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Acute intermittent porphyria

Inflammation, diet and biomarkers in acute intermittent porphyria

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A dissertation for the degree of Philosophiae Doctor – August 2020

Cover: Illustration by Bjørnar K. Meisler

"…Whenever I choose I can see before me her distorted features where she lay there in exceptional pain but otherwise obviously healthy without any temperature and without abdominal tenderness or muscular defence. I remember her gradual decay, her hopelessness when she in spite of the many journeys to the hospital never could get her health back between the attacks, her increasing nervousness and so the tottering gait, the ascending paralysis, the sloppy hands, the rigid facial expression, and finally the resignation when she after a final try had again been sent home from hospital without any other diagnosis than – only nervousness. Now she had not any attacks anymore, she just deteriorated and got more and more dependent on help from her surroundings. At last she stayed silent and immovable in her bed, tired of living and probably welcoming death when he one late afternoon came in disguise of a respiratory paralysis."

Einar Wallquist: from "Få mans land" (A land of few), 1939.
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Porphyria in Sweden by Thunell S. et al. (1) with permission from the editorial office of Physiol. Res.

Table of contents

List of tables

List of figures

Acknowledgements

This thesis has been made possible by the support of a number of people. Foremost, I thank my main supervisor, Ole-Lars Brekke, who gave me the opportunity and resources to be able to follow my ideas. He guided me to stay on track during the long journey through the PhD road and shared my joy when the ideas materialised. Also, I thank Ole-Lars for his endurance throughout the years and his persistent interest in research on acute intermittent porphyria.

Secondly, I thank my co-supervisor, Erik Waage-Nielsen, who introduced me to research at the anaesthesiology department, and who inspires me to gain knowledge in the interest of patients. I also thank my co-supervisor, Tom Eirik Mollnes, who let me in as a member of his excellent research group, and the leader of the research section, Petter R. Øien, for facilitation of research at Nordland Hospital.

I thank all the excellent personnel at the research laboratory: Anne Landsem, Dorte Christiansen, Hilde Fure, Judith K. Ludviksen, Corinna Lau and Åse Emblem. Also, I want to thank Bård Ove Karlsen, who helped me out with the illustrations and statistics and Kristin S. Berg for her statistical help. I thank Linda T. Hennø, co-author on Paper III. I thank Marlene Blomstereng Karlsen for helping me with the precise collection of nutritional data. Also, I want to thank Jim A. Dahl, Anne Landsem and the nurses at the day care unit at Nordland Hospital for their help with the practical tasks. I am thankful to Inger Aagnes and Hilde Ofte for their cooperation during the PhD courses and Gro Aandahl for supporting me. I thank Amy Dickey and Padmini Pillai, who collaborated with me on the talk at Massachusetts General Hospital. I thank Ida and Lisa for making me think about things other than research. I thank the Nordland Hospital Trust and Health North Authorities for their funding, the library personnel at Nordland Hospital Trust for their cooperation and the Diagnostic Clinic for providing me the much-needed leave from my job to pursue my research.

A heartfelt thanks to the study participants, who spent a day at the hospital for this study, and afterwards at home filling out the diet logbook for one week. I especially thank the Porphyria Association in Nordland (PIN) for their kind and lasting cooperation. Further, I am very grateful for the resolute and most important support, love, patience, inspiration and practical help from my husband, Bjørn, my daughter, Adele, my mother, Aud Karin, my brother, my in-laws Marit and Ove and my entire family!

Selected abbreviations

AIP	Acute intermittent porphyria
ALA	5-Aminolevulinic acid
ALAS1	5-Aminolevulinic acid synthase 1
ALT	Alanine aminotransferase
BMI	Body mass index
CXCL	Chemokine (C-X-C)-motif
CYP	Cytochrome p450
DAMPs	Damage-associated molecular patterns
EDTA	Ethylenediaminetetraacetic acid
eGFR	Estimated glomerular filtration rate
ELISA	Enzyme-linked immunosorbent assay
EPNET	European Porphyria Network
HCC	Hepatocellular carcinoma
HMBS	Hydroxymethylbilane synthase
IL	Interleukin
IR	Insulin resistance
NAPOS	The Norwegian Porphyria Centre
NNR12	Nordic nutrition recommendations 2012
PAMP	Pathogen-associated molecular pattern
PAL	Physical activity level factor
PBG	Porphobilinogen
PBGD	Porphobilinogen deaminase
PGC-1α	Peroxisome proliferator-activated receptor-γ coactivator 1α
PUFA	Polyunsaturated fatty acids
PTX3	Long-pentraxin 3
PTF1.2	Prothrombin fragment 1+2
RMR	Resting metabolic rate
RT	Room temperature
SFA	Saturated fatty acids
TCC	Terminal complement complex
T2D	Type 2 diabetes

List of Papers

Paper I: **Systemic inflammation in acute intermittent porphyria: a case-control study.**
Elin Storjord, Jim A. Dahl, Anne Landsem, Hilde Fure, Judith K. Ludviksen, Sandra Goldbeck-Wood, Bård O. Karlsen, Kristin S. Berg, Tom E. Mollnes, Erik Waage Nielsen, Ole-Lars Brekke *Clin. Exp. Immunol*. 2017;187(3):466–479.

Paper II: **Lifestyle factors including diet and biochemical biomarkers in acute intermittent porphyria: Results from a case-control study in northern Norway.**
Elin Storjord, Jim A. Dahl, Anne Landsem A, Judith K. Ludviksen, Marlene B. Karlsen, Bård O. Karlsen, Ole-Lars Brekke *Mol. Genet. Metab.,* 2019;128(3):254–270.

Paper III: **Effect of the anticoagulant, storage time and temperature of blood samples on the concentrations of 27 multiplex assayed cytokines - Consequences for defining reference values in healthy humans.** Linda Torrissen Hennø, **Elin Storjord**, Dorte Christiansen, Grete Bergseth, Judith K Ludviksen, Hilde Fure, Svein Barene, Erik Waage Nielsen, Tom E. Mollnes, Ole-Lars Brekke. *Cytokine*. 2017;97:86–95.

Sammendrag

Akutt intermitterende porfyri (AIP) er en sjelden autosomal dominant arvelig metabolsk sykdom med nedsatt funksjon på grunn av mutasjon i enzymet hydroksymetylbilan syntase (HMBS) i hemsyntesen. Dette kan gi opphopning av porfyrinforstadiene - aminolevulinsyre (ALA) og porfobilinogen (PBG) som kan gi akutte magesmerter, pareser, psykiske plager og fatigue samt økt risiko for nyresvikt og hepatocellulært carcinom (HCC). AIP anfall kan utløses av noen medikamenter, faste, infeksjoner og stress.

Metabolske sykdommer som diabetes mellitus er assosiert med inflammasjon, og derfor undersøkte vi om det også var inflammasjon ved AIP utenom anfall målt som økte cytokiner i plasma. Siden prøvetakingsbetingelsene påvirker analyseresultatet for cytokiner, så sammenlignet vi verdiene av 27 ulike cytokiner i serum og i fire ulike plasmatyper fra friske personer tilsatt forskjellige antikoagulantia for å finne optimal prøvebehandling. I tillegg undersøkte vi effekten av lagringstemperatur, frysing og tining av prøvene. Vi konkluderte med at cytokiner bør analyseres i EDTA plasma lagret på is og sentrifugert innen fire timer. I tillegg etablerte vi et referanseområde for cytokiner fra 162 friske personer. Ved bruk av multiplex analyse fant vi signifikant økte cytokiner i gruppen med 50 personer med AIP som uttrykk for lavgradig inflammasjon sammenlignet med de 50 personene i kontrollgruppen som var matsjet for alder, kjønn og bosted. Inflammasjonen kan muligens være med på å forklare den høyere risiko for leverkreft ved AIP. Det ble funnet lavere nivå av prealbumin (transtyretin) og lavere nyrefunksjon hos symptomatiske vs. asymptomatiske personer med AIP. Fastende C-peptid og Insulin verdier var lavere i gruppen med symptomatisk AIP sammenlignet med sine matsjede kontroller. Personer med AIP spiste mindre karbohydrat enn det som er anbefalt i retningslinjer, til tross for anbefalinger om et karbohydratrikt kosthold da glukose hemmer første steg i hemsyntesen. Forskningsdeltakerne fylte ut 7 dagers kostdagbok, fikk gjort antropometriske mål, fylte ut spørreskjema inneholdende bl.a. spørsmål om livsstil og ble intervjuet av lege. Blodprøver ble analysert for vitaminer samt markører for glukosemetabolismen og fettstoffskiftet, markører for jernmetabolismen, komplementaktiverings-produkter og immunglobuliner, og urin ble analysert for PBG, ALA og katekolaminer.

Abstract

Acute intermittent porphyria (AIP) is a rare autosomal dominant inherited metabolic disease with lowered function of the enzyme, hydroxymethylbilane synthase (HMBS) in the haem synthesis, due to mutation in its gene. The resulting excess of the porphyrin precursors, ALA and PBG, can result in acute abdominal pain, paresis and fatigue, as well as increased risk of kidney failure and hepatocellular carcinoma (HCC). Certain drugs, fasting, infections and stress can trigger AIP attacks. Metabolic diseases like diabetes mellitus are associated with inflammation. Therefore, we investigated if there also were inflammation in AIP outside attacks measured as elevated levels of plasma cytokines. Since the handling of blood samples can affect the cytokine levels, we compared the levels of 27 different cytokines in serum and in plasma from healthy controls using four different anticoagulants. The effects of storage temperature, freezing and thawing were also examined. We concluded that one should analyse cytokines in EDTA plasma samples stored at 4 ºC for up to four hours before centrifugation. Also, we established reference ranges for cytokines from 162 healthy persons. By using a multiplex assay, we found significantly elevated levels of cytokines in the 50 AIP cases as a sign of low-grade inflammation as compared with the 50 controls matched for age, gender and place of residence. The inflammation may be one of the explanations for the higher risk for HCC in AIP. The cases and controls filled out a seven-day diet logbook, had their anthropometric measures recorded, filled out a questionnaire on lifestyle factors and were interviewed by a physician. The blood was also analysed for vitamins, glucose metabolism measures, fats, iron metabolism markers, complement activation markers and immunoglobulins, and the urine was analysed for PBG, ALA and catecholamines. We found lower levels of prealbumin (transthyretin) and lower kidney function in symptomatic vs. asymptomatic AIP cases. The fasting C-peptide and insulin levels were lower in the symptomatic AIP cases as compared with their matched controls. Notably, people with AIP had a lower carbohydrate intake than the quantity recommended by guidelines; hence, they were recommended a higher carbohydrate intake because glucose inhibits the first step in haem synthesis.

1 Background

1.1 Introduction

Acute intermittent porphyria (AIP) is a rare autosomal dominant inherited metabolic disease caused by an impaired function of the enzyme, hydroxymethylbilane synthase (HMBS, EC 2.5.1.61), also called porphobilinogen deaminase (PBGD). AIP occurs most often due to a heterozygote mutation in the gene encoding of HMBS during haem synthesis. AIP has the phenotype Mendelian Inheritance in Man (MIM)–number, #176000 (Omim.org, accessed December 2019). The disease causes neurovisceral attacks manifesting as acute abdominal pain, nausea, vomiting, obstipation, tachycardia, high blood pressure, decreased muscle strength and fatigue. In seriously ill patients, paresis and involvement of the respiratory muscles can give rise to the need for respiratory help (2, 3). Insomnia, depression and anxiety may also occur in association with acute pain during an AIP attack (4, 5). Rarely, psychosis and hallucinations may be seen during an AIP attack, but these resolve completely on remission (4).The urine can become dark red, particularly if standing in light for some hours (6). Although the disease is rare, it is relatively prevalent in the area around Nordland Hospital, with Saltdal municipality having a prevalence of 600/100,000 (7). This high prevalence gives us a special opportunity and responsibility to study AIP.

Most persons with AIP remain asymptomatic, while certain others exhibit the afore-mentioned symptoms of AIP repeatedly (6, 8). To investigate the reason for this difference in symptoms, we compared symptomatic cases and asymptomatic cases, and cases with high and low PBG. By adding a matched control group, we could also test our hypotheses on health-related differences between the cases and controls since we know that the symptomatic AIP cases have elevated porphyrin precursor levels. Some AIP cases have elevated porphyrin precursor levels all the time, while certain others show elevated levels only sometimes, and this might have several consequences other than an AIP attack (9). There is increased risk for hepatocellular carcinoma (HCC) and impaired kidney function in AIP (10). This study aimed to examine if there were differences in the status of low-degree inflammation, kidney and liver function, glucose metabolism in symptomatic cases, asymptomatic cases and matched controls, as inflammation and glucose metabolism can affect the AIP disease activity. We also wanted to scrutinize the role of nutritional factors, how they differed (if they did) between symptomatic cases, asymptomatic cases and matched controls and if these factors affected the AIP disease activity. The purpose was to contribute to better general health of patients suffering from AIP by increasing the knowledge on

the disease that, in the future, can contribute to better treatment of AIP. It was very important to record reliable cytokine measurements, for which we performed a methodological study on how to best handle blood samples for cytokine analysis to get a correct answer and to find reference values for cytokines in plasma samples of healthy adults.

1.1.1 History of acute intermittent porphyria

Hippocrates (460–277 BC) is cited as being the first to recognize porphyria; he described a woman from Thasos with abdominal pain and red urine (11, 12). The Dutch physician, B. J. Stokvis, described in 1889 a woman with dark urine who died after taking Sulfonal (1, 13). Quantitative methods for measuring PBG and ALA in urine were described in 1955 (14). In 1970, it was found that AIP was related to a deficiency of HMBS (15). In 1971, hematin was introduced to treat acute porphyria (16); it was given to a woman with a serious AIP attack, after she failed to respond to other treatments. It resulted in a decline in ALA and PBG, but unfortunately, she died six weeks later of CNS failure attributed to AIP and uraemia (16). In 1980, the gene for HMBS on chromosome 11 was found (17), and in 1984, cDNA for HMBS mRNA was found (18). Later, mutation analyses became available (19, 20).

1.1.2 Local aspects of AIP history

In Sweden, there has been extensive research on AIP since J. Waldenström and E. Wahlquist did family investigations in northern Sweden. They found 103 cases by testing their urine using para-dimethylaminobenzaldehyde in HCl diluted with distilled water (Ehrlich's reagent), and then noting the red colour (1, 13) as reported in Waldenströms dissertation in 1937. The disease was called Swedish Porphyria (21). In 1935, A. Engel and E. Wahlquist provided evidence of acute intermittent porphyria, then called hematoporphyrinuria, as an inherited disease (13, 22). In around 1830, Swedes from this area migrated and likely brought the W198X AIP mutation to Nordland county in northern Norway.

In 1982, Eldøen and Sandvik published an article on AIP in Saltdal community, Nordland county in northern Norway (23). They performed biochemical analysis of urine samples collected from 91 persons above 13 years of age in Saltdal and surrounding areas and found 17 persons with certain AIP diagnosed with both high PBG and low enzyme activity, among whom eight people classified as possible AIP cases based on either high PBG or low enzyme activity. Of the 17 confirmed cases, only two were known beforehand. A total of 108 persons filled out questionnaires about symptoms, out of which 13 persons were found to have symptoms resembling AIP (23).

Tollåli, Waage Nielsen and Brekke reported in 2002 that after three years of studying families in Nordland in cooperation with general practitioners, they found 59 persons with AIP (7). This gave a prevalence of 25 per 100 000 in Nordland county, and 600 per 100.000 in Saltdal municipality (7). In 1999, The Porphyria Association in Nordland (PIN) was established. The local aspects of AIP history, the local high AIP prevalence and the presence of a local AIP patient association made it possible and important for us to study AIP to gain knowledge in the interest of patients.

1.1.3 Mutation, prevalence, incidence and penetrance of AIP

AIP is a disease distributed worldwide (24). The incidence of symptomatic AIP reported in 2013 was 0.13 new cases per year per million inhabitants in Europe, while Sweden had 0.51 and Norway 0.14 (25). The high incidence of W198X mutation in northern Sweden and northern Norway is possibly explained (partly) by a founder effect that arose in northern Sweden in the 1700s (26, 27). The Norwegian Porphyria Centre (NAPOS) reported that the number of registered AIP cases in Norway till 2012 were 326 persons, of whom 189 were symptomatic and 137 were predictively identified as asymptomatic (27). The data from NAPOS indicates a prevalence ratio of AIP in Norway of approximately 4:100,000 of symptomatic AIP cases (40 per million in Norway), and a prevalence ratio of 7:100,000 for AIP when predictively identified genetic predisposition were also included (27). This number is higher than the 6.3 per million in Norway that was calculated from the incidence in a study by Elder et al. (25). Puy et al. reported in 2010 that in European countries, 1:75,000 are affected by AIP, except northern Sweden which has a higher prevalence (2).

A study of blood collected from donors in 1997 in France found a high prevalence of mutations in the HMBS gene in the ratio of 1:1675 (28). A clinical penetrance of around 1–2% was estimated when comparing the prevalence of likely pathogenic HMBS mutations among Caucasians, with the prevalence estimate of symptomatic European heterozygotes (6, 28-30). An oligogenic model of heredity as the cause of AIP is suggested (31), and the low penetrance suggests the importance of environmental modifiers and predisposing or protective genes (30). In families with known mutations, the clinical penetrance is higher, hence the percentage of symptomatic persons in known AIP families ranging from approximately 10% (32), to 23% (25), and up to 42% (33), 44% (34) and 52% (35). These findings possibly point toward other factors that are shared in a family, or that these families were very well mapped and diagnosed. This raises the question as to why only some of them contract AIP symptoms and some do not, which is reflected in our hypotheses. In Norway, more than 30 HMBS mutations have been found (36), and 454 mutations have been reported globally so far (29, 30, 37-39). However, the prevalence of pathogenic HMBS

mutations and the actual disease penetrance are uncertain (30). Some mutations reported as disease-causing did not have sufficient supportive evidence for verifications, and has later been classified as questionably pathogenic (40). There is recently initiated an International Porphyria Diagnostic Collaborative that is working on an evidence-based database of verified pathogenic and benign variants for the porphyrias (40). Different mutations could have different pathogenicity (35), and there is also variable expression of the clinical phenotype within the same family (5). The penetrance of different mutations may also be different (34), and out of W198X, R173W and R167W, the last one showed a lower clinical penetrance (26).

The HMBS enzyme consists of three domains, and HMBS-mutations are found on all three domains. Many of these mutations are single base changes (missense), while others form a truncated protein (nonsense) (41). The W198X (Trp198Term) mutation is a nonsense mutation, which results in a truncated protein where both folding and stability is affected since the mutation leads to a premature stop codon (41). In persons showing W198X mutation, a base substitution of G to A in exon 10 in the HMBS gene is observed (42). The protein is easily degraded and possibly becomes inactive (41). This lower than normal activity of HMBS is often sufficient, but when AIP patients encounter a trigger that increases the demand for haem, then the first and rate-limiting step in the haem biosynthesis, ALAS1, is induced (43). HMBS becomes rate-limiting (43) and ALA and PBG build up, which can induce an AIP attack.

1.2 Pathophysiology of AIP

AIP is classified as an acute hepatic porphyria. The symptoms most often manifest as acute attacks (43). AIP mutations affect the synthesis of haem, a cofactor for many haemoproteins such as haemoglobin, myoglobin, cytochrome p450 (CYP), mitochondrial cytochrome, catalase and peroxidase (6). The formation of haem is a process involving several steps and eight different enzymes (6). The mutation which is required for an AIP diagnosis, decreases approximately 50% of the functioning capability of the third enzyme involved in the haem synthesis, HMBS or PBG deaminase, potentially resulting in a build-up of ALA and PBG upfront (43, 44). In humans, 75–80% of the haem is synthesized by erythroblasts in the bone marrow, while 15–20% is formed in the liver (6). The haem biosynthesis in the liver is of main importance in AIP, as this is where ALA, PBG and porphyrins build up (43). The ALAS enzyme has two isoforms: ALAS1 and ALAS2. The ubiquitous housekeeping form called ALAS1 is present in virtually all cells, including hepatocytes, while ALAS2 is an erythroid form, which is the main form for developing erythrocytes. ALAS1 is

the enzyme of interest for AIP, especially in the hepatocytes (43). The ALAS enzyme in the mitochondria catalyses the condensation of glycine and succinyl-CoA and forms ALA, which then enters the cytosol. By the actions of the enzyme, δ–aminolevulinic acid dehydratase (ALAD), two ALA molecules join and form PBG. After that, HMBS polymerizes four PBG molecules to form the linear tetrapyrrole hydroxymethylbilane (HMB). Then, HMB readily cyclizes to form uroporphyrinogen I, but little of it is made because of the activity of the uroporphyrinogen III synthase (43). Uroporphyrinogens are then subjected to decarboxylation by uroporphyrinogen decarboxylase that resides in the cytoplasm (43). This forms coproporphyrinogen. The latter converts into protoporphyrinogen because of the activity of coproporphyrinogen oxidase (43). Then, protoporphyrin oxidase in the cytosol carries out sequential decarboxylation reactions to form protoporphyrin (43). Finally, ferrochelatase catalyses the insertion of ferrous iron in protoporphyrin to produce haem (43).

1.2.1 Regulation of haem biosynthesis and haem catabolism

Haem-mediated feedback inhibition regulates ALAS1 activity in the hepatocytes (45). Glucose downregulates ALAS1 activity through peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α) (43). Drugs and chemicals stimulate transcription of ALAS1 through pregnane X receptor (PXR) (43). In hepatic cells, there are two forms of ALAS1: major and minor. Haem regulates the major form through increased breakdown of mRNA, while the minor form is resistant (43). Also, haem decreases the transportation of preALAS1 into mitochondria (43). To sum up, deficiency of glucose, deficiency of haem or presence of porphyrinogenic medications can upregulate ALAS1 – probably mainly in the liver (46). Stress, infections and porphyrinogenic drugs metabolizing through CYP can increase haem consumption, thereby indirectly triggering ALAS1 (47). It is not clearly known how the menstrual cycle and female hormones affect ALAS1 activity (47). Nevertheless, it is known that progesterone is an inducer of ALAS1, and it was reported that persons with AIP that was given ethinyl estradiol, got higher levels of ALA and PBG (48, 49).

The spleen and the liver degrade haem, resulting in the formation of biliverdin, iron and carbon monoxide (CO). The breakdown of haem is regulated by haem oxygenase (HO), which is the first and rate-controlling enzyme in haem degradation (43). Haem oxygenase-1 (HO-1) is inducible by factors causing physical and chemical stress, e.g. reactive oxygen species, heat shock, transition metals and by it´s substrate haem (43). Notably, haem oxygenase-2 (HO-2) is almost non-inducible (43). Small non-coding RNAs that regulate gene expression, miRNAs, also play a role in the regulation of HO-1 gene expression (43).

1.2.2 Predisposition and initiation of AIP attack

In addition to the pathogenic mutation, there are possibly other genetic or environmental modifiers that predispose some to AIP attack and protect others. Moreover, even diet affects ALAS1 and AIP disease activity (50, 51) (Fig. 1). Alcohol, smoking and marijuana could trigger an AIP attack due to their known induction of hepatic ALAS1 (6, 52, 53). Psychological, emotional and physical stress can be triggers for AIP attacks as well (6).

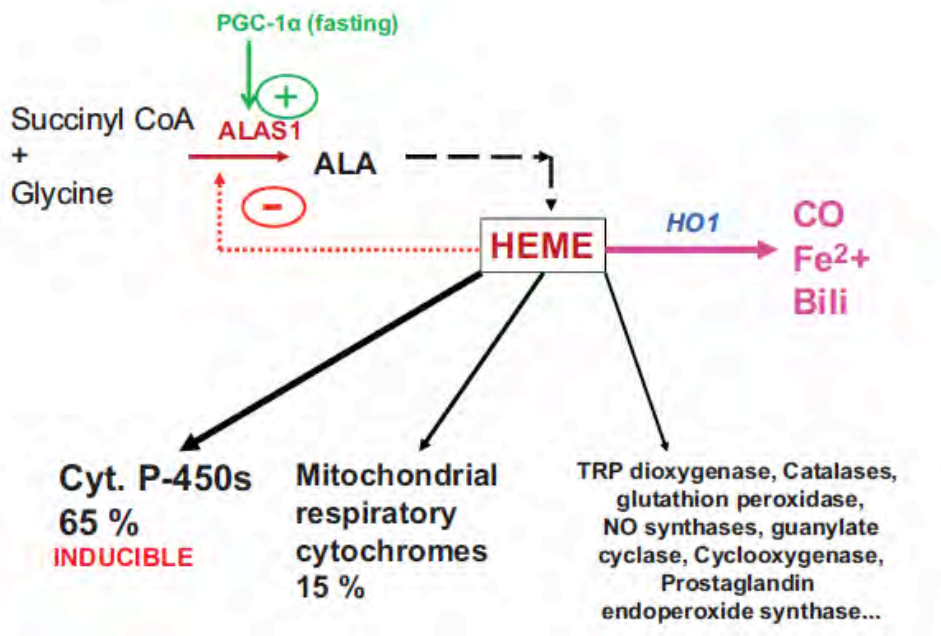

Fig. 1. Regulation of haem biosynthesis in the liver and haem proteins.
The illustration shows potential targets for ALAS1 induction causing acute attacks in AIP. Abbreviations: CO = carbon monoxide, HO = haem oxygenase, NO = nitric oxide, PGC-1α = Peroxisome proliferator-activated receptor-γ coactivator 1α, TRP = tryptophan. Reprint of Fig. from Clinics and Research in Hepatology and Gastroenterology (2015) 29, Karim et al., Porphyrias: A 2015 Update, pages 412–425 (54) with permission from Elsevier.

The level of PBG has been shown to be associated with emotional stress (55). The functioning of the liver CYP is affected by the central and peripheral nervous systems and neurotransmitters (56). A study on AIP carriers suggested that CYP2D6 genotype could be a susceptibility factor that modified the penetrance (35). Metabolic stress caused by infections, acute inflammation or other diseases are possible triggers of AIP attacks, and appropriate vaccines should be administered to AIP patients (6). The nuclear receptor, constitutively active receptor (CAR), is associated with control of the ALAS1-transcription. The expression of CAR is increased by xenobiotics and steroid-hormones such as cortisol, which is generated during different forms of stress, infections

and fasting (57). Many of the hypothalamic-pituitary-adrenal-axis stressors are clinically recognized as porphyrinogenic (57). Hormones play a crucial part in the fluctuation of AIP activity, and the second part of the menstrual cycle, the luteal phase, during which the progesterone is elevated, is especially prone to attacks (48, 58). Pregnancy is most often tolerated well, but some can get more frequent attacks during pregnancy (6). Medications that may trigger AIP disease activity include oestrogens (49), progestogens, barbiturates, sulfonamides and others that induce CYP and ALAS1 (6, 59). The porphyrogenicity of the drug is partly related to the effect on the CYP system, which increases the hepatic turnover of haem and gives feedback to ALAS1 to speed up the haem synthesis (59) (Fig. 2). If drugs have side effects that patients with AIP are vulnerable to, such as nausea or decreased appetite, it can possibly also trigger an AIP attack. ALA and PBG may enter neural cells and induce porphyrin accumulation that may lead to transient reversible protein-porphyrin aggregates, which could contribute to acute porphyria attacks (60).

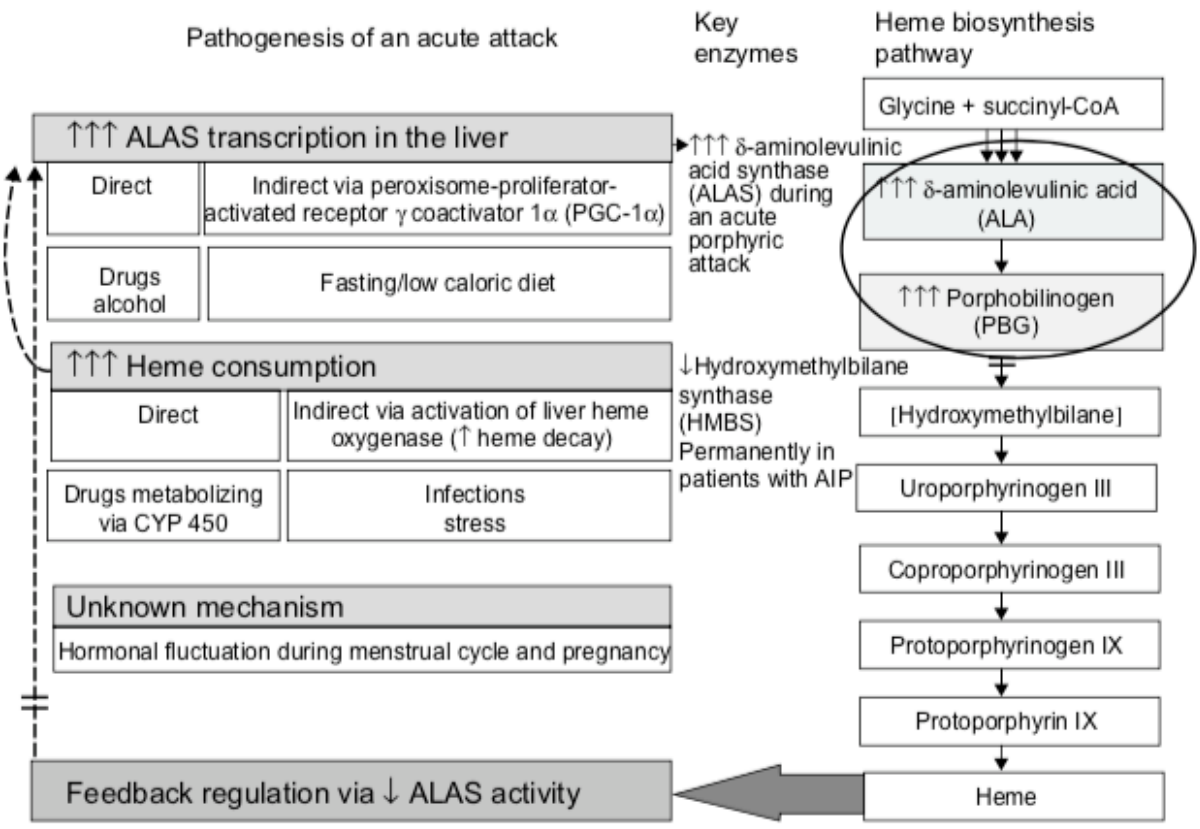

Fig. 2. Precipitating factors and pathogenesis of an acute attack in AIP.
The illustration shows several exogenic and endogenic factors that induce haem biosynthesis via direct or indirect activation of ALA synthase. Reprint of Fig. from: The Application of Clinical Genetics 2015:8. An update of clinical management of acute intermittent porphyria, Pischik E., Kauppinen R., pages 201–214, 2015 (47), with permission from Dove Medical Press, a part of Taylor & Francis Group.

1.2.3 Porphyrin precursor toxicity causing symptoms in AIP

The first compound in the porphyrin synthesis pathway is ALA, and it is produced by ALAS1 from glycine and succinyl CoA, through the reaction called the Shemin pathway (61). PBG is a pyrrole-containing intermediate in the biosynthesis of porphyrins, and it is generated from ALA by enzyme ALA dehydratase (61). Under certain conditions, it can act as a neurotoxin that causes damage to nerve cells and tissue, and also as a metabotoxin, which is an endogenously produced metabolite that causes adverse health effects at chronically high levels (61). There have been different hypotheses on what causes the symptoms of AIP. The pathophysiological mechanisms causing pain and other clinical manifestations of AIP are not completely understood (54).

In AIP, the autonomic neuropathy and autonomic dysfunction are thought to be responsible for most of the symptoms (62). The peripheral nervous system can be affected in AIP, and signs of central nervous system involvement have also been seen (47, 63). The AIP attack is reported to be a neurovisceral crisis including acute abdominal pain; acute peripheral neuropathy and encephalopathy may develop in severe AIP attacks (62). The probable cause of the neuropathic symptoms and pain in acute porphyria has been attributed to either direct toxic effect of PBG, especially ALA (62, 64-66), possibly in combination with depletion of the "free" haem pool, or because of neural haem deficiency (62, 66).

In homozygous dominant AIP, MRI suggested selective cerebral oligodendrocyte postnatal involvement, whereas most structures that developed prenatally were intact (67). Symptomatic AIP cases that had high ALA and PBG and got a liver transplant became asymptomatic, with normalized ALA and PBG levels, supporting the toxic effect of ALA and PBG (65, 67, 68). The neuropathy is suggested to be linked to impaired Na^+/K^+ pump function (69). Lead poisoning and hereditary tyrosinemia, which also show perturbed haem synthesis and high ALA, present with a similar pain syndrome (65). There are several different hypotheses of how excess ALA could cause pain, including direct toxicity. There is also the possibility of demyelination secondary to the formation of free radicals, and that excess ALA could cause vasospasm (70). Bylesjø et al. did not find evidence to support for the theory of demyelination from investigations of cerebrospinal fluid of eight symptomatic and eight asymptomatic AIP cases, finding no oligoclonal immunoglobulin bands, although they were checked outside AIP attacks. However, four of the 16 AIP cases had multiple white matter lesions in the brain seen on magnetic resonance imaging (63). On the contrary, the report by Marsden et al. did not support the idea of ALA as a neurotoxin that might be responsible for many of the AIP symptoms, unless it is proposed that the body adapts fairly quickly

to elevated urine ALA, and it is the rapid changes in ALA concentrations which cause complications (71). The reason for their dismissal of the idea of ALA being a neurotoxin is that AIP symptoms often resolve in few weeks, while the marked biochemical abnormalities persist for more than 10 years (71). The long-standing elevated porphyrin precursor levels do not fit well with the model of acute induction of ALAS and toxic porphyrin precursors explaining the complications of AIP, according to Marsden et al. (71). Interestingly, in line with this assumption, recombinant human HMBS that successfully decreased the level of PBG did not relieve symptoms (72).

A study of an explanted liver from an AIP patient supports the hypothesis that the neurovisceral symptoms of AIP are not associated with generalized hepatic haem deficiency, rather they are related to a depletion of hepatic haem confined to the "free" haem pool, leading to induction of hepatic ALAS1 and accumulation of neurotoxic ALA and/or PBG (68). ALA and especially PBG concentrations were high in the explanted liver, but microsomal haem content was sufficient, and representative CYP activities were essentially normal (68). In the perineural fluid, the ALA concentration is approximately 30% of that in serum; this is because of a blood-nerve-barrier (62). Even so, this amount is sufficient to cause both axonal degeneration and polyneuropathy in AIP patients (62). An unaffected blood-brain barrier results in only 1% of ALA entering the brain, but there is possibly a lower barrier protection in the limbic area, which perhaps could explain mild mental changes like anxiety and insomnia during the initial part of an AIP attack (62). Autonomic nerves do not have this barrier, and this could be the reason for dysautonomia during AIP attacks (62). ALA is structurally similar to gamma-aminobutyric acid (GABA), a major inhibitory neurotransmitter in the central nervous system (73). ALA interacts with GABA receptors and can inhibit the potassium induced release of GABA (73). Regarding the potential lack of haem proteins, there has been evidence of decreased function of some haem proteins, particularly hepatic tryptophan dioxygenase, and of hepatic cytochromes, P450, while the question of neuronal haem deficiency was unsettled (66). Pischik et al. suggested that a combination of overproduction of ALA via direct neurotoxicity, oxidative damage and modification of glutamatergic release may initiate the neuronal damage (62).

1.2.4 Impact of AIP on mitochondrial energetic metabolism

It has been seen that the treatment of rats with ALA caused fatigue (74). Homedan et al. performed a study on 34 mice, of which 16 were controls and the rest were HMBS-/- (75). They suggested a cataplerosis of the tricarboxylic acid cycle (TCA cycle), in this case induced by phenobarbital (75), and that the bioenergetic failure is part of the reason for muscular dysfunction (76) (Fig. 3). The

reason for the cataplerosis is that succinyl-CoA is withdrawn by ALAS induction. In this way, the TCA cycle cannot supply the respiratory chain with the reduced cofactors (75). A pilot study was performed on cellular bioenergetics measuring oxygen consumption rate in monocytes and neutrophils in peripheral blood in humans with different kinds of porphyria, including AIP (77). They suggest an existence of mitochondrial abnormalities in porphyria during active disease and less so during remission (77). Recently, a depression-like behaviour and mitochondrial dysfunction was found in a mouse model of homozygous AIP, which suggested a mechanistic role for the disrupted mitochondrial energy production in the pathogenesis of the behavioural consequences of severe HMBS deficiency (78). Urinary metabolomics profiling of asymptomatic AIP has shown higher concentrations of acetate, citrate and pyruvate as compared with PCT patients (79). In asymptomatic AIP patients, these results suggest metabolic reprogramming "and supports the relationship that occur between haem synthesis and mitochondrial energetic metabolism" (79).

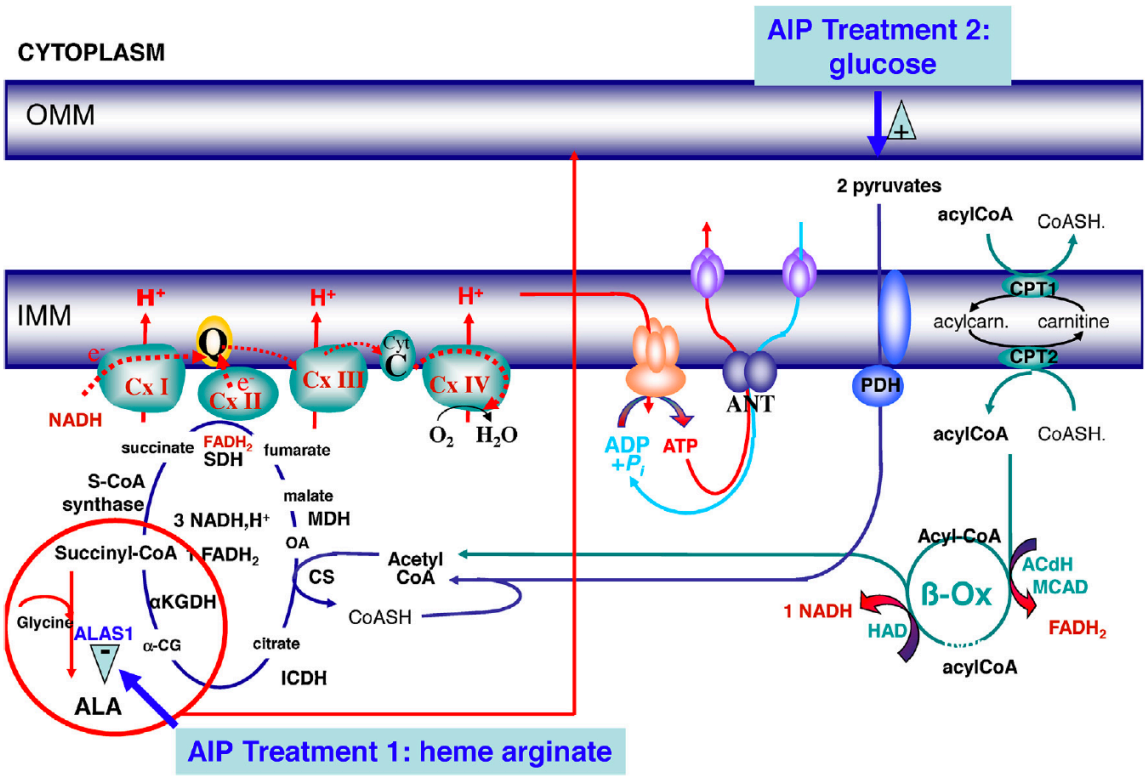

Fig. 3. Interconnections between haem synthesis, the respiratory chain and tricarboxylic acid cycle during phenobarbital exposure and the treatment with haem arginate and glucose.

Abbreviations: α-CG = α-ketoglutarate, αKGDH = alpha-ketoglutarate dehydrogenase, ACdH = acyl-CoA dehydrogenase, ADP = adenosine diphosphate, ALA = 5-aminolevulinic acid, ALAS = ALA synthase, ANT = adenine nucleotide translocator, ATP = adenosine triphosphate, CoASH = coenzyme A, CS = citrate synthase; Cx1 = complex I or NADH ubiquinone reductase, Cx II =

complex II or succinate ubiquinone reductase, Cx III = complex III or ubiquinone-cytochrome c reductase, Cx IV = complex IV or cytochrome-c-oxydase, CPT1 = carnitine palmitoyl transferases, $FADH_2$ = flavin adenine dinucleotide (hydroquinone form), HAD = 3-hydroxyl-CoA dehydrogenase, ICDH = isocitrate dehydrogenase, IMM = inner mitochondrial membrane, MCAD = medium-chain acyl-coenzyme A dehydrogenase, NADH = nicotinamide adenine dinucleotide – hydrogen (reduced form), OA = oxaloacetate, OMM = outer mitochondrial membrane, OXPHOS = oxidative phosphorylation; PDH = pyruvate dehydrogenase, SDH = succinate dehydrogenase. Figure reprinted from The International Journal of Biochemistry & Cell Biology, 2014, June; 51, Homedan et al., Acute intermittent porphyria causes hepatic mitochondrial energetic failure in a mouse model, pages 93-101, copyright (75), with permission from Elsevier.

1.2.5 Ischemia and vascular reactivity

It has been suggested that the abdominal pain could be partly due to intestinal angina, as exemplified by an AIP patient whose autopsy showed intestinal gangrene (80). Vasospasm has been suggested as the cause of transient cortical blindness in a few AIP cases (81, 82). It was reported an increased maximal contraction in the arteries of AIP mice during an induced attack as a response to phenylephrine (PE), and the AIP arteries in female mice had increased sensitivity to PE (83). The AIP mice model had ~30% of normal PBGD activity (83). Hemin induced greater relaxation in arteries in AIP mice than in wild type mice (83).

1.3 Diagnosing AIP

There is no definite consensus on what an AIP attack is, according to an oral statement made by Sverre Sandberg, the President of the European Porphyria Network, at the International Conference on Porphyrins and Porphyrias (ICPP) 2019. To diagnose an acute porphyria attack, there are different pieces of a puzzle that should be put together, without all being necessary present: 1) Positive anamnesis of triggering factors such as drugs, starvation, stress, infection; 2) Known AIP history in the family, being a known carrier; 3) Presence of AIP symptoms; 4) Presence of hyponatremia; 5) Positive urinary PBG and ALA quantitative test; 6) Levels of urine ALA, PBG above the limit; and 7) normal stool porphyrins and 8) New biomarkers (84). According to a study on the biological variation of urine ALA and PBG, a rise of 50% of PBG is considered significant to diagnose an AIP attack in a known carrier of an HMBS mutation (9).

1.3.1 ALA, PBG and porphyrins analysis and genetic testing

Biochemical methods are applied to monitor disease activity of AIP, by studying the levels of PBG especially, along with ALA and porphyrins (85-87). Gene analysis is used for carrier detection of HMBS mutations (85-87). There is increased PBG also in variegate porphyria (VP) and hereditary

coproporphyria (HCP). Plasma fluorescence scanning separate VP from AIP and HCP and faecal porphyrin fractionation of coproporphyrin III and I show greatly elevated coproporphyrin isomer III levels in HCP (88). The urine porphyrins are usually increased in AIP and other acute porphyrias, mainly because of *in vitro* polymerization of PBG to uroporphyrin. Urine porphyrins consist of uro-porhyrins I/III, hepta-carboxyl-porphyrin I, hexa-carboxyl-porphyrin I, penta-carboxyl-porphyrin I, copro-porphyrin I/III and very little or no proto-porphyrin IX. Urine porphyrins can be elevated in conditions such as hepatobiliary disease, infections and alcohol abuse (84). PBG, ALA and the porphyrins generate a pattern that points to different porphyrias. Normal urine PBG and ALA excretion in an adult person excludes an AIP attack with high probability, given that that the sample is taken during or in the days right after typical symptoms. Receiver operating characteristic (ROC) curve analysis for PBG helps to find the optimal cut-off for PBG and gives the associated sensitivity and specificity (85). During the follow up of an AIP patient, if an AIP attack is suspected, the current PBG/mmol creatinine is compared to PBG outside-attacks.

In accordance with the regulations in Norway, predictive testing for first-degree relatives involves genetic testing for the mutation of interest in persons above 18 years of age, but it is done only if they consent after genetic counselling (6, 89). If the presymptomatic genetic testing is performed in children below 16 years of age, the parents must first provide a written informed consent and attend genetic counselling. In Norway, genetic testing of adults with symptomatic AIP does not require genetic counselling, but it is mandatory to make a note of the symptoms and signs of AIP and get an oral consent from the patient. There is no convincing genotype-phenotype correlation in AIP since the mutation analysis does not predict the disease severity; moreover, most carriers of HMBS mutations are asymptomatic.

1.4 Symptoms and signs

AIP is called the ‘little imitator’ due to its variety of symptoms and the possibility to imitate or be confused with other diseases (90). The signs and symptoms of AIP attacks can mimic a range of conditions (84). There is no pathognomonic symptom for AIP (46). Symptoms can arise at all ages and sexes, but women tend to have more AIP disease activity than men, and the symptoms often arise during the reproductive years in women and at old age in men (48). This is possibly linked to menstruation in fertile women and changes in hormones in elderly men. The symptoms in AIP can manifest recurrently in some patients, but only as one or a few acute AIP attacks in most symptomatic cases. No official scaling system of AIP patients is available. However, the AIP cases

are characterized as asymptomatic when they have no symptoms and no history of attacks (Table 1). Symptomatic cases have had at least one porphyric attack. Symptomatic AIP cases with more than four AIP attacks per year was classified as recurrent AIP by Neeleman et al. (91), while many classify recurrent AIP as four or more AIP attacks per year (25, 92-94). Severe AIP is characterized as recurrent AIP if more than 24 attacks per year occur.

Table 1. Clinical characteristics of patients with acute intermittent porphyria

	Asymptomatic AIP	**Symptomatic AIP**	**Recurrent AIP**
HMBS mutation	Yes	Yes	Yes
At least one proven AIP attack during life	No	Yes	Yes
Four or more AIP attacks per year	No	No	Yes

1.4.1 Abdominal pain, nausea and vomiting

The key symptoms indicating AIP, if no other cause is evident, are acute attacks with abdominal pain without peritoneal signs, nausea, vomiting, hypertensive crisis and polyneuropathies (47, 54). Abdominal pain is especially AIP-like if it is associated with nausea, vomiting, constipation, tachycardia and hypertension as a sign of dysautonomia (3). The abdominal pain is severe and poorly localized, and the pain can also affect legs, back and other sites (4).

1.4.2 Autonomic neuropathy and peripheral neuropathy

Abdominal pain, constipation, decreased gastric motility, tachycardia and hypertension could all possibly be related to autonomic neuropathy (64), and e.g. hypertension and tachycardia being caused by sympathetic hyperactivity (46). Arrhythmia also occurs during AIP attacks (95). In a study of 30 AIP patients, 83.3% had cardiac dysautonomia (96). It has been suggested that a parasympathetic dysfunction in AIP could be linked to arrhythmias (97). Porphyric neuropathy presents typically as a motor neuropathy of axonal type (64). The peripheral motor neuropathy may progress to difficulties in swallowing, flaccid paralysis and the need for support by a ventilator due to respiratory insufficiency (4, 64). Also, the peripheral motor neuropathy has been linked to the development of urinary retention or incontinence in some AIP cases (4).

1.4.3 Seizures, encephalopathy, hyponatremia and fatigue

Insomnia, agitation, confusion, psychosis with hallucinations (4, 98), acute encephalopathy, epileptic seizures and rhabdomyolysis can occur in relation to an AIP attack (47, 54, 99). The encephalopathy could be explained by posterior reversible encephalopathy syndrome, and it could include both mental symptoms, seizures and too much ADH in the syndrome of inappropriate anti-diuretic hormone secretion (SIADH) (62). Fatigue, irritability and confusion are common symptoms in patients with recurrent AIP (100). Convulsions (95) and acute encephalopathy (47, 101) in an AIP attack is often associated with hyponatremia (95), and it could be drug-resistant and premenstrual (47, 101). The hyponatremia could be caused by vomiting, diarrhoea or poor oral intake of sodium and could potentially be dangerous. Another possible cause of hyponatremia is the presence of SIADH, which is related to the hypothalamus, but could also be an adverse effect of prolonged treatment with glucose intravenously if sodium is not added to the glucose (102, 103). It is hypothesized that in AIP, the SIADH sometimes rather could be renal salt wasting (RSW), according to an oral statement by professor R. Hift at ICPP 2019. SIADH and RSW have similar characteristics including e.g. hyponatremia and concentrated urine, while differing e.g. in that SIADH have normal to high extra-cellular fluid volume, while it is decreased in RSW(104).

1.4.4 Red urine

Red urine without blood can lead to a suspicion of AIP. High PBG concentrations in the urine of AIP patients may develop a red colour, either when the urine is fresh, or typically after some hours of storage, exposing it to air and light (Fig. 4). The red-purple-brown colour is due to formation of porphobilin, oxidized porphyrins and other compounds produced by condensation of the urine PBG (3, 43, 105).

Fig. 4. Urine from an AIP patient at intervals of 0 hours, 2 hours, 4 hours and > 24 hours after urine void developing the characteristic dark red-purple colour after 4 hours of storage.

1.4.5 Recurrent attacks and chronic symptoms

Recurrent attacks are defined as more than or similar to four attacks per year (94). In France, where patients with recurrent attacks were rare, it was reported that recurrent attacks had increased as they found that this percentage in later years was 7.6% of the symptomatic AIP cases (< 10%), and this had happened during the same time period as treatment with haem arginate was introduced (2, 94). The estimated percentage of AIP patients that would develop recurrent attacks was 3–5% (25, 106). In a Spanish study it was reported that 23% of 35 symptomatic AIP patients had recurrent attacks that required hemin infusions and were without long-term biochemical remission (107). Approximately 20% of the persons with AIP who have recurrent symptoms develop chronic and ongoing pain and other symptoms (6) such as fatigue and nausea (108) and incomplete recovery of paresis (109). Frequency of symptoms was a predictor of fatigue score in a model from a study of patients, which reported outcome measurement information system scales in AIP (110). In Sweden, chronic impairment in 18% of 149 symptomatic cases was reported, including moderate abdominal pain, muscle pain or paraesthesia, muscle weakness, fatigue and mild psychiatric symptoms. Also, one patient developed chronic paresis (33).

1.5 Complications and associated diseases

Complications associated with AIP are chronic peripheral neuropathy, hypertension, renal impairment and hepatocellular carcinoma (HCC) (10). These complications are further discussed in relation to inflammation in sections 1.7.8 and 1.7.9.

1.5.1 Hypertension

Hypertension is more prevalent in persons with symptomatic AIP than in the general population (47). Of 108 persons with acute porphyria, of whom 90 had AIP, it was found that 41% had systemic arterial hypertension as compared with 23% of the controls (5). In a study from Sweden, where the vast majority had the W198X mutation, 40% of the persons with symptomatic AIP had hypertension as compared with 10% of the asymptomatic AIP carriers (33). The risk of renal failure may be correlated with the occurrence of hypertension during AIP attacks, although vasospasm during attacks and nephrotoxicity of ALA are also possible contributors (10). The underlying pathogenesis of hypertension in AIP is poorly understood, although recent experiments in a mouse model of AIP showed an increased local vasoconstrictor response, a lower sensitivity to vasodilatation in vessels from female mice and significant vasodilatation to hemin (83). Also,

hypertension and tachycardia have been shown to be related to increased levels of circulating catecholamines, assumed to be due to stimulation of the sympathetic nervous system (111). It is also suggested that elevated circulating catecholamine levels during acute attacks of porphyria are caused at least partially by blockade of re-uptake into the sympathetic neurons (112).

1.6 Treatment

Removing triggering factors and increasing glucose intake is the first line of treatment of AIP symptoms. In the initial phases of an AIP attack, the patient should try to increase the oral intake of carbohydrates, especially the sugar intake. Removing any triggering factors such as alcohol, smoking, prolonged fasting and getting treated for known triggers such as ongoing infections is important. If feasible, any porphyrinogenic medications should be removed or replaced with safer options (3).

1.6.1 Standard treatment in hospital

Intravenous glucose and haem arginate are part of the recommended treatment modality for acute AIP attacks in the hospitals from both the Norwegian and Swedish competence centres. The glucose should be given intravenously with the addition of fast-acting insulin (3, 6, 113). Addition of sodium is also recommended because there has been an example where this was not done and profound hyponatremia led to death (105). A combination of anti-nausea medication and pain medication, often intravenous morphine, are often needed during AIP attacks (114). Regular meals with a high carbohydrate content are recommended, although vomiting, nausea and pain may perturb regular meals. Pain, poor appetite, changes in taste and early satiety has been reported as predictors for malnutrition in a study of patients with chronic liver disease (115).

1.6.2 Haem preparations

The haem preparations used for treatment of acute attacks of AIP are haem arginate (Normosang® Recordati Rare Diseases, SARL, Puteaux, France) and lyophilized hematin (Panhematin® Ovation Pharmaceuticals Inc., Deerfield, IL, USA). Haem arginate and hemin act by replenishing the hepatocyte haem pool and downregulating ALAS1, thereby decreasing the production of ALA and PBG and bettering the clinical condition (116). A severe AIP attack or moderate attacks where glucose infusion is not efficient should be treated with haem preparations, possibly promptly (6, 47, 117). Hemin is clinically more effective than glucose (118), and has possibly reduced the mortality

rate of AIP attacks (1), as it was 50-60% around hundred years ago, while seldom lethal now (13). Anyhow, the improved prognosis is also likely due to avoidance of known precipitating factors like e.g. barbiturates (13). Causes of death in AIP attacks can e.g. be paralysis of the breathing musculature because of neurological dysfunction orheart rhythm disturbances (13) while also complications such as kidney failure or hepatocellular carcinoma can add on to morbidity and mortality (13). In a review from 2017, Stein et al. stated that "Clinical experience gathered in many different countries over the past 25 years suggests that patients treated with hemin at an early stage in their attack have faster resolution of symptoms, shorter hospital stays and a lower incidence of complications, including neuropathy and seizures, than those who did not receive hemin" (4). However, Stein et al. and the European Porphyria Network (porphyria.eu) suggest that carbohydrate loading and intravenous glucose have been replaced by haem preparations as the treatment of choice for an acute attack of AIP (114, 119).

In an open-label study on hemin, the treatment was found to be successful in all the cases of acute attacks in 73% of the patients (120). A study of 108 Americans with acute porphyria, of whom 90 had AIP, showed that 74% felt that hematin was very effective as a treatment of their attacks of acute porphyria (5). On the other hand, in a double-blind study comparing placebo and haem arginate in 12 patients with AIP, it was found that it effectively reduced ALA and PBG, but that this reduction was not accompanied with striking resolution of the clinical manifestations of the attack (121). However, several previous reports had suggested that the treatment was associated with clinical improvement (122, 123).

The use of haem preparations not only for an acute AIP attack but also as prophylaxis of AIP attack has propelled its use and is recommended for AIP patients with recurrent attacks (106, 120). However, only AIP attacks, and not long-term treatment with haem, has been an indication from the pharmaceutical companies such as Recordati (124). Whether AIP patients with chronic symptoms or recurrent AIP attacks should get prophylactic haem therapy is debatable, as some recommend it to be restricted to those with severe neurovisceral crisis (94).

1.6.3 Treatment of pain, nausea, tachycardia, hypertension and electrolyte imbalances

Opiates are most often necessary to treat the pain during acute AIP attacks (4). Decisions about the type of analgesics and anti-emetics must be individualized and always checked against the drug-database for acute porphyria. Balwani et al. recently stated: "The cardinal symptom of acute hepatic porphyria, neuropathic pain, is often refractory to treatment and requires complex analgesic regimens" (125). Seizures would warrant anticonvulsive medication, and if relevant, careful

correction of hyponatremia and hypomagnesemia must be done (113). If tachycardia and systemic arterial hypertension arise during an AIP attack, and if treatment with drugs such as beta-adrenergic blockers is needed, this should be performed with caution, taking into account the possibility of hypovolemia and a compensatory catecholamine secretion (113).

1.6.4 Removing triggering factors and long-term management

A cornerstone in the treatment of AIP attack is to remove present triggering factors, such as treating an ongoing bacterial infection (113). Also, since stress is a very common trigger of AIP attacks (95), unnecessary stress should be avoided by the patient (6, 95). Removing or replacing porphyrinogenic medications is important. Persons with symptomatic AIP should get yearly check-up of biomarkers of kidney and liver function, plasma sodium and magnesium, cell blood count and measurement of urine PBG and ALA. Persons with AIP should be monitored for the potential development of long-term complications (6), especially those with frequent attacks, and patients with complications require close follow up and referral to subspecialists when needed (106). Persons with AIP should get a physical examination including measurement of blood pressure and assessment of kidney function, and they should be asked about the quality of life. Further, persons with AIP above 50 years of age should get a liver ultrasound done twice every year due to the high risk of HCC (106). Those with asymptomatic AIP in Norway are recommended to get a regular check-up at their G.P. every third year. A dialogue with a medical doctor, where medication use is checked against a relevant database, is warranted. Also, a discussion of how a precipitating factor such as stress should be handled is important. If relevant, a talk on how to avoid alcohol, drugs and smoking must also be performed. The patients should be informed about the importance of sharing information about AIP with their relatives, giving them the opportunity to decide if they want to get tested for AIP. AIP in pregnancy should be closely monitored, as excess risk of perinatal death in first-time mothers with active acute porphyria have been observed (126). A previous report suggested that in AIP, pregnancy can be associated with an increased rate of hypertension, spontaneous abortion, infants with low birth weight and increased mortality (127).

1.6.5 Givosiran, liver transplantation or gonadotropin-releasing hormone analogue

Givosiran, which is available in the market in the US and EU, is an option to be considered in patients aged 12 years and older, especially in patients with recurrent attacks, as it is shown to reduce the AIP attack rate (92) and porphyrin precursor levels (128). Givosiran is an RNAi, that is, RNA interference or silencing RNA that targets the hepatic ALAS1 mRNA. It has been tested in

mice, where it prevented and treated AIP attacks (129, 130). A phase I study on humans showed that once-monthly injections of givosiran in AIP patients with recurrent AIP attacks nearly normalized ALA and PBG and gave a lower AIP attack rate than in patients treated with placebo (131). In November 2019, the Food and Drug Administration (FDA) approved givosiran in USA under the name, GIVLAARI®, for adults with acute hepatic porphyria (92, 93). GIVLAARI® was recently approved by the EU. The efficacy and safety was reported in ENVISION, a randomized-double-blind placebo-controlled, multinational phase 3 trial enrolling 94 patients with acute hepatic porphyria (93, 125). They found a mean annualized attack rate of 3.2 in the givosiran group and 12.5 in the placebo group, hence a 74% lower rate in the group receiving givosiran (125).

Further, it should be evaluated when the criteria for a liver transplant is met for patients. If there are serious and prolonged symptoms, high biochemical disease activity and organs such as the kidneys start to get affected. A liver transplantation could be indicated in such situations (132, 133). Others have stated that when neurological symptoms progress despite ovulatory suppression and/or prophylactic hemin, then liver transplant should become a consideration (8). A liver transplant in AIP gives the patient a liver without the genetic AIP mutations, thereby normalising the level of HMBS and subsequent normalization of ALA, PBG and resolution of symptoms (134). Liver transplant for AIP cured a patient in 2002 (133), and combined liver and kidney transplants in AIP have also been performed (135). By now, several liver transplantations have been performed on AIP patients with good results (68). In total, 34 patients who had acute porphyria and got a liver transplant have been included in the European Liver Transplant Registry (ELTR) according to an oral presentation by Lissing M. (ICPP conference, Milano 2019). A disease-specific complication after liver transplantation in AIP is possibly hepatic artery thrombosis (136). Another medication used in the treatment of AIP are gonadotropin-releasing hormone (GnRH)–analogues given to prevent cyclical attacks of AIP in women (137, 138).

1.7 Inflammation

1.7.1 Could inflammation be a trigger, part of symptoms and a consequence of AIP?

The clinical symptoms of inflammation were defined during the 1st century AD as heat (calor), pain (dolor), redness (rubor) and pain (dolor) and thereafter with the addition of loss of function (functio laesa). A controlled inflammatory response is beneficial to fight back certain triggers, but becomes damaging if dysregulated. Inflammation is a host response to various stimuli, and it is now

considered as complex immune processes and having complex consequences (139). Inflammation affects haem synthesis, and infections and/or inflammation are known to trigger AIP attacks (47). Surgery and infections can induce HMBS indirectly by inducing HO (Fig. 2), which is an enzyme that catabolizes haem and causes and increased demand (2). A possible mechanism for developing porphyria symptoms is that the inflammation activates the transcription factor NF-κB. This, in turn, influences various CYP enzymes in liver (140), which affects haem synthesis (141). CYP are also involved in the initiation of pain by directly activating sensory neurons and cytokines (142). AIP has many known trigger factors, but some AIP patients may suffer acute attacks without any identifiable trigger other than physical and mental stress (100). In the liver cells, acute phase proteins and Interleukin-(IL)-6 may increase ALAS1 activity (143). Also, inflammation may possibly affect the haem synthesis, perhaps, through hepcidin, a key regulator of the entry of iron into the circulation (144). One could, therefore, speculate that inflammation could be a part of a possible vicious cycle in AIP, and that inflammation is partly responsible for the symptoms in AIP. Inflammation increases ALAS1 activity and can trigger increased AIP biochemical activity and symptoms, and this might again trigger and enhance inflammation due to organ damage induced by ALA, PBG or porphyrins. In addition, other metabolic diseases such as diabetes are associated with inflammation (139, 145, 146). This urged us to investigate the role of inflammation in AIP.

1.7.2 Haem preparations as a possible trigger of inflammation

Free Normosang solution is mildly caustic and hypertonic, which can cause painful inflammation, venous thrombosis and thrombophlebitis in the small peripheral veins (65). A study of a human hepatoma cell line to which haem was added concluded that haem exerts a variety of effects on mRNA and miRNA profiling, including effect on oxidative/stress response (147). From a study of livers in a HMBS-/- mouse model and five human explanted livers from recurrent AIP patients, it was reported that frequent hemin infusions generated a chronic inflammatory hepatic disease, and that this inflammation induced HO-1 remotely to the hemin treatment (94). The induced HO-1 maintains a high ALAS1 level that could be responsible for recurrent attacks, which has, according to Schmitt et al., increased 4.4-fold after hemin was introduced as a treatment option (94). This increase could have many explanations, including both induction of HO-1 and improved survival by heme therapy (94, 148). Further, inflammation and liver deposits of iron in the explanted liver of patients who received intravenous haem have been reported (106, 149). Based on these findings (94, 106, 149), a restrictive use has been called upon regarding long-term treatment with intravenous haem (106). On the contrary, it was recently stated by Yarra et al. that: "..,we have not

found that the administration of intravenous haem for valid cause and reasons [treatment of acute attacks and prevention of recurrent attacks of acute hepatic porphyrias] leads to any increased incidence of recurrent attacks" (150). Yarra et al. further stated that: "Their suggestion [Schmitt et al.] seems rather unlikely a priori because only patients with pre-existent recurrent and frequent symptoms will be those who will be treated with repeated haem infusions", and Yarra et al. noted that in the key figure of Schmitt et al. the apparent frequency of recurrent attacks in France already was increased *before* the institution of prophylactic haem arginate therapy (150). Anyway, in our study, none were on prophylactic or long-term Normosang treatment, and none were treated with Normosang at the time of study (151). Hence, we would look at the other possible causes of inflammation.

1.7.3 Metaflammation, low-grade inflammation, sterile inflammation

Metaflammation, meaning chronic metabolic inflammation, is a term that has emerged in recent years in relation to metabolic diseases (152). Both tissue homeostasis and metabolic diseases' pathogenesis are determined partly by inflammatory cells' actions and interactions with stromal components in metabolic organs such as the liver, brain, pancreas and adipose tissue (152). Elevated levels of cytokines has been shown to be associated with increased risk for type 2 diabetes (T2D) (145). Increased expression of tumour necrosis factor (TNF) in human adipose tissue and in persons with obesity is seen (153). T2D is related to lifestyle and genetic factors (154). The disease is caused by insufficient insulin production from beta cells in the setting of IR, but not all persons with IR get T2D since an impairment of insulin secretion from dysfunctional pancreatic beta cells is required (154). Then, hyperglycaemia follows, and eventually T2D develops. Contributing factors to the pancreatic islet dysfunction may be deposits of amyloid polypeptide (154, 155), branched chain amino acids associated with the microbiome in the gut (156), metaflammation and obesity (157). The root cause of this error in stimulus-secretion coupling is not totally understood (158).

Sterile inflammation is inflammation that occurs typically in the absence of any microorganisms. It can be the result of trauma, ischemia-reperfusion injury, atherosclerosis, gout, chemically induced injury and certain metabolic diseases (159). It can lead to recruitment of neutrophils and macrophages, and production of cytokines, chemokines and induction of T cell-mediated adaptive immune responses (159). Sterile inflammation can be a result of damage-associated molecular patterns (DAMPs) released into the extracellular environment during necrotic cell death (159). A part of the rationale for one of our hypothesis is that AIP is a metabolic disease,

and many metabolic diseases in the later years have been linked to low-grade sterile inflammation, including atherosclerosis and T2D (139, 146).

Although others have talked about the possibility of inflammation in AIP and the increased risk for HCC indicating a possibility for chronic low-grade inflammation, there were no studies where this was investigated in AIP. Inflammation could possibly also be associated with kidney failure in AIP. Further, infections and/or acute inflammation are known to cause AIP attacks (54). Part of the rationale for investigating inflammation in AIP is that symptomatic AIP cases, perhaps, could exhibit a chronic inflammation that could explain why some have symptoms while others do not. Furthermore, we hypothesized that the inflammation could affect haem synthesis in the liver, thereby increasing the biochemical disease activity and symptoms.

Potential information regarding inflammation and its effects could give new insight into pathogenesis of AIP and its symptoms and complications. We found it quite interesting to test the hypothesis regarding the role of low-grade inflammation in AIP and whether they had higher number of inflammatory diseases as compared with the controls.

1.7.4 Cytokines and long-pentraxin 3 (PTX3)

Cytokines are small signal proteins that include interleukins, chemokines, lymphokines and interferons (160). Cytokines can be excreted from different cells such as macrophages, mast cells, fibroblasts and leucocytes (160-162). They can affect cells all over the body, and they also have autocrine and paracrine effects. Cytokines can act pro-inflammatory or anti-inflammatory. They regulate a variety of immune responses through binding to receptors on different cells (160). A part of the rationale for measuring cytokines in AIP is that T2D, which also is a metabolic disease, has been shown to be associated with elevated cytokines levels (145, 163). The cytokine concentrations have also been shown to be elevated acutely after induced hyperglycaemia (164). Another rationale for measuring cytokines was that AIP is linked to increased prevalence of kidney damage (165), and kidney damage has earlier been shown to be linked to inflammation in other diseases (166). IL-17 is a proinflammatory cytokine derived from Th17 helper T cells and natural killer cells and is involved in the Th17 response in many chronic inflammatory conditions (167). Long-pentraxin 3 is a soluble pattern-recognition molecule that is present in the blood and body fluids and is a part of the innate immunity (168). It has the ability to recognise pathogens and promote their disposal (168). PTX3 is produced locally in a number of tissues, mostly in macrophages and neutrophils. Further, pentraxins interact with components of the complement system, and thereby has

implications for host defence and regulation of inflammation (168). We, therefore, aimed to measure cytokines in AIP patients and controls.

1.7.5 Damage-associated molecular patterns (DAMPs)

There are many potential sources of inflammation in AIP, enhanced concentrations of ALA and PBG in plasma being one possibility (129, 169). If ALA and PBG act as DAMPs, they could be one of the causes of inflammation in AIP, since it is known that circulating DAMPs cause inflammatory response to injury (170, 171). However, this has not been extensively investigated, although incubation with ALA increased lipid peroxidation in a cancer cell line (172). On the other hand, ALA decreased the LPS-induced cytokines in a macrophage cell line *in vitro*, indicating that ALA may have anti-inflammatory effects on some cell types (173). It has been reported that ALA treatment of a normal gastric cell line enhanced reactive oxygen species generation and induced apoptosis associated with p53, whereas cancer-like mutant cells were unaffected (174). Porphyrins have been reported to activate the complement system (175), and one could speculate that porphyrins act as DAMPs.

The term DAMPs is used for self-molecules that can initiate inflammatory response without any pathogens or other external antigen (176). DAMPs remain unrecognized by the immune system under normal physiological conditions, but during cellular stress or tissue injury, these molecules are exposed (170). There are a growing number of known DAMPs such as uric acid crystals, nuclear DNA, histones, mitochondrial DNA, high-mobility group protein B1, heat shock protein (176), RNA, ATP and others (177). We cannot totally rule out that ALA and PBG can act as DAMPs directly. However, ALA has several anti-inflammatory effects, indicating that it may be unlikely that ALA acts as DAMP in AIP (173, 178). We speculate that the damage in tissues, possibly caused by ALA and PBG giving free radicals and oxidative stress (179, 180) and related inflammation could release DAMPs, , which have been shown to stimulate immune cells and inflammation in many diseases (170, 181, 182). In sterile inflammatory liver diseases such as non-alcoholic steatohepatitis (NASH) or hepatic ischemia-reperfusion injury (I/R), first line and second line DAMPs have been reported (183). The immune system does not only discriminate between self and non-self but also between self-safe and self-dangerous entities (183).

1.7.6 Complement

Some DAMPs, including histones, heat-shock proteins and biglycane, have been shown to activate the complement cascade during reperfusion after ischemia (184). Uric acid might also activate the

complement pathway to induce inflammatory response (185). Complement plays an important role in the innate immunological recognition of DAMPs. The complement system is a part of the human innate immunity system, protecting the body from invading microbes and other threats; it also takes part in immune surveillance and housekeeping tasks (186). Normally, specialized pattern recognition receptors detect DAMPs and initiate enzymatic cascades, activating one of three main initiation routes that are called the classical (CP), lectin (LP) and alternative pathways (AP) (186). CP is activated by pentraxins and surfaces covered with antibodies. LP is activated by ficolins, mannose binding lectin (MBL) or collectins that are bound to carbohydrates. Foreign and damaged cells activate AP. Importantly, C3b that comes from initial activation of CP and LP also initiates the AP, in which C3 activation is amplified by formation of a C3 convertase (186, 187). In the complement cascade, the next step is the production of C3 convertases. Then, C3 is cleaved to form the anaphylatoxin C3a and C3b, which further activate the cascade (187). Then C5 convertase is formed, which is cleaved to form the anaphylatoxin C5a and C5b (188). The component C5b together with one unit of C6, C7, C8, and several C9 molecules, form the membrane attack complex (MAC) or a soluble form sC5b9 (189). When the balance between complement activation and regulation is disturbed, it plays a role in many clinical disorders (186). The complement system is involved in many diseases including paroxysmal nocturnal haemoglobinuria (PNH), atypical haemolytic uremic syndrome (aHUS), ischemia-reperfusion injuries, cardiac infarction, trauma and stroke (186, 187, 190). Haem and porphyrins, although not officially listed as DAMPs, have been reported to activate the alternative complement pathway (175, 191, 192). This added to the rationale for investigating levels of complement markers in AIP and controls. This is since it is known that porphyrin precursors ALA and PBG build-up in AIP, that these components and porphyrins are elevated in the urine, and that part of the treatment for AIP is giving haem-containing products. Dietary supplementation with ALA has been shown to induce T-cell responses in chickens through oxidative stress (193). On the other hand, ALA has antioxidant properties; 5-aminolevulinic acid–sodium ferrous citrate (5-ALA-SFC) has been reported to activate electron transport chain in mitochondria and further induce haem oxygenase-1, resulting in prevention of ROS production and elimination of ROS (194). Also, ALA is studied for possible use in diabetes management (195).

1.7.7 Pattern recognition receptors and the inflammasome

The pattern recognition receptors can be localized on the cellular membrane surface, such as on the Toll-like receptors (TLR) recognizing pathogen-associated molecular patterns (PAMPS) (196) or DAMPs (177). Furthermore, there are similar receptors on the cellular inside, such as the Nod-like

receptors (NLRs) recognizing DAMPs (197). Both infection-associated PAMPs and host-derived DAMPs can impact several metabolic diseases that are associated with inflammation (198). A subgroup of NLRs is called NOD-like receptor protein 3 (NLRP3), and this is one of the proteins that is able to assemble to a structure called inflammasome, a multimolecular protein (199, 200). The NLRP3 inflammasome recognizes PAMPs and DAMPs and other inflammation-inducing stimuli, and then activates the caspace-1 cascade, leading to the production of proinflammatory cytokines, especially IL-1β and IL-18 (200). The inflammasome can be activated by a wide spectrum of danger signals coming not only from microorganisms but also from metabolic dysregulation (199). Gout-associated uric acid crystals can activate the NLRP3 inflammasome (201). The NLRP3 inflammasome has been shown to sense obesity-associated signals and contribute to obesity-induced inflammation and IR (202). Further, the NLRP3 inflammasome is also activated by cholesterol crystals and required for atherogenesis (203). Further, the NLRP3 inflammasome can be regulated by dietary metabolites, and this adds to our rationale for looking into both diet and inflammation in AIP (204).

1.7.8 Hepatocellular carcinoma and inflammation

There is high prevalence of hepatocellular carcinoma (HCC) in AIP (205-208), especially without preceding cirrhosis (209). HCC was found in 3% of symptomatic AIP and in 1% of latent AIP in a study from Sweden (33). Although in some AIP patients whose liver could be examined, a concomitant existence of portal cirrhosis was found in 5 of 11 AIP patients with HCC (206). The annual incidence rates range from 0.16 to 0.35% and are higher in Sweden and Norway (210). In the global incidence of HCC, there is male dominance, but in AIP, it is the opposite. High levels of ALA and PBG seem to be more common in the AIP cases that gets cancer, although porphyria specific risk factors are still unclear (208, 209, 211). The risk of HCC is higher among persons with AIP as compared with the average population, especially after the age of 50 (10, 211, 212). In France, a 36-fold increased risk was found (213), and in Norway, eight persons had primary liver cancer out of the 91 persons with symptomatic acute hepatic porphyria, which was relatively higher than in the reference population (212). Non-resolving inflammation increases the risk for HCC (214). An intrinsic pathway where genetic events lead to activation of transcription factors such as NF-κB and an extrinsic pathway where PAMPs and DAMPs also lead to activation of transcription factors can lead to cytokine production (214). In AIP, the mechanism(s) of carcinogenesis leading to HCC are unknown. However, many growth factors such as fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) may act as

possible pro-angiogenic factors secreted by stromal cells after chronic liver injury, and IL-17 may be involved in tumour progression (215). ALA may also be a carcinogenic factor in AIP (10). It was found that the mRNA expression of proinflammatory cytokines, IL-1β, IL-6 and TNF, were elevated in the liver of AIP patients as compared with the mean control levels (68). Possible pathophysiology of HCC in AIP could be either a direct toxic effect of the haem precursors on the liver, expecially ALA, compound heterozygosity for genes that possibly are involved in haem biosynthesis, or maybe a relative lack of haem could cause loss of oxidative stress homeostasis (210). Liver injury that leads to cell death, inflammation and compensatory regeneration of hepatocytes is a charachteristic lesion underlying hepatic carcinogenesis. This takes place in the context of oxidative stress, providing a mutagenic environment, thereby increasing the risk for HCC (210). A histological study of an explanted liver from an AIP patient showed that most portal areas had mild nonspecific lymphocytic inflammation and mild ductular reaction but no cirrhosis (68), and there was expression of HO-1. Haem accumulation, inflammatory cytokines, hypoxia and oxidative stress can induce HO-1 (216).

1.7.9 Impaired kidney function and inflammation

Chronic kidney disease is seen in up to 59% of the symptomatic AIP patients (165, 217), with a decline in the glomerular filtration rate by ~ 1 mL/min per 1.73 m^2 annually (165). In a study of 108 cases with acute porphyria, 29% had chronic kidney disease as against 1% of the controls (5). Histopathologically, two types of lesions characterized porphyria associated kidney disease (PAKD) (165). According to Pallet et al. these were: “A chronic tubulointerstitial nephropathy, which is often associated with mild and nonspecific arteriosclerosis, and a chronic fibrous intimal hyperplasia associated with focal cortical atrophy” (165). It is suggested that in proximal tubular cells, ALA and PBG can promote endoplasmic reticulum stress, apoptosis and changes in epithelial phenotype (165). Tubulointerstitial nephritis was described in a case of AIP (218). While apoptosis is well tolerated by the immune system, other types of cell deaths cause DAMP release and cytokine production (219).

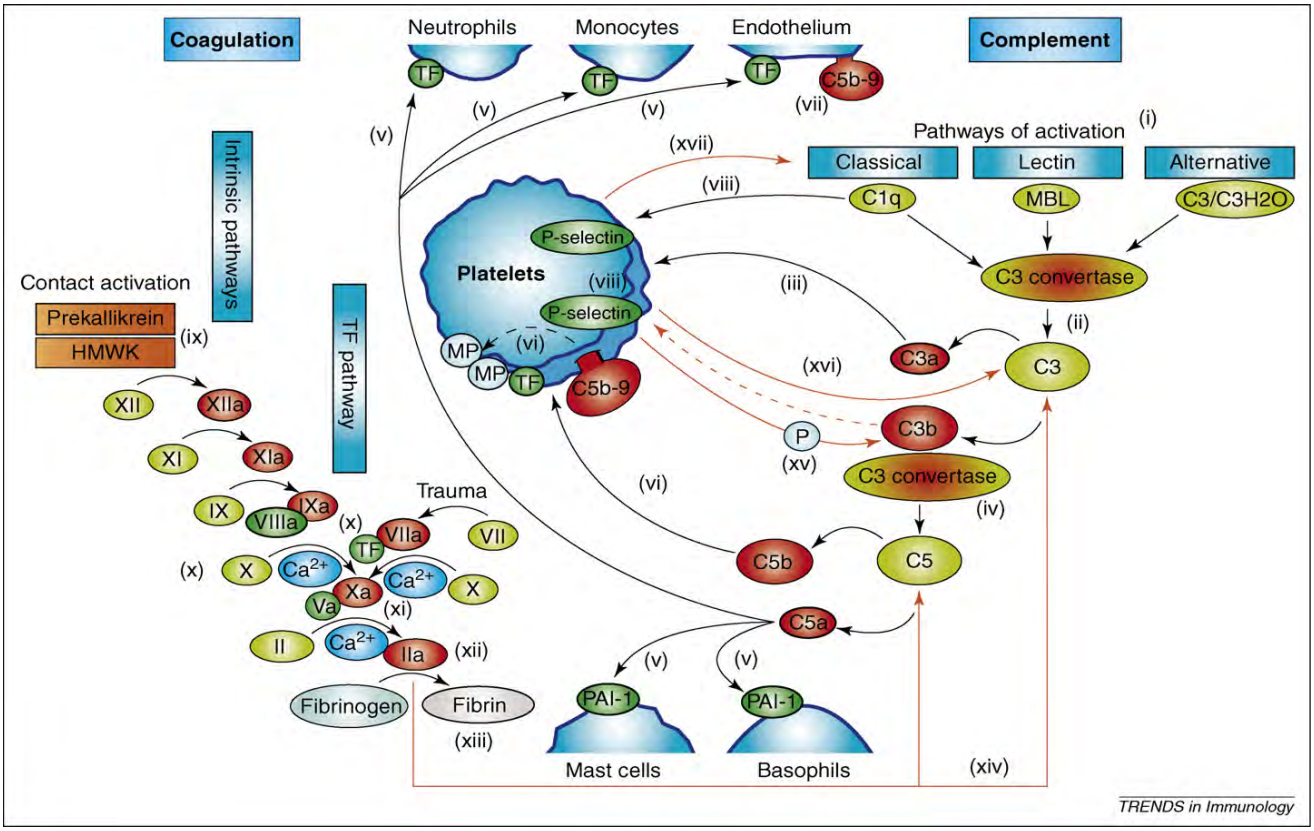

Fig. 5. Complement–coagulation reciprocal interactions.

Zymogens are marked in light green, and active components are shown in red.
Black arrows illustrate the interactions of complement with coagulation, increasing the propensity of blood to clot. The amplification of complement activation exerted through the components of the coagulation system is shown as red arrows. Abbreviations: HMWK = high molecular weight kininogen, MBL = mannose binding lectin, P = P-selectin, PAI = plasminogen activator inhibitor, TF = Tissue factor. Fig. reprinted from Trends in Immunology, 2007, Markiewski et al., Complement and coagulation: strangers or partners in crime? 28: pages 184-192, copyright (2007)(220) with permission from Elsevier.

1.7.10 Thromboinflammation and prothrombin fragment 1.2 (PTF1.2)

Thromboinflammation is the result of activation of the cascade systems in the blood (190), including the complement, contact, coagulation and fibrinolytic systems (Fig. 5). Together, these systems lead to activation of polymorphonuclear cells, platelets, monocytes and endothelial cells that line the blood vessels (190). Some of the AIP patients receiving Normosang encounter thrombophlebitis in the peripheral vein where Normosang is given. AIP patients who have received a liver transplant might have increased risk of thrombus in the hepatic arteries and are, therefore, advised to use acetyl salicylic acid (136). PTF1.2 is a marker for hypercoagulable state and may reflect an increased risk for thrombosis, and it is cleaved from the end of human

prothrombin when activated by FXa to yield thrombin (221). Since we suspected inflammation in AIP, it gave us the rationale for also measuring a marker of coagulation, PTF1.2 .

1.8 Diet and glucose metabolism

The low penetrance of AIP points to a critical role of modifying factors, either genetic or environmental (30). Since glucose already is a part of treatment for acute attacks (3, 6, 113), we surmised that studying diet and hormones related to glucose metabolism in AIP could possibly give new insights into the disease activity and its treatment modalities.

1.8.1 Glucose, sugar, carbohydrates

During an AIP attack, nausea and vomiting often contribute to the lack of nutrition. Glucose reduces the AIP disease activity by downregulating ALAS1, the first and rate-limiting step in the haem synthesis (222), through effects on PGC-1α (223-225). A high carbohydrate diet might, therefore, contribute to the decrease in the level of AIP disease activity through the inhibiting effect of glucose on haem synthesis (224). The American Porphyria Foundation states on their internet pages that, "If an acute attack is severe or prolonged, sufficient glucose can be given by vein to meet the total energy requirements of a patient. This is accomplished best by using a catheter that is inserted into a large central vein. Additional nutrients, including vitamins, minerals, amino acids and fat can also be given to maintain all dietary requirements. Provision of total nutritional needs in this manner by vein is commonly called total parenteral nutrition (TPN). Oral feedings can be introduced gradually as recovery from an attack begins, and when there are signs that functions of the stomach and intestines are improving." Balwani et al. were in line with this regarding recommendation of a balanced diet, avoiding prolonged fasting or crash dieting, and recommending carbohydrate loading for early stages of an acute attack, but somewhat to the contrary they also stated that: "Moreover, a sustained adherence to a high-carbohydrate diet does not prevent attacks and is not recommended (106)." Anyhow, the Nordic nutrition recommendations state that for the general population, 45–60% of the total energy intake (E%) from carbohydrates is recommended (226). Studies of seven AIP patients who did not have symptoms at the outset of the study, but five of them previously had experienced symptoms, suggest that increased carbohydrate content in the diet reduced ALA and PBG excretion (51). Glucose given, mainly intravenously, reduced the excretion of ALA and PBG in 15 AIP patients after 3–5 days of treatment (227). Glucose also reduced the induction of ALAS1 because of the barbiturate meprobamate in avian liver cells (227).

In four patients in an acute AIP attack and one in remission who was treated with laevulose (fructose) intravenously the treatment was associated with a fall in urinary PBG and ALA (228). The AIP symptoms also decreased in a woman who got total parenteral nutrition with 62% carbohydrate (223). In Sweden, sixteen persons with symptomatic AIP who later developed type 2 diabetes (T2D) no longer had porphyria symptoms (229). Taken together, this gave us a rationale for our hypotheses regarding possible differences in the diet, glucose intake and carbohydrate intake in AIP cases as compared with controls and in the symptomatic as compared with asymptomatic AIP cases.

1.8.2 Fat and protein

Generally, the amount of saturated fat intake in the Norwegian population is excessive (226). In AIP patients, this might be of particular importance because a high-fat diet has fewer carbohydrates, which could provide less glucose effect on the haem synthesis, which is not good for AIP patients (51). The typical AIP trigger factor, low glucose level in hepatocytes, gives us a rationale to also look into the amount of fat as not only a competitive nutrient to carbohydrates but also as an energy-dense nutrient which might result in obesity. Chronic elevated fatty acids are linked to inflammation and insulin resistance (IR), and saturated fatty acids (SFA) can activate the NLRP3 inflammasome (204). IL-1β inhibits the signalling of insulin in both hepatocytes, pancreatic cells and adipocytes (204). These complicated fat-inflammation-insulin connections add to the rationale for investigating all these markers in AIP, where the patients are better off without fasting and experience fewer AIP attacks if they develop T2D (229). Protein should provide 10–20% of the total energy intake in the general population and 15–20% for the elderly (226), and persons with gout should reduce intake of purine-rich food (230).

1.8.3 Insulin and C-peptide

Insulin is an endocrine peptide hormone that is secreted by the β-cells of the pancreatic islets of Langerhans. The half-life of insulin is 3–5 min. Wilcox stated that insulin "maintains normal blood glucose levels by facilitating cellular glucose uptake, regulating carbohydrate, lipid and protein metabolism and promoting cell division through its mitogenic effects" (231). Insulin "binds plasma membrane-bound receptors in target cells to orchestrate an integrated anabolic response to nutrient availability" (232). Insulin regulates the metabolism of carbohydrates and also of lipids and proteins (233). IR means impaired signal transduction and biological actions as a response to insulin stimulation (233), and when the secretion of insulin no longer can compensate for the IR, a person

can develop T2D (233). There is a complex interplay of multiple metabolic pathways on insulin; lipids, amino acids and bile acids also have roles in modulation of the insulin sensitivity (233). C-peptide, a 31-amino-acid peptide with a half-life of 20–30 min, is a part of proinsulin, and it is cleaved before co-secretion with insulin from pancreatic beta cells (234). Plasma C-peptide is a measure of insulin secretion and pancreatic beta-cell function (234).

1.8.4 Visfatin, Resistin, Ghrelin, Leptin and Gastric inhibitory polypeptide

Visfatin is a protein of 52-kDa and is synthesized in visceral adipose tissue (235) and secreted by macrophages. Also, the bone marrow, liver and muscles have high expression of visfatin (235). Its functions are regulation of adipogenesis, angiogenesis, insulin sensitivity and glucose metabolism. Further, it upregulates the gene expression of both pro- and anti-inflammatory cytokines (235). Visfatin is identical to nicotinamide phosphoribosyl transferase, and it is found upregulated in diabetes and atherosclerosis (236).

Resistin got its name because of its ability to resist or interfere with insulin action (237). Human resistin is a peptide of 12.5 kDa. In humans, it is primarily produced by peripheral blood mononuclear cells, macrophages and bone marrow cells but also by hepatocytes (237). Its physiological functions have been reported to be regulation of glucose metabolism, insulin sensitivity, adipogenesis and angiogenesis (235). Positive correlations have been reported between IR and obesity and serum resistin (237). Further, resistin is an adipokine and as such has been found to induce cytokine expression in chondrocytes in humans (237). Resistin is involved in the increased release of cytokines, as it can activate TLR4 on macrophages (238). Thus, since resistin is linked to insulin, dietary factors and inflammation, and since we suspected that there could be differences in diet, insulin and inflammation among the asymptomatic AIP cases and symptomatic AIP cases and the controls, it gave us the rationale to investigate both resistin and the hormones and biomarkers related to diabetes.

Ghrelin is a peptide of 28 amino acids and is a gut-derived hormone (239). It is derived from preproghrelin that is produced by cells within the gastric oxyntic glands of the stomach (239). Ghrelin has a variety of functions: it impacts glucose metabolism, both during fasting and fed states; influences to the effect of stress as; affects the energy expenditure, appetite and nutrient intake (240). This gave us a rationale for analysing this marker in AIP. It was initially known to be a growth hormone releasing peptide; however, later it was found to increase body weight through its action on the hypothalamic melanocortinergic system and by stimulation of lipogenesis (239).

Leptin is an adipocytokine-like hormone, and it increases the secretion of proinflammatory cytokines and chemokines (235). It is a 16 kDa protein (235). In humans, it is produced mainly by adipocytes but also by T cells, basophils and mast cells (235). Its physiological functions are regulation of food intake, energy expenditure, body weight, angiogenesis, blood pressure, reproduction and haematopoiesis (235).

Gastric inhibitory polypeptide (GIP), also called gastroinhibitory peptide or glucose-dependent insulinotropic peptide, is a hormone belonging to the secretin family (241). It is a stimulator of insulin secretion and a weak inhibitor of gastric acid secretion. It is an incretin. Another incretin is glucagon-like peptide-1 (GLP-1). GIP and GLP-1 are accountable for the "incretin -effect", meaning a greater stimulation of insulin release after peroral intake of glucose as compared with intravenous glucose intake (241).

1.8.5 Vitamins and micronutrients

The overall health can be influenced by low levels of vitamins and micronutrients (226, 242, 243), as can also the haem biosynthesis (43). It has been suggested that the cobalamin-folate system has a role in the acute porphyric process (244). Thunell et al. found higher levels of vitamin B12 in persons with symptomatic acute intermittent porphyria than in the controls (244). It is also known that the key enzyme ALAS1 has pyridoxal phosphate, i.e. the active form of vitamin B6 as prostethic group suggesting a possible role of vitamin B6 in AIP (245). Lack of vitamin D is associated with increased inflammation since vitamin D can inhibit monocyte/macrophage cytokine production (246).

1.9 Iron metabolism and biomarkers of liver and kidney function

AIP attacks can be provoked by factors that induce the haem biosynthesis (45). Haem consists of iron and protoporphyrin IX; therefore, it might be possible that iron supplements taken because of iron deficiency can induce increased haem synthesis, thereby triggering or aggravating AIP symptoms (144, 247). Of interest in this aspect is that the first step in haem synthesis has two different possible isoenzymes, ALAS1 and ALAS2, one expressed in erythroid cells in the bone marrow, and one liver form of the enzyme ALAS1 expressed ubiquitously, suggesting a nonspecific form (248).

AIP is a liver disease with possible kidney complications (217), whose mechanisms are not fully understood. We hypothesized differences between liver and kidney parameters in the AIP cases against the controls. Based on a clinical follow-up of AIP cases, it was suggested that

studying the hepatic markers insulin-like growth factor 1 and prealbumin (transthyretin) in AIP (249) would be of help, and we analysed prealbumin. In addition, we measured regular liver damage markers such as ALT, AST and ferritin, and liver failure related biomarkers such as bilirubin and platelets. Gamma–glutamyl transferase (GT) and alkaline phosphatase (ALP) activity are related to cholestatic liver disease. In addition, kidney function and kidney damage biomarkers such as Cystatin C and creatinine and calculated eGFR were included.

1.10 Effect of anticoagulant, storage time and temperature of blood samples on cytokine concentrations, and reference range for plasma cytokine

1.10.1 Sampling and processing of cytokines

Cytokines are extensively used for research purposes as biomarkers of inflammation (250-252), but the sampling and processing techniques are not standardized. Under suboptimal circumstances, these sampling and processing techniques could potentially affect the results since cytokines can be released from blood leucocytes even after sampling. Furthermore, coagulation and thrombin can probably elicit the release of cytokines from peripheral blood mononuclear cells (253). Sepsis with bacteria is still a serious condition with substantial mortality, and there are partly uncertain diagnostic methods (254, 255). Modern research on sepsis and a long row of other studies on inflammation measure many biomarkers, including cytokines, chemokines and growth factors made by leukocytes and other cells in the blood. Several of these cytokines and growth factors are potential new markers for inflammatory diseases such as sepsis in humans, and interleukin-6 and interleukin-1β and TNF are considered markers for neonatal sepsis (256). The effect of different anticoagulants that are added to the blood during sampling and the appropriate storage conditions for the blood sample after sampling (time, temperature) were not investigated sufficiently previously. We aimed to study the effect of different anticoagulants and sampling conditions on venous blood sampling for analysis of a host of cytokines, chemokines and growth factors in humans. The research group has a whole blood model of inflammation in humans using the anticoagulant lepirudin that does not affect the complement system (257). The complement system has a great impact on the activation of leucocytes and cytokine release (258). Also, there is interaction between the complement system and coagulation (190). Surfaces such as plastic, PVC and the tube wall in glass tubes and blood sampling tubes and the surface of alginate microcapsules (259) can activate complement and contribute to cytokine synthesis. Coagulation-activation due to

upregulation and activation of tissue factor (TF) can also be increased because of complement activation and increased cytokine synthesis (260). TF mRNA is upregulated spontaneously when the whole blood is incubated *in vitro* in plastic sampling tubes (260). We investigated the impact of the sampling conditions and different anticoagulants on cytokine levels in fresh human venous blood samples (Paper III).

1.10.2 Reference ranges for plasma cytokines

There were no specific reference ranges for plasma cytokines in a large number of healthy controls, with what we found as the most reasonable sampling conditions, including choice of anticoagulant, time and temperature (Paper III). Reference intervals are the most common decision support tool used for the interpretation of numerical reports from medical biochemistry (261). We aimed to calculate the reference ranges for 27 different cytokines based on venous blood samples collected from 162 healthy Norwegians. The different tubes with anticoagulants that were tested to find the optimal sampling conditions for cytokines were lepirudin-containing tubes, to which EDTA was added to stop complement activation immediately before centrifugation, heparin tubes, citrate tubes and EDTA tubes. The mechanism by which these anticoagulants work is that both sodium citrate and EDTA bind to calcium. In comparison, lepirudin is a direct and specific thrombin inhibitor, which has the effect that the coagulation cascade is functional upstream of thrombin. Lepirudin is a hirudin analog, and it has no effect on complement activation (257). Lithium–heparin accelerates the effect of antithrombin III, which neutralizes thrombin, thereby preventing the formation of fibrin from fibrinogen.

1.11 Hypotheses

1.11.1 Inflammation, glucose metabolism and diet

H0-1: There is similarity in the content of energy, protein, carbohydrates, sugar, alcohol, folate, thiamin and iron between the diet of the symptomatic AIP and the asymptomatic AIP and the control groups.
Ha-1: There is a difference in the content of energy, protein, carbohydrates, sugar, alcohol, folate, thiamin and iron between the diet of the symptomatic AIP, the asymptomatic AIP and the control groups.

H0-2: There is similarity in the body mass index (BMI) and waist–hip ratio between the symptomatic AIP, the asymptomatic AIP and the control groups.
Ha-2: There is a difference in the BMI and waist/hip-ratio between the symptomatic AIP, the asymptomatic AIP and the control groups.

H0-3: The biomarkers in the blood/serum/plasma related to inflammation, iron metabolism and glucose metabolism, vitamins and stress (see variables) show the same levels in the symptomatic AIP, the asymptomatic AIP and the control groups.
Ha-3: The biomarkers in the blood/serum/plasma related to inflammation, iron metabolism and glucose metabolism, vitamins and stress (see variables) show difference in their levels in the symptomatic AIP, the asymptomatic AIP, and the control groups.

H0-4: The number of persons in the symptomatic AIP group with gout or other chronic inflammatory conditions are not more than those in the asymptomatic AIP group or the control group.
Ha-4: The number of persons in the symptomatic group AIP with gout or other chronic inflammatory conditions are more than those in the asymptomatic AIP or the control group.

H0-5: The number of persons in the symptomatic AIP group with possible complications such as high blood pressure and reduced kidney function are not more than those in the asymptomatic AIP group or the control group.
Ha-5: The number of people in the symptomatic AIP group with possible complications such as high blood pressure and reduced kidney are more than those in the asymptomatic AIP group or the control group.

H0-6: There are no significant correlations between markers of biochemical AIP disease activity and dietary intake, biomarkers of glucose metabolism, stress, inflammation, kidney failure or liver function markers.
Ha-6: There are significant correlations between markers of biochemical AIP disease activity and dietary intake, biomarkers of glucose metabolism, stress, inflammation, kidney failure or liver function markers.

Further, we wanted to map the use of porphyrinogenic medications and herbal remedies, and then register which medications were used by the AIP cases and controls. We wanted to measure urine ALA and PBG in both the cases and controls, map the percentage of symptomatic AIP cases with different AIP symptoms and different triggers of the AIP symptoms.

1.11.2 Cytokine measurements and reference ranges

H0-7: Time and storage temperature between sampling and centrifugation have no effect on plasma cytokine values in venous blood samples.
Ha-7 Time and storage temperatures between sampling and centrifugation will affect plasma cytokine values in venous blood samples.
H0-8: Plasma obtained using different anticoagulants give similar levels of cytokine values in venous blood samples.
Ha-8 Plasma obtained using different anticoagulants give different levels of cytokine values in venous blood samples.

1.12 Aim of thesis

The overarching aim was to investigate inflammation, diet and related biomarkers to try to find differences between symptomatic AIP cases and asymptomatic AIP cases, along with the possible differences between the AIP cases and the controls. To achieve this aim, we had to ensure we had the best way to handle the cytokine analyses, and sampling conditions and the reference ranges of several cytokines were examined. Thus, specifically we intended to do the following:

- Paper I: Study inflammation and glucose metabolism in AIP and correlations between inflammatory markers, including cytokines, and AIP disease activity measured in terms of PBG.
- Paper II: Examine the role of diet, particularly carbohydrates and sugar, in the AIP cases and correlations between nutritional markers and AIP disease activity measured in terms of PBG and inflammatory markers, including cytokines.
- Paper III: Find the optimal conditions for sampling, handling and storage for cytokine analysis of venous blood samples. Establish reference ranges for 27 cytokines in healthy controls.

2 Materials and methods

2.1 Study participants and design of papers

In total, 50 AIP cases and 50 controls matched for age, gender and place of residence participated in the study (Paper I). Exclusion criteria were age below 18 years and lacking consent competence. The dietary intake was registered using a standardized seven-day diet logbook by 46 of the total 50 pairs of AIP cases and controls matched for age, gender and place of residence (Paper II). Of interest were the intake of sugar, carbohydrates, fat, protein and iron, constituents that could impact haem synthesis. Asymptomatic AIP was defined as a condition with an AIP mutation but not exhibiting symptoms of AIP ever. Symptomatic AIP was defined as a condition with an AIP mutation and exhibiting typical AIP symptoms at least once. Blood samples from 10 healthy blood donors, five women and five men, were collected to investigate the optimal conditions for sampling, handling and storage of the analysis of 27 cytokines (Paper III). Blood samples from a total of 162 healthy persons in the range of 18–65 years of age, of whom 107 were women and 55 men were used for calculating the reference interval for cytokines. This included 49 healthy blood donors from the Blood Bank of Nordland Hospital Trust Norway, 42 healthy controls in a clinical epidemiological study performed in Nordland county Norway (262) and 71 healthy persons from a study on health care workers in Nordland county (263).

For papers I and II, the study participants with AIP were sent invitation letters. One matched control for each AIP participant were randomly picked from the same population by Evry.com based on gender, age and postal code of cases. Some persons of the first draw declined to participate in the study, so we asked Evry.com to do one additional draw. The controls also got an invitation letter and signed consent forms. Three of the consenting matched controls did not show up, so they were replaced by other persons who matched by age, gender and postal code. One of the possible AIP patients and her control were excluded since she neither had a positive genetic test nor high biochemical disease activity. The reason for postal code being matched was that matching for place of residence is a common design of epidemiological studies since health issues might be affected by the place of residence. One could also assume that dietary habits are similar among people living in the same geographic area.

2.2 Questionnaires, structured interview and diet logbook

The participants filled out a questionnaire at home (Appendix I and II). Another questionnaire in Appendix III, IV and V was a modification of a previously used patient-filled questionnaire made by NAPOS for use in the national registry (27), and in this research project, the MD filled it out during the interview. There were two different versions of the patient questionnaire, one for AIP (Appendix I) and one for the matched controls (Appendix II) and three different versions of the questionnaire filled out by the MD: one for manifest or symptomatic AIP (Appendix III), one for latent or asymptomatic AIP (Appendix IV) and one for the matched controls (Appendix V). Symptomatic AIP cases were asked about the time of AIP diagnosis, AIP symptoms, number and duration of attacks, triggering and relieving factors related to attacks. All the participants were questioned about information on symptoms, regular medication, nutritional supplements, lifestyle factors, present and chronic diseases, any surgeries prior to the interview, smoking and alcohol habits, physical activity and emotional stress.

A clinical nutritionist gave the same instructions to all the participants beforehand, both in writing (Appendix VI), by pictures (Appendix VII) and orally, on how to fill in the seven-day diet logbook (Appendix VIII) to make sure all the participants filled out the logbook correctly. Appendix VII contains only some examples of the pictures given to the participants. She was available on telephone for follow-up questions through the week they filled out their diet logbook. The logbook was, afterwards, scanned using the Teleform program, version 6.0. Daily intakes of energy and nutrients were computed using a food database and software system developed at the Department of Nutrition, University of Oslo. Sugar intake was regarded as intake of "added sugar" and did not include naturally occurring sugars. Vitamin A intake was calculated as the sum of retinol and 1/12 beta-carotene.

2.3 Clinical examinations, blood and urine samples

2.3.1 Blood pressure and anthropometric measures

Serial systolic and diastolic blood pressure was automatically measured over a 20–30 min period, after every second minute, using a CAS 740 monitor (CAS Medical Systems Inc., Branford, CT, USA). Nurses at the hospital measured and registered the bodyweight, length and waist/hip-ratio for each participant, according to the Norwegian written summary of the WHO recommendations (Appendix IX).

2.3.2 Energy intake, resting metabolic rate and physical activity level factor

The total energy intake was calculated as (g carbohydrate x 17 kJ/g) + (g protein x 17 kJ/g) + (g fat x 27 kJ/g) + (g fibre x 8 kJ/g). Resting metabolic rate (RMR) was calculated by employing Mifflin's formula; for women: RMR (kcal) = 10 x weight (kg) + 6.25 x height (cm) - 5 x age (years) – 161; for men: RMR (kcal) = 10 x weight (kg) + 6.25 x height (cm) - 5 x age (years). Energy requirement was calculated by multiplying RMR with a physical activity level factor (PAL) (226). Energy requirement was converted from kcal to kJ by multiplying with 4.184. The physical activity level (PAL) factors for the participants were set by individual evaluation of the description of their work and leisure physical activity.

2.3.3 Analysis of aminolevulinic acid, porphobilinogen and porphyrins in urine

The participants delivered fresh urine samples collected from the morning urine void, in light protected containers; light protection was used to avoid a decrease in urinary porphyrin concentrations (264). We used the cut-off for PBG/creatinine < 1.5 μmol/mmol creatinine (44, 264-266). PBG and ALA were analysed using a kit from BioRad Laboratories (Munich, Germany) and Ehrlich's reagent, a column test, (Paper I and II); such tests are widely used for analysis of ALA and PBG (267, 268). The PBG ratio (μmol porphobilinogen/mmol creatinine) was calculated. The analytic method chosen was column chromatography, which is read spectrophotometrically. The urine is separated using mini chromatographic columns. Carbamide is removed, and ALA and PBG are separated. Then, ALA is eluated and spectrophotometrically measured, and the same was followed for PBG. The addition of Ehrlich's reagent gives a red colour, and the amount of coloured product and ALA and PBG standards are detected spectrophotometrically (14). According to the EPNET, the preferred method for measurement of PBG is quantification of the red product formed because of its reaction with Ehrlich's reagent after removal of urobilinogen and other interfering substances by anion exchange chromatography. Urine total porphyrins were analysed spectrophotometrically after extraction on a BioRad column containing anion exchange resin (269).The determination of fractionated urine porphyrins by HPLC was performed using HPLC columns and reagents obtained from Recipé GmbH (München, Germany), as previously described (270). All the participants with AIP had their genetic analyses for AIP performed prior to inclusion in the study using routine laboratory methods.

All the blood samples were obtained using venipuncture between 8 and 9 a.m. after an overnight fast. Vacuette citrate, EDTA and serum tubes were used for collecting the samples.

2.3.4 Complement and coagulation analyses

Total complement activation measured as the soluble terminal complement complex (TCC) was analysed by enzyme-linked immunosorbent assay (ELISA) (271). C3bc was analysed as previously described with ELISA (271). BN Prospec® nephelometer was used to measure the complement components, C3 and C4. The Enzygnost F1 + 2 (monoclonal) kit (Dade Behring, Marburg GmbH, Germany) was used to analyse prothrombin fragment 1 + 2 (PTF1.2) in EDTA plasma. The optical density was measured on an MRX microplate reader (Dynex Technologies, Denkendorf, Germany). Long-pentraxin 3 (PTX3) was analysed with enzyme-linked immunosorbent assay (ELISA) kit from R&D Systems Inc. (Minneapolis, MN, USA). C3bc and sC5-b9 (TCC) were analysed using ELISA (271). These are both assays based on monoclonal antibodies to neoepitopes exposed in the activation products and is not present in the native components. Further, our measurements of anti-transglutaminase, anti-endomysium, anti-nuclear antibody screen, rheumatoid factor and anti-CCP was performed using ELISA kits (262). Anti-cardiolipin was analysed using indirect EIA.

2.3.5 Analysis of cytokines using multiplex technology

The cytokines were analysed in EDTA plasma using a Bio-Plex Human Cytokine 27-plex kit from BioRad Laboratories Inc. (Hercules, CA, USA). The EDTA blood was immediately cooled on crushed ice, immediately centrifuged and stored at -80 °C (Paper I and, II) as recommended (272). The multiplex technology consists of bead-based analyses, measuring all the 27 cytokines (antigens) at the same time. The beads are distinguished from each other by colour. An antibody that emits phycoerythrin targets the antigen of interest and is conjugated to the surface of the beads. Flow cytometric technology is used to determine the type of bead and the fluorescence intensity. For testing of cytokine stability, the impact of storage, temperature and how different anti-coagulants could affect the cytokine results in venous blood samples were examined (Paper III). We, in addition, measured cytokines also from the following: (1) lepirudin-containing tubes, to which EDTA (10 mM final concentration) was added immediately before centrifugation to prevent further complement activation, (2) citrate tubes (3.2% sodium citrate) and (3) EDTA tubes (4.43 mM final concentration) and (4) heparin tubes. Further, for testing of cytokine stability, lepirudin-anticoagulated whole blood samples were incubated with *E. coli* (1 x 10^7/mL) in Nunc™ cryotubes. The Nunc tubes were incubated for two hours at 37 °C before centrifugation (Paper III). Control samples were obtained by immediate centrifugation (T0). To examine the stability of cytokines in the plasma samples, the samples were stored in Nunc tubes in the dark at either room temperature (RT) or at +4 °C for two, four and 24 hours. The *E. coli* strain LE392 (ATCC 33572) was obtained

from the American Type Culture Collection (Manassas, VA, U.S.A.). *E. coli* was grown overnight on a lactose dish, after which 5–10 colonies were transferred to LB-medium [1% tryptone, 0.5% (w/v) yeast extract, 1% (v/v) NaCl] purchased from Becton Dickinson (Sparks, MD) and grown overnight. The bacteria were harvested and washed once with Dulbecco's PBS without Ca^{2+} and Mg^{2+} by centrifugation at 3220xg for 10 min at +4 °C. The bacteria were aliquoted, heat inactivated for one hour at 60 °C and stored at -80 °C. A frozen ampoule was thawed at ambient temperature, and the cells were washed six times with PBS by centrifugation at 3220xg for 10 min at +4 °C.

2.3.6 Diabetogenic hormones analysed by multiplex technology, and other immunoassays

Plasma (P)-insulin, visfatin, resistin, ghrelin and leptin and C-peptide in EDTA plasma were measured on a Bio-Plex 200 system from BioRad using a pro human diabetes 10-plex kit from Bio-Rad Laboratories Inc. (Hercules, CA, USA). The EDTA tubes for analysing diabetogenic hormones in plasma were immediately placed on crushed ice, centrifuged at 1500x*g* for 15 min at +4 °C, and the plasma was stored at -80 °C until analysis. Multiplex also has a sandwich format: capture antibody, sample addition and detection antibody, and then a chemiluminescent/fluorescent reporter system. Chemiluminescence means the emission of light as the result of a chemical reaction that could be mediated by, for instance, luminol or acridinium esters. For the specific Bio-Plex multiplex immunoassays used in our study for cytokines, chemokines and growth factors, and for T2D-related markers and obesity markers, biotin labelled detection antibodies are specific for the epitopes on each target, and a fluorescent labelled streptavidin reporter binds to the biotin-labelled detection antibodies. There is a dual detection flow cytometer that in one channel is used to sort the different assays by bead colour; a bead is a fluorescently dyed microsphere with a distinct colour code or spectral address. In another channel, the analyte concentration is determined by measuring the reporter dye fluorescence (bead region). Phycoerythrin is the fluorescent reporter. The principle is similar to sandwich ELISA. In general, immunoassays have a sandwich format: capture antibody, sample addition and detection antibody, addition of colorimetric enzymatic substrates. Immunoassays compare the immunogenicity of the analyte that you want to measure, with a particular defined standard. Enzyme immunoassay (EIA) and enzyme-linked immunosorbent assay (273, 274), singleplex, are methods using the immunoassay principle with an enzyme attached to the antigen or antibody, as per the reporter label (275).

2.3.7 Biochemical analyses

Serum ferritin, folate and vitamin B12 and anti-thyroid peroxidase antibodies (anti-TPO) were analysed by immunoassays on an ADVIA Centaur® XP system (Siemens Healthineers). Haematology tests were performed using a Siemens ADVIA 2120i Hematology Systems (Siemens Healthineers). The ADVIA 2120i system has flow cytometry based analysis using light scatter, differential white blood cell (WBC) lysis and myeloperoxidase and oxazine 750 staining to provide a complete blood count, a WBC differential and reticulocyte count (276). Electrolytes such as (S)-Na^+, K^+, Mg^{2+}, immunoglobulins and albumin, liver parameters and kidney parameters such as S-creatinine and urine creatinine, S-iron and other routine clinical chemistry parameters were analysed on an ADVIA®1800 system (Siemens Healthineers, Siemens Medical Solutions Diagnostics, Japan) using reagents from Siemens Healthcare Diagnostics Ltd.

B-HbA1c (% or mmol/L) was analysed on a Tosoh G8 high-performance liquid chromatography (Tosoh Bioscience, Inc., San Fran, CA, US). For total 25-OH-vitamin-D (25-hydroxy vitamin-D), a Thermo HPLC system with a liquid chromatography-mass spectrometry (LC-MS/MS) detector was used, which can detect analytes that are able to ionize based on the relationship between mass and charge. Total vitamin D kits from Chromsystems and an Ultimate 3000 HPLC system (Thermo Scientific, Waltham, MA, USA) equipped with a TSQ Quantum Access MAX triple quadrupole mass spectrometry detector were used. Chromatography is based on two phases that cannot merge, one mobile and one stationary. A sample is injected in the mobile phase and then transported through a column along the stationary phase. The different analytes distribute between or interact with the two phases to a different degree, thereby getting separated. Liquid chromatography has liquid during the mobile phase. In liquid chromatography (LC), the difference in the size of the particles is used in the separation column. At the end of the separation column, there is a detector that gives an electrical signal when different analytes eluate from the column. This gives a concentration profile, a chromatogram along a time frame. High performance LC (HPLC) has particles of 3.5 to 10 μm used in the separation column.

Catecholamines such as epinephrine and norepinephrine and vanillylmandelic acid (VMA) in random morning urine samples were analysed by HPLC using kits from Chromsystems Instruments & Chemicals GmbH (Munich, Germany). The analytical principle is that an electrochemical detector is used that can detect components that are able to oxidize or reduce.

A BN ProSpec® nephelometer (Siemens Healthcare Diagnostics Ltd.) was used to measure S-prealbumin, alpha-1 antitrypsin, complement components C3 and C4 and S-CDT% (percentage of carbohydrate-deficient transferrin). The nephelometry principle is measurement of the scattered

light intensity from a fixed angle (for BN ProSpec of 13–24 degrees) for quantitative measurement of the analytes. Depending on how muddy/turbid the fluid (serum) is, the light will spread in different ways, and this is measured. The "muddiness" depends on the size of the particles, the shape of the particles and the number of them. A nephelometer measures the "muddiness" of the fluid sample, where the light is spread and a photometer measures its intensity. If you measure the intensity of light that is let directly through the sample, the instrument is then called a turbidimeter.

2.4 Statistical analyses

A few normally distributed data were presented as mean and standard deviation. Nonparametric data were presented as median and interquartile range (IQR). Categorical data were presented in terms of percentage. Statistical significance was considered at $p < 0.05$. Most of the statistical analysis was performed using Prism version 6.0 from GraphPad Software Inc. (CA, USA). The Spearman's rank correlation coefficient was calculated, giving r and two-tailed p. for the AIP cases since the data were not distributed normally. The Mann-Whitney U-test was used on non-matched data and nonparametric data, that is, on symptomatic and asymptomatic cases. The Fischer's exact test was used on categorical variables. Multiple linear regression analyses were performed on data from the AIP cases using IBM SPSS Statistics for Macintosh version 23 (Armonk, NY, USA).
In the hierarchical cluster analysis, the data were imported into R version 3.1.3, Bug in Your Hair. The matrix results were then transformed using natural logarithm. The Bioconductor (version 3.3) library complex heatmap (version1.10.2) was used to cluster and distance the cytoplex matrix for both row and columns with the following parameters: cluster = complete linkage, distance = Euclidian (277).

2.5 Ethical aspects of paper I–III

Paper I–II
The Regional Ethics Committee of South East approved the study before inclusion of the study participants (2011/2197/REK sør-øst D). The participants signed a written, informed consent. The study followed the World Medical Association Declaration of Helsinki ethical principles on medical research involving human subjects. All procedures in this trial were in accordance with these ethical standards. The bio-bank was created and treated according to the chapter 6 in the Health Research Act of 2008 (278). The samples were de-identified. This study is registered with the ClinicalTrials.gov number NCT01617642. A possible benefit for the individual research participant was the possibility of detecting pathological blood tests, which would not otherwise

have been detected. The benefit of this could be debatable, as unnecessary findings and time-consuming follow-up by a general practitioner could be a result (GP). The fact that there are relatively many people with AIP in our hospitals area gives us a special responsibility to advance the knowledge about AIP. Enhanced knowledge about AIP is of major importance, both for the patients and the health service.

Paper III

The blood donors provided written informed consent. The regional ethics committee of the Northern Health Region of Norway approved the study (PREK Nord 32/2004), and the biobank was approved by the Department of Health in Norway. The study by Barene et al. was approved by The Regional Ethics Committee of the Northern Health Region of Norway (PREK Nord 2010/2385-8).

3 Summary of results

3.1 Paper I

In this study of 50 AIP cases – 35 symptomatic and 15 asymptomatic – and 50 matched controls, the levels of 27 cytokines, chemokines and growth factors were significantly increased in the cases with AIP as compared with the matched controls. The complement activation markers, including plasma C3bc and C3bc/C3 ratio, were also increased in the AIP cases as compared with the controls. ALT, IgG level and blood monocyte count were significantly higher. The levels of 27 cytokines, chemokines and growth factors and the C3bc/C3 ratio were also significantly enhanced in the symptomatic AIP cases versus their matched controls. The levels of 23 of the 27 cytokines were significantly enhanced in the asymptomatic AIP cases as compared with matched controls. Hierarchical cluster analyses of cytokines of AIP cases and matched controls divided them into two main clusters: (a) and (b). Cluster (b) had high levels of many cytokines and included 30 AIP cases and five controls. The five controls in cluster (b) had inflammatory diseases. The 30 AIP cases in cluster (b) had significantly higher cytokine and visfatin levels than the 20 AIP cases in cluster (a).

PBG levels in the 50 AIP cases correlated positively with the inflammatory biomarkers, PTX3, IL-9, IL-7, S-Cystatin C, CCL4 and with the biomarkers of complement activation, C3bc/C3 ratio and TCC levels. PBG correlated negatively with prealbumin levels. PTX3 was independently associated with PBG in the AIP cases after adjusting for potential confounders in a multiple linear regression analysis. Kidney function expressed as relative estimated glomerular filtration rate

(eGFR) Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) based on cystatin C levels was a significant predictor of the biochemical disease marker PBG in the multiple linear regression analysis. Serum prealbumin levels and kidney function (eGFR) were both significantly lower in the symptomatic than in the asymptomatic AIP cases. C-peptide and insulin levels after an overnight fast were significantly lower in the symptomatic AIP cases than in the matched controls, while plasma visfatin levels were significantly increased in the symptomatic AIP cases as compared with the matched controls. The results indicated that AIP cases had significantly elevated plasma cytokine levels as compared with the controls, and that the PBG levels were associated with the PTX3 levels and eGFR based on Cystatin C levels.

3.2 Paper II

The dietary intake of carbohydrates was lower than recommended in NNR12 in 91% of the controls and 78% of the AIP cases, while the intake of added sugar was higher than recommended in 37% of the cases and in 26% of the controls (226). The intake of carbohydrates and added sugar were different neither between the case and control groups nor between AIP cases with high and low PBG levels, and the intake of sugar/candies and carbohydrates was not significantly different between the symptomatic and asymptomatic AIP cases. The intake of sugar/candies in g/day was significantly higher in the AIP cases with low ALA levels, and ALA correlated significantly and negatively with the sugar/candy intake. The intake of fibre was lower than recommended in both the cases and the controls. The intake of total fat and SFA were similar in both the groups but higher than recommended. In comparison, the serum total cholesterol, LDL cholesterol and triglyceride levels were significantly higher in the AIP cases than in the controls. The intake of polyunsaturated fatty acids (PUFA) was significantly lower in the cases than in the controls. The intake of vitamin D and folate was below the recommended quantity in more than half of both the cases and the controls. The PBG levels correlated significantly and positively with total cholesterol levels and significantly and negatively with total energy intake and PUFA intake.

The creatinine and homocysteine levels were significantly higher in the AIP cases with high PBG levels than in those with low PBG levels. The homocysteine levels were significantly higher in the symptomatic AIP cases as compared with their matched controls. Kidney function, measured as eGFR CKD-EPI with the creatinine equation, was significantly lower in the symptomatic than in the asymptomatic AIP cases. The PBG levels correlated significantly and positively with P-homocysteine, S-creatinine and PTX3. Stress was the most prevalent trigger factor for AIP attacks. ALA correlated significantly and positively with serum cortisol levels and with smoking, depending

on the number of cigarettes smoked/day. Both urine PBG and ALA correlated with P-PTF1.2 levels. P-leptin, P-ghrelin and P-resistin correlated positively and significantly with PTF1.2 levels. The median plasma insulin level was significantly higher, and the glucose/insulin-ratio was significantly lower in the AIP cases with low PBG levels than those with high PBG levels. The P-resistin level was significantly higher in the symptomatic than in the asymptomatic AIP cases. ALA and PBG correlated significantly and negatively with S-prealbumin. The results indicated that diet and energy intake affect the AIP disease activity. Smoking and the levels of some hormones were associated with enhanced AIP disease activity.

3.3 Paper III

In this paper, we investigated the effect of storage time, temperature and different anticoagulants, including EDTA, heparin, citrate and lepirudin, on the level of multiplex assayed cytokines in human venous blood samples. Most serum samples showed significantly higher cytokine levels than plasma samples consistent with *in vitro* release during coagulation. CCL2 and CCL4 were significantly increased in heparin plasma.

Storing plasma at RT for one hour increased most cytokines as compared with the baseline T0 sample. Stable cytokine levels were observed in whole blood anticoagulated with EDTA, citrate and lepirudin when the samples were stored at 4 °C for one hour, but when stored at RT, a significant increase in some of the cytokine levels was observed after one hour, and even more so after four hours. There was no significant change in the cytokine levels in plasma samples frozen and thawed up to three times.

We defined the reference ranges for the cytokines in EDTA plasma obtained from 162 healthy blood donors. Both the 2.5–97.5th percentile and the < 95th percentile levels of the cytokines were calculated. In conclusion, cytokines should be analysed in EDTA (or citrated blood) samples, immediately cooled and rapidly centrifuged (within four hours) to obtain EDTA (or citrated plasma) to be stored at -80 °C.

4 Discussion

4.1 General discussion

The studies in this thesis examined the presence of and possible interactions between inflammation, lifestyle factors including diet, biomarkers and AIP disease activity in acute intermittent porphyria. In addition, the sampling conditions for analysing plasma cytokines in venous blood samples were examined. The most common symptoms and signs during AIP attacks were abdominal pain (91%), dark urine (80%), tiredness (71%), muscle ache and muscle weakness (both 60%) (Paper II), and this was in line with other reports on AIP attacks (33, 279). We found, as hypothesized, elevated ALA and PBG in the AIP cases and discussed if these could be associated with tissue damage and the measured elevated cytokines and inflammation, and if glucose metabolism is disturbed.

4.1.1 Cytokines, complement, kidney damage and possible DAMPs in AIP

AIP was associated with a low-grade systemic inflammation, and some inflammatory markers and markers of complement activation correlated positively with AIP biochemical disease activity (Paper I). PTX3 and relative eGFR based on Cystatin C levels were associated independently with disease activity measured as PBG in multiple linear regression analysis (Paper I). The cytokine levels were elevated in the AIP cases, and biomarkers of complement activation were slightly activated in these AIP cases. This could possibly contribute to the understanding of the pathophysiology of AIP and its complications and symptoms. We speculate that a part of the cytokine elevation seen in AIP cases is a result of the neurotoxic porphyrin precursors ALA and PBG inducing organ damage in the liver and kidney or other tissues. The inflammation could possibly be due to DAMPs (159, 170). The toxicity of ALA causing symptoms such as pain (65) and possible organ damage in AIP (210) is supported by many but not all studies (71), while the possibility of organ damage/complications in AIP is described widely (10, 62, 165, 210, 211). Further, prealbumin and kidney function were decreased in the symptomatic AIP cases but not in the asymptomatic cases, suggesting inflammation in the liver and organ damage in the kidneys (Paper I). C-peptide and insulin were lower in the symptomatic cases as compared with the matched controls (Paper I), and insulin was lower in those with high PBG levels (Paper II). From these results, we suggest that AIP is associated with a systemic low-grade inflammation, possibly due to organ damage by the porphyrin precursors or other molecules, and most likely the lower prealbumin in the symptomatic cases is not a sign of under-nutrition since these persons were not malnourished

or starved (Paper II). Although systemic low-degree inflammation has not previously been found in AIP, our results are partly in line with a suggestion that decreased prealbumin levels in AIP cases could be due to under-nutrition or hepatic inflammation due to sustained accumulation of porphyrin precursors (249). Another study reported that chronic inflammation in the liver of some AIP patients may be due to iron overload after repeated haem infusions (94). However, none of the AIP cases in our study had received repeated haem injections in the last six months before blood sampling.

Relevant DAMP candidates in symptomatic AIP cases could be ALA, PBG, porphyrins, also uric acid crystals due to kidney failure, although the difference in the number of persons with gout in AIP cases as compared to the controls was not statistically significant (Paper I). DAMPs can stimulate immune competent cells directly by receptor binding (139, 159, 170) or by damaging the organs, thereby activating complement and activation of immune cells (175, 191). Our finding could fit with the speculation that ALA is the primary causal factor of the AIP, and that ALA induces inflammation, oxidative stress, vascular damage and cell death in multiple cell types (65, 280). However, others have reported that ALA has anti-inflammatory effects (178). The cause of elevated cytokine levels in AIP thus remains to be investigated.

Although the AIP cases had higher cytokine levels than their controls, there was no increase in other inflammatory diseases in AIP, such as rheumatoid arthritis (Paper I). There was neither any significant difference in number of persons with inflammatory diseases between symptomatic vs. asymptomatic AIP cases (not shown). We chose to compare the cytokine levels in AIP cases with the 50 controls since these were matched for age, gender and place of residence. If we instead had compared the cytokine levels in the AIP cases with the cytokine values of the 162 healthy controls (Paper III), we would have seen that the median of eight – IL-1β, IL-4, IL-5, IL-7, IL-12, CCL4, CXCL8 and VEGF – of the 27 cytokines in AIP cases were above the 95 percentile of the cytokine levels in the 162 healthy controls, perhaps pointing toward the extra importance of these in AIP (comparing Paper I and Paper III). When comparing the cytokine levels of the 162 healthy controls (Paper III) with the 50 AIP cases using the Mann Whitney *U*-test, we found that 25 of the 27 cytokines were significantly higher in the AIP cases ($p < 0.0001$). The exceptions were CCL5 and PDGF-BB, which did not reach significance.

In the symptomatic AIP cases, eGFR was reduced, possibly due to kidney damage (Paper I, II). The median eGFR was similar between the AIP cases and controls, as AIP is only one of the many causes of kidney failure in the general population. The prevalence of CKD in the general population in Norway is steady at around 11% despite increasing obesity and diabetes, perhaps

linked to the improved treatment of hypertension and hypercholesterolemia and higher physical activity (281). Persons with AIP, in addition to having per se increased risk of CKD due to AIP, may also have increased risk associated with the hypertension that often accompanies AIP attacks (10). The diastolic blood pressure was higher in the symptomatic than in the asymptomatic cases (Paper II). The increased PTH levels (Paper II) could be due to reduced kidney function or insufficient 1,25-dihydroxy vitamin D levels; however, the latter was not analysed in this study. We speculate that the PTH elevation observed in AIP patients with CKD in this study is most likely due to the CKD because the number of persons with vitamin D deficiency were not different between the AIP cases and controls (Paper II). Low vitamin D levels may also be associated with enhanced inflammation (246). We speculate that the end-organ effects of AIP, such as CKD and liver damage, may be a better indicator of AIP disease activity over time as compared with single measurements of urine ALA and PBG, since the levels of porphyrin precursors tend to vary around the same levels in each patient (9).

Further, our results suggest that in a cluster of AIP cases, the inflammatory cytokine response is dominated by a Th17 response. Other inflammatory diseases such as rheumatoid arthritis (RA) also have a Th17 response, and a cluster of AIP cases with high cytokine levels were clustered together with controls with RA and ankylosing spondylitis (Paper I). Further, IL-1β, IL-6 and TNF that were elevated in the AIP cases, are known to be involved in the development of Th17 cells (167).The link between organ damage and inflammation in AIP has not been thoroughly elucidated, although a possible association of inflammation in the liver in AIP cases was reported after repeated use of haem arginate treatment (94). We hypothesize that other pathways are involved in the cytokine release in AIP cases, including DAMPs from the cells damaged by ALA, PBG or porphyrins. Release of DAMPs from damaged cells, including uric acid crystals in gout due to reduced kidney function, has been shown to directly or indirectly activate immune cells to release cytokines, interleukins and growth factors (159). Whether enhanced levels of ALA, PBG and porphyrins may act as DAMP themselves remains to be elucidated. Interestingly, different types of cell death affect the immune system differently; necroptosis and necrosis gives inflammation (282, 283), while apoptosis can give anti-inflammation from metabolites (284).

However, the idea of liver-derived PBG and ALA being toxic and causing symptoms is supported by the observations of patients who got liver transplant from donors with AIP, and then developed AIP symptoms (285). Interestingly, a woman with AIP who got a liver transplant from her brother with unknown asymptomatic AIP, got symptoms of AIP after a few years, showing the need for modifying factors (286). Further, it has been shown that dietary supplements with ALA

produce an inflammatory response in broiler chickens (193). The low-grade inflammation per se could also add on to the symptoms of AIP. One possible mechanism could be that inflammation and cytokines activate NF-κB, which again affects different CYP enzymes in the liver that speeds up the haem synthesis in the liver (140). Repeated hemin infusions can create iron overload, and excess iron in the liver can also generate inflammation in the liver, which induces HO and can maintain a high ALAS1 level that could be responsible for recurrent attacks (94). In comparison, in our study, only 21% of the symptomatic AIP cases had ever received Normosang, and of these only 3% got Normosang more than three times, and none received Normosang during the study period. The half-life, t ½, of elimination for haemarginate is 10.8 ± 1.6 hour, which increases to 18.1 hours after the 4th infusion (124, 287), suggesting that previous use of Normosang cannot be the whole reason for, if any, increase in the cytokines in our study. The iron levels in the AIP cases were not higher than the reference level, and we found equal serum levels of ferritin, iron and soluble transferrin receptor in the cases and controls, ruling out iron overload. However, a tendency to higher S-TIBC levels in the cases than in the controls, and a significantly higher S-iron level in the cases with high PBG compared to those with low PBG may suggest that iron status may play a role in the disease activity. S-soluble transferrin receptor, which is more reliable marker under current inflammation, and % hypochromic RBCs (long-term parameter) and reticulocyte haemoglobin (short-term parameter), which are helpful for the diagnosis of functional iron deficiency in patients with kidney failure, were not different between cases and controls. Notably, a link between inflammation and iron metabolism is hepcidin. Hepcidin – we will analyse in the future – is produced by hepatocytes and is an iron regulatory hormone that increases in persons with inflammation, especially because of IL-6 (288). The iron homeostasis is important for haem synthesis, implying that it possibly influences the biochemical AIP disease activity, too. Since the AIP cases with high PBG levels had higher S-iron, this could mean a speedy haem synthesis. In line with this, a previous study reported a higher level of leukocyte iron in persons with AIP than in the controls (244).

Non-resolving inflammation increases the risk for HCC (214). In AIP, the mechanism(s) of carcinogenesis of HCC is unknown. In general, the driver for HCC could be both an intrinsic pathway, where genetic events leads to activation of transcription factors such as NF-κB and cytokine release, and an extrinsic pathway, where PAMPs and DAMPs also lead to activation of transcription factors and cytokine release (214). Our findings of low-grade inflammation in AIP could possibly support the suggestion of inflammation as being one of the contributors for HCC in AIP (210).

4.1.2 Complement, coagulation and metabolic disease

Organ damage may activate complement, which is measured as increased levels of TCC and C5a (187). In turn, complement may activate cells to release cytokines (187, 188). There is possible organ damage in AIP, both in the kidneys (165) and liver (94, 101). In line with Cystatin C, TCC and C3bc/C3-ratio, the latter two markers for complement activation, all correlated with PBG (Paper I). On the other hand, the elevation in C3bc and ALT in AIP when compared with matched controls was slight, so there are probably other mechanisms involved, too. In general, the complement system has many roles in health and disease, one of them being participating in the clearance of damaged cells and tissues, and another being prevention and development of chronic inflammation and cancer (289). We speculate that both the roles are relevant for damaged cells and inflamed tissues in patients with AIP. Further, complement prevents spread of infection to other cells and tissues (289). Many tissues such as those in the liver, kidneys and the nervous system and other organs can be affected and damaged in AIP. In AIP, complement activation is sparsely reported in the literature, but in porphyria cutanea tarda, photodynamic action of uroporphyrin on the complement system has been seen (175).We found low-degree complement activation and correlations between PBG and plasma TCC levels in AIP (Paper I). There is communication between the complement system, the pattern recognition receptors TLR and haemostasis (290). Since the complement system is slightly activated, cytokines are elevated in AIP and ALA and PBG correlate positively with PTF 1.2 (Paper II), we speculate that there could be a slightly increased risk of thrombosis in AIP. In line with this, a possible increased risk for hepatic artery thrombosis in liver is found in recipients with AIP as compared with non-AIP liver recipients (136). In T2D, elevated cytokines were possible facilitators of hypercoagulation and abnormal clot formation (291). It is reported that diabetes likely enhanced the capacity of platelets to mediate microvascular thrombosis and inflammation during ischemia-reperfusion injury (292). Also, in a study of acute ischemic stroke in rats it was reported that hyperglycemia primed the thromboinflammataory cascade, amplifying middle cerebral artery occlusion –induced downstream microvascular thromboinflammation (293). Interestingly, a high glucose level may itself increase cytokine release through an oxidative mechanism and epigenetic changes (164, 294), which are different kind of modifications of the DNA, such as DNA methylation, caused by environmental factors (295). The epigenome is the compound layer which surrounds the DNA, that in response to different environmental signals, has the capacity to alter the gene expression within the genome, without changing the DNA sequence (295), thereby impacting the disease risk (296). However, in AIP cases

with T2D, the beneficial effect of slightly higher levels of glucose on the AIP disease activity dominates the clinical picture (229).

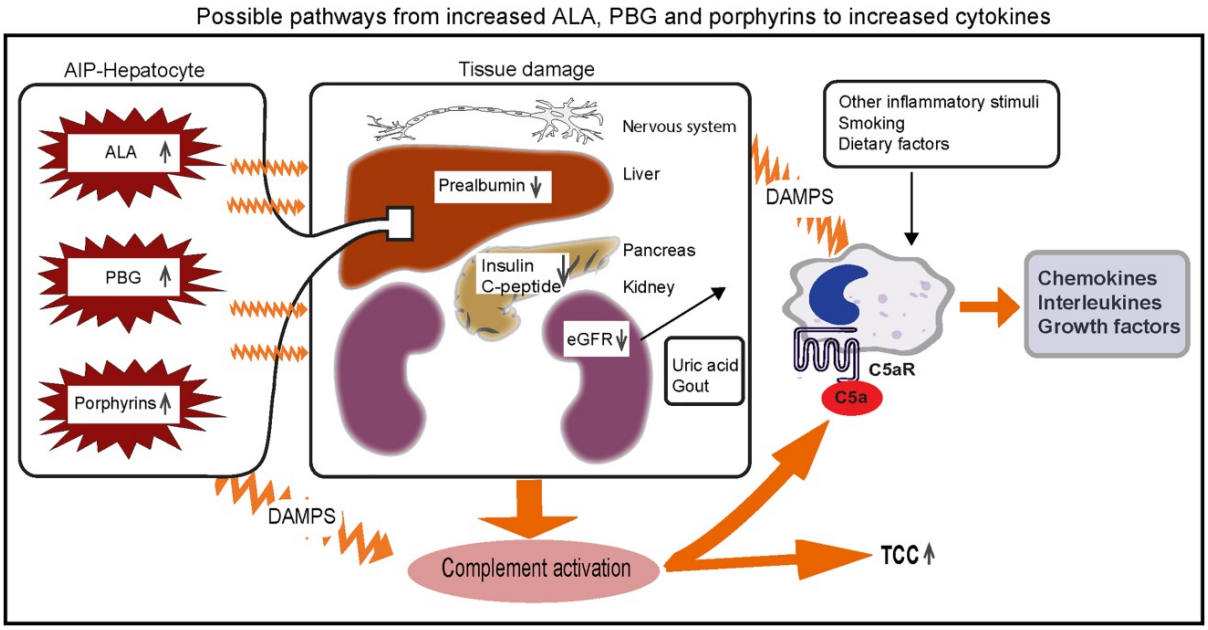

Fig. 6. Possible pathways from increased ALA, PBG, and porphyrins to increased cytokines. Abbreviations: AIP = acute intermittent porphyria, ALA = 5-aminolevulinic acid, DAMPs = damage-associated molecular pattern, eGFR = estimated glomerular filtration rate, PBG = porphobilinogen, TCC = terminal complement complex. Fig. reprinted from Clinical & Experimental Immunology, 2016, Storjord et al., Systemic inflammation in acute intermittent porphyria: a case control study,187: pages 466–479, copyright (2016) (262) with permission from John Wiley and Son.

4.1.3 Inflammation, organ damage, lifestyle and glucose metabolism

Lifestyle factors such as smoking, alcohol and dietary factors may activate cells to release cytokines (297, 298). Smoking causes cellular damage, oxidative stress, cell death, DAMP release and inflammation (299), and it may contribute partly to inflammation in AIP (Fig. 6), as smoking correlated positively with some cytokines (results not shown). The number of cigarettes per day and the ALA level were significantly correlated (Paper II). This finding is in line with earlier reports where smoking was reported to be a triggering factor of AIP attacks (33, 300), although AIP cases did not smoke more than the controls (Paper II). A recent study concluded that self-efficacy among Norwegian HMBS mutation carriers is high, and that they try to avoid possible triggers and implement recommended eating habits after diagnosis and information (301). Total cholesterol and triglyceride were higher in AIP cases than in controls (Paper II). This is in line with the study by Stein and Tschudy, who found elevated cholesterol levels in many of the symptomatic and asymptomatic AIP cases (101). In comparison, in a study of asymptomatic acute porphyria, only

HDL was found to be elevated in AIP (302). Further, the insulin and C-peptide levels were lower in the symptomatic cases than in the matched controls (Paper I). We speculate that the pancreas could be affected by DAMPs in AIP, and that symptomatic AIP is associated with disturbed glucose metabolism and possibly reduced uptake of glucose into cells due to decreased insulin levels. This is most likely in line with the study by Stein and Tschudy, who found elevated glucose levels two hours after oral glucose tolerance test in many symptomatic AIP cases (101). In a study of a mother with compensated AIP compared to a daughter with decompensated AIP and high ALA and PBG, it was found hyperinsulinemia during a glucose tolerance test only in the daughter, from which Sixel-Dietrich et al. suggested a cellular insulin resistance and altered hormonal homeostasis (303). Anyhow, T2D is also a metabolic disease with low-grade inflammation and decreased insulin secretion (146, 163, 295). It is alleged that components of nutrition, such as glucose and fatty acids, can act as DAMPs, and by-products of metabolites such as cholesterol, ceramide and uric acid are DAMPs (204). Total cholesterol correlated positively with PBG (Paper II). DAMPs generate chronic inflammation that can be involved in development and progression of IR, T2D and kidney failure (146). Also, chronic consumption of a Western diet, typically described as high in terms of calories and rich in sugars, trans and saturated fats, salt and food additives, but low in complex carbohydrates, fibres, vitamins and minerals, along with a sedentary behaviour can cause metaflammation (157). The intake of saturated fatty acids was excessive in both cases and controls (Paper II), and the intake of SFA can, therefore, not explain the difference in cholesterol levels and cytokine levels between the cases and controls. The energy requirement calculated from RMR and PAL was also the same in the cases and controls (Paper II). We found reduced fasting insulin levels in symptomatic cases and slightly enhanced HbA1c and triglyceride levels in AIP cases, which could suggest that DAMPs such as glucose and lipids may play a role in AIP.

4.1.4 Disturbed glucose metabolism

There are a variety of signals and receptors that are involved in different physiological aspects of glucose metabolism, regulation of glucose and insulin level, such as glucose transporters, glucose receptors, insulin receptors, a host of diabetogenic hormones, diet and lifestyle (304-307).

4.1.5 Insulin, C–peptide and factors affecting level of glucose

The participants had fasted overnight (Paper I, II). In the fasting state, the insulin and C-peptide levels are naturally lower than after a meal (234). Insulin regulates gluconeogenesis, and the control of gluconeogenesis depends on the availability of substrates, allostery from metabolites and transcriptional mechanisms such as PGC-1α and cellular redox state (308). It is alleged that already

after 3 hours of fasting you get a fasting blood glucose level (309). During fasting, the glucagon-effects prevent hypoglycaemia by stimulating glycogenolysis and gluconeogenesis and by initiating hepatic glucose release (310). Gluconeogenesis contributes approximately half of the total hepatic glucose production after an overnight fast (308). Most of gluconeogenesis occurs in the liver (310). The kidneys can also make new glucose (311). The liver, which is the culprit organ in AIP and the "gluco-stat" of the body, is exposed to insulin concentrations two- to three-fold higher than in the general circulation (232). The symptomatic AIP cases evidence reduced fasting insulin release when compared with the matched controls (Paper I); which may indicate that decreased glucose uptake into cells may explain the accelerated haem synthesis in at least the symptomatic AIP cases. In addition to this, C-peptide was also lower in the symptomatic AIP cases as compared with their matched controls (Paper I). This could suggest a reduced beta cell function in the symptomatic cases, and that reduced insulin release is associated with enhanced disease activity. On the other hand, we found no significant difference between plasma insulin and plasma C-peptide levels between symptomatic and asymptomatic AIP cases (Paper I), although C-peptide has a large intra-individual biological variation. The level of plasma insulin was lower and glucose/insulin ratio was higher in those with high PBG levels as compared with those with low PBG levels (Paper II), suggesting that hormones regulating glucose uptake may be disturbed in the AIP cases with increased PBG excretion, and that pancreas function might be affected. We speculate that the reduced insulin and C-peptide secretion from the pancreas in the symptomatic cases (Paper I, II) may be due to organ damage, reduced hormone secretion or disturbed regulation. It is suggested that fasting, which reduces the insulin levels, gives higher PGC-1 α, which gives higher ALAS1, thereby resulting in higher PBG (224, 303, 312). PGC-1α-induced ALAS1 transcript levels are reduced by insulin (224). Further, hepatic PGC-1α is a major determinant of the severity of acute porphyric attacks in a mouse model (224). An increase in fatty-acid β-oxidation and elevation of hepatic gluconeogenesis are both controlled by PGC-1α, and PGC-1α has a key role in liver energy homeostasis and targets haem proteins (224). Interestingly, Andersson et al. suggested that T2D could reduce symptoms in AIP, showing an example of reduced PBG and ALA in an AIP patient who had T2D, and reporting about 16 AIP patients who had T2D and no longer had AIP symptoms (229). Although there is no known increase of T2D in AIP, we speculate that the diabetogenic hormones are disturbed in symptomatic AIP cases. This shows that the lower insulin levels in those with an increased AIP disease activity (Paper I, II) is in line with the findings that low insulin level affects PGC-1α (308), thereby affecting ALAS1 (224). The combination of glucose and insulin is more potent in inhibiting fasting-mediated induction of PGC-1α and ALAS1 (224). Glucose loading

reduces ALAS1 transcript levels 30 min after injection (224). Our findings (Paper I, II) may support the addition of insulin to the glucose given to AIP patients during a porphyric attack. This is also in line with the findings in diabetic mice with decreased insulin levels that got an insulin-mimetic, and then got reversed ALAS1 induction and reduction of PGC-1α (313). Further, altered glucose homeostasis and hepatic carbohydrate metabolism has been observed during fasting in HMBS-deficient mice (314). After 14 hours of fasting, HMBS-deficient mice have shown activated gluconeogenesis and ketogenesis due to inability to use stored glycogen (314). However, in studies on fasting HMBS-deficient mice (314) and in a rat model of acute porphyria, elevated levels of insulin were seen (315). The insulin sensitivity can also be associated with lipids and related metabolites (233). We speculate that ALA can induce liver and kidney damage and, perhaps, even pancreas damage, secondary elevated cytokines and perhaps a disturbance in diabetogenic hormones, both contributing to disease activity and complications. The use of different medications (Paper I and II) adds to the complexity of interpreting the insulin result.

Further, we speculate if potentially different diet and eating patterns over time could have impacted the levels of glucose, insulin and C-peptide, as long-term metabolic effects of the diet could lead to metabolic changes. A long-term Western diet and sedentary lifestyle can create metaflammation that is "memorized" by innate immune cells through long-lasting metabolic and epigenetic cellular reprogramming (157), and metaflammation is known to be associated with insulin resistance (152). The sensitivity of glucose – how sensitive the body's cells are in response to insulin – can be affected by, for instance, the physical activity level in the last 72 hours (316). According to Homedan et al. (Fig. 3), giving glucose is a way of helping the TCA, through pyruvate and then acetyl CoA, which gives extra substrate to the depleted TCA cycle (75). An affected respiratory chain perhaps fits with being tired or fatigued as some of the symptomatic cases were (Paper II). Chronic inflammation in the liver causes insulin resistance, leading to increased hepatic glucose production (317). Further, the gut microbiota, which we did not measure, can also impact on the liver glucose and lipid metabolism (317).

The sympathetic nervous system and the parasympathetic nervous system can also affect glucose levels, although we found similarity in terms of epinephrine and norepinephrine in cases and controls in our study (Paper II). In comparison, the breakdown product of catecholamines, urinary vanillylmandelic acid, was lower in cases than in controls, and stress was a common triggering factor of AIP attacks (Paper II). Cortisol levels, which are generally higher in the morning, affect glucose metabolism. We drew the blood samples in the morning and found no significant difference in serum cortisol levels between cases and controls (Paper II), but we did not

measure urine cortisol. Stress could have affected cortisol, thereby affecting the glucose output. Pozo et al. found lower urine cortisol levels in AIP cases (318), speculating that this could be a physiological compensation mechanism due to long lasting ALAS1 induction in the liver (318).

In AIP, the level of glucose particularly inside the hepatocyte is of importance for the AIP disease activity. Glucose is hydrophilic, so transport of glucose across the plasma membrane and into the cells depends on the carrier proteins expressed in the plasma membrane since the lipid bilayer of plasma membrane is impermeable for glucose (311, 319). Glucose transporter 2 (GLUT2), which is present in the liver, is insulin-independent and constitutively present at the plasma membrane; it maintains an equilibrium between intracellular and extracellular glucose (310). Thus, insulin is not needed for the glucose to enter the hepatocyte; hence, the influx of glucose into the hepatocyte in AIP may, therefore, not be directly affected by the low insulin levels found in some AIP cases. However, insulin has important functions for the metabolism in the liver and other organs. Insulin promotes glucose uptake and protein synthesis in muscles. Also, insulin promotes glucose and fatty acid uptake and inhibits lipolysis in adipose tissue. In the liver, insulin promotes glucose utilization, suppresses glucose production and promotes triglyceride synthesis (320). Levels of HbA1c, cholesterol and triglycerides were slightly higher in the AIP cases vs. control (Paper I). We speculate that the lower levels of insulin in the symptomatic cases gives a lower level of lipoprotein lipase, which again gives a higher level of triglycerides, as found in AIP cases (Paper I, II), and from that, perhaps, an increased risk of nephrosclerosis or coronary disease emerges. In a muscle cell, the binding of insulin to the insulin receptor is needed for GLUT4 to be translocated to the surface, and then for the glucose uptake to take place (232). We, therefore, speculate that the influx of glucose could be lower in some cells in symptomatic AIP cases, such as in muscles, adipose tissue and heart, since they depend on GLUT4, and this again could affect the glucose metabolism.

There is a range of possible implications of a potentially disturbed insulin level in AIP, as insulin targets many tissues, the major being muscle, adipose tissue, liver and neurons (320). Further, the kidneys also have GLUTs and SGLTs, and contributes to the glucose homeostasis, by renal gluconeogenesis that prevents hypoglycaemia (311). Interestingly, it is suggested that a brain-centred glucoregulatory system can also lower blood glucose levels via both insulin-dependent and -independent mechanisms, also having interactions with pancreatic islets (321). Further, the brain has an emerging role in the homeostatic regulation and glucose metabolism due to insulin, leptin and ghrelin acting on the hypothalamic arcuate nucleus (322). Also, insulin receptor substrate proteins are involved in metabolic homeostasis and are important transducers of the insulin signal in

the liver (320). Immune cell activation in the liver, muscle, pancreas and brain can impair the local insulin signalling and lead to metabolic dysfunction (323). It is suggested that fibroblast growth factor (FGF)-21 participates in regulation of glucose metabolism in liver (324), from which we could speculate that the low grade inflammation in AIP could cause metabolic dysfunction, or that the lower insulin in the symptomatic cases is a form of complex adaption to the circumstances that has not yet been understood. In neurons, insulin promotes anorexigenic/appetite-suppressant and locomotor signals (320). The insulin receptor signalling is of great importance in T2D, obesity and insulin resistance, and perhaps in AIP, too. In addition to the direct effects of insulin on signalling kinases and metabolic enzymes, it also affects insulin-regulated gene transcription (320). Hence, we speculate that the slightly lower insulin may be a sign of a slightly damaged pancreas, maybe as a consequence of increased ALA, PBG or inflammation in AIP, or that the low level of insulin is due to perturbed signalling of the other hormones affecting insulin levels, which again can be associated with inflammation related to AIP (Table 2).

Table 2. Diabetogenic hormones at normal weight, overweight, type 2 diabetes and in AIP

Hormone		Normal weight/ healthy	Overweight/ IR	T2D	Symptomatic AIP or cluster with AIP
C-peptide	F	**N**	↑	↑	↓
	P	↑	↑	↑ Insufficient	**ND**
Insulin	F	**N**	↑	↓ or (↑) Insufficient	↓
	P	↑	↑	↓	**ND**
Resistin	F	**N**	↑	↑	↑
	P	↑			**ND**
Visfatin	F	**N**	↑	↑	↑
	P				**ND**

IR = insulin resistance, N = Normal, F = Fasting, P = postprandial (carbohydrate intake), ↑= increased/high, ↓= Decreased/low, ND = not determined in our study.

4.1.6 Visfatin

We surmise a role of the adipose tissue in the inflammation observed in some of the AIP cases, as there was an increased visfatin level seen in the AIP cases in cluster (b) with high cytokine levels (Paper I). Visfatin is known to act on insulin sensitivity and glucose metabolism, and it is upregulated in humans with T2D (235). In line with this, insulin correlated positively with the

levels of plasma visfatin and leptin (Paper II). It is suggested that hepatocyte-secreted dipeptidyl peptidase 4 in obesity promotes adipose inflammation and IR (325). Although the BMI, waist- and hip-circumference were not different between the AIP cases and controls (Paper I, II), both the groups had a high percentage of persons with overweight where one could speculate that the adipose tissue adds to the low-grade inflammation.

4.1.7 Resistin

Resistin was higher in symptomatic than in asymptomatic AIP cases after an overnight fast, and there was a positive correlation between resistin and leptin (Paper II). This may suggest that the effect of inflammation on AIP (Paper I), and that adipokines and hormones affecting insulin resistance or a disturbed glucose metabolism may be involved in a high AIP disease activity (101, 237). Lee et al. showed that resistin levels did not significantly change even after 48 hours of fasting (326), and Gruendel et al. showed that serum resistin levels increased postprandially (327). We speculate that the elevated fasting resistin levels found in the AIP cases could be an effect of the AIP disease itself since it was observed during periods between AIP attacks and outside acute inflammation, and since there were not any difference in number of persons that had inflammatory diseases in AIP cases compared to controls (Paper I). This is in line with the claim that resistin activates TLR4 on macrophages and increases cytokine release (238). The higher resistin levels in the symptomatic as compared with asymptomatic AIP cases may also imply that hormones regulating the glucose uptake may be disturbed in the symptomatic AIP cases (Paper I and II). The higher resistin levels in the symptomatic than in the asymptomatic cases (Paper II) and the higher cytokine levels in AIP cases than in the controls (Paper I) might indirectly impact the glucose metabolism, for instance, IL-6 is known to suppress gluconeogenesis in the liver by activating a transcription factor (317). In the symptomatic AIP cases, the higher resistin production was not followed by higher insulin release as it is in persons with IR and excess body weight (Table 2). Resistin exerts its glucoregulatory effects by stimulating hepatic glucose production (305). Furthermore, resistin has an anorexic effect, decreasing body mass and increasing lipogenic enzymes and inflammatory cytokines in the liver (305). This is partly in line with our findings regarding increased resistin in the symptomatic AIP cases, although the cytokines were increased in both asymptomatic and symptomatic AIP cases (Paper I, II). Further, it is stated that resistin can directly activate the complement system, and this is justified by the slightly elevated markers of complement in AIP cases who also have elevated resistin (Paper I, II). The slightly lower prealbumin levels in the symptomatic AIP cases as compared with the asymptomatic cases may

indicate liver inflammation in the symptomatic AIP cases, and this may be a partial cause of the elevated cytokines (Paper I). A study on Hispanic non-porphyric persons reported that higher resistin levels were associated with low insulin sensitivity and higher adiposity (328). Elevated levels of resistin have been shown to be related to increased risk for T2D, but the potential effect of resistin on risk for T2D decreases after adjustment for overweight and inflammation (329). Others have found that serum resistin levels increased in patients with T2D, but they did not find any link to markers of IR or adiposity (330). There was no significant difference in the number of persons with T2D between AIP cases and controls (Paper I). Interestingly, weakening of porphyria symptoms has been reported in AIP patients having T2D (229). The role of resistin in AIP thus needs further investigation.

4.1.8 Diet and lifestyle

We speculate that a low energy intake and a low carbohydrate intake is detrimental in AIP since we found that carbohydrate intake of the AIP cases was lower than recommended, and that a low energy intake was correlated with higher biochemical disease activity (Paper II). This fits with the beneficial effect of glucose in AIP due to the glucose and insulin's effects on PGC-1α (224); it also fits with the rise in ALA and PBG when the caloric intake was decreased and a decline in ALA and PBG when carbohydrate and energy intake was increased in a study of seven persons with AIP who received meticulous attention in a hospital metabolic unit (51). In a recent review, Wang et al. stated that extreme dieting and starvation should be avoided in AIP (6). Furthermore, persons with AIP should be offered a consultation with a dietitian "to promote and maintain a well-balanced diet somewhat high in carbohydrates (60–70% of total calorie intake)" (6). Actually, charcoaled food, cabbage and Brussel sprouts contain chemical substances that could upregulate ALAS1, but the amount needed to do so would probably be far above what is normally consumed (porphyriafoundation.org, accessed November 2019). The dietary intake of most dietary variables were not different between the cases and controls, which fits with what was found in a group of 20 persons with AIP and 20 controls in northern Sweden; however, the latter was based on a retrospective interview filling in a standardized questionnaire (244), whereas our study used a prospective diet logbook (Paper II). A higher intake of marmalade and jam in the symptomatic cases was found (244). The cases showed a mild but not significant tendency to consume more added sugar as compared with the controls (Paper II). This possibly reflects that the AIP patients followed the advice of eating carbohydrate-rich food, perhaps incorrectly interpreted by them as added sugar. Also, the campaigns against consumption of excess sugar might have lead some of

them to incorrectly assume that carbohydrates are not good for the health, while actually most persons do not reach the recommended 45–60 E% of the diet from carbohydrates (226). Both too high and too low percentages of carbohydrates have been associated with increased mortality, with a minimal risk observed at 50–55% carbohydrate intake (226). What one eats instead of the recommended carbohydrates affects mortality (331). Eating more of the healthy carbohydrates in general and restricting the increased added sugar intake to periods when there is a risk of an attack would be in line with the recommendations. However, since we found a significantly higher intake of sugar/candies in AIP cases with low urine ALA (Paper II), we speculate that this otherwise considered unhealthy dietary habit is, in some ways, beneficial in AIP not only during attacks, but increasing the healthy carbohydrates would perhaps be better. Clinical improvement by glucose, although given intravenously, was shown in a study on eight patients with attacks of acute porphyrias and three in remission (222). It has been postulated that high glucose exposure may play a role in the etiology of metabolic diseases, the background being studies on human adipose tissue stromal cells, where high levels of extracellular glucose sensitized an inflammatory response gene expression program and exposure to high glucose altered histone methylation in inflammation response genes (332). Further, our findings of elevated cytokines (Paper I) and possibly disturbed hormones (Paper II) fit with the idea of AIP being a chronic disorder in at least the symptomatic patients, meaning that a person with AIP is not a completely healthy person, even though they experience only a few days of attack a couple of times during their lifespan. An analogy would be addressing T2D as acute intermittent ketoacidosis, which would not be a good idea.

The AIP cases and controls had the common task of consuming little fibre and a lot of saturated fat (Paper II), even if the contrary is advised in general (226). We found a lower intake of PUFA in cases when compared with controls, and that the intake of PUFA correlated negatively with PBG (Paper II). This fits with the suggestion given by a case-control diet study on 16 AIP patients, which said that the intake of PUFA, complex carbohydrates, MUFA and folic acid must be increased in AIP, and the intake of protein, lipid and SFA should be decreased (333). Also, nutrients are used as substrates in metabolic pathways and can also directly regulate the cell's fate through different mechanisms, and these activities can cause the response to inflammatory insults (204). As AIP patients are prone to hypertension and the risk of chronic kidney disease, for some of them, it could be important that the dietary recommendations also include their vulnerability, which should be manageable since a diet emphasizing vegetables, fruits and low-fat dairy foods and moderate amounts of whole grains, fish, poultry and nuts are beneficial for patients with CKD or hypertension (334). In recent years, carbohydrates has been discredited by some, and the a "low-

carb, high-fat" diet has spread (335) even though there is lack of data supporting its long-term efficacy, safety and health benefits (336). The American Porphyria Foundation (porphyriafoundation.org, accessed November 2019) stated that when the Atkins Diet – high fat, high protein, low carbohydrate-diet – became popular, many persons with AIP who adhered to this diet became ill, (337); moreover, the trending ketogenic diet has porphyria as a contraindication (338). We speculate that intermittent fasting diets could also trigger AIP attacks (339).The intake of polyunsaturated fatty acids (PUFA) was significantly lower in the cases than in the controls (Paper II), which we speculate could be unfortunate since PUFAs are mainly anti-inflammatory (233). In animal studies; PUFAs have been associated with improved insulin sensitivity, albeit this effect is controversial in humans (233). Also, the PBG levels correlated negatively with the total PUFA intake. SFAs are found to be worsening the insulin sensitivity in animal studies, but in humans, the role of SFAs on IR is conflicting (233).

Low-grade inflammation in AIP and the recommended increased carbohydrate intake give rise to the question on how to eat both to reduce inflammation and the AIP disease activity. It is reported that anti-inflammatory diets, which also stabilize insulin levels, should be rich in colourful, non-starchy vegetables, as they consist of polyphenols that could inhibit the nuclear factor, NF-κB (298). This would hold true if our speculation that chronic low-degree inflammation in AIP is more of a burden in the long run than a harmless accommodation due to the enhanced levels of toxic porphyrin precursors. In the case of smoking-induced inflammation, exercise produces increased anti-oxidative defence and decreased expression of inflammatory cytokines (299). Hence, AIP patients must also be encouraged to exercise. Stress has been found to be a frequent triggering factor of AIP attacks (Paper II) (100), and it may increase proinflammatory cytokines, which, in turn, could give rise to depression and fatigue (340). Hence, we speculate that avoiding or combating stress is important. Also, PTX3 worked as a significant predictor of PBG after adjustment of confounders in the regression analysis (Paper I). Also, elevated inflammation biomarkers were found in serum of persons who dealt with multiple stressors as compared with a group without as many stressors (341). Even so, counselling AIP patients on how to avoid or handle stress is not widely practiced; but they are informed that stress is a potential triggering factor. In my opinion, this should be addressed in the future. There is no risk calculator/risk chart to use with suggested interventions for persons with AIP outside AIP attacks, such as those existing for coronary heart disease and T2D. Due to the rarity of the disease, there are no large studies to use as a background for advice to persons with AIP, such as the Framingham study used for coronary disease risk stratification; nevertheless, a multinational natural history study including 112 patients

of whom 104 have recurrent AIP attacks has been described (279). Lifestyle factors, including psychological stress, have been shown to be associated with shortening of telomere length (342). Even though previous studies on clinical and biochemical characteristics of AIP attacks have not reported cytokine measurements, we speculate that some of the physical signs and symptoms during an AIP attack, such as pain and a reduced functioning level and fatigue could perhaps also be associated with sterile inflammation. We speculate that the cytokine levels are even higher during many AIP attacks than in "remission", but this remains to be examined.

4.1.9 Micronutrients, vitamins and herbal remedies

We did not find a significant difference regarding intake of either vitamin B12 and folate or S-B12 or S-folate levels between the case and control group, although there was a higher median for both in the cases (Paper II), whereas Thunell et al. had found higher levels of vitamin B12 in persons with symptomatic acute intermittent porphyria when compared with the controls (244). Vitamin B6, also called pyridoxine, is a water-soluble vitamin (343). It is important for the normal functioning of many organs (343). It is metabolized to pyridoxal 5-phospate, an active molecule that serves as a co-enzyme for many metabolic enzymatic reactions, and it is directly involved in the first step of haem synthesis as a co-factor of ALAS1. ALA and PBG levels declined during vitamin B6 deficiency in a study of one asymptomatic AIP patient and increased following the administration of vitamin B6 (343). This impairment of ALA and PBG was not found in starved animals with severe vitamin B6 deficiency (343). We do not have the results of the effect of vitamin B6 levels from our study available yet. The level of vitamin D – which functions as a nutrient, a hormone and an immuno-modulator (344) – was insufficient in many cases and controls (Paper II). This could perhaps contribute to the inflammation, but can not explain the enhanced inflammation in the AIP cases. Use of complementary and alternative medicine is common in patients with inborn defects of metabolism (345), as some of them are not satisfied with conventional therapy (321). Use of vitamin supplements, fish oil and herbal remedies was relatively common in AIP cases (Paper II), and should be discussed between them and their doctor.

4.1.10 Link between different factors

There occurs constant communication between the complement system, the PRRs, haemostasis, haem synthesis, diabetogenic hormones and the nervous system. This creates a mesh of signals, but also gives rise to a wide range of possible treatment modalities, for instance, from preventing stress or consuming carbohydrates to giving haem arginate or using small interfering RNA. Irrespective of

their lifestyle and dietary routine, people with AIP are just as much prone to stress and infections as others. We must acknowledge the unpredictability and probability of this disease attacking people. Availability of treatment modalities and knowledge is important to help people experiencing an AIP attack. This project has tried, in addition to gaining knowledge, to spread knowledge regarding AIP to relevant persons in the local health care and to AIP patients. We found stress to be a common triggering factor, and suggest the presence of an increased inflammation and a disturbed glucose metabolism in AIP cases. Based on this, we speculate that it is beneficial in people suffering from AIP to get enough sleep, adopt a healthy diet with lots of carbohydrates of high quality (e.g. vegetables, berries, whole grains, etc.) which maintain a steady insulin level, find ways of coping with stress and the right amount and type of physical activity. Anyhow, a recent study highlights the need for individualized advice, as they observed large inter-individual variability in postprandial responses of blood triglyceride, glucose and insulin following identical meals (346). Berry et al. report that person-specific factors, like the gut microbiome, had a greater influence than did meal macronutrients for postprandial lipemia, but not for postprandial glycemia, while genetic variants had a modest impact on predictions (346). Further, the oral health should also be recognized as a possible contributor to the general health, inflammation and the "blurred nexus" regarding IR (347), with a potential to affect the haem synthesis.

Although AIP is a chronic enzyme deficiency increasing the risk of HCC and possible inflammation in the body, most persons today are not using any long-term medication for AIP. It is thought-provoking that the AIP cases have low-grade inflammations outside attacks, perhaps because of the elevated ALA and PBG levels that cause organ damage. This upholds the need for devising treatment plans for dormant AIP, such as in the case of diabetic patients. Having high ALA and PBG without obvious symptoms should also be taken seriously in AIP, such as elevated blood sugar levels in diabetes. For those who show symptoms, there are many possible interventions and precautionary measures, which give most of persons with AIP the hope to lead a normal life. One should acknowledge the suffering of patients with recurrent and chronic AIP symptoms; they need treatment now as they did a hundred years ago (see quote, Dr Einar Wallquist, page 3).

4.1.11 Sampling conditions and reference values of cytokines

Correct sampling conditions for cytokines in venous blood samples are important in good clinical and research practice. Our recommendation for cytokine analyses obtained from this study are EDTA or citrated blood immediately cooled on crushed ice, and immediately centrifuged and

plasma stored at -80°C (Paper III). EDTA blood is preferred since it can be stored for up to four hours at 4 °C before centrifugation. Cytokine levels were lowest in the EDTA tubes as compared with all the other tubes when the blood samples were centrifuged at T0 (Paper III). Further, we think that complement activation cannot explain the difference in cytokine concentration between serum and plasma because TCC was only elevated in the serum tube with gel and clot activator while the cytokine levels were increased in both the serum tubes; this shows that the cytokine level were elevated with or without the presence of clot activator (Paper III). Regarding storage of samples, we found that cytokine concentrations in anti-coagulated whole blood increased significantly after one hour at room temperature. We, therefore, suggest storing samples at +4 °C for up to four hours since it had little or no effect on cytokine levels (Paper III). The increase of cytokines at room temperature could be due to *in vitro* synthesis or release of pre-synthesized cytokines by blood leukocytes (348). Cytokine levels measured in EDTA and citrate plasma stayed stable despite several rounds of thawing and freezing; hence, we suggest that it is acceptable to thaw and freeze the sample maximally three times. In our opinion, the sampling conditions may influence the results substantially. If future studies analysing cytokines follow these advices, it would ensure better comparability and validity of the results. For optimal use of cytokine analysis in clinical practice as a biomarker of conditions such as sepsis or post-traumatic complications, further research is needed. The cytokine concentrations in serum were significantly higher than in plasma. This is in line with several previous reports (349). This may implicate that the cytokines are synthesized or released from blood cells during coagulation activation *in vitro*. We postulated that the coagulation process and thrombin activation enhance cytokine release, which also fits with previous findings (350, 351). Coagulation itself may induce cytokine release from whole blood leukocytes, which is consistent with earlier findings showing that thrombin activates leukocytes and cytokine release through activation of protease-activated receptors PAR-1 and PAR-4 (253). Use of serum for cytokine analyses should, therefore, be avoided. Another finding in paper III in addition to the recommended guidelines for cytokine analysis was the estimation of reference ranges for cytokines in humans. We consider the upper 95th percentile values to be the best estimate of the reference ranges, and they are common for women and men (Paper III). Whether the reference ranges should be separate for women and men could be argued, but for practical reasons, and since the differences were small and only existed on a minority of the 27 cytokines, we chose common reference values for both the sexes. Since the 2.5 percentile was not measurable in some of the participants, and since an increase in cytokines is more logically related to the disease than a low value, we chose the upper 95th percentile as the value of choice. In healthy individuals, the

concentration of many cytokines were either immeasurable or very low. These reference ranges for plasma cytokines were not previously described in the literature in a large number of healthy individuals. However, the sampling conditions, handling and storage of samples may vary among the studies, which may explain at least some of the different results obtained from different studies (352). To interpret the results of cytokine analyses, one must also take into account the large biological variation (160), which could cause the need for repeated measurements. In addition, the levels of cytokines can be affected by many environmental factors such as age, gender, smoking, depression, medication, the microbiome, time of year, time of day and genetic factors (353, 354).

4.1.12 Strengths

The study's strength is that it has analysed a relatively large group of AIP patients considering the fact that it is a rare disease. Also, it has measured many relevant inflammatory biomarkers on which the local research laboratories have expertise. The standardized sampling conditions and preparations improve the strength of the study; for instance, the cytokine analyses and other analyses on fasting patients were all performed at the same time in the morning by skilled technicians at the same laboratory and during the same time of the year. The set up with controls matched for age, gender and place of residence is also a great advantage since the afore-mentioned factors are no longer the reason for differences between cases and controls.

4.1.13 Limitations of the study

Doing several different group stratifications and multiple hypotheses testing on a relatively small number of participants may be a weakness. Performing many correlations between many biomarkers from the results of an analysis on a relatively low number of participants is a weakness and could lead to false-positive results. Hence, one should acknowledge the preliminary nature of some of the results and understand that there is a possibility that we may have over-interpreted some of the data. Also, in the interview-setting, there was no blinding, indicating that we knew who had AIP and who did not, and this could have perhaps led to bias in our questions and their answering. When comparing asymptomatic and symptomatic patients, the age and sex distribution was no longer matched between the groups. A weakness is the cross-sectional way of the study, only giving an instant picture of how the biomarkers and clinical measurements were at one point of time instead of over a significant period of time. However, the participants provided information through questionnaires and interviews on previous diseases and AIP attacks. Also, even if this study analysed the urine ALA and PBG levels only at one point, which could be a weakness, another

study investigating biological variation in AIP suggests that AIP patients vary around their own typical porphyrin precursor level, making this less of a problem (9). Since this study was not undertaken on AIP cases during the attacks, it is not possible for us to elucidate how the cytokine levels and other biomarkers and clinical measurements would be during an porphyric attack. Regarding our grouping of AIP cases based on high and low biochemical disease activity (ALA and PBG), and then comparing different dietary markers and other markers in these groups with those in others, it is to be noted that a high PBG or ALA level does not necessarily correlate to AIP symptoms.

The lack of international consensus on precisely what an AIP attack is proves to be a limitation regarding the correct classification of persons with AIP as asymptomatic or symptomatic. The low power can reduce the chance of detecting a true effect. Also, it reduces the likelihood that a statistically significant result would reflect a true effect. Although no official scaling system is available for AIP, there are four major subgroups of acute porphyria patients: symptomatic patients with sporadic attacks (< four attacks/year) or symptomatic cases with recurrent acute attacks (≥ four attacks/year), asymptomatic high porphyrin precursor excretors and asymptomatic latent patients without symptoms or porphyrin precursor elevations (106). We grouped the AIP cases based on whether they had a history of AIP attack (symptomatic) or not (asymptomatic). Further, we also compared the AIP cases with high and low PBG levels.

To classify a case as symptomatic, we relied on previous routine genetic and biochemical testing and previous clinical symptoms and diagnosis perceived as AIP attacks. In addition to this, many of the symptomatic AIP cases and some of the asymptomatic AIP cases also had present verified elevated PBG levels. Given the continuum between total asymptomatic and substantially symptomatic, the classification of the cases becomes troublesome. However, when a patient presents with a history of previous attack with high PBG levels, typical symptoms and positive genetic testing, there is very little doubt about its classification. Anyhow, the distinction against the matched controls was clear.

4.2 Methodological considerations: external and internal validity

External validity: sampling bias

Research on rare diseases such as AIP can often be hindered due to small sample sizes (Paper I, II). However, this limitation can be overcome by multicentre studies. Almost all, except two participants, had the same AIP mutation (Paper I, II), and all the included participants were from Norway (Paper I, II, III). This renders the external validity of the study questionable. On the other

hand, the same mutation is also prevalent in Sweden, and all the AIP mutations lead to reduced function of the same enzyme. Showing many aspects in depth on these particular AIP cases could prove to be the strength of the study, but some might regard it as a limitation since it could be argued that the possibility to transfer the results to patients with other AIP mutations is less.

Regarding inclusion, of the persons with AIP, it could be likely that those who thought it would be most useful to participate were the ones with symptoms, therefore wanting to participate. The sampling bias can thus affect the external validity by lowering the generalizability of the study. Also, persons who were very much affected by the AIP could hesitate to participate since the travel to the hospital could be too much of a stressor. Thus, 35 symptomatic and 15 asymptomatic people were included in the study. When stratifying the asymptomatic and symptomatic AIP cases based on clinical history and interview, there were possibilities for misclassifications since there is no international consensus on what an AIP attack is. The symptomatic AIP patients had AIP mutation, at least one AIP attack with typical symptoms and elevated PBG levels. An advantage of this presumably overrepresentation of symptomatic cases is that we got a thorough look at the symptoms and related diseases that can arise in symptomatic AIP persons. Regarding the matched controls, they were randomly selected based on their age, gender, and place of residence. Some persons from the first batch of controls rejected the invitation, so a second random selection was necessary to obtain 50 matched controls.

Internal validity: selection bias and recall bias

Internal validity can be affected by systematic errors such as selection bias, information bias, incorrect statistical methods and confounding factors. Of the AIP cases who got information about the study, only three did not want to participate, among whom one was too ill to join, creating a healthy user bias or non-respondent bias (355). All persons with AIP who consented to participate in the study showed up for the interview and clinical examination. Of the controls, three men did not show up for interview and clinical examination, but they were replaced by other men with matched age and postal code.

By doing a matched case control study, we avoided some of the selection bias, producing as similar groups as possible for comparison. One person was classified as having AIP on the basis of the information they provided, but genetic analyses and urine analyses ruled out the provisional diagnosis. This person and the matched control were thereafter removed from the study. The response rate for using the diet logbook was high, giving a good impression of the dietary habits in the cohort. Our study asked about previous attacks and specific symptoms that the patients may

have experienced long time ago, which made the study vulnerable to recall bias (355), as the question might lead to overestimation of the symptoms. A prospective study of AIP attacks could have given more accurate results. Regarding information bias, all the participants were given the same information, but one could assume that AIP cases had more information and awareness on dietary factors and general knowledge on AIP beforehand.

4.3 Considerations regarding statistics and statistical power

For PBG, a difference of 9 μmol/mmol creatinine was anticipated between the mean value of the healthy control group and the AIP cases. A minimum of 20 persons were needed to find a statistical difference between the groups with 95% probability ($p < 0.05$) at previously observed SD for mean value. A limitation is the relatively low number of participants, that may render the study vulnerable for random error (356), (Papers I and II). The relatively high number of hypotheses leads to carefulness in the interpretation of the set threshold for the p-value, since if Bonferroni correction had been used, the p-value needed for statistical significance would be lower. When multiple hypotheses are tested, the likelihood for incorrectly rejecting a null hypothesis increases (Type I error, false positive). The advantage of using PBG as a means in the power calculations was that we compared the AIP cases with the controls. The power is the probability that the test will correctly identify the difference, if any. It might be that, for some comparisons, we were not able to detect a difference, such as between the asymptomatic and the symptomatic AIP cases, if any. If so, we would fail to reject the null hypothesis (H0), even though H0 is false (Type II error, false negative) (357). To decide the sample size, we also had to look to at how many participants could be included, knowing the rarity of the disease, costs and workload. A case control study, in itself, generates new hypotheses and points to a direction for new research but does not find the causality. It rather pinpoints significant correlations, and from them, one could speculate on the causative factor and its outcome, based on the existing knowledge on AIP. However, it is important to keep in mind that correlations do not imply causation (358).

We performed multiple linear regression analyses to describe the relationship between PBG and inflammatory molecules. Nevertheless, a pitfall exists; since the dataset included 50 patients with latent or symptomatic AIP, the number of observations is too small for running a linear regression analyses, and we encountered quite a few obstacles in order to fulfil the underlying assumptions for linear regression. The assumption of normal distribution was tested for all independent variables using q-q-plots and, to some extent, the Shapiro-Wilk test. Standardized residual plots were generated for all the analyses in order to evaluate the underlying assumptions of a linear regression analyses, namely normality, homoscedasticity and linearity. When the

assumptions were not met, we transformed the variables using natural logarithm, which was sufficient to achieve satisfactory residual plots. As a result, most variables were transformed using natural logarithm, including the dependent variables PBG. The following factors were hypothesized to influence the disease activity in the linear regression analysis and were tested for being potential confounders: liver function/damage, kidney function, fasting, mental stress, smoking, alcohol consumption, pancreas function, sex hormones, age and gender. Additionally, we tried to test all the cytokines in the model, but we encountered problems with multicollinearity. We, therefore, did not make a model including all the cytokines and confounders because the dataset included only 50 patients and we could not present a model including 30 variables. Moreover, several of the independent variables were highly correlated. We, therefore, thought that the best way to present the results is to present the adjusted p-value of PTX3, pre-albumin and eGFR, instead of producing a large table with confusing results that cannot be trusted. Anyhow, the fact that there was an unknown confounder that was not considered is not dismissed. To achieve slightly more reliable results in the linear regression analysis, we should have recorded more than 110 observations.

We chose a model in hierarchical clustering analysis that provided information and clustered persons in groups which produced a pattern that made sense by using a clustering method described before (277). This analysis is a statistical classification technique where cases or biomarkers are divided into groups and subgroups (clusters) in a way that the biomarkers (cytokines levels) in a cluster resemble each other and are different from the biomarkers (cytokine levels) in other clusters. This is a tool for finding associations, patterns and structures within big datasets. We chose an agglomerative way of making the hierarchy of clusters, meaning “bottom up”, that every observation starts in its own cluster, and that pairs of clusters are put together when going up in the hierarchy. Before clustering, the dataset was distanced, for instance, by measuring the distance between different cytokines using Euclidian distance – the shortest distance between two points. Higher in the hierarchy, the distance between a pair and another sample must be measured, which is more complicated. Hence, we chose a method called maximum or complete linkage method, where the distance between the coupled pair and the other is set as the maximum of the pair of differences in each case.

4.4 Pros and cons regarding main group of the used methods and analysis

The laboratory was participating in external quality assessment and is also performing internal quality controls, where applicable, which is important to ensure good analysis quality (267). Certain interfering substances can affect the binding between analyte and antibody and thereby

produce analytical errors in immunoassays (359). Selectivity is only as good as the antibody can offer, and the results can possibly be affected by autoantibodies and matrix effects. Another problem is that some antibodies do not identify small differences in antigens, such as different protein isoforms. The manual processes can lead to relatively high interlaboratory variability and intra- and inter-assay variability. Advantages of immunoassays include the fact that because they are so widely used, they are often already fully validated and accepted. The microplate format makes a relatively high throughput possible because of many samples in parallel; but with multiplex, multiple analytes in each sample can also be measured. Immunoassays are relatively sensitive when coupled with good signal amplification. With multiplex (ELISA) technology, multiple biomarkers are measured at once, minimizing time and sample volume required. It can, in total, give biomarker profiles in addition to each single analyte. Notably, multiplexing has the possibility for cross-reactivity. The advantage of LC-MS/MS analysis used for measuring serum total vitamin D level is its selectivity. It can (a) distinguish between quite similar biomolecules, (b) shows sensitivity in detection of very small analytes and (c) the intra- and inter-assay reproducibility is high. Flowcytometry gives quick analyses of large cell populations but no information on individual cells. Its accuracy depends on how well trained the operators are and using the correct reagents and techniques. The presence of extreme lipemia, chylomicrons or extremely high bilirubin might interfere with and elevate the haemoglobin levels (according to the Advia 2120i Operator´s guide). However, none of the participants in our study had extreme lipemia or extremely high bilirubin. Regarding nephelometry analysis of proteins, a limitation could be the antigen excess or matrix effects, but this was not a problem in our study.

Using a seven-day diet logbook has a potential for underreporting (360), and the dietary intake is, therefore, probably slightly (17%) underreported. Advantages with our method were that a trained clinical nutritionist had conducted an instruction meeting with all the participants beforehand, and she also conducted follow-ups along the way when the participants had any questions. The nutrition institute in Oslo analysed the diet logbooks using their regular methods and gave us systemized data.

We used the PBG ratio and urine creatinine to correct urine concentration, and we set an upper reference limit of PBG at 1.5 μmol/mmol creatinine (44, 264-266). ALA, PBG and total porphyrins can remain elevated for many years, and some persons are constant high excretors of porphyrins precursors (71). The half-life of PBG is 10.6 years, highlighting the concerns of using these analyses for diagnosis of recurrent attacks (71). A two-fold increase in PBG (U-PBG/creatinine-ratio) in combination with symptoms are suggestive of an AIP attack (9). However,

it has previously been suggested that an AIP attack can be confirmed by a PBG concentration (not ratio) at least ten times the upper limit of normal (361) within one week of the onset of neurovisceral symptoms (31, 265, 362). During an AIP attack, the PBG concentration is typically 10 mg/L (44.2 μmol/L) (363). None of the AIP cases experienced an AIP attack at the time of the study (Paper I, II), but several cases had PBG levels above the cut-off, being high excretors without present apparent symptoms, either with or without a history of previous symptoms, as ALA and PBG can also be significantly elevated above the upper reference limit in asymptomatic AIP cases. A less than 50% change of PBG/creatinine can be due to analytical and biological variation (9). In a short-term setting, the reference change value (RCV) of PBG has been approximately 50% and 70% in a long-term setting (9). Other causes of increased ALA are lead poisoning and tyrosinemia (65). Methenamine hippurate intake can give false-negative PBG results (268). Although rare, AIP symptoms can also arise in children (364), but ALA and PBG do not always increase substantially (364, 365). In our study, only adults had participated. It is stated regarding children that, "Acute porphyrias should be considered in the differential diagnosis of cases with unexplained neurological weakness and myalgias, as well as in children with hypertension in association with behavioral abnormalities, including irritability and lethargy" (364). Qualitative tests for PBG are rapid and cheap, whereas screening tests have low sensitivity and poor specificity and should always be confirmed using a specific, quantitative method (95, 265). In our study, we relied on the quantitative ALA and PBG tests (Paper I, II). Further, most persons with AIP mutation have a lowered enzyme activity of the HMBS in their RBCs, but this is variable and not a reliable test for AIP (44), thus was not used in the study.

4.5 Clinical and other implications

Paper I and II: One of the current clinical practice recommendations from NAPOS is that insulin should be added to the 10% glucose solution when given intravenously to AIP patients to counter AIP attacks. During AIP attacks, the transcription of ALAS1 can, to some extent, be downregulated by giving glucose and the subsequent increased endogen production of insulin and/or the given insulin added to glucose (224, 225). Our finding that fasting insulin was lower in the symptomatic cases supports this clinical practice, but more research is needed on glucose metabolism and insulin effects during AIP attacks. Insulin is needed for glucose uptake into many cells and for metabolic purposes (320), but glucose has to be given at the same time in large enough amounts. Haem arginate treatment is assessed as very effective in improving the symptoms of acute AIP attacks (5), but the inflammatory potential and the possibility of adding to potential chronicity advocates a more

cautious way of using it as a long-term treatment (94). However, haem is still recommended as a treatment modality for AIP attacks. The potential effects of low-grade inflammation and lifestyle factors, including diet, in AIP have been widely discussed in meetings with the local porphyria association and at international conferences.

Paper III: This paper has contributed to the knowledge regarding optimal sampling methods, enhanced in terms of accuracy, for cytokines, chemokines and growth factors in venous human blood samples. The project has laid the foundation for future research projects regarding the use of cytokines, growth factors and other biomarkers for detecting cytokines in different diseases and conditions in humans. Notably, some cytokines are promising biomarker for assessment of neonatal sepsis, although conflicting cut-off levels were reported and potential confounders should be considered (255).

4.6 Way forward

For some, the burden of AIP is high (279, 366). This highlights the need for novel therapies and better knowledge on prevention of AIP attacks and the modifying factors that impact breakthrough of symptoms. Research on inflammation and diabetogenic hormones could be extended, such as by studying givosiran's impact. Mapping and changing the diet in AIP patients with chronic symptoms and studying PBG and ALA changes after intake of carbohydrates or glucose with and without insulin-treatment could be fruitful. Also, of interest are genetic studies, including studies of mRNA and other RNA molecules, to examine the role of other related mutations and epigenetic changes. In an animal model, one could use CRISPRCas9 gene editing to study the effect of AIP on the HMBS enzyme activity, metabolomics and transcriptomics. In a whole blood model, the possible roles of PBG, ALA, porphyrins and haem arginate as possible DAMPs on cytokine release and complement activation can be studied. To analyse serum concentrations of different environmental pollutants, new liver markers, TSH, FT4, plasma hepcidin, adiponectin and faecal microbiome could be compared with PBG, ALA and inflammatory markers. It would also be interesting to analyse cytokine levels and hepcidin during AIP attacks. Gut microbes have been shown to impact insulin sensitivity (156) and affects health and disease (367). We will complete the studies about enhanced liver fibrosis test (ELF) in AIP, dental health in AIP, ROC-curves for ALA and PBG in diagnosing AIP and the role of different B-vitamins, including B6, in AIP. With interest, I will follow the work of research groups focusing on AIP therapy in the form of liver directed gene-therapy (116, 368-370), hepatocyte transplantation (371), pharmacological chaperones (372), systemic mRNA (373, 374) and inhibition of β–catenin (375). I will also follow the planned international genome-wide

associations study and those studying modifiers that could impact AIP disease activity, such as a polymorphism in the peptide transporter 2 (PET2T) gene (376). Also, I will follow the search for better diagnostic tools for AIP and AIP disease activity, such as cellular bioenergetics (77), Vitamin D-binding protein (377) and Protein Induced by Vitamin K absence or Antagonist II (PIVKA-II) (210). The application of machine learning and knowledge engineering to electronic health records will also give interesting results (378).

4.7 Conclusions

The thesis brings to the research area the idea of low-grade inflammation as a part of the pathogenesis and consequence of AIP. AIP cases had low-grade inflammation measured as higher levels of plasma cytokines. We speculate that the low-grade inflammation could possibly be involved in the carcinogenesis of HCC in AIP cases. The lower prealbumin levels in the symptomatic AIP cases as compared with the asymptomatic cases may suggest inflammation in the liver. PBG correlated positively with PTX3, suggesting a link between AIP disease activity and inflammation. However, cytokines were increased in asymptomatic cases also. Prealbumin correlated negatively with PBG, pointing towards increased hepatic inflammation at higher PBG levels. We suggest porphyrin-precursor-related organ damage in the liver and other organs that may drive inflammation. Also, kidney function was decreased in the symptomatic AIP cases, and this could contribute to inflammation as well as perhaps being at least partly caused by inflammation. In some of the cases and controls who smoked, the habit seemed to influence inflammation.

We also found that fasting insulin and C-peptide levels were lower in the symptomatic AIP cases as compared with the matched controls, and insulin was lower in those with high PBG compared to the insulin level in those with low PBG, while P-resistin was higher in the symptomatic AIP cases as compared with the asymptomatic cases. Lower insulin levels could lead to decreased uptake of glucose into cells and accelerated haem synthesis in some AIP cases. Visfatin was higher in a cluster of AIP cases with high cytokines. Dietary challenges were present in both the case and control groups; they consumed a lot of saturated fatty acids and quite less fibre. The total and LDL cholesterol levels were higher in the AIP cases when compared with matched controls. The carbohydrate intake was lower than recommended, and AIP cases could benefit from increasing this intake. Moderate alcohol intake was reported among the AIP cases, even though they were recommended against it. A high intake of energy, sugar and candies and a higher insulin level were associated with a lower biochemical disease activity. The influence of diabetogenic hormones along with insulin level and lifestyle factors in relation to AIP disease activity needs

further investigation. Cytokines should be analysed in EDTA (or citrate) blood samples, immediately cooled and rapidly centrifuged (within four hours) to obtain EDTA (or citrate) plasma to be stored at -80 °C. We hope that standardization of sampling conditions and methods to analyse cytokines will make it easier to use cytokines as inflammatory biomarkers in the future.

5 References

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Errata

Published paper II: Fig. legend to Fig. 9: “P-gastrointestinal peptide (GIP)”, should have been written “P-gastric inhibitory polypeptide (GIP)”.

Published paper III: Table 4: The correct 97.5 percentile for IL-9 is 173

Paper I

Clinical & Experimental Immunology
The Journal of Translational Immunology

Clinical and Experimental Immunology ORIGINAL ARTICLE doi:10.1111/cei.12899

Systemic inflammation in acute intermittent porphyria: a case–control study

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Accepted for publication 2 November 2016
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Summary

This study aimed to examine whether acute intermittent porphyria (AIP) is associated with systemic inflammation and whether the inflammation correlates with disease activity. A case–control study with 50 AIP cases and age-, sex- and place of residence-matched controls was performed. Plasma cytokines, insulin and C-peptide were analysed after an overnight fast using multiplex assay. Long pentraxin-3 (PTX3) and complement activation products (C3bc and TCC) were analysed using enzyme-linked immunosorbent assay (ELISA). Urine porphobilinogen ratio (U-PBG, μmol/mmol creatinine), haematological and biochemical tests were performed using routine methods. Questionnaires were used to register AIP symptoms, medication and other diseases. All 27 cytokines, chemokines and growth factors investigated were increased significantly in symptomatic AIP cases compared with controls ($P < 0{\cdot}0004$). Hierarchical cluster analyses revealed a cluster with high visfatin levels and several highly expressed cytokines including interleukin (IL)-17, suggesting a T helper type 17 (Th17) inflammatory response in a group of AIP cases. C3bc ($P = 0{\cdot}002$) and serum immunoglobulin (Ig)G levels ($P = 0{\cdot}03$) were increased significantly in cases with AIP. The U-PBG ratio correlated positively with PTX3 ($r = 0{\cdot}38$, $P = 0{\cdot}006$), and with terminal complement complex (TCC) levels ($r = 0{\cdot}33$, $P = 0{\cdot}02$). PTX3 was a significant predictor of the biochemical disease activity marker U-PBG in AIP cases after adjustment for potential confounders in multiple linear regression analyses ($P = 0{\cdot}032$). Prealbumin, C-peptide, insulin and kidney function were all decreased in the symptomatic AIP cases, but not in the asymptomatic cases. These results indicate that AIP is associated with systemic inflammation. Decreased C-peptide levels in symptomatic AIP cases indicate that reduced insulin release is associated with enhanced disease activity and reduced kidney function.

Keywords: chemokines, complement, cytokines, human, inflammation

Introduction

Acute intermittent porphyria (AIP) is an autosomal dominant metabolic disorder caused by a mutation in the hydroxymethylbilane synthase gene, which encodes an enzyme involved in haem synthesis [1]. A rare condition worldwide, with a prevalence in European populations of only 1/75 000 to 10–20/100 000 [2,3], AIP is prevalent in Nordland county in Norway, with a prevalence as high as 600/100 000 in one municipality [4]. Many patients have only one porphyria attack over a lifetime, and few suffer chronic AIP symptoms. Individuals carrying the AIP gene mutation are classified as symptomatic (15–20%) or asymptomatic (80–85%) [1]. Symptomatic AIP presents typically with acute, severe abdominal pain, nausea, vomiting, constipation, dark red urine and muscle weakness [1]. Most attacks last from a few days up to 2 weeks. Bulbar or phrenic nerve paresis may ensue occasionally and lead to life-threatening respiratory failure [1,5]. A high prevalence of hypertension and kidney impairment has been documented [3]. Some mutations, including the W198X

mutation, carry an increased risk of hepatocellular carcinoma (HCC), which is found in approximately one-third of patients with this mutation [3,6].

Established triggers for AIP attack include infections, alcohol, smoking, hormonal factors, physical or mental stress and medications affecting haem synthesis [1,2]. In terms of the mechanisms whereby these triggers cause illness, fasting, fever and stress are known to induce haem oxygenase-1 activity, leading to a reduction in the free haem pool and induction of 5-aminolevulinic synthase-1 (ALAS1) [1]. Acute phase proteins and interleukin (IL)-6 may also increase ALAS1 synthetase activity in liver cells [7]. ALAS1 is increased in the liver in AIP patients, due probably to intracellular haem deficiency [8]. Some patients with AIP, however, may suffer severe forms of the disease without any known triggers. Treatment for this group is demanding, and requires the avoidance or treatment of triggering factors, a high oral sugar intake, glucose and insulin infusion or infusion of haem. In these patients, ALAS1 mRNA in the liver is decreased by insulin through the peroxisome proliferator-activated receptor co-activator 1α [9]. The fact that liver transplantation is an efficient cure for AIP strengthens the evidence that the role of liver metabolism in AIP pathogenesis is central [10]. In addition, inflammation may possibly affect haem synthesis through hepcidin [11].

Low-grade inflammation has been reported to play a role in several systemic diseases, such as atherosclerosis and type II diabetes [12,13]. AIP could therefore be associated with low-grade inflammation. The accumulation of certain damage-associated molecular patterns (DAMPs) has been shown to stimulate the immune cells and inflammation in various diseases [14]. Haem and porphyrins have been reported to activate the complement system [15,16]. Sato *et al.* showed that dietary supplementation with 5-aminolevulinic acid (ALA) induced T cell responses in chickens through oxidative stress [17]. Delaby *et al.* reported lower prealbumin levels in patients with symptomatic AIP, probably as a result of malnutrition or hepatic inflammation [18].

Although there is evidence for a possible role of inflammation in AIP, it has not been determined whether patients with AIP have increased systemic inflammation. The aims of this study were to examine systemic inflammation in AIP by plasma biomarkers and biochemical disease activity in the form of urine porphyrin precursor levels, and to determine whether inflammatory markers are associated with biochemical disease activity, insulin release and kidney function.

Materials and methods

Study design and participants

A case–control study was conducted in 50 individuals with AIP, 15 asymptomatic and 35 symptomatic carriers of the AIP gene mutation and 50 controls matched for age, sex and place of residence (Table 1). Of the 50 AIP patients, 48 had the W198X mutation. The patients and matched controls were recruited from the Norwegian counties of Nordland, Troms, Trøndelag and Oslo. The 42 healthy controls consisted of 22 men and 20 women, with a mean age of 48.8 years, without inflammatory diseases. The single-draw blood samples were obtained in a period outside AIP attacks. Only one patient had chronic AIP symptoms.

Ethics, consent and permission

The Regional Committee for Medical and Health Research Ethics approved the study. Written informed consent was obtained from all participants. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration. This trial is registered with ClinicalTrials.gov number NCT01617642.

Medical evaluation

A physician completed a questionnaire for each participant during the interview on the same day that the blood samples were taken. Individuals with the AIP gene mutation were questioned about the presence or absence of AIP symptoms, time of diagnosis, number and durations of attacks and suspected triggering and relieving factors. They were asked specifically about abdominal pain, vomiting, constipation, muscle ache, muscle weakness, decreased sensitivity, paresis, headache, tiredness, epilepsy, palpitations, dark or red urine and psychiatric symptoms. Participants were also asked about their smoking and alcohol habits, physical activity and emotional stress. All participants gave a past medical history, history of current illness and drug history before the interview. Dietary intake was registered using a 7-day food diary. Trained nurses measured height and weight and calculated body mass index (BMI).

Blood sampling and laboratory methods

Blood samples were obtained by venepuncture between 8 a.m. and 9 a.m. after an overnight fast using Vacuette citrate and ethylenediamine tetraacetic acid (EDTA) serum tubes (Greiner Bio-one GmbH, Frickenhausen, Germany). The EDTA tubes for plasma cytokine and complement analysis were placed immediately onto crushed ice and centrifuged at 1500 ***g*** for 15 min at +4°C, and the plasma was stored at −80°C until analysis.

Multiplex technology

The cytokines were analysed in EDTA plasma using a Bio-Plex Human Cytokine 27-plex kit (Bio-Rad Laboratories Inc., Hercules, CA, USA). The following cytokines, chemokines and growth factors were analysed: interleukin (IL)-1β, IL-1RA (IL-1 receptor antagonist), IL-2, IL-4, IL-5, IL-

Table 1. Baseline patient demographic characteristics of the study population

	Controls	[a]AIP cases	OR (CI)	*P*
Demographic features				
Age, years (s.d.)	50·4 (18·6)	50·6 (18·4)		0·38
Height, cm (s.d.)	171·6 (9·5)	172·1 (9·7)		0·84
Weight, kg (s.d.)	80·2 (14·0)	81·0 (15·7)		0·60
Body mass index, kg/m^2 (s.d.)	27·2 (3·9)	27·2 (3·9)		0·92
Woman, *n* (%)	21 (42%)	21 (42%)		
Men, *n* (%)	29 (58%)	29 (58%)		
Symptomatic [a]AIP, *n* (%)		35 (70%)		
Asymptomatic [a]AIP, *n* (%)		15 (30%)		
Inflammatory diseases				
Gout, *n* (%)	2 (4%)	8 (16%)	4·57	0·09
Inflammation in the musculoskeletal system, *n* (%)	6 (12%)	3 (6%)	0·47	0·49
Rheumatoid arthritis, *n* (%)	1 (2%)	1 (2%)	1·00	1·00
Ankylosing spondylitis, *n* (%)	2 (4%)	1 (2%)	0·49	1·00
Gastrointestinal inflammatory disease, n (%)	3 (6%)	3 (6%)	1·00	1·00
Bacterial infection recently, *n* (%)	1 (2%)	1 (2%)	1·00	1·00
Viral infection recently, *n* (%)	1 (2%)	0 (0%)	0·33	1·00
Diabetes mellitus, *n* (%)	3 (6%)	4 (8%)	1·36	1·00
Anti-inflammatory drugs and allopurinol, current use				
Allopurinol, *n* (%)	1 (2%)	4 (8%)	4·26	0·36
NSAIDs, *n* (%)	5 (10%)	3 (6%)	0·57	0·72
Immunosuppressants, *n* (%)	3 (6%)	1 (2%)	0·32	0·62
Prednisolone, *n* (%)	2 (4%)	1 (2%)	0·49	1·00
Penicillin V, *n* (%)	1 (2%)	0 (0%)	0·33	1·00
Autoantibodies and biomarkers				
S[c]-Rheumatoid factor $\geq$ 6 U/l, *n* (%)	7 (14%)	4 (8%)	0·53	0·53
S[c]-Anti-nuclear ab[b] > 20 U/l, *n* (%)	8 (16%)	10 (20%)	1·31 (0·47–3·66)	0·80
S[c]-Anti-thyroid peroxidase ab[b] > 60 kU/l, *n* (%)	2 (4%)	6 (12%)	3·27	0·27
S[c]-Anti-citrullinated peptide ab[b] > 20 U/l, *n* (%)	2 (4%)	0 (0%)	0·19	0·50
S[c]-Anti-cardiolipin, U/l, *n* (%)	1 (2%)	0 (0%)	0·33	1·00
S[c]-Urate > 400 μmol/l, *n* (%)	12 (24%)	15 (30%)	1·35 (0·56–3·30)	0·65
S[c]-Cystatin C, mg/l (IQR)	0·97 (0·85–1·03)	0·98 (0·86–1·11)		0·43
Relative eGFR[d] CKD-EPI, ml/min/1·73 m^2 (IQR)	87 (69–99)	83 (63–100)		0·54
Ethanol intake and smoking				
Ethanol intake, g per day (IQR)	6·3 (0–14·7)	3·2 (0–11·9)		0·28
Never smokers, *n* (%)	19 (38%)	19 (38%)		
Former smokers, *n* (%)	25 (50%)	21 (42%)	0·84 (0·35–1·99)	0·83
Current smokers, *n* (%)	6 (12%)	10 (20%)	1·67 (0·50–5·51)	0·55

The data represent the mean values (s.d.), *n* (%) or median values and interquartile range (IQR). Wilcoxon's matched-pairs signed-rank test was used for the demographic features; Fisher's exact test was used for all other variables. OR = odds ratio; CI = confidence interval; $n = 50$ matched pairs, except for daily alcohol intake $n = 46$ matched pairs. OR for smoking was calculated by comparing former smokers against never smokers in cases *versus* controls and current smokers against never smokers in cases *versus* controls. [a]AIP = acute intermittent porphyria; [b]ab = antibodies; [c]S = serum; [d]relative estimated glomerular filtration rate (eGFR) Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) based on cystatin C levels; NSAIDs = non-steroidal anti-inflammatory drugs; s.d. = standard deviation.

6, IL-7, chemokine (C-X-C) motif 8 (CXCL8), IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, chemokine (C-C motif) ligand 5 (CCL5), chemokine ligand 11 (CCL11), basic fibroblast growth factor (FGF), granulocyte colony-stimulating factor (G-CSF), granulocyte–macrophage colony-stimulating factor (GM-CSF), interferon (IFN)-γ, CXCL10, CCL2, CCL3, CCL4, platelet-derived growth factor-BB (PDGF-BB), tumour necrosis factor (TNF) and vascular endothelial growth factor (VEGF). Insulin, visfatin and C-peptide were analysed using the human Bio-plex Pro human diabetes immunoassay kit from Bio-Rad. The analysis was performed using the Bio-Plex 200 system (Bio-Rad).

Enzyme immunoassay

Long-pentraxin 3 (PTX3) was analysed in EDTA plasma using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems Inc., Minneapolis, MN, USA). The complement activation products C3bc and soluble C5b-9 (sC5b-9)

were analysed in EDTA plasma using enzyme-linked immunosorbent assays (ELISAs), as described previously [19]. Both assays are based on monoclonal antibodies to neoepitopes exposed in the activation products and not present in the native components. Optical density was measured using an MRX microplate reader (Dynex Technologies, Denkendorf, Germany) [20]. Anti-transglutaminase and anti-endomysium were analysed using Quanta Lite™ h-tTG immunoglobulin (Ig)A ELISA kits (Inova Diagnostics, Inc., San Diego, CA, USA). Anti-nuclear antibody (ANA) screen, rheumatoid factor and anti-CCP were analysed in serum using Quanta Lite™ ANA, rheumatoid factor (RF) IgM and CCP3 IgG ELISA kits, respectively (Inova Diagnostics, Inc.). Anti-cardiolipin was analysed using the indirect enzyme immunoassay (EIA) test RELISA® cardiolipin IgG and IgM (Immuno-Concepts N.A. Ltd., Sacramento, CA, USA).

Routine biochemistry tests

Routine haematology parameters were analysed using a Siemens ADVIA 2120 Hematology System (Siemens Healthcare Diagnostics Ltd, Camberley, UK). HbA1c was analysed using a Tosoh G8 high-performance liquid chromatography (HPLC) system. Serum levels of urate, IgA, IgG and IgM, urine creatinine and other clinical chemistry parameters in urine and serum were measured using an Advia®1800 system (Siemens Medical Solutions Diagnostics, Tokyo, Japan) and reagents from Siemens Healthcare Diagnostics Ltd. Anti-thyroid peroxidase antibodies (anti-TPO) levels were analysed using an Advia Centaur® system (Siemens). Levels of C3, C4 and high-sensitivity C-reactive protein (CRP) in serum were analysed using a BN ProSpec® nephelometer (Siemens Healthcare Diagnostics Ltd). Urine porphobilinogen (U-PBG) levels were analysed using a kit from BioRad Laboratories (München, Germany) and Ehrlich reagent, and urine porphyrins were analysed as described previously [21]. The U-PBG ratio (μmol porphobilinogen/mmol creatinine) was subsequently calculated.

Statistical analyses

The Wilcoxon matched-pairs signed-rank test was used on the matched case–control data. The non-detectable cytokine values were set to zero. The Mann–Whitney *U*-test was used on the non-matched case data. Spearman's rank correlation coefficient was used for the cases with AIP and in the calculations of the *r*- and two-tailed *P*-values because the data were not distributed normally. $P < 0{\cdot}05$ was considered to be statistically significant. The statistical analysis was performed using Prism version 6.0 from GraphPad Software Inc. (San Diego, CA, USA). Multiple linear regression analyses were performed on data from the AIP cases using IBM SPSS Statistics for Macintosh version 23 (Armonk, NY, USA). In the hierarchical cluster analysis, the cytokine levels in each AIP case and matched controls were divided on the median levels of each cytokine in the 50 matched controls. Individuals with undetectable cytokine values were replaced with a random number below the lowest detection limit of each assay. The data were then imported into R version 3.1.3, Bug in Your Hair. The matrix results were then transformed using natural logarithm. The Bioconductor (version 3.3) library complex heatmap (version 1.10.2) was used to cluster and distance the cytoplex matrix for both row and columns with the following parameters: cluster = complete linkage, distance = Euclidian [22].

Results

Baseline characteristics and demographic features

The demographic features and baseline characteristics were similar in AIP patients and matched controls (Table 1). Of the 50 patients, 35 had symptomatic AIP and 48 had the W198X mutation. There was no difference in the prevalence of inflammatory diseases and the use of anti-inflammatory medication between AIP cases and controls (Table 1). Finally, the levels of autoantibodies, biomarkers related to inflammatory diseases and the kidney function did not differ between the matched controls and AIP cases (Table 1).

Plasma levels of cytokines, chemokines, growth factors and other biomarkers in cases with acute intermittent porphyria compared with controls

The levels of all 27 cytokines, chemokines and growth factors were increased significantly ($P < 0{\cdot}0004$) in the cases with AIP compared with the matched controls (Table 2). Median concentrations of proinflammatory cytokines, including TNF, IL-1β, CXCL8, CCL2 and IL-6, were increased significantly ($P < 0{\cdot}0001$ for all) by 4·1-, 1·8-, 1·6-, 1·5- and 2·5-fold, respectively, in the cases with AIP compared with the matched controls (Table 2). Furthermore, median levels of IL-7, IL-12 (p70), IL-17 and IFN-γ (Table 2) were increased significantly ($P < 0{\cdot}0001$ for all) by 2·3-, 3·8-, 24·6- and 7·7-fold, respectively. The IL-15 level (Table 2) was increased in cases with AIP, but with undefined fold-change because the median concentration in the matched controls was undetectable.

Median levels of the anti-inflammatory cytokines, including IL-1RA and IL-10, were also increased significantly ($P < 0{\cdot}0001$ for both) by 2·3- and 10·1-fold, respectively, in AIP cases compared with matched controls (Table 2). The levels of several chemokines, including CCL3 and CCL4, CCL5, CXCL10 and CCL11 (Table 2), were all increased significantly ($P < 0{\cdot}001$ for all) in AIP cases compared with matched controls. Median levels of the growth factors FGF basic, VEGF, G-CSF, GM-CSF and PDGF-BB were also increased significantly ($P < 0{\cdot}0001$ for

Table 2. Biomarker levels in acute intermittent porphyria cases and controls

	Controls ($n = 50$)	[a]AIP cases ($n = 50$)	[$]Fold increase	[$]Healthy controls
U[b]-PBG[c] (μmol/mmol creatinine)	0·4 (0·3–0·5)	2·6 (0·9–8·5)***	6·5	
U[b]-total porphyrins (nmol/mmol creatinine)	6·7 (4·4–10·6)	25·7 (8·5–83)***	3·8	
S[d]-ALT (IU/l)[e]	24·5 (18·8–34)	31·0 (23–45)#	1·3	
P[f]-IL-1β[g](pg/ml)	0·9 (0·65–1·2)	1·6 (0·9–3·3)***	1·8	0·9 (0·4–2·0)
P[f]- IL-1RA[g] (pg/ml)	28·0 (13–50)	64·0 (23–197)***	2·3	21 (0–117)
P[f]-IL-2[g] (pg/ml)	1·0 (0·0–2·0)	6·0 (1·0–16)***	6·0	0·03 (0·0–7·9)
P[f]-IL-4[g](pg/ml)	1·0 (0·2–1·0)	2·0 (1·0–4·0)***	2·0	1·0 (0·0–2·0)
P[f]-IL-5[g] (pg/ml)	1·1 (0·7–1·4)	1·7 (1·3–4·0)***	1·6	1·0 (0·5–3·9)
P[f]-IL-6[g] (pg/ml)	2·0 (0·6–3·0)	5·0 (2·0–9·3)***	2·5	2·0 (0·02–6·9)
P[f]-IL-7[g] (pg/ml)	3·0 (1·4–5·0)	7·0 (5·0–14)***	2·3	3·0 (0·0–11)
P[f]-CXCL8[g] (pg/ml)	5·0 (3·0–7·0)	8·0 (6·0–14)***	1·6	4·5 (0·8–19)
P[f]-IL-9[g] (pg/ml)	5·5 (4·0–8·3)	14·0 (8·8–22)***	2·6	5·5 (2·0–32)
P[f]-IL-10[g] (pg/ml)	0·14 (0·0–0·53)	1·4 (0·2–6·0)***	10·1	0·1 (0·0–9·5)
P[f]-IL-12(p70)[g] (pg/ml)	2·0 (0·2–5·3)	13·5 (6·8–25)***	6·8	1·8 (0·0–32)
P[f]-IL-13[g] (pg/ml)	2·2 (1·7–4·0)	5·0 (2·2–8·3)***	2·3	2·1 (0·9–14)
P[f]-IL-15[g] (pg/ml)	0·0 (0–0·12)	1·25 (0·0–5·0)***	[j]ND	0·0 (0·0–2·0)
P[f]-IL-17[g] (pg/ml)	0·65 (0·0–9·3)	16·0 (3·5–55)***	24·6	0·5 (0·0–54)
P[f]-CCL2[h] (pg/ml)	11·0 (8·0–14·0)	16·5 (13·0–22)***	1·5	10·0 (3·0–28)
P[f]-CCL3[h] (pg/ml)	1·0 (0·15–3·0)	3·0 (2·0–6·0)***	3·0	1·0 (0·0–11)
P[f]-CCL4[h] (pg/ml)	35·0 (29–46)	57·5 (45–69)***	1·6	34·0 (21–157)
P[f]-CCL5[h] (ng/ml)	0·22 (0·11–0·52)	1·13 (0·17–10·7)***	5·1	0·21 (0·02–4·0)
P[f]-CCL11[h] (pg/ml)	43·0 (20–67)	79·0 (59–105)***	1·8	35·5 (0·5–153)
P[f]-FGF basic (pg/ml)	12·0 (4·0–23·5)	31·0 (10–70)***	2·6	12·0 (0·0–71)
P[f]-G-CSF (pg/ml)	7·0 (1·5–15·0)	29·5 (4·8–68)***	4·2	7·0 (0·0–60)
P[f]-GM-CSF (pg/ml)	4·0 (0·14–6·3)	14·0 (5·0–31)***	3·5	3·0 (0·0–15)
P[f]-IFN-γ (pg/ml)	13·5 (0–53)	104 (13·5–264)***	7·7	13·5 (0·0–117)
P[f]-CXCL10 (pg/ml)	601 (402–914)	963 (711–1162)**	1·6	597 (205–2238)
P[f]-TNF (pg/ml)	8·0 (4·6–21)	33 (12–84)***	4·1	8·0 (0·02–54)
P[f]-VEGF (pg/ml)	2·0 (0·42–5·0)	16·5 (6·8–29)***	8·3	1·8 (0·0–17)
P[f]-PDGF-BB (pg/ml)	2·0 (0·27–11·3)	19·0 (3·5 −155)***	9·5	1·5 (0–117)
P[f]-C3bc (CAU[i]/ml)	5·7 (4·8–7·6)	7·8 (6·4–9·1)*	1·4	
P[f]-C3bc/C3 ratio (CAU[i]/ml/g/l)	5·4 (4·5–7·2)	7·0 (5·7–8·0)*	1·3	
S-Immunoglobulin G (g/l)	10·0 (8·5–11)	10·9 (10–12)#	1·1	
B-HbA1c (%)	5·3 (5·0–5·6)	5·5 (5·3–5·7)*	1·1	
S[d]-Triglyceride	1·0 (0·7–1·4)	1·2 (1·0–1·9)#	1·2	
S[d]-Total cholesterol	5·1 (4·6–5·7)	5·7 (5·1–6·4)*	1·1	

The data represent the median values and interquartile range (IQR). The data were analysed using the Wilcoxon matched-pairs signed-rank test on AIP cases ($n = 50$) *versus* matched controls. The *P*-values are exact, two-tailed. #$P < 0{\cdot}05$; *$P < 0{\cdot}01$; **$P < 0{\cdot}001$; ***$P < 0{\cdot}0001$ on AIP cases ($n = 50$) *versus* matched controls. All *P*-values $< 0{\cdot}001$ when comparing the cytokines in the AIP cases with the 42 healthy controls. [$]Fold increase, AIP cases median value ($n = 50$)/controls median values ($n = 50$), [$]cytokine values, median and 2·5–97·5 percentiles in the 42 healthy controls. [a]AIP = acute intermittent porphyria; [b]U = urine; [c]PBG = porphobilinogen; [d]S = serum; [e]IL = interleukin; [f]P = ethylenediamine tetraacetic acid (EDTA) plasma; [g]IU/l = international units per litre; [h]CCL = chemokine ligand; [i]CAU/ml = complement arbitrary units per millilitre; [j]n.d. = not determined; [k]B = EDTA blood.

all) by 2·6-, 8·3-, 4·2-, 3·5- and 9·5-fold, respectively, in AIP cases compared with matched controls (Table 2). IL-2, IL-4, IL-5, IL-9 and IL-13 were increased significantly ($P < 0{\cdot}0001$ for all) in cases with AIP (Table 2). The levels of most cytokines were also increased in the AIP cases compared to the median level and the reference ranges in the 42 healthy controls (Table 2). The *P*-values remained on the same level when we compared the cytokine levels with the 42 healthy controls instead of the 50 matched controls, except that the *P*-values for CCL5 and FGF basic against the 42 healthy controls were $P < 0{\cdot}001$ instead of $P < 0{\cdot}0001$.

Serum ALT, IgG level and blood monocyte count were slightly, but significantly higher ($P < 0{\cdot}05$) in AIP cases than matched controls (Table 2). Complement activation markers, including plasma C3bc and C3bc/C3 ratio, were higher ($P < 0{\cdot}01$) in cases than matched controls (Table 2). Serum cholesterol and triglyceride levels were also significantly higher in cases than matched controls (Table 2). The biomarkers of AIP disease activity U-PBG and total

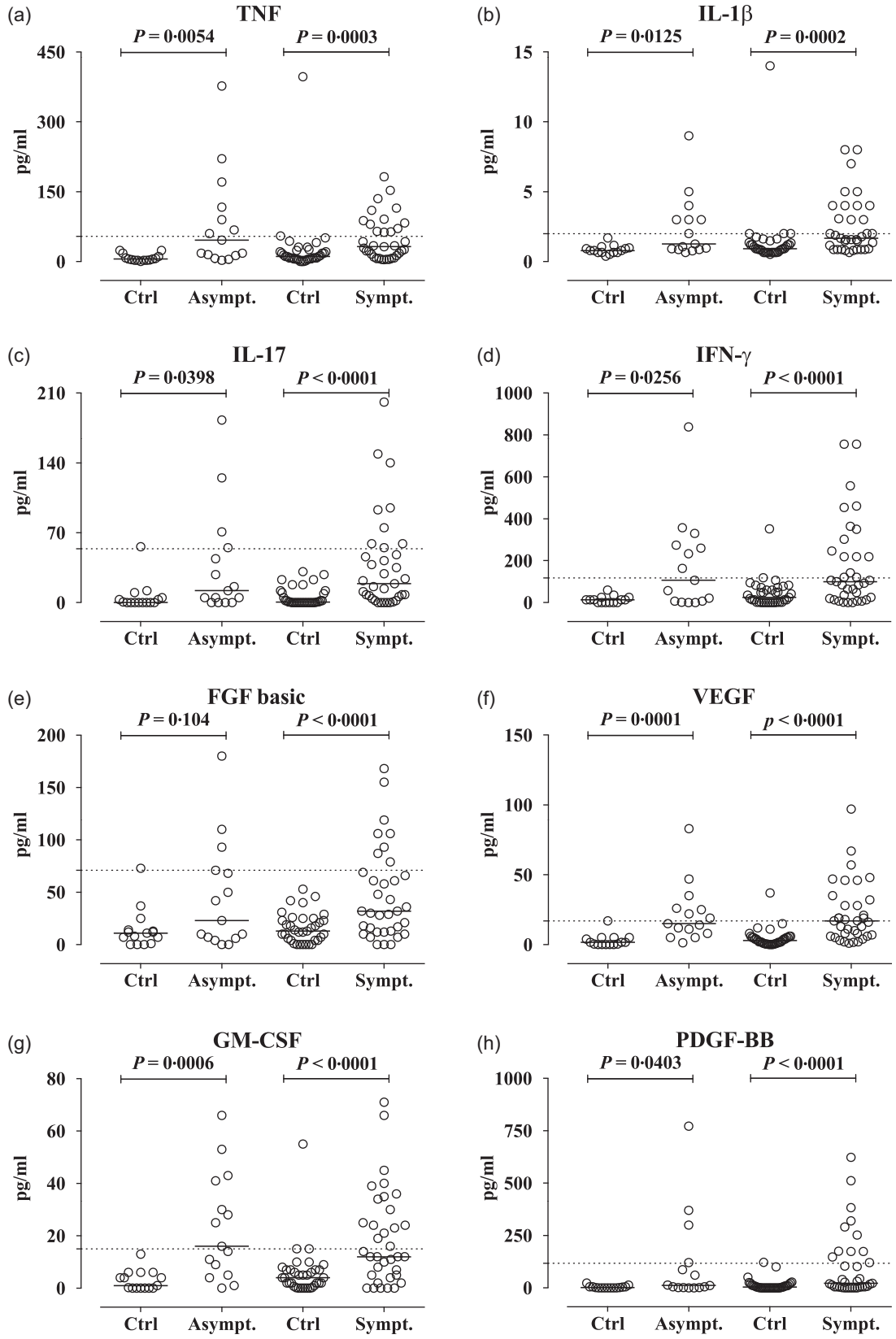
(a)
TNF
P = 0·0054
P = 0·0003
450
300
150
0
pg/ml
Ctrl
Asympt.
Ctrl
Sympt.
(b)
IL-1β
P = 0·0125
P = 0·0002
15
10
5
0
pg/ml
Ctrl
Asympt.
Ctrl
Sympt.
(c)
IL-17
P = 0·0398
P < 0·0001
210
140
70
0
pg/ml
Ctrl
Asympt.
Ctrl
Sympt.
(d)
IFN-γ
P = 0·0256
P < 0·0001
1000
800
600
400
200
0
pg/ml
Ctrl
Asympt.
Ctrl
Sympt.
(e)
FGF basic
P = 0·104
P < 0·0001
200
150
100
50
0
pg/ml
Ctrl
Asympt.
Ctrl
Sympt.
(f)
VEGF
P = 0·0001
p < 0·0001
150
100
50
0
pg/ml
Ctrl
Asympt.
Ctrl
Sympt.
(g)
GM-CSF
P = 0·0006
P < 0·0001
80
60
40
20
0
pg/ml
Ctrl
Asympt.
Ctrl
Sympt.
(h)
PDGF-BB
P = 0·0403
P < 0·0001
1000
750
500
250
0
pg/ml
Ctrl
Asympt.
Ctrl
Sympt.

Fig. 1. Increased levels of cytokines and growth factors in asymptomatic (Asympt.) and symptomatic (Sympt.) acute intermittent porphyria (AIP) cases compared with matched controls (Ctrl). (a) Increased levels of tumour necrosis factor (TNF), (b) interleukin (IL)-1β and (c) IL-17, (d) interferon (IFN)-γ and the growth factors (e) basic fibroblast growth factor (FGF basic), and (f) vascular endothelial growth factor (VEGF), (g) granulocyte–macrophage colony-stimulating factor (GM-CSF) and (h) platelet-derived growth factor (PDGF)-BB in asymptomatic and symptomatic AIP cases compared with their respective matched controls (Ctrl). The concentration of cytokines and chemokines were measured using a multiplex cytokine assay. The results are expressed as pg/ml. The results from the age- and sex-matched controls ($n = 50$, including 35 controls for the symptomatic cases) and the cases with AIP ($n = 50$, 35 symptomatic) are shown as scatter-plots with the median. The horizontal dotted gridline indicate each cytokine's upper reference value (97·5 percentile) in 42 healthy controls. The data were analysed using the Wilcoxon matched-pairs signed-rank test.

porphyrins were, as expected, significantly higher ($P < 0{\cdot}0001$ for both) in cases than controls (Table 2). High-sensitivity CRP, PTX3, serum IgA and IgM levels, total leucocyte count, platelet count and haemoglobin levels were not significantly different between the AIP cases and the matched controls (data not shown).

Cytokine levels in asymptomatic and symptomatic acute intermittent porphyria cases

We next examined whether cytokine levels were increased both in asymptomatic and symptomatic AIP cases. The levels of all 27 cytokines, chemokines and growth factors were increased significantly in cases with symptomatic AIP compared with matched controls, and eight of these are shown (Fig. 1). Furthermore, the levels of 23 of these 27 analytes were increased significantly in cases with asymptomatic AIP compared with matched controls, and seven of these are shown (Fig. 1). However, G-CSF, CCL3, FGF-basic and CXCL8 were not increased in asymptomatic AIP cases. The levels of cytokines and growth factors were also increased in many AIP cases compared to their respective reference ranges in 42 healthy controls (Fig. 1). The C3bc/C3 ratio was enhanced significantly by 1·3-fold ($P < 0{\cdot}005$) in the symptomatic AIP cases *versus* their matched controls. However, the levels of the complement activation markers terminal complement complex (TCC), C3bc/C3 ratio, C3 and C4 levels were not significantly different between the symptomatic and asymptomatic AIP cases (data not shown).

Hierarchical cluster analyses of cytokines

The hierarchical cluster analyses of cytokines in each AIP case and 50 matched controls divided the AIP cases and matched controls into two main clusters, (a) and (b) (Fig. 2). Cluster (b) featured high levels (red) of many cytokines and included five controls and 30 AIP cases, 21 of which were symptomatic AIP cases and nine were asymptomatic (Fig. 2). All five controls in cluster (b) with high cytokine levels had inflammatory diseases, such as undiagnosed rheumatoid arthritis and ankylosing spondylitis. Cluster (b2-2) contains one control with undiagnosed, untreated rheumatoid arthritis and AIP cases. Many AIP cases in cluster (b2-2) with high cytokine levels also had elevated U-PBG and U-ALA levels. The 30 AIP cases in cluster (b) had significantly higher cytokine and visfatin levels than the 20 AIP cases in cluster (a) (Supporting information, Table S1). However, the PBG ratio and kidney function were not significantly different in the AIP cases in cluster (b). The cytokines clustered in three groups, and the cluster 1 cytokines most elevated in the AIP cases were IL-17, PDGF-BB, IL-10 and IL-15. Finally, the AIP cases with the R167W mutation had similar cytokine levels to those with the W198X mutation (Fig. 2).

Correlations between disease activity, inflammatory markers and kidney function in cases with acute intermittent porphyria

Biochemical disease activity in AIP was measured by U-PBG ratio. U-PBG levels correlated positively with the inflammatory biomarker PTX3 ($r = 0{\cdot}38$, $P = 0{\cdot}01$) (Fig. 3). U-PBG levels also correlated positively with IL-9 ($r = 0{\cdot}31$, $P = 0{\cdot}03$) and IL-7 levels ($r = 0{\cdot}30$, $P = 0{\cdot}03$) (Fig. 3). U-PBG levels correlated positively with CCL4 levels ($r = 0{\cdot}30$, $P = 0{\cdot}03$, Fig. 3) and negatively with prealbumin levels ($r = -0{\cdot}31$, $P = 0{\cdot}03$) (data not shown). The U-PBG level also correlated positively with the biomarkers of complement activation, including the C3bc/C3 ratio ($r = 0{\cdot}35$, $P = 0{\cdot}01$) and TCC levels ($r = 0{\cdot}33$, $P = 0{\cdot}02$) (Fig. 3). Examining the relationship between AIP disease activity and kidney function, we found that U-PBG levels correlated positively with the levels of S-cystatin C, a biomarker that is increased in the presence of impaired kidney function ($r = 0{\cdot}41$, $P = 0{\cdot}003$) (Fig. 3).

Multiple regression analyses of inflammatory markers and potential confounding parameters that were associated independently with the biochemical disease activity

Multiple linear regression analyses were performed to describe further the relationship between the biochemical disease activity measured as the U-PBG ratio and inflammatory markers in the AIP cases. PTX3 was associated independently with the disease activity in the AIP cases after adjusting for potential confounders and other inflammatory markers (Table 3). We tested several regression models, including various individual or pairs of cytokines, and the coefficient estimates of PTX3 remained unchanged. In addition, kidney function expressed as relative estimated glomerular filtration rate (eGFR) Chronic Kidney Disease

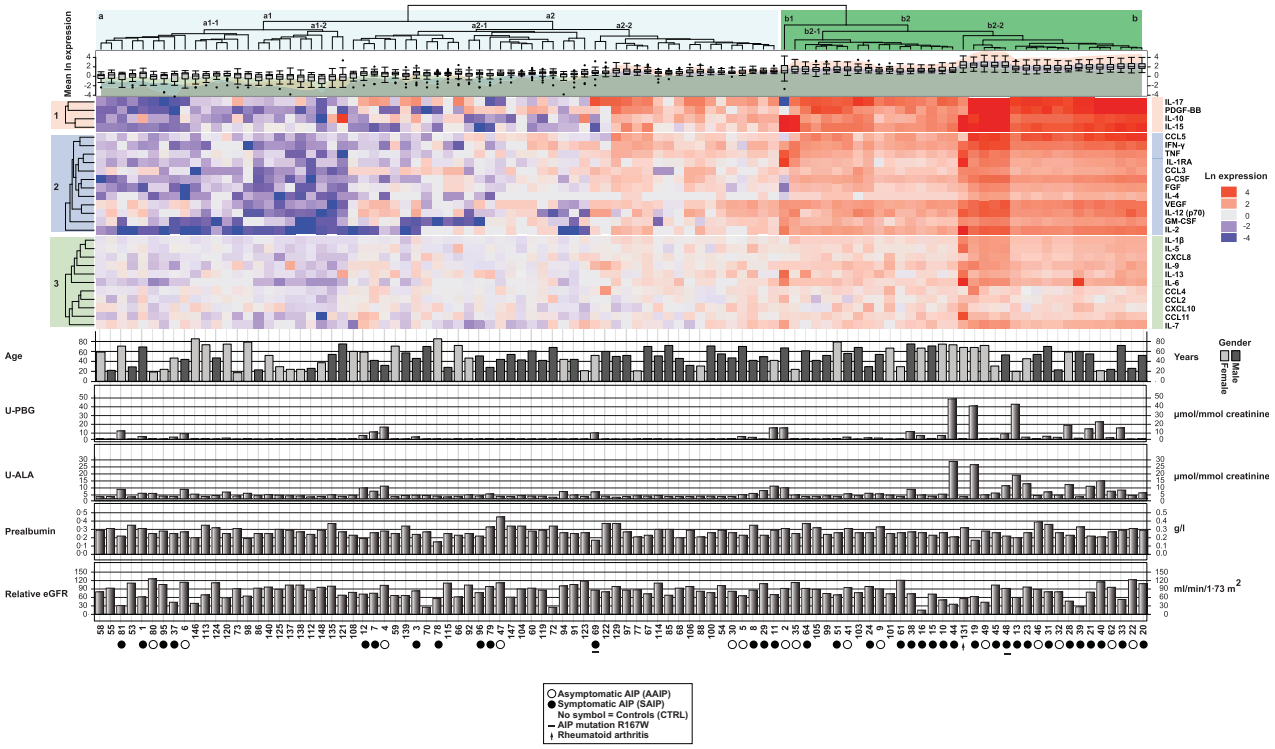

Fig. 2. Hierarchical cluster analysis of cytokines in acute intermittent porphyria (AIP) cases and matched controls. The cytokine levels in each AIP case and matched controls were divided on the median levels of each cytokine in the 50 matched controls. The matrix results were then transformed using natural logarithm. The ln expression levels of individual cytokines are represented by a colour scale, with high values in red, intermediate levels in white and low cytokine levels in blue. The cluster analysis was performed using R with Euclidian distance and complete clustering. The main clusters of AIP cases and controls are named (a) and (b), and the subclusters are named (a1), (a2), (b1) and (b2) on the dendrogram. The cytokines clustered into three clusters named 1, 2 and 3. The mean ln expressions of all the cytokines levels are given as box-plots below the dendrogram. The age in years, gender, U-porphobilinogen (U-PBG) and U-ALA ratio expressed as μmol/mmol creatinine, pre-albumin (g/l) and the relative estimated glomerular filtration rate (eGFR) Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) (ml/min/1·73 m^2) based on cystatin C levels, are shown as a bar graphs below the heatmap for each AIP case and control. Asymptomatic (open circles) and symptomatic (filled circles) AIP cases are indicated below the heatmap, those with the R167W mutation are indicated with underscored symbols.

Epidemiology Collaboration (CKD-EPI) based on cystatin C was a significant predictor in the multiple linear regression analysis (Table 3). The confounders tested including prealbumin, the ratio between energy consumption and energy requirements, catecholamines in urine, smoking pack-years, percentage of alcohol of total energy consumption, fasting C-peptide levels, glucose insulin ratio, age and gender were not significant themselves and altered neither the coefficient estimates nor the *P*-values significantly (data not shown). We did not test drugs as a confounder, as only one AIP case received a porphyrinogenic drug.

Prealbumin, kidney function, C-peptide, insulin and porphyrin precursor levels in symptomatic and asymptomatic cases with acute intermittent porphyria

Serum prealbumin levels (Fig. 4a) and kidney function measured as eGFR (Fig. 4b) were both significantly lower in symptomatic compared to asymptomatic AIP cases. C-peptide (Fig. 4c) and insulin levels (Fig. 4d) after an overnight fast were significantly lower in the symptomatic AIP cases compared to their matched controls. The glucose/insulin ratio (Fig. 4e) and plasma visfatin levels (Fig. 4f) were increased significantly in symptomatic AIP cases compared to matched controls. In comparison, plasma glucagon levels were decreased in both asymptomatic and symptomatic AIP cases compared to their controls (data not shown). Levels of U-ALA and U-PBG were, as expected, increased significantly in both symptomatic and asymptomatic AIP cases (Fig. 4g,h). The number of symptoms in AIP correlated negatively with kidney function and pre-albumin levels, but were not correlated significantly with the inflammatory markers (data not shown).

Discussion

This study found evidence of higher levels of systemic inflammation in AIP cases than in controls matched for

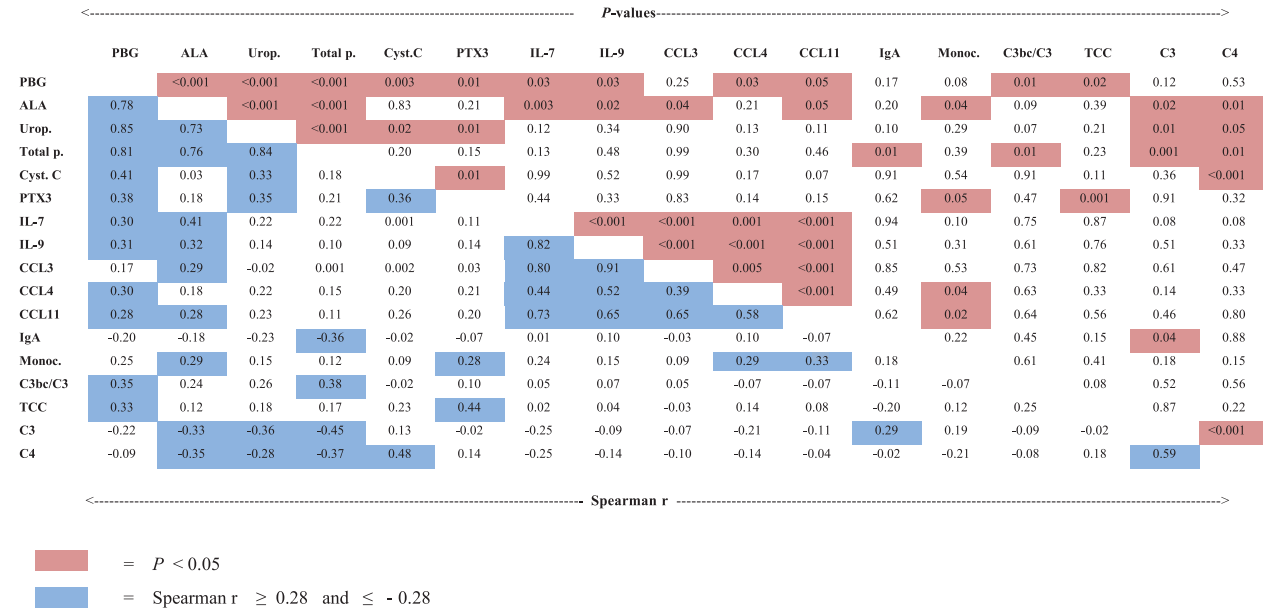

Fig. 3. Correlation matrix of porphyrins and precursors, biomarkers of inflammation and kidney function. The data in the left lower part are Spearman's correlation coefficients, *r*, in the acute intermittent porphyria group ($n = 50$), $r \geq 0{\cdot}28$ and $r \leq -0{\cdot}28$ (blue colour). The pairwise correlation between the different variables is depicted. The corresponding significant *P*-values ($P < 0{\cdot}05$) are indicated by red colour in the upper right part. The following variables were included: urine porphobilinogen ratio (PBG); urine 5-aminolevulinic acid ratio (ALA); uroporphyrin (Urop.); total porphyrins (Total p.); serum cystatin C (Cyst. C); plasma long pentraxin 3 (PTX3); interleukin (IL)-7 and -9; chemokine ligand (CCL) 3, 4 and 11; serum immunoglobulin A (IgA); blood (B) monocyte count (Monoc.); serum C3bc/C3 ratio (C3bc/C3); the plasma levels of the terminal complement complex (TCC); complement C3 and C4.

age, sex and place of residence. Virtually all inflammatory markers, including cytokines, chemokines and growth factors, were elevated significantly in individuals with AIP compared with the matched controls. Many AIP cases had cytokine levels above the reference ranges in the 42 healthy controls. Furthermore, levels of several inflammatory biomarkers were correlated with the U-PBG ratio, a biomarker of AIP disease activity. AIP disease activity was associated with decreased insulin levels.

The association we found between AIP and inflammation may have several explanations. One probable explanation for the increased levels of the proinflammatory cytokines such as TNF and IL-1β, the latter reflecting inflammasome activation [23], may be the accumulation of DAMPs [14,23]. Possible DAMPs candidates in AIP might be ALA, PBG and/or various porphyrins, in addition to tissue injury (Fig. 5). As uric acid, and especially uric acid crystals, are well-known inflammatory stimuli, we cannot rule out totally that they may play a role as DAMPs in AIP cases. DAMPs may stimulate immunocompetent cells either directly by binding to receptors on the immunecompetent cells [12,14,23], or secondarily through proinflammatory, organ damage-related complement or immune cell activation [15,16]. By analogy, diabetes mellitus is another metabolic disease in which DAMPs including glucose, cholesterol and fatty acids are known to be involved in insulin resistance and kidney failure [13]. The reduced insulin levels and slightly enhanced HbA1c and triglyceride levels we found in AIP cases may be due to tissue damage, and indicate that DAMPs play a similar role in AIP to the role they are known to play in diabetes. Given the reduced insulin levels we found in symptomatic AIP cases – accompanied presumably by reduced cellular glucose uptake, reduced negative feedback on ALAS1 and enhanced haem synthesis and release of ALA and PBG – it seems reasonable to argue that symptomatic AIP seems to be associated with disturbed glucose metabolism.

Table 3. Multiple linear regression of *U-PBG (μmol/mmol creatinine), model $R^2 = 0{\cdot}308$

	‡B	95% CI	*P*-value
*P-long pentraxin-3	0·591	0·054 to 1·129	0·032
†Relative eGFR CKD-EPI	−0·014	−0·028 to −0·001	0·036
S-Prealbumin	−5·085	−11·357 to 1·188	0·110

The data are regression coefficients, B, in the acute intermittent porphyria cases ($n = 50$). *Ln-transformed. †Relative estimated glomerular filtration rate (eGFR) calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) cystatin C equation. ‡B = regression coefficients; CI = confidence interval.

The hierarchical cluster analysis of cytokines indicated that many of the AIP cases with the highest levels of several cytokines, especially IL-17, PDGF-BB, IL-10 and IL-15, also had high biochemical disease activity measured by U-PBG and ALA. Gender, age, kidney function and prealbumin could not explain the distribution of AIP cases into clusters (a) and (b). The increased visfatin levels in AIP cases in cluster (b) with high cytokine levels indicate a role of the

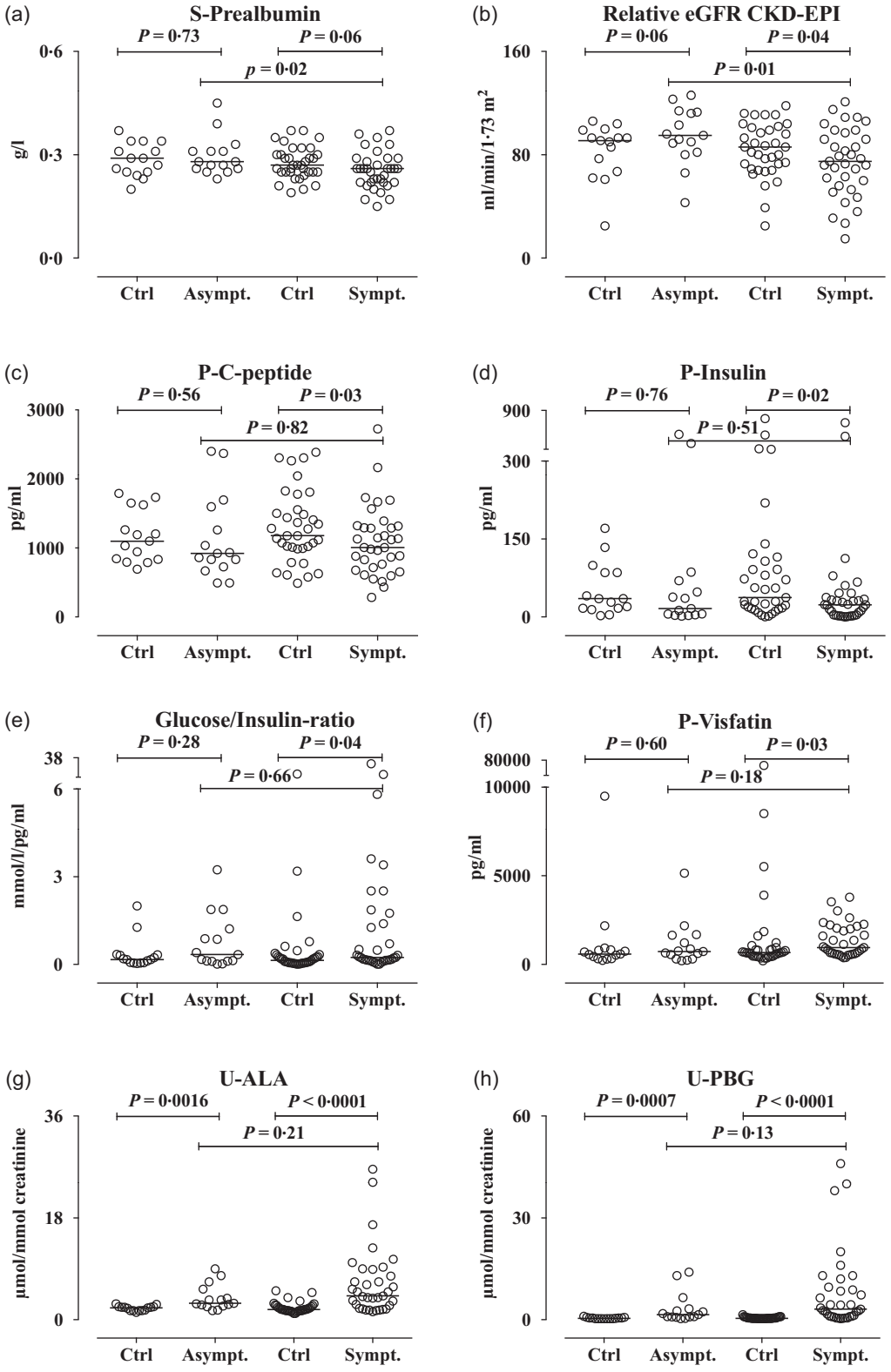

Fig. 4. Prealbumin levels, kidney function, C-peptide, insulin and glucose/insulin ratio, visfatin and porphyrin precursor levels in asymptomatic (Asympt.) and symptomatic (Sympt.) acute intermittent porphyria (AIP) cases compared with controls (Ctrl). (a) Serum prealbumin levels (g/l), (b) relative eGFR (estimated glomerular filtration rate) Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) (ml/min/1·73 m^2), (c) plasma C-peptide (pg/ml), (d) plasma insulin (pg/ml), (e) glucose/insulin ratio (mmol/l/pg/ml), (f) plasma visfatin levels (pg/ml), (g) urine delta-amino levulinic acid (ALA, μmol/mmol creatinine), (h) urine porphobilinogen (PBG, μmol/mmol creatinine), in asymptomatic (Asympt.) and symptomatic (Sympt.) acute intermittent porphyria cases compared with their respective matched controls (Ctrl). Prealbumin, cystatin C, C-peptide, insulin and visfatin were analysed using immunoassays. Glucose, ALA and PBG were analysed using standard biochemical assays. The results from the age- and sex-matched controls ($n = 50$; of these 35 were controls for the symptomatic cases) and the cases with AIP ($n = 50$, 35 symptomatic) are shown as scatter-plots with the median. The paired data were analysed using the Wilcoxon's matched-pairs signed-rank test. The asymptomatic and symptomatic AIP cases were compared using the Mann–Whitney *U*-test.

adipose tissue in the inflammation observed in these cases. The AIP cases with the most elevated cytokines clustered together with controls with inflammatory diseases, e.g. untreated rheumatoid arthritis and ankylosing spondylitis. IL-17 was the cytokine with the highest relative fold increase in the AIP cases and was also identified in the most highly expressed cytokine cluster in the cluster analysis. IL-17 is a proinflammatory cytokine derived from type 17 helper T cells and natural killer cells and is involved in the Th17 response in several chronic inflammatory conditions [24]. IL-1β, IL-6 and TNF, which are involved in the development of Th17 cells, were also elevated significantly in the AIP cases [24]. IL-2 and IFN-γ, which are involved in Th1 responses, were increased moderately in the AIP cases and were also identified in cytokine cluster 2. In comparison, IL-5 and IL-13, which are involved in Th2 responses were only slightly elevated in the AIP cases and were located in cytokine cluster 3. The inflammatory cytokine response in a cluster of AIP cases therefore appears to be dominated by a Th17 response similar to other chronic inflammatory conditions, such as rheumatoid arthritis and multiple sclerosis.

Complement plays an essential role in the innate immunological recognition of DAMPs. Frimat *et al.* showed that

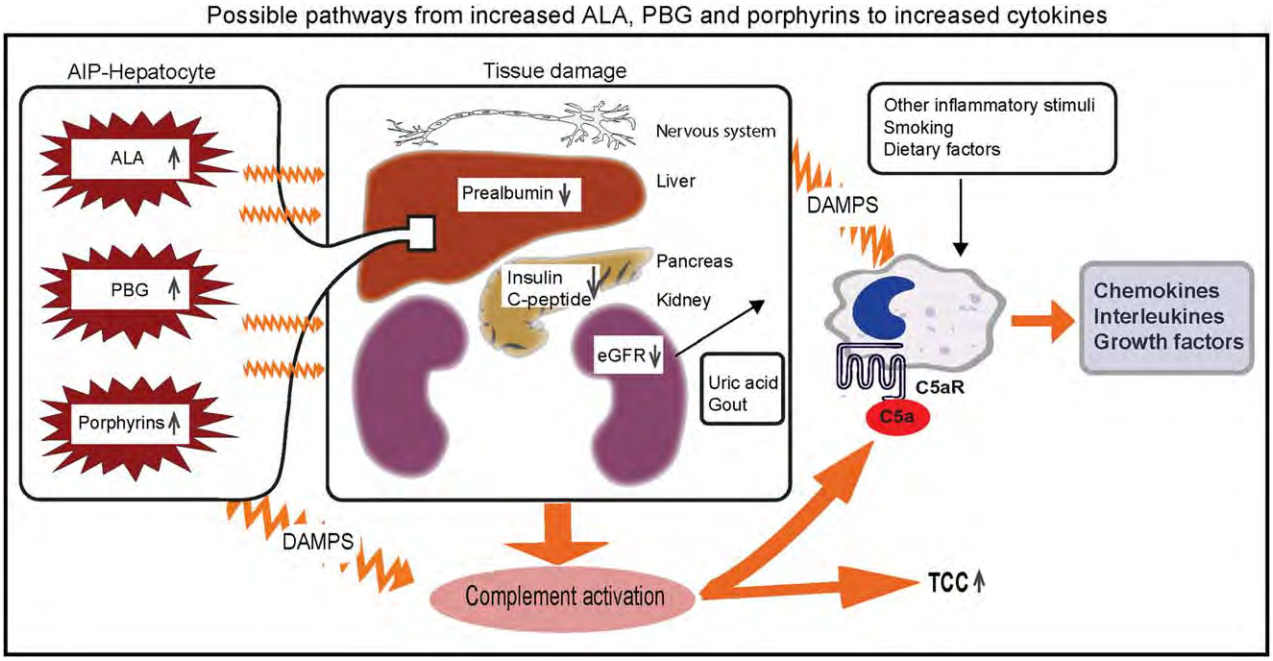

Fig. 5. Possible pathways from increased ALA, PBG and porphyrins to increased cytokines. Increased levels of delta-aminolevulinic acid (ALA), porphobilinogen (PBG) and porphyrins from the liver of acute intermittent porphyria (AIP) patients may cause tissue damage in several tissues, including the nervous system, the liver itself, pancreas and kidneys. Reduced prealbumin indicates inflammation in the liver, and reduced eGFR (estimated glomerular filtration rate) is due to kidney damage. Reduced insulin and C-peptide secretion from the pancreas may be due to reduced hormone secretion or organ damage. Organ damage may activate complement measured as increased levels of the terminal complement complex (TCC) and C5a, which activates cells to release cytokines, interleukins and growth factors. Another pathway of cytokine release may possibly be the release of damage-associated molecular patterns (DAMPS) from damaged cells including uric acid crystals in gout due to reduced kidney function which activate directly or indirectly immune cells to release cytokines, interleukins and growth factors. Whether enhanced levels of ALA, PBG and porphyrins may act as DAMPS themselves remains to be elucidated. Other inflammatory stimuli due to smoking and dietary factors, etc. may also activate cells to release cytokines.

haem, which consists of protoporphyrin IX and iron, activates the alternative complement pathway in serum and on endothelial surfaces [15]. It is well known that complement activation, in particular through the release of C5a, induces cytokine release in immune-competent cells [25]. We observed enhanced complement activation consistently in the AIP cases revealed by increased levels of C3bc and C3bc/C3 ratio in plasma. Thus, we provide new evidence that the complement system may also play a hitherto unrecognized role in the pathophysiology of AIP. The inflammatory response in AIP may be due partly to damage in the liver, kidney or other organs induced by ALA, PBG or porphyrins [12]. Organ damage and cell death may activate complement, leading to immune cell activation and cytokine release similar to the complement activation during sterile inflammation, e.g. myocardial infarction [23,25]. Consistent with this, the AIP disease activity marker U-PBG was correlated with the kidney function biomarker cystatin C, TCC levels and the C3bc/C3 ratio; the last two are indicators of ongoing complement activation. The negative correlation between C3, C4 and the levels of ALA, coproporphyrin and uroporphyrin in urine may be due to complement activation with consumption of C3 and C4 or liver damage with reduced synthesis of C3 and C4. However, the increases in serum ALT reflecting liver cell damage and plasma C3bc levels reflecting complement activation were modest in the cases with AIP compared with the controls, suggesting that other additional mechanisms are probably involved.

The multiple linear regression analyses showed that PTX3 was associated independently with AIP biochemical disease activity. This association of AIP with smouldering inflammation might provide pathophysiological insights into the long-term consequences of AIP, including the enhanced risk of HCC and chronic kidney disease. PTX3 is produced by innate immune cells such as granulocytes, macrophages and dendritic cells and is involved in inflammation, arteriosclerosis and tissue repair [26]. PTX3 is a pattern recognition molecule able to interact with several complement proteins, including binding to C1q, which leads to activation of the classical pathway. As PTX3 was correlated with enhanced disease activity, enhanced complement activation and decreased kidney function, we speculate that PTX3-induced complement activation may be involved in the organ damage and/or tissue repair observed in the AIP cases [26]. Therefore, our data indicate that PTX3 may play a key role in the low-grade inflammation observed in the AIP cases. However, as the data set included 50 AIP patients, the results of the multiple linear regression analysis must be confirmed in larger studies. In this study, we held the number of variables in the multiple regression model low because of the limited number of cases and because it is highly probable that several of the cytokines expressions are inter-related. Furthermore, we cannot exclude an unknown confounder that we have not taken into account in the multiple linear regression analyses.

Our data indicate that the basal level of inflammation might be a driving factor in disease development. First, many AIP cases had cytokine levels above the reference ranges in the healthy controls. Secondly, the PTX3 level was associated independently with disease activity in the multiple regression analysis. Thirdly, this study indicates that low-grade inflammation is associated with enhanced biochemical disease activity in AIP. Furthermore, inflammation induced by bacterial and viral infections may provoke acute attacks in AIP. We speculate that this low-grade inflammation may be involved in the development of organ damage, HCC, reduced kidney function and enhanced disease activity in some of the AIP cases. The decreased kidney function in AIP was shown previously to be associated with the development of tubulointerstitial nephropathy and focal cortical atrophy with progressive arteriosclerosis in the kidney [27]. The prevalence of HCC is high among AIP patients [3,6], but the mechanism(s) of carcinogenesis in AIP are unknown. HCC usually arise in a diseased liver with a dynamic inflammatory microenvironment. Several growth factors, including FGF, PDGF and VEGF, may act as possible pro-angiogenetic factors secreted by stromal cells after chronic liver injury, and IL-17 may be involved in tumour progression [28]. In this study, we determined that several of these cytokines and growth factors were elevated outside attacks, also compared with the reference ranges, and we therefore speculate that they may be involved in HCC development in AIP patients. However, other mechanisms may also possibly explain the enhanced risk of HCC in AIP patients, including the direct or indirect carcinogenicity of ALA [3].

Immune-competent cells in the liver may also contribute to inflammation in AIP. The innate hepatic immune system plays a role in defence against infection, liver damage and repair [29]. Furthermore, prealbumin levels correlated negatively with the number of symptoms (data not shown) and with several inflammatory variables. In line with this, prealbumin levels were decreased in symptomatic AIP cases, due most probably to inflammation in the liver. The level of prealbumin correlated negatively with levels of the AIP disease activity biomarker U-PBG, confirming that hepatic inflammation is important in the pathophysiology of AIP [18]. It is unlikely that these lower prealbumin levels relate to impaired nutritional status in AIP cases, as BMI and nutritional intake (data not shown) were similar in both groups. Dowman *et al.*'s finding that two of three liver graft recipients from a patient with AIP developed AIP symptoms and increased U-PBG levels suggests that AIP symptoms are derived only from metabolites from the liver [30]. The finding that liver transplantation is a cure for AIP and normalizes the excretion of ALA and PBG confirms this hypothesis [10]. Both porphyrins or porphyrin precursors from the liver may thus induce inflammation and organ damage and mediate the symptoms in AIP. However, most inflammatory biomarkers and cytokines

were not correlated with ALA, U-PBG or porphyrin levels, suggesting that the direct activation of immune-competent cells by porphyrins and porphyrin precursors may only partially explain our results. It thus remains to be elucidated whether enhanced levels of ALA, PBG and porphyrins may act as DAMPs themselves in AIP.

The inflammation found in AIP patients may also be due to ALA, PBG and porphyrin-induced free radical release, and consequent cytokine release [17,31,32]. Free radicals may stimulate inflammation through direct activation of the transcription factor nuclear factor kappa B (NF-κB), leading to the release of IL-6, IL-8 and other cytokines [23]. The role of free radicals in the AIP-associated inflammation should be examined in future experimental and clinical studies. Furthermore, the cytokine profile during AIP attacks and in other AIP mutations should be addressed in future studies.

Because the activation of adaptive immunity also stimulates cytokine release, we searched for autoimmune diseases and examined the levels of different autoantibodies in the AIP cases and controls. No significant differences were found in the frequency of inflammatory diseases and anti-inflammatory medications between the controls and cases with AIP, indicating that autoimmunity and adaptive immunity responses do not account for the inflammatory response. However, the prevalence of gout was enhanced non-significantly in AIP cases. Furthermore, the reduced baseline kidney function in the symptomatic AIP patients might have had an impact upon the levels of some cytokines. The accumulation of uric acid and other metabolites in kidney failure may induce inflammation. Reduced kidney function has been shown to increase haem synthesis and the excretion of ALA and PBG in porphyric mice [33]. This is in line with our finding that reduced kidney function was the only confounding factor that predicted the biochemical disease activity in the AIP cases.

In conclusion, in this first study of its kind, to our knowledge, we found that numerous inflammatory markers, including all 27 cytokines measured, were increased in individuals with AIP compared with matched controls, and that several of these correlated with the biomarker of disease activity U-PBG. Taken together, these two findings point towards a new pathophysiological mechanism in AIP. Our results suggest that probable porphyrin/precursor-related hepatic damage may drive inflammation in AIP. The fact that symptomatic AIP cases also had evidence of reduced insulin release may indicate that decreased glucose uptake into cells may explain accelerated haem synthesis in at least some AIP cases. This metabolic similarity between diabetes mellitus and AIP, both of which show decreased insulin release and low-grade inflammation, could usefully be studied further. In particular, the effect of treatment with a high carbohydrate intake and a small dosage of insulin in symptomatic AIP patients to lower the disease activity and prevent attacks should be examined in future studies.

Acknowledgements

This work was supported by grants from Northern, Southern and Eastern Norway Regional Health Authorities, The Research Council of Norway and the European Community's Seventh Framework Program under grant agreement no. 602699 (DIREKT).

Disclosure

The authors declare that they have no competing financial or other interest in relation to their work.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's website.

Table S1. Cytokine levels in the acute intermittent porphyria (AIP) cases in clusters (a) and (b).

Supplementary Table 1. Cytokine levels in the AIP cases in cluster (a) and (b)

	AIP cases in cluster (a)	AIP cases in cluster (b)	P-value	[§]Fold-change
Age (years)	47 (42-57)	56 (30-70)	0.23	1.2
U[b]-PBG[c] (µmol/mmol creatinine)	2.7 (0.6-7.1)	2.4 (0.9-12.3)	0.43	1.1
U-ALA (µmol/mmol creatinine)	3.8 (1.9-6.5)	4.0 (2.6-9.0)	0.42	1.1
S-Prealbumin (g/L)	0.26 (0.22-0.29)	0.26 (0.23-0.31)	0.44	1.0
Relative eGFR[d] CKD-EPI, (ml/min/1.73 m^2)	83 (67-105)	85 (58-98)	0.77	1.0
P[f]-Visfatin (pg/ml)	587 (406-666)	1479 (800-2164)	0.0001	2.5
P[f]-IL-1β[e] (pg/ml)	0.86 (0.81-0.95)	3.0 (1.9-4.3)	<0.0001	3.5
P[f]- IL-1RA[e] (pg/ml)	18 (8.4-28)	154 (99-286)	<0.0001	8.5
P[f]-IL-2[e] (pg/ml)	0.72 (0.02-1.8)	14 (6.0-20)	<0.0001	19
P[f]-IL-4[e](pg/ml)	1.0 (0.2-1.0)	3.0 (2.0-5.0)	<0.0001	3.0
P[f]-IL-5[e] (pg/ml)	1.3 (0.9-1.4)	3.0 (1.7-5.0)	<0.0001	2.3
P[f]-IL-6[e] (pg/ml)	1.6 (0.9-2.8)	9.0 (5.0-14)	<0.0001	5.6
P[f]-IL-7[e] (pg/ml)	4.5 (2.3-6.8)	11.0 (6.8-19)	<0.0001	2.4
P[f]-CXCL8[g] (pg/ml)	5.0 (4.0-6.0)	12. (8.0-18)	<0.0001	2.4
P[f]-IL-9[e] (pg/ml)	7.5 (4.3-10.9)	19 (15-27)	<0.0001	2.5
P[f]-IL-10[e] (pg/ml)	0.13 (0.02-0.73)	4.0 (1.8-9.0)	<0.0001	31
P[f]-IL-12(p70)[e] (pg/ml)	5.5 (1.6-9.0)	22 (14-36)	<0.0001	4.0
P[f]-IL-13[e] (pg/ml)	2.6 (2.0-4.8)	6.0 (4.0-12)	0.0002	2.3
P[f]-IL-15[e] (pg/ml)	0.009 (0.005-0.1)	4.0 (1.9-8.0)	<0.0001	444
P[f]-IL-17[e] (pg/ml)	2.0 (0.02-5.8)	45.0 (18-80)	<0.0001	23
P[f]-CCL2[g] (pg/ml)	13.5 (10.0-19.0)	19.0 (13.0-23.5)	0.0276	1.4
P[f]-CCL3[g] (pg/ml)	2.0(0.89-2.0)	5.0 (3.0-7.3)	<0.0001	2.5
P[f]-CCL4[g] (pg/ml)	48.5 (34.0-60.0)	66.0 (51.8-76.2)	0.0044	1.4
P[f]-CCL5[g] (pg/ml)	142 (103-378)	7504 (1929-14215)	<0.0001	53
P[f]-CCL11[g] (pg/ml)	55 (44-72	92 (77-120)	<0.0001	1.7
P[f]-FGF basic (pg/ml)	10 (4.5-13)	64 (35-96)	<0.0001	6.4
P[f]-G-CSF (pg/ml)	3.5 (1.4-7.8)	67 (35-108)	<0.0001	19
P[f]-GM-CSF (pg/ml)	4.0 (0.1-7.8)	27 (16-40)	<0.0001	6.8
P[f]-IFN-γ (pg/ml)	6.8 (2.2-24)	226 (107-359)	<0.0001	33
P[f]-CXCL10 (pg/ml)	837 (540-992)	1071 (801-1258)	<0.0259	1.3
P[f]-TNF (pg/ml)	7.5 (4.8-16)	70 (41-116)	<0.0001	9.3
P[f]-VEGF (pg/ml)	5.5 (2.3-8.0)	26 (18-46)	<0.0001	4.7
P[f]- PDGF-BB (pg/ml)	2.0 (0.1-5.0)	113 (26-293)	<0.0001	57

The data represent the median values and IQR. The data were analyzed using the Mann-Whitney U test on AIP cases (n= 20) in cluster (a) vs. the AIP cases (n = 30) in cluster (b) in the hierarchical cluster analysis. The *P* values are exact, two-tailed. [§]Fold-change, median value in cluster (b)/median value in cluster (a). [a]AIP, acute intermittent porphyria; [b]U, urine; [c]PBG, porphobilinogen; [d]S, serum; [e]IL, interleukin; [f]P, EDTA plasma; [g]CCL, chemokine ligand.

Paper II

Molecular Genetics and Metabolism 128 (2019) 254–270

Contents lists available at ScienceDirect

Molecular Genetics and Metabolism

journal homepage: www.elsevier.com/locate/ymgme

Lifestyle factors including diet and biochemical biomarkers in acute intermittent porphyria: Results from a case-control study in northern Norway

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ARTICLE INFO

Keywords:
Diet
Alcohol
Smoking
Porphyria
Resistin
Human

ABSTRACT

Background: Lifestyle factors, including a low intake of carbohydrates, dieting, alcohol consumption, cigarette smoking and stress are some of the possible triggers of attacks in acute intermittent porphyria (AIP). The influence of lifestyle factors, including energy intake, diet and alcohol consumption on the biochemical disease activity in AIP and biochemical nutritional markers were examined.

Methods: A case-control study with 50 AIP cases and 50 controls matched for age, sex and place of residence was performed. Dietary intake was registered using a food diary in 46 matched pairs. Symptoms, alcohol intake, stress and other triggering factors of the last AIP attack were recorded on questionnaires. Porphyrin precursors, liver and kidney function markers, vitamins, diabetogenic hormones and other nutritional biomarkers were analyzed by routine methods. The Wilcoxon matched-pairs signed rank test was used to compare the cases vs. controls. The Spearman's rank correlation coefficient was used on the cases.

Results: Increasing total energy intake was negatively correlated with the biochemical disease activity. The intake of carbohydrates was lower than recommended, i.e., 40 and 39% of total energy intake in the AIP cases and controls, respectively. The plasma resistin level was significantly higher ($p = .03$) in the symptomatic than asymptomatic cases. Plasma insulin was lower in those with high porphobilinogen levels. The intake of sugar and candies were higher in the AIP cases with low U-delta aminolevulinic acid (ALA) levels ($p = .04$). Attacks were triggered by psychological stress (62%), physical strain (38%), food items (24%) and alcohol (32%) in the 34 symptomatic cases. Alcohol was used regularly by 88% of the cases (3.2 g ethanol/day) and 90% of the controls (6.3 g/day), but the intake was significantly lower in symptomatic than in asymptomatic cases ($p = .045$).

Conclusion: A high intake of energy, sugar and candies and a higher insulin level were associated with a lower biochemical disease activity. The resistin level was higher in the symptomatic than the asymptomatic cases. AIP patients drink alcohol regularly, but the intake was significantly lower in the symptomatic cases.

Trial registration: ClinicalTrials.gov Identifier: NCT01617642.

1. Introduction

Acute intermittent porphyria (AIP) is an autosomal, dominant, inherited and rare metabolic disorder. The disease is caused by a mutation in the hydroxymethyl bilan synthase (HMBS) gene, which encodes an enzyme in the heme biosynthesis [1,2]. The prevalence of heterozygous AIP mutations in the European population is between 1/75,000 to 10–20/100,000 [3,4], and about 600/100.000 in the Saltdal area in Nordland County, Norway. A recent study has shown a higher prevalence of 1/1675 of the HMBS mutation in the general population in

Abbreviations: AIP, Acute intermittent porphyria; U-ALA, urine 5-Aminolevulinic acid; ALAS1, 5-Aminolevulinic acid synthase 1; BMI, Body mass index; HCC, Hepatocellular carcinoma; U-PBG, Urine porphobilinogen; PTX3, Long-pentraxin 3

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https://doi.org/10.1016/j.ymgme.2018.12.006
Received 29 August 2018; Received in revised form 28 November 2018; Accepted 9 December 2018
Available online 10 December 2018

Europe [5]. AIP is typically asymptomatic, and approximately 90% of those with AIP mutations never experience symptoms [2].

AIP symptoms may be triggered by mental or physical stress, alcohol, smoking, certain medications, infections or hormonal factors [1–3]. Fasting and fever can also induce heme oxygenase activity; the free heme pool is then reduced, and 5-aminolevulinic acid (ALA) synthase-1 is induced [1]. Common symptoms during the acute neurovisceral attacks include abdominal pain, nausea, vomiting, constipation, dark red urine and muscle weakness [1,6]. Bulbar or phrenic nerve paresis may occasionally lead to life-threatening respiratory failure [1,7]. A high prevalence of hypertension and renal impairment has been documented [4]. A few patients, mainly females, experience recurrent attacks. Patients who have a AIP gene mutation and have experienced symptoms of AIP are referred to as symptomatic AIP, while those who never had symptoms are referred to as asymptomatic [1]. A recent study by Lenglet et al. indicated oligogenic inheritance for AIP, and this could be the reason why only a few patients are symptomatic and why some are high and some are low excreters of ALA and PBG [8].

The first line of treatment is to remove any known triggering factor. In hospital, intravenous infusion of glucose and/or of synthetic heme can be administered [9]. The inhibiting effect of glucose on the heme synthesis is probably related to peroxisome proliferator activated cofactor 1α (PGC-1α) [10]. Liver transplantation cures AIP completely, indicating that liver metabolism plays an important part in pathogenesis [11].

The dietary advice from the American Porphyria Foundation (APF) to patients with AIP is to avoid prolonged fasting, and to have a carbohydrate intake of 55–60% of the total energy intake (E%). Further, "The carbohydrates should preferably not include large amount of refined sugars, but during less severe attacks intake of 300 g/day of dextrose or other metabolizable carbohydrate is recommended." The Nordic Nutrition recommendations for the general population recommends a carbohydrate intake of 45–60 E% [12]. A recent national diet survey (Norkost 3) showed that the average diet in Norway consisted of 43–44 E% as carbohydrates [13]. Balwani et al. stated that a "sustained adherence to a high-carbohydrate diet does not prevent attack and is not recommended" [14]. Crash dieting and bariatric surgery can precipitate porphyric attacks [15].

Ethanol may precipitate porphyric attacks in some, but not all persons with AIP mutations, and they are advised to avoid alcohol [16–18]. Smoking has been reported to be associated with a high frequency of AIP attacks [19,20]. Low levels of vitamins and micronutrients can influence overall health [12,21,22], and especially the heme biosynthesis [23]. Repeated hemin infusions may have adverse effects in AIP patients, possibly through a high iron load and inflammation [24]. Naik et al. reported that stress was a direct contributor to attacks in 56% of the examined 16 AIP cases with recurrent attacks [25].

Few studies have examined the role of the dietary intake of sugar, total carbohydrates and other dietary factors on the disease activity in AIP and simultaneously analyzing biochemical biomarkers. We hypothesized that the total energy intake and intakes of carbohydrates, sugar, alcohol, iron or diabetogenic hormones could be different in the asymptomatic vs. the symptomatic AIP cases.

2. Methods

2.1. Participants and study design

A case-control study was conducted, comparing 50 AIP cases with 50 controls matched for age, sex and place of residence. Of these cases, 15 were asymptomatic and 35 were symptomatic. Most cases had the AIP mutation W198X (n = 48), while two patients had the R167W mutation. The study was approved by the Regional Ethics Committee for Medical and Health Research. We recruited all the participants from September to November 2012, and all participants were examined once. The controls were randomly selected from the registry of residents from the Norwegian Tax Administration. The exclusion criteria for both groups were as follows: age under 18 or lacking competence to provide consent to participate in the study. An additional exclusion criterion for the AIP cases was absence of the AIP gene mutation, and one person was excluded for this reason. The exclusion criteria for the controls were also proven AIP or other porphyria, none were excluded. Four members of the control group withdrew from the study immediately before the study began and they were replaced by new matched controls, while all AIP cases agreed to participate.

2.2. Recording of symptoms, triggering factors and diet

The cases with AIP were questioned by a medical doctor regarding the presence or absence of AIP symptoms, the time of diagnosis, the number and durations of attacks, and about triggering and relieving factors during attacks. All participants were asked about their smoking and alcohol habits, physical activity, emotional stress, medications, nutritional supplements, present and chronic diseases and any surgeries prior to the interview. The dietary intake was registered using a 7-day diet logbook and was obtained from 46 cases and 46 matched controls. Of the 35 symptomatic AIP cases, 34 filled out a diet log-book. A clinical nutritionist instructed the participants on how to use the 7-day diet logbook. The logbook was scanned using the Teleform program, version 6.0 (Datascan, Oslo, Norway). Daily intakes of energy and nutrients were computed using the food database and software system (KBS, version 7) developed at the Department of Nutrition, University of Oslo. The food database (AE-10) is based mainly on the official food composition table. Energy was calculated as (g carbohydrate × 17 kJ/g) + (g protein × 17 kJ/g) + (g fat × 27 kJ/g) + (g fiber × 8 kJ/g). Resting metabolic rate (RMR) was calculated by Mifflin's formula, for women: RMR (kcal) = 10 × weight (kg) + 6.25 × height (cm) – 5 × age (years) – 161; for men: RMR (kcal) = 10 × weight (kg) + 6.25 × height (cm) – 5 × age (years). Energy requirement was calculated multiplying RMR with a physical activity factor (PAL) [12]. The PAL factors for the participants were set by individual evaluation of the description of their work and leisure physical activity. Energy requirement was converted from kcal to kJ by ×4.184. Sugar was regarded as "added sugar" and did not include naturally occurring sugars. Vitamin A was calculated as the sum of retinol and 1/12 beta-carotene. Nutritional supplements were included in the dietary calculations. Serial diastolic blood pressure was automatically measured over a 20–30 min period, every second minute using a CAS 740 monitor (CAS Medical Systems Inc., Branford, CT, USA). The body weight, length and waist/hip-ratio were measured according to the WHO recommendations.

2.3. Blood sampling

Blood samples were obtained between 8 and 9 am by venipuncture after an overnight fast. Vacuette citrate, EDTA and serum tubes were used (Greiner Bio-one Gmbh, Frickenhausen, Germany). The EDTA tubes for analyzing diabetogenic hormones in plasma were immediately placed on crushed ice, centrifuged at 1500*g* for 15 min at +4 °C, and the plasma was stored at −80 °C until analysis.

2.4. Routine biochemistry tests

Hematology parameters were analyzed on a Siemens ADVIA 2120i Hematology System (Siemens Healthcare Diagnostics Ltd., Camberly, UK). Serum (*S*)-Na^+, K^+, Mg^{2+}, creatinine, urine creatinine, S-iron and other routine clinical chemistry parameters were analyzed on a ADVIA®1800 system (Siemens Medical Solutions Diagnostics, Japan) using reagents from Siemens Healthcare Diagnostics Ltd. The relative estimated glomerular filtration rate (eGFR) was calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) using

the creatinine equation and expressed as ml/min/1.73 m^2 [26]. Serum ferritin, folate and vitamin B12 were analyzed on a ADVIA Centaur® XP system (Siemens). A BN ProSpec® nephelometer (Siemens Healthcare Diagnostics Ltd.) was used to measure S-prealbumin and alpha-1 antitrypsin. Urine porphobilinogen (U-PBG) and U-ALA were analyzed using a kit from Bio-Rad Laboratories (Munich, Germany). Total and fractionated porphyrins were analyzed by high-performance liquid chromatography (HPLC) as previously described [27]. Epinephrine, norepinephrine and vanillylmandelic acid (VMA) in a random morning urine sample were analyzed by HPLC using kits from Chromsystems Instruments & Chemicals GmbH (Munich, Germany). Total 25-OH Vitamin D were analyzed using kits from Chromsystems and an Ultimate 3000 HPLC system (Thermo Scientific, Waltham, MA, USA) equipped with a TSQ Quantum Access MAX triple quadrupole mass spectrometry detector.

2.5. Enzyme immunoassay

A Bio-Plex pro human diabetes 10-plex kit from Bio-Rad Laboratories Inc. (Hercules, CA, USA) was used to measure plasma (P)-insulin, visfatin, resistin and C-peptide in EDTA plasma on a Bio-Plex 200 system from Bio-Rad. The Enzygnost F1 + 2 (monoclonal) kit (Dade Behring, Marburg GmbH, Germany) was used to analyze prothrombin fragment 1 + 2 (PTF1.2) in EDTA plasma. Long-pentraxin 3 (PTX3) was analyzed by an enzyme-linked immunosorbent assay (ELISA) kit from R&D Systems Inc. (Minneapolis, MN, USA). The optical density was measured on a MRX microplate reader (Dynex Technologies, Denkendorf, Germany).

2.6. Statistical analyses

The sample consisted initially of 50 cases with AIP and 50 controls matched for age, sex and residence. The dietary data arise from (n = 46) AIP-cases and their 46 matched controls. When comparing groups of AIP cases with high (> 1.5) and low (≤1.5 μmol U-PBG/mmol creatinine) U-PBG levels and high (> 3.9) and low (≤3.9 μmol U-ALA/mmol creatinine) ALA levels, we used data from all 47 AIP cases that had filled in the dietary logbook. We used the Wilcoxon matched-pairs signed rank test on the matched case-control data. The Spearman's rank correlation coefficient was calculated, giving an r and two-tailed p. The Mann-Whitney *U* test was used on non-matched data, i.e., on symptomatic vs. asymptomatic cases. The Fischer's exact test was used on categorical variables. Statistical significance was considered at $p < .05$. The statistical analysis was performed using Prism version 6.0 from GraphPad Software Inc. (CA, USA).

3. Results

3.1. Baseline characteristics of cases with acute intermittent porphyria and matched controls

The baseline characteristics of the study population, which consisted of AIP cases and controls matched for age, sex and place of residence, are shown in Table 1. Most variables were similar in the two groups (Table 1). Of the 50 controls and 50 AIP cases, 6 and 10 persons, respectively, were current smokers (p = .55) [26]. Of the 34 symptomatic AIP cases, 15% had experienced one AIP attack ever, and 85% have had more than one porphyria attack. Some of them have had more than twenty AIP attacks during their life. Of the 34 symptomatic cases 50% had been treated with glucose intravenously and 21% with Normosang intravenously, during their AIP attacks. None of the AIP cases were under an AIP attack, and none were having glucose or Normosang treatment at the time of study.

Overweight defined as BMI 25–29.9, was found in 52% of the AIP cases and 38% of the matched controls. Similarly, 24% of the cases and 30% of the controls had obesity (BMI ≥ 30). Of the 50 controls and 50 cases, 3 controls and 4 cases had diabetes mellitus. No significant differences in the frequency of inflammatory diseases were observed, except for a near-significant difference in the number of persons with gout [26]. Statins were used by 7 AIP cases and 8 of their matched controls. However, only 5 of 46 (11%) of the AIP cases had an adequate total energy intake, i.e. 89% had a too low energy intake according to the dietary logbook. Of the controls, 4 of 46 (9%) had an adequate total energy intake, while 42 (91%) had a too low energy intake.

3.2. Dietary intake of carbohydrates, sugar, protein, fat and fatty acids in cases with acute intermittent porphyria and matched controls

The dietary intake of carbohydrates as E% was similar, but lower than the Nordic 2012 recommendations in 78% of the AIP cases and 91% of the controls (Fig. 1A). The intake of added sugar was similar between the groups but was higher than the recommendations in 37% of cases and 26% of the controls (Fig. 1B). The intake of protein (Fig. 1C), fiber (Fig. 1D), total fat (Fig. 1E), saturated fatty acids (SFA, Fig. 1F) and monounsaturated fatty acids (MUFA, Fig. 1G) was similar in the cases and controls. However, the intake of fiber given as g/MJ (Fig. 1D) was lower and the intakes of total fat and SFA given as E% (Fig. 1E, F) were higher than recommended. The intake of polyunsaturated fatty acids (PUFA) was significantly lower (p = .04) in cases than in the controls, and 15% of cases and 9% of controls had a lower intake of PUFA given as E% intake than recommended (Fig. 1H).

3.3. Dietary intakes of vitamin D, calcium, vitamin B12, folate and the corresponding serum levels in cases with acute intermittent porphyria and matched controls

The intake of vitamin D was similar in the two groups, but below the recommended 10 μg/day in 63% of the AIP cases and 54% of the controls (Fig. 2A). The intake of calcium was insufficient in 65% of cases and 50% of controls. The total S-calcium levels were within the normal reference limits in 98% of cases and 96% of the controls. The vitamin B12 intake was similar and sufficient in both groups (Fig. 1E), and S-vitamin B12 levels were above the lower reference limit in both cases and controls (Fig. 1F). One case and one control used vitamin B12 injections (Fig. 1F). In comparison, the intake of folate was lower than recommended in 82% of cases and 78% of the controls, when considering men and postmenopausal women (Fig. 2G), and 88% of cases and 78% of controls, when considering premenopausal women. However, only 8% of the controls had S-folate levels below the lower reference limit (Fig. 2H).

3.4. Intake of vitamin supplements

Doctor-prescribed regular vitamin B12 injections and vitamin D medications was used by 2% of cases and 2% of controls (App Table A). Of the cases, 2% used isotretinoin (vitamin A), and 2% of controls used magnesium supplements (App. Table A). Iron medication was taken by 6% of cases and 12% of the controls. Over-the-counter vitamin supplements were taken by several cases and controls, including vitamin B, vitamin D, multi vitamins, vitamin K and herbal remedies (Appendix Table A).

3.5. Homocysteine levels, kidney function, calcium, albumin, parathyroid hormone and porphobilinogen levels in asymptomatic and symptomatic cases and matched controls

The homocysteine levels in plasma were significantly higher in the symptomatic AIP cases compared to their matched controls, but were similar in the asymptomatic cases and controls (Fig. 3A). The kidney function given as eGFR was significantly lower (p = .0008) in the symptomatic than in the asymptomatic AIP cases (Fig. 3B). Diastolic

Table 1
Baseline characteristics of the study population.

Characteristics	Controls			Cases			p	RI[a] (Norway)	RDA[b], AI (Am.)
	25%ile	Median	75%ile	25%ile	Median	75%ile			
Age, years	31.8	52	68	31.8	52	68.3	0.39		
Height, cm	163	172	179	164	171	180	0.84		
Weight, kg	71.5	80.3	91.4	69.4	81	92.3	0.6		
BMI, kg/m^2	23.8	26.8	30	25	27	29.8	0.92	20–25[c]	18.5–24.9[c]
Hip circumference, cm	93.9	98.8	106	93.9	99.4	107	0.75		
Waist circumference, cm	89	94.6	104	89.3	94.8	106	0.84	≤93/≤79[d,e]	
Waist/Hip –ratio	0.9	0.97	1.01	0.88	0.98	1.02	> 0.99		
RMR[f] W[g], kJ/day	5054	5448	6125	4971	5515	6268	0.67		
Energy req.[h], W[g] kJ/day	9117	9690	10,933	7786	9590	11,343	0.45		
Energy intake W[g], kJ/day	5532	7266	9001	5978	7141	7947	0.84		
RMR[f] M[i], kJ/day	6335	7289	7540	6519	7234	7728	0.12		
Energy req.[h], M[i] kJ/day	10,899	11,983	13,539	11,288	13,188	13,866	0.30		
Energy intake M[i], kJ/day	7735	8988	11,111	8209	9383	12,058	0.53		
RMR[f] all, kJ/day	5581	6318	7355	5535	6523	7397	0.40		
Energy req.[h], all, kJ/day	9786	11,016	12,426	9678	11,527	13,334	0.81		
Energy intake all, kJ/day	6996	8367	10,621	7099	8162	10,763	0.72		
Protein, g/day	65.6	80.6	105	68.8	77.6	98.9	0.92		56/46[e]
Fat, g/day	72	86.8	112	70	84.5	115	0.76		
Saturated fat, g/day	29	36.8	43.9	28.7	36	47.6	0.38		
C-MUFA[l], [m], g/day	23	29.1	36.9	21.9	28.4	37.1	0.58		
C-PUFA[k], g/day	12.5	16.7	20.7	10.8	13.8	20.5	0.19		
Cholesterol, mg/day	264	356	434	280	383	483	0.56		
Carbohydrates, g/day	156	190	211	166	201	248	0.12		130
Sugar, g/day	27.3	36.6	50	30.7	42.8	56	0.18		
Vitamin A, µg/day	645	929	1074	707	909	1160	0.25		
Retinol, µg/day	515	701	893	568	750	945	0.31	900/700[e]	900/700[e]
B-carotene, µg/day	1194	1875	3104	1101	1728	2368	0.69		
Tocopherol, mg/day	8.0	14.2	20.8	9.35	12.3	17.3	0.79	10/8[e]	15
Thiamin, mg/day	1.00	1.31	1.85	1.01	1.32	1.92	0.94	[l]	1.2/1.1[e]
Riboflavin, mg/day	1.27	1.55	2.42	1.26	1.66	2.52	0.77	[m]	1.3/1.1[e]
Vitamin B6, mg/day	1.03	1.44	2.13	1.12	1.49	2.32	0.67	1.5/1.2[n]	1.3[n]
Vitamin C, mg/day	44.8	63.5	145	51	73.5	134	0.72	75	90/75[e]
Fe, mg/day	7.8	9.4	14.4	7.7	9.8	14.3	0.48	9/15[e,o]	8/18[e,p]
Potassium, mg/day	2716	3537	4325	2637	3201	4108	0.49	3500/3100[k]	4700
Mg, mg/day	283	332	412	258	289	406	0.33	350/280 [e]	420/320[q,e]
Zn, mg/day	8.0	10.6	13.3	7.9	10.5	15.8	0.39	9/7[e]	11/8[e]
Se, µg/day	34.5	49.5	73.3	37.5	52.5	75.5	0.77	60/50[e]	55
Cu, mg/day	0.81	1.1	1.34	0.87	1.08	1.61	0.59	0.9	0.9
Phosphorus, mg/day	1147	1457	1733	1156	1392	1799	0.84	600[r]	700
Sugar, E%[s]	6.3	7.5	10.6	6.1	9.1	12.4	0.1	< 10	< 10
Alcohol, E%[s]	0	2.5	4.8	0	0.95	4.1	0.55	< 5	

The data represent median values and interquartile ranges; 25%ile and 75%ile. The data were analyzed using the Wilcoxon matched-pairs signed-rank test on the AIP cases (n = 46) versus matched controls on all data except age, height, weight, BMI, hip circumference, waist circumference and Waist/Hip-ratio (n = 50) AIP cases and 50 matched controls. Of the 46 matched pairs 20 are women and 26 are men. The p-values are exact, two-tailed.

[a] RI = Recommended intake, from Norwegian guidelines on diet, nutrition and physical activity, The Norwegian Directorate of Health, 2014.

[b] RDA = Recommended Dietary Allowance, AI = Adequate intake, from Dietary Guidelines for Americans. 8th Edition 2015–2020, January 2016, US Department of Health and Human Services and US Department of Agriculture.

[c] Normal body mass index, BMI. For persons above 70 years (y): 22–27.

[d] Indicates low risk level metabolic disease.

[e] Men/Women.

[f] RMR, Resting metabolic rate calculated by Mifflin's formula.

[g] W = women.

[h] Energy requirement = RMR* PAL.

[i] M = men.

[j] C-MUFA = Cis-mono unsaturated fatty acids, [k]C-PUFA = Cis-poly unsaturated fatty acids.

[l] Thiamin: M: 1.4 mg/d for 18–30 y 1.3: 31–60 y, 1.2: > 60. W: 1.1 mg/d for 18–60 y, 1.0: > 60 y.

[m] Riboflavin; M: 1.6 mg/d for 18–30 y, 1.5: 31–60 y, 1.4: 61–74, 1.3: > 75 y, W: 1.3 mg/d for 18–30 y, 1.2 > 30 y.

[n] Vitamin B6 Nordic rec.: W > 60 y: 1.2, [n] Vitamin B6 American rec.: W > 51 y: 1.5, M > 51 y: 1.7.

[o] Iron, Women: > 61 y: 9.

[p] Iron, Women, American rec. > 51 y: 8.

[q] Mg: M:19–30: 400 W:19–30 y: 310.

[r] P: 18–20 y: 700.

[s] E% = % of total energy intake.

blood pressure was significantly higher in the symptomatic than in the asymptomatic cases (Fig. 3C), while the U-epinephrine level was lower (Fig. 3D). S-calcium and albumin were significantly lower (Fig. 3E, F) and PTH levels in plasma were significantly higher (Fig. 3G) in the symptomatic cases than in the asymptomatic cases. As expected, U-PBG levels were significantly higher in the asymptomatic and symptomatic cases compared with their respective matched controls (Fig. 3H).

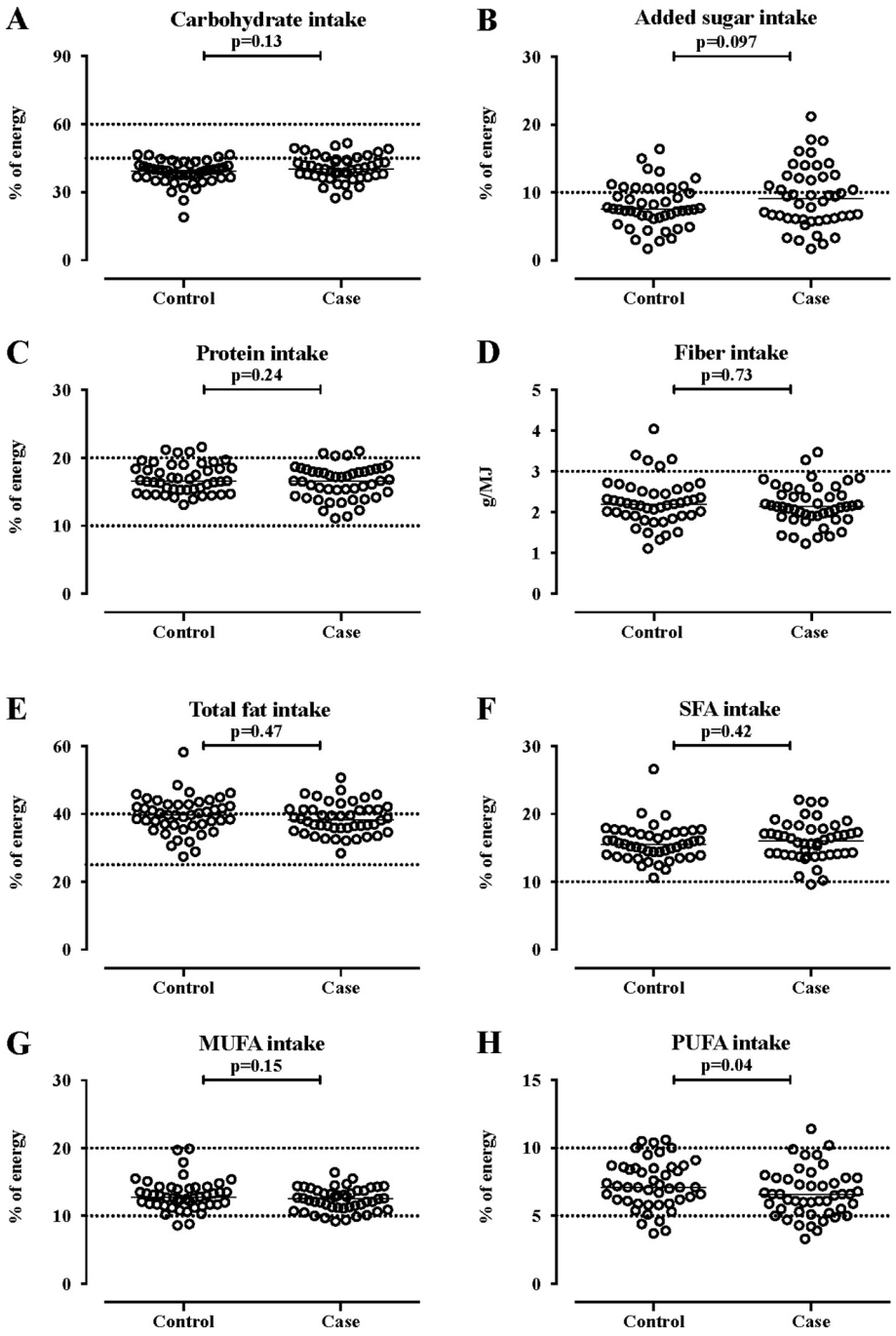


Fig. 1. The dietary intake of carbohydrates, added sugar, protein, fiber, total fat, saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) in 46 acute intermittent porphyria (AIP) cases (Case) compared with their matched controls (Control, n = 46). The dietary intake of (A) carbohydrates, (B) added sugar, (C) protein, (E) total fat, (F) saturated fatty acids (SFA), (G) MUFA and (H) PUFA are expressed as % of total energy intake, except for fiber intake (g/MJ). The dietary intake was calculated from a seven-day dietary logbook. The results are shown as scatterplots with the median. The two horizontal dotted gridlines show the recommended intake (RI)-interval for adults above 18 years for carbohydrate, protein, fiber, total fat, MUFA and PUFA. The one horizontal dotted gridline for added sugar and SFA indicates the upper recommended intake level. All dietary recommendations in the figure are from the current Norwegian guidelines on diet, nutrition and physical activity. The data were analyzed using the Wilcoxon matched-pair signed rank test. The p-values are exact, two-tailed.

3.6. Intake of sugar/candies, carbohydrates, protein, meat and alcohol and plasma resistin and alpha-1 antitrypsin levels in asymptomatic and symptomatic cases and matched controls

The intake of sugar/candies and carbohydrates was not significantly different between the symptomatic and asymptomatic AIP cases (Fig. 4A, B). The median intake of protein was close to be significantly different (p = .052) between the symptomatic and asymptomatic cases (Fig. 4C). The intake of meat given as g/day was significantly lower in the symptomatic than in the asymptomatic cases (Fig. 4D). The P-resistin level were significantly higher (p = .03) in the symptomatic than in the asymptomatic AIP cases and were significantly lower (p = .004) in the asymptomatic cases than in their matched controls (Fig. 4E). Interestingly, the intake of alcohol both given as g/day (Fig. 4G) and as E% (Fig. 4F) was significantly lower in the symptomatic than in the asymptomatic AIP cases. However, the alcohol intake was not significantly different from the matched controls (Fig. 3E, F).

3.7. Alcohol intake, cigarette smoking and intake of carbohydrates, added sugar and sugar/candies in acute intermittent porphyria cases with low and high levels of porphyrin precursors

We next examined if lifestyle factors, including alcohol, cigarette smoking and intake of carbohydrates and sugar/candies in the diet were different in the AIP cases with low and high biochemical disease activity. The intake of alcohol given as g/day (Fig. 5A) and E% (Fig. 5B) was similar in the AIP cases with low and high U-PBG levels. The number of cigarettes smoked per day (Fig. 5C), fat intake as E% (Fig. 5D), carbohydrate intake as E% (Fig. 5E) and added sugar intake as E% (Fig. 5F) were not different in the AIP cases with high and low U-

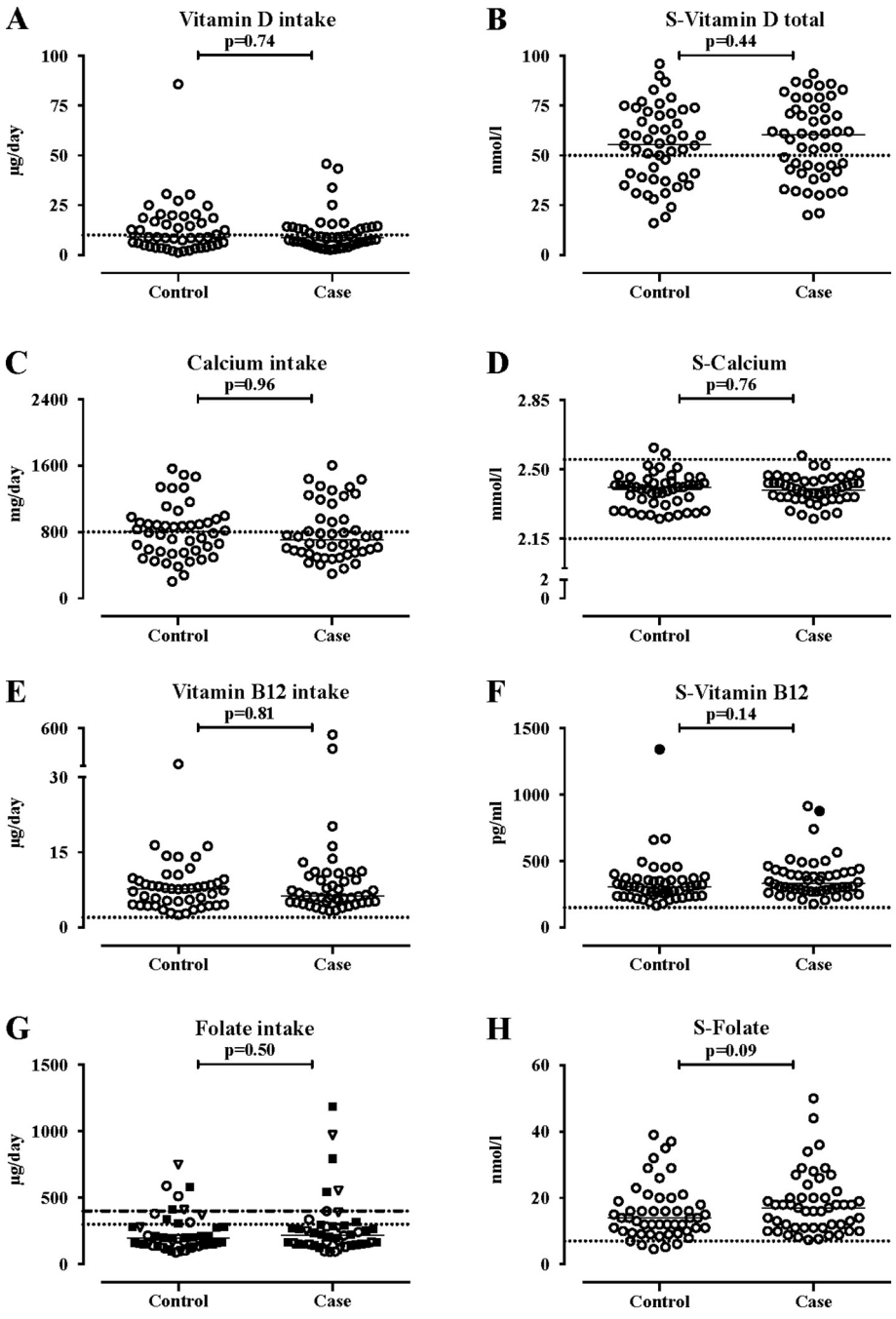


Fig. 2. Dietary intake of vitamin D, calcium, vitamin B12 and folate and the corresponding serum (S) levels in acute intermittent porphyria (AIP) cases and matched controls. The intake of (A) vitamin D, (C) calcium, (E) vitamin B12 and (G) folate in AIP cases (n = 46) was compared with their matched controls (n = 46). The dietary intakes were calculated from a seven-day dietary logbook and expressed as µg/day, except for the calcium intake (mg/day). The horizontal dotted gridline shows the recommended intake (RI) for adults above 18 years for the following: (A) vitamin D intake: 10 µg/day, but for persons > 75 years, the RI is 20 µg/day; (C) calcium intake: 800 mg/day, but for persons 18–20 years, the RI is 900 mg/day, and for postmenopausal women, a supplement of calcium of 500–1000 mg/day might reduce age-related bone loss and (E) Vitamin B12 intake: 2 µg/day, (G) The RI for folate intake is 300 µg/day for postmenopausal women (open circles) and men (black squares). The upper horizontal dot-line gridline in panel (G) shows the RI of folate 400 µg/day for women in fertile age (open triangles). All dietary recommendations are from the current Norwegian guidelines on diet, nutrition and physical activity. The serum level of (B) total 25-OH vitamin D was analyzed by liquid chromatography with tandem mass spectrometry (LC-MS/MS) and expressed as nmol/l. The serum levels of (D) total calcium (mmol/l), (F) S-vitamin B12 (pg/ml) and (H) S-folate (nmol/l) were analyzed using standard biochemical methods and expressed as mmol/l, pg/ml and nmol/l, respectively. "The control and the AIP case who received regular vitamin B12 injections are marked as black dots." The results are shown as scatter-plots with the median. The horizontal dotted gridlines indicate the reference limits for: (B) S-25 OH vitamin D total (50 nmol/l), (D) S-calcium (2.15–2.55 mmol/l), (F) S-vitamin B12 (150 pmol/l) and (H) S-folate (7 nmol/l). The data were analyzed using the Wilcoxon matched-pair signed rank test. The p-values are exact, two-tailed.

PBG levels. Interestingly, the intake of sugar/candies given as g/day (Fig. 5G) was significantly higher (p = .04) in the AIP cases with a low U-ALA levels (Fig. 5G). However, the sugar/candies intake was not significantly different in the AIP cases with low and high PBG levels (Fig. 5H).

3.8. Meat intake and serum iron levels, folate intake and folate levels, and creatinine and homocysteine levels in acute intermittent porphyria cases with low and high levels of porphyrin precursors

The intake of meat given as g/day (Fig. 6A) and folate given as µg/day (Fig. 6C) was similar in the AIP cases with low and high U-PBG levels. The S-iron levels (Fig. 6B) were close to be significantly different (p = .053) between the AIP cases with low and high U-PBG levels. The intake of folate (Fig. 6C) and the S-folate levels (Fig. 6D) were similar in the AIP cases with low and high PBG levels. However, the S-creatinine (Fig. 6E) and P-homocysteine levels (Fig. 6F) were significantly higher in the AIP cases with high U-PBG levels than in those with low PBG levels.

3.9. Serum glucose, plasma insulin, glucose/insulin ratio, pre-albumin and plasma C-peptide levels in acute intermittent porphyria cases with low and high levels of porphyrin precursors

The glucose levels were similar in the AIP cases with low and high U-PBG levels (Fig. 7A). The median plasma insulin level was significantly (p = .02) higher (Fig. 7B) and the glucose/insulin-ratio (Fig. 7C) was significantly lower (p = .02) in the AIP cases with low compared to those with high U-PBG levels. The pre-albumin levels (Fig. 6D) and plasma C-peptide levels (Fig. 6E) were similar in the AIP cases with low and high U-PBG levels. The C-peptide levels were not significantly different between the cases with low and high U-ALA

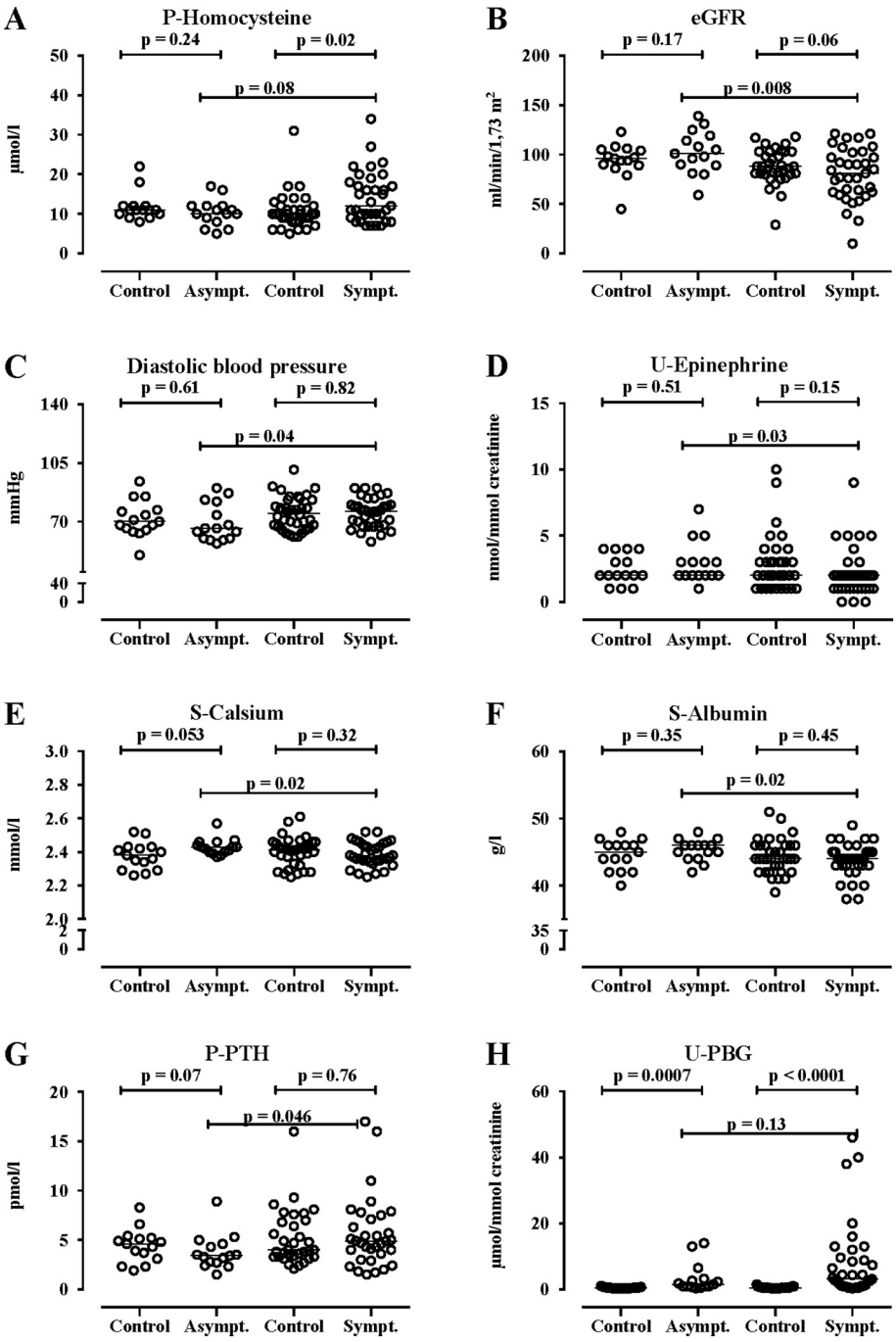


Fig. 3. Levels of homocysteine, kidney function, diastolic blood pressure, urine (U)-epinephrine, serum (S)-calcium, S-albumin, plasma (P)-PTH and U-porphobilinogen (U-PBG) levels in asymptomatic (Asympt.) and symptomatic (Sympt.) acute intermittent porphyria (AIP) cases (n = 50) and matched controls (Control, n = 50). The analytes were analyzed using standard biochemical methods. (A) P-homocysteine is expressed as µmol/l, (B) relative eGFR (estimated glomerular filtration rate) chronic kidney disease (CKD-EPI) as ml/min/1.73 m^2 with the creatinine equation, (C) diastolic blood pressure as mm Hg, (D) U-epinephrine as nmol/mmol creatinine, (E) S-calcium as mmol/l, (F) S-albumin as g/l, (G) P-PTH as pmol/l and (H) U-PBG as µmol PBG/mmol creatinine. Serial diastolic blood pressure was automatically measured over a 20–30 min period, every second minute using a CAS 740 blood pressure monitor. The paired case-control data were analyzed using the Wilcoxon matched-pair signed rank test. The asymptomatic and symptomatic AIP cases were compared using the Mann-Whitney *U* test.

levels (Fig. 7F). The AIP cases (n = 2) who received anti-diabetic medications are indicated with black dots (Fig. 7). When the p values for the comparison of biomarkers in the AIP cases with low and high U-PBG were calculated after the exclusion of those who received anti-diabetic medication, the results were; S-glucose (p = .11) as before, P-insulin (p = .04) and Glucose/Insulin-ratio (p = .04).

3.10. Porphyrin precursors, serum cholesterol, triglycerides and other biochemical biomarkers in cases with acute intermittent porphyria and controls

The levels of all porphyrins and porphyrin precursors were, as expected, significantly higher in the AIP cases than in the controls (Table 2). The S-total cholesterol, S-LDL cholesterol, and S-triglyceride levels were significantly higher in AIP cases than in the controls (Table 2). The S-phosphate levels were lower in the cases than in the controls (Table 2). The VMA levels in random urine samples were significantly lower in the AIP cases than in the controls, but the levels of U-norephinephrine and U-ephineprine were similar. All biomarkers of iron deficiency, including hemoglobin and erythrocyte numbers, were similar in the AIP cases compared to those of the controls (Table 2). The S-CDT%, S-electrolytes and cortisol levels were similar in cases and controls (Table 2).

3.11. Correlations between dietary intake, alcohol, smoking, biochemical biomarkers of kidney and liver function, and porphyrin precursors in cases with acute intermittent porphyria

The U-PBG levels correlated significantly and positively with P-homocysteine, S-creatinine, PTX3, HDL and total cholesterol levels

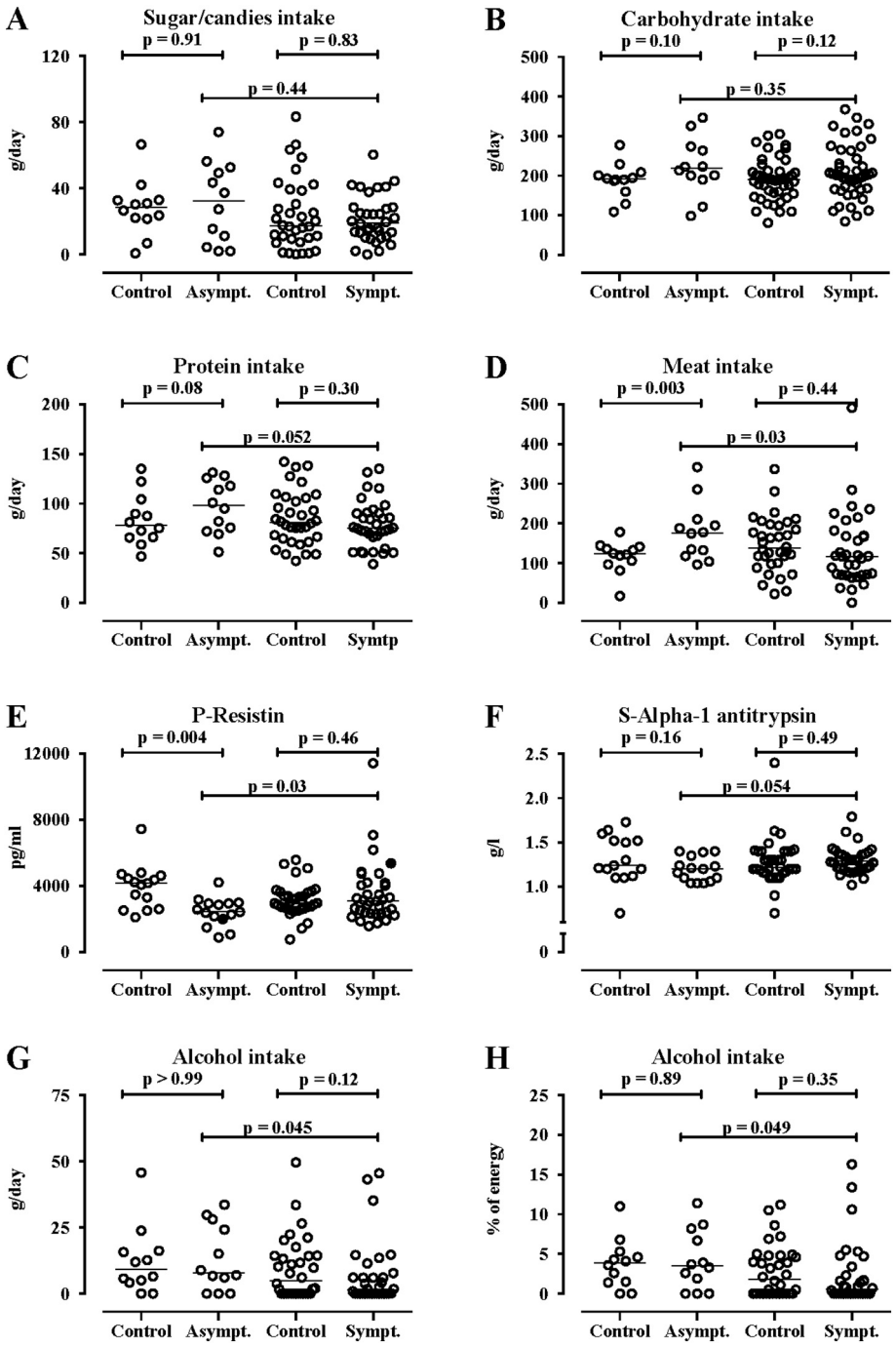


Fig. 4. Dietary intake of sugar and candies, carbohydrates, protein, meat, alcohol and urine-porphobilinogen (U-PBG) levels in asymptomatic (Asympt.) and symptomatic (Sympt.) acute intermittent porphyria (AIP) cases and matched controls (Control). (A) Intake of sugar and candies is given as g/day, (B) carbohydrate intake as g/day, (C) protein intake as g/day, (D) meat intake as g/day, (E) P-resistin as pg/ml, and the AIP cases and controls who received antidiabetic medications are indicated by black dots, (F) S-alpha-1 antitrypsin as g/l, (G) alcohol intake as g/day and (H) alcohol intake as % of total energy intake. The dietary intake was calculated from a seven-day dietary logbook from 46 matched pairs, i.e., 12 asymptomatic and 34 symptomatic cases and 12 and 34 matched controls. S-alpha-1 antitrypsin was analyzed using standard biochemical methods and P-resistin was analyzed using a Diabetes 10-plex assay in 15 asymptomatic and 35 symptomatic cases and matched controls. The paired case-control data were analyzed using the Wilcoxon matched-pair signed rank test. The asymptomatic and symptomatic AIP cases were compared using the Mann-Whitney *U* test.

(Fig. 8). U-PBG levels correlated significantly and negatively with pre-albumin, total energy intake and PUFA intake (Fig. 8). In comparison, U-ALA correlated significantly and negatively with S-pre-albumin and sugar/candies intake (Fig. 8). U-ALA correlated significantly and positively with smoking given as cigarettes smoked/day (Fig. 8). Furthermore, the inflammatory marker PTX3 correlated significantly and negatively with the carbohydrate intake given as g/day, but not with carbohydrates as E% (Fig. 8). As expected, the alcohol intake correlated positively and significantly with the CDT% (data not shown).

3.12. Correlations between biomarkers of glucose metabolism, diabetogenic hormones and biomarkers of disease activity in cases with acute intermittent porphyria

The porphyrin precursor U-PBG correlated positively and significantly with U-ALA and S-iron levels (Fig. 9). U-ALA correlated significantly and positively with S-cortisol levels (Fig. 9). Both U-PBG and U-ALA correlated with P-PTF1.2 levels. B-reticulocytes, P-leptin, P-ghrelin and P-resistin also correlated positively and significantly with PTF1.2 levels (Fig. 9). Furthermore, resistin and U-VMA showed a positive correlation. Plasma resistin levels in the cases were not correlated with BMI (data not shown). As expected, S-glucose correlated significantly and positively with HbA1c levels (Fig. 9).

3.13. Triggering factors for attacks and symptoms in cases with acute intermittent porphyria

The most frequent AIP symptoms and signs were abdominal pain (91%), dark/red urine (80%), tiredness (71%), muscle ache (60%) and muscle weakness (60%), followed by vomiting, headaches, decreased

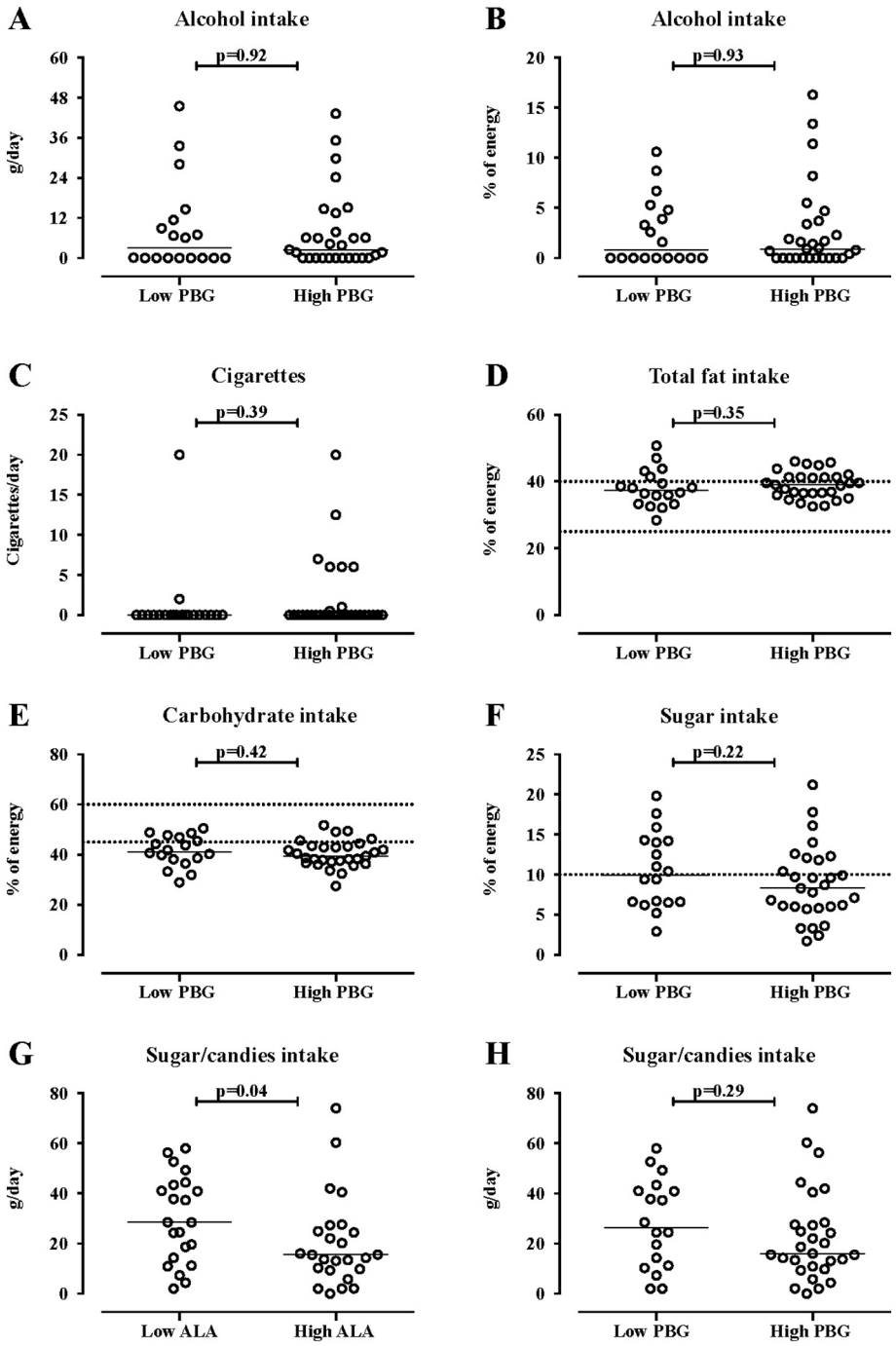


Fig. 5. Lifestyle factors, including (A, B) alcohol intake, (C) cigarette smoking, (D) total fat intake, and (E) intake of carbohydrates and (F) total sugar in acute intermittent porphyria (AIP) cases with low urine (U)-porphobilinogen (PBG) compared with AIP cases with high U-PBG levels. (G) Intake of sugar and candies in AIP cases with low (< 3.9) U-delta-aminolevulinic acid (ALA) compared with AIP cases with high U-ALA (> 3.9 μmol ALA/mmol creatinine), i.e., the reference limit for this assay. A low U-PBG level was defined as values ≤ 1.5 μmol PBG/mmol creatinine, i.e., the reference limit for this assay. The dietary components were calculated from a seven-day dietary logbook. The results are shown as scatter-plots with the median. The two horizontal dotted gridlines on total fat intake (D) and carbohydrate intake (E) indicate the recommended intake (RI) range, and the horizontal dotted gridline (F) for added sugar intake indicates the upper RI, as % of total energy for adults above 18 years. All dietary recommendations are from the current Norwegian guidelines on diet, nutrition and physical activity. The data were analyzed using the Mann-Whitney *U* test. The P-values are exact, two-tailed.

awareness, palpitations, psychological symptoms, constipation, paresis and epilepsy (Table 3). The most prevalent triggering factors for porphyric attack in the AIP cases were psychological stress (62%), followed by work environment (41%), which was described in most cases as stress at work or stressful working (Table 3). Physical strain were the third most prevalent trigger (38%), followed by other diseases including infections, sleep deprivation, food items, hunger, alcohol, dieting and medications. Cigarette smoking was a triggering factor in 6 % of the symptomatic AIP cases. The menstrual cycle was a trigger for 17% of the attacks in female AIP cases. Of the asymptomatic and symptomatic AIP males, 75% and 69%, respectively, had finished one year of military service.

4. Discussion

To our knowledge, this is the first report that both analyzes the dietary intake using a dietary logbook and simultaneously analyzes biochemical nutritional markers and diabetogenic hormones in blood samples in AIP cases and matched controls. The results indicate that the intake of slow-release carbohydrates was lower, and the intakes of sugar and saturated fatty acids was higher than recommended for the Nordic countries, both in the AIP cases and controls. Vitamin D deficiency was the most common vitamin deficiency in both the cases and controls. The intake of alcohol was unexpectedly high in the AIP cases, but lower in the symptomatic than in the asymptomatic cases. Insulin was higher in the cases with low U-PBG levels. Plasma resistin levels were higher in the symptomatic than the asymptomatic cases. These

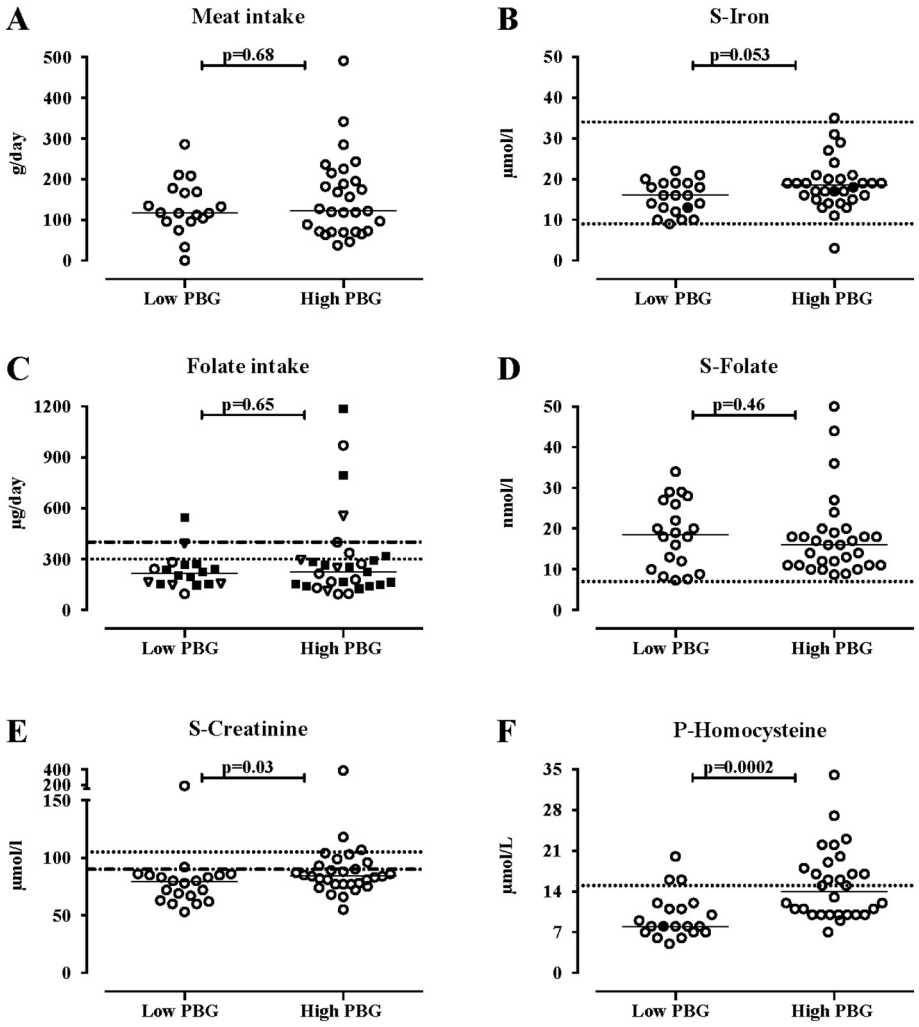


Fig. 6. Intake of meat and folate and the serum (S) levels of iron, folate, plasma (P) homocysteine and kidney function markers in acute intermittent porphyria (AIP) cases with low U-porphobilinogen (PBG) compared with AIP cases with high U-PBG. Low U-PBG was defined as ≤1.5 µmol PBG/mmol creatinine, corresponding to the upper reference limit. The dietary components were calculated from a seven-day dietary logbook including intake of (A) meat and (C) folate. (B) S-iron, (D) S-folate, (E) S-creatinine and (F) P-homocysteine levels were analyzed using standard biochemical assays and the results are shown as scatter-plots with the median. The two horizontal dotted gridlines in panel B indicates the reference values for S-iron (9–34 µmol/l) and black dots indicate AIP cases who received iron supplements. (C) The RI for folate intake is 300 µg/day for postmenopausal women (open circles) and men (black squares). The upper horizontal dot-line gridline in panel (C) shows the RI of folate 400 µg/day for women in fertile age (open triangles). All dietary recommendations in the figure are from the current Norwegian guidelines on diet, nutrition and physical activity. The horizontal dotted gridlines indicate the reference value for: (D) S-folate (> 7 nmol/l), (E) S-creatinine in women (lower line, 90 µmol/l) and for men (upper line, 105 µmol/l), and (F) P-homocysteine (> 15 µmol/l) and a black dot indicates the AIP case who received B12 injections. The data were analyzed using the Mann-Whitney U test. The P-values are exact, two-tailed.

findings may have several explanations and implications.

The energy intake was sufficient in all AIP cases and controls since the BMI results indicate that half of the AIP cases and about 4 out of 10 of the matched controls in this study were overweight. Similarly, 1 out of 4 of the cases and 3 out of 10 of the controls had obesity in line with the high and increasing frequency of obesity in Norway. However, when the energy intake was calculated from the diet logbook, most cases and controls had an insufficient energy intake. However, the dietary intake is probably slightly (17%) underreported according to unpublished data from a validation study of the 7- day diet log book among adults (personal communication from A.M. Wetting Johansen, the Department of Nutrition, Univ. of Oslo). Interestingly, an increasing energy intake was negatively correlated with the U-PBG levels in the AIP cases suggesting that the total energy intake affects the biochemical disease activity also in periods with no dieting.

The intake of carbohydrates in most AIP cases and controls was lower than the recommended 45–60 E% in Norway and other Nordic countries [12,22] and in the US [21]. Thus, the AIP cases did not have a high intake of slow-release carbohydrate foods, in line with a previous report on 16 Spanish AIP cases [28]. In addition, the intake of added sugar was higher than the recommended maximum 10% of total energy intake in many AIP cases and controls, and this may lead to obesity. The intake of sugar and candies was higher in those with low ALA levels than in the group with high ALA levels, suggesting an effect of this dietary factor on the biochemical disease activity. However, no difference in sugar and candies or carbohydrate intake was found between the asymptomatic and symptomatic AIP cases. Finally, no significant correlation was found between the intake of carbohydrates and disease activity in the AIP cases, although a near significant correlation was found between carbohydrate intake in g/day and U-PBG. This may be due to a too low consumption of carbohydrates in the AIP cases, since the carbohydrate intake in two previous studies were up to 500 g/day [29,30], compared to a median intake of 201 g carbohydrates/day in the cases in this report. However, the previous finding that the AIP cases who acquired type 2 diabetes mellitus did not have symptoms of AIP supports the protective role of elevated glucose levels [31]. Furthermore, glucose reduced the synthesis of ALA and PBG in a mouse model of AIP [10]. However, the plasma glucose level is regulated by multiple hormones, including insulin, resistin, glucagon, cortisol and catecholamines. A previous study indicated that the S-glucose level was unchanged, but the fasting levels of insulin were higher and the glucagon was lower in AIP mice than in wild-type mice [32]. We previously found that the fasting insulin levels were decreased in the symptomatic AIP cases compared to the matched controls, but no difference was found between asymptomatic and symptomatic cases [26]. This report suggests that the fasting insulin was lower and the glucose/insulin ratio was higher in the AIP cases with high compared to the group with low U-PBG level. This finding, and the report by Handschin et al. [10] indicating an inhibitory effect of insulin on porphyrin precursor levels in mice, suggests that glucose should be administered together with insulin during porphyric attacks.

The higher resistin level in the symptomatic than the asymptomatic cases, and the positive correlation with leptin levels may indicate that inflammation [26], adipokines and hormones affecting insulin

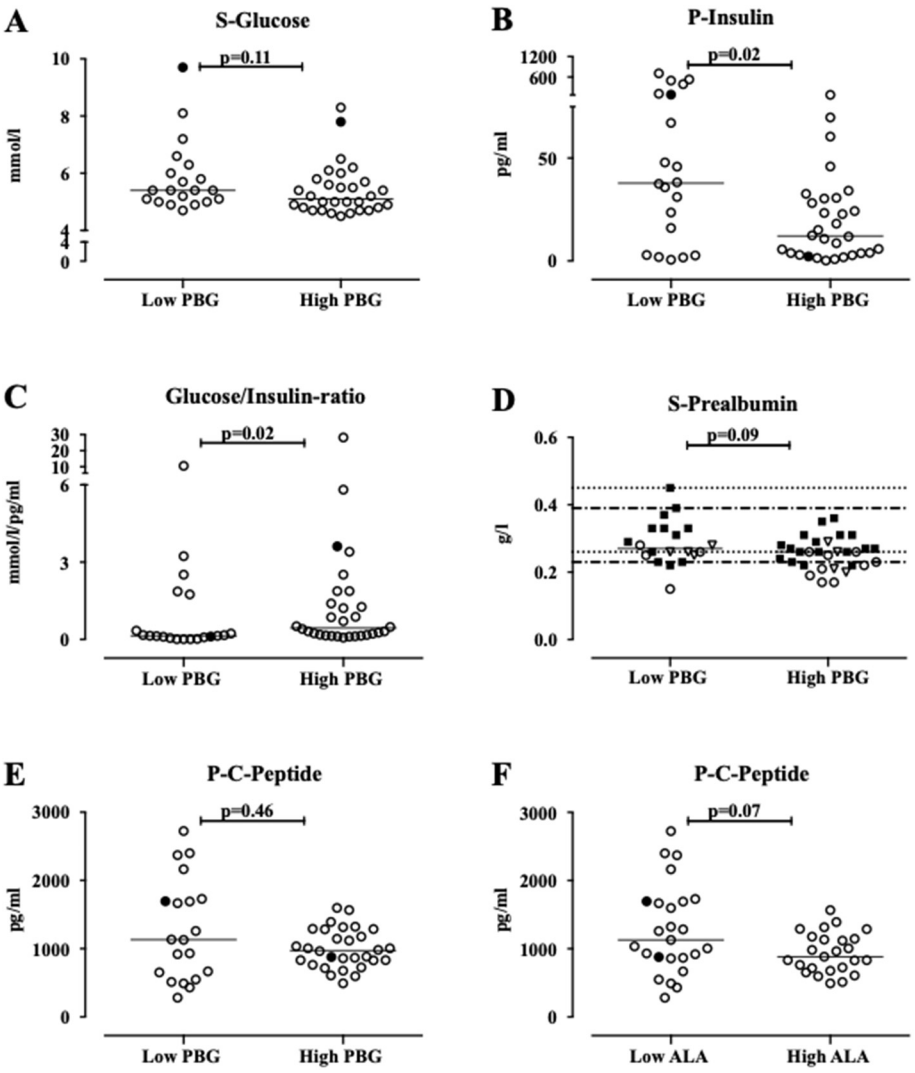


Fig. 7. Glucose, insulin, glucose/insulin ratio, prealbumin and C-peptide levels in acute intermittent porphyria (AIP) cases with low U-porphobilinogen (PBG) compared with acute intermittent porphyria (AIP) cases with high U-PBG. (A) serum glucose levels (mmol/l), (B) insulin (pg/ml), (C) glucose/insulin ratio (mmol/l/pg/ml), (D) S-pre-albumin (g/l), (E) C-peptide levels (pg/ml) in AIP cases with low (n = 20) and high (n = 30) levels of porphyrin precursors in urine. The black dots in panels A, B, C, E and F, indicate AIP cases who received anti-diabetic medications. A low U-porphobilinogen (PBG) level was defined as ≤1.5 μmol PBG/mmol creatinine, i.e., the reference value in urine. (F) C-peptide levels in AIP cases with low U-delta aminolevulinic acid (ALA) defined as < 3.9 μmol ALA/mmol creatinine (n = 25). S-glucose and pre-albumin were analyzed using standard biochemical assays, while the other markers were analyzed using a Bio-PlexPro Human Diabetes 10-plex immunoassay from Bio-Rad. The results are shown as scatter-plots with the median. The two horizontal dotted gridlines in panel (D) prealbumin are the normal reference values for men (black squares) and for women > 50 years (open circles). For women < 50 years (open inversed triangles) the reference values are 0.23–0.39 g/l (dotline gridline). The data were analyzed using the Mann-Whitney U test. The P-values are exact, two-tailed.

resistance may possibly be involved in a high disease activity [33].

However, we have no explanation for these differences, but this indicate that the role of hormones regulating glucose uptake such requires further investigation. Acute inflammation can trigger AIP attacks [1], but the inflammation observed as increased resistin levels found in the AIP cases could be an effect of the AIP disease itself, since it was observed in periods between attacks and outside acute inflammation. Resistin activates TLR4 on macrophages and increases the release of cytokines [34]. Furthermore, increased resistin levels is probably not an effect of other inflammatory diseases, as we did not find enhanced frequency in inflammatory diseases that could explain the difference in cytokines [26]. On the other hand, the slightly higher frequency of smoking, higher LDL levels and lower prealbumin levels indicating liver inflammation in the AIP cases, may partly explain the enhanced inflammation [26]. Another possible pathway from AIP with increased ALA, PBG and porphyrins to inflammation could be that ALA, PBG and porphyrins cause tissue damage that creates elevated cytokines [26]. Additionally, damage associated patterns (DAMPS) such as elevated uric acid due to reduced kidney function in some AIP cases could lead to cytokine production [26].

The intake of fat, and especially saturated fatty acids, was higher than recommended in both the AIP cases and controls and is associated with enhanced risk of cardiovascular disease [35]. The higher cholesterol levels in the cases can not be explained by a higher intake of SFA in the AIP cases, since the intake of SFA was elevated in both groups. Replacement of dietary saturated fats with PUFA normally reduces cholesterol levels and the CVD risk [35], and the lower PUFA intake in the AIP cases, may partly explain the higher total cholesterol and triglyceride levels in the AIP cases than in the controls. The intake of protein was within the national recommendations both in the AIP cases and the controls. The fiber intake was similar but was lower than recommended in both AIP cases and controls, in line with a previous report [28]. The intake of meat was lower in the AIP cases than in the controls. This may have several reasons, but we speculate that this may be due to the enhanced prevalence of reduced kidney function and maybe gout [26] in the AIP cases since patients with gout are advised to reduce the intake of purine-rich foods [36].

The intake of some vitamins, including vitamin D, was insufficient in many AIP cases and controls, and vitamin D deficiency was common in both groups. This may partly explain the enhanced PTH levels in some AIP cases, although reduced kidney function may also increase PTH levels [37]. Vitamin D deficiency is very common in Norway due to insufficient dietary sources and a lack of sunlight in northern Norway [38]. Vitamin D deficiency may have several negative effects, including increased risk of osteoporosis [37]. S-iron was close to be significantly higher in the AIP cases with high PBG levels, but iron deficiency was rare in the AIP cases. The homocysteine levels were increased in the symptomatic AIP cases compared to their matched controls, but this was probably not due to a severe deficiency of folate or vitamin B12. The increased homocysteine levels in the symptomatic cases was most likely due to reduced kidney function since homocysteine and creatinine levels were highly and positively correlated. However, a deficiency

Table 2
Biomarker levels in acute intermittent porphyria cases and controls.

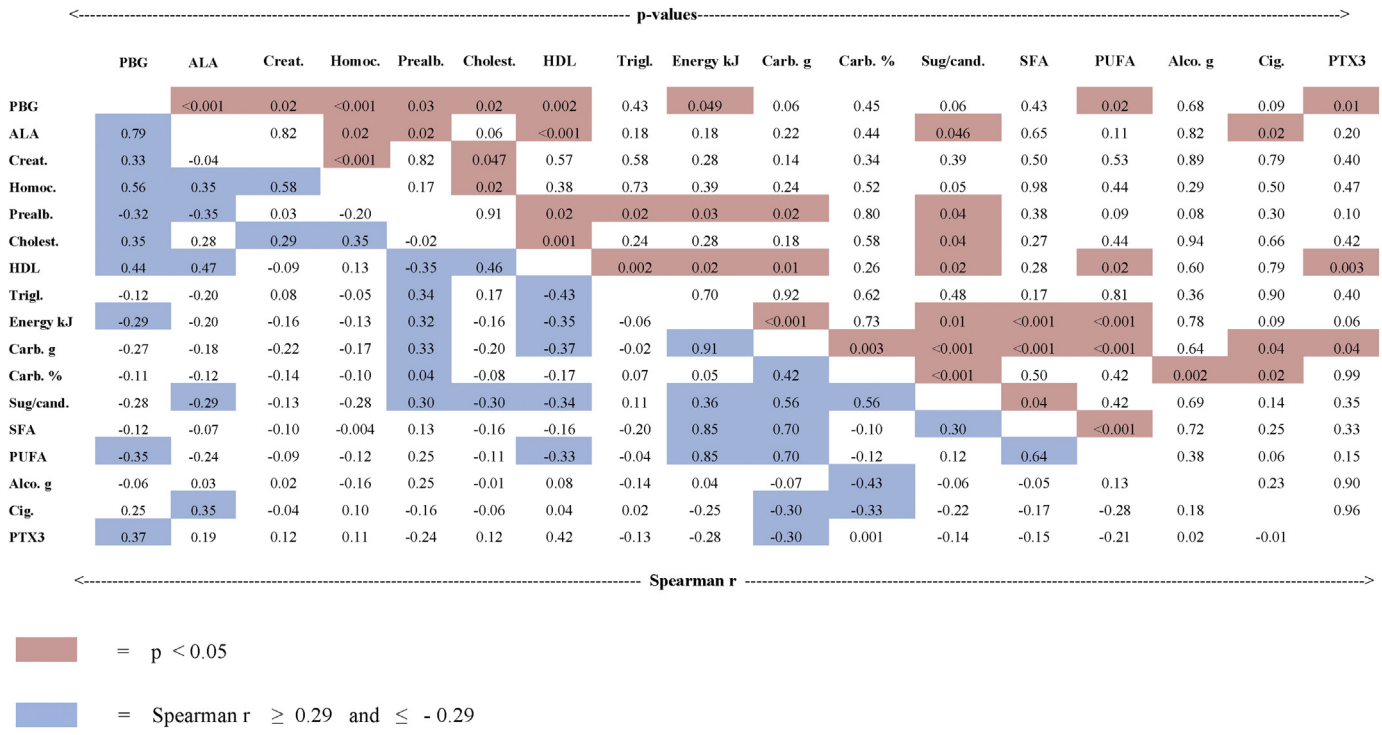
	Controls			Cases			p
	25%ile	Median	75%ile	25%ile	Median	75%ile	
U-ALA[a], μmol/mmol creatinine	1.6	1.9	2.3	2.4	3.9	7.0	< 0.0001
U-PBG[b], μmol/mmol creatinine	0.3	0.4	0.5	0.9	2.6	8.5	< 0.0001
U-Total porphyrins, nmol/mmol creatinine	4.4	6.8	11	8.5	26	83	< 0.0001
U-Uroporphyrins, nmol/mmol creatinine	1	1.25	1.7	1.3	2.8	19.7	< 0.0001
U-Heptaporphyrins, nmol/mmol creatinine	0.2	0.3	0.5	0.3	0.7	1.3	< 0.0001
U-Hexaporphyrins, nmol/mmol creatinine	0.1	0.1	0.3	0.0	0.2	0.7	0.045
U-Pentaporphyrins, nmol/mmol creatinine	0.0	0.1	0.2	0.1	0.4	1.4	< 0.0001
U-Coproporphyrins, nmol/mmol creatinine	3.0	4.5	8.8	6.3	14	44	< 0.0001
S-Total Cholesterol, mmol/l	4.6	5.1	5.7	5.1	5.7	6.4	0.002
S-LDL[c]-Cholesterol, mmol/l	2.3	3.1	3.7	3.0	3.5	3.9	0.02
S-HDL[d]-Cholesterol, mmol/l	1.3	1.5	1.8	1.1	1.4	1.8	0.50
S-Triglycerides, mmol/l	0.7	1.0	1.4	1.0	1.2	1.85	0.01
S-CDT[e], %	1.2	1.4	1.7	1.3	1.5	1.6	0.49
B-Hemoglobin, g/dl	13.7	14.4	15.3	13.6	15.0	15.8	0.19
B-EVF[f]	0.40	0.42	0.45	0.41	0.43	0.47	0.08
Erc-MCV[g], fl	85	87	91	86	89	93	0.18
Erc-MCH[h], pg	29	30	31	29	30	32	0.57
B-EPK[i], $\times 10^{12}$/l	4.6	4.9	5.1	4.7	5	5.3	0.25
Erc-Reticulocytes, %	0.8	0.9	1.2	0.8	1	1.2	0.47
Rtkc-CHr[j], pg	32.6	33.7	34.7	32.7	33.9	35	0.71
Erc-Hypo[k], %	0.1	0.2	0.3	0.1	0.2	0.4	0.59
B-Platelets, $\times 10^{9}$/l	224	266	299	194	243	287	0.05
S-Ferritin, μg/l	49	120	186	57	111	222	0.76
S-Iron, μmol/l	13	19	22	14	17	19	0.81
S-TIBC[l], μmol/l	59	62	70	63	67	75	0.06
S-Soluble transferrin receptor, mg/l	1.0	1.2	1.5	1.0	1.23	1.5	0.70
S-Sodium, mmol/l	140	142	143	140	141	142	0.20
S-Potassium, mmol/l	4.1	4.2	4.4	4.2	4.3	4.5	0.11
S-Magnesium, mmol/l	0.85	0.90	0.93	0.88	0.91	0.96	0.31
S-Phosphate, mmol/l	0.99	1.13	1.25	0.87	1.06	1.21	0.03
S-Cortisol, nmol/l	385	460	587	406	549	584	0.45
U-Norepinephrine, nmol/mmol creatinine	12	16	23	9	14	20	0.09
U-Epinephrine, nmol/mmol creatinine	1.75	2.0	3.25	1.0	2.0	3.0	0.37
U-VMA[m], μmol/mmol creatinine	1.1	1.6	2.1	0.8	1.3	1.9	0.01

The data represent the median values and interquartile range; 25 percentile (25%-ile) and 75 percentile (75%-ile).
Wilcoxon's matched-pairs signed-rank test was used on all case-control data in this table. N = 50 matched pairs.

[a] U-ALA = Urine-delta aminolevulinic acid.
[b] U-PBG = Urine-porphobilinogen.
[c] S-LDL = Serum–low-density lipoproteins.
[d] S-HDL = Serum–high-density lipoproteins.
[e] S-CDT% = percentage of carbohydrate-deficient transferrin.
[f] B-EVF = Whole blood erythrocyte volume fraction.
[g] Erc-MCV = Erythrocyte mean corpuscular volume.
[h] Erc-MCH = Erythrocyte mean corpuscular hemoglobin.
[i] B-EPK = Whole blood erythrocyte-particle concentration.
[j] Rtkc-Chr = Reticulocyte hemoglobin content.
[k] Erc-Hypo = Percentage of hypochromic red cells.
[l] S-TIBC = Serum-total iron-binding capacity.
[m] U-VMA = Urine vanillylmandelic acid.

of vitamin B1 and B6 cannot be excluded since they were not measured. The diastolic blood pressure and PTH was higher and S-albumin and calcium were slightly lower in the symptomatic compared to the asymptomatic AIP cases, probably also due to a reduced kidney function in the symptomatic AIP cases.

The use of alcohol in the AIP cases was much higher than expected, and was higher than the generally recommended upper limit of 1 alcohol unit (12 g) per day in a few cases. However, the alcohol intake was significantly lower in the symptomatic AIP cases than in the asymptomatic cases. Since alcohol had triggered attacks in 32% of the AIP cases in line with a previous study [18], we speculate that the symptomatic cases reduced the intake to avoid attacks. No correlation was found between the alcohol intake and disease activity. The finding that most AIP cases in this study used moderate amounts of alcohol regularly may indicate that a regular, but moderate intake of alcohol is less porphyrinogenic than an acute intake of alcohol [17,39]. However, the alcohol intake observed in both the AIP cases and controls, may partially hide the real impact of alcohol in the AIP group. AIP cases are advised to avoid alcohol. According to a WHO report from 2016, the mean total alcohol intake per capita in Norwegian adults above 15 years age was 7.5 l pure alcohol per year, compared to 9.8, 9.8, 6.3 and 7.3 in the whole European region, the US, the African and the Western Pacific Regions, respectively [40]. The mean total alcohol intake was 5.7 and 4.3 l per year in the controls for the asymptomatic and symptomatic AIP cases, respectively, and 6.1 and 3.1 l in the asymptomatic and symptomatic AIP cases, respectively. This indicates that the alcohol intake in the cases was not high compared to the rest of Norway and other countries. Furthermore, the alcohol habits vary between different countries, and different individuals, and this should be kept in mind when our results from Norway are being evaluated. It is well known that alcohol is toxic to the liver and is associated with enhanced risk of hepatocellular carcinoma (HCC) and liver cirrhosis [41].



	PBG	ALA	Creat.	Homoc.	Prealb.	Cholest.	HDL	Trigl.	Energy kJ	Carb. g	Carb. %	Sug/cand.	SFA	PUFA	Alco. g	Cig.	PTX3
PBG		<0.001	0.02	<0.001	0.03	0.02	0.002	0.43	0.049	0.06	0.45	0.06	0.43	0.02	0.68	0.09	0.01
ALA	0.79		0.82	0.02	0.02	0.06	<0.001	0.18	0.18	0.22	0.44	0.046	0.65	0.11	0.82	0.02	0.20
Creat.	0.33	-0.04		<0.001	0.82	0.047	0.57	0.58	0.28	0.14	0.34	0.39	0.50	0.53	0.89	0.79	0.40
Homoc.	0.56	0.35	0.58		0.17	0.02	0.38	0.73	0.39	0.24	0.52	0.05	0.98	0.44	0.29	0.50	0.47
Prealb.	-0.32	-0.35	0.03	-0.20		0.91	0.02	0.02	0.03	0.02	0.80	0.04	0.38	0.09	0.08	0.30	0.10
Cholest.	0.35	0.28	0.29	0.35	-0.02		0.001	0.24	0.28	0.18	0.58	0.04	0.27	0.44	0.94	0.66	0.42
HDL	0.44	0.47	-0.09	0.13	-0.35	0.46		0.002	0.02	0.01	0.26	0.02	0.28	0.02	0.60	0.79	0.003
Trigl.	-0.12	-0.20	0.08	-0.05	0.34	0.17	-0.43		0.70	0.92	0.62	0.48	0.17	0.81	0.36	0.90	0.40
Energy kJ	-0.29	-0.20	-0.16	-0.13	0.32	-0.16	-0.35	-0.06		<0.001	0.73	0.01	<0.001	<0.001	0.78	0.09	0.06
Carb. g	-0.27	-0.18	-0.22	-0.17	0.33	-0.20	-0.37	-0.02	0.91		0.003	<0.001	<0.001	<0.001	0.64	0.04	0.04
Carb. %	-0.11	-0.12	-0.14	-0.10	0.04	-0.08	-0.17	0.07	0.05	0.42		<0.001	0.50	0.42	0.002	0.02	0.99
Sug/cand.	-0.28	-0.29	-0.13	-0.28	0.30	-0.30	-0.34	0.11	0.36	0.56	0.56		0.04	0.42	0.69	0.14	0.35
SFA	-0.12	-0.07	-0.10	-0.004	0.13	-0.16	-0.16	-0.20	0.85	0.70	-0.10	0.30		<0.001	0.72	0.25	0.33
PUFA	-0.35	-0.24	-0.09	-0.12	0.25	-0.11	-0.33	-0.04	0.85	0.70	-0.12	0.12	0.64		0.38	0.06	0.15
Alco. g	-0.06	0.03	0.02	-0.16	0.25	-0.01	0.08	-0.14	0.04	-0.07	-0.43	-0.06	-0.05	0.13		0.23	0.90
Cig.	0.25	0.35	-0.04	0.10	-0.16	-0.06	0.04	0.02	-0.25	-0.30	-0.33	-0.22	-0.17	-0.28	0.18		0.96
PTX3	0.37	0.19	0.12	0.11	-0.24	0.12	0.42	-0.13	-0.28	-0.30	0.001	-0.14	-0.15	-0.21	0.02	-0.01	

= $p < 0.05$

= Spearman r ≥ 0.29 and ≤ -0.29

Fig. 8. Correlation matrix of porphyrin precursors, diet, alcohol, cigarette smoking, diet-related biomarkers and PTX3 in the acute intermittent porphyria (AIP) cases (n = 47). The data in the left lower part are Spearman's correlation coefficients, in the AIP cases (n = 47), r ≥ 0.29 and r ≤ −0.29 (blue color). The correlation matrix shows pairwise correlations of the different variables. In the upper right part the corresponding significant p values ($p < .05$) are indicated by red color. The variables included are: urine porphobilinogen ratio (U-PBG); urine 5-aminolevulinic acid ratio (ALA); S-creatinine (Creat.); P-homocysteine (Homoc.); S-pre-albumin (Prealb.); S-total cholesterol (Cholest.); S-high-density lipoprotein (HDL); S-triglycerides (Trigl.); intake of energy kJ/day (Energy KJ); carbohydrates g/day (Carb. g); Carbohydrates % of total energy (Carb. %); sugar/candy intake g/day (Sug/Cand.); saturated fatty acids (SFA) (g/day); polyunsaturated fatty acids (PUFA) g/day; alcohol g/day (Alco. g); current smoking of cigarettes per day (Cig.); and pentraxin 3 (PTX3), ng/ml. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

However, in the study by Innala et al., none of the AIP cases developing HCC were abusing alcohol [42]. This indicates that the general recommendation of no or a very low alcohol intake in AIP cases should be continued.

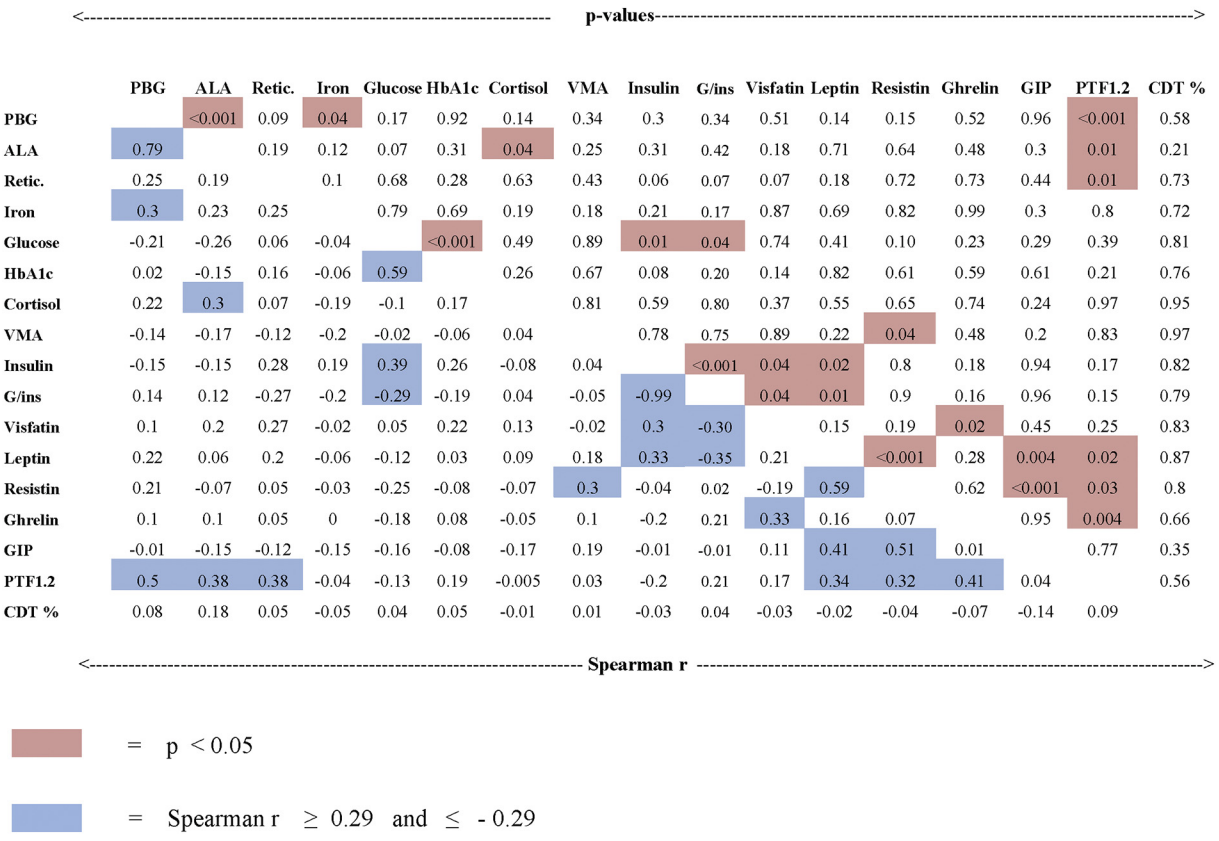
Psychological stress and physical strain was reported as triggers of attacks in 62% and 38%, respectively, of the symptomatic AIP cases. This result is in line with the report by Naik et al. who reported that 56% of patients with acute hepatic porphyrias said that stress, mainly emotional stress or caused by work, was a direct contributing factor to their acute attacks [25]. Several stress hormones were analyzed in this study, outside of AIP attacks, but only the levels of VMA were decreased in the AIP cases compared to the controls. In comparison, the levels of epinephrine and norepinephrine were similar in the cases and controls. We have no explanation for these findings, but speculate that the catecholamine release or VMA formation might be slightly decreased in the AIP cases in periods outside attacks. Stress may also affect the levels of stress hormones, which in turn affect the metabolism of glucose and fat. A previous report by Pozo et al. showed reduced levels of urine adrenal steroid hormones, including cortisol metabolites, in AIP cases compared to controls [43]. In this report, S-cortisol levels were similar in the AIP cases and controls, but urine metabolites of cortisol were not examined.

This report indicates that 2%, 6% and 20% of the AIP cases used porphyrinogenic, probably porphyrinogenic or possibly porphyrinogenic medications, respectively. Furthermore, 29% of the symptomatic cases reported that medications had triggered attacks. This is an unexpected finding since it is well known that correct medication is important in AIP cases and that many medications are porphyrinogenic [44]. The most likely explanation for this is that some medical doctors still have limited knowledge about AIP despite repeated information given regarding correct medication.

Current smoking was slightly more common among the AIP cases than among the controls. However, smoking had triggered attacks in only 6 % of the symptomatic AIP cases in this report. Among the asymptomatic and symptomatic cases, one out of five were smoking tobacco. Furthermore, the number of cigarettes smoked per day was correlated with the biochemical disease activity of ALA, but not of PBG, in the AIP cases. This is partially in agreement with Bylesjo et al. who reported that smoking was associated with a high AIP attack frequency [20]. In addition, even low cigarette usage is associated with an enhanced risk of several diseases, including lung cancer, coronary heart disease and stroke and should be avoided [45].

The kidney function was lower in the symptomatic AIP cases, which is in agreement with several previous reports indicating that the porphyrin precursors are toxic to the kidney tubuli and endothelial cells causing a chronic tubulointerstitial nephropathy [5,6]. In line with this, the S-albumin and P-PTH levels were slightly lower and higher in the cases than controls, respectively. Furthermore, the pre-albumin levels were reduced in the AIP cases, indicating reduced liver function, malnutrition or inflammation of the liver [26,46].

This study has several limitations that might have affected the results. First, most of the AIP cases had the same AIP mutation. Second, many different parameters were analyzed, implying that the risk of false positive results in the statistical analysis is high. Third, the number of AIP cases was limited to 50, mainly due to the rarity of the disease. However, to our knowledge this is the first report on the role of dietary factors in AIP cases using a dietary logbook and biochemical analysis in blood samples for many of the same nutrients and vitamins. The registration of food intake, however, may have been underreported since the reported energy intake was lower than the calculated energy



	PBG	ALA	Retic.	Iron	Glucose	HbA1c	Cortisol	VMA	Insulin	G/ins	Visfatin	Leptin	Resistin	Ghrelin	GIP	PTF1.2	CDT %
PBG		<0.001	0.09	0.04	0.17	0.92	0.14	0.34	0.3	0.34	0.51	0.14	0.15	0.52	0.96	<0.001	0.58
ALA	0.79		0.19	0.12	0.07	0.31	0.04	0.25	0.31	0.42	0.18	0.71	0.64	0.48	0.3	0.01	0.21
Retic.	0.25	0.19		0.1	0.68	0.28	0.63	0.43	0.06	0.07	0.07	0.18	0.72	0.73	0.44	0.01	0.73
Iron	0.3	0.23	0.25		0.79	0.69	0.19	0.18	0.21	0.17	0.87	0.69	0.82	0.99	0.3	0.8	0.72
Glucose	-0.21	-0.26	0.06	-0.04		<0.001	0.49	0.89	0.01	0.04	0.74	0.41	0.10	0.23	0.29	0.39	0.81
HbA1c	0.02	-0.15	0.16	-0.06	0.59		0.26	0.67	0.08	0.20	0.14	0.82	0.61	0.59	0.61	0.21	0.76
Cortisol	0.22	0.3	0.07	-0.19	-0.1	0.17		0.81	0.59	0.80	0.37	0.55	0.65	0.74	0.24	0.97	0.95
VMA	-0.14	-0.17	-0.12	-0.2	-0.02	-0.06	0.04		0.78	0.75	0.89	0.22	0.04	0.48	0.2	0.83	0.97
Insulin	-0.15	-0.15	0.28	0.19	0.39	0.26	-0.08	0.04		<0.001	0.04	0.02	0.8	0.18	0.94	0.17	0.82
G/ins	0.14	0.12	-0.27	-0.2	-0.29	-0.19	0.04	-0.05	-0.99		0.04	0.01	0.9	0.16	0.96	0.15	0.79
Visfatin	0.1	0.2	0.27	-0.02	0.05	0.22	0.13	-0.02	0.3	-0.30		0.15	0.19	0.02	0.45	0.25	0.83
Leptin	0.22	0.06	0.2	-0.06	-0.12	0.03	0.09	0.18	0.33	-0.35	0.21		<0.001	0.28	0.004	0.02	0.87
Resistin	0.21	-0.07	0.05	-0.03	-0.25	-0.08	-0.07	0.3	-0.04	0.02	-0.19	0.59		0.62	<0.001	0.03	0.8
Ghrelin	0.1	0.1	0.05	0	-0.18	0.08	-0.05	0.1	-0.2	0.21	0.33	0.16	0.07		0.95	0.004	0.66
GIP	-0.01	-0.15	-0.12	-0.15	-0.16	-0.08	-0.17	0.19	-0.01	-0.01	0.11	0.41	0.51	0.01		0.77	0.35
PTF1.2	0.5	0.38	0.38	-0.04	-0.13	0.19	-0.005	0.03	-0.2	0.21	0.17	0.34	0.32	0.41	0.04		0.56
CDT %	0.08	0.18	0.05	-0.05	0.04	0.05	-0.01	0.01	-0.03	0.04	-0.03	-0.02	-0.04	-0.07	-0.14	0.09	

= $p < 0.05$

= Spearman r ≥ 0.29 and ≤ -0.29

Fig. 9. Correlation matrix of porphyrin precursor and biomarkers of glucose metabolism- and diet-related biomarkers, prothrombin factor 1 + 2 (PTF1.2) and CDT%. In the lower left part are Spearman correlation coefficients, r, in the acute intermittent porphyria group (n = 47), $r \geq 0.29$ and $r \leq -0.29$ (blue color). The correlation matrix shows pairwise correlations of the different markers. In the upper right part are the corresponding significant p values ($p < .05$), which are indicated by red color. The variables included are the following: urine porphobilinogen ratio (PBG); urine 5-aminolevulinic acid ratio (ALA); Erc-Reticulocytes % (Retic.); S-iron (Iron); S-glucose (glucose); B-HbA1c (HbA1c); S-cortisol (cortisol); urine vanillylmandelic acid (VMA); P-insulin (Insulin); S-glucose/P-insulin ratio (G/ins-r); P-visfatin (Visfatin); P-leptin (Leptin); P-resistin (Resistin); P-ghrelin (Ghrelin); P-gastrointestinal peptide (GIP); P-prothrombin F1 + 2 (PTF1.2) and percentage of carbohydrate deficient transferrin (CDT%). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 3
Symptoms and triggering factors of attacks in acute intermittent porphyria cases.

Symptoms during AIP attacks[a]	%	Triggering factors of attacks[b]	%	Triggering factors of attacks,women[c]	%	Military service accomplished[d]	%
Abdominal pain	91	Psychological stress	62	Birth control pills	0	Asymptomatic men	75
Dark/red urine	80	Work environment (mainly stress)	41	Menstrual cycle	17	Symptomatic men	69
Tiredness	71	Physical strain	38	Menstrual cycle, first day	17		
Muscle ache	60	Other disease	32	Menstrual cycle, ovulation	0		
Muscle weakness	60	Sleep deprivation	32	Menstrual cycle, other	0		
Other[e]	43	Alcohol	32				
Vomiting	43	Medication	29				
Headaches	43	Food items	24				
Decreased sensibility	40	Hunger	24				
Palpitations	40	Dieting	9				
Psychological symptoms	26	Other (alternative medications)	6				
Constipation	23	Smoking	6				
Paresis	17						
Epilepsy	6						

The data were obtained from interviews done by medical doctors and questionnaires.
[a] % of AIP symptomatic cases, (n = 35), 18 women, 17 men.
[b] % of AIP symptomatic cases, (n = 34), one data missing.
[c] % of (n = 18) symptomatic women, regarding menstrual cycle.
[d] % of 12 asymptomatic and % of 16 symptomatic men regarding military service (one of 17 symptomatic men had missing data on question on military service).
[e] e.g: Diarrhea, visual disturbances, fecal incontinence, impaired memory.

requirement, and the RMR-factor (energy intake divided by RMR) was lower than expected from their activity level in some of the cases and controls [47]. However, the underreporting seems to be similar in both cases and controls, indicating that the intake in the cases and controls can still be compared. Another limitation is that the comparison against the reference energy and nutritional recommendations are for Norway, Nordic Countries and Americans, and it may, thus, be questioned with respect to the extent that this issue may limit the generalization of

results to other countries, for instance, in Asia and Southern Europe, etc. However, the dietary recommendations do not vary much between the Nordic countries, European countries and the US [48], indicating that the comparison of intakes in the AIP case and control group with the recommended intake is valid for many countries. Furthermore, the comparison of the intake in the AIP case and control groups is also valid, although the actual dietary habits may vary in different countries. The differences in dietary habits between the Nordic area [12], the Mediterranean area [49], and other parts of the world should be kept in mind when our results are generalized to other AIP patients. In general, the individual dietary pattern should be assessed when individualized dietary advice are given to AIP patients. Furthermore, the biomarkers on glucose metabolism were possibly affected by the antidiabetic medication used by two AIP cases..

In summary, several lifestyle factors, including total energy intake, alcohol, stress and medications may affect the disease activity of AIP cases. The distinction between slow-release carbohydrates and added sugar is not always precisely explained to the AIP patients, leaving some of them on a diet high in added sugar which may lead to obesity. Resistin levels were higher in the symptomatic than asymptomatic AIP cases, and insulin was higher in the cases with low U-PBG levels suggesting that hormones regulating glucose uptake may be disturbed in AIP. Several of these lifestyle factors, including diet, alcohol intake and stress, are known to affect the expression of genes through epigenetic changes. A recent study suggested oligogenic inheritance in AIP, pointing to several factors that dictate who manifests symptoms [8]. Future studies should therefore examine the role of other related mutations and other epigenetic changes that could explain why only a few of those with an AIP mutation have symptoms of the disease, while most cases are asymptomatic.

Acknowledgements

This work was supported by grants from Northern, Regional Health Authorities, and from the Somatic research fund at the Nordland Hospital Trust.

Disclosure

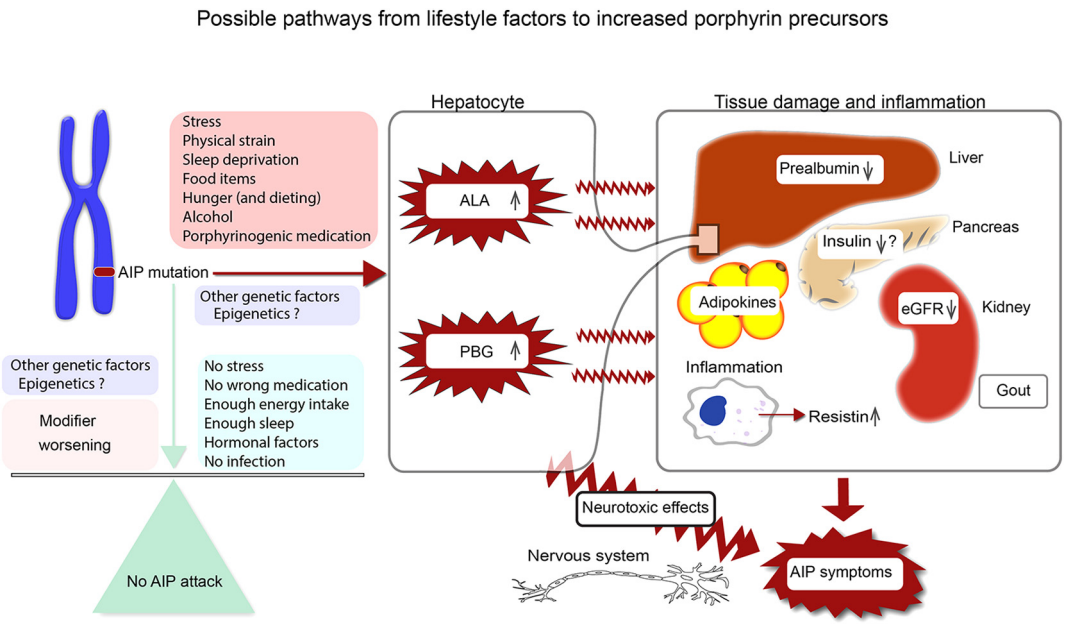
Dr. E. Storjord has received research grants from the Somatic research fund at the Nordland Hospital Trust and has received grants from the Northern Regional Health Authorities in Norway. Dr. Ole-L. Brekke has received grants from the Northern Regional Health Authorities in Norway.

Appendix A. Appendix Table A Porphyrinogenic medications, vitamin supplements, dietary supplements and herbal remedies in acute intermittent porphyria cases and controls

	Controls n (%)	Cases n (%)	p
Medications			
PSP[a] medications	4 (8)	10 (20)	0.15
PRP[b] medications	12 (24)	3 (6)	0.02
P[c] medications	1 (2)	1 (2)	1.00
Medications containing vitamins, Mg or iron			
Vitamin B12 injection	1 (2)	1 (2)	1.00
Etalpha (1α(OH)-vitamin D_3)	1 (2)	1 (2)	1.00
Isotretinoin (Vitamin A)	0 (0)	1 (2)	1.00
Magnesium	4 (8)	0 (0)	0.12
Iron	6 (12)	3 (6)	0.27
Supplements			
Vitamin B	6 (12)	4 (8)	0.74
Vitamin C	5 (10)	2 (4)	0.44
Vitamin D	4 (8)	5 (10)	1.00
Multi-vitamins	4 (8)	5 (10)	1.00
Calcium	3 (6)	2 (4)	1.00
Vitamin K	0 (0)	1 (2)	1.00
Fish oil	18 (36)	15 (30)	0.67
Persons using herbal remedies	8 (16)	7 (14)	1.00

The data were obtained from questionnaires from all participants, and from the 7-day diet logbook from n = 47 cases and 48 controls, and are given as n and % of AIP cases (n = 50) and of their age-, sex- and place of residence-matched controls (n = 50).
The herbal remedies are not counted in the PSP, PRP and P-data. Herbal remedies containing vitamins, calcium, magnesium or iron are also counted in supplements where applicable. Two-tailed P values were calculated using Fischers exact test.

[a] PSP = Possibly porphyrinogenic medication.
[b] PRP = Probably porphyrinogenic medication.
[c] P = Porphyrinogenic medication.



Appendix Fig. A. Possible pathways from lifestyle factors to increased porphyrin precursor levels and symptoms in acute intermittent porphyria. The possible role of lifestyle factors in the biochemical disease activity and symptoms in AIP cases are summarized. An AIP mutation is the prerequisite for AIP disease. To acquire elevated porphyrin precursor levels often requires several triggering factors at the same time such as stress, physical strain, dieting, alcohol and/or porphyrinogenic medications (red box), which may partly explain the low manifestation rate of symptoms in the AIP cases. Many factors may contribute to the worsening or improvement of porphyrin precursor levels and symptoms, such as genetic and epigenetic modifiers (blue box), the intensity of triggers as the level of protective factors such as a higher sugar intake and relaxation. Other diseases, such as diabetes mellitus, may also protect those with an AIP mutation. High levels of ALA and PBG affects the nervous system, the liver and kidney and result in abdominal pain, and vomiting etc. during the acute nevrovisceral attacks. Enhanced U-PBG levels are possibly associated with lower insulin levels. Resistin levels are higher in the symptomatic than the asymptomatic cases, and are associated with inflammation. As illustrated, most AIP cases tolerate different amounts of single triggering factors and probably have sufficient protective factors (marked in green) to avoid an increase in the porphyrin precursors and attacks. This conforms to what we have found, for example, with alcohol intake, since there was no difference in the PBG levels in those who drank more or less alcohol.

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Paper III

Cytokine 97 (2017) 86–95

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.elsevier.com/locate/cytokine

Effect of the anticoagulant, storage time and temperature of blood samples on the concentrations of 27 multiplex assayed cytokines – Consequences for defining reference values in healthy humans

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ARTICLE INFO

Keywords:
Anticoagulant
Complement
Cytokines
Lepirudin
Plasma

ABSTRACT

Cytokines are potentially useful biomarkers of sepsis and other inflammatory conditions. Many cytokines can be released by leukocytes and platelets after sampling. The sampling and processing techniques are consequently critically important to measure the *in vivo* levels. We therefore examined the effects of four different anticoagulants, EDTA, citrate, lepirudin, heparin compared to serum, on the levels of 27 different cytokines. The effects of storage temperature, freezing and thawing on the plasma cytokines were examined. Cytokines were analysed using a multiplex immunoassay. The cytokine levels in serum were significantly higher compared with plasma, consistent with release of cytokines *in vitro* during coagulation. In general, the lowest values for all cytokines were found in EDTA samples, stored on crushed ice, centrifuged within 4 h and thereafter stored at −80 °C. MCP-1 and MIP-1β levels were highest in heparin plasma and storage of blood for up to 4 h at room temperature significantly increased the interleukin (IL)-2, IL-6, IL-8, IFN-γ and GM-CSF levels in EDTA plasma, indicating post-sampling release. In contrast, the IP-10 levels were unaffected by sample storage at both temperatures. Our results indicate that the cytokines were more stable in plasma than in whole blood after sampling. Thus, cytokines should be analysed in EDTA plasma samples stored on ice and centrifuged within 4 h. Based on these data, the reference ranges of 27 cytokines in EDTA plasma in 162 healthy human donors were calculated.

1. Introduction

Cytokines, chemokines and growth factors are potential biomarkers of sepsis and other inflammatory diseases [1–3]. These compounds are extensively used as biomarkers of inflammation in a number of human diseases and inflammatory conditions [4–10]. In the case of many analytes in general, much attention has been focussed on the pre-analytical sampling conditions [11,11–13]. Although poorly studied, the sampling and processing techniques used for analysis of the cytokine levels in plasma and/or serum samples might also be important. To obtain cytokine values representative of the *in vivo* conditions, the sampling techniques must prevent cytokines from being released by blood leukocytes after sampling. The time and the storage temperature between the sampling and centrifugation steps might therefore be important.

Human blood leukocytes, including monocyte-macrophages and neutrophils, synthesize a number of cytokines after stimulation with an endotoxin or bacteria through Toll-like receptor activation [14]. A number of other stimuli, including bacterial products [15], complement fragment C5a [16], immune complexes, complement activation, C5a [17] and calcium ionophores also stimulate cytokine synthesis [18]. Coagulation activation itself and thrombin can also enhance the release

Abbreviations: EDTA, ethylenediamine tetraacetic acid; IL, interleukin; RT, room temperature

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http://dx.doi.org/10.1016/j.cyto.2017.05.014
Received 23 December 2016; Received in revised form 14 May 2017; Accepted 17 May 2017

of IL-1β, IL-6 and TNF by monocytes through activation of protease-activated receptors [19]. Thrombin can activate protease-activated receptor (PAR)-1 and PAR-4, leading to leukocyte activation and cytokine release [19].

When blood is drawn into plastic tubes, complement and coagulation activation by the plastic surface leads to the activation of immune-competent cells and platelets, in part due to their recognition of this foreign surface [20]. This phenomenon of activation by foreign surfaces is termed bioincompatibility and opposite biocompatibility [20]. Complement is rapidly activated by plastic and other foreign surfaces and participates in the activation of platelets and leukocytes [21]. Coating plastic tubing with heparin effectively reduces complement activation and cytokine release [22]. The release of the anaphylatoxin C5a is involved in cytokine release triggered by several different stimuli [17,23]. In addition, coagulation activation and thrombin can also stimulate immune-competent cells, including leukocytes, leading to cytokine release [24].

Previously, most studies used serum for cytokine measurements [25]. The manufacturer of many ELISA and immunoassay kits for cytokine analyses also recommends using serum samples for the analyses. However, several studies indicated that coagulation activation is linked to inflammation in a process called immunothrombosis [26]. The potential role of thrombin in coagulation activation leading to cytokine release in human whole blood samples is yet to be examined. Several anticoagulants, such as EDTA and citrate, bind calcium and thereby inhibit both coagulation and the activation of the complement system. However, newer anticoagulants, such as hirudin and its recombinant analogue lepirudin, which specifically inhibit thrombin, have not been evaluated as anticoagulants for use in cytokine analyses. In contrast to heparin and calcium-binding anticoagulants, such as EDTA and citrate, lepirudin has no adverse effects on complement activation [27]. Lepirudin has therefore been considered the preferred anticoagulant for whole blood when the role of complement activation *in vitro* is examined [27]. The levels of 27 different cytokines, interleukins and growth factors in EDTA, citrate, lepirudin and heparin plasma and serum obtained from ten healthy blood donors were therefore examined. The influence of storage time and temperature before and after centrifugation on cytokine levels were also studied.

2. Materials and methods

2.1. Study population

To examine the effects of temperature and storage time on cytokine levels, venous blood was collected from ten healthy blood donors (five females and five males). The donors were without any medication and did not experience fever or other symptoms of infectious or inflammatory illness during the two weeks prior to blood sampling. The blood donors provided written informed consent. The study was approved by the regional ethics committee of the northern Health Region of Norway. To determine the reference ranges, EDTA plasma samples from the following groups of healthy donors were included: (1) 49 healthy blood donors from the Blood Bank of Nordlandssykehuset Bodø, (2) 42 healthy controls in a clinical epidemiological study performed in Nordland County, Norway, and (3) 71 healthy persons from a local study on health care workers; giving a total of 162 healthy Norwegian persons (107 females and 55 males, aged range 18–85). The samples were obtained by venipuncture after an overnight fast, between 8 and 9 a.m. and were immediately placed on ice, centrifuged within 15 min at +4 °C and stored at −80 °C until analysis.

2.2. Blood sampling and sample tubes

To prevent contamination by tissue thromboplastin during sampling, blood was first drawn into one dry 5-mL Vacutainer® tube (Becton Dickinson (BD), Plymouth, UK), which was discarded. Winged blood collection sets (BD Vactuainer® Safety-Lok™, needle gauge 21, needle length 0.75 inches, tubing length 7 inches, with luer locks (Franklin Lakes, NJ, U.S.A.) were used to collect the blood. Tubes were filled in the following order: blood collected in lepirudin-containing tubes (Refludan®, 50 μg/mL) was added to 4.5-mL Nunc™ polypropylene CryoTubes™ (NUNC A/S, Roskilde, Denmark) using lids from dry 4.5-mL BD Vacutainer® tubes, blood for serum samples was collected in tubes without an additive (Vacuette®, 2 mL), in 3.2% sodium citrate Vacuette® tubes (2 mL), in lithium heparin tubes (Vacuette®, 4 mL), in K3EDTA tubes (Vacuette®, 2 mL), and in serum sep clot activator tubes (Vacuette®, 4 mL). All Vacuette® tubes were purchased from Greiner Bio-One GmbH (Kremsmünster, Austria). All of the tubes were held vertically during sampling to prevent contamination by anticoagulants between using the different tubes. All tubes were gently rotated eight times by hand after sampling.

2.3. Whole blood storage conditions

After the blood samples were collected, one tube (T0) was immediately centrifuged at 3220*g* for 15 min at 4 °C and stored at −80 °C. Two tubes were stored at +4 °C and two tubes were stored at room temperature (RT), one for one hour (T1 h) and one for four h (T4 h). In the case of the two serum sampling tubes, the same procedure was initiated after one h of coagulation at room temperature.

2.4. Treatment of plasma samples

Four healthy blood donors were selected and using the same blood sampling technique. We chose four different plasma collection tubes, as follows: (1) lepirudin-containing tubes, to which EDTA (10 mM final concentration) was added immediately before centrifugation to prevent further complement activation, (2) citrate-containing tubes (3.2% sodium citrate), (3) EDTA-containing tubes (4.43 mM final concentration)

To obtain higher levels of cytokines for testing cytokine stability, some of the lepirudin anticoagulated whole blood samples were incubated with *E. coli* (1×10^7/mL) in Nunc™ cryotubes. The Nunc tubes were incubated for 2 h at 37 °C before centrifugation. Control samples were obtained by immediate centrifugation (T0). To examine the stability of cytokines in the plasma samples, the samples were stored in Nunc tubes in the dark at either RT or at +4 °C for two, four and 24 h. The *E. coli* strain LE392 (ATCC 33572) was obtained from American Type Culture Collection (Manassas, VA, U.S.A.). *E. coli* was grown overnight on a lactose dish, after which 5–10 colonies were transferred to LB-medium (1% tryptone, 0.5% (w/v) yeast extract, 1% (v/v) NaCl) purchased from Becton Dickinson (Sparks, MD) and grown overnight. The bacteria were harvested and washed once with Dulbecco's PBS without Ca^{2+} and Mg^{2+} by centrifugation at 3220*g* for 10 min at 4 °C. The bacteria were aliquoted, heat inactivated for 1 h at 60 °C and stored at −80 °C. A frozen ampoule was thawed at ambient temperature and the cells were washed six times with PBS by centrifugation at 3220*g* for 10 min at 4 °C.

2.5. Freezing and thawing of plasma

The effect of freezing and thawing on the cytokines was examined using the T0 plasma samples (sodium citrate, 4.43 mM EDTA samples). The samples were frozen (−80 °C) and then thawed on ice (0 °C) for 20 min, followed by one h of storage on ice to simulate standard sample handling. The samples were then maintained in a −80 °C freezer for a minimum of 2 h to ensure freezing. The samples were then frozen and thawed 1, 3 and 6 times.

2.6. Cytokine analyses

The cytokines in the plasma and serum samples were analysed using a Bio-Plex Human Cytokine 27-Plex Panel kit purchased from Bio-Rad

Laboratories Inc. (Hercules, CA, U.S.A.). The analyses was performed using high-sensitivity detection according to the manufacturer's instructions. Estimated values below the lowest standard were used. The following cytokines were analysed: interleukin (IL)-1 beta (IL-1β), IL-1 receptor antagonist (IL-1RA), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8 (C-X-C motif chemokine ligand 8; CXCL8), IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, eotaxin-1 (C-C motif chemokine ligand 11; CCL11), basic fibroblast growth factor (FGF-basic), granulocyte colony stimulating factor (G-CSF), granulocyte–macrophage colony stimulating factor (GM-CSF), interferon gamma (IFN-γ), interferon-γ-inducing protein 10 or IP-10 (CXCL10), monocyte chemoattractant protein 1 (MCP-1 or CCL2), macrophage inflammatory protein-1-alpha (MIP-1α or CCL3), macrophage inflammatory protein-1-beta (MIP-1β or CCL4), platelet-derived growth factor-BB (PDGF-BB), RANTES (CCL5), tumour necrosis factor (TNF) and vascular endothelial growth factor (VEGF).

2.7. Statistical analyses

GraphPad Prism 5 (GraphPad Software, CA) was used for the statistical calculations. A non-parametric, one-way repeated measurement ANOVA with Dunn's post-test was used to compare the pre-analytical sampling conditions. To analyse the effects of whole blood incubation, all of the T0 samples were compared to the T0 tube with the highest median concentration, which was the control. The dilution of the plasma due to the volume of citrate added to the citrate-containing tubes was corrected for and each donor's haematocrit was corrected according to the method of Van Beaumont [28]. The data regarding storage of whole blood and plasma at different temperatures (4 °C and RT) in each type of sample tube were analysed separately, leaving time as the only variable, by comparing the T1 h and T4 h samples to the T0 sample in the same series. $P < 0.05$ was considered to indicate significant differences. The Excel program (Microsoft Corp., Redmond, WA) replaced immeasurable cytokine levels with random numbers between 0.001 and the lower detection limit (LDL) value. Measurable cytokine levels that were lower than that of LDL were accepted as valid numbers. Some of the levels of the cytokine RANTES were greater than the highest detection limit (HDL). Excel replaced these values with random numbers between the level of HDL and the highest measured concentration of RANTES that we measured. The data for the MIP-1β, IL-1β and IP-10 levels included one extreme outlier that exceeded the mean value +/−3 SD of the other data, which were therefore excluded from the T0 figures without affecting the results of the statistical analyses. The cytokine levels in females and males were compared using the Mann-Whitney *U* test after replacing the values below the lowest measurable values with zero.

3. Results

We first aimed to investigate the optimal conditions for sampling, handling and storage of samples to be analysed for the 27 cytokines. Ten blood donors were selected for these experiments.

3.1. Effect of plasma anticoagulants on cytokine levels in immediately cooled and centrifuged blood samples compared with serum samples

The cytokine levels in EDTA, citrate, lepirudin and heparin-plasma obtained from immediately cooled and centrifuged blood samples and compared them with the levels in serum samples were studied. Two different serum-collection tubes were used, one was a plain tube lacking a gel and the other contained a gel and a clot activator. Of the 27 cytokines analysed, all were detected above the lower detection limit in some controls (see Table 4 below for details). Twelve of these were representative for the group and are presented in detail (Figs. 1 and 2). In general serum samples showed significantly ($P < 0.05$) higher levels than plasma samples (Fig. 1), whereas CXCL10 and CCL11 did not show these major difference between plasma and serum (Fig. 2). Notably, two of the cytokines, CCL2 and CCL4, were markedly and significantly increased in heparin plasma (Fig. 2). Two of the cytokines, RANTES (CCL5) and PDGF-BB, were markedly higher in serum than in plasma samples (Fig. 1), consistent with *in vitro* release during coagulation.

Upon correcting for the time during which the serum samples coagulated at RT, reached by comparing the T0 serum with plasma that had been stored at RT for 1 h, it was found that the differences in all of the cytokine levels, except for those of IL-5 and CCL3, were significant.

All together, these results indicate that cytokines should be analysed in EDTA or citrated blood samples, immediately cooled and rapidly centrifuged to obtain EDTA or citrate plasma to be stored.

3.2. Effect of storage time and the temperature during the storage of whole blood samples on plasma cytokine levels

The effect of storage time and temperature on the cytokine levels in EDTA-, citrate- and lepirudin-anticoagulated whole blood was investigated (Table 1). The here listed 15 cytokines were selected based on their robust measurable amount in the samples from tubes that were stored for 1 and 4 h at RT or 4 °C. Stable cytokine levels were observed in whole blood anticoagulated with EDTA, citrate and lepirudin when the samples were stored at 4 °C for one h. However, when EDTA-, citrate- or lepirudin-anticoagulated whole blood was stored at RT, a significant increase in some of cytokine levels were observed after only one h of storage (Table 1). An even more pronounced increase in the levels of several cytokines was observed after 4 h of incubation at RT, indicating an *in vitro* cytokine release during storage at RT. The cytokine release in heparinized whole blood was not examined.

3.3. Effect of storage temperature on plasma cytokines

We next examined the stability of the cytokines in plasma during storage. Plasma obtained from *E. coli*-incubated whole blood contained high levels of 13 different cytokines (Table 2). The cytokine levels were stable for up to four h at 4 °C in the lepirudin- and EDTA-plasma, but after 24 h, the levels of some of the cytokines decreased. Upon incubation at RT, a decrease in some of the cytokine levels was observed after only 4 h. and the levels of some cytokines even increased with increasing incubation time. In sodium citrate plasma, the CCL3 (MIP-1α), and PDGF-BB levels significantly increased after only 2 h of incubation at 4 °C, whereas that of RANTES increased after 24 h of incubation. The highest concentration of EDTA examined (10 mM) did not influence the stability of any of the cytokines in plasma (data not shown). The results indicate that EDTA plasma samples can be stored for up to 4 h at 4 °C after centrifugation and before freezing.

3.4. The effect of freezing and thawing on plasma cytokine levels

The effect of freezing and thawing on cytokine stability were then examined in EDTA and citrate plasma (Table 3). There was no significant change in the cytokine levels in plasma frozen and thawed up to three times. After freezing and thawing 6 times, a slight but significant decrease in the IL-1β level and an increase in the CCL5 (RANTES) level were observed in EDTA plasma, suggesting a maximum of three freezing and thawing in the recommendations.

We second aimed to define a reference range in a healthy human population. Based upon the above findings, we decided to test EDTA plasma, immediately stored cold, centrifuged within one h and stored at −80 °C.

3.5. Reference ranges of the cytokine levels in EDTA plasma obtained from 162 healthy controls

The reference ranges for the cytokines in EDTA plasma obtained from 162 healthy blood donors were then established (Table 4). Both

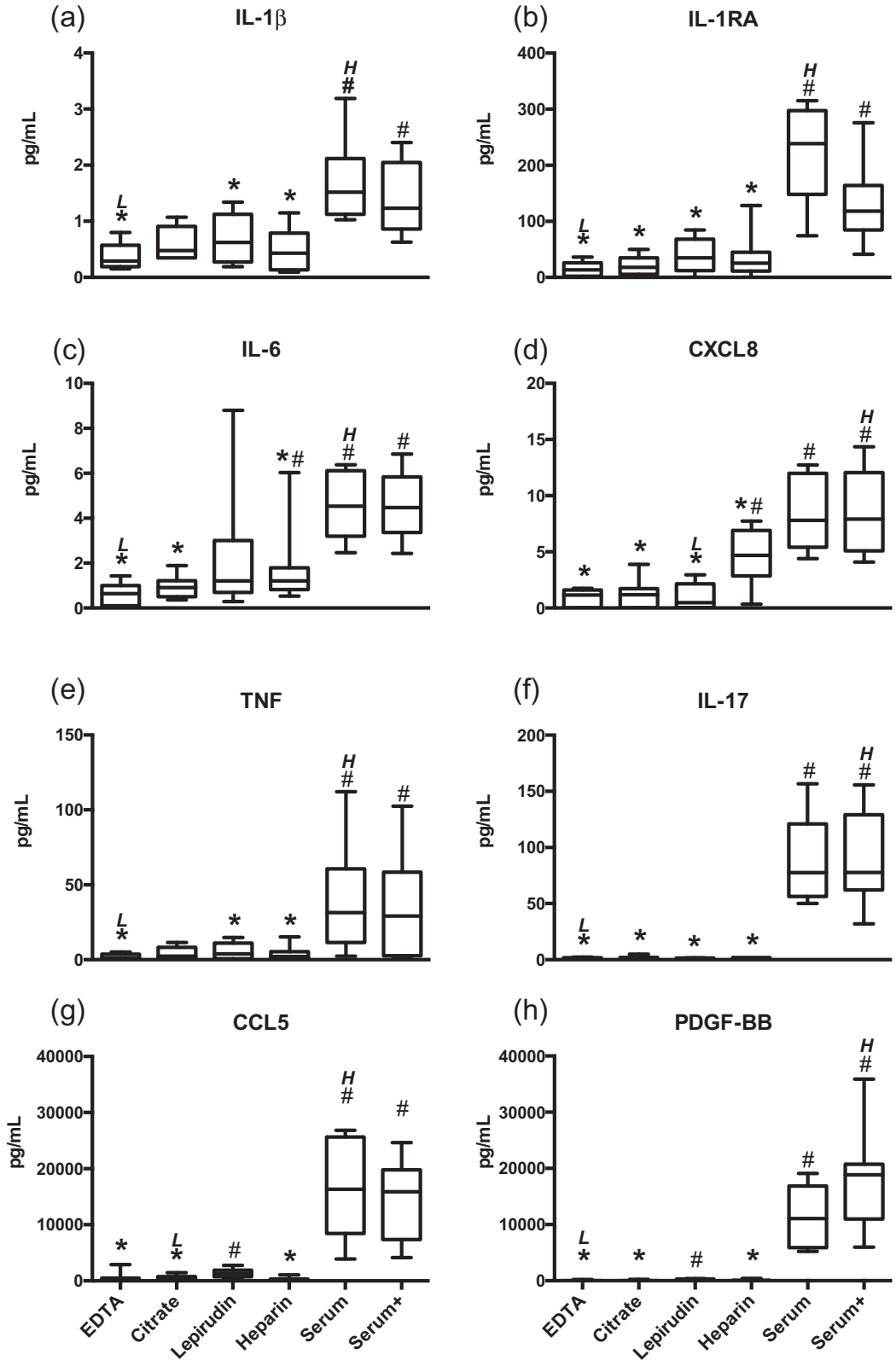

Fig. 1. Cytokine levels in serum compared with those in immediately centrifuged and frozen EDTA, citrate, lepirudin and heparin plasma samples. The levels of (a) IL-1β, (b) IL-1RA, (c) IL-6, (d) IL-8 or C-X-C motif chemokine ligand 8 (CXCL8), (e) TNF, (f) IL-17, (g) C-C motif chemokine ligand 5 (CCL5) and (h) PDGF-BB were analysed using Multiplex technology and expressed as pg/mL on the Y axis. The data are given as median values with whiskers (10 and 90 percentiles, n = 10). Serum +; serum tubes containing a gel and a clot activator. $^{*}P < 0.05$ compared with the tube in which the highest median concentration of cytokine was detected (H). $^{\#}P < 0.05$ compared with that detected in the tube with lowest median cytokine concentration (L).

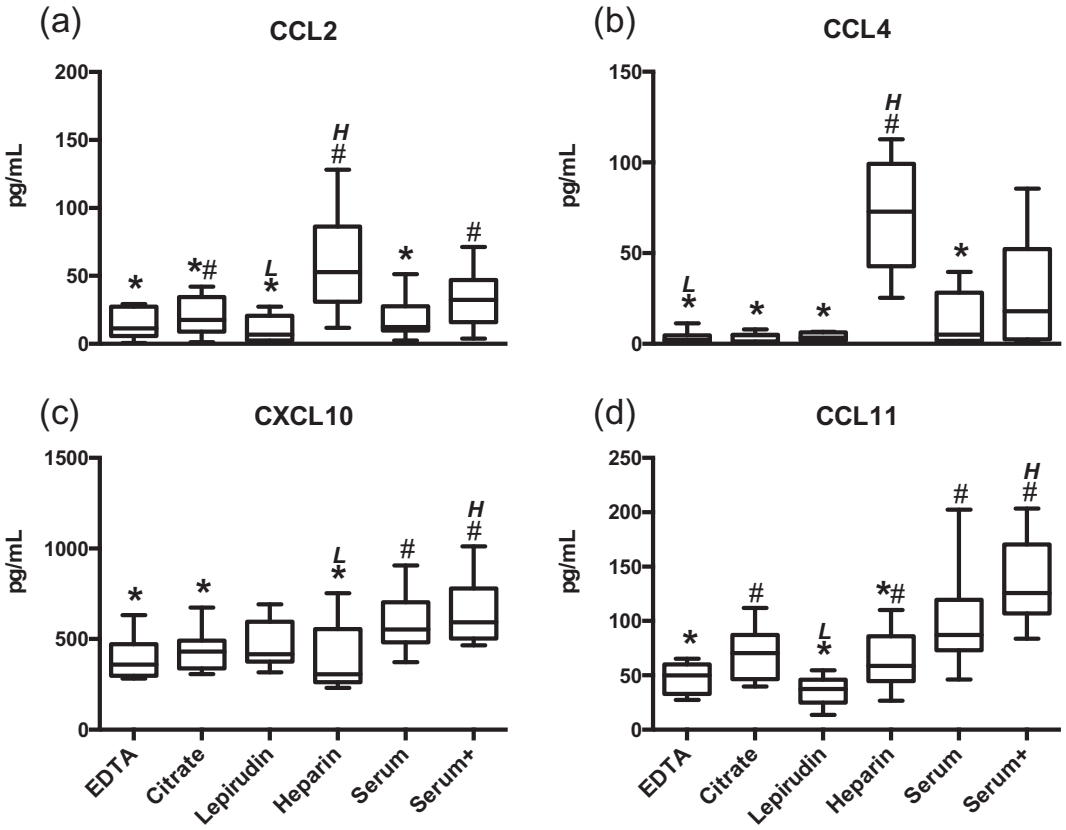

Fig. 2. Cytokines elevated in heparin plasma (a, b) and those detected at similar levels in serum and the immediately centrifuged and frozen EDTA, citrate, lepirudin and heparin plasma samples (c, d). The levels of (a) C-C motif chemokine ligand 2 or CCL2, (b) CCL4, (c), C-X-C motif chemokine ligand 10 or CXCL10, and (d) CCL11 were analysed using Multiplex technology and expressed as pg/mL. The data are given as median values with whiskers (10 and 90 percentiles, n = 10). Serum +: serum tubes containing a gel and a clot activator. $^{*}P < 0.05$ compared with the tube in which the highest median concentration of cytokine was detected (H). $^{\#}P < 0.05$ compared with that detected in the tube with lowest median cytokine concentration (L).

the 2.5–97.5th percentile and the < 95th percentile levels of the cytokines are shown. Some of the cytokines were detected in samples obtained from all of the blood donors, implying that both the 2.5–97.5th percentile values could be calculated. The frequency distribution and statistical analysis of these cytokines indicated that the upper limits were similar in females and males (Supplementary Table 1 and supplementary Fig. 1). The frequency distribution and statistical analyses of these 27 biomarkers indicated that only one of these inflammatory biomarkers, CXCL8 in males were normally distributed (Fig. 3 and Supplementary Fig. 1). The percentage of individuals (%) with undetectable cytokines (n, number of cytokines) in > 75% of the donors were: n = 0; 50–75%: n = 5; 25–50%: n = 9 and < 25% undetectable values: n = 13 different cytokines.

4. Discussion

The findings of this study indicate that the sampling conditions greatly affect the cytokine levels. In general, serum showed considerably higher levels in samples taken from both types of serum collection tubes as compared with those in plasma. After centrifugation and separation the cytokine levels were stable in plasma samples. In contrast, storage of whole blood samples, particularly when the latter were stored at room temperature, lead to increase in many of the cytokines, consistent with *in vitro* release from the cells. EDTA-plasma, obtained from blood stored on slushed ice < one h, consistently contained the lowest levels of the cytokines and could be recommended as guidelines for sampling. Thus, the tentative reference ranges for the cytokines were calculated using EDTA plasma obtained according to these guidelines from 162 healthy controls.

The higher cytokine levels observed in serum compared with those in plasma suggest that the coagulation process and thrombin activation enhances cytokine release. This result is consistent with those of previous studies [29,30]. The cytokine levels in samples taken from both types of serum collection tubes after one h of coagulation at RT were significantly higher than the levels in the baseline anticoagulated samples (T0) and anticoagulated whole blood that was stored for one h at RT. When the levels of the soluble terminal complement complex (sTCC) as a measure of complement activation were analysed using an ELISA, we found that complement was activated only in the serum collection tube containing a gel (data not shown). This result indicates that complement activation is not responsible for the high concentration of cytokines in serum. Thus, we suggest that coagulation itself induce cytokine release from whole blood leukocytes. This hypothesis is in accordance with a previous observation that thrombin activates leukocytes and cytokine release through the activation of PAR-1 and PAR-4 [31].

In anticoagulated whole blood, the levels of many cytokines increased when the samples were stored at RT, even in the presence of EDTA. These results are likely due to the *in vitro* synthesis or release of pre-synthesized cytokines by leukocytes [32], a process that is prevented at low temperature. Interestingly, in the lepirudin-anticoagulated whole blood in which thrombin activity was specifically inhibited, only a small increase in the release of PDGF-BB, IL-6, IL-8 and IL-13 was observed, indicating that thrombin is involved in the release of these cytokines. Further studies are needed to explain these findings. Due to the temperature effect, we recommend that whole blood samples be placed on ice (0 °C) after collection and be immediately centrifuged. If centrifugation is delayed, we recommend storage for up to one h and a maximal storage of 4 h at +4 °C before centrifugation.

The use of heparin plasma should be avoided because the levels of

Table 1
Cytokine levels in plasma obtained from whole blood containing different anticoagulants that was stored at 4 °C and room temperature before centrifugation.

Cytokine[a]	EDTA[b]				Citrate[b]				Lepirudin[b]			
	4 °C		RT[c]		4 °C		RT[c]		4 °C		RT[c]	
	T1h[d]	T4h[d]	T1h[d]	T4h[d]	T1h[d]	T4h[d]	T1h[d]	T4h[d]	T1h[d]	T4h[d]	T1h[d]	T4h[d]
IL-1β	0.84 (0.28–2.87)	1.1 (0.3–22)	**3.8*** (1.2–32)	**5.1*** (1.2–73)	1.0 (0.9–2.3)	1.2 (0.9–15)	**1.4*** (1.2–12)	**1.7*** (1.1–13)	1.1 (0.7–3.0)	1.6 (0.5–2.2)	**1.8*** (1.1–3.0)	**1.9*** (1.0–4.3)
IL-1RA	0.8 (0.4–4.8)	1.4 (0.3–4.4)	**6.6*** (3.8–96)	**16*** (1.8–215)	1.0 (0.3–2.7)	1.17 (0.36–2.17)	**2.0*** (1.5–5.9)	**3.9*** (0.6–29)	1.1 (0.2–73)	1.1 (0.8–29)	**2.8*** (1.4–106)	**3.2*** (1.1–180)
IL-4	1.0 (0.2–2.2)	1.0 (0.04–121)	**54*** (2.1–257)	**50*** (1.2–392)	1.7 (1.0–26)	**1.7*** (1.0–97)	**57*** (1.9–87)	**29*** (1.0–128)	1.4 (0.8–25)	2.0 (0.7–15)	**3.1*** (1.8–24)	**3.6*** (1.1–27)
IL-5	1.2 (0.8–3.8)	0.9 (0.5–66)	**9.6*** (2.5–210)	**12*** (1.2–344)	1.0 (0.8–1.6)	0.9 (0.1–96)	1.7 (1.0–43)	**2.1*** (1.0–19)	1.4 (0.5–4.8)	1.3 (0.4–61)	**2.7*** (1.5–20)	**3.1*** (1.2–25)
IL-6	1.0 (0.5–3.9)	1.2 (0.14–4.0)	6.5 (1.5–210)	**11*** (0.9–290)	1.0 (0.8–2.3)	1.1 (0.6–1.8)	**2.7*** (1.4–4.8)	**2.5*** (0.9–5.8)	1.0 (0.5–3.4)	1.5 (0.3–3.1)	**1.8*** (0.8–4.1)	1.8 (0.4–3.3)
IL-7	1.0 (0.07–2.7)	1.2 (0.1–7.9)	18 (0.8–33)	**27*** (2.1–131)	1.3 (0.08–3.1)	0.9 (0.08–3.0)	1.6 (0.8–22)	1.2 (0.9–6.5)	**1.7*** (0.5–34)	1.5 (0.2–29)	3.3 (1.0–75)	2.1 (0.6–31)
IL-8	0.7 (0.08–12)	0.9 (0.05–20)	7.6 (0.4–121)	**11*** (0.36–189)	1.1 (0.1–28)	0.8 (0.01–2.5)	1.9 (0.5–23)	1.2 (0.03–39)	1.2 (0.5–50)	1.0 (0.24–1.9)	**4.1*** (1.0–99)	1.5 (0.6–132)
IL-9	0.8 (0.08–1.4)	1.1 (0.08–3.3)	5.1 (0.17–225)	21 (0.17– > 1000)	1.0 (0.6–4.0)	1.0 (0.02–4.3)	2.1 (0.5–9.6)	2.1 (0.7–39)	1.4 (0.3–171)	1.1 (0.5–22)	**2.6*** (0.9–323)	2.4 (0.8–144)
IL-13	1.1 (0.18–2.1)	1.1 (0.34–9.8)	3.7 (0.7–126)	**12*** (1.3–380)	1.4 (0.09–27)	0.8 (0.02–2.8)	1.9 (0.06–29)	1.1 (0.08–19)	0.34 (0.12–4.3)	0.6 (0.06–3.4)	1.5 (0.1–3.4)	1.2 (0.06–38)
CCL11	**0.8*** (0.7–1.1)	0.9 (0.7–1.2)	1.3 (0.8–1.7)	**1.5*** (1.0–2.1)	**0.9*** (0.8–1.1)	0.9 (0.6–1.5)	0.9 (0.7–1.1)	0.9 (0.6–1.3)	0.7 (0.5–1.4)	**0.7*** (0.3–1.4)	0.9 (0.6–2.2)	0.7 (0.5–1.7)
G-CSF	1.0 (0.01–1.3)	1.0 (0.06–15)	**6.9*** (1.4–465)	**7.0*** (2.0–787)	1.1 (0.6–36)	1.0 (0.9–21)	**7.4*** (1.3–362)	**3.7*** (0.7–432)	1.7 (0.5–483)	1.47 (0.36–257)	**2.8*** (0.9–586)	2.5 (0.4–265)
CXCL10	1.0 (0.9–1.2)	1.1 (0.8–1.4)	1.0 (0.8–1.3)	0.9 (0.8–1.1)	0.9 (0.8–1.2)	0.9 (0.7–1.2)	0.8 (0.8–1.6)	0.8 (0.5–2.0)	0.9 (0.8–1.2)	**0.9*** (0.8–1.1)	0.9 (0.8–1.3)	0.8 (0.6–1.0)
CCL2	0.8 (0.5–2.8)	0.9 (0.3–1.1)	0.6 (0.4–2.9)	**0.4*** (0.13–2.9)	0.9 (0.6–2.3)	0.8 (0.14–1.2)	**0.7*** (0.1–0.9)	**0.36*** (0.1–0.7)	0.8 (0.03–1.1)	**0.5*** (0.04–1.2)	0.8 (0.35–1.3)	**0.6*** (0.02–0.9)
PDGF-BB	1.7 (0.36–18)	2.6 (0.05– > 1000)	743 (3.6– > 1000)	**760*** (1.7– > 1000)	4.1 (0.3–506)	**6.1*** (1.8– > 1000)	**84*** (3.9– > 1000)	**203*** (2.3– > 1000)	2.8 (0.4–13)	4.4 (0.13–11)	**7.3*** (2.3–30)	**8.1*** (1.4–42)
CCL5	1.2 (0.7–2.1)	0.9 (0.1–70)	**16*** (1.3–92)	**14*** (1.5–206)	**4.0*** (2.1–10)	**5.9*** (1.2–83)	**20*** (2.3–75)	**18*** (2.19–241)	2.4 (0.7–5.3)	2.8 (0.4–3.8)	**3.3*** (1.1–8.5)	1.5 (0.6–3.5)

[a] The cytokine levels are given as the median fold change (10–90 percentile) of their respective T0 values.
[b] The effect on cytokine levels of storing EDTA-, citrate- and lepirudin-anticoagulated whole blood at 4 °C and room temperature for one and four h. One sample of each anticoagulant was immediately centrifuged and stored at −80 °C. Two samples containing each anticoagulant were stored at 4 °C and two samples were stored at room temperature, one for one h and one for four h.
[c] RT = room temperature.
[d] T1h: one h of storage before centrifugation. T4h: four h of storage before centrifugation.
* $P < 0.05$ compared to its respective T0 basal sample (values in bold).

Table 2
Stability of cytokines in plasma obtained from *E. coli*-stimulated whole blood stored at 4 °C or room temperature after centrifugation.

Cytokine[a]	EDTA[b]						Citrate[b]					
	4 °C			RT[c]			4 °C			RT[c]		
	T2h[d]	T4h[d]	T24h[d]	T2h[d]	T4h[d]	T24h[d]	T2h[d]	T4h[d]	T24h[d]	T2h[d]	T4h[d]	T24h[d]
IL-1β	1.0 (0.9–1.2)	0.9 (0.7–0.9)	**0.7**[*] (0.6–1.0)	1.0 (0.9–1.1)	0.9 (0.8–1.0)	0.9 (0.8–1.0)	1.1 (0.9–1.2)	1.0 (0.8–1.1)	0.8 (0.6–0.9)	1.1 (0.9–1.2)	1.0 (0.8–1.2)	**0.8**[*] (0.6–0.9)
IL-1RA	1.3 (1.0–1.6)	0.7 (0.6–0.8)	0.6 (0.4–1.5)	1.0 (0.9–1.1)	0.8 (0.7–0.9)	0.7 (0.6–1.0)	1.0 (0.7–1.3)	0.9 (0.8–1.2)	0.8 (0.6–1.1)	1.1 (0.6–1.4)	0.9 (0.8–0.9)	0.8 (0.6–0.9)
IL-6	1.1 (1.1–1.3)	1.0 (0.8–1.1)	1.1 (0.9–1.2)	1.1 (1.0–1.2)	1.0 (0.9–1.2)	1.0 (0.9–1.1)	1.1 (1.0–1.2)	1.1 (1.0–1.2)	1.2 (1.0–1.4)	1.2 (0.9–1.4)	1.1 (1.1–1.2)	1.1 (1.0–1.1)
IL-8	1.1 (1.0–1.8)	0.9 (0.6–1.0)	0.7 (0.3–0.9)	1.1 (0.9–1.2)	0.8 (0.5–1.1)	1.1 (0.8–1.4)	1.1 (1.0–1.1)	1.1 (1.0–1.2)	1.1 (0.9–1.2)	1.2 (1.0–1.4)	1.1 (1.1–1.2)	1.0 (0.9–1.2)
IL-9	1.2 (0.8–1.5)	0.6 (0.5–1.1)	0.8 (0.7–1.2)	0.8 (0.6–1.1)	0.7 (0.44–1.2)	0.8 (0.7–1.0)	1.2 (1.0–1.2)	0.9 (0.8–1.1)	0.8 (0.55–0.9)	0.9 (0.8–1.3)	0.9 (0.8–1.2)	0.8 (0.8–0.9)
CCL11	0.9 (0.5–1.1)	0.7 (0.6–0.9)	0.9 (0.8–1.1)	0.9 (0.5–1.0)	**0.8**[*] (0.4–1.0)	0.9 (0.8–1.0)	1.2 (1.0–1.3)	1.0 (0.8–1.2)	0.9 (0.8–1.1)	1.0 (0.9–1.3)	0.9 (0.9–1.1)	0.9 (0.8–1.0)
IFN-γ	0.8 (0.7–1.8)	0.8 (0.45–1.3)	0.9 (0.6–1.3)	0.9 (0.6–1.3)	0.7 (0.5–1.3)	1.0 (0.9–1.2)	1.0 (1.0–1.2)	0.9 (0.8–1.1)	0.8 (0.6–1.1)	0.9 (0.7–1.2)	0.8 (0.7–1.1)	**0.7**[*] (0.7–0.8)
CXCL10	1.0 (0.8–1.2)	0.9 (0.8–1.1)	1.0 (0.9–1.9)	1.0 (0.9–1.0)	0.8 (0.7–1.0)	1.1 (0.9–1.2)	1.0 (1.0–1.1)	1.0 (0.8–1.1)	0.9 (0.8–1.1)	0.9 (0.9–1.2)	0.9 (0.8–1.0)	**0.8**[*] (0.7–0.9)
CCL3	1.0 (0.9–1.4)	1.0 (0.8–1.3)	1.1 (0.9–1.5)	1.0 (0.9–1.2)	1.0 (0.8–1.2)	1.2 (1.0–1.4)	**1.1**[*] (1.1–1.2)	1.0 (1.0–1.2)	1.1 (0.8–1.1)	1.1 (1.0–1.3)	1.0 (1.0–1.2)	1.0 (0.8–1.1)
CCL4	1.1 (1.0–1.2)	1.0 (0.9–1.1)	1.1 (0.9–1.2)	1.2 (1.1–1.2)	1.1 (1.0–1.2)	**1.3**[*] (1.2–1.5)	1.3 (0.7–1.6)	1.1 (0.8–1.7)	1.1 (0.7–1.7)	1.3 (0.8–1.6)	1.2 (1.0–1.3)	1.2 (0.9–1.4)
PDGF-BB	1.0 (0.7–1.1)	0.9 (0.8–1.0)	1.0 (0.5–1.3)	1.0 (0.8–1.2)	0.7 (0.6–1.1)	1.1 (0.9–1.1)	**1.2**[*] (1.2–1.3)	1.2 (0.8–1.2)	1.0 (1.0–1.2)	1.1 (1.0–1.3)	1.0 (0.9–1.3)	1.1 (1.1–1.1)
CCL5	1.1 (1.1–1.1)	1.0 (0.9–1.1)	1.1 (1.0–1.3)	1.3 (1.1–1.4)	1.1 (1.1–1.5)	**1.3**[*] (1.2–1.9)	1.2 (0.9–1.3)	1.2 (1.0–1.3)	**1.4**[*] (1.0–1.4)	1.4 (1.0–1.4)	1.2 (1.1–1.7)	1.3 (1.2–2.2)
TNF	1.0 (0.9–1.2)	0.8 (0.7–1.1)	0.7 (0.6–1.0)	1.0 (0.8–1.2)	0.8 (0.6–1.2)	0.9 (0.6–1.0)	1.0 (1.0–1.1)	1.0 (0.9–1.3)	1.0 (0.7–1.3)	1.2 (0.8–1.4)	1.0 (1.0–1.0)	0.7 (0.7–1.0)

[a] The cytokine levels are given as the median fold change (10–90 percentiles) of their respective T0 values.
[b] The effect on cytokine levels of storing 4.43 mM EDTA- and lepirudin-anticoagulated with citrated and plasma at 4 °C and room temperature for one and four h. One sample of each anticoagulant was immediately centrifuged and stored at −80 °C. Two samples containing each anticoagulant were stored at 4 °C and two samples were stored at room temperature, one for one h and one for four h.
[c] RT = room temperature.
[d] T1h: one h of storage before freezing. T4h: four h of storage before freezing.
* $P < 0.05$ compared to its respective T0 basal sample (values in bold).

Table 3
The effect of freezing and thawing on cytokine stability.

Cytokine[a]	EDTA[b]		Citrate[b]	
	3 times[c]	6 times[c]	3 times[c]	6 times[c]
IL-1β	0.9 (0.9–0.9)	**0.8**[*] (0.7–0.8)	1.0 (0.9–1.0)	0.9 (0.8–1.1)
IL-1RA	1.1 (0.9–1.1)	0.9 (0.9–1.0)	1.0 (0.9–1.1)	0.9 (0.8–1.2)
IL-6	1.0 (1.0–1.1)	1.0 (1.0–1.1)	1.0 (0.9–1.1)	1.0 (0.9–1.3)
IL-8	1.1 (0.9–1.2)	1.0 (1.0–1.0)	1.0 (0.9–1.1)	1.1 (0.9–1.3)
IL-9	1.1 (0.7–1.5)	1.0 (1.0–1.2)	1.1 (0.8–1.1)	0.9 (0.8–1.1)
CCL11	1.1 (0.9–1.2)	1.1 (1.0–1.2)	1.0 (1.0–1.1)	1.1 (0.8–1.1)
IFN-γ	1.0 (1.0–1.6)	1.0 (0.6–1.6)	0.9 (0.9–1.1)	0.9 (0.8–1.0)
CXCL10[d]	0.9 (0.8–1.0)	0.9 (0.8–1.0)	1.0 (0.9–1.2)	1.0 (0.8–1.2)
CCL3	1.1 (1.0–1.2)	1.0 (0.9–1.0)	1.0 (0.9–1.1)	1.0 (0.9–1.2)
CCL4	1.3 (0.8–1.7)	1.1 (0.7–1.2)	0.9 (0.4–1.1)	1.4 (0.9–2.5)
PDGF-BB	1.2 (0.7–1.2)	1.0 (0.8–1.1)	1.0 (0.8–1.2)	1.0 (0.8–1.3)
CCL5[e] S	1.3 (1.1–1.6)	**1.4**[*] (1.4–1.7)	1.1 (1.0–1.2)	**1.4**[*] (1.3–1.5)
TNF	1.0 (0.84–1.04)	1.0 (0.88–1.02)	1.1 (0.9–1.2)	0.9 (0.7–1.1)

[a] The cytokine levels are given as the median fold change (10–90 percentiles) of their respective T0 values.
[b] The effect of freezing and thawing on cytokines in 4.43 mM EDTA or citrate plasma.
[c] Samples were frozen and thawed three or six times before cytokine analyses.
[d] CXCL10, C-X-C motif chemokine ligand 10.
[e] CCL5, C-C motif chemokine ligand 5.
[*] $P < 0.05$ compared to its respective T0 value (values in bold).

Table 4
Reference ranges of cytokines in EDTA plasma.

	Median	Reference ranges[d] 2.5–97.5 percentiles	< 95 perc.	% values < LDL[e]	LDL[e] (pg/mL)
IL-1β[a]	0.7	0.1–2.0	< 1.6	0	0.03
IL-1RA	26	0–352	< 147	11	1.2
IL-2	0.03	0–10	< 7.2	49	0.03
IL-4	0.5	0–2.0	< 1.5	17	0.02
IL-5	0.7	0–2.2	< 1.6	22	0.02
IL-6	1.5	0–9.3	< 5.4	20	0.02
IL-7	0.5	0–7.6	< 6.0	46	0.06
IL-8 (CXCL8)[b]	2.8	0–11	< 7.9	14	0.01
IL-9	5.1	0–17	< 59	21	0.04
IL-10	0	0–6.6	< 3.0	61	0.03
IL-12(p70)	0.2	0–20	< 9.4	49	0.2
IL-13	1.3	0–11	< 5.9	7.4	0.03
IL-15	0	0–2.9	< 2.2	54	0.01
IL-17	0	0–69	< 31	62	0.01
CCL2 (MCP-1)[c]	13	3.1–31	< 27	1.2	0.6
CCL3 (MIP-1α)	0	0–6	< 4.2	70	0.2
CCL4 (MIP-1β)	21	0–63	< 50	46	0.5
CCL5 (RANTES)	824	20–8030	< 5379	0	3.4
CCL11 (Eotaxin-1)	38	0–120	< 91	19	0.3
FGF-basic	0	0–51	< 40	63	0.5
G-CSF	5.7	0–47	< 43	13	0.3
GM-CSF	2.0	0–37	< 24	40	0.2
IFN-γ	11	0–121	< 115	25	0.9
CXCL10	439	184–1398	< 1202	0	0.9
TNF	6.4	0–52	< 44	25	0.08
VEGF	0.4	0–12	< 6.0	49	0.2
PDGF-BB	17	0–907	< 762	28	0.1

[a] IL, interleukin.
[b] CXCL, C-X-C motif chemokine ligand.
[c] CCL, C-C motif chemokine ligand.
[d] The cytokine reference ranges were calculated in immediately cooled EDTA plasma samples obtained from 162 healthy individuals. All cytokines and biomarkers are expressed as pg/mL.
[e] LDL, Lower detection limit, defined as the lowest measurable concentration of each cytokine, or the lowest given assay LDL corresponding to the lowest standard in the assay for CCL2,4,5 and 11, FGF-basic, G-CSF, IFN-γ, CXCL10 and VEGF.

several cytokines, in particular CCL4 (MIP-1β) and CCL2 (MCP-1), were elevated in such samples. While the reason for this phenomenon remains to be elucidated, we speculate that heparin may activate monocytes and cytokine release in whole blood, as indicated in a previous report [33]. Furthermore, the anticoagulant that was used significantly affected the upregulation of tissue factor and platelet activation in fresh human whole blood [34]. This result supports the observation that the anticoagulant used significantly affects coagulation, platelet activation, and the cytokine levels in fresh human whole blood and it is reasonable to suggest that among the available anticoagulants, EDTA is the one to be preferred for cytokine analysis. However, citrated plasma can be used, but has the disadvantage that the plasma needs to be immediately separated from the cells.

The levels of cytokines in plasma were more stable than were those in whole blood. Therefore, EDTA plasma should be separated from the cellular fraction optimally within one h and maximally four h after blood sampling. After separation of plasma, the cytokines were relatively stable although time and temperature influenced the levels. Storage at RT for up to 24 h lead to a decrease in some cytokines and increase in others. A decrease may be related to the lower level of stability of some cytokines in plasma, their degradation by proteases or the binding of the cytokines to their respective soluble cellular receptors. Changes in this binding over time could also possibly explain the changes in cytokine levels that occur during sample storage. Notably, none of the cytokines changed during storage of EDTA plasma for up to 4 h at 4 °C.

Finally, the reference ranges of the cytokines in 162 healthy controls were estimated using the upper 95th percentile and the 2.5–97.5th percentile values from EDTA plasma samples, obtained in the morning and treated according to the conditions described above. We suggest the upper 95th percentile values to be the best estimate of the reference range instead of 2.5th and 97.5th for two reasons. First, from a pathophysiological point of view an increased value of a cytokine would be more frequent and reflect a disease state than a low value. Second, for most of the cytokines there were some individuals with undetectable levels, precluding an exact 2.5th percentile to be calculated. Although the distribution of cytokine values were statistically different in males and females for nine of the cytokines, most of them had very similar upper 95th percentile values. Thus, the significant difference was due to a different distribution pattern, only slightly influencing the 95th percentile. We therefore suggest that common reference range can be used for males and females.

In conclusion, our data indicate that the cytokine levels in several plasma samples, including EDTA and citrate plasma samples, are significantly lower than those in serum. Previous studies using serum should be interpreted with some caution. The mechanisms underlying the greater cytokine levels in serum compared with those in plasma remain to be elucidated, but this study indicates that thrombin-induced cytokine release might be involved. Recommended guidelines for cytokine analyses based on our data would be: EDTA or citrate blood immediately cooled on crushed ice, and immediately centrifuged and stored at −80 °C. EDTA blood is preferred since it can be stored for up to 4 h at 4 °C before centrifugation. The plasma samples can be thawed and frozen up to three times. The role of thrombin-induced cytokine release should be addressed in future studies.

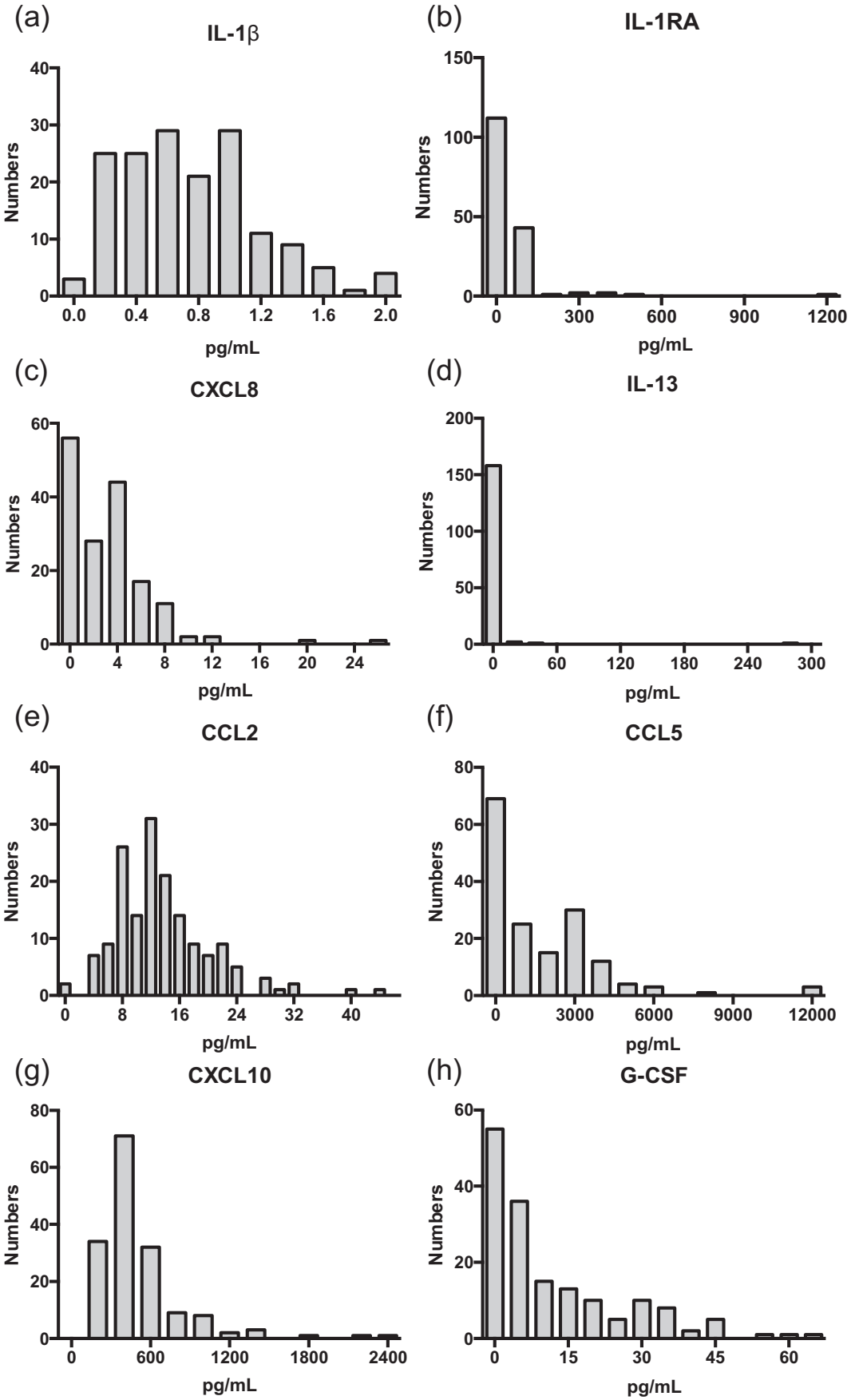

Fig. 3. Frequency histograms of selected cytokines and biomarkers in 162 healthy individuals. (a) Interleukin (IL)-1β, (b) IL-1RA, (c) C-X-C motif chemokine ligand 8 or CXCL8, (d) IL-13, (e) C-C motif chemokine ligand 2 (CCL2), (f) CCL5, (g) CXCL10 and (h) G-CSF were measured using a multiplex cytokine assay. The histogram contains grey bar graphs showing the frequency distribution of the cytokines with fewest zero values. On the x-axis is the value of the cytokine in pg/mL. On the y-axis is the number of persons with these values. The data were analysed using Prism 6 for Mac OS X.

Acknowledgements

This study was supported by the Helse Nord RHF and the National Research Council of Norway.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cyto.2017.05.014.

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Appendices

Appendix I

Egenrapportskjema for AIP

Skjemaet tas med til sykehuset undersøkelsesdagen, og leveres til lege under legekonsultasjonen.

1. Bruk av faste medisiner, helsekostpreparat og vitamin-tilskudd

	MEDIKAMENTNAVN	TYPE	DOSERING
Eksempel: Paracet		Eksempel: tablett	Eksempel: 500 mg morgen 500 mg kveld
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			

2. Tidligere påviste kroniske tilstander

(Hvis det ikke er nok plass i feltet for å beskrive nærmere, kan du gi ytterligere kommentarer på baksiden av arkene)

Spørsmål:	Hvis Ja, kryss av	Hvis Nei, kryss av	Beskriv nærmere
Har du eller har du hatt urinsyregikt?			
Har du noen kroniske betennelser nå?			Evnt. hvilke?
Har du nedsatt nyrefunksjon?			
Har du vært plaget med munntørrhet?			
Har du vanskeligheter med å tygge?			

SYKDOMMER OG TIDLIGERE OPERASJONER		DIAGNOSEÅR/ OPERERT ÅR
Eksempel: høyt blodtrykk		Eksempel: 2009
1		
2		
3		
4		
5		
6		
7		
8		
9		
10		

3. Spørsmål vedr. akutt intermitterende porfyri

Spørsmål:	Hvis Ja, kryss av	Hvis Nei, kryss av	Beskriv nærmere
Går du til regelmessig kontroll hos fastlegen angående porfyri?			Hvor ofte evnt. ?
Har du tatt regelmessige urinprøver til analyse av porfyriner?			Hvor ofte evnt. ?
Har du hatt porfyri-anfall?			Når?
Hvilke porfyri-symptomer har du hatt?			Evnt. beskriv?
Hvis alder > 50 år: Har du tatt årlig ultralyd av lever?			
Hvis > 50 år: Ønsker du at vi henviser deg til ditt lokale sykehus for årlig ultralyd-kontroll?			
Ønsker du at vi foreslår et kontrollopplegg til din fastlege ang. AIP?			
Ønsker du tilsendt et egenkontrollskjema for oppfølging av AIP tilstanden?			

.

Appendix II

Egenrapportskjema for kontrollgruppe

Skjemaet tas med til sykehuset undersøkelsesdagen, og leveres til lege under legekonsultasjonen.

1. Bruk av faste medisiner, helsekostpreparat og vitamin-tilskudd

	MEDIKAMENTNAVN	**TYPE**	**DOSERING**
Eksempel: Paracet		Eksempel: tablett	Eksempel: 500 mg morgen 500 mg kveld
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			

2. Tidligere påviste kroniske tilstander

(Hvis det ikke er nok plass i feltet for å beskrive nærmere, kan du gi ytterligere kommentarer på baksiden av arkene)

Spørsmål:	**Hvis Ja, kryss av**	**Hvis Nei, kryss av**	**Beskriv nærmere**
Har du eller har du hatt urinsyregikt?			
Har du noen kroniske betennelser nå?			Evnt. hvilke?
Har du nedsatt nyrefunksjon?			
Har du vært plaget med munntørrhet?			
Har du vanskeligheter med å tygge?			

SYKDOMMER OG TIDLIGERE OPERASJONER		DIAGNOSEÅR/ OPERERT ÅR
Eksempel: høyt blodtrykk		Eksempel: 2009
1		
2		
3		
4		
5		
6		
7		
8		
9		
10		

.

Appendix III

UTFYLLINGSSKJEMA LEGEKONSULTASJON, MANIFEST AIP

NAVN:_____

FØDSELSDATO:_____

Når ble AIP-diagnosen stilt?_____

Hvilken lege fikk du din diagnose av:_____

Hvilket sykehus fikk du din diagnose av:_____

Ble prøvene sendt til NAPOS: Ja eller Nei (ring rundt riktig)

Ble prøvene sendt til annet sykehus; hvilket i så fall:_____

Har du vært i militæret: Ja eller Nei (ring rundt riktig)

Hvis ja, hadde du noen spesielle plager i forbindelse med din militærtjeneste?

Har du brukt medisiner som du mener har utløst porfyrisymptomer hos deg?

Nei (ring rundt riktig)

Ja (ring rundt riktig)

I så fall hvilke:_____

Har du brukt medisiner som du vet at du tåler (som ikke har utløst porfyrisymptomer) ?

I så fall hvilke: _____

Bare kvinner:

Alder ved første menstruasjon:_____

Evt alder ved menopause:_____

Antall graviditeter:_____

Sykdomsopplysninger

Anfall

8. Hvor mange korte anfall (varighet mindre enn 12 timer) har du hatt?

☐ Ingen ☐ 7 - 10
☐ 1 ☐ 11 - 20
☐ 2 - 3 ☐ Mer enn 20
☐ 4 - 6

9. Hvor lenge varer vanligvis disse korte porfyrianfallene?

☐ Under 1 time
☐ 1-3 timer
☐ 4-12 timer

10. Hvor mange lengre anfall (varighet mer enn 12 timer) har du hatt?

☐ Ingen ☐ 7 - 10
☐ 1 ☐ 11 - 20
☐ 2 - 3 ☐ Mer enn 20
☐ 4 - 6

11. Hvor lenge varer vanligvis disse lengre porfyrianfallene?

☐ 12-24 timer ☐ 4-10 dager
☐ 1-3 dager ☐ Mer enn 10 dager

12. I hvilken alder har din porfyrisykdom vært mest plagsom/besværlig? (du kan sette flere kryss)

☐ 0-9 år ☐ 20-24 år ☐ 40-49 år
☐ 10-14 år ☐ 25-29 år ☐ 50-59 år
☐ 15-19 år ☐ 30-39 år ☐ 60 år og eldre

13. Hvor mange ganger har du vært innlagt på sykehus ved anfall pga. porfyri de siste 2 årene?

Antall ganger: ☐☐

14. Beskriv kort dine tre siste sykehusinnleggelser pga. porfyrisykdom:

Årstall: ☐☐☐☐ Sykehus: _____ Varighet (dager): ☐ 1-7 ☐ 8-14 ☐ >14

Årstall: ☐☐☐☐ Sykehus: _____ Varighet (dager): ☐ 1-7 ☐ 8-14 ☐ >14

Årstall: ☐☐☐☐ Sykehus: _____ Varighet (dager): ☐ 1-7 ☐ 8-14 ☐ >14

Plager

15. Hvilke plager har du opplevd i forbindelse med dine porfyrianfall? (du kan sette flere kryss)

☐ Magesmerter
☐ Oppkast
☐ Forstoppelse
☐ Muskelverk
☐ Muskelsvakhet
☐ Nedsatt følelse
☐ Lammelser
☐ Hodepine
☐ Tretthet
☐ Epilepsi
☐ Hjertebank
☐ Mørk/rødfarget urin
☐ Psykiske plager, hvilke:
☐ Annet:

(bruk kommentarfeltet på siste side dersom det blir for liten plass)

16. Hvilke plager hadde du ved ditt hittil sterkeste anfall, og hvor plagsomme var de?

Plager:	**Ikke tilstede**	**Lite plagsom**	**middels plagsom**	**plagsom**	**svært plagsom**
Magesmerter	☐	☐	☐	☐	☐
Oppkast	☐	☐	☐	☐	☐
Forstoppelse	☐	☐	☐	☐	☐
Muskelverk	☐	☐	☐	☐	☐
Muskelsvakhet	☐	☐	☐	☐	☐
Nedsatt følelse	☐	☐	☐	☐	☐
Lammelser	☐	☐	☐	☐	☐
Hodepine	☐	☐	☐	☐	☐
Tretthet	☐	☐	☐	☐	☐
Epilepsi	☐	☐	☐	☐	☐
Hjertebank	☐	☐	☐	☐	☐

☐ Psykiske plager, hvilke:

☐ Mørk/rødfarget urin

☐ Andre plager:

(bruk kommentarfeltet på siste side dersom det blir for liten plass)

17. Hvilke faktorer mener du kan ha forårsaket porfyrianfall hos deg en eller flere ganger? (Du kan sette flere kryss)

- ☐ Slanking
- ☐ Sult
- ☐ Fysiske påkjenninger
- ☐ Psykisk stress
- ☐ Søvnmangel
- ☐ Alkohol
- ☐ Tobakk
- ☐ Medisiner (Se også spørsmål 34)
- ☐ P-piller, hvilke:

☐ Menstruasjonssyklus, når:
- ☐ første menstruasjonsdager
- ☐ ved eggløsning (ca. 14 dager før menstruasjon)
- ☐ annet:

☐ Arbeidsmiljø, hva:

☐ Andre sykdommer, hvilke (f.eks. influensa, lungebetennelse):

☐ Mat, hvilken:

☐ Andre ting, hva:

☐ vet ikke (bruk kommentarfeltet på siste side dersom det blir for liten plass)

18. Har du eller har du noen gang hatt langvarige plager (varighet mer enn en måned) som forbindes med porfyrisykdommen?

☐ Nei
☐ Ja, hvilke:

(bruk kommentarfeltet på siste side dersom det blir for liten plass)

19. Mener du at du i perioder også (uavhengig av anfall):

- har nedsatt følelse i armer eller ben?	☐ Ja	☐ Nei
- har nedsatt kraft i armer eller ben?	☐ Ja	☐ Nei
- er overfølsom for sollys?	☐ Ja	☐ Nei
- har fatigue/utmattelse	☐ Ja	☐ Nei
- har synsfeltutfall	☐ Ja	☐ Nei
- har inkontinens	☐ Ja	☐ Nei

Sykdomsopplysninger - siste to år

20. Har du hatt anfall de siste to årene? ☐ Ja ☐ Nei (gå videre til spørsmål 23)

21. Hvis ja; hvilke plager hadde du ved ditt siste anfall, og hvor plagsomme var de?

Plager:	**Ikke tilstede**	**Lite plagsom**	**middels plagsom**	**plagsom**	**svært plagsom**
Magesmerter	☐	☐	☐	☐	☐
Oppkast	☐	☐	☐	☐	☐
Forstoppelse	☐	☐	☐	☐	☐
Muskelverk	☐	☐	☐	☐	☐
Muskelsvakhet	☐	☐	☐	☐	☐
Nedsatt følelse	☐	☐	☐	☐	☐
Lammelser	☐	☐	☐	☐	☐
Hodepine	☐	☐	☐	☐	☐
Tretthet	☐	☐	☐	☐	☐
Epilepsi	☐	☐	☐	☐	☐
Hjertebank	☐	☐	☐	☐	☐

☐ Psykiske plager, hvilke:

☐ Mørk/rødfarget urin

☐ Andre plager:

(bruk kommentarfeltet på siste side dersom det blir for liten plass)

Dersom du har hatt anfall de siste to årene:

22. Hvilke faktorer mener du utløste plager ved ditt siste porfyrianfall? (Du kan sette flere kryss)

- ☐ Slanking
- ☐ Sult
- ☐ Fysiske påkjenninger
- ☐ Psykisk stress
- ☐ Søvnmangel
- ☐ Alkohol
- ☐ Tobakk
- ☐ Medisiner (Se også spørsmål 34)
- ☐ P-piller, hvilke:
- ☐ Menstruasjonssyklus, når:
  - ☐ første menstruasjonsdager
  - ☐ ved eggløsning (ca. 14 dager før menstruasjon)
  - ☐ annet:
- ☐ Arbeidsmiljø, hva:
- ☐ Andre sykdommer, hvilke (f.eks. influensa, lungebetennelse):
- ☐ Mat, hvilken:
- ☐ Andre ting, hva:
- ☐ vet ikke

(bruk kommentarfeltet på siste side dersom det blir for liten plass)

Behandling

23. Hvor mange ganger har du tatt sukker for å forebygge et anfall?

- ☐ aldri
- ☐ 1-10 ganger
- ☐ 11-50 ganger
- ☐ Mer enn 50 ganger

24. Når var siste gang du tok sukker:

måned: ☐☐ årstall: ☐☐☐☐

25. Hvor mange ganger har du fått behandling med glukose iv.(intravenøst)?

- ☐ aldri
- ☐ 1-3 ganger
- ☐ 4-10 ganger
- ☐ Mer enn 10 ganger

26. Når var siste gang du fikk behandling med glukose iv.?

måned: ☐☐ årstall: ☐☐☐☐

27. Hvor fikk du denne behandlingen:

28. Hvor mange ganger har du fått behandling med Normosang (Hemearginat) iv.(intravenøst)?

- ☐ Aldri
- ☐ 1-3 ganger
- ☐ 4-10 ganger
- ☐ Mer enn 10 ganger

29. Når var siste gang du fikk behandling med Normosang iv.? (Hemearginat)

måned: ☐☐ årstall: ☐☐☐☐

30. Hvor fikk du denne behandlingen:

31. Har du fått annen behandling for din porfyrisykdom?

- ☐ Nei
- ☐ Ja; angi hvilken behandling:

32. Hvor mange ganger har du fått denne behandlingen?

- ☐ 1-3 ganger
- ☐ 4-10 ganger
- ☐ Mer enn 10 ganger

Andre forholdsregler

33. Hvilke andre forholdsregler tar du i din hverdag, for å unngå symptomer av din porfyrisykdom?

(bruk kommentarfeltet på siste side dersom det blir for liten plass)

Levevaner

Røyking og alkoholinntak er to faktorer som kan utløse porfyrianfall, og det er derfor ønskelig at du nedenfor kort beskriver dine røykevaner og ditt alkoholforbruk.

Røyking

58. Røyker du eller har du røykt?
- ☐ Har aldri røykt (gå til spørsmål 63)
- ☐ Røykte daglig før, har sluttet
- ☐ Røykte av og til før, har sluttet
- ☐ Røyker av og til
- ☐ Røyker daglig

59. Alder da du begynte å røyke: .. ☐☐ år

60. Alder ved røykestopp: ..... ☐☐ år

61. Hva og hvor mye røyker du vanligvis?
Ferdigsigaretter, antall pr. dag: ..... ☐☐
Rulletobakk, antall sigaretter pr. dag: ..... ☐☐
Sigar, antall pr. dag:..... ☐☐

62. Hva og hvor mye røykte du vanligvis før du sluttet?
Ferdigsigaretter, antall pr. dag: ..... ☐☐
Rulletobakk, antall sigaretter pr. dag: ..... ☐☐
Sigar, antall pr. dag:..... ☐☐

Alkohol

63. Drikker du alkohol?
- ☐ Nei (gå til spørsmål 67)
- ☐ Ja

64. Hvor ofte drikker du vanligvis alkohol?
- ☐ Sjeldnere enn 1 gang pr. måned
- ☐ Omtrent 1-3 ganger pr. måned
- ☐ Omtrent 1-3 ganger pr. uke
- ☐ Mer enn 3 ganger pr. uke

65. Hvor mange "enheter" alkohol drikker du i gjennomsnitt pr. uke?
(1 enhet alkohol = 1 liten flaske øl = 1 glass vin = 1 drink (brennevin), 1 flaske vin = 6 enheter)
- ☐ Mindre enn 1
- ☐ 1-5
- ☐ 6-10
- ☐ Mer enn 10

66. Er det noen typer alkoholholdige drikker som du mener kan utløse porfyrisymptomer hos deg?
- ☐ rødvin
- ☐ hvitvin
- ☐ øl
- ☐ brennevin
- ☐ annet:

Fysisk aktivitet og dagligliv

67. Hvis du er i lønnet eller ulønnet arbeid; hvordan vil du beskrive arbeidet ditt?
- ☐ For det meste stillesittende arbeid (f.eks. skrivebordsarbeid, montering)
- ☐ Arbeid som krever at jeg går mye (f.eks. ekspeditørarbeid, lett industriarbeid, undervisning)
- ☐ Arbeid hvor jeg går og løfter mye (f.eks. postbud, pleier, bygningsarbeid)
- ☐ Tungt kroppsarbeid (f.eks. skogsarbeid, tungt jordbruksarbeid, tungt bygningsarbeid)

68. Hvor mye fysisk aktivitet bedriver du i fritiden din? (inkl. reise til og fra arbeid)

	Ingen	**under 1 time pr. uke**	**1-2 timer pr. uke**	**>3 timer pr. uke**
Lett fysisk aktivitet (ikke svett/andpusten, f.eks. turgåing).....	☐	☐	☐	☐
Hard fysisk aktivitet (svett/andpusten).....	☐	☐	☐	☐

69. Hvordan vil du beskrive din hverdag?
- ☐ Jevnt over rolig og lite stressende
- ☐ Jevnt over rolig og lite stressende, men mye stress i perioder
- ☐ Jevnt over aktiv og middels stressende
- ☐ Jevnt over aktiv og middels stressende, men mye stress i perioder
- ☐ Jevnt over høyt tempo og svært stressende

Livskvalitet

70. Mener du at sykdommen og de begrensninger den medfører går utover din livskvalitet?
- ☐ Nei, ikke i det hele tatt
- ☐ Nei, i liten grad
- ☐ Ja, delvis
- ☐ Ja, i stor grad

Problemer

71. Hva synes du er mest problematisk med det å ha sykdommen AIP, sett i forhold til din hverdag? (arbeid, skole, hjem og fritid)

Appendix IV

UTFYLLINGSSKJEMA LEGEKONSULTASJON, LATENT AIP

NAVN:_____

FØDSELSDATO:_____

Når ble AIP-diagnosen stilt?_____

Hvilken lege fikk du din diagnose av:_____

Hvilket sykehus fikk du din diagnose av:_____

Ble prøvene sendt til NAPOS: Ja eller Nei (ring rundt riktig)

Ble prøvene sendt til annet sykehus; hvilket i så fall:_____

Har du vært i militæret: Ja eller Nei (ring rundt riktig)

Hvis ja, hadde du noen spesielle plager i forbindelse med din militærtjeneste?

I så fall hvilke:_____

Har du brukt medisiner som du mener har utløst porfyrisymptomer hos deg?

Nei (ring rundt riktig)

Ja (ring rundt riktig) I så fall hvilke:_____

Har du brukt medisiner som du vet at du tåler (som ikke har utløst porfyrisymptomer)?

I så fall hvilke:_____

Bare kvinner:

Alder ved første menstruasjon:_____

Evt alder ved menopause:_____

Antall graviditeter:_____

Tar du noen forholdsregler i din hverdag, for å unngå symptomer av din porfyrisykdom?

Nei (ring rundt riktig)

Ja (ring rundt riktig)

I så fall, hvilke _____

_____

_____

_____

_____

Opplever du at din porfyrisykdom setter begrensninger for deg når det gjelder enkelte aktiviteter (i forbindelse med f.eks arbeid, skole, hjem og husholdning, generelt dagligliv eller sport og fritid)?

Nei (ring rundt riktig)

Ja (ring rundt riktig)

I så fall hvilke aktiviteter gjelder dette:_____
(bruk baksiden hvis for lite plass)

Har du noen gang vært innlagt på sykehus for magesmerter?

Nei (ring rundt riktig) Ja (ring rundt riktig)

Har du noen gang blitt operert i magen: Nei (ring rundt riktig) Ja (ring rundt riktig)

I så fall hvorfor:_____

Har du noen gang fått narkose? Nei (ring rundt riktig) Ja (ring rundt riktig)

Hvis ja, har du fått noen reaksjoner i forbindelse med narkose?

I så fall hvilke reaksjoner: _____

Har du fått noen reaksjoner i forbindelse med medisiner du bruker nå fortiden, fast eller av og til?

Nei (ring rundt riktig) Ja (ring rundt riktig)

Hvis ja, hvilken medisin og hvilken reaksjon:_____

_____

Har du tidligere brukt medisiner du har reagert på?

Nei (ring rundt riktig) Ja (ring rundt riktig)

Hvis ja, hvilken medisin og hvilken reaksjon:

_____

_____

Har du vært spesielt utsatt for noen av plagene som er nevnt nedenfor: (det kan settes flere kryss)

- ☐ Magesmerter
- ☐ Oppkast
- ☐ Forstoppelse
- ☐ Muskelverk
- ☐ Muskelsvakhet
- ☐ Nedsatt følelse
- ☐ Lammelser
- ☐ Hodepine
- ☐ Tretthet
- ☐ Epilepsi
- ☐ Hjertebank
- ☐ Mørk/rødfarget urin
- ☐ Fatigue/utmattelse
- ☐ Synsfeltutfall
- ☐ Inkontinens
- ☐ Psykiske plager, i så fall hvilke _____ _____
- ☐ Annet: _____

Levevaner

Røyking og alkoholinntak er to faktorer som kan utløse porfyrianfall, og det er derfor ønskelig at du nedenfor kort beskriver dine røykevaner og ditt alkoholforbruk.

Røyking

58. Røyker du eller har du røykt?
- ☐ Har aldri røykt (gå til spørsmål 63)
- ☐ Røykte daglig før, har sluttet
- ☐ Røykte av og til før, har sluttet
- ☐ Røyker av og til
- ☐ Røyker daglig

59. Alder da du begynte å røyke: .. ☐☐ år

60. Alder ved røykestopp: ..... ☐☐ år

61. Hva og hvor mye røyker du vanligvis?
Ferdigsigaretter, antall pr. dag: ..... ☐☐
Rulletobakk, antall sigaretter pr. dag: ..... ☐☐
Sigar, antall pr. dag:..... ☐☐

62. Hva og hvor mye røykte du vanligvis før du sluttet?
Ferdigsigaretter, antall pr. dag: ..... ☐☐
Rulletobakk, antall sigaretter pr. dag: ..... ☐☐
Sigar, antall pr. dag:..... ☐☐

Alkohol

63. Drikker du alkohol?
- ☐ Nei (gå til spørsmål 67)
- ☐ Ja

64. Hvor ofte drikker du vanligvis alkohol?
- ☐ Sjeldnere enn 1 gang pr. måned
- ☐ Omtrent 1-3 ganger pr. måned
- ☐ Omtrent 1-3 ganger pr. uke
- ☐ Mer enn 3 ganger pr. uke

65. Hvor mange "enheter" alkohol drikker du i gjennomsnitt pr. uke?
(1 enhet alkohol = 1 liten flaske øl = 1 glass vin = 1 drink (brennevin), 1 flaske vin = 6 enheter)
- ☐ Mindre enn 1
- ☐ 1-5
- ☐ 6-10
- ☐ Mer enn 10

66. Er det noen typer alkoholholdige drikker som du mener kan utløse porfyrisymptomer hos deg?
- ☐ rødvin
- ☐ hvitvin
- ☐ øl
- ☐ brennevin
- ☐ annet:

Fysisk aktivitet og dagligliv

67. Hvis du er i lønnet eller ulønnet arbeid; hvordan vil du beskrive arbeidet ditt?
- ☐ For det meste stillesittende arbeid (f.eks. skrivebordsarbeid, montering)
- ☐ Arbeid som krever at jeg går mye (f.eks. ekspeditørarbeid, lett industriarbeid, undervisning)
- ☐ Arbeid hvor jeg går og løfter mye (f.eks. postbud, pleier, bygningsarbeid)
- ☐ Tungt kroppsarbeid (f.eks. skogsarbeid, tungt jordbruksarbeid, tungt bygningsarbeid)

68. Hvor mye fysisk aktivitet bedriver du i fritiden din? (inkl. reise til og fra arbeid)

	Ingen	**under 1 time pr. uke**	**1-2 timer pr. uke**	**>3 timer pr. uke**
Lett fysisk aktivitet (ikke svett/andpusten, f.eks. turgåing).....	☐	☐	☐	☐
Hard fysisk aktivitet (svett/andpusten).....	☐	☐	☐	☐

69. Hvordan vil du beskrive din hverdag?
- ☐ Jevnt over rolig og lite stressende
- ☐ Jevnt over rolig og lite stressende, men mye stress i perioder
- ☐ Jevnt over aktiv og middels stressende
- ☐ Jevnt over aktiv og middels stressende, men mye stress i perioder
- ☐ Jevnt over høyt tempo og svært stressende

Livskvalitet

70. Mener du at sykdommen og de begrensninger den medfører går utover din livskvalitet?
- ☐ Nei, ikke i det hele tatt
- ☐ Nei, i liten grad
- ☐ Ja, delvis
- ☐ Ja, i stor grad

Problemer

71. Hva synes du er mest problematisk med det å ha sykdommen AIP, sett i forhold til din hverdag? (arbeid, skole, hjem og fritid)

Appendix V

UTFYLLINGSSKJEMA LEGEKONSULTASJON, KONTROLLGRUPPE

NAVN:_____

FØDSELSDATO:_____

Har du vært i militæret: Ja eller Nei (ring rundt riktig)

Hvis ja, hadde du noen spesielle plager i forbindelse med din militærtjeneste?

I så fall hvilke:_____

Bare kvinner:

Alder ved første menstruasjon:_____

Evt alder ved menopause:_____

Antall graviditeter:_____

Har du noen gang vært innlagt på sykehus for magesmerter?

Nei (ring rundt riktig) Ja (ring rundt riktig)

Har du noen gang blitt operert i magen: Nei (ring rundt riktig) Ja (ring rundt riktig)

I så fall hvorfor:_____

Har du noen gang fått narkose? Nei (ring rundt riktig) Ja (ring rundt riktig)

Hvis ja, har du fått noen reaksjoner i forbindelse med narkose?

I så fall hvilke reaksjoner: _____

Har du fått noen reaksjoner i forbindelse med medisiner du bruker nå fortiden, fast eller av og til?

Nei (ring rundt riktig) Ja (ring rundt riktig)

Hvis ja, hvilken medisin og hvilken reaksjon:_____

_____

Har du tidligere brukt medisiner du har reagert på?

Nei (ring rundt riktig) Ja (ring rundt riktig)

Hvis ja, hvilken medisin og hvilken reaksjon:

_____

_____

Har du vært spesielt utsatt for noen av plagene som er nevnt nedenfor: (det kan settes flere kryss)

- ☐ Magesmerter
- ☐ Oppkast
- ☐ Forstoppelse
- ☐ Muskelverk
- ☐ Muskelsvakhet
- ☐ Nedsatt følelse
- ☐ Lammelser
- ☐ Hodepine
- ☐ Tretthet
- ☐ Epilepsi
- ☐ Hjertebank
- ☐ Mørk/rødfarget urin
- ☐ Fatigue/utmattelse
- ☐ Synsfeltutfall
- ☐ Inkontinens
- ☐ Psykiske plager, i så fall hvilke _____ _____

- ☐ Annet: _____

Levevaner

Røyking og alkoholinntak er to faktorer som kan utløse porfyrianfall, og det er derfor ønskelig at du nedenfor kort beskriver dine røykevaner og ditt alkoholforbruk.

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- ☐ Har aldri røykt (gå til spørsmål 63)
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- ☐ Røykte av og til før, har sluttet
- ☐ Røyker av og til
- ☐ Røyker daglig

59. Alder da du begynte å røyke: .. ☐☐ år

60. Alder ved røykestopp: ..... ☐☐ år

61. Hva og hvor mye røyker du vanligvis?
Ferdigsigaretter, antall pr. dag: ..... ☐☐
Rulletobakk, antall sigaretter pr. dag: ..... ☐☐
Sigar, antall pr. dag:..... ☐☐

62. Hva og hvor mye røykte du vanligvis før du sluttet?
Ferdigsigaretter, antall pr. dag: ..... ☐☐
Rulletobakk, antall sigaretter pr. dag: ..... ☐☐
Sigar, antall pr. dag:..... ☐☐

Alkohol

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- ☐ Nei (gå til spørsmål 67)
- ☐ Ja

64. Hvor ofte drikker du vanligvis alkohol?
- ☐ Sjeldnere enn 1 gang pr. måned
- ☐ Omtrent 1-3 ganger pr. måned
- ☐ Omtrent 1-3 ganger pr. uke
- ☐ Mer enn 3 ganger pr. uke

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- ☐ 1-5
- ☐ 6-10
- ☐ Mer enn 10

66. Er det noen typer alkoholholdige drikker som du mener kan utløse porfyrisymptomer hos deg?
- ☐ rødvin
- ☐ hvitvin
- ☐ øl
- ☐ brennevin
- ☐ annet:

Fysisk aktivitet og dagligliv

67. Hvis du er i lønnet eller ulønnet arbeid; hvordan vil du beskrive arbeidet ditt?
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- ☐ Arbeid som krever at jeg går mye (f.eks. ekspeditørarbeid, lett industriarbeid, undervisning)
- ☐ Arbeid hvor jeg går og løfter mye (f.eks. postbud, pleier, bygningsarbeid)
- ☐ Tungt kroppsarbeid (f.eks. skogsarbeid, tungt jordbruksarbeid, tungt bygningsarbeid)

68. Hvor mye fysisk aktivitet bedriver du i fritiden din? (inkl. reise til og fra arbeid)

	Ingen	**under 1 time pr. uke**	**1-2 timer pr. uke**	**>3 timer pr. uke**
Lett fysisk aktivitet (ikke svett/andpusten, f.eks. turgåing).....	☐	☐	☐	☐
Hard fysisk aktivitet (svett/andpusten).....	☐	☐	☐	☐

69. Hvordan vil du beskrive din hverdag?
- ☐ Jevnt over rolig og lite stressende
- ☐ Jevnt over rolig og lite stressende, men mye stress i perioder
- ☐ Jevnt over aktiv og middels stressende
- ☐ Jevnt over aktiv og middels stressende, men mye stress i perioder
- ☐ Jevnt over høyt tempo og svært stressende

Livskvalitet

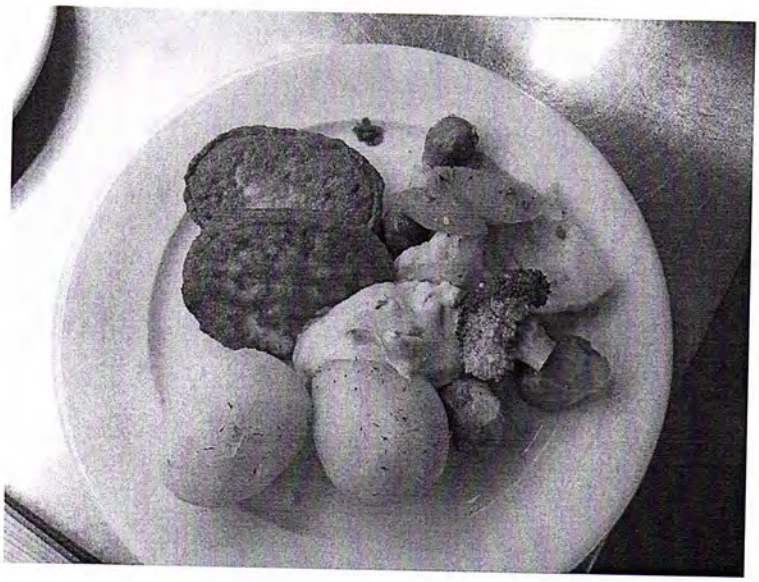
70. Mener du at sykdommen og de begrensninger den medfører går utover din livskvalitet?
- ☐ Nei, ikke i det hele tatt
- ☐ Nei, i liten grad
- ☐ Ja, delvis
- ☐ Ja, i stor grad

Problemer

71. Hva synes du er mest problematisk med det å ha sykdommen AIP, sett i forhold til din hverdag? (arbeid, skole, hjem og fritid)

Appendix VI

Råd om utfylling av kostdagboken



Råd om utfylling av kostdagboken

For å få en best mulig oversikt over det du spiser og drikker ønsker vi å vite HVA og HVOR MYE du spiser og drikker i løpet av syv dager.

I mappen finner du:

- Råd om utfylling av dagboken
- Syv dagbøker, en dagbok for hver registreringsdag
- Et bildehefte til hjelp ved beskrivelse av HVOR MYE som blir spist/drukket
- En frankert svarkonvolutt med påført adresse

Kosten skal registreres syv sammenhengende dager.

Du skal registrere alt du spiser og drikker fra du våkner om morgenen første registreringsdag til du sovner for natten siste dag i registreringsperioden.

Du skal spise akkurat som du pleier. Det er MEGET VIKTIG at du IKKE ENDRER noe på vanene dine i forbindelse med denne undersøkelsen (dagen kan selvsagt være "uvanlig" ved sykdom, bursdager og lignende).

Det er MEGET VIKTIG at du skriver ned ALT du spiser og drikker i løpet av disse dagene.

Hvordan fyller du ut dagboken?
Bla gjennom dagboken og bildeheftet slik at du blir kjent med innholdet. På de siste sidene i denne informasjonen, er det eksempler på hvordan dagboken fylles ut.

Forsiden
På forsiden av hver dagbok, skal det fylles inn kjønn, alder, hvilken ukedag det er, dato og om det var en vanlig eller uvanlig dag. På forsiden finner du også en oversikt over hvor du finner de ulike matvarene i dagboken.

Tidsbolker
Legg merke til at en dag er delt inn i 5 tidsbolker (eks. kl. 6-10, kl. 10-14). Fire av disse er på 4 timer, mens den siste strekker seg fra kl. 22 om kvelden til kl. 06 neste morgen.

Du skal skrive ned hvor mye du har spist eller drukket i de aktuelle tidsbolkene. Har du begynt å spise i en tidsbolk og sluttet i neste, skriver du alt i den tidsbolken du begynte.

ABSOLUTT ALT du spiser og drikker skal registreres
For hver matvare/drikke er det oppgitt en enhet. For eksempel skal drikke angis i antall glass, og brød i antall skiver. Du skal for alle matvarer angi hvor mange enheter du har spist/drukket. Du kan skrive hele tall som 1, 2, 3 eller deler som ¼ eller 1 ½ for alle matvarer og drikker i dagboken. Antallet skal fylles inn i de sorte rutene.

ABSOLUTT BARE bokstaver i de orange rutene
For noen matvarer må du se i bildeboken, for å angi hvor mye du har spist. Ved disse matvarene er det vist til den bildeserien du må se på.

Bildeseriene består av 4 alternativer merket A, B, C og D. Velg det alternativet som stemmer best med hvor mye du har spist. Du skriver bokstaven inn i den orange ruten.

Enkelte matvarer skal du sammenligne med bilder som ikke ligner på det du har spist. Her skal du kun bruke bildene til å se hvor stor plass matvaren du spiser tar på tallerkenen.

Spiser du flere porsjoner av ulik størrelse, må du tenke hvordan alle porsjonene ville sett ut til sammen. Har du eksempelvis spist to porsjoner av spaghetti, en som ligner på B og en annen på A, kan du skrive 1 ½ B. Skriv det som ligner mest på det du til sammen spiste.

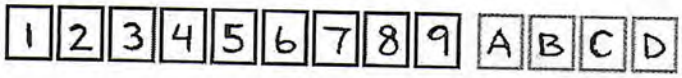
ABSOLUTT IKKE kryss i dagboken
Det skal aldri brukes kryss i dagboken. Det skal kun skrives bokstaver og tall.

Når matvaren ikke er i dagboken
Spiser du matvarer/matretter som ikke finnes oppført i dagboken, må du beskrive nøye det du har spist, hvor mye og når du har spist i de åpne boksene "Annet – beskriv best mulig hva, hvor mye og når".

Praktisk gjennomføring
Om du skriver nøyaktig hva du har spist/drukket like etterpå, eller om du vil notere ned på et hjelpeark og "føre inn" på kvelden, er opp til deg. Men ikke vent til neste dag, da kan det være lett å glemme noe. Hjelpearket kan være greit å ha med seg når en er på besøk eller andre steder hvor det kan bli spist/drukket noe.

- Det er viktig at du ikke bretter eller krøller dagbøkene.
- Bruk gjerne myk blyant å skrive med.
- Du kan også bruke blå eller sort penn.

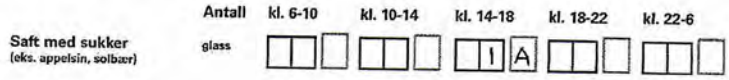
- Du skal skrive tall og bokstaver som vist her:



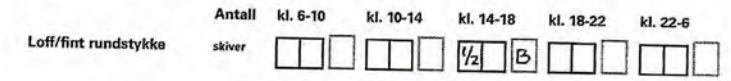
Lurer du på noe?
Marlene Blomstereng Karlsen ringer andre dagen i registreringsperioden for å oppklare eventuelle spørsmål eller problemer. Du kan også ringe **Marlene Blomstereng Karlsen** på telefon **995 26 607** mellom kl. 09-20 hvis det er noe du lurer på i forbindelse med utfylling av dagboken. Hvis jeg ikke kan ta telefonen, send en sms så ringer jeg tilbake så snart jeg kan.

Eksempel
Ingrid begynner å spise middag kl. 17.45. Hun spiser en porsjon med spaghetti og tomatsaus med pølsebiter, og litt revet ost på toppen. I tillegg spiser hun en halv skive loff med Soft Flora margarin. Til middag drikker hun ett glass saft. Hun spiser en liten bit mango til dessert.

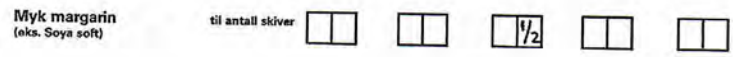
Ingrid tar frem både dagboken og bildeboken. Hun blar opp på siden for drikke. Hun finner linjen for "Saft med sukker" og i kolonnen "kl. 14-18" skriver hun 1 i en av de sorte rutene og etter å ha sett i bildeboken skriver hun "A" i den orange ruten, da glasset hun brukte lignet mest på A-glasset.



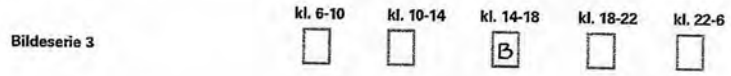
Ingrid spiste en halv skive loff med Soft Flora margarin. Ingrid blar opp på siden med brød. Hun finner linjen med "Loff/fint rundstykke" og skriver ½ i de sorte rutene i kolonnen "kl. 14-18". Hun ser på tykkelsen i bildeheftet og finner at tegning B passer best til brødskiven hun spiste. Hun skriver B i den orange ruten.



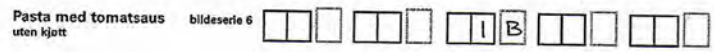
Ingrid finner linjen med "Myk margarin" og skriver ½ i kolonnen "kl. 14-18".



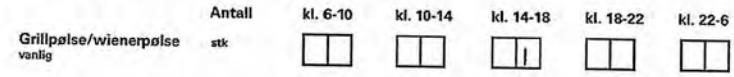
Deretter ser hun i bildeboken og finner at hun brukte samme mengde som på bilde B. Under "Hvor mye smurte du på brødet?" skriver Ingrid B i den orange ruten.



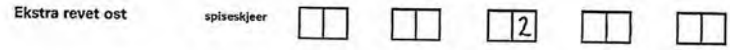
For å registrere porsjonen med spaghetti og tomatsaus med pølsebiter, blar Ingrid opp på siden med kjøttretter. Det er flere pastaretter å velge mellom. Hun velger "Pasta med tomatsaus". Deretter slår hun opp i bildeboken for å anslå hvor mye pasta og saus hun spiste. Porsjonen lignet mest på bilde B. Hun skriver derfor 1 i den sorte ruten og B i den orange.



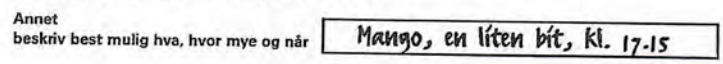
I tillegg skriver Ingrid hvor mye pølser hun hadde i tomatsausen. Hun finner linjen med "Grillpølse/wienerpølse vanlig" og skriver 1 i kolonnen "kl. 14-18".



Ingrid hadde også 2 spiseskjeer ost på pastaen. Hun finner linjen for "Ekstra revet ost" og skriver 2 i kolonnen "kl. 14-18".



Ingrid finner ikke mango blant fruktene som er nevnt i dagboken. I boksen "Annet" etter frukt, skriver hun "Mango, en liten bit, kl. 17.15".



Retur av dagbøker

Send inn de syv utfylte dagbøkene **med en gang** etter at registreringsperioden er over. Bruk den ferdig frankerte svarkonvolutten. Send bare inn de syv utfylte dagbøkene.

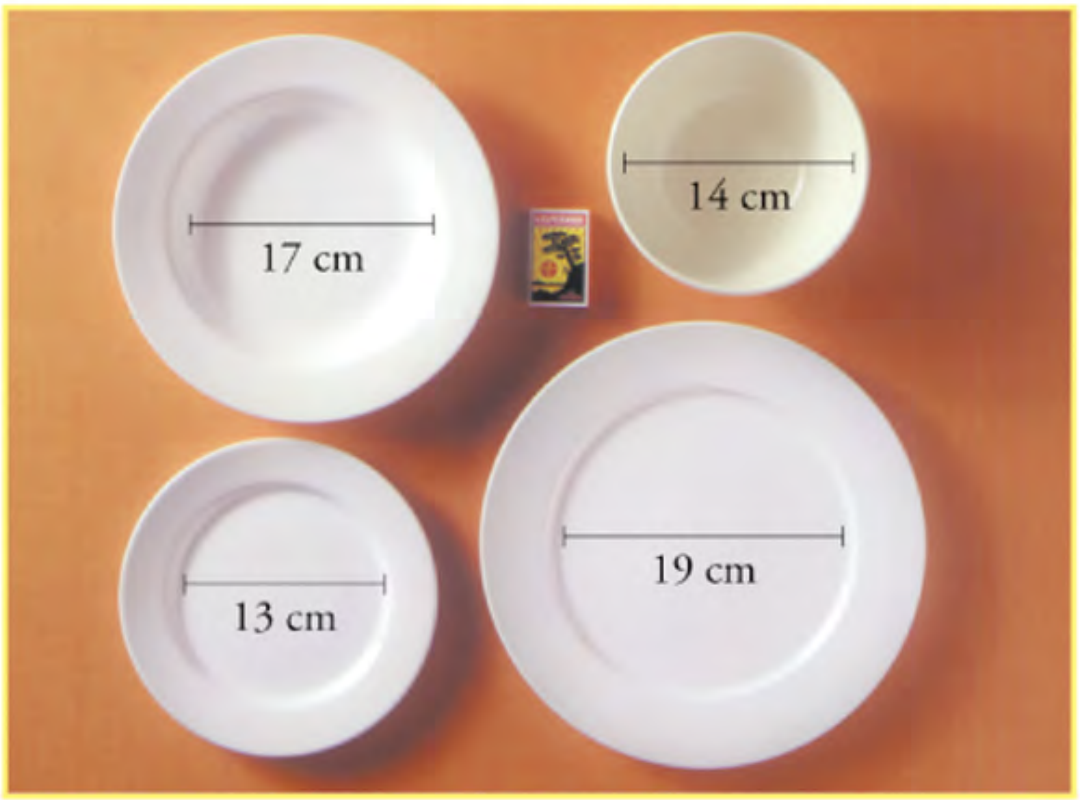
Hvis du har mistet konvolutten, bruk følgende adresse:

Marlene Blomstereng Karlsen
Klinisk ernæringsfysiolog
Regionalt senter for spiseforstyrrelser (RESSP)
Nordlandssykehuset HF
8092 Bodø

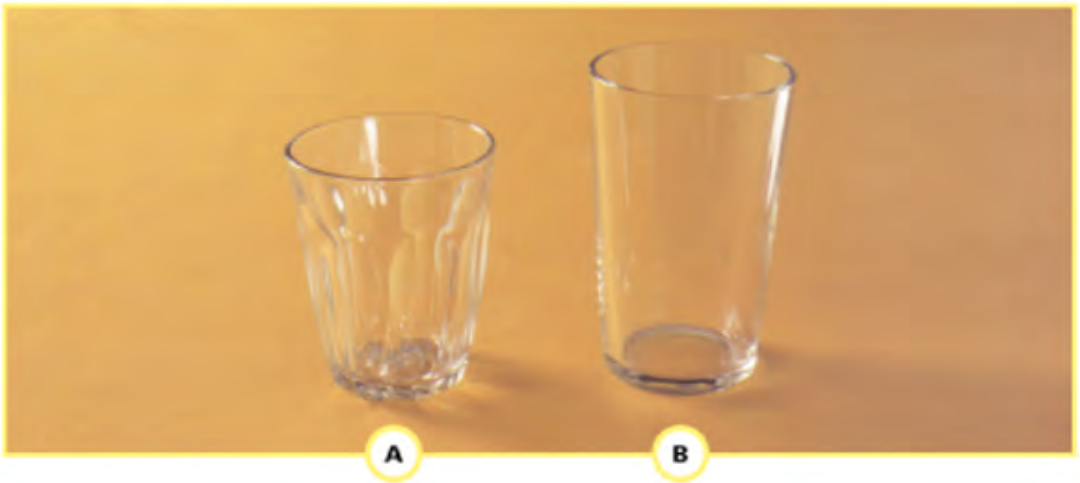
Appendix VII

Bildehefte med porsjonsstørrelser

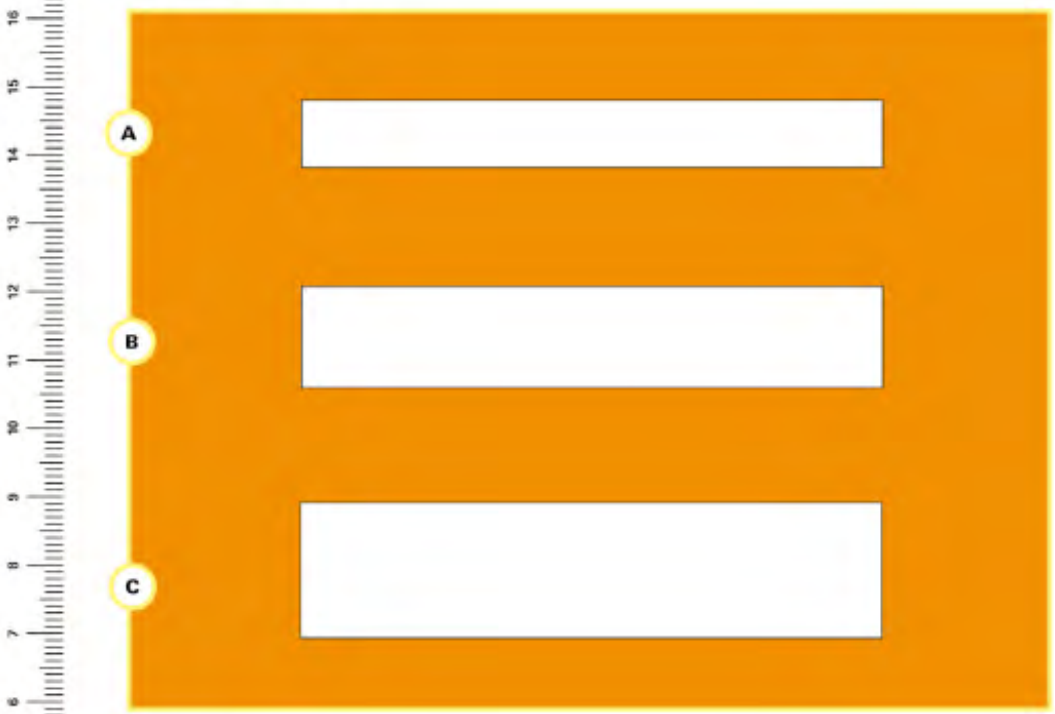
DETTE BILDET VISER STØRRELSEN PÅ TALLERKENENE SOM ER BRUKT I BILDEHEFTET



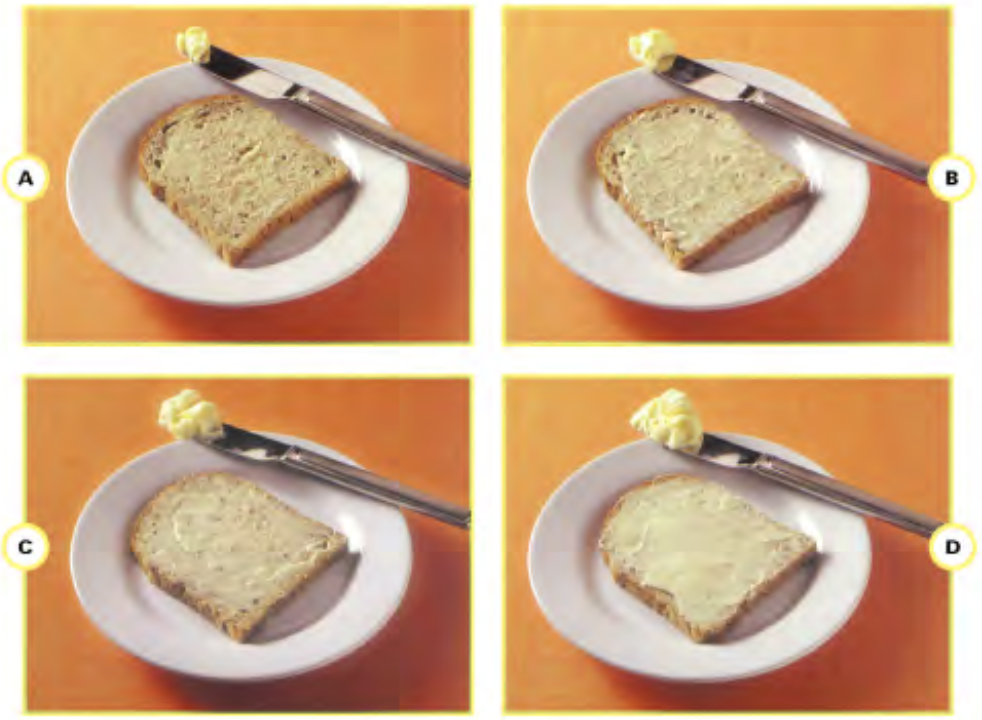
1. GLASS



2. BRØDTYKKELSE

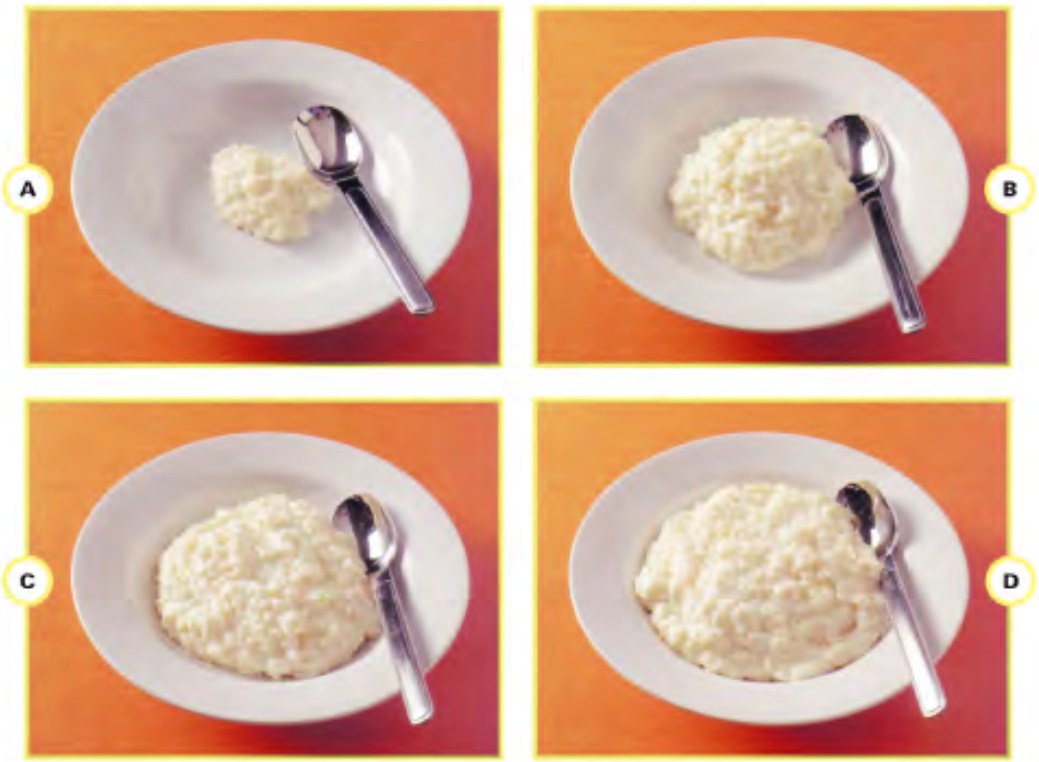


3. SMØR/MARGARIN PÅ BRØD



4. CORNFLAKES (FROKOSTBLANDING)

5. GRØT



10. SALAT

11. KJØTTSAUS (LAPSKAUS)

12. PIZZA, TREKANTSTYKKER

13. PIZZA, FIRKANTSTYKKER

UTVIKLET AV:

STATENS RÅD FOR ERNÆRING OG FYSISK AKTIVITET

SNT Statens næringsmiddeltilsyn

INSTITUTT FOR ERNÆRINGSFORSKNING

Appendix VIII

Dagbok

Fyll inn:

Kjønn ☐ Skriv 1 hvis gutt/mann, 2 hvis jente/kvinne

Alder ☐☐ år

Ukedag ☐ 1=mandag, 2=tirsdag, 3=onsdag, 4=torsdag, 5=fredag, 6=lørdag og 7=søndag

Dato ☐☐.☐☐.☐☐

Var denne dagen en vanlig dag? Skriv ja eller nei i rutene.

☐☐☐

Hvis det var en uvanlig dag, forklar hvorfor denne dagen var uvanlig:

Hvor finner jeg matvarene i dagboken?

HUSK:

Alt du spiser/drikker skal skrives opp

Sett ikke kryss i dagboken

Sett bare bokstaver i de orange rutene

Sett bare tall i de sorte rutene

Drikke

For størrelsen på glasset du drikker av, se bildeserie 1.
Fyll inn bokstaven i den orange ruten.

	Antall	kl. 6-10	kl. 10-14	kl. 14-18	kl. 18-22	kl. 22-6
Vann	glass					
Helmelk, søt/sur (eks. helmelk, kefir)	glass					
Lettmelk, søt/sur (eks. lettmelk, Cultura)	glass					
Ekstra lett lettmelk	glass					
Skummet melk	glass					
Drikkeyoghurt	glass					
Sjokolademelk av helmelk (eks. O'boy, Nesquick)	glass					
Sjokolademelk av lettmelk (eks. Nesquick, Litago)	glass					
Sjokolademelk av ekstra lett lettmelk (eks. O'boy)	glass					
Sjokolademelk av skummet melk (eks. O'boy, Nesquick)	glass					
Litago sjokolademelk	1/2 liter					
Kakao av helmelk	kopp					
Kakao av lettmelk	kopp					
Kakao av ekstra lett lettmelk	kopp					
Kakao av skummet melk	kopp					
Appelsinjuice	glass					
Eplejuice/eplemost	glass					
Nektar (eks. eple, tropisk frukt, annen frukt)	glass					
Brus med sukker (eks. Cola, Solo)	glass					
Brus med sukker (eks. Cola, Solo)	1/2 liter					
Brus, kunstig søtet (eks. Cola light, Solo lett)	glass					
Brus, kunstig søtet (eks. Cola light, Solo lett)	1/2 liter					

Drikke forts.

	Antall	kl. 6-10	kl. 10-14	kl. 14-18	kl. 18-22	kl. 22-6
Saft med sukker (eks. husholdning, appelsin, solbær)	glass					
Saft, kunstig søtet (eks. Fun light)	glass					
Mineralvann (eks. Farris)	glass					
Mineralvann (eks. Farris)	1/2 liter					
Energidrikk (eks. Battery)	boks (330 ml)					
Te, vanlig (eks. Earl Grey, solbær)	kopp					
Fruktte (eks. nype, kamille)	kopp					
Iste/urtete med sukker	glass/pakning (250 ml)					
Kaffe, kokt (eks. presskanne)	kopp					
Kaffe, traktet/filter	kopp					
Kaffe, pulver (instant)	kopp					
Caffe latte, Cappucino	glass/kopp (4dl)					
Espresso	kopp (1dl)					
Sukketter/Natrena/ Canderel	stk					
Sukker til te/kaffe	teskje/ biter					
Melk til te/kaffe	spiseskje					

Annet
beskriv best mulig hva, hvor mye og når:

Øl, vin, brennevin

	Antall	kl. 6-10	kl. 10-14	kl. 14-18	kl. 18-22	kl. 22-6
Alkoholfritt øl, vørterøl (eks. Clausthaler, Munkholm)	glass					
Lettøl	boks/flaske (330 ml)					
Pilsner	boks/flaske (330 ml)					
Sterkøl	boks/flaske (330 ml)					
Rusbrus (eks. Cider, Bacardi breezer)	boks/flaske (330 ml)					
Hvitvin	glass					
Rødvin	glass					
Brennevin	dram (4 cl)					

Annet
beskriv best mulig hva, hvor mye og når:

Brød m.m.

Skriv antall skiver i sort rute. For tykkelse på brødskiven se bildeserie 2 og fyll inn bokstaven i orange rute.

1 skive = 1/2 rundstykke

	Antall	kl. 6-10	kl. 10-14	kl. 14-18	kl. 18-22	kl. 22-6
Loff/fint rundstykke	skiver					
Mellomgrovt brød, grovt rundstykke, kneippbrød	skiver					
Grovt brød	skiver					
Dansk rugbrød, Pumpernikkel	skiver					
Baguette/Ciabatta	stk					
Knekkebrød lyst, skonrok, kavring	stk					
Knekkebrød, mørkt	stk					
Lompe, potetlefse	stk					
Pølsebrød, hamburgerbrød	stk					
Pitabrød	stk					
Flatbrød (eks. Mors flatbrød, Ideal)	stk					

Smør eller margarin på brød.

1 skive = 1/2 rundstykke = 1 knekkebrød
= 2 vaffelhjerter = 2 kjeks = 1/2 ciabatta

	Antall	kl. 6-10	kl. 10-14	kl. 14-18	kl. 18-22	kl.22-6
Meierismør	til antall skiver					
Bremykt	til antall skiver					
Brelett	til antall skiver					
Margarin (eks. Soya, Per, Melange)	til antall skiver					
Lettmargarin (eks. Soft light)	til antall skiver					
Annet beskriv best mulig hva, hvor mye og når:						

Hvor mye smurte du på brødet?

Se bildeserie 3 og skriv bokstaven for det bildet som ligger nærmest opp til den smør-/margarinmengden du brukte på brødet. Hvis du hadde forskjellig mengde smør/margarin på de brødskivene du spiste innenfor det angitte tidsrommet, kan du anslå et gjennomsnitt for skivene.

	kl. 6-10	kl. 10-14	kl. 14-18	kl. 18-22	kl. 22-6
Bildeserie 3					

Pålegg

Du skal oppgi mengde pålegg i forhold til brødskiver. Har du spist to typer pålegg på samme brødskive, fører du opp begge (eks. 1 hvitost helfet og 1 skinke). Hvis du bare har spist pålegg og ikke brød, anslå til hvor mange skiver du kunne brukt dette pålegget.

1 skive = 1/2 rundstykke = 1 knekkebrød
= 2 vaffelhjerter = 2 kjeks = 1/2 ciabatta

Kjøttpålegg

	Antall	kl. 6-10	kl. 10-14	kl. 14-18	kl. 18-22	kl. 22-6
Servelat, vanlig	til antall skiver					
Kokt skinke, spekeskinke, lett servelat	til antall skiver					
Salami, spekepølse, fårepølse	til antall skiver					
Leverpostei, vanlig	til antall skiver					
Leverpostei, mager	til antall skiver					
Kalkun-/ kyllingpålegg	til antall skiver					

Ost

	Antall	kl. 6-10	kl. 10-14	kl. 14-18	kl. 18-22	kl. 22-6
Hvitost helfet 27% fett (eks. Jarlsberg, Norvegia)	til antall skiver					
Hvitost halvfet 16% fett (eks. Norvegia lettere)	til antall skiver					
Brunost helfet (eks. Geitost, G35, Fløtemysost)	til antall skiver					
Brunost halvfet, prim	til antall skiver					
Smøreost, vanlig (eks. Baconost, Snøfrisk)	til antall skiver					
Smøreost, mager (eks. mager skinkeost)	til antall skiver					
Kremost (eks. Philadelphia, Gourmetoster)	til antall skiver					
Dessertost (eks. Brie, Gräddost, Ridderost)	til antall skiver					

Fiskepålegg

	Antall	kl. 6-10	kl. 10-14	kl. 14-18	kl. 18-22	kl. 22-6
Kaviar	til antall skiver					
Røkt laks/ørret	til antall skiver					
Makrell i tomat, røkt makrell	til antall skiver					
Sardiner, sursild, ansjos	til antall skiver					

Syltetøy/søtpålegg

	Antall	kl. 6-10	kl. 10-14	kl. 14-18	kl. 18-22	kl. 22-6
Syltetøy vanlig, gelé, marmelade	til antall skiver					
Syltetøy lett, frysetøy	til antall skiver					
Honning	til antall skiver					
Peanøttsmør	til antall skiver					
Sjokolade-/nøttepålegg	til antall skiver					
Hapå/Litagopålegg	til antall skiver					

Annet
beskriv best mulig hva, hvor mye og når:

31963

Annet pålegg

1 skive= 1/2 rundstykke= 1knekkebrød
=2 vaffelhjerter= 2 kjeks= 1/2 ciabatta

	Antall	kl. 6-10	kl. 10-14	kl. 14-18	kl. 18-22	kl. 22-6
Egg, kokt/stekt	til antall skiver					
Majonessalat (eks. italiensk salat, rekesalat)	til antall skiver					
Majonessalat, lett (eks. italiensk salat, lett)	til antall skiver					
Tomat som pålegg	til antall skiver					
Banan som pålegg	til antall skiver					

Annet
beskriv best mulig hva, hvor mye og når:

Pynt på brødskiver

	Antall	kl. 6-10	kl. 10-14	kl. 14-18	kl. 18-22	kl. 22-6
Majones/remulade, vanlig	til antall skiver					
Majones/remulade, lett	til antall skiver					
Agurk (frisk/syltet)	til antall skiver					
Rødbeter (syltet)	til antall skiver					
Paprika	til antall skiver					

Annet
beskriv best mulig hva, hvor mye og når:

Yoghurt

	Antall	kl. 6-10	kl.10-14	kl.14-18	kl.18-22	kl.22-6
Yoghurt med frukt	beger (175 ml)					
Yoghurt 0,1% fett	beger (125 ml)					
Yoplait frukt	beger (125 ml)					
Litago yoghurt	beger (125 ml)					
Litago yoghurt m/müsli	beger inkl. müsli					
Go'morgen yoghurt m/müsli	beger inkl.müsli					
Piano Duo Yoghurt	beger (125 ml)					

Appendix IX

REGISTRERING AV MÅLINGER HOS FORSKNINGSDELTAKER

Navn: _____

Fødselsdato: _____

Høyde i cm: _____

Vekt i kg: _____

Hofteomkrets i cm:

Hofteomkretsmåling 1: _____

Hofteomkretsmåling 2: _____

Dersom mer enn 1 cm forskjell på hofteomkretsmåling 1 og hofteomkretsmåling 2, så skal de to målingene gjentas og noteres her som ekstra-hofteomkretsmåling:

Ekstramåling 1:_____

Ekstramåling 2: _____

Livvidde/Midjemål i cm :

Livviddemåling 1: _____

Livviddemåling 2: _____

Dersom mer enn 1 cm forskjell på livviddemåling 1 og livviddemåling 2, så skal de to målingene gjentas og noteres her som ekstra-livviddemåling:

Ekstra-livviddemåling 1:_____

Ekstra-livviddemåling 2:_____

2.5 Oppsummering og konklusjoner

Livvidde bør måles ved midtpunktet mellom nedre grensen til
nederste ribben som en kjenner, og toppen av hoftekam

med et strekkresistent målebånd som gir en
konstant 100 g spenning. (målebåndet skal brukes tett/stramt, men ikke
snurpe/sammentrekke)

Hofteomkrets skal måles rundt den bredeste delen
av setet, med målebånd parallellt med gulvet.

For begge målinger bør personen stå
med føttene tett sammen,
armene i siden
og kroppsvekt jevnt fordelt,
og skal ha på seg lite klær.

Personen skal være
avslappet,
og målingene bør tas på slutten av en normal utpust.

Hver måling bør gjentas to ganger,
hvis det er mindre enn 1 cm forskjell mellom de to målingene
så skal gjennomsnittet beregnes.

Dersom forskjellen mellom de tomålingene overstiger 1 cm,
bør de to målingene gjentas.

Ref:
Waist circumference and waist–hip ratio: report of a WHO expert consultation, Geneva, 8–11 December 2008

(Målebånd til omkretsmålinger Undersøkelsen krever målebånd av god kvalitet som ikke endrer seg over tid. Målebånd av glassfiber er å foretrekke. Målebåndet må være flatt og lett å bøye, og optimalt ha en blank første del (ca 10 cm).
Ref: helsedirektoratet Nasjonale og faglige retningslinjer for veiing og måling i helsestasjons- og skolehelsetjenesten. 2010.)