal-to-noise ratio of the peak shown in **Figure 1B**.

Table 2. ***Lower limits of detection (LOD) of seven peptides used as internal standards***

No.	Peptide	m/z values of EICs[a]	LOD (concentration)	LOD (amount)
	(sequence)	*($[M+H]^{1+}$, $[M+2H]^{2+}$)*	*(nmol/L)*	*(fmol injected)*
1	VYV	380.2	19[b]	186[b]
2	GYYPT	600.3	21	204
3	YPFPG	580.3	12	113
4	DRVYIHPF	1046.5, 523.8	12	114
5	YGGFL	556.3	5.7	54
6	YGGWL	595.3	15	143
7	YPFPGPI	790.4, 395.7	5.7	55

[a] Extracted ion chromatogram.

[b] The reported LOD, calculated from the intercept of the y-axis with the upper limit of the 95% confidence interval of the calibration curve, appears to be lower than the LOD derived from the signal-to-noise ratio of peak VYV depicted in Figure 1B.

The within-day and between-day analytical variation of the method were determined by repetitive analyses (n=5) of the pooled urine sample (equivalent to 50 nmol creatinine) injected together with 5 µL of a low (1:2000 diluted peptide stock solution, 0.25–0.73 pmol) and a high (1:500 diluted peptide stock solution, 1.0–2.9 pmol) amount of added peptides on the same day and on five consecutive days, respectively. Outliers were removed ($\alpha=0.05$) using the Dixon test [43] and Cochran's maximum variance test [44]. Overall, the within-day and between-day variation ranged between 2.9 and 19% for the various peptides (**Table 3**).

Table 3. *Within-day (n=5) and between-day (5 days, n=5) variation of internal standard peptides added to a pooled urine sample*

		within-day			between-day		
peptide	amount injected	area	intensity	RT [a]	area	intensity	RT
(sequence)	*(pmol)*	*CV [b] (%)*	*CV (%)*	*SD [c] (min)*	*CV (%)*	*CV (%)*	*SD (min)*
VYV	0.73	12	11	0.36	18	14	0.48
	2.9	6.0	12		7.2	9.5	
GYYPT	0.48	16	11	0.43	19	17	0.35
	1.9	7.2	9.3		12	14	
YPFPG	0.53	12	7.3	0.25	13	10	0.37
	2.1	3.2	4.4		6.5	8.7	
DRVYIHPF	0.25	19	12	0.23	5.3	6.0	0.43
	1.0	7.4	13		8.1	9.1	
YGGFL	0.50	6.2	5.8	0.26	6.2	8.3	0.44
	2.0	2.9	3.1		7.3	7.8	
YGGWL	0.50	3.0	4.5	0.27	4.8	5.3	0.47
	2.0	3.5	3.6		6.6	6.8	
YPFPGPI	0.40	6.5	8.3	0.29	8.5	6.6	0.52
	1.6	2.9	4.4		5.2	7.0	

[a] RT, retention time; [b] CV, coefficient of variation; [c] SD, standard deviation.

3.2. Data processing and multivariate statistical analysis of LC-MS profiles of urinary compounds

3.2.1. Data processing and analysis

Binning from an instrumental resolution of 0.1 amu to 1 amu reduced the size of the dataset to reach an acceptable data processing and analysis time on a normal desktop computer (see 'Experimental' for specifications). The M-N rule proved to be a simple, fast and robust peak picking algorithm, which generated a peak list within one hour per sample. The input, i.e. the number of samples, determined the time required for peak matching, missing peak allocation and for construction of the final peak matrix (see 'Experimental' for details).

3.2.2. Multivariate statistical comparison of pooled urine samples to assess the effect of analytical variation

LC-MS data from repetitive analyses (n=6) of 11.1 µL injections of blank pooled urine (AUC_{214}= $1.02x10^5$ AU) and of the same urine spiked at 2-10 times the LOD (n=6) were processed as described before to obtain a final matrix containing 10029 peaks. This peak matrix was used to construct a PC score plot of the two first principal components (PC 1 and PC 2) best explaining the total variance of the data

(**Figure 2A**). Using all 10029 peaks, the plot showed a strong overlap between the blank (◆) and spiked (★) group, which was confirmed by a MD of 0.43 (non-significant).

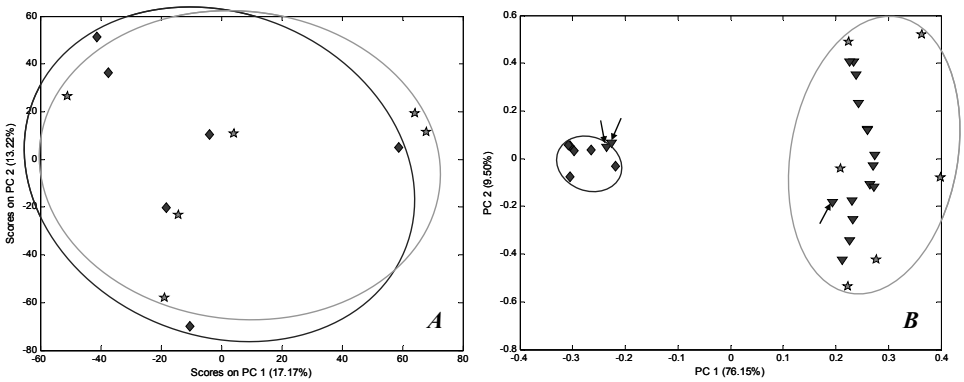


Figure 2. ***Description of the analytical variation in urine profiles from a pooled urine sample analyzed six times.***

A. The principal component (PC) analysis plot using the complete peak matrix of 10029 peaks shows overlap between repetitions of the blank (◆) and spiked (★; 2-10 times lower limit of detection of standard peptides) pooled urine sample. Together PC 1 and PC 2 explain only 30.39% of the variation between the samples.

B. The biplot depicts the same blank (◆) and spiked (★) samples as in panel A together with 17 of the 10029 most discriminating peaks (▼) selected by the nearest shrunken centroid classification method at a leave-one-out cross-validation error of 0. Arrows indicate peaks that do not belong to the added peptides. In contrast to **Figure 2A**, PC 1 and PC 2 now explain a much larger portion (85.65%) of the variation between the samples. Ellipses circle groups of samples belonging to the same class, i.e. blank and spiked.

Application of the NSC classification method yielded 17 highly discriminatory peaks (▼) out of the original peak matrix at a shrinkage value of 3.0 with a LOOCV (see 'Experimental') error of 0 (**Figure 2B**). Fourteen (82%) of these 17 peaks could be traced back to the added standard peptides based on m/z-values and retention times. These 14 peaks were elevated in the spiked group as expected (data not shown). Of the remaining three peaks (arrows), two were located in the region of the blank urine sample and one was located in the spiked samples area. Univariate comparison revealed no significant difference between peak areas ($P>0.5$) indicating that these peaks were selected by chance. The higher number of selected peaks relative to the number of spiked peptides is due to the fact that the original data were not deconvoluted with respect to charge state or isotopic distribution. This did, however, not affect the classification of samples. The PCA biplot, based on the 17 selected peaks, showed clear discrimination between the blank and the spiked samples, which was confirmed by a MD of 9.91 (significant). Urine samples spiked

at nM concentrations with peptides can thus be discriminated using the developed LC-MS method followed by supervised peak selection with cross-validation and visualization of the first two principal components.

3.2.3. Multivariate statistical comparison of urine samples from different individuals to assess the effect of biological variation

LC-MS data obtained from the analysis of blank and spiked (2-10 times LOD) urine samples (6.6-19.8 µL injected; AUC_{214} = $1.02x10^5$ AU) of six healthy individuals (male: female / 3:3; median age [range] 27 years [25-30]) yielded a matrix containing 14234 peaks. To relate analytical variation to biological variation, we generated a common matrix of 14234 peaks including the samples described under section 3.2.2, which reflect analytical variation only.

Figure 3A shows a PC score plot generated with the entire peak matrix. Blank (●) and spiked (■) samples of the same individual co-localized in 5 out of 6 cases. This indicates that the analytical variation is much smaller than the biological variation between different healthy individuals. Co-localization of the data points from the repetitive analyses of blank (◆) and spiked (★) pooled urine samples (analytical variation only) emphasizes the importance of biological variation relative to analytical variation further. Moreover, pooling the urine of these six healthy individuals averaged the explained variation represented by PC 1 and PC 2 (both around 0) out.

It was thus of importance to assess whether spiking of the seven standard peptides at 2-10 times the LOD could still be discriminated despite the observed biological variation. The NSC algorithm yielded 16 discriminatory peaks (▼) from the original peak matrix at a shrinkage value of 1.85 with an LOOCV error of 0. Fourteen (88%) of these 16 peaks could again be related to 6 of the 7 added peptides based on m/z-values and retention times (**Figure 3B**).

Peak areas and intensities, derived from the EIC of the first two isotopic peaks of the NSC-selected peptide peaks, were compared by univariate statistics (**Figure 4**). P-values were less than 0.001 for area (**Figure 4A)** and intensity (**Figure 4B**) for 5 out of the 7 spiked peptides, whereas no significant difference was found for the two other peaks that the NSC algorithm had selected as being discriminatory. One of these peaks (peak X in **Figure 3B**; 694.6 m/z) co-eluted with one of the spiked peptides (YPFPGPI; $[M+H]^+$=790.4 m/z). The non-significant decrease in peak area and intensity of this signal after spiking suggests an ion-suppression effect. We could not clarify the reason why the peak at 80.85 min (peak Y in **Figure 3B**; m/z = 356.5) was selected as being discriminatory by the NSC algorithm.

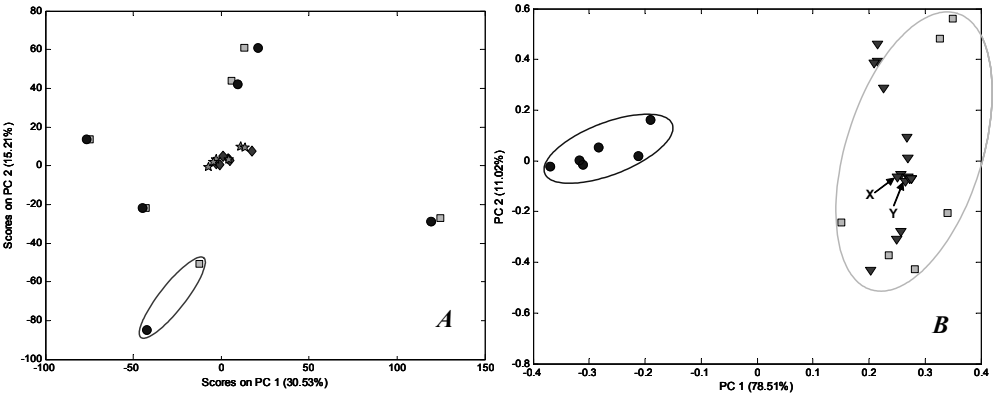


Figure 3. *Description of the analytical and biological variation in urine profiles from six healthy individuals.*

A. The principal component (PC) analysis plot using the complete peak matrix of 14234 peaks shows overlap between the blank (●) and spiked (■; 2-10 times lower limit of detection of standard peptides) urine samples of six apparently healthy adults, and six repetitive analysis of a blank (n=6; ◆) and a spiked (n=6; ★) pooled urine sample. Co-localization (red ellipses) of the blank and spiked samples from the same individual (for 5 out of 6 individuals) and the pool, suggests biological variation to be the main determinant of the observed variation between samples. Unsupervised PC analysis obviously provides insufficient discriminatory power to detect the variation caused by spiking. Together PC 1 and PC 2 explain 45.74% of the variation between the samples.

B. The biplot depicts the same blank (●) and spiked (■) samples as in panel A together with 16 of the 14234 most discriminating peaks (▼) selected by the nearest shrunken centroid classification method at a leave-one-out cross-validation error of 0. PC 1 in the biplot explains 78.51% of the variation between blank (●) and spiked (■) samples. Two peaks (X and Y) could not be related to the added peptides.

The NSC algorithm missed the added peptide GYYPT as being discriminatory probably because it eluted early in the gradient and was added to the urine at a concentration equivalent to 2.2 times the LOD. Despite this fact, it was clearly possible to discriminate the two groups of samples based on a PCA score plot, explaining 89.6% of the variation between blank and spiked samples, using the 16 selected peaks (MD of 9.10) in the presence of the observed biological variation.
Classification of the repetitions of the blank and spiked pooled urine samples, using the 16 peaks selected by the NSC algorithm, resulted also in assignment of the samples to the appropriate classes (MD of 8.04; not shown).

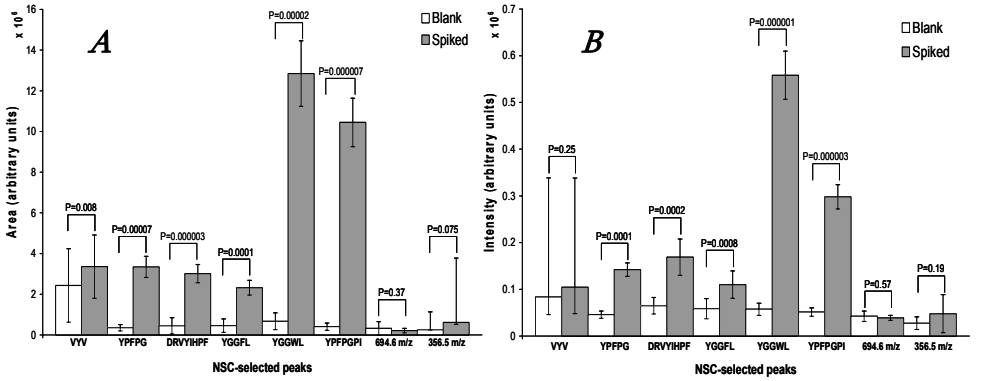


Figure 4. ***Univariate comparison of peak areas and intensities of peptides/peaks discriminating blank from spiked urine samples from different individuals.***

Graphs represent univariate paired comparisons (P-values) of peak area (**A**) and intensity (**B**) of smoothed and baseline subtracted extracted ion chromatograms from raw LC-MS data of the peptides/peaks selected by the nearest shrunken centroid classification method. Bars represent the mean values (error bars: +/- 1 x SD). The median (error bars: minimum-maximum) is presented for the area of 356.5 m/z and the intensity of peptide VYV. Comparison was performed with the Wilcoxon signed ranks test (non-parametric) and the paired Student's T-test (parametric) dependant on the normality of the distribution (Shapiro-Wilk's test).

3.2.4. Application of the developed methodology to urine from patients with and without proteinuria

In order to evaluate the applicability of the described methodology, we studied urine of hospitalized patients with established proteinuria (P; male: female / 5:1; median age 60 years [60-75]; median (range) total protein concentration in a random portion of urine: 7.6 g/L [6.5-10]) due to primary or secondary renal morbidity, and we studied urine of hospitalized patients with a medical history of renal morbidity but without proteinuria at the time of sampling (NP; male: female / 5:1; median age 63 years [56-72]; total protein concentration in portion urine: 0.1 g/L [0.0-0.2]). Proteinuria was defined as having a total urinary protein concentration above 1 g/L in a random portion. The total peak matrix contained 11867 peaks and did not allow discrimination between the proteinuric and non-proteinuric samples (MD=1.84) without supervised classification.

The NSC classifier yielded 92 discriminatory peaks at a shrinkage value of 1.88 with a LOOCV error of 0. The urine samples with and without proteinuria were discriminated (MD = 6.95) in the PC 1 dimension of the biplot (**Figure 5A**). Ninety-one out of 92 discriminatory peaks (▼) increased in intensity in proteinuria (■), whereas one peak seemed to be increased (arrow) in the samples without proteinuria (●). Classification of the urine samples from six healthy individuals described in sections 3.2.2 and 3.2.3 (spiked or blank; total of 24 samples; ◆) using

the model generated with the variables selected from the 12 chromatograms described in this section, resulted in the formation of a dense cluster close to the non-proteinuric samples (**Figure 5B**), discriminating them even more strongly from the proteinuric samples (MD =15.40) notwithstanding the fact that some of them contained spiked peptides. Most remarkable was the difference (MD =3.39) between the urine samples of apparently healthy individuals without known renal morbidity used for spiking experiments and the patients with (a history of) renal morbidity but without proteinuria. A trend of progressing renal disease may be implied, as patients shift to the right along the PC 1-axis.

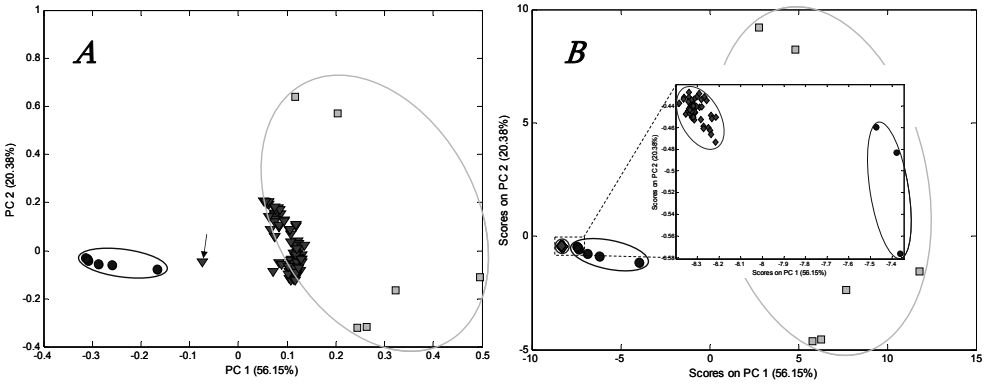


Figure 5. *Comparison of urine samples from proteinuric and non-proteinuric patients with renal disorders.*

The biplot (**A**) shows the urine samples from 6 patients with proteinuria (■; ≥1 g/L total protein concentration in a random portion of urine) and from 6 patients without proteinuria (●; ≤0.1 g/L) but with a medical history of renal morbidity. 92 peaks (▼) out of 11867 were selected for their discriminatory properties by the nearest shrunken centroid classification method at a leave-one-out cross-validation error of 0. The arrow indicates an upregulated peak in the non-proteinuria group. Using this model, classification of 24 samples from apparently healthy individuals (◆) resulted in a dense cluster (purple ellipse) that was separated from the non-proteinuric patients (**B**) implying large differences in the composition of urine between healthy individuals and hospitalized patients with or without proteinuria. The enlarged area in panel B shows that some patients without overt proteinuria show a trend in PC 1 placing them at various levels towards overt proteinuria.

3.2.5. Selection of discriminatory peaks and identification by tandem mass spectrometry

The 92 discriminatory peaks obtained by NSC from the total peak matrix were curated by manual de-isotoping and charge deconvolution. From the resulting 54 curated peaks we selected 6 peaks for further univariate statistical comparison, based on their elevated levels in the majority of the proteinuric samples. **Figure 6**

shows the box and whisker plots and corresponding P-values for univariate comparison of the peak areas (**A**) and intensities (**B**) of these 6 peaks for the two patient groups. The overlaid EIC of 2 of the 6 peaks (**Figure 6C**) showed that both of these peaks were increased in 3 out of 6 proteinuric urine samples, but not in the non-proteinuric samples. This indicates a considerable variability in the proteinuric patient group, which is also evident from the PCA score plot (see **Figure 5**), relative to patients with kidney disease but no proteinuria. Interestingly, there was no correlation ($P>0.05$, $R^2 \leq 0.0276$) between total protein content in urine and peak area or intensity of these 6 peaks in patients with proteinuria, suggesting that the selected peaks discriminate patients not simply based on the total protein concentration in urine. Remarkably, most of the selected peaks did not reach statistical significance ($P<0.05$) on their own when comparing proteinuric and non-proteinuric patient samples due to the large variation in the proteinuric patient group. This adds further support to the value of a multivariate statistical comparison for discrimination between complex samples.

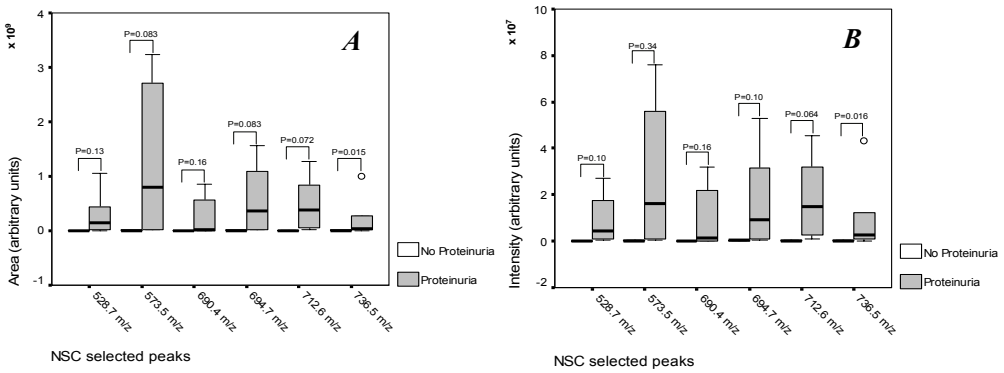
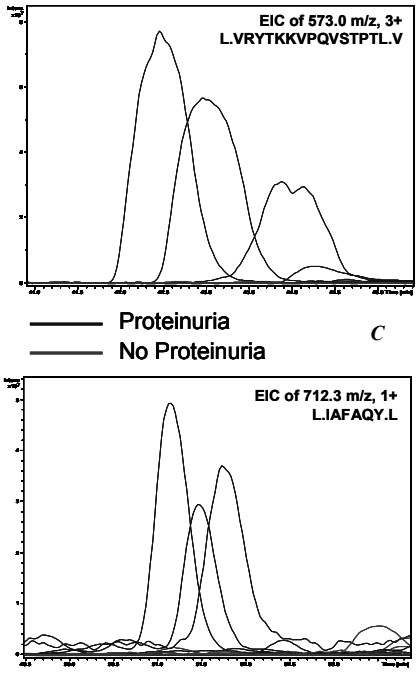


Figure 6. ***Univariate comparison of peak area and intensity of 6 peaks discriminating proteinuria from non-proteinuria.***

Represented are box and whisker plots and the corresponding P-values for univariate comparisons of peak area (**A**) and intensity (**B**) of smoothed and baseline subtracted extracted ion chromatograms (EIC) from raw LC-MS data of 6 discriminating peaks selected by the nearest shrunken centroid (NSC) classification method. Comparison was performed with the Mann-Whitney U test (non-parametric) and the Student's T-test (parametric) dependant on the normality of the distribution (Shapiro-Wilk's test). Panel (**C**, next page) shows the EIC of two discriminatory peaks selected by the NSC algorithm for the proteinuric (blue) and non-proteinuric (red) samples.



The proteinuric urine sample containing the highest level of these six peaks was analyzed in duplicate by nanoLC ESI-Q-TOF MS/MS to identify the parent protein(s) from which the discriminating peaks were derived. LC-MS data were processed as described under 'Experimental'. **Table 4** and **5** list the most significant hits of identified proteins for MASCOT- and Phenyx-algorithm based Uniprot/SwissProt database searches, respectively. Most of the identified proteins are derived from blood.

Table 4. *Proteins in a proteinuric urine sample identified by LC-MS/MS and subsequent UniProt database search by the MASCOT algorithm*

Protein	Accession no.	Score [a]	Cov.(%) [b]	# Peptides [c]
α-1-antitrypsin precursor [d]	P01009	1090	43	16/35
serum albumin precursor [d]	P02768	445	11	4/19
haptoglobin precursor [d]	P00738	173	14	1/9
serotransferrin precursor[d]	P02787	153	7	2/7
α-1-acid glycoprotein 1 precursor [d]	P02763	117	12	1/7
apolipoprotein A-I precursor [d]	P12270	103	9	1/3
hemoglobin β-subunit [d]	P68871	102	21	2/3
α-1-antichymotrypsin precursor [d]	P01011	99	10	1/5
transthyretin precursor (prealbumin) [d]	P02766	96	28	2/5
Collagen α-1 (XIV) chain precursor	P02766	73	1	1/5

[a] Listed are proteins associated with the ten highest probability based Mowse scores (remaining identified proteins with a Mowse score ≥61 (P<0.001) are listed under 'Supporting Information'). An in-house version of the Mascot search engine (v1.9.05) was used to search the UniProt (release 7.7) database. Enzyme 'none' was specified for the search. [b] Percent ratio of all amino acids from valid peptide matches to the total number of amino acids in the protein. [c] Number of unique peptide matches (ion score >34 [P<0.05] was considered significant) followed by the total number of peptide matches found for the given protein. [d] Proteins were also identified by Phenyx (**Table 5**). Further detailed information is available under 'Supporting Information'.

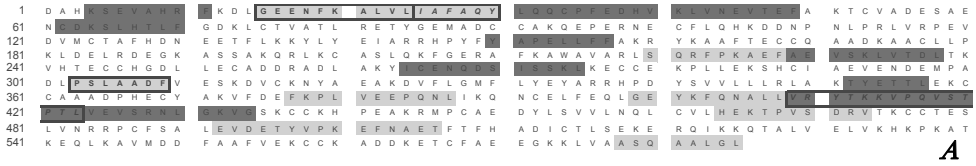
Table 5. *Proteins in a proteinuric urine sample identified by LC-MS/MS and subsequent UniProt/SwissProt databases search by the Phenyx algorithm*

Protein	Accession no.	Score [a]	Cov. (%) [b]	# Peptides [c]
α-1-antitrypsin precursor [d]	P01009_WOSIG0	187.8	35	39/57
serum albumin precursor [d]	P02768_WOSIG0	47.43	4	9/21
haptoglobin-related protein precursor [d]	P00739_WOSIG0	40.49	12	8/10
α-1-acid glycoprotein 1 precursor [d]	P02763_WOSIG0	28.28	14	6/10
hemoglobin β-subunit [d]	P68871	25.33	16	4/6
serotransferrin precursor [d]	P02787_WOSIG0	24	3	4/9
α-1-acid glycoprotein 2 precursor	P19652_WOSIG0	14.95	9	3/3
apolipoprotein A-I precursor [d]	P02647_WOSIG0	13.93	8	2/5
α-1b-glycoprotein precursor	P04217_WOSIG0	9.33	2	1/3
zinc-α-2-glycoprotein precursor	P25311_WOSIG0	8.87	3	3/3
transthyretin precursor (prealbumin) [d]	P02766_WOSIG0	8.82	8	1/4
α-1-antichymotrypsin precursor [d]	P01011_WOSIG0	8.37	3	2/7
angiotensinogen precursor [e]	P01019_PEPT0	8.29	80	1/1

[a] Listed are the proteins with AC-scores ≥8 (remaining identified proteins with an AC-score ≥5 are listed under 'Supporting Information'). A web-based version of the Phenyx search engine (v2.1) was used to search the UniProt_Sprot (r. 48.8 of 10-Jan-2006) at AC-scores ≥5. Enzyme 'Chymotrypsin (FYL)' was specified for the search. [b] Percent ratio of all amino acids from valid peptide matches to the total number of amino acids in the protein. [c] Number of valid peptide matches followed by the total number of peptide matches found for the given protein. Further detailed information is available under 'Supporting Information'. [d] Proteins were also identified by MASCOT (**Table 4**). [e] Angiotensin II was spiked into the urine sample at a concentration of 0.5 μM (2.5 pmol).

Two of the peaks selected by the NSC algorithm to be elevated in proteinuric urine samples (Ion-Trap 573.0 m/z, 3+ and 712.3 m/z, 1+ **Figure 6C**) were identified as possible breakdown products of human serum albumin by both algorithms (**Figure 7A**: serum albumin precursor protein, P02768-CHAIN0 in uniprot_sprot, L.VRYTKKVPQVSTPTL.V, and L.IAFAQY.L position 31-36; **Table 6**), a protein that is known to be increased in concentration in glomerular proteinuria [5]. Manual conversion of the monoisotopic multiply-charged fragment ions to monoisotopic singly-charged fragment ions confirmed the identity of one peptide with a higher score (**Table 6**). To evaluate peak selection by the NSC algorithm, we compared the respective EIC of two other albumin-derived peptides that were not selected by the NSC algorithm (**Table 6** and **Figure 7B**). The levels of the two discriminatory peptides were elevated in at least 3 out of 6 proteinuric patients (for both peptides; $P>0.05$), while the other two non-discriminatory peptides showed increased intensities in only 2 out of 6 patients (for both peptides; $P>0.05$). The fact that the NSC algorithm did not select these two peptides indicates that it was not able to detect them as discriminatory due to the large biological variability in the

proteinuric patient group. The observation that there are proteolytic fragments of albumin with a higher discriminatory value than others points towards the existence of 'disease-specific' proteolytic degradation of albumin and urinary excretion of its fragments. This idea is supported by recent data of Osicka and Comper [45] and Villanueva et al. [46].



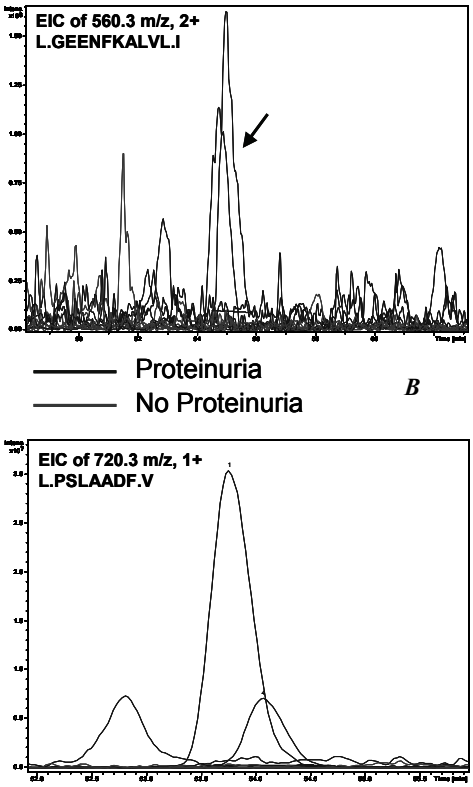


Figure 7. ***Serum albumin peptides identified by LC-MS/MS***

The amino acid sequence of serum albumin precursor (Uniprot_Sprot AC no. P02768-CHAIN0) is shown (**A**). Peptide matches are listed and highlighted in colored boxes (green for valid peptides, red for invalid peptides, and orange for valid half cleaved peptides). Two of the serum albumin precursor peptides (boxed, ***amino acids*** in italic and bold) were selected by nearest shrunken centroid (NSC) classification to be discriminatory between proteinuria and non-proteinuria, whereas two (boxed, **amino acids** in bold) of the other albumin peptides were not selected by NSC. Panel (**B**) depicts the extracted ion chromatograms of these two non-discriminatory albumin peptides for the proteinuric (blue) and non-proteinuric (red) samples indicating that also these peptides are elevated in some proteinuric patients (see **Fig. 6C** for discriminatory peptides).

Table 6. *Peaks identified by LC-MS/MS as serum albumin derived peptides*

sequence	cleavage specificity	modifications	precursor mass (Da)	charge	mass error (Da)	score	position
selected by NSC [a]							
L.VRYTKKVPQVSTPTL.V [b]	1 MC [c]	none	1715.99	3+	0.02	40[d]	415-429
L.IAFAQY.L [e]	1 MC	none	711.359	1+	0.036	4.99 [f]	31-36
not selected by NSC							
L.GEENFKALVL.I [e]	2 MC	none	1118.597	2+	0.014	5.1 [f]	21-30
L.PSLAADF.V [e]	half	none	719.349	1+	0.03	4.73 [f]	303-309

[a] Peptides selected by nearest shrunken centroid (NSC) classifier. [b] Peptide identified using MASCOT. [c] MC, missed cleavage; [d] Individual ion score >33 indicates identity or extensive homology ($p<0.05$); [e] Peptides identified using Phenyx. [f] Peptides with z-scores ≥4 and $p<0.0001$ were considered significant. Further detailed information is available under 'Supporting Information'.

4. Discussion and conclusion

The aim of this work was to develop a platform for the comparative profiling of urine by microbore, reversed-phase HPLC coupled on-line to ESI-Ion Trap MS. Dedicated data preprocessing followed by statistical classification (with cross-validation) and PCA were used to assess the relative contributions of analytical and biological variation to the obtained results as well as to apply the methodology to a sample set of kidney patients with and without proteinuria.

4.1. Method development and evaluation

Urine has been the biofluid of choice in many clinical and pharmacological studies focusing on diseases of the genitourinary tract [47-50]. Profiling urinary (trypsin-digested) proteins and peptides, usually starts with prefractionation or (affinity-) enrichment followed by separation based on physico-chemical properties (e.g. by HPLC and/or 1D/2D electrophoresis [1DE/2DE]) and detection/identification by mass spectrometry [18;51-53]. While 1DE/2DE are often used in urinary proteomics, their application is limited by poor coverage of proteins with extreme properties (e.g. low molecular weight; high isoelectric point) or low concentration, a relatively low sample throughput and the difficulty to automate handling of large numbers of clinical samples [51]. Profiling by LC-MS provides an alternative with advantages in areas where 1DE/2DE is weak, notably the low-molecular weight region of the proteome also termed the peptidome. The urinary peptidome can be considered complementary to the urinary proteome, since most peptides are derived from higher molecular weight proteins through proteolytic cleavage [18]. Since there is evidence that the "degradome" of high molecular weight proteins may give insights into disease mechanisms and provide new diagnostic biomarkers [46;54], we have developed a simple, rapid and robust on-line reversed phase LC

ESI Ion Trap MS-based profiling method with minimal sample pretreatment directed at low-molecular weight compounds in urine.

Validation parameters such as the LOD, linearity, within- and between-day analytical variation and standard deviation for retention time and peak area were used to characterize the method. Sensitivity for peptides (3-8 amino acids) is comparable to what can be routinely attained with a microbore-LC ESI-Ion Trap MS configuration, i.e. detection of low nM (<25 nmol/L) concentrations and femtomole quantities (<250 fmole) of peptides [55]. However, it must be remarked that small peptides, such as one of the standard peptides VYV, that elute at the beginning of the gradient are prone to higher analytical variation in terms of peak area and retention time, especially at low concentrations. This is likely due to some chromatographic migration under the isocratic sample loading conditions affecting retention time and possibly small losses during loading of the trap column. Very small peptides, however, tend to be less disease-specific, as they may be derived from a wide range of precursor proteins. Since our method does not rely on stable isotope labeled internal standards, it was critical to evaluate its linearity, which was larger than $R^2 = 0.9$ for each of the 7 added peptides tested over a concentration range from 10 to 500 nM supporting the presumed linear relationship between peptide concentration and MS response [28]. A linear correlation between peptide concentration and detected signal allows compositional analyses not only from a qualitative but also from a quantitative point of view enabling us to address changes in concentrations of the detected compounds [56]. Randomization of the order of analysis was used to level out systematic errors due to unavoidable between-day and within-day analytical variation, which might otherwise confound the ensuing statistical analysis. Together, these performance characteristics can be used for power calculations when setting up biomarker discovery projects [18]. Guidelines for power calculations in LC-MS driven biomarker research are lacking. However, experimental data from microarray studies provide some guidelines with respect to power calculations, i.e. sample size, variability of the population, desired detectable differences, acceptable error rate, experimental design, technical variability and data pre-processing [57;58].

In contrast to most published studies, we opted for preanalytical normalization of the amount of injected sample as compared to post-analytical data normalization, since post-analysis normalization cannot account for non-linear effects such as ion suppression. There are still many issues to address in post-analysis data normalization like global vs. local normalization, replicate filtering and averaging [34;38;59]. The urinary creatinine concentration is generally used as normalization factor in comparative studies [17;18]. Normalization based on creatinine was compared with a multi-compound normalization strategy based on the AUC_{214} of

the chromatographic region of interest (excluding amino acids, small hydrophilic peptides and other early-eluting UV-absorbing compounds). The latter approach seems better adapted to normalizing the amount of urine over a wide range of compounds, as was supported by the lower CV of the AUC_{TIC} and the peak areas of the added peptides. The drawback of this approach is that duplicate analyses are required and that the relatively large contribution of one peak/compound to the total UV-area suggests in essence normalization to only a few high abundant UV-absorbing compounds. Development and evaluation of other preanalytical multi-compound normalization strategies for the comparative profiling of urine remains therefore an issue.

4.2. Data (pre-)processing and multivariate statistical comparison of LC-MS profiles of urinary compounds in different experimental settings

The performance of the developed analytical platform was evaluated by (i) assessing the effect of analytical and biological variation on the comparison of urine samples spiked with exogenous peptides and (ii) by a preliminary study comparing urine samples of hospitalized patients with and without proteinuria due to various causes.

Unsupervised multivariate statistical analysis by PCA using the complete peak matrix of about 14000 peaks was unable to discriminate blank from spiked pooled urine samples. The observed variation between the samples is in this case composed of analytical variation and variation introduced by the added peptides (low nM concentrations). Our results suggest that spiking affects the linear combination of all elements composing the data vectors in an unsupervised setting only minimally. It is therefore necessary to apply a supervised classification algorithm (the NSC with permutation-based cross-validation in our case) to select discriminating elements prior to PCA, which resulted in clear separation between spiked and blank samples in the PC 1 dimension. We employed the NSC classifier, because of its simplicity and the fact that Tibshirani et al. [39] found it to perform as well or even better than several other classifiers. The validity of the described approach was shown by the fact that the NSC algorithm was capable of extracting the added peptides as discriminatory peaks out of the 14000 feature containing peak matrix. However, 3 peaks selected by the classifier could not be related to the added peptides through possible ion-suppression due to or in-source fragmentation products of the added peptides. Analytical artifacts and suboptimal data-processing issues, e.g. binning [56], might have played a role. These issues are presently being addressed.

Comparing blank and spiked urine samples of different healthy individuals by unsupervised PCA proved that variation in urinary peptide profiles between-

subjects (i.e. biological variation) was much larger than within-subjects (i.e. analytical variation), irrespective of spiking. However, the correct classification of blank versus spiked urine samples from different individuals using the NSC-selected peaks (14 out of 16 were related to the spiked peptides) emphasizes the applicability of the platform also in the presence of considerable biological variation. Nevertheless some issues, e.g. the selection of false-positive discriminatory peaks, remain to be addressed.

Our preliminary study comparing urine samples from hospitalized patients with a normal and high protein content resembled a study of Jurgens et al. [18]. Using LC combined with off-line MALDI-TOF MS detection and differential peptide display to compare pathological with healthy urines, they observed a substantial number of peptides in post-renal disease, which are absent in normal urine. Ninety-two peaks were selected from the LC-MS data to obtain a zero classification error. The larger portion (75%) of these peaks eluted late in the gradient, which suggests that they are rather hydrophobic. Clustering of the non-proteinuric samples from healthy individuals apart from the non-proteinuric samples from hospitalized patients, indicates that there is a trend of increased renal dysfunction in these patients as visualized by a right-shift in PC 1. Our current platform appears to be suitable in the study of early-stage renal disease but larger sets of samples need to be analyzed to substantiate these findings. The identified precursor proteins in the proteinuric urine samples are in good agreement with other studies profiling the urinary proteome [3;10;16;51] and peptidome [18]. The identification of two discriminatory peaks as serum albumin derived peptides is in accordance with the expected rise of albumin during glomerular proteinuria. Albumin is the first protein to rise in proteinuria and is degraded intracellular in the lysosomes after which the fragments are being exocytosed to the apical and basolateral sides of the renal tubular cells [60]. Recently it has also been suggested that significant amounts of albumin fragments are excreted in urine, possibly resulting from tubular degradation of filtered albumin, followed by luminal secretion of its fragments [61;62]. Disease-specific proteolytic degradation products of tissue or biofluid proteins are possible biomarker candidates [46]. However, most discriminatory peaks, including the albumin-derived peptide peak, were not significantly elevated in proteinuria when analyzed by univariate statistics due to a very large biological variation amongst the proteinuric patient group. The use of higher-order interactions between features in multivariate statistical analysis thus provides more discriminative power than univariate statistics. Manual univariate comparison of selected peaks remains, however, imperative for biomarker selection and further identification.

In summary, we have developed an analytical platform for the comparative analysis of urine samples by LC-MS followed by dedicated data preprocessing and multivariate statistical analysis of the obtained profiles. The analytical and biological variation did not adversely affect the performance of the method with respect to the classification of blank and spiked samples of different origin. The preliminary study of pathological and healthy urine samples emphasizes the potential of the platform not only for patient classification but also for detecting trends. However, some issues remain to be addressed and improved to enhance the performance of the platform. Currently we are implementing more advanced data processing and multivariate statistical analysis approaches such as: data meshing instead of binning, optimized M-N rule filtering for peak detection and improved peak matching methods together with de-isotoping and charge-deconvolution. Advanced retention time alignment and possibly m/z alignment algorithms are also being considered as well as other classifying algorithms and methods. Combined with larger and better defined urine sample sets this will allow us to do advanced proof-of-principle studies towards the goal of defining reliable biomarkers suitable for clinical validation.

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Supporting Information

Supporting Information Available: Detailed experimental description of the urine analysis method and MS/MS database queries for protein and peptide identification. This material is available free at http://pubs.acs.org.

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Chapter 6

Evaluation of a biomarker discovery platform for low-molecular weight urinary compounds:

comparative analysis of urine from pregnant and non-pregnant females by liquid chromatography – mass spectrometry and multivariate statistical data analysis

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Abstract

Introduction: This paper describes the application and evaluation of a comparative urine analysis platform employing reversed-phase liquid chromatography-mass spectrometry (LC-MS) and multivariate statistical data analysis for the discovery of low-molecular weight (LMW) urinary compounds that are differentially excreted in human pregnancy. *Methods and materials:* Urinary compounds were separated by gradient elution and subsequently detected by electrospray ionization (ESI) Ion-Trap mass spectrometry. Normalization of the injected amount was based on the creatinine concentration and on the area under the curve of the UV-chromatogram at 214nm (AUC_{214}). Data processing included 2-dimensional smoothing (meshing), peak detection with a geometrical algorithm based on local slope, and time alignment of the peaks using correlation optimized warping with a two-dimensional correlation signal based on the peaks themselves. Discriminatory peaks were selected from the final peak matrix by supervised classification using the nearest shrunken centroid (NSC) algorithm. Final dimensionality reduction and visualization were performed using principal component analysis (PCA). Using this methodology, urine samples from 7-16 weeks pregnant females (n=25) and age-matched controls (n=25) were comparatively analyzed. *Results:* Sample classification using all peaks (15876) already showed clear separation of the pregnant group from the control group with a sensitivity of 96% and a specificity of 100%. At a shrinkage of 3.11 (186 discriminatory peaks) sensitivity and specificity were 100%. When increasing the shrinkage further to 7.52 (10 discriminatory peaks) and to 12.55 (1 discriminatory peak), the sensitivity decreased from 96% to 92%, respectively, while the specificity remained 100%. Deconvolution of multiple charge states and isotopic distributions of the 186 discriminatory peaks resulted in a list of 38 discriminatory peaks that also contained the discriminatory peaks from higher shrinkage values. *Discussion:* The current method is capable of discriminating urine samples from pregnant and non-pregnant females. Optimization of data-processing improved the quality of the selected peaks, although a minor number of noisy peaks and redundancy in the peak list of discriminatory peaks at low shrinkage values remained. Other classification algorithms will be explored as well as improving the currently used NSC, to address this problem. Relating sensitivity and specificity to shrinkage value can assist in determining the most discriminatory biomarker candidates. Efforts are presently directed at identifying discriminatory compounds and at validating the classification model in an independent test-set.

1. Introduction

At least some of the pathological changes in human organs are reflected in urine. Thus urine-analysis can aid in diagnosis, treatment monitoring and prognosis of notably diseases of the genitourinary tract [1]. Biomarker discovery studies have used urine as sample matrix, because it can be obtained in large quantities by non-invasive sampling, it is less complex than blood, and urinary peptides/proteins tend to be relatively small in size, water-soluble and rather stable. Compounds in urine show furthermore fewer intermolecular interactions than blood compounds [2], making their separation, and thus detection and quantification, more easy. However, the composition of urine varies greatly between and within individuals because of differences in age and gender [3] as well as factors of cultural and dietary nature [4]. If possible, these factors should be controlled for by careful matching of patients and controls. Recent reviews emphasize important aspects regarding urinary biomarker discovery studies and achievements [5;6]. A recent viewpoint paper defines some essential factors that should be considered in the design and execution of biomarker discovery studies including study group selection, sample collection, choice of technology and quality control [7]. This requires close interdisciplinary collaboration.

Compositional analysis of urine can be done using numerous analytical techniques [8] (e.g. 2D gel electrophoresis, separation techniques hyphenated to mass spectrometry (MS) and their off-line variants. Liquid chromatography (LC) coupled to MS using electrospray ionization (ESI) is a frequently used analytical technique to profile urinary compounds for biomarker research, which has the advantages of automation, high sensitivity, high resolution and the feasibility of detecting thermolabile, water-soluble compounds without the need for chemical derivatization. LC-MS is, however, time-consuming and suffers from ion-suppression effects of interfering compounds. Urine analysis by LC-MS has been applied in many fields: peptidomics [9-19], proteomics [1;20-26], metabolomics [27-29] as well as in drug metabolism research. The use of internal standards and/or standardization of the injected amount is necessary in order to correct for possible interferences of matrix components with the ionization of compounds of interest in quantitative studies to generate comparable LC-MS data [30]. A quantitative label-free profiling method of complex biological samples seems feasible as long as there is linearity of signal versus concentration and a high degree of reproducibility of sample processing (i.e. pre-analytical phase) and the LC-MS method (i.e. analytical phase) itself [31;32].

In a previous paper [33] we have described the development, evaluation and application of a comparative urine analysis platform for the discovery of low-

molecular weight (LMW) urinary biomarkers using LC-MS and multivariate statistical data analysis. We observed that normalization of the injected amount to the area under the curve (AUC) of the chromatogram at 214 nm (AUC_{214}) instead of creatinine levels was better adapted to the profiling of a wide range of molecules with different physico-chemical properties (e.g. molecular weight, pK_a, hydrophobicity), which was reflected in the lower relative standard deviation (RSD) of individual peaks and the total chromatographic peak area. Processing of LC-MS data prior to multivariate statistical analysis was implemented in the workflow, because this is critical when comparing LC-MS data sets that may reach 10^7 to 10^8 data points per analysis. Data processing has the goal to correct for unwanted variations (analytical noise) in data sets [34-36], thus generating a peak matrix that contains only relevant information that is comparable. This peak matrix is obtained by matching common peaks in different samples and by filling locations that 'miss' peaks with background subtracted local data. After data processing the nearest shrunken centroid (NSC) classification algorithm [37] is used to reduce the dimensionality and to select discriminatory peaks. Visual inspection of patterns in the high-dimensional data space was done using principal component analysis (PCA), a technique that further reduces dimensionality to two dimensions [38]. Using this methodology we were able to extract discriminatory peaks from the LC-MS data of spiking-experiments despite biological and analytical variation down to the low nM concentration range. The methodology was applied to identify differences in the urinary composition of patients with kidney disease that is accompanied by normal protein excretion or proteinuria. This resulted in the identification of differentially excreted albumin-derived peptides amongst other compounds that allowed discrimination.

In this paper we describe the application and evaluation of this comparative urine analysis platform for the discovery of LMW biomarkers that are differentially excreted in urine from pregnant as compared to non-pregnant females. The methodology was improved with respect to data processing and data analysis compared to our previous work [33]. The discriminatory peaks that were selected by the classification model were evaluated for their discriminatory properties in terms of sensitivity and specificity.

2. Experimental

2.1. Subjects: pregnant and non-pregnant females

Urine samples from 25 pregnant females [median (range) age 38.7 years (29.2-41.3)] were retrieved from a biobank of frozen (-20 °C) urine samples from the department of Obstetrics and Gynecology of the University Medical Center in Groningen (The Netherlands). Urine samples in this biobank originated from pregnant females that attended the clinic for prenatal screening because of their age. The median (range) duration of gestation of the 25 pregnant females was 9.8 weeks (7.6-15.7). Samples of first-void midstream morning urines that were brought to the hospital, were aliquoted and stored at -20 °C for a median (range) period of 7.1 years (7.1-7.2) before analysis by LC-MS. We prospectively collected first-void midstream morning urine samples from 25 non-pregnant females [median (range) age 27.4 years (21.0-45.5)], notably colleagues and friends. The median (range) storage time at -20 °C for the samples from non-pregnant females was 11 days (3-30) before analysis by LC-MS.

Pregnancy was established by measurement of urinary free beta subunits of the human chorionic gonadotropin (β-hCG). Urine samples of 25 pregnant females had a median (range) β-hCG of 16396 mIU/mL (2713-62103). For 24 controls β-hCG levels were below the lower detection limit of 1.2 mIU/mL with one non-pregnant control showing a β-hCG level of 22 mIU/mL, because she gave birth 18 days before the date of urine collection. All samples were collected in polypropylene containers (Sarstedt, Nümbrecht, Germany) and kept at 4 °C for a maximum of 1 day, after which they were aliquoted in 10 mL polypropylene tubes (Becton Dickinson, Heidelberg, Germany) and stored at -20 °C. None of the subjects was fasting at time of urine collection.

All subjects that participated in this study gave their oral and/or written informed consent. The study protocol was in agreement with local ethical standards and the Helsinki declaration of 1964, as revised in 2004.

2.2. Chemicals

Acetonitrile (ACN) HPLC-S gradient grade (Biosolve; Valkenswaard, The Netherlands), ultra pure water (18.2 MΩ/cm), trifluoroacetic acid (TFA) 99% spectrophotometric grade (Aldrich; Milwaukee, USA), formic acid (FA) 98-100% pro analysis (Merck; Darmstadt, Germany) were used for reagent preparation. A peptide stock solution [peptide, concentration in mmol/L; VYV, 0.29; YGGFL (leucine enkephalin), 0.20; DRVYIHFP (angiotensin II), 0.10; YPFPGPI (β-casomorphin 7), 0.16; YPFPG (β-casomorphin 5), 0.21; GYPPT (gluten exorphin

A5), 0.19; YGGWL (gluten exorphin B5), 0.20] was used to evaluate the repeatability of the analytical LC-MS system. The first five peptides were obtained from Sigma (Zwijndrecht, The Netherlands) and the latter two from PepScan (Lelystad, The Netherlands).

2.3. Sample Preparation

Urine samples were thawed resulting in a minimum of 1 and maximum of 3 freeze-thaw cycles, then they were mixed, acidified with 1% TFA, stored overnight on melting ice, and centrifuged to remove precipitate (10 min at 1500 *g* and 4 °C). The supernatant was diluted 1:1 with 0.2% FA in 10% ACN and stored at 4 °C until analysis.

Urinary creatinine concentrations were assayed on a Merck MEGA (Merck, Darmstadt, Germany) and on a Roche/Hitachi Modular P (Roche Diagnostics, Basel, Switzerland) analyzer using the alkaline picrate (Jaffe) and enzymatic creatinine (creatinine plus) assay, respectively. Urinary levels of β-hCG were measured on an AutoDELFIA (Wallac Oy, Germany) with a solid-phase, two-site fluoroimmunometric assay.

2.4. Reversed Phase LC-MS analysis

The injection order of the urine samples was randomized. Five µL of the 500-times diluted peptide stock solution (in 0.1% FA and 5% ACN) were injected onto the precolumn prior to injection of each urine sample. Between each injection the injection system was cleaned by rinsing with 70% ACN and filled again with 0.1% FA in 5% ACN. System stability was monitored and evaluated by 5 µL injections of the 500-times diluted peptide stock solution before and after each series of 10 urine samples. An equivalent of each urine sample corresponding to 50 nmol creatinine was injected in a first instance (creatinine-batch). In second instance, we calculated a median AUC_{214} and normalized all AUC_{214}-based injection volumes to this value (AUC_{214}-batch). The AUC_{214} was calculated between 20 and 80 min retention time, which corresponds to 4 - 64 min for mass spectrometric data acquisition (MS data acquisition was started 16 min later than the gradient program). The majority of UV-absorbing compounds eluted in between 20 and 80 min retention times [33].

All LC-MS analyses were performed on an 1100 series capillary HPLC system equipped with a cooled autosampler (4 °C), a UV detector (λ=214 nm) and an SL Ion Trap mass spectrometer (Agilent Technologies). A urine volume equivalent to 50 nmol of creatinine or the median AUC_{214} was desalted on an Atlantis™ dC_{18} precolumn (2.1 × 20 mm, 3 µm particles and 10 nm pore diameter; Waters, Milford, Massachusetts, USA) using 0.1% FA in 5% ACN at a flow rate of 50 µL/min for 16 min. Urinary compounds were back-flushed from the precolumn onto a

thermostated (25 °C) Atlantis™ dC_{18} analytical column (1.0 × 150 mm, 3 µm particles, 30 nm pores; Waters) and separated in 90 minutes at a flow rate of 50 µL/min during which the percentage of solvent B (0.1% FA in ACN) in solvent A (0.1% FA in ultra pure H_2O) was increased from 5.0 to 43.6% (0.43%/min). During these 90 minutes UV absorption and positive mode ESI-MS spectra were acquired. Settings for ESI and MS were as follows: 16.0 psi N_2; drying gas: 6.0 L/min N_2; T: 325 °C; cap. voltage: 3.8 kV; skimmer: 57.5 V; cap. exit: 190.7 V; oct. 1: 4.12 V; oct. 2: 2.49 V; oct. RF: 190.7 V; lens 1: -4.9 V; lens 2: -37.7 V; trap drive: 52.5; scan speed: 5500 m/z s^{-1}; trap loading: 50 ms accumulation time or 30000 ions; scan range: 100-1500 m/z; Gaussian acquisition filter (width 0.1 m/z) of each scan; rolling average of 5 spectra. Spectra were saved in the centroid/line mode. Following each gradient, both columns were washed with 95% B for 5 min and equilibrated with 5% B for at least 10 min prior to the next injection.

2.5. Data processing and analysis

2.5.1. Data analysis for method evaluation

LC-MS chromatographic data were analyzed with the Data Analysis software provided with the LC/MSD Trap (version 3.4 build 181) (Bruker Daltoniks, Bremen, Germany). For raw (unprocessed) LC-MS data, peak areas, intensities and retention times were obtained from the respective smoothed and baseline subtracted extracted ion chromatograms (EIC). One cycle of smoothing with a Gaussian filter at a width of 1 sec (± 1.8 points) preceded baseline subtraction with a flatness of 1.

Univariate statistical analyses were performed with the Statistical Product and Service Solutions package version 14.0 (SPSS Inc., Chicago, IL, USA).

2.5.2. Data processing

For processing and multivariate statistical analysis the original Bruker Daltoniks line-mode LC-MS data files were converted into ASCII-format with the Bruker Data Analysis software using an in-house developed visual basic script. The data processing workflow was developed and compiled using Visual Studio 2005, version 8.0.50727.762 (SP.050727-7600, Microsoft Corp, Redmond, WA, USA). For further data analysis Matlab [version 7.3.0.267 (R2006b) Mathworks, Natick, MA, USA] and the PLS toolbox (version 4.0, Eigenvector Research Inc., Wenatchee, WA, USA) were used.

Initially the m/z ratios were meshed using a 2-dimensional (2D) Gaussian filter with sigma values of 0.05 min and 0.25 amu for the retention time and m/z dimension, respectively. Meshing partially compensated for the slight shift in m/z values as a result of trap overfilling that occurred during elution of abundant

compounds [39] and was used to smooth and fit deficient line data into a regular grid. Peak picking was performed using a geometrical algorithm based on local slope. The algorithm scans the mesh for local maxima. Starting at and descending from the peak maximum the algorithm then detected changes in the slope (from negative to positive) of the path of connected points thereby assigning the end/border of the peak. Connected points for one maximum were then considered to belong to one peak and the connected points in the perimeter of the peak were determined. This algorithm enabled the separation of overlapping peaks in particular shoulder peaks. After the algorithm has completed for a mesh, all peaks are output along with their height, width, volume, and extents in both m/z and retention time. Furthermore, each peak has a local estimate of the background value. In this work, the peak height was used for all analysis. **Figure 1** shows a typical 3-dimensional plot of LC-MS data after the data processing.

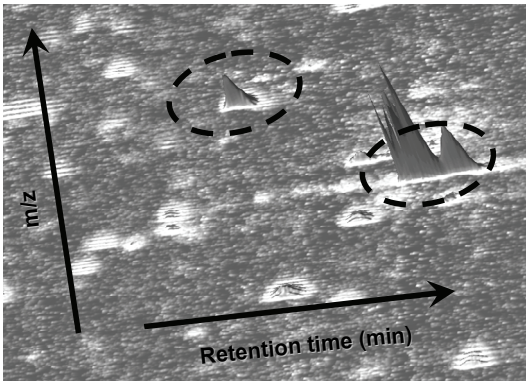

Figure 1.
Example of a bird's view (3-dimensional plot) of typical LC-MS data after data processing. Data were processed using 2-dimensional meshing without data reduction and peak picking using a geometrical algorithm based on local slope.

After having defined all peaks accurately, time alignment was performed using 2D correlation optimized warping (COW) of peak locations stored in the peak list of each chromatogram [40]. Then the full set of peaks from all samples was searched for local clusters of peaks, referred to as "MetaPeaks." The generated final peak matrix of the complete study consisted of an index(*row*)-sample(*column*)-height(*value*) matrix. The index corresponds to one unique combination of a retention time and m/z value from a particular metapeak. This final peak matrix was used for multivariate statistical analysis by NSC and PCA.

All data processing was done on a personal computer equipped with a +3800 MHz AMD processor with 4 GB of RAM.

2.5.3. Classification and multivariate statistical methods

To select the most discriminating peaks, we applied the NSC classification algorithm [37;41]. NSC regularizes data whereby class-specific centroids are "shrunk" toward the overall (nonclass-specific) centroid, which has the effect of eliminating the influence of the most weakly correlated peaks, thereby reducing the capacity to overfit [42]. This algorithm is used to select peaks that are relevant for the discrimination of the predefined classes in conjunction with permutation tests to validate the classification algorithm [43] using leave-one-out cross-validation (LOOCV) to avoid overfitting due to single outliers. The optimal shrinkage value was the value at which LOOCV showed the lowest classification error. In LOOCV one observation per class is iteratively omitted from the data set that is used to construct the classification model, which is then used to classify the omitted observation as case or control (e.g. pregnant or non-pregnant in this study). Variables selected at the highest shrinkage value (lowest number of peaks) and lowest LOOCV error, were employed for construction of the final classification model. The selected peaks were then analyzed and visualized by plotting the first two principal components obtained after PCA [38]. As a measure for class separation the Mahalanobis distance (MD) was calculated [44]. We consider an MD above 4.0, corresponding to a difference of 4 sigma between the mean centroids of the classes [43], as indicative for significant class separation.

3. Results

3.1. Evaluation of the normalization strategy and LC-MS system

In pregnant females the urine volume equivalent to 50 nmol creatinine corresponded to a higher mean (±SD) of the AUC of the total chromatogram (TIC) between 20 and 105 min retention time than in non-pregnant female controls: 5.4 (±0.8) $*10^9$ arbitrary units (AU) vs. 4.7 (±0.6) $*10^9$ AU, respectively ($p<0.001$). There was no significant difference ($p>0.2$) between the median (range) AUC_{TIC} of the creatinine- (4.9 (3.7-7.4) $*10^9$ AU) and the AUC_{214}-normalized (4.7 AU (3.2-8.1) $*10^9$ AU) batches. The RSD of the AUC_{TIC} was lower for the AUC_{214}-normalized data than for creatinine-normalized data, 15 vs. 24%, respectively. **Figure 2** shows histograms of the RSD for all peak intensities in the final peak matrix of the creatinine- and AUC_{214}-normalized data. The AUC_{214}-normalized LC-MS data (11639 peaks; median RSD 96.0%) had a higher ($p<0.001$) median RSD of peak height than the creatinine-normalized LC-MS data (12233 peaks; median RSD 91.8%).

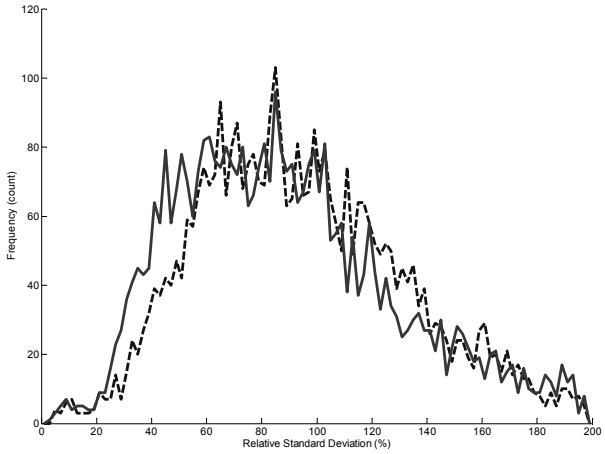

Figure 2.
Histogram of the relative standard deviation (RSD) of peaks in the final peak matrix from the batch that was normalized to creatinine (a urine volume corresponding to 50 nmol creatinine was injected; 12233 peaks; **filled line**) and the batch that was normalized to the area under the curve at 214 nm between 20 and 80 min retention time (AUC_{214}; 11639 peaks; **striped line**). Each batch consisted of urine samples from 25 pregnant females and from 25 apparently healthy non-pregnant females.

Normalization to creatinine (median RSD 88.7%) better reduced the variation in the pregnant group ($p<0.001$) than normalization to AUC_{214} (median RSD 92.3%). Also in the non-pregnant group, normalization to creatinine (median RSD 90.1%) better reduced the variation ($p<0.001$) than normalization to AUC_{214} (median RSD 95.3%). Normalization of the injection volume to the AUC_{214} better reduced the RSD of the AUC_{TIC} compared to normalization to the creatinine concentration. Remarkably, creatinine-normalization resulted in a lower median RSD of peak heights in each group (pregnant/non-pregnant) than AUC_{214}-normalization.

System-stability was evaluated by calculating the within-series correlation of six 5 μL injections of the 500-times in 0.1% FA and 5% ACN diluted peptide stock solution (IS) before and after each series of 10 urine samples. **Figure 3** shows a high correlation ($R^2 > 0.95$) of peak intensities for creatinine-normalized samples as well as for AUC_{214}-normalized samples ($R^2 > 0.9$), between the six individual samples, except for the first file in the creatinine-batch (absence of peak for peptide VYV from the peak list) and the last file in the AUC_{214}-batch (absence of peak for peptide YGGWL from the peak list). The latter finding is remarkable, because the peak is clearly visible in the raw LC-MS data, which suggests a data processing error. This reasoning is not valid for the missing VYV peak, because the raw MS data lacks this peak at the expected retention time.

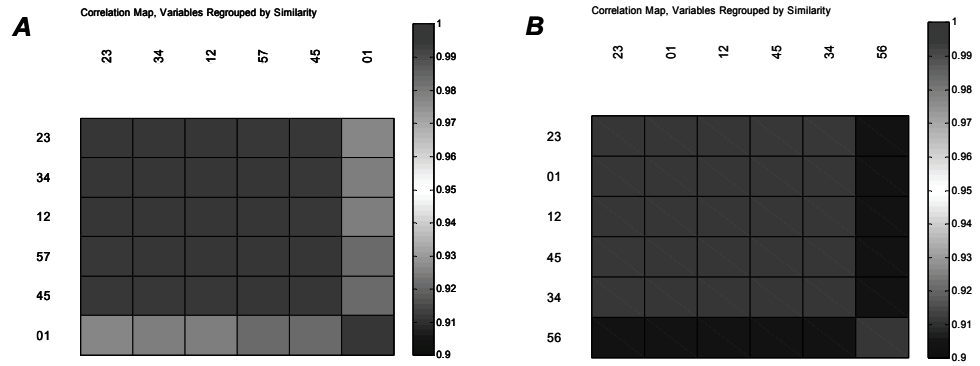

Figure 3

Correlation map of the final peak matrix (obtained as described in 'Experimental') from six 5 µL injections of 500-fold diluted peptide stock solution (1.0-2.9 pmol injected). These samples were analyzed before and after each series of 10 samples (equivalent to ±24h of analysis time) of the creatinine-normalized batch (**A**) and the batch of samples that was normalized to the area under the curve at 214 nm (**B**). Samples are regrouped by similarity. The numbers correspond to the order of analysis (e.g. 23 means that this sample was the 23rd sample that was analyzed in this batch.

3.2. Data processing and multivariate statistical analysis of LC-MS profiles

3.2.1. Data processing and analysis

The data processing workflow was significantly improved in comparison to our previous report (33). The previous workflow included binning from an instrumental resolution of 0.1 amu to 1 amu. This was done to reduce the size of the datasets (approximately by a factor 10) to reach an acceptable data processing and analysis time with Matlab on a desktop computer (see 'Experimental' for specifications). The new data processing workflow (C++) compared to the old data analysis processing ((33); Matlab) is improved by the following: (i) C++ provides much greater performance, particularly with large spectral datasets. (ii) Data meshing is used to address the problem of the irregular distribution of peak intensities of line data, and to detect peaks by assigning the top of a peak. (iii) Higher performance when working with regular, meshed data rather than irregular points. (iv) Calculation of peak parameters (height, volume, extent, background) along with the separation of overlapping peaks. (v) Finally, the peak picking algorithm is better capable of detecting and accurately quantifying relatively small peaks (low S/N), thus enlarging the dynamic range.

The most important improvement is, however, the inclusion of 2D alignment (retention time and m/z) in the workflow. In the previous study the SD for

retention time was ≤0.52 min (33). Comparing peak heights of batches (e.g. a training-set and a test-set) that are analyzed interspaced in time (e.g. months to years between analyses) requires proper peak matching in both the time- and m/z-dimension. First, COW (42) was applied to align the TIC of different samples. COW is suitable for single signal detectors (e.g. UV, fluorescence, flame-ionization etc.) and for samples that contain low numbers of peaks. We adapted the COW algorithm to calculate the correlation between the TIC (hereafter referred to as the 'COW-TIC') of different samples in a similar manner as for single signal detectors. However, urine is a complex biological sample and its composition is highly variable, qualitatively and quantitatively. The biological variance of the concentration of individual compounds is high, resulting in markedly different TIC of samples from different individuals. Therefore, the COW-TIC algorithm, a 1-dimensional alignment algorithm, was not able to align the 50 chromatograms, because retention time shifts within-batch and biological variation led to dissimilar TIC.

The time alignment algorithm was then changed to calculate correlations based on the peak list of each chromatogram. By doing this, 2 dimensions (2D: retention time and the mean m/z-value of the intensity distribution, including the measured widths of each peak in m/z and retention time) are taken into account in contrast to the COW-TIC, that uses only 1 dimension (1D; the TIC is a summed intensity) for COW. The new 2D-alignment algorithm corrects for large time shifts. In addition, inclusion of peak matching using metapeak clusters, and implementation of a 2D 'shift-area' to compensate for shifts in retention time and m/z decreases the probability of peak mismatching compared to the 1D sliding windows peak matching algorithm used previously (33). In summary the new data processing workflow resulted in a well-aligned peak matrix with a more accurate measure for peak intensity – all with significantly improved performance.

To evaluate whether data processing reduced the variation in retention time between the different chromatograms, the TIC was divided into 10 segments of 8 min between 25 and 105 min retention time. This is the region where the majority of peaks detected by MS elute (see example of TIC in (33)). From each of these segments, the four highest peaks were extracted from the final peak matrix of the AUC_{214}-batch. From these four highest peaks, the extracted ion chromatogram (EIC) of the peak with the 'poorest' alignment before and after 2D-alignment is depicted in **Figure 4**.

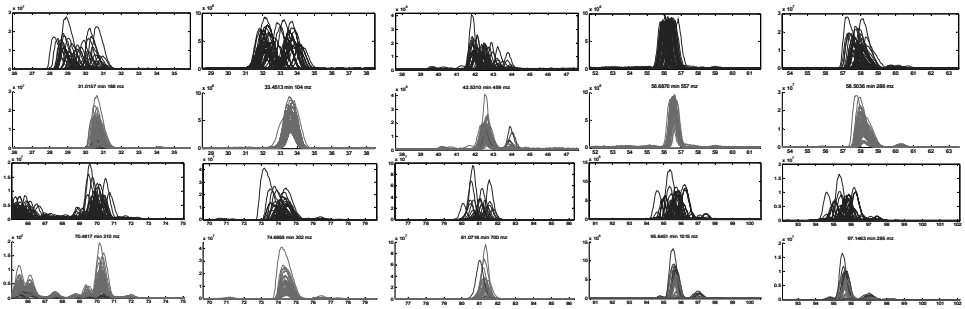

Figure 4.

Each panel represents an extracted ion chromatogram (EIC) of the peak with the 'poorest' time alignment derived from the four highest peaks in each of the ten 8 minute segments between 25 and 105 minutes retention time. The **upper trace** in each panel represents meshed LC-MS data before 2-dimensional (2D: retention time and m/z) alignment using correlation-optimized warping, while the **lower trace** represents meshed data after 2D-alignment. The final peak matrix from the analysis 50 urine samples (25 pregnant females; 25 non-pregnant females) that were normalized to the area under the curve at 214 nm was used.

3.2.2. Multivariate statistical comparison of urine from pregnant and non-pregnant females

The final peak matrix of the AUC_{214}-batch contained 15876 peaks. By varying the shrinkage value of the NSC classifier and following the LOOCV error, we obtained a region with zero cross-validation error (**Figure 5A)**. The plot of all 15786 peaks in the final peak matrix explains only 11.5% of the variance on principal component (PC) 1 and PC 2 (**Figure 5B**), although there was little overlap between the pregnant and non-pregnant groups (MD of 2.45). This resulted in a sensitivity of 96% and a specificity of 100% when using all peaks in the peak matrix for sample classification.

The NSC algorithm selected 186/15876 (1.2%) peaks at a shrinkage of 3.11 that separated (MD is 5.39) the two classes without any LOOCV error (specificity and sensitivity of 100%) **(Figure 5A and 5C)**. Using these 186 discriminatory peaks, PC 1 and PC 2 explain 52.1% of the variance in the data (**Figure 5C)**. Increasing the shrinkage to 7.52 resulted in the selection of 10/15876 (0.063%) discriminatory peaks that explained 90.6% of the variance in the data through PC 1 and PC 2 (**Figure 6A**). The MD was 4.16, which indicates significant separation of the classes, with a sensitivity of 96% and specificity of 100%. **Figure 6B** shows the individual values in a Box-Whisker plot for the only peak (514.6 m/z, 95.65 min; $p=6.8*10^{-10}$) that remains at a shrinkage value of 12.55 (92% sensitivity, 100% specificity).

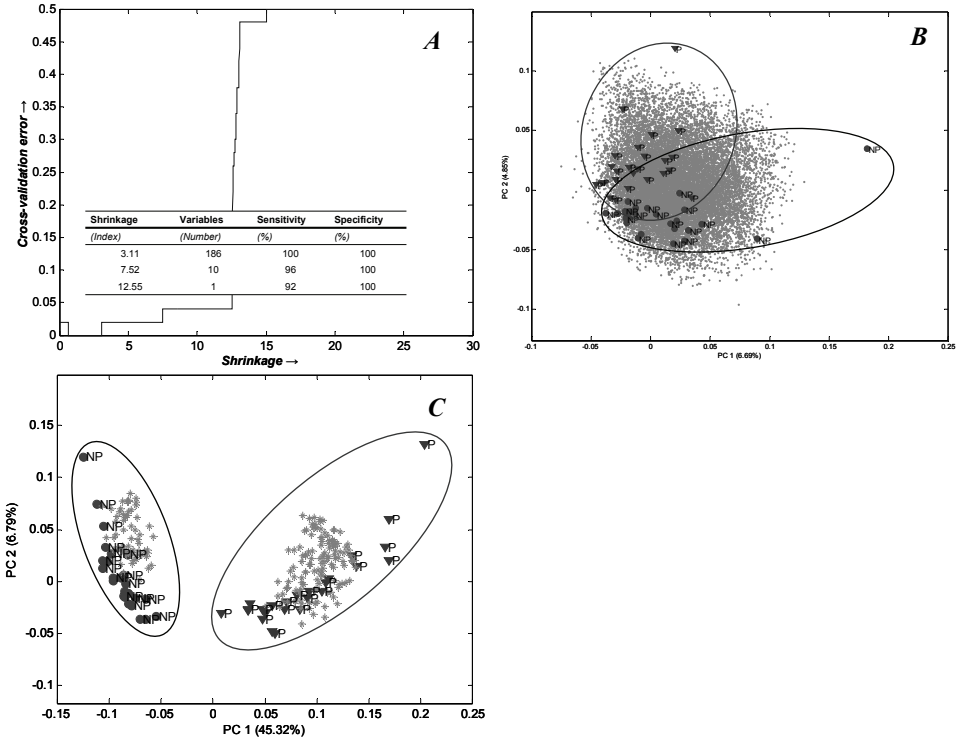

Figure 5.

Leave-one-out cross validation (LOOCV) error plot (**panel A**) of the classification model derived from the analysis of the final peak matrix (15876 peaks) from data of 50 urine samples (25 pregnant females; 25 non-pregnant females) that were normalized to the area under the curve at 214 nm. Cross-validation error (Y) was calculated at each shrinkage value (X) using the nearest shrunken centroid algorithm. The inserted table reports the number of discriminatory peaks, sensitivity and specificity of the classification model at three shrinkage values. Plot of principal component (PC) 1 and PC 2 using all peaks (15876) in the final peak matrix (**panel B**). Plot of PC 1 and PC 2 using 186/15876 peaks (1.2%) that were selected by the nearest shrunken centroid (NSC) algorithm at a shrinkage value of 3.11 (**panel C**). Pregnant females (▼), non-pregnant females (●), discriminatory peaks (✳).

We used the list of 186 discriminatory peaks that was obtained at a shrinkage value of 3.11 (sensitivity 100%, specificity 100%) as starting point to select biomarker candidates. A histogram of the ^{10}log of the p-values from the non-parametric comparison (Mann-Whitney U test; MWU-test) of discriminatory peak heights in the final peak matrix between pregnant and non-pregnant females is depicted in **Figure 7**. All selected peaks had p-values < 0.01 with some p-values reaching down to 10^{-10}. One selected peak had a p-value of 0.4 and was, according to the MWU-test, not significantly different between pregnant and non-pregnant females. This peak was probably selected because it is a Na^+-adduct (81.25 min, 722.7 m/z) of a highly discriminatory peak (81.26 min, 700.6 m/z; p= $3.17*10^{-07}$).

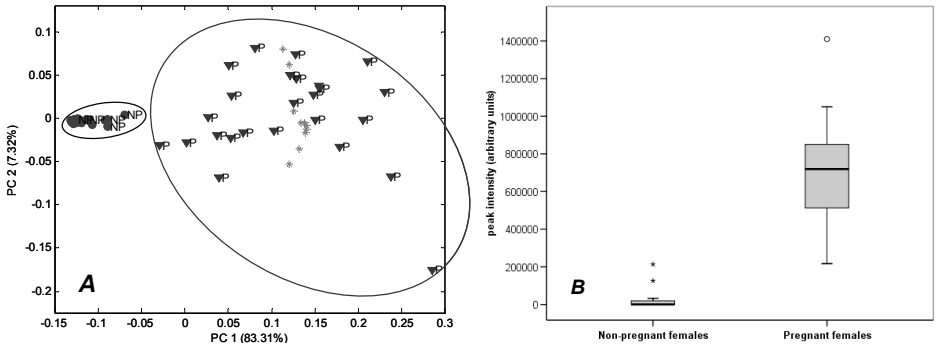

Figure 6.

Plot of principal component (PC) 1 and PC 2 using 10/15876 peaks (0.063%) that were selected by the nearest shrunken centroid (NSC) algorithm at a shrinkage value of 7.52 from the final peak matrix (15876 peaks) from data of 50 urine samples (25 pregnant females; 25 non-pregnant females) that were normalized to the area under the curve at 214 nm (**panel A**). Box-and-Whisker plot (**panel B**) of the only peak (514.6 m/z; 96.7 min) that was selected by the nearest shrunken centroid algorithm at a shrinkage value of 12.55 (p-value = 6.18×10^{-10}; see Table 1). Pregnant females (▼), non-pregnant females (●), discriminatory peaks (✳).

In addition, the use of a non-parametric test like the MWU-test, which compares mean ranks, may be less appropriate when many values in both groups are set at 0 (equal ranks). Visual inspection of both the raw and processed data show a clear difference in peak height of this peak, and a Student's t-test of the non-Gaussian distributed data resulted in $p=3.95*10^{-4}$. Interestingly, in the urine of pregnant females we found 12/186 (6.5%) discriminatory peaks to correlate ($p<0.05$; 5 negatively, 7 positively) with free β-hCG subunit levels. The free β-hCG concentration in the urine samples of pregnant females ranged between 0.12 and 2.8 µmol/L, which would be equivalent to 0.36 to 17 pmol on-column, based on the conversion table of the WHO of 1975 (1 IU = 0.045 nmol free β-hCG). The β-hCG subunit, however, was not identified by LC-MS/MS (unpublished observation).

Redundancy in the peak list was explored and reduced by manual deisotoping and charge-state deconvolution. Plotting the raw m/z values against retention time revealed furthermore Na^+ adducts and H_2O-loss due to "in-source" fragmentation of certain discriminatory peaks. Correlation analysis of the intensity of discriminatory peaks showed that some are quantitatively related (unpublished observations), implying structural or functional similarities. The curated peak list contained 38/186 (20.4%) peaks with most of these peaks being related to a different compound.

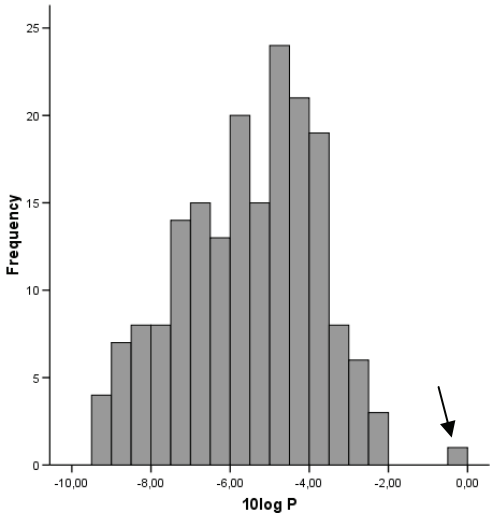

Figure 7.
Histogram of the ^{10}log plot of p-values obtained by non-parametric comparison of the peak-volumes of 186 peaks that discriminate urine of 25 pregnant females from urine of 25 non-pregnant females with 100% sensitivity and 100% specificity. One peak was not statistically significant at α=0.05 (arrow).

Ten of these 38 peaks were the principal discriminatory peaks at a shrinkage level of 7.52 (sensitivity 96%, specificity 100%) (**Table 1**). Univariate statistical comparison (**Table 1**) and EIC (**Figure 8**) of these discriminatory peaks revealed strongly elevated levels in the majority of the urine samples from pregnant females making them candidate biomarkers for pregnancy.

Table 1. ***Characteristics of 10 peaks that discriminate between urine samples from pregnant and non-pregnant females at a shrinkage value of 7.52 (sensitivity 96%, specificity 100%).***

Peak #	RT [a]	m/z [b]	Peak height pregnancy (P) [c]	Peak height controls (NP) [d]	p-value [e]
	(min)	*$(M+1H)^{1+}$*	*Arbitrary units*	*Arbitrary units*	
1	78.3	604.6	1390000 (0-3100000)	34549 (0-192143)	1,79E-008
2	95.6	285.4	1140000 (353106-2730000)	0 (0-324000)	3,68E-010
3	95.6	1015.9[f]	903843 (0-2220000)	7364 (0-246307)	2,24E-007
4	95.7	203.4	44150 (14448-95888)	3996 (0-17652)	1,35E-009
5	95.7	514.6	719223 (216616-1410000)	0 (0-213025)	6,18E-010
6	95.7	519.7	432093 (0-533025)	64568 (0-259183)	2,29E-008
7	97.0	519.7	180159 (0-347504)	8948 (0-35279)	1,79E-007
8	98.3	301.6	223725 (0-392491)	4459 (0-35978)	6,42E-006
9	98.3	477.7	221690 (0-379820)	7231 (0-52876)	2,23E-006
10	98.3	517.6	246524 (91532-559978)	9057 (0-78090)	1,23E-009

Data represent median values (minimum-maximum). **Figure 8** depicts the extracted ion chromatograms of the discriminatory peaks. [a] RT, retention time; [b] Mass-to-charge ratio derived from final peak matrix generated as described under 'Experimental'; [c] Peak height derived from the final peak matrix of AUC_{214}-normalized LC-MS data of 25 pregnant females (P); [d] Peak height derived from the final peak matrix of AUC_{214}-normalized LC-MS data of 25 female controls (C); [e] p-value derived from Mann-Whitney U tests for non-Gaussian distributed data (tested with the Shapiro-Wilk test and normality plots); [f] z=2.

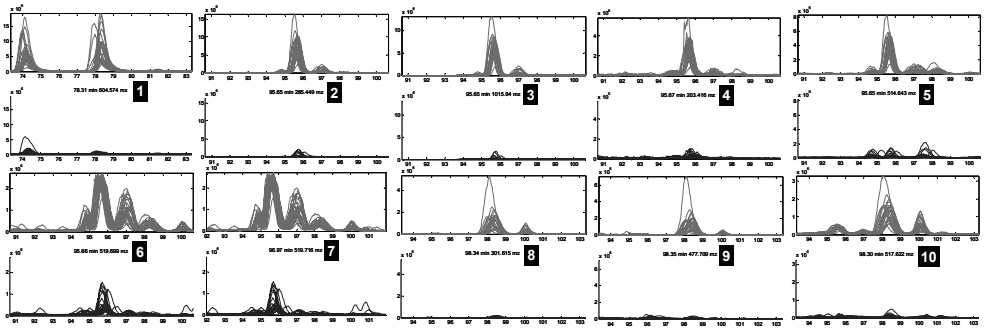

Figure 8.

Overlaid extracted ion chromatograms (EIC) of 10 peaks that discriminate between urine samples from pregnant and non-pregnant females with a sensitivity of 96% and specificity of 100%. The **upper panel** shows EICs of peaks from 25 pregnant females. The **lower panel** shows EICs of peaks from 25 non-pregnant females. The data is derived from the final peak matrix of the AUC_{214}-normalized batch. **Table 1** reports the characteristics of the discriminatory peaks. In most panels additional peaks are present with similar m/z-values but different retention times than the discriminatory peaks (centered peak in each panel). These additional peaks also differentiate between the samples of pregnant and non-pregnant females. More than 95% of these additional peaks have discriminatory properties (included in list of 186 discriminatory peaks at a shrinkage of 3.11), but they are not selected at the shrinkage level of 7.52.

4. Discussion and conclusion

The aim of this work was to evaluate our comparative urine analysis platform for its potential to differentiate between two physiological states. For this we have used profiling of LMW urinary compounds from pregnant and non-pregnant females by LC-MS and subsequent multivariate statistical analysis of processed data.

Normalization of the injected amount to the AUC_{214} did not result in a lowering of the peak height variance compared to normalization of creatinine, although this did reduce the variance in the AUC_{TIC}. This suggests that biological variation is not compromised by normalization to the AUC_{214}. Moreover, AUC_{214}-normalization better normalizes the injected amount if the AUC_{TIC} is considered to be representative for this. It should, however, be remarked that the peaks in the peak matrix of the AUC_{214}-batch were not matched to those of the creatinine-batch. An alternative, and even better approach is to run quality control (QC) samples (e.g. a pooled urine sample) before and after a predefined number of samples (e.g. 10). These QC could be analyzed at different creatinine concentrations (e.g. 25, 50 and 75 nmol creatinine) to evaluate the biological and analytical variation of the method and its quantitative properties. Furthermore, it can be questioned whether normalization to the AUC_{214} is appropriate in case of renal dysfunction. Large amounts of UV-absorbing compounds are excreted during renal pathology which will then lead to erroneous normalization if compared to urine samples from

controls with normal renal function. Thus, in each biomarker discovery study for disorders of the genitourinary tract the normalization strategy should be dependent on the disease under study.

Our new data processing workflow improved the quality of the final peak matrix with respect to the comparability and accuracy. Respectively, this was done by geometrical peak picking and peak measurement, and COW in 2D (retention time and m/z). In addition, full-resolution Ion-Trap MS data was used instead of binned Ion-Trap MS data to avoid loss of information during data processing. However, some peaks were not properly aligned (**Figure 3**) and peaks with low S/N were not properly picked and thus not included in the peak matrix. Adaptation of certain settings, e.g. 2D peak detection window, 2D alignment algorithm window, to the quality of the data is needed to optimize the output of the algorithms.

By assessing the relation between shrinkage value, cross-validation error, sensitivity, specificity and the number of discriminatory peaks we were able to optimize and improve the selection of discriminatory peaks, notably by reduction of the number of candidate biomarkers. Manual univariate comparison and visualization of selected peaks for both classes aided also in biomarker selection. In addition, we observed that the model at a shrinkage value of 3.11 (186 peaks) with both a specificity and sensitivity of 100% contained many 'noisy' and redundant peaks which might be caused by the narrow window that was used to match peaks to the metapeaks. The trade-off between a decrease in sensitivity and an increase in number of discriminatory peaks, might be a good strategy for selecting 'real' candidate biomarkers that are characterized by a strong discriminatory capacity. Implementing sensitivity, specificity and MD as measures for proper sample classification should improve the statistical evaluation and thus the quality of the selected biomarker candidates. The next step is to test the performance of the final classification model and the associated candidate biomarkers in larger independent test sets that include related 'disorders' such as extra-uterine pregnancies and testis carcinoma. We will also investigate the effects of short- and long-term storage (-20 °C) of urine samples from pregnant and non-pregnant females on the classification. However, the aim of this study was not to find a biomarker for pregnancy, but merely to obtain an advanced-proof-of principle and to optimize the comparative analysis platform.

At present we are trying to identify the discriminatory peaks by LC-MS/MS. The majority of the discriminatory peaks (independent of the shrinkage value) eluted late in the gradient, which suggests that they are rather hydrophobic. In addition, the low molecular masses of the discriminatory peaks and their non-peptide like fragmentation in MS/MS experiments (unpublished observations) suggests that we

are dealing with metabolites of pregnancy-related hormones and possibly post-translationally modified proteins (e.g. glycoproteins). The anticipated variation in the concentration of these discriminatory peaks because of the large variation in gestational age, is clearly exemplified by the larger distribution of the samples in the 'pregnancy' cluster compared to the 'non-pregnant' cluster (**Figure 6A**).

Pregnancy is a physiological condition in which modified immune responses allow a semi-allogenic fetus to survive within the uterine environment. The gestational tissues, including the uterine myometrium, the decidua, the placenta and fetal membranes, allow the fetus to survive and considerably modify the composition of blood, urine, amniotic fluid, cervicovaginal fluid etc. during gestation [45]. These biofuids are increasingly investigated for early diagnostic markers of preterm labor, pre-eclampsia and fetal growth restriction [46-48]. Compounds that are differentially expressed in pregnancy and of which some are even pregnancy specific are placental lactogen, chorionic gonadotropin (of which β-hCG is a subunit), fetal fibronectin, α-fetoprotein, dimeric inhibin A, unconjugated estriol, placental peptides, pregnancy-associated placental protein-A [3]. In our group of pregnant females the gestation duration ranged from 7.6-15.7 weeks, which leads to a considerable variation in the concentrations of these pregnancy specific compounds in blood. This concentration variation of hormones, proteins, their proteolytic fragments or conjugates is probably reflected in the composition of urine. Comparing the urinary composition of pregnant with non-pregnant females we expected on forehand to find clear differences, because the analytical method (reversed phase LC – positive mode MS) is capable of detecting those compounds.

The finding of correlations between β-hCG in urine and peak heights of certain discriminatory peaks in the urine of pregnant females suggests that some of the selected peaks discriminate patients based on an indirect relationship, either metabolic, functional or structural, with the total content of free β-hCG in urine. However, some correlations may be false because of a type-II error. The sample pretreatment procedure that consisted of at least 1 freeze-thaw cycle and sample acidification for mild protein precipitation could have removed pregnancy-related proteins or their fragments. Also, most pregnancy-related proteins are glycoproteins, which may have hampered the identification of these proteins by 'routine' proteomics, i.e. analysis by LC-MS/MS and protein database queries.

In summary the comparative urine analysis method has benefited from improvements in the data processing workflow. It was possible to discriminate urine samples of pregnant females from those of non-pregnant females with a sensitivity of 96% and specificity of 100% using only 10 discriminatory peaks.

Identification of the discriminatory peaks and validation of the classification model in an independent test-set is imperative.

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Part III.

Appendices

Summary and Future Perspectives

In the **Introduction** I give a description of the burden, epidemiology, classification and implicated causes of psychiatric disorders such as schizophrenia and autism. The etiology of these disorders is complex and only partially understood. At the same time mental disorders contribute enormously to psychological, social and economic suffering, on a global and individual level. Having an affected relative can have detrimental effects on future perspectives of a family, notably in third-world countries, because most disorders are characterized by early onset and chronicity with little hope of full recovery. The predicted rise in prevalence of certain psychiatric disorders by the WHO is worrying. Despite the relatively wide availability of psychiatric services and psychiatrists in Western countries, under-diagnosis and under-treatment are still common. Currently available diagnostic and treatment (psychosocial and/or medication) modalities are not perfect. Diagnostic modalities have poor consistency in the short-term, while treatment modalities have poor efficacy and significant side-effects. Metabolic side-effects burden the lives of patients with psychiatric disorders extra, because they are associated with increased risk of cardiovascular disease (CVD) in a population that is already at risk. Risk factors for CVD are difficult to treat in patients with schizophrenia, because of substance abuse and self-neglect. Few patients with schizophrenia recover sufficiently to have a normal societal life. The largely unknown disease etiology and a diagnostic system that is solely based on observations of symptom clusters seem to be responsible for a poor prognosis of patients with psychiatric disorder. It is surprising, in this context, that the many epidemiological studies and the studies of animal models (e.g. for anxiety, depression and addiction) have not resulted in drugs other than those that were discovered by serendipity. These drugs have, however, increased our knowledge about mental disorders on a molecular and cellular level with respect to neuronal signaling, and insight into their modes of action is increasing. So far, it seems that hypothesis-driven research has failed to provide novel solutions for psychiatric disorders, while identifying many risk factors related to psychosocial, cultural, and early-life events. While genetic studies have uncovered predisposing gene candidates (many of which could not be replicated in subsequent studies), their overall contribution to the susceptibility to mental disorders appears to be small or negligible compared to the contribution of non-genetic risk factors. Despite these efforts in fundamental research, no single gene has been identified that explains the complex etiology of mental disorders and that might function as a lead towards more effective therapies.

More recently altered gene expression due to epigenetic regulation has been implicated in the development of mental disorders. Our environment (e.g.

nutrition), and even some of the putatively involved mutations (e.g. MECP2) and polymorphisms (e.g. MTHFR), can affect gene expression via epigenetic mechanisms. This partly explains why genetic findings are not easily replicated and possibly also the way psychosocial and cultural factors increase the risk for the development of a psychiatric disorder. Therefore, it seems to make more sense to study health and disease at the 'executive' level of a cell or organism, i.e. quantitative and qualitative changes at the protein level are likely to better reflect the dynamic processes that are characteristic for health and disease than is done by our 'static' genome. The same reasoning is valid if we are trying to increase insight into the process(es) that lead(s) to the development of a mental disorder.

Modern non-hypothesis driven technologies, such as genomics, transcriptomics, proteomics and metabolomics, are likely to increase our comprehension of mental disorders through the (expression-) profiling of hundreds to thousands of genes, gene-transcripts (mRNA), proteins and metabolites, respectively. Powerful bioinformatics approaches are essential for the integration of these results in functional correlation networks, thereby offering the possibility to study mental disorders in a systems biology approach. If non-hypothesis driven research is used in such a way that it complements hypothesis driven studies, it can be expected that in the near future new prognostic, diagnostic and therapeutic biological markers (biomarkers) or panels of markers for mental disorders will be discovered. These markers should have superior sensitivity and specificity and relate in a causative manner to the organ, tissue, cell or molecular pathway that is part of the pathophysiology. In the end it may even be possible to make mental disorders run a less severe course, to prevent or delay the onset in susceptible individuals, to decrease the presence or effect of environmental risk-factors, to identify highly-susceptible individuals, and to prevent or even cure mental disorders in generations to come. The latter is a utopia if we appreciate the thought that psychiatric disorders are a an inseparable part of the spectrum of humanity.

In **Chapter 1** we elude on the role of folate, notably its role in carbon-1 metabolism and epigenetics, and long-chain polyunsaturated fatty acids (LCPUFA) in schizophrenia, autism and depression. As described in the **Introduction**, these complex disorders do not inherit by Mendel's law and the search for a genetic basis has remained unsuccessful. The relation of low birth weight and pregnancy complications with schizophrenia and autism suggests developmental adaptations by fetal 'programming'. Epigenetics might constitute the basis of such programming and depends on folate status and carbon-1 metabolism in general. In key animal experiments dietary carbon-1 substrate availability during pregnancy was found to affect gene expression in the offspring. This led to the idea that early folate status of

patients with schizophrenia might be compromised, which is supported by (i) coinciding incidences of schizophrenia and neural tube defects (NTDs) during the Dutch hunger winter of 1944-1945, (ii) coinciding seasonal fluctuations in birth of patients with schizophrenia and NTDs, and higher schizophrenia incidence in (iii) immigrants and (iv) methylene tetrahydrofolate reductase 677C→T homozygotes. Recent studies in schizophrenia and autism point at epigenetic silencing of genes (e.g. reelin) or chromosomal loci that are crucial for e.g. brain development. The product of the reelin gene, for example, is involved in neural plasticity and neurodevelopment. Findings of a low status of carbon-1 substrates in adults with schizophrenia with DNA hypomethylation and altered gene expression as possible consequences, add to the idea of aberrant carbon-1 metabolism in certain psychiatric disorders. Low folate status is also associated with the severity of the negative symptoms of schizophrenia. The same has been suggested for the LCPUFAs arachidonic (AA, from meat) and docosahexaenoic (DHA, from fish) acid, which are components of brain phospholipids, and modulators of signal transduction and gene expression. Patients with schizophrenia and possibly autism, exhibit abnormal phospholipid metabolism that might cause local depletion of AA and impaired eicosanoid-mediated neuronal signal transduction. National fish intakes relate inversely with major and postpartum depressions, which suggests a relation between the intake of the LCPUFA eicosapentaenoic acid (EPA, from fish) and the incidence of depression. Five out of six randomized controlled trials with add-on EPA have shown positive effects in schizophrenia and four out of six were favorable in depression and bipolar disorders. From the presented evidence we conclude that folate and LCPUFAs may be important in both the etiology and severity of at least some psychiatric diseases. These findings together with the fact that low status of B-vitamins and LCPUFA are associated with increased risk of cardiovascular disease, led to the study described in **Chapter 2**.

In **Chapter 2** we assessed the essential fatty acid (EFA) and B-vitamin status, together with their anthropometrical, lifestyle and biochemical determinants, in 61 patients with schizophrenia and established whether those with a very poor status of these important nutritional constituents respond biochemically to the appropriate dietary supplements. This study also aimed to test the membrane-phospholipid hypothesis in schizophrenia, which suggests altered rates of incorporation and removal of EFA from phospholipids in neural cell membranes. The fatty acid composition of erythrocytes (peripheral) was assumed to reflect the fatty acid composition of neurons (central) according to results from previous studies. We found that as a group, patients had high erythrocyte saturated fatty acid (FA) and monounsaturated FA levels but low levels of the important polyunsaturated FAs of the ω3 and ω6 series. Patients reporting not to take vitamin

supplements had low concentrations of serum vitamin B_{12} and high plasma homocysteine (Hcy). In a multivariate analysis Hcy variance proved best explained by serum folate in both the total group and in male patients, and by vitamin B_{12}- and B_6-blood levels in female patients. Alcohol consumption and duration of illness were found to be risk factors for low polyunsaturated FA status (<2.5th percentile of the reference range), while male gender and absence of fish consumption predicted hyperhomocysteinemia (>97.5th percentile of the reference range). To our astonishment we found two patients exhibiting biochemical EFA deficiency (20:3ω9 above the cut-off value), while 7 patients had biochemical signs of ω3/DHA marginality, which was defined as moderately increased 22:5ω6/DHA ratio. In addition, four patients exhibited intermediate hyperhomocysteinemia (30-100 μmol/L) with plasma values ranging from 57.5-74.8 μmol/L. Such severe abnormalities are rare in the general population suggesting a metabolic origin or the long-term consumption (many years) of a diet deficient in these essential nutrients. The fact that none of these 5 patients, with either intermediate hyperhomocysteinemia, biochemical EFA deficiency, or both, was assessed by their clinicians to have a poor diet, is worrying and indicative of suboptimal risk factor assessment and treatment in patients with schizophrenia. That diet was at the basis of these abnormalities was confirmed after supplementing 4 of these 5 patients with B-vitamins and with soybean and fish oils. Although, we did not measure psychiatric symptoms during supplementation, in the long-term the treating psychiatrist noticed some improvements. We concluded that a subgroup of patients with schizophrenia suffers from biochemical EFA deficiency, ω3/DHA marginality, moderate hyperhomocysteinemia, or combinations thereof. Correction is indicated in view of the possible relation of poor EFA and B-vitamin status with psychiatric symptoms, but notably to reduce their high risk of cardiovascular disease.

In **Chapter 3** we describe a study on the relationship between platelet (PLT) serotonin (5-HT) and intestinal permeability in children with pervasive developmental disorders (PDD) in Curaçao. Platelet hyperserotonemia is observed in 30-40% of patients with PDD, and we hypothesized that PLT 5-HT levels in PDD are mainly determined by intestinal motility (see **Chapter 4**), for which increased intestinal permeability might be a proxy. Previous studies reporting increased intestinal permeability in approximately half of the patients with PDD, are in support of the existence of a 'leaky gut syndrome' in a subgroup of children with PDD. The aim of this study was to assess whether a leaky gut is related to increased PLT 5-HT contents. For this, differential sugar absorption and PLT 5-HT were determined in 23 children with PDD. Platelet 5-HT (2.0-7.1 nmol/10^9 PLT) was elevated in 4 or 6 out of 23 patients, depending on the employed cut-off value. Two cut-off values were used: 5.4 nmol/10^9 PLT, is used in the diagnosis of

carcinoid tumors; and 4.55 nmol/10^9 PLT, is used to classify patients with PDD as being normo- or hyperserotonemic. Remarkably, none of the patients exhibited elevated intestinal permeability (urinary lactulose/mannitol ratio: 0.008-0.035 mol/mol). Also, no correlation between PLT 5-HT and intestinal permeability or GI tract complaints was observed, which led us to reject our initial hypothesis. PLT 5-HT correlated with 24h urinary 5-hydroxyindoleacetic acid (5-HIAA; p=0.034), which is the principal metabolite of 5-HT. Also urinary 5-HIAA and urinary 5-HT were interrelated (p=0.005) suggesting increased production of 5-HT to be responsible for PLT hyperserotonemia in a subgroup of children with PDD. To study the (in)consistency of intestinal permeability and PLT 5-HT, we suggest monthly monitoring in a well-defined patient and control group, notably to confirm or reject the presence of increased intestinal permeability in this group.

The usefulness of PLT 5-HT as a marker of intestinal motility is the topic of **Chapter 4**. To study the effects of intestinal motility on PLT 5-HT in a non-invasive indirect manner, we determined whether PLT 5-HT is lower at a condition of relative gut motor activity quiescence (i.e. in newborns at birth) compared with a condition of normal gut motor activity (i.e. in their mothers at birth), and whether in newborns institution and discontinuation of enteral feeding coincide with increases and decreases of PLT 5-HT, respectively. For this PLT 5-HT was determined in 17 mothers and their 18 healthy full-term newborns at birth in Curaçao. To support a role of intestinal motility as determinant of PLT 5-HT, longitudinal PLT 5-HT data and data of feeding modes (enteral or parenteral) in 5 out of 20 included preterm-born infants were evaluated. We replicated the findings from previous studies that newborns exhibit about two-fold lower PLT 5-HT compared with their mothers (medians: 1.5 and 2.9 nmol/10^9 PLT, respectively). In a multivariate analysis we found newborn PLT 5-HT to be positively related with maternal PLT 5-HT and newborn mean PLT volume, and negatively with newborn whole blood tryptophan. We have no explanation for the relation between newborn and mother PLT 5-HT. The negative correlation of newborn PLT 5-HT with newborn tryptophan suggests that PLT 5-HT is largely independent of tryptophan-availability. In the longitudinally investigated preterm-born infants we observed 7 increases and 1 decrease of PLT 5-HT during institution of enteral feeding (observed in 5/5 infants), and 2 decreases and 1 increase of PLT 5-HT during parenteral feeding (observed in 3/5 infants). Despite the low number of included newborns in the longitudinal study we expect that the lower PLT 5-HT at birth, and its change in response to the institution or discontinuation of enteral feeding, is related to the relatively gut motor quiescence at birth, and the increase/decrease of intestinal motility as reaction to the institution/discontinuation of enteral feeding, respectively. These results suggest gut motor activity to be an

important determinant of PLT 5-HT in early postnatal life. However, to support this notion, *in vivo* intestinal motility measurements should be correlated with changes in PLT 5-HT over time (e.g. some months).

In **Chapter 5** we describe the development, evaluation and application of a biomarker discovery platform for urine as a non-hypothesis driven approach. Low-molecular weight urinary compounds were analyzed by reversed-phase liquid chromatography coupled to mass spectrometry (LC-MS) and data were compared using multivariate statistical data analysis. More specifically, separation by gradient elution with acetonitrile and subsequent detection by UV-absorbance at 214nm in-line with electrospray Ion-Trap MS was employed. Using this technique we were able to resolve thousands of compounds with good sensitivity and selectivity. The method was evaluated for its lower limit of detection (5.7-21 nmol/L), within-day (2.9-19%) and between-day (4.8-19%) analytical variation of peak areas, linearity (R^2: 0.918-0.999), and standard deviation for retention time (<0.52 min) by means of addition of seven 3-8 amino acid peptides (0-500 nmol/L) to determine the possibilities and limitations of this biomarker discovery platform. Relating the amount of injected urine to the area under the curve (AUC) of the chromatographic trace at 214 nm better reduced the coefficient of variation (CV) of the AUC of the total ion chromatogram (CV =10.1%) than relating it to creatinine (CV =38.4%). This suggests that for specific biomarker discovery studies in urine from patients with normal renal function, a multi-compound normalization strategy is preferred over a single-compound normalization strategy. LC-MS data (retention time; mass-to-charge ratio; peak intensity) were subsequently preprocessed to improve data-handling (data reduction) and to render data comparable (peak matching) and free of noise (peak selection). The common peak matrix, containing all peaks for all samples, was analyzed by dimension-reducing principal component analysis (PCA) after supervised classification and variable selection by the nearest shrunken centroid (NSC) algorithm. The NSC algorithm removes peaks from the peak matrix that do not contribute significantly to the separation of groups of samples (e.g. spiked and non-spiked) with simultaneous control for classification errors by leave-one-out cross-validation. The feasibility of the method to discriminate urine samples of differing compositions was evaluated by (i) addition of seven peptides at nM concentrations to blank urine samples of different origin and by (ii) a study of urine from kidney patients with and without proteinuria. (i) The added peptides were ranked as highly discriminatory peaks despite significant biological variation. (ii) Ninety-two peaks were selected as discriminating proteinuric from non-proteinuric samples. Removal of redundant peaks from the peak list of 92 discriminatory peaks resulted in a list of 54 peaks of which 6 were more intense in

the majority of the proteinuric samples. Two of these 6 peaks were identified as albumin derived peptides by LC-MS/MS. This was expected, because of the early rise of albumin during the onset of glomerular proteinuria. Interestingly, other albumin derived peptides were non-discriminatory indicating the possibility of preferential proteolysis at certain cleavage sites. An advanced proof-of-principle of the comparative urine analysis platform was obtained by the study of urine samples from pregnant and non-pregnant females, as described in **Chapter 6**.

Chapter 6 is a sequel of **Chapter 5**. In this chapter we describe the application and evaluation of our non-hypothesis-driven comparative urine analysis platform to select urinary compounds that are differentially excreted in human pregnancy. A somewhat similar method to that described in **Chapter 5** was used. Data processing, however, was optimized and included peak meshing, peak detection using a geometrical algorithm, and peak-alignment in the 2-dimensions (retention time and m/z) through correlation optimized warping. This resulted in better comparable and more accurate data that were almost devoid of irrelevant peaks coming from analytical noise. Discriminatory peaks were selected by supervised classification with the leave-one-out cross-validated NSC algorithm and visualized by PCA. Urine samples of 7.6-15.7 weeks pregnant females (n=25) and non-pregnant females (n=25) were comparatively analyzed. Sample classification using all peaks (15876) in the final peak matrix showed only little overlap of the pregnant and non-pregnant group (sensitivity 96%, specificity 100%). Using the NSC algorithm 186 discriminatory peaks were selected at a shrinkage of 3.11 to fully separate the pregnant from non-pregnant samples (sensitivity and specificity of 100%). Increasing the shrinkage value to 7.52 (10 discriminatory peaks) and 12.55 (1 discriminatory peak) decreased the sensitivity only slightly to 96% and 92%, respectively, while the specificity remained 100%. The 186 selected peaks were evaluated by univariate comparison, visual inspection and deconvoluted for multiple charge-states and isotopic distribution to reduce redundancy. Results from correlation analysis of discriminatory peaks suggest structural and/or functional similarities. Relating specificity, sensitivity and additional measures for class separation to the shrinkage value and the number of discriminatory peaks can aid in improving the quality (i.e. discriminatory power) of the selected biomarker candidates. Work along these lines is in progress. The comparative analytical platform was able to discriminate urine samples from pregnant and non-pregnant females with an acceptable sensitivity and specificity using only a few peaks (10). Efforts now focus on the identification of discriminatory peaks and validation of the classification model using an independent test-set. The method will be applied to the discovery of markers related to diseases of the genitourinary tract and adverse

pregnancy outcomes, but it may also prove useful in studies related to mental disorders, especially those where a nutritional component is suspected.

In conclusion, this thesis aimed to evaluate certain biochemical markers of nutrition and presumably of intestinal permeability and motility in schizophrenia and autism, respectively (**Chapters 2 and 3**). Further in-depth analysis of fatty acid and carbon-1 metabolism in schizophrenia and autism (**Chapter 1**) is warranted, and so are studies to the role of the intestine and its biochemistry in autism. These studies should be complemented by symptom ratings. It is worrying that low statuses of LCPUFA and B-vitamins in patients with schizophrenia go largely undetected and untreated, because these low statuses are easily correctable by nutritional supplementation. The monitoring and treatment of comorbidity in psychiatric patients is of special importance, because comorbidity adds to the existing heavy disease burden.

To determine the meaning and usefulness of certain markers (e.g. PLT 5-HT), and to determine the role of factors that are likely to affect the concentration of these markers (e.g. intestinal motility) in autism, other groups of subjects were studied such as newborns and their mothers (**Chapter 4**). The study of other groups of subjects that are characterized by a greater homogeneity, absence of confounding co-morbidity, and the presence of the physiological condition of interest (e.g. normal or sub-normal intestinal motility) can facilitate the interpretation of phenomena observed in mental disorders.

Probably none of the investigated biochemical parameters are specific for a diagnostic psychiatric entity, and it seems that we are only able to (biochemically) characterize subgroups of patients. Present psychiatric nosology is not based on etiology, and until now no marker, mechanism or risk factor is able to explain the onset and development of psychiatric disorders. This fact, together with the enormous socioeconomic and psychological burden of psychiatric disorders, the predicted rise in prevalence, and availability of rather suboptimal diagnostic and therapeutic modalities calls for another view of researchers on research of complex diseases such as mental disorders (**Introduction**). Alternative approaches using non-hypothesis driven methods that have a valid underlying clinical question are therefore welcome to complement hypothesis driven research, although their potential should not be overestimated until proven otherwise.

Modern '-omics' technologies that enable the unbiased, comprehensive and simultaneous study of large numbers of genes, mRNA (gene-transcripts), proteins and metabolites in a systems biology approach, will certainly influence the way mental disorder research is conducted. The development, evaluation and application of an '-omics' technique for biomarker discovery in urine is described in

Chapter 5. Comparative analysis of low-molecular weight urinary compounds using state-of-the-art separation (liquid chromatography; LC) and detection (mass spectrometry; MS) techniques followed by tailored multivariate analysis of LC-MS data that contain thousands of compounds, is a good example of how non-hypothesis driven research can be performed. Optimization and further evaluation of this method in a group of pregnant and non-pregnant females exemplifies the possibilities and pitfalls of this methodology (**Chapter 6**). A stepwise approach in the development, evaluation and application of these '-omics' methods to more complex clinical research questions is necessary to convince the end-users of their potential and validity. Going down in concentration sensitivity (by e.g. prefractionation, affinity extraction), the application of more selective detectors, and the use of optimized algorithms for data-processing and -analysis are ways to improve the success rate of cross-sectional and longitudinal biomarker discovery studies. If these studies are complemented and integrated with results from hypothesis-driven research we might be able to unravel some of the mysteries of complex diseases including mental disorders such as autism and schizophrenia.

Samenvatting en Toekomstperspectief

In de **Inleiding** beschrijf ik de ziektelast en -kosten, de epidemiologie, diagnostiek en mogelijke oorzaken van psychiatrische stoornissen zoals schizofrenie en autisme. De etiologie van deze stoornissen is complex en wordt slechts deels begrepen. Tegelijkertijd dragen psychiatrische stoornissen enorm bij aan het lijden in psychologisch, sociaal en economisch opzicht, op een mondiaal en individueel niveau. Het hebben van een familielid met een psychiatrische stoornis kan een negatieve invloed hebben op het toekomstperspectief van een familie, voornamelijk in de derde wereld, omdat de meeste stoornissen zich openbaren in de jeugd of adolescentie, chronisch van aard zijn en er een weinig hoop op volledig herstel is. De door de Wereldgezondheidsorganisatie voorspelde stijging van het aantal personen dat op een bepaald moment lijdt aan een psychiatrische stoornis is zorgwekkend. Ondanks de relatief goede beschikbaarheid van psychiatrische zorg en psychiaters in de westerse wereld, zijn er nog steeds veel patiënten die niet gediagnosticeerd zijn of suboptimaal behandeld worden. De opties die op dit moment beschikbaar zijn voor de diagnostiek en behandeling (psycho-/gedragstherapie en/of medicamenteus) lijken niet optimaal. Zo wordt op de korte termijn de gestelde diagnose frequent gewijzigd en daarnaast is medicamenteuze therapie maar matig effectief en gaat deze gepaard met vervelende bijwerkingen. De metabole bijwerkingen zijn extra belastend voor patiënten met psychiatrische stoornissen, omdat deze een haast onvermijdelijk verhoogd risico op het ontwikkelen van cardiovasculaire aandoeningen met zich meebrengen in groep die al een verhoogd risico heeft. Cardiovasculaire risicofactoren zijn moeilijk te behandelen in patiënten met schizofrenie, vanwege middelenmisbruik en zelfverwaarlozing. Compleet herstel dat leidt tot normaal maatschappelijk functioneren is dan ook voor maar weinig patiënten weggelegd. De onbekende etiologie en de enkel en alleen op observaties van symptoomclusters gebaseerde diagnostiek lijken deels debet aan de slechte prognose van psychiatrische patiënten.

Het is verrassend dat de vele epidemiologische studies en studies van diermodellen (bijvoorbeeld voor angst, depressie en verslaving) nog niet geleid hebben tot de ontdekking van geneesmiddelen anders dan door serendipiteit en verbetering van de bestaande middelen. Geneesmiddelen, echter, hebben onze kennis over zenuwprikkeloverdracht op een moleculair en cellulair niveau vergroot, en het begrip van het werkingsmechanisme van deze geneesmiddelen groeit. Tot dusver lijkt het erop dat hypothesegedreven onderzoek er niet in geslaagd is om met nieuwe antwoorden te komen voor psychiatrische stoornissen, ondanks dat dit soort onderzoek geleid heeft tot de ontdekking van vele risicofactoren die psychosociaal of cultureel van aard zijn of verband houden met

gebeurtenissen in het vroege leven. Genetisch onderzoek heeft vele genen opgeleverd die predisponeren voor het ontstaan van psychiatrische stoornissen (in vervolgstudies, echter, kon van vele genen de betrokkenheid niet gerepliceerd worden). Globaal genomen lijkt de bijdrage die 'foute' genen leveren aan de kans op het ontwikkelen van een psychiatrische stoornis klein of verwaarloosbaar in vergelijking met de bijdrage van andere niet-genetische risicofactoren. Ondanks deze inspanningen op het gebied van fundamenteel onderzoek is er geen enkel gen gevonden welke de complexe etiologie van psychiatrische stoornissen verklaart en die kan dienen als aanknopingspunt voor de ontwikkeling van meer werkzame therapieën.

Recentelijk is gesuggereerd dat veranderde expressie van genen door epigenetische regelmechanismen een rol zou kunnen spelen bij het ontstaan van een psychiatrische stoornis. Onze omgeving (vb. voeding) en zelfs enkele 'verdachte' genmutaties (vb. MECP2) en genpolymorfismen (vb. MTHFR) kunnen genexpressie beïnvloeden door middel van epigenetische mechanismen. Dit verklaart gedeeltelijk waarom bevindingen van genetisch onderzoek moeilijk te repliceren zijn en eventueel ook hoe psychosociale en culturele factoren het risico vergroten op het ontwikkelen van een psychiatrische stoornis. Het lijkt dan ook verstandiger om gezondheid en ziekte te bestuderen op het 'uitvoerende' niveau van een cel of organisme, met andere woorden kwalitatieve en kwantitatieve veranderingen op eiwitniveau lijken een betere afspiegeling van de dynamische processen die kenmerkend zijn voor gezondheid en ziekte dan gedaan wordt door ons 'statische' genoom. Dezelfde redenering gaat op als we trachten ons inzicht te vergroten in het traject dat leidt tot het ontstaan van een psychiatrische stoornis.

Moderne technologieën, zoals (epi-)genomics, transcriptomics, proteomics en metabolomics die vaak op een niet hypothese gedreven wijze gebruikt worden zullen ons begrip van psychiatrische stoornissen verbeteren door het respectievelijk in kaart brengen van honderden tot duizenden genen, gen-transcripten (mRNA), eiwitten en metabolieten. Het gebruik van geavanceerde bio-informatica is essentieel voor integreren van deze resultaten in functionele correlatienetwerken, waardoor de mogelijkheid ontstaat om psychiatrische stoornissen vanuit de systeembiologie te benaderen. Wanneer de kennis die voortvloeit uit niet-hypothese gedreven onderzoek gecomplementeerd wordt met resultaten van hypothese gedreven onderzoek, dan mag verwacht worden dat in de nabije toekomst prognostische, diagnostische en therapeutische biologische markers (biomarkers) of combinaties daarvan voor psychiatrische stoornissen ontdekt zullen worden. Deze markers dienen wel superieure sensitiviteit en specificiteit te hebben en dienen bij voorkeur gerelateerd te kunnen worden aan het orgaan, het weefsel, de cel of moleculaire cascade die onderdeel is van het pathofysiologisch proces. Op

de lange termijn is het misschien zelfs mogelijk om de ernst van het verloop van psychiatrische stoornissen te verminderen, om het ontstaan ervan in individuen die verhoogd vatbaar zijn voor het ontwikkelen van een psychiatrische stoornis te voorkomen of te vertragen, om de aanwezigheid van risicofactoren in de omgeving te verminderen of hun impact te reduceren, om vatbare/gevoelige individuen te identificeren, en om het ontstaan van psychiatrische stoornissen in latere generaties te voorkomen of zelfs te genezen. Dit laatste is een utopie als we psychiatrische stoornissen beschouwen als een onlosmakelijk deel van het spectrum van menselijkheid.

In **Hoofdstuk 1** wordt uitgewijd over de rol van folaat als substraat in het koolstof-1 metabolisme en epigenetica, en over langeketen meervoudig onverzadigde vetzuren (LCPUFA) in schizofrenie, autisme en depressie. Zoals beschreven wordt in de **Inleiding,** worden complexe stoornissen zoals schizofrenie niet overgeërfd volgens de wet van Mendel, en het onderzoek naar een genetische basis is niet succesvol gebleven. De relatie van een laag geboortegewicht en zwangerschapscomplicaties met schizofrenie en autisme veronderstelt aanpassingen van de ontwikkeling van de foetus door `programmering'. Epigenetica staat mogelijk aan de basis van dit zogenaamde 'programmeren' en lijkt nauw verbonden met de folaat-status en het koolstof-1 metabolisme in het algemeen. In begripsbepalende proeven in zwangere ratten werd gevonden dat de beschikbare hoeveelheid koolstof-1 substraat (bijvoorbeeld folaat) in de voeding expressie van genen in het nageslacht kan beïnvloeden. Dit leidde tot de gedachte dat de folaat-status van patiënten met schizofrenie in hun perinatale leven gecompromitteerd zou kunnen zijn, wat ondersteund wordt door (i) samenvallende pieken in de incidentie van schizofrenie en neurale buisdefecten (NBD) na de Nederlandse Hongerwinter van 1944-1945, (ii) samenvallende seizoensgebonden schommelingen van het aantal geboortes van patiënten met schizofrenie en NBD, en een hogere incidentie van schizofrenie in (iii) immigranten en (iv) in mensen die homozygoot zijn voor het gen methyleen tetrahydrofolaat reductase 677C→T. Recente studies in patiënten met schizofrenie en autisme laten zien dat expressie van genen (vb. reeline) of chromosomale loci die cruciaal zijn voor bijvoorbeeld de ontwikkeling van de hersenen, gedereguleerd worden door epigenetische processen. Zo is het product van het reeline-gen betrokken bij het tot stand brengen van neurale plasticiteit en de neuro-ontwikkeling. Het feit dat in volwassen patiënten met schizofrenie een lage status van koolstof-1 substraten gevonden wordt, welke als mogelijke consequenties DNA hypomethylering en veranderde genexpressie heeft, versterkt het idee van een afwijkend koolstof-1 metabolisme in bepaalde psychiatrische stoornissen. Een lage folaat-status wordt ook geassocieerd met de ernst van negatieve symptomen in

schizofrenie. Een zelfde effect is voorgesteld voor een lage status van de LCPUFA arachidonzuur (AA, uit vlees) en docosahexaeenzuur (DHA, uit vis). Deze vetzuren maken deel uit van hersenfosfolipiden en modulatoren van signaaltransductie en genexpressie. Patiënten met schizofrenie, en mogelijk ook patiënten met autisme, vertonen een abnormaal fosfolipidenmetabolisme, wat lokaal uitputting van AA kan geven en een verslechterde eicosanoïd-gemedieerde zenuwprikkeloverdracht zou kunnen veroorzaken. Verder blijkt dat de inname van vis op nationaal niveau omgekeerd evenredig gerelateerd is aan de incidentie van postpartum-depressies en depressies in engere zin, wat een relatie suggereert tussen de inname van de ω3-LCPUFA eicosapentaeenzuur (EPA, uit vis) en de incidentie van depressie. Vijf van de 6 gerandomiseerde gecontroleerde studies naar de effecten van suppletie met EPA naast het gebruik van antipsychotica in patiënten met schizofrenie lieten positieve effecten zien, en 4 van de 6 studies toonden gunstige effecten voor patiënten met een depressie en/of bipolaire stoornis. Uit het hiervoor vermelde, kan men concluderen dat folaat en LCPUFA een belangrijk rol zouden kunnen spelen in zowel de etiologie als ernst van in ieder geval enkele psychiatrische ziekten. Deze bevindingen samen met het feit dat een lage status van B-vitaminen en LCPUFA geassocieerd wordt met een verhoogd risico op cardiovasculaire ziekten, leidde tot de uitvoering van de studie die beschreven wordt in **Hoofdstuk 2** van dit proefschrift.

In **Hoofdstuk 2** onderzochten we de status van essentiële vetzuren (EFA) en B-vitaminen en hun anthropometrische, leefstijl en biochemische determinanten in 61 patiënten met schizofrenie. We stelden vast of die patiënten met slechte status van deze belangrijke voedingsbestanddelen in biochemisch opzicht verbeterden na inname van de juiste voedingssupplementen. Daarnaast werd deze studie uitgevoerd om de membraanfosfolipiden-hypothese van schizofrenie te testen, welke stelt dat de inbouw en verwijdering van EFA-bevattende fosfolipiden uit neuronale celmembranen veranderd is in patiënten met schizofrenie. De vetzuursamenstelling van erytrocyten (perifeer) zou volgens eerder onderzoek een afspiegeling zijn van de vetzuursamenstelling van neuronen (centraal). Wij vonden dat in de groep patiënten als geheel hun erytrocyten veel verzadigde vetzuren (FA) en mono-onverzadigde FA bevatten en weinig LCPUFA van de ω3 en ω6 reeksen. De patiënten die geen vitaminesupplementen-gebruik meldden, hadden lage serum vitamine B_{12} en hoge plasma homocysteine (Hcy) spiegels. Uit een multivariate analyse bleek dat de variantie in Hcy het best verklaard werd door serum folaat in zowel de gehele groep patiënten als de mannelijke patiënten. De variantie van Hcy in vrouwelijke patiënten werd het best verklaard door vitamine B_{12} en B_6. Alcoholconsumptie en ziekteduur werden gevonden als risicofactoren voor het hebben van een lage status van meervoudig onverzadigde FA (<2.5 percentiel van

de referentiewaarden), terwijl "man-zijn" en de afwezigheid van vis in de voeding hyperhomocysteïnemie (>97.5 percentiel van de referentiewaarden) voorspelden. Tot onze verbazing vonden wij twee patiënten die biochemische EFA-deficiëntie (20:3ω9 boven de afkapwaarde) vertoonden en 7 waren biochemisch ω3/DHA marginaal, wat gedefinieerd was als een matig verhoogde 22:5ω6/DHA ratio. Bovendien vonden we vier patiënten met matig verhoogde Hcy-concentraties (30-100 μmol/L) met plasmawaarden die zich bevonden tussen 57.5-74.8 μmol/L. Zulke bevindingen zijn zeldzaam in de doorsnee bevolking. Dit suggereert dat deze bevindingen een metabole oorzaak hebben, en/of dat deze te wijten zijn aan de langdurige inname (vele jaren) van voeding die ontoereikende hoeveelheden B-vitaminen en LCPUFA bevat. Het feit dat geen van deze 5 patiënten met hun matig verhoogde Hcy, biochemische EFA-deficiëntie, of beiden, door hun behandelaren aangemerkt werd als een patiënt met een slechte voeding, is zorgwekkend, en wijst op suboptimaal monitoren van risicofactoren en de behandeling daarvan in patiënten met schizofrenie. Dat voeding aan de basis lag van deze afwijkende bevindingen werd bevestigd nadat vier van de vijf patiënten werden behandeld met B-vitaminen en met sojaboon- en visoliën. Hoewel psychiatrische symptomen niet gemeten werden tijdens de suppletie, bemerkte de behandelende psychiater wel verbetering op de lange termijn. Concluderend kan gesteld worden dat een subgroep van patiënten met schizofrenie lijdt aan biochemische EFA-deficiëntie, ω3/DHA marginaliteit, matig verhoogde Hcy, of combinaties daarvan. Behandeling van deze biochemische afwijkingen is geïndiceerd vanwege de mogelijke relatie van slechte EFA en B-vitamine status met psychiatrische symptomen, maar in het bijzonder om hun verhoogd risico op hart- en vaatziekten te verminderen.

In **Hoofdstuk 3** wordt een onderzoek beschreven naar het verband tussen het trombocyten (PLT) serotonine (5-HT) en de darmdoorlaatbaarheid in kinderen met pervasieve ontwikkelingsstoornissen (PDD) op Curaçao. Een verhoogde hoeveelheid 5-HT in de PLT wordt gevonden in 30-40% van kinderen met PDD. Onze hypothese was dat PLT 5-HT in PDD hoofdzakelijk bepaald wordt door darmmotiliteit (zie ook **Hoofdstuk 4**) en dat een verhoogde darmdoorlaatbaarheid hiervoor een proxy kan zijn. In eerdere studies werd gevonden dat ongeveer de helft van de patiënten met PDD een verhoogde darmdoorlaatbaarheid heeft, wat een argument is voor de aanwezigheid van het `lekke darmsyndroom' in een subgroep van kinderen met PDD. Het doel van deze studie was te beoordelen of er een verband bestaat tussen een 'lekke' darm en een verhoogde hoeveelheid 5-HT in PLT. Om dit te onderzoeken, werden de differentiële suikerabsorptie en het PLT 5-HT bepaald in 23 kinderen met PDD. Trombocyten 5-HT (2.0-7.1 nmol/10^9 PLT) was, afhankelijk van de gebruikte afkapwaarde, verhoogd in 4 of 6 van de 23 patiënten. Twee afkapwaarden werden gebruikt: 5.4 nmol/10^9 PLT, wordt gebruikt

voor diagnostiek van carcinoïdtumoren; en 4.55 nmol/10^9 PLT, wordt gebruikt om kinderen met PDD te classificeren als normo- of hyperserotonemisch. Het was opmerkelijk dat geen van de onderzochte patiënten een verhoogde darmdoorlaatbaarheid bleek te hebben (lactulose/mannitol ratio in urine: 0.008-0.035 mol/mol). Tevens werden er geen correlaties gevonden tussen het PLT 5-HT, de darmdoorlaatbaarheid en maag-darmklachten. Dit leidde tot het verwerpen van onze eerste hypothese. Trombocyten 5-HT correleerde met 5-hydroxyindoleazijnzuur (5-HIAA) in 24-uurs urine (p=0.034). 5-HIAA is een voornaamste metaboliet van 5-HT. Ook waren 5-HIAA in de urine en 5-HT in de urine aan elkaar gerelateerd (p=0.005), wat suggereert dat een verhoogde aanmaak van 5-HT verantwoordelijk is voor de verhoging van 5-HT in de PLT in een subgroep van kinderen met PDD. Om de (in)consistentie van darmdoorlaatbaarheid en PLT 5-HT te bestuderen, stellen we voor om deze maandelijks te meten in een goed gedefinieerde groep patiënten en controles, in het bijzonder om de aanwezigheid van een 'lekke' darm in deze groep te bevestigen of te verwerpen.

De bruikbaarheid van PLT 5-HT als parameter voor darmmotiliteit is het onderwerp van de studie die beschreven wordt in **Hoofdstuk 4**. Om de invloed te bestuderen van darmmotiliteit op PLT 5-HT op een niet-invasieve, indirecte manier, bepaalden wij of PLT 5-HT lager is in een toestand van relatief lage darmmotiliteit (d.w.z. in pasgeborenen bij de geboorte) in vergelijking tot een toestand van normale darmactiviteit (d.w.z. in hun moeders). Daarnaast bepaalden we of het starten of stoppen met enteraal voeden gepaard gaat met respectievelijk een stijging of daling van PLT 5-HT. Trombocyten 5-HT werd bepaald in 17 moeders en in hun 18 gezonde, a-term geboren kinderen bij de geboorte op Curaçao. Om de rol van darmmotiliteit als determinant van PLT 5-HT te ondersteunen, werd van 20 vroegtijdige geboren zuigelingen longitudinaal informatie verzameld over de voedingsroute (enteraal of parenteraal) en het PLT 5-HT. In 5 van de 20 zuigelingen was longitudinale informatie beschikbaar. Evenals eerdere studies vonden we dat pasgeborenen twee maal lagere PLT 5-HT waarden hebben in vergelijking tot hun moeders (medianen: 1.5 en 2.9 nmol/10^9 PLT, respectievelijk). Multivariate analyse liet zien dat PLT 5-HT van pasgeboren positief gerelateerd is aan het PLT 5-HT van de moeder en het gemiddelde PLT-volume van pasgeborenen, en negatief gerelateerd aan de tryptofaan concentratie in bloed van pasgeborenen. Wij hebben geen verklaring voor de relatie tussen het PLT 5-HT van pasgeborenen en hun moeders. De negatieve correlatie van PLT 5-HT met tryptofaan in pasgeborenen suggereert een tryptofaanonafhankelijkheid van PLT 5-HT in het vroege leven. In de longitudinaal onderzochte prematuur geboren zuigelingen vonden wij 7 verhogingen en 1 vermindering van het PLT 5-HT tijdens het starten van het enteraal voeden (in 5/5 zuigelingen), en 2 verminderingen en 1

verhoging van PLT 5-HT tijdens het stoppen van enteraal voeden (starten van parenteraal voeden) in 3/5 zuigelingen. Ondanks het kleine aantal bestudeerde pasgeborenen in de longitudinale studie verwachten we dat het lagere PLT 5-HT bij de geboorte en de verandering hiervan bij het starten en/of stoppen met enteraal voeden verband houdt met respectievelijk de relatief lage darmmotiliteit bij de geboorte, en de verhoging/vermindering van de darmmotiliteit als reactie op het starten/stoppen met enteraal voeden. Het lijkt erop dat darmmotiliteit een belangrijke determinant is van PLT 5-HT in het vroege postnatale leven. Echter, om dit idee te ondersteunen dienen *in vivo* metingen van de darmmotiliteit verricht te worden en deze dienen gerelateerd te worden aan veranderingen in PLT 5-HT in de tijd (bijvoorbeeld enkele maanden).

In **Hoofdstuk 5** beschrijven wij de ontwikkeling, de evaluatie en de toepassing van een platform voor de ontdekking van biomarkers in urine. Laag-moleculaire stoffen in urine werden geanalyseerd door middel van reversed-phase vloeistofchromatografie, gekoppeld aan massaspectrometrie (LC-MS) en data werden geanalyseerd door middel van multivariate statistische analyse van de van ruis 'gezuiverde' data. Specifieker, stoffen in urine werden gescheiden door middel van gradiënt-elutie met acetonitrile en detectie vond vervolgens plaats met UV-absorptie bij 214nm en elektrospray ionisatie Ion-Trap MS. Door gebruik te maken van deze techniek konden we duizenden stoffen scheiden met een goede gevoeligheid en selectiviteit. De methode werd geëvalueerd door middel van toevoeging van zeven 3-8 aminozuur lange peptides (0-500 nmol/L). Prestatie-indicatoren waren: detectielimiet (5.7-21 nmol/L), binnen-dag (2.9-19%) en tussen-dag (4.8-19%) analytische variatie van piekoppervlakten, lineariteit (R^2: 0.918-0.999) en standaarddeviatie van retentietijd (<0.52 min). Deze prestatie-indicatoren bepalen de (on)mogelijkheden van dit platform voor de ontdekking van biomarkers in urine. Het aanpassen van het (op de chromatografische kolom geïnjecteerde) urine volume aan het oppervlakte onder de curve (AUC) van het UV-chromatogram bij 214nm, verlaagde de variatiecoëfficiënt (CV) van de AUC van het chromatogram van de totale hoeveelheid ionen (CV =10.1%) meer dan wanneer het urine volume aangepast werd aan de creatinine concentratie (CV =38.4%). Dit suggereert dat voor specifieke studies naar de ontdekking van biomarkers in de urine van patiënten met een normale nierfunctie, aanpassing/normalisering van het urine volume op basis van meerdere stoffen te verkiezen is boven de aanpassing/normalisering op basis van één stof. LC-MS data, bestaande uit retentietijden, massa-lading (m/z) ratios en piekintensiteiten, werden vervolgens voorbewerkt met algoritmen om de verwerking van de data te verbeteren (data-reductie), en om ruis te verwijderen (piekselectie) en data vergelijkbaar te maken

(piekgroepering/-clustering). De uiteindelijke, gemeenschappelijke piekenmatrix die alle pieken van alle urinemonsters bevatte, werd geanalyseerd met principale componentenanalyse (PCA) na gesuperviseerde classificatie en piekenselectie door het "nearest shrunken centroid" (NSC) algoritme. Principale component analyse vermindert de dimensionaliteit van de data zodat visualisatie mogelijk is. Het NSC algoritme verwijdert die pieken uit de piekenmatrix die niet significant bijdragen aan de scheiding/classificatie van de gedefinieerde groepen monsters (vb. 'spiked' vs. blanco) en tegelijkertijd corrigeert dit algoritme voor classificatiefouten door een laat-er-één-uit kruisvalidatie. De haalbaarheid van de methode om urinemonsters van verschillende samenstellingen te onderscheiden werd onderzocht door (i) toevoeging van zeven peptiden (nM concentraties) aan blanco urinemonsters van verschillende individuen en een urine-'pool', en door (ii) een studie naar de urine samenstelling van nierpatiënten met en zonder proteïnurie. We vonden dat de methode (i) de toegevoegde peptiden rangschikte als sterkst onderscheidende pieken ondanks de grote biologische variatie die aanwezig is in de samenstelling van urine. (ii) Tweeënnegentig pieken werden geselecteerd met de methode als zijnde het meest onderscheidend tussen nierpatiënten met en zonder proteïnurie. Het opschonen van de piekenlijst met 92 onderscheidende pieken resulteerde in een lijst van 54 pieken waarvan er 6 intenser waren in de meerderheid van de urinemonsters die afkomstig waren van nierpatiënten met proteïnurie. Twee van deze 6 pieken werden geïdentificeerd met LC-MS/MS als peptiden die afkomstig waren van albumine. Dit is in overeenstemming met de verwachte vroege stijging van albumine tijdens glomerulaire proteïnurie. Het was opmerkelijk dat andere van albumine afkomstige peptiden niet onderscheidend waren, wat kan duiden op ziektespecifieke eiwitafbraak. De ontwikkelde methode werd vervolgens toegepast op een enigszins complexere vraagstelling waarbij de verschillen in samenstelling van de urinemonsters van zwangere en niet-zwangere vrouwen onderwerp van studie was (**Hoofdstuk 6**).

In **Hoofdstuk 6** beschrijven wij de toepassing en de evaluatie van ons niet-hypothesegedreven platform voor de vergelijking van urinemonsters, en de selectie van onderscheidende pieken, van zwangere en niet-zwangere vrouwen. Op enkele summiere veranderingen van het LC-MS systeem na werden de urinemonsters geanalyseerd zoals beschreven in **Hoofdstuk 5**. De verwerking van de LC-MS data, echter, werd geoptimaliseerd en bestond uit piekenschakeling ('meshing'), piekdetectie met een geometrisch algoritme, en 2-dimensionale (retentietijd en m/z) piekgroepering/-clustering door correlatiegeoptimaliseerd clusteren. Dit resulteerde in beter vergelijkbare, meer precieze en juiste data met minder pieken die afkomstig waren van analytische ruis. De onderscheidende pieken werden vervolgens geselecteerd met behulp van gesuperviseerde classificatie door het NSC

algoritme en laat-er-één-uit kruisvalidatie. De resultaten hiervan werden gevisualiseerd door middel van PCA. Urinemonsters van 7.6-15.7 weken zwangere vrouwen (n=25) en niet-zwangere vrouwen (n=25) werden geanalyseerd en vergeleken. Bij het gebruik van alle pieken in de uiteindelijke piekenmatrix (15876) voor de classificatie van de urinemonsters vonden we nauwelijks overlap tussen zwangere en niet-zwangere groep (sensitiviteit 96%, specificiteit 100%). Door gebruik te maken van het NSC algoritme werd een optimale shrinkage-waarde van 3.11 gevonden waarbij het aantal pieken in het classificatiemodel teruggebracht werd tot 186 (1.2%). Dit model had geen kruisvalidatie-fout, en een sensitiviteit en specificiteit van 100%. Het vergroten van de shrinkage-waarde tot 7.52 (10 onderscheidende pieken in het model) en 12.55 (1 onderscheidende piek in het model) leidde slechts tot een afname van de sensitiviteit tot respectievelijk 96% en 92%, terwijl de specificiteit 100% bleef. De lijst met 186 onderscheidende pieken werd geëvalueerd door middel van univariate toetsing en visuele inspectie, en opgeschoond voor wat betreft meerdere ladingstoestanden van 1 onderscheidende piek en isotopen-verdeling om het aantal mogelijke biomarker kandidaten te verminderen. Resultaten van de analyse van correlaties tussen onderscheidende pieken suggereren structurele en/of functionele relaties. Het relateren van de specificiteit, de sensitiviteit of andere maten voor het onderscheid tussen groepen aan de shrinkage-waarde en het aantal onderscheidende pieken, kan helpen bij het verbeteren van de kwaliteit (onderscheidend vermogen) van de geselecteerde biomarker-kandidaten. Hier wordt aan gewerkt. Het vergelijkende urineanalyse platform kon urinemonsters van zwangere en niet-zwangere vrouwen onderscheiden met een acceptabele sensitiviteit en specificiteit op basis van slechts een klein aantal pieken (10). Momenteel wordt gewerkt aan de identificatie van de onderscheidende stoffen en aan het valideren van het classificatiemodel met een onafhankelijke test-set. De uiteindelijke methode zal verder geoptimaliseerd worden en worden ingezet voor de ontdekking van biomarkers voor ziekten van de urogenitale tractus en ongunstige zwangerschapsuitkomsten, maar de methode zou ook bruikbaar kunnen zijn voor de bestudering van psychiatrische stoornissen waarbij een voedingscomponenten mogelijk een rol spelen.

Samenvattend, het doel van dit proefschrift was om markers van micronutriënten-status en darmmotiliteit te bestuderen in patiënten met schizofrenie en autisme (**Hoofdstukken 2 en 3**). Meer gedetailleerde studies naar het vetzuur- en koolstof-1 metabolisme in schizofrenie en autisme (**Hoofdstuk 1**) zijn gewenst, evenals studies naar de rol van de biochemie van de darm in autisme. Deze studies dienen bij voorkeur gecomplementeerd te worden met maten/scores van de psychiatrische symptomatologie. Het is zorgelijk dat lage statussen van zowel LCPUFA als B-

vitaminen in patiënten met schizofrenie grotendeels niet onderkend en dus onbehandeld worden, omdat deze lage statussen makkelijk corrigeerbaar zijn door middel van suppletie. Het monitoren en behandelen van co-morbiditeit in psychiatrische patiënten is van wezenlijk belang, omdat co-morbiditeit bovenop de reeds bestaande hoge ziektelast komt.

Om in autisme de betekenis en bruikbaarheid van bepaalde markers (vb. PLT 5-HT) te bepalen, en om de rol van factoren die de concentratie van deze markers waarschijnlijk beïnvloeden (vb. darmmotiliteit) te bepalen, werden andere groepen proefpersonen bestudeerd, zoals pasgeborenen en hun moeders (**Hoofdstuk 4**). Het bestuderen van andere groepen proefpersonen die gekenmerkt worden door een grotere homogeniteit, door afwezigheid van storende co-morbiditeit, en door de aanwezigheid van de te onderzoeken fysiologische toestand (vb. normale of sub-normale darmmotiliteit), kan de interpretatie van (epi-)fenomenen die waargenomen worden in psychiatrische ziekten vergemakkelijken.

Waarschijnlijk is geen van de door ons onderzochte stoffen/markers specifiek voor een psychiatrische diagnostische entiteit, en het lijkt er op dat we slechts in staat zijn om subgroepen van patiënten (biochemisch) te karakteriseren. De hedendaagse psychiatrische nosologie is niet gebaseerd op de etiologie, en tot nu toe is geen enkele marker, mechanisme of risicofactor in staat om het ontstaan en de ontwikkeling van deze aandoeningen te verklaren. Dit feit, samen met de grote socio-economische en psychologische last van psychiatrische stoornissen, de voorspelde stijging van de prevalentie, en de beschikbaarheid van suboptimale diagnostische en therapeutische modaliteiten, vraagt om een andere blik van onderzoekers op hypothesegedreven onderzoek van complexe ziekten zoals psychiatrische ziekten (**Inleiding**). Alternatieve onderzoekstrategieën die niet-hypothesegedreven zijn en een valide klinische vraagstelling hebben, zijn een welkome aanvulling op hypothesegedreven onderzoek, hoewel de mogelijkheden van niet-hypothese gedreven onderzoek niet overschat dienen te worden tot het tegendeel bewezen is.

Moderne '-omics' technologieën die de gelijktijdige en onbevooroordeelde bestudering (kwalitatief en kwantitatief) van zeer grote aantallen genen, mRNA (gen-transcripten), eiwitten en metabolieten mogelijk maken in een systeembiologie georiënteerde aanpak, zullen zeker de manier waarop onderzoek naar psychiatrische ziekten gedaan wordt, beïnvloeden. De ontwikkeling, de evaluatie en de toepassing van een '-omics' techniek voor de ontdekking van biomarkers in urine wordt beschreven in **Hoofdstuk 5**. Vergelijkende analyse van urinemonsters met state-of-the-art technieken voor het scheiden (vloeistofchromatografie; LC) en detecteren (massaspectrometrie; MS) van laag-moleculaire stoffen gevolgd door multivariate analyse van LC-MS data die

tienduizenden pieken bevat, is een goed voorbeeld van hoe niet-hypothesegedreven onderzoek zou kunnen worden uitgevoerd. Bij de optimalisering en verdere evaluatie van deze methode in zwangere en niet-zwangere vrouwen zullen de verdere mogelijkheden en problemen van dit soort methodes duidelijk worden (**Hoofdstuk 6**). Nochtans, is de stapsgewijze ontwikkeling, evaluatie, en toepassing van deze '-omics' methodologie op complexere klinische onderzoekvragen noodzakelijk om de eindgebruiker te overtuigen van de mogelijkheden en betrouwbaarheid. Het vergroten van de concentratiegevoeligheid (door vb. prefractionering, affiniteitextractie), de toepassing van meer selectieve detectors, en het gebruik van geoptimaliseerde algoritmen voor de dataverwerking en –analyse zijn manieren om het succes van cross-sectionele en longitudinale studies voor de ontdekking van biomarkers te vergroten. Als deze studies gecomplementeerd en geïntegreerd worden met resultaten van hypothesegedreven onderzoek, dan zouden wij misschien in staat zijn een deel van de mysteries te kunnen ophelderen van complexe ziekten waaronder psychiatrische stoornissen zoals autisme en schizofrenie.

Samenvatting voor de Leek

Voordat ik begin met iedereen te bedanken voor de spreekwoordelijke bijgedragen steentjes en pallets met stenen lijkt dit me dè plaats om iets over mijn onderzoek te schrijven.

Ik heb dus onderzoek gedaan naar de rol van voeding en biomarkers binnen de psychiatrie en dan met name bij autisme en schizofrenie. De oorzaken van deze psychiatrische ziekten zijn onbekend. Er zijn geen laboratoriumtesten om autisme of schizofrenie mee aan te tonen en de huidige behandelingsmogelijkheden zijn beperkt effectief. Patiënten met een psychiatrische ziekte en hun familie hebben vaak psychosociale en/of financiële problemen. Om psychiatrische patiënten beter te kunnen helpen is er meer onderzoek nodig naar de oorzaken en het verloop van deze ziektes, en naar betere therapieën. Dit onderzoek kan zowel hypothese gedreven (deel I van mijn proefschrift) als niet-hypothese gedreven (deel II van mijn proefschrift) gedaan worden. Bij hypothese gedreven onderzoek is er voorafgaand aan het onderzoek een duidelijke vraagstelling/idee, terwijl dit bij niet-hypothese gedreven onderzoek niet het geval is.

Een voorbeeld van een hypothese gedreven onderzoek is ons onderzoek naar langeketen meervoudig onverzadigde ω3- en ω6-vetzuren (belangrijk voor de opbouw van de hersenen en zenuwprikkeloverdracht) en B-vitaminen (foliumzuur, B_6 en B_{12}; belangrijk voor celgroei in de hersenen) bij patiënten met schizofrenie. Het bleek dat veel patiënten met schizofrenie milde tot ernstige tekorten hebben van deze micronutriënten. Deze tekorten worden in verband gebracht met meer symptomen van schizofrenie en met een verhoogd risico op het krijgen van hart- en vaatziekten. Een klein groepje patiënten met ernstige tekorten kreeg vis- en slaolie, en B-vitamine-tabletten. Hiermee konden we hun tekorten aanvullen. Of de psychiatrische symptomen verbeterden, weten we niet, omdat deze destijds niet gemeten werden. Ook blijkt uit literatuuronderzoek dat tekorten van ω3-vetzuren en B-vitaminen in verband gebracht worden met autisme en depressie en dat toediening van deze micronutriënten meestal positieve effecten laat zien op de symptomen van patiënten met een depressie en schizofrenie.

Op Curaçao heb ik onderzoek gedaan naar de relatie tussen de darmdoorlaatbaarheid en de hoeveelheid serotonine (een stof die o.a. de darm prikkelt om te bewegen) in bloedplaatjes bij kinderen met autisme, vanwege de 'lekke darm'-hypothese van autisme. Ons idee was dat een meer doorlaatbare ('lekke') darm meer serotonine in de bloedbaan uitscheidt, waardoor het bloedplaatjes-serotonine verhoogd wordt. Echter, alle onderzochte kinderen hadden een normale darmdoorlaatbaarheid, maar 25% had wel een verhoogd

bloedplaatjes-serotonine. Ook dachten we dat naarmate de darm meer beweegt (darmmotiliteit) het bloedplaatjes-serotonine stijgt. Daarom deden we onderzoek bij pasgeborenen (relatief weinig darmmotiliteit) en hun moeders (normale darmmotiliteit), en deden we onderzoek naar het effect van infuusvoeding (parenteraal) en voeding via de mond (enteraal) op het bloedplaatjes-serotonine in te vroeg geborenen. Moeders hadden een tweemaal zo hoog bloedplaatjes-serotonine als hun pasgeborenen. Daarnaast zagen we dat het bloedplaatjes-serotonine hoger wordt als pasgeborenen enteraal gevoed worden en dat dit lager wordt als hiermee gestopt wordt en overgegaan wordt op parenterale voeding. We denken dus dat darmmotiliteit gestimuleerd wordt in pasgeborenen door enterale voeding en dat de serotonine die vrijkomt door en tijdens de vertering van voeding een belangrijke bijdrage levert aan de hoeveelheid serotonine in de bloedplaatjes.

Het tweede deel van dit proefschrift gaat over een methode die gebruikt wordt voor het op een niet-hypothese gedreven manier ontdekken van biomarkers in urine. Biomarkers zijn stoffen die gebruikt kunnen worden om aan te tonen of iemand ziek zal worden, ziek is, of beter wordt. We maakten gebruik van vloeistofchromatografie gekoppeld aan massaspectrometrie voor het resp. scheiden en zichtbaar maken van stoffen in urine als pieken (± 5-15 duizend!). Om te zien welke pieken afwezig/aanwezig zijn of een lagere/hogere concentratie hebben in de urine van iemand die ziek is, analyseerden we urinemonsters van zieke en gezonde mensen. Vervolgens vergeleken we de hoogtes van alle pieken van gezonden met die van zieken, natuurlijk met de computer. De pieken werden daarvoor eerst voorbewerkt om ruis te verminderen en de pieken vergelijkbaar te maken. Daarna zochten we met diverse wiskundige methoden naar pieken die sterk verschillen tussen ziek en gezond. Om onze methode te testen, hebben we bijvoorbeeld urine met en zonder toegevoegde peptiden (stukjes eiwit) geanalyseerd om te zien of onze methode de peptiden die we toegevoegd hadden eruit kon vissen als die pieken die het belangrijkste verschil maken. Ook hebben we de methode toegepast op urinemonsters van zwangere en niet-zwangere vrouwen, om te zien of we deze twee groepen van elkaar konden onderscheiden op basis van de stoffen in hun urine. Met 10 van de ±15.000 pieken konden we bepalen of een vrouw zwanger was. Nu willen we nog weten wat deze 10 pieken zijn, met andere woorden: welke stoffen (vb. zwangerschapshormoon) passen bij die 10 pieken? Ons doel was natuurlijk niet om een zwangerschapstest te ontwikkelen, maar om te zien of onze niet-hypothese gedreven methode werkt. Het uiteindelijke doel is om met deze methoden meer inzicht te krijgen in ziekteprocessen, en om laboratoriumtesten (biomarker-testen) voor ziektes te ontwikkelen, vb. voor vroeggeboorte. Een dergelijke methode kan natuurlijk ook gebruikt worden om psychiatrische ziekten mee te onderzoeken.

Dankwoord

Hatsikidee, ik stop ermee. Het einde is in zicht, maar ik kan natuurlijk niet afsluiten met een simpel 'Iedereen bedankt!', alhoewel dit wel een aantrekkelijke optie is. Een groot aantal mensen verdient het om persoonlijk bedankt te worden en degenen die ik hierbij vergeet alvast een welgemeend 'ut spiet mie donders'. Het is misschien raar om mezelf te bedanken, maar ik doe het toch, omdat ik het bijltje er op sommige momenten niet bij neergegooid heb. Ramses, bedankt!

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Over de auteur

Ramses Kemperman werd geboren op vrijdag 10 maart in 1978 in Nijmegen (NL). Tot aan de derde klas van de basisschool bracht hij zijn levensjaren door in Elst (Gelderland), waarna hij naar Roden (Drenthe) verhuisde. Het Atheneum werd in 1996 afgerond aan het Nienoordcollege in Leek (Groningen), waarna hij Farmacie ging studeren aan de Rijksuniversiteit in Groningen. Het doctoraal in de Farmacie werd in 2001 behaald met als titel van het afstudeeronderzoek 'Ontwikkeling en optimalisering van immunofixatie-gel electroforese assays voor de detectie en kwantificering van β2 transferrine in neus- en oorvocht en Bence-Jones eiwitten in urine'. Het apothekersexamen werd afgelegd in 2002, waarna gestart werd aan promotietraject in januari 2003.

Het promotieonderzoek werd zowel uitgevoerd bij de vakgroep Analytische Biochemie (destijds Bioanalyse en Toxicologie) van de Rijksuniversiteit Groningen als bij de afdeling Laboratoriumgeneeskunde van het Universitair Medisch Centrum in Groningen (destijds Academisch Ziekenhuis Groningen). Er werd onderzoek verricht aan de biochemie van psychiatrische stoornissen waaronder autisme en schizofrenie. Daarnaast was hij betrokken bij het ontwikkelen, evalueren en toepassen van een methode voor de niet-hypothese gedreven ontdekking van biologische markers in urine. Zijn interesse gaat uit naar de klinische chemie van psychiatrische stoornissen. In zijn vrije tijd doet hij meestal leuke dingen.

Lijst van publicaties

1. **Kemperman RFJ**, Bruins S, te Lintelo JTV, van der Dijs FPL, Erwich JJHM, Muskiet FD, Landman H, Kema IP, Muskiet FAJ. Relation between platelet serotonin and feeding mode in newborns suggests that gut motor activity is a determinant of platelet serotonin content. *accepted for publication in* Biogenic Amines

2. **Kemperman RFJ**, Muskiet FD, Boutier AI, Kema IP, Muskiet FAJ. Brief report: Normal intestinal permeability at elevated platelet serotonin levels in a subgroup of children with pervasive developmental disorders in Curaçao (Netherlands Antilles). J Autism Dev Disord. 2007 Jul 28; [Epub ahead of print]

3. **Kemperman RFJ**, Horvatovich PL, Hoekman B, Reijmers TH, Muskiet FAJ, Bischoff R. Comparative urine analysis by liquid chromatography-mass spectrometry and multivariate statistics: method development, evaluation, and application to proteinuria. J Proteome Res. 2007 Jan;6(1):194-206.

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5. **Kemperman RFJ**, Veurink M, van der Wal T, Knegtering H, Bruggeman R, Fokkema MR, Kema IP, Korf J, Muskiet FAJ. Low essential fatty acid and B-vitamin status in a subgroup of patients with schizophrenia and its response to dietary supplementation. Prostaglandins Leukot Essent Fatty Acids. 2006 Feb;74(2):75-85. Epub 2005 Dec 27.

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