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 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 B₁₂ 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 B₁₂- and B₆-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 Curacao. 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/10⁹ 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/10⁹ PLT, is used in the diagnosis of
carcinoid tumors; and 4.55 nmol/10⁹ 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/10⁹ 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.