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ALEKSEI RAKITIN

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Metabolic effects of acute and chronic treatment with valproic acid in people with epilepsy

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Department of Neurology and Neurosurgery, University of Tartu, Estonia

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Dedicated to my grandfather
Mikhail Chunikhin
and to my grandmother
Natalia Chunikhina

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Paper II: conception of idea, study design, data collection, assessment of patients, data analysis, and writing the manuscript

Paper III: study design, data collection, assessment of patients, data analysis, and writing the manuscript

Paper IV: study design, data collection, assessment of patients, data interpretation, and writing the manuscript

ABBREVIATIONS

AED	Antiepileptic drug
AUC	Area under the curve
BMI	Body mass index
CBZ	Carbamazepine
CI	Confidence interval
FDR	False discovery rate
FFA	Free fatty acid
GABA	γ-Aminobutyric acid
HDAC	Histone deacetylase
HDL-C	High-density lipoprotein cholesterol
HOMA-IR	Homeostatic model assessment-insulin resistance
IGF-1	Insulin-like growth factor 1
ILAE	International League Against Epilepsy
IQR	Interquartile range
IR	Insulin resistance
IV	Intravenous
K_{ATP}	ATP-sensitive potassium channels
LTG	Lamotrigine
mRNA	Messenger ribonucleic acid
MS	Metabolic syndrome
NAFLD	Nonalcoholic fatty liver disease
OGTT	Oral glucose tolerance test
OR	Odds ratio
PCOS	Polycystic ovary syndrome
POLG	Polymerase γ
ROC	Receiver operating characteristic
SD	Standard deviation
SHBG	Sex hormone-binding globulin
TG	Triglycerides
TPM	Topiramate
VPA	Valproic acid

1. INTRODUCTION

Valproate, also known as valproic acid (VPA), is one of the most frequently prescribed antiepileptic drugs (AEDs) (Perucca, 2002), with more than one million people around the world estimated to be taking VPA every day (Farinelli et al., 2015). VPA was first synthesized in 1882 by Beverly S. Burton as an analogue of valeric acid, which is naturally produced by *Valeriana officinalis* (Burton, 1882) (Figure 1). VPA is a simple-branched short-chain fatty acid that is a clear liquid at room temperature (Figure 2). Initially, VPA was used as a solvent for organic compounds. There was no known clinical use of this drug until its anticonvulsant activity was serendipitously discovered by Pierre Eymard in 1962 (Chateauvieux et al., 2010).

After being approved as an anticonvulsant in France in 1967, VPA was marketed in more than 100 countries for the treatment of epilepsy (Levy, 2002). Since then, more than 10 new AEDs have been discovered and marketed around the globe. Despite this fact, VPA remains a first-choice agent for most idiopathic and symptomatic generalized epilepsies (Perucca, 2002). In recent years, VPA has been successfully used for other indications, including bipolar disorder, migraine headache, and pain related to diabetic neuropathy (Bril et al., 2011; Nanau and Neuman, 2013). VPA has been used in the treatment of various cancers (Weller et al., 2011) and has shown possible neuroprotective potential in patients with Alzheimer disease (Zhang et al., 2010). These chronic conditions usually require long-term treatment, which emphasizes the importance of the long-term safety of the drug.

VPA has a broad range of side effects, leading to its frequent designation as a "dirty drug" (Panayiotopoulos, 2010). Frequently reported adverse effects include postural tremor, hyperammonemia, alopecia, liver toxicity, and gastrointestinal and hematological effects (Perucca, 2002). VPA influences embryogenesis, causing an increased rate of major congenital malformations and behavioral anomalies among children exposed to the drug *in utero* (Tomson and Battino, 2012). These effects could be due to VPA's role as a potent promotor of histone acetylation and its ability to influence gene transcription directly (Chateauvieux et al., 2010). The most common side effect of VPA treatment is a weight gain, which occurs in about half of patients, and is associated with important metabolic and endocrine abnormalities (Verrotti et al., 2009).

Metabolic syndrome (MS) refers to a cluster of metabolic risk factors, such as glucose intolerance, dyslipidemia, central obesity, and hypertension, which are major risk factors for cardiovascular and cerebrovascular diseases (Reaven, 1988). MS is a major economic burden and public health problem (Fu et al., 2007; Wang et al., 2010). In people with epilepsy, a sedentary lifestyle and AED use contribute to obesity and MS (Ben-Menachem, 2007; Steinhoff et al., 1996). Numerous studies have explored the effects of different AEDs on specific metabolic and anthropometric parameters, such as the plasma lipid concentration and occurrence of obesity. However, few studies have examined

the presence of MS in patients with epilepsy. Verrotti *et al*. showed that the presence of MS in VPA-treated children and adolescents who became obese did not differ from MS prevalence in otherwise healthy overweight subjects (Verrotti et al., 2010). On the other hand, Kim and Lee reported that women with epilepsy on VPA monotherapy more frequently suffered from MS compared to women treated with carbamazepine (CBZ), lamotrigine (LTG), or topiramate (TPM) (Kim and Lee, 2007).

No study to date has explored the risk of MS in VPA-treated patients at the population level. Thus, the aim of this study was to evaluate the prevalence of MS and possible relevant factors for MS development in VPA-treated patients with epilepsy in Estonia, compared to the general population and to CBZ-treated patients, as well as to explore possible molecular mechanisms of metabolic changes in these patients.

Figure 1. *Valeriana officinalis*

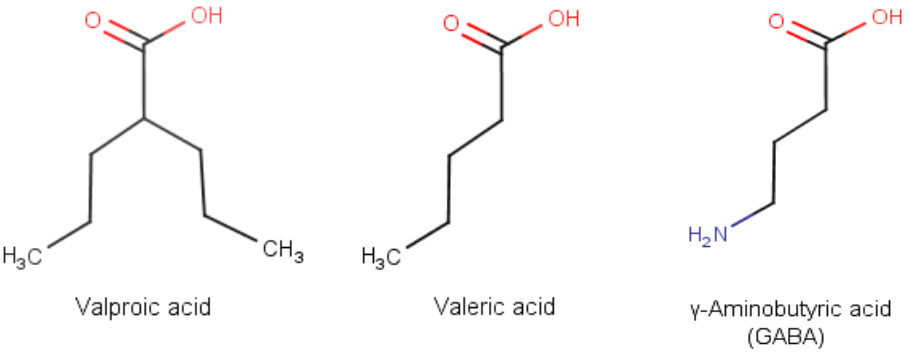

Figure 2. Structure of valproic, valeric and γ-aminobutyric acid

2. LITERATURE REVIEW

2.1. Use of valproic acid in treatment of epilepsy

Despite extensive research, the precise mechanism underlying the anticonvulsive activity of VPA is not fully understood (Perucca, 2002). VPA increases activity of gamma amino butyrate (GABA) through increased synthesis, decreased turnover, and reduced degradation, thereby potentiating GABAergic transmission in specific brain regions (Chateauvieux et al., 2010). VPA attenuates N-methyl-D-aspartate-mediated excitation (Zeise et al., 1991) and participates in blockade of voltage-dependent sodium channels (McLean and Macdonald, 1986). There is some evidence that VPA could modulate dopaminergic and serotoninergic transmission (Loscher, 1999). Thus, the anticonvulsive property of VPA is probably backed by multiple complex modes of action.

VPA is extensively (≥90%) bound to plasma proteins, mainly albumin, and metabolized in the liver. There are at least three routes of VPA metabolism in humans. Microsomal glucuronide conjugation and β-oxidation in mitochondria are major routes, accounting for metabolism of 50% and 40% of the VPA dose, respectively. As a minor route, cytochrome P450-dependent oxidation accounts for ~10% of dose metabolism (Ghodke-Puranik et al., 2013). VPA has the widest spectrum of use among AEDs, being effective against all seizure types at efficacies comparable to those of alternative AEDs. At the start of treatment, especially when the precise epileptic syndrome is unclear, VPA may be started in all cases. Tolerability issues and adverse effects determine whether an alternative AED would be preferred in an individual patient (Perucca, 2002). VPA is especially effective against absence seizures and juvenile myoclonic epilepsy (Glauser et al., 2013). Intravenous (IV) infusion of VPA at doses of 15–45 mg/kg is widely used in the treatment of established status epilepticus. Safety studies of IV VPA in patients with status epilepticus showed a low overall incidence (<10%) of adverse events, which mainly encompassed dizziness, mild hypotension, and thrombocytopenia (Trinka et al., 2014). Thus, VPA represents a safe and effective alternative to phenobarbital and phenytoin in the treatment of status epilepticus (Trinka et al., 2015).

2.2. Use of valproic acid for other indications

VPA is a first-line drug for the treatment of bipolar disorder (Johannessen and Johannessen, 2003) and is efficacious for treating acute episodes of mania (Macritchie et al., 2003). VPA is used in the acute treatment of severe migraine attacks (Mulleners et al., 2015; Shahien et al., 2011; Sheridan et al., 2015). In the prophylactic treatment of migraine, VPA was found to reduce the mean monthly headache frequency by ~4 days (Mulleners et al., 2015). Evidence-based guidelines of the European Federation of Neurological Societies / European

Academy of Neurology suggest that VPA be used as a first-choice drug for migraine prophylaxis (Evers et al., 2009). VPA has been approved for the treatment of trigeminal neuralgia (Ross, 2000). According to guidelines of the American Academy of Neurology, VPA may be effective for the treatment of diabetic polyneuropathy (Bril et al., 2011).

A relatively new clinical application area of VPA is the treatment of leukemia and some solid tumors (Chen et al., 2012). In a recent retrospective study of survival among patients with glioblastoma, subjects who received VPA concomitantly with temozolomide had longer survival times compared to subjects who received temozolomide monotherapy. The precise mechanism of this phenomenon is unclear; however, the antitumor effect of VPA could be mediated through increased bioavailability of temozolomide or inhibition of histone deacetylation (HDAC), as the latter effect probably potentiates the benefit from radiochemotherapy (Weller et al., 2011). For brain-tumor patients with epilepsy, the preferred treatment is a non-enzyme-inducing AED (Perucca, 2013). Although there are no prospective randomized trials to determine which AED provides the best risk-benefit ratio in individual patients, VPA as a first-line non-enzyme-inducing anticonvulsant could have the advantage. Promising results have been obtained in preclinical studies assessing the potential of VPA in the treatment of Duchenne muscular dystrophy, HIV infection, and Parkinson, Huntington, and Alzheimer diseases. However, there is insufficient evidence to determine whether VPA could be used in the clinical practice for any of these indications (Chateauvieux et al., 2010).

2.3. Adverse effects of valproic acid

2.3.1. Gastrointestinal effects

Gastrointestinal side effects, such as nausea, vomiting, and gastrointestinal distress, occur in up to 25% of patients who take VPA (Dreifuss et al., 1987). Diarrhea, abdominal cramps, and constipation are more rarely observed at the start of treatment (Wagner et al., 2000). Manifestations of gastric intolerance are less pronounced when the drug is an enteric-coated formulation or administered with food (Perucca, 2002).

2.3.2. Hair loss

Hair loss, hair thinning, or regrowth of curly or differently colored hair may occur with VPA use (Johannessen and Johannessen, 2003). Alopecia due to telogen shedding may appear within 3 months of treatment initiation (Chateauvieux et al., 2010). This effect is usually reversible and may remit even when continued VPA therapy (Davis et al., 1994).

2.3.3. Hematological disorders

VPA can affect pro- and anti-coagulatory factors, leading to thrombocytopenia, impaired platelet function, reduced serum fibrinogen and von Willenbrand factor antigen levels (Zeller et al., 1999), and factor XIII deficiency (Pohlmann-Eden et al., 2003). Some reports found that VPA increases the risk of post-surgical bleeding (Pohlmann-Eden et al., 2003; Tetzlaff, 1991), leading authors to recommend that VPA be discontinued before surgery (Anderson et al., 2003). However, the increased risk of postoperative bleeding in patients undergoing neurosurgery was not confirmed in clinical trials (Anderson et al., 1997; Psaras et al., 2008; Ward et al., 1996). A recent study by Zighetti *et al*. monitored primary hemostasis in VPA-treated patients with epilepsy and healthy controls. Those authors found no significant differences in coagulation or primary hemostasis (Zighetti et al., 2015). Nevertheless, some neurosurgeons are still reluctant to operate on patients who are using VPA. In this case, an appropriate alternative could be levetiracetam, which has a low interaction potential, good tolerability, and can be uptitrated relatively rapidly (Perucca, 2013).

2.3.4. Hyperammonemia

There have been frequent reports of hyperammonemia among patients on VPA therapy (Murphy and Marquardt, 1982; Zaccara et al., 1987). The hyperammonemia is typically asymptomatic and detected only by blood tests, although symptoms of encephalopathy, confusion, vomiting, and ataxia have been noted occasionally (Davis et al., 1994). The mechanism of hyperammonemia is related to the accumulation of toxic levels of VPA metabolites subsequent to the reduction of levels of hepatic N-acetylglutamate, which catalyzes the first step in urea biosynthesis (Deutsch et al., 2009). High concentrations of ammonia in the brain cause elevated levels of glutamine within astrocytes, resulting in astrocyte swelling, cerebral edema, and metabolic consequences (Brusilow, 2002). Polytherapy may exacerbate hyperammonemia (Johannessen and Johannessen, 2003). VPA may decrease plasma carnitine levels, thereby enhancing hyperammonemia (Nanau and Neuman, 2013). VPA can form an ester with carnitine, and this ester has a high renal clearance rate. Carnitine deficiency inhibits the urea cycle secondary to a lack of precursor molecules and adenosine triphosphate (LaBuzetta et al., 2010). Levocarnitine supplementation is recommended for VPA-induced liver toxicity, hyperammonemic encephalopathy, and VPA overdose (Perucca, 2002).

2.3.5. Hepatotoxicity and pancreatitis

Transient elevations of hepatic enzymes without clinical symptoms are seen in 15–30% of VPA-treated patients (Anderson, 2002). The overall prevalence of severe liver toxicity is about 1:20,000 among VPA-treated patients, but may be

as high as 1:600 or 1:800 among infants under 2 years of age, with incidence decreasing with increasing age (Perucca, 2002). For this reason, VPA use is contraindicated in this population (Nanau and Neuman, 2013). Factors that increase the risk of liver toxicity are polytherapy, especially in children with mental retardation, coexistence of certain metabolic defects (β-oxidation disorders, mitochondrial diseases), pre-existing liver disease, and elevated liver enzyme levels (Bryant and Dreifuss, 1996; Dreifuss et al., 1989; Konig et al., 1994). In recent years, there has been a remarkable decrease in the incidence of VPA-associated hepatic injury. This drop may be related to better recognition of hepatic injury-related symptoms by physicians, avoidance of VPA in high-risk groups, and rapid discontinuation of VPA after early diagnosis of hepatic injury (Konig et al., 1994).

Long-term VPA treatment is associated with nonalcoholic fatty liver disease (NAFLD), a group of diseases characterized by hepatic steatosis and hepatic fat accumulation without an underlying secondary cause (Farinelli et al., 2015). Prevalence of NAFLD in the general population is about 20–25% (Chalasani et al., 2012; Loria et al., 2010). NAFLD has two subsets: nonalcoholic fatty liver and nonalcoholic steatohepatitis. Nonalcoholic fatty liver is characterized by the presence of intrahepatic fat accumulation without evidence of hepatic injury. On the other hand, nonalcoholic steatohepatitis is associated with inflammation and hepatic injury, and poses a risk for development of cirrhosis and hepatic malignancy (Farinelli et al., 2015). Luef *et al*. observed ultrasound features of fatty liver disease in 61% of epilepsy patients treated with VPA monotherapy for at least 2 years, compared to 23% of patients treated with CBZ (Luef et al., 2004). The pathogenic mechanism of VPA-induced NAFLD is not fully understood, but it is likely a consequence of VPA-induced weight gain and MS (Farinelli et al., 2015). The flux of fatty acids to the liver through the portal vein causes hepatic lipogenesis (Fishbein et al., 2006). In a study by Verrotti *et al*., ultrasound-diagnosed NAFLD was found more frequently among VPA-treated patients compared to normal-weight controls, but was found at similar rates among VPA-treated patients and weight-matched controls. This finding suggests that the development of NAFLD is not a consequence of the direct action of VPA metabolites, but rather the result of weight gain and MS (Verrotti et al., 2011a). VPA-associated pancreatitis is a relatively rare condition. Features of this adverse effect include elevated serum amylase and/or lipase levels and epigastric abdominal pain. After discontinuation of VPA, symptoms usually disappear (Nanau and Neuman, 2013).

2.3.6. Mitochondrial toxicity

As a simple fatty acid, VPA is a substrate for the fatty acid β-oxidation pathway in mitochondria. Thus, VPA toxicity could be related to its interference with mitochondrial β-oxidation (Silva et al., 2008). Among the AEDs, VPA has the highest potential to induce mitochondrial toxicity. VPA treatment could be fatal

for patients with some mitochondrial diseases, such as inborn errors of metabolism that affect β-oxidation (Finsterer and Zarrouk Mahjoub, 2012). Certain mitochondrial disorders are associated with epilepsy and sometimes are treated with VPA. However, VPA treatment is contraindicated for patients with the mitochondrial diseases, including MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis and stroke syndrome); MEMSA (myoclonic epilepsy, myopathy, and sensory ataxia); MERRF (myoclonic epilepsy with ragged red fibers); Leigh syndrome; and Alpers-Huttenlocher syndrome (Finsterer and Segall, 2010). This contraindication is particularly important in patients with catalytic deficiency in the mitochondrial DNA polymerase γ (POLG) activity, caused by mutation of nuclear gene *POLG1* (Nanau and Neuman, 2013). POLG is the only DNA polymerase found in mitochondria, and its deficiency results in refractory seizures, progressive neuronal degeneration, and liver disease (Palin et al., 2010).

2.3.7. Neurological side effects

Central nervous system adverse effects of VPA treatment, such as sedation and cognitive dysfunction, are observed but not prominent and may result from interactions with concomitantly given AEDs. The most common neurological adverse effect is postural tremor, a dose-related side effect that resembles essential tremor (Perucca, 2002). Less-frequent central nervous system adverse effects of VPA include dizziness, mood changes, extrapyramidal symptoms, nystagmus, and headache (Chateauvieux et al., 2010). VPA treatment is occasionally related to encephalopathy, which may involve development of a confused state, stupor, or coma, and is fully reversible after VPA discontinuation. This condition needs to be differentiated from symptoms caused by VPA-induced hyperammonemia (Perucca, 2002).

2.3.8. Polycystic ovary syndrome (PCOS)

Reproductive endocrine disorders have frequently been reported in women with epilepsy (Verrotti et al., 2011c). Polycystic ovaries are found on ultrasonography in ~20% of normal women and generally are not considered an abnormal feature (Taylor, 1998). On the other hand, PCOS is a disorder characterized by polycystic ovaries associated with hyperandrogenism, hypofertility, menstrual irregularities, hirsutism, acne, obesity, and reduced glucose tolerance. PCOS is the most common endocrine disorder in women of reproductive age, with a general prevalence among premenopausal women of 5–6% (Bilo and Meo, 2006).

A 1993 report from Isojärvi *et al*. was the first to describe the possible association between VPA use and PCOS in a large series of patients (Isojarvi et al., 1993). After this report, the possible association between PCOS, epilepsy, and VPA received widespread attention from the scientific community (Bilo

and Meo, 2006). A large body of evidence suggests that VPA treatment increases the incidence of PCOS (Isojarvi et al., 1996; Isojarvi et al., 1993; Isojarvi et al., 2001a; Morrell et al., 2002; Morrell et al., 2008; Sahota et al., 2008), although some studies have reported no significant association (Bauer et al., 2000; Bilo et al., 1988; Luef et al., 2002a; Luef et al., 2002c; Murialdo et al., 1998). This inconsistency has led to lively debate between research groups (Genton et al., 2001; Isojarvi et al., 2001b). Possible explanations for the discrepancy may be the small sample size of some studies, lack of randomization, and differences in PCOS definitions (Verrotti et al., 2011c). A recent meta-analysis of 11 studies showed that women with epilepsy who were taking VPA had an ~1.95-fold risk of PCOS compared to women treated with other AEDs. However, due to the heterogeneity of studies included in the meta-analysis, the authors recommended that the result be confirmed in further prospective, randomized studies (Hu et al., 2011).

Several theories have been proposed to explain the possible association of VPA treatment with PCOS. One theory is that VPA-induced weight gain leads to decreased peripheral insulin sensitivity and hyperinsulinemia. Insulin inhibits production of insulin-like growth factor 1 (IGF-1) binding protein 1 and sex hormone-binding globulin (SHBG) in the liver (Hamilton-Fairley et al., 1993), leading to an increased ovarian concentration of free IGF-1 (Cataldo, 1997). Insulin and IGF-1 stimulate thecal androgen production and reduce SHBG levels, thereby increasing free plasma testosterone levels (Hopkinson et al., 1998). Therefore, hyperinsulinemia increases the secretion of ovarian androgen and promotes androgen bioavailability (Verrotti et al., 2011c). Moreover, VPA may directly stimulate ovarian androgen biosynthesis, probably by HDAC inhibition, which promotes transcription of steroidogenic genes (Nelson-DeGrave et al., 2004). Finally, due to its inhibitory effect on the hepatic P450 enzyme system, VPA may impair the metabolism of sex steroids such as testosterone, hence provoking increased androgen levels (Isojarvi, 2008).

2.3.9. Effects on offspring

The first observations of teratogenic effects of VPA were reported in the early 1980s (Gomez, 1981; Robert and Guibaud, 1982). Since then, data from different pregnancy registries have consistently shown that mothers who use VPA during pregnancy have a higher risk of major congenital malformations among their offspring (Campbell et al., 2014; Hernandez-Diaz et al., 2012; Tomson et al., 2011). Exposure to VPA during the first trimester is associated with increased risks of spina bifida, craniosynostosis, cleft palate, hypospadias, atrial septal defect, and polydactyly (Tomson et al., 2015b). The risk of major congenital malformations is generally two-to-three times higher for VPA compared to other AED monotherapies (Tomson et al., 2015b). Risk of fetal malformations among women treated with polytherapy depends mainly on whether the regimen includes VPA (Holmes et al., 2011; Vajda et al., 2010). Recent reports

emphasize that the teratogenic effect of VPA is dose related; the risk of major congenital malformations with VPA doses less than 700 mg/d seems to be comparable to that of other AEDs (Tomson et al., 2015a).

There are reports of impaired intellectual and behavioral development among children who were exposed to VPA *in utero*. School-aged children of mothers who were treated with VPA during pregnancy had significantly lower verbal intelligence quotient scores than children exposed to CBZ, phenytoin, or not exposed to AEDs (Adab et al., 2004). In response to these data, in 2014 the European Medicines Agency strengthened its warnings on VPA use by females. However, epileptologists raised concerns about some of the recommendations, which would require women with epilepsy to fail on several less-appropriate medications before being prescribed VPA, which is the most effective AED for some syndromes (Tomson, 2015). Thereafter, the Commission on European Affairs of the International League Against Epilepsy and the European Academy of Neurology developed specific recommendations. Current recommendations maintain that VPA should be avoided in women with childbearing potential and should not be prescribed as a first-line treatment for focal epilepsy. For epilepsy syndromes that are most effectively treated by VPA, this drug could be offered to females after a risk-benefit assessment and discussion with the patient and/or her representatives. When possible, VPA doses not exceeding 500–600 mg/day are recommended, although higher doses may be necessary to attain seizure control (Tomson et al., 2015c).

2.3.10. Inhibition of histone deacetylase

VPA acts directly at the level of gene transcription by inhibiting HDAC and increasing access to transcription sites (Chateauvieux et al., 2010). HDAC inhibitors and VPA regulate innate and adaptive immune pathways, with possible anti-inflammatory effects (Shakespear et al., 2011). VPA-induced inhibition of HDAC can result in cell-cycle disruption, growth arrest, and apoptosis, which may explain the teratogenic action of the drug (Ornoy, 2009). *In vitro* and *in vivo* preclinical studies demonstrated strong antitumor effects of VPA against various cancers by modulating multiple pathways, including cell-cycle arrest, angiogenesis, apoptosis, and differentiation (Chateauvieux et al., 2010). VPA inhibits proliferation and induces differentiation of malignancies, such as leukemia, lymphoma, teratocarcinoma, and medulloblastoma. Clinically, VPA has been used to treat leukemia and some solid tumors (Chen et al., 2012).

2.3.11. Weight gain

In the medical community, VPA is widely thought to cause a weight gain (Table 1). Around 10–70% of patients with epilepsy on VPA treatment experience problems with body weight increase (Belcastro et al., 2013). Only two double-blind clinical studies have assessed weight change in VPA-treated

patients. Privitera *et al*. compared patients with new-onset seizures treated by CBZ, TPM, or VPA for 1 year, reporting mean weight increases of 2 and 5 kg in VPA-treated adults and children, respectively. CBZ was weight-neutral and TPM was related to weight loss (Privitera et al., 2003). Another study found significantly higher median weight gain after 32 weeks of treatment with VPA (5.4 ± 4.1 kg vs. baseline) compared to LTG (0.5 ± 5.0 kg vs. baseline) among patients with epilepsy (Biton et al., 2001). Results from other studies on weight gain in VPA-treated patients are rather ambiguous. Some support the occurrence of this side effect (Dinesen et al., 1984; El-Khatib et al., 2007; Isojarvi et al., 1996; Luef et al., 2002b; Morrell et al., 2003; Prabhakar et al., 2007), whereas others find no difference in weight among VPA-treated patients compared to controls (de Vries et al., 2007; Luef et al., 2002c; Pylvanen et al., 2003; Pylvanen et al., 2006b; Stephen et al., 2001). Considering all of the published data on this topic, we assume that probably VPA causes weight gain. However, the real incidence and magnitude of this problem are unknown and should be investigated in specially designed, well-controlled, prospective clinical trials. With regard to potential risk factors, results from clinical studies suggest that women seem to be more prone to weight gain during VPA treatment compared to men. For example El-Khatib *et al*. showed that women experienced weight gain more frequently and to a greater extent than did men (43.6% of women *vs*. 23.5% of men on VPA therapy had weight gain) (El-Khatib et al., 2007). In female patients, the increase in body weight occurs more frequently in postpubertal girls taking VPA (Biton et al., 2003; Prabhakar et al., 2007; Rattya et al., 1999), leading some authors to suggest that a mature (adult) endocrine system is necessary for development of VPA-related obesity (Verrotti et al., 2011b). Weight gain is usually observed within the first 3 months of VPA therapy and peaks by 6 to 12 months (Verrotti et al., 2011b). Some authors suggest that patients may have gradual but progressive weight gain over years after VPA medication is started (Rattya et al., 1999). The influence of daily VPA dosage on weight gain was not specifically investigated. Available data suggest that there is no correlation between the degree of weight gain and the daily VPA dosage or serum VPA concentration (Verrotti et al., 2011b). Some studies showed that the risk of obesity on VPA therapy is higher in patients with higher weight at the start of treatment (Novak et al., 1999; Verrotti et al., 2009).

Table 1. Weight change in VPA-treated adults and adolescents in recently published studies.

Study	No. of patients	Age, years	Treatment duration, years	Weight increase	Groups for comparison	Incidence study	Classi-fication of evidence
Privitera et al., 2003	34 (M) 44 (F)	25*	0.67*	+	CBZ, TPM	+	II
Biton et al., 2001	31 (M) 37 (F)	30.1 ± 14.0	0.6*	+	LTG	+	I
Biton et al., 2003	9 (M) 11 (F)	16 ± 3.0	0.67*	+	LTG	+	I
Morrell et al., 2003	103 (F)	24.9 ± 5.8	<5	+	LTG	–	III
Dinesen et al., 1984	20 (M) 43 (F)	19.1–79.0**	0.75–6.2**	+	No	+	IV
Isojarvi et al., 1996	22 (F)	29 ± 7.0	7 ± 5.0	+	CBZ, healthy controls	–	III
Stephen et al., 2001	17 (M) 23 (F)	17–47**	≥2	–	LTG	–	III
Pylvanen et al., 2003	37 (M)	18–43**	0.5–15**	–	CBZ, OXC, healthy controls	–	III
Pylvanen et al., 2006b	31 (M) 20 (F)	31.4 ± 11.9	6.4 ± 5.0	–	Healthy controls	–	III
El-Khatib et al., 2007	51 (M) 55 (F)	34 ± 10** (F) 35 ± 13** (M)	1.5 ± 0.7 (F) 1.8 ± 0.5 (M)	+	No	–	IV
Luef et al., 2002b	22 (F)	31.2 ± 4.2	3–27**	+	CBZ, LTG	–	III
Luef et al., 2002c	22 (F)	31 ± 4.4	13.5 ± 6.5	–	CBZ, LTG, PRM	–	III
Prabhakar et al., 2007	25 (F)	15–28**	1	+	No	+	IV
de Vries et al., 2007	43 (F)	14.9 ± 3.3	1–9.5**	–	Untreated	–	III

* Median values; ** Range values. Rating scale of evidence for relevant studies: Class I – an adequately powered prospective, randomized, controlled clinical trial in a representative population, primary outcome (weight gain) is clearly defined; Class II – randomized controlled trial in a representative population, primary outcome (weight gain) is not clearly defined; Class III – all other controlled trials in a representative population, case-control or open-label studies, major confounding differences between risk groups that could affect outcome are described; Class IV – uncontrolled studies, case series, or expert reports (adopted from European Federation of Neurological Societies scoring system (Brainin et al., 2004).

2.4. Possible mechanisms relating valproic acid and weight gain

According to the first law of thermodynamics, body weight cannot change if energy intake and energy expenditure are equal over time. Humans take in energy with food in the form of protein, carbohydrate, or fat. They expend energy through the resting metabolic state, which is the amount of energy necessary to fuel the body at rest and the energy expended through physical activity. Energy intake is fully controlled by human behavior, whereas energy expenditure is approximately 20–40% related to behavior (Hill et al., 2012). The weight change in patients on VPA therapy should be caused by a change in behavior: they will either eat more or decrease their physical activity. There are no published data suggesting that the level of physical activity in VPA-treated patients should change. However, there are reports showing that these patients have increased appetite and higher frequency of carbohydrate cravings (Dinesen et al., 1984; El-Khatib et al., 2007; Verrotti et al., 1999). Thus, VPA most likely changes patient behavior by increasing the appetite, resulting in energy balance disruption and subsequent weight gain.

Pathogenic mechanisms underlying VPA-induced weight gain are still unclear. Most likely, these mechanisms are multifactorial. The balance between energy intake and expenditure is a complex process that is regulated by various appetite-regulating neuropeptides and cytokines that act within the hypothalamus. Some hypotheses have been proposed to explain the effect of VPA on weight increase (Belcastro et al., 2013; Verrotti et al., 2011b; Verrotti et al., 2009) (Figure 3).

1. VPA is a well-known promotor of histone acetylation and modulates expression of many genes. In adipocytes, VPA suppressed the gene expression of adiponectin (Qiao et al., 2006), a biologically active mediator that modulates insulin sensitivity. Adiponectin plasma concentrations were negatively correlated with weight and were significantly lower in obese patients with epilepsy compared to patients who did not gain weight (Greco et al., 2005). It is not clear, however, whether hypoadiponectinemia in patients with epilepsy was the cause or consequence of increased weight. Another important signaling peptide that regulates body weight is leptin, which is produced by adipocytes. Leptin regulates energy balance by inhibiting hunger. Leptin plasma concentration and mRNA expression in adipose tissue were directly related to obesity severity, as an increase of fat mass was associated with an increase of leptin levels (Verrotti et al., 2011b). *In vitro*, VPA reduced leptin mRNA levels and secretion of leptin protein, which probably can induce enhanced appetite in patients on VPA treatment (Lagace et al., 2004). Many clinical studies, however, reported increased serum levels of leptin in patients who gained weight during VPA treatment (Greco et al., 2005; Hamed et al., 2009; Rauchenzauner et al., 2008; Verrotti et al., 1999). This finding could be explained by leptin resistance secondary to weight increase. Leptin resistance is reduced sensitivity to leptin action, resulting in an inability to

detect satiety despite high energy storage. In the absence of VPA-induced obesity, no significant changes in leptin levels have been observed (Verrotti et al., 2009).

2. VPA has been suggested to cause dysregulation of the hypothalamic system (Lakhanpal et al., 2011; Lakhanpal and Kaur, 2007). VPA-mediated enhancement of GABAergic transmission within the hypothalamic axis may increase appetite (Biton et al., 2003). VPA may also modify transcription of adipokine (resistin, fasting-induced adipose factor) genes, which are expressed in the brain and pituitary and are involved in central energy metabolism (Brown et al., 2008; Munzberg and Myers, 2005).
3. Turnbull *et al*. demonstrated a moderate fall in blood glucose levels after oral or intraperitoneal administration of VPA in Wistar rats (Turnbull et al., 1985). Subsequent preclinical studies confirmed this effect (Akindele et al., 2015; Khan and Jena, 2016; Khan et al., 2016; Terasmaa et al., 2011). Several clinical studies reported lower blood glucose levels in VPA-treated patients compared to controls (Aydin et al., 2005; Demir and Aysun, 2000; Martin et al., 2009; Pylvanen et al., 2006a). In some patients, lower blood glucose concentration was independent of insulin concentration. This finding suggests that VPA may modulate glucose homeostasis by an insulin-independent mechanism (Demir and Aysun, 2000; Martin et al., 2009). Mild hypoglycemia in VPA-treated patients can cause an increase in appetite, leading to weight gain.
4. Many studies have reported hyperinsulinemia among patients on VPA treatment (Verrotti et al., 2011b). There is no general agreement on whether hyperinsulinemia is a result of weight gain and insulin resistance (IR) following VPA treatment (Belcastro et al., 2013) or whether VPA treatment directly causes hyperinsulinemia and IR, which lead to weight gain in some patients (Luef et al., 2003; Pylvanen et al., 2006a). Some studies showed that high blood insulin concentrations occur more frequently among patients who have already gained weight compared to those who have not, suggesting that hyperinsulinemia is secondary to weight gain (Isojarvi et al., 1996; Luef et al., 2002c; Pylvanen et al., 2002; Stephen et al., 2001; Verrotti et al., 1999). On the other hand, Pylvanen *et al*. found that both obese and lean patients taking VPA had hyperinsulinemia, suggesting that IR development is a factor that leads to weight increase. Other authors proposed that hyperinsulinemia in these patients may be the result of disturbed insulin metabolism in liver, caused by VPA, resulting in higher insulin concentrations in peripheral circulation (Pylvanen et al., 2006a). Luef *et al*. reported that VPA can directly stimulate pancreatic β-cells and cause insulin secretion *in vitro* (Luef et al., 2003). Both theories are probably true; at the start of treatment, VPA might increase pancreatic insulin secretion and/or decrease insulin degradation in the liver, leading to hyperinsulinemia and decreased blood glucose levels, resulting in enhanced appetite and energy storage. In patients who exhibit weight increase, IR could develop, which also might lead to an elevation of the blood insulin level.

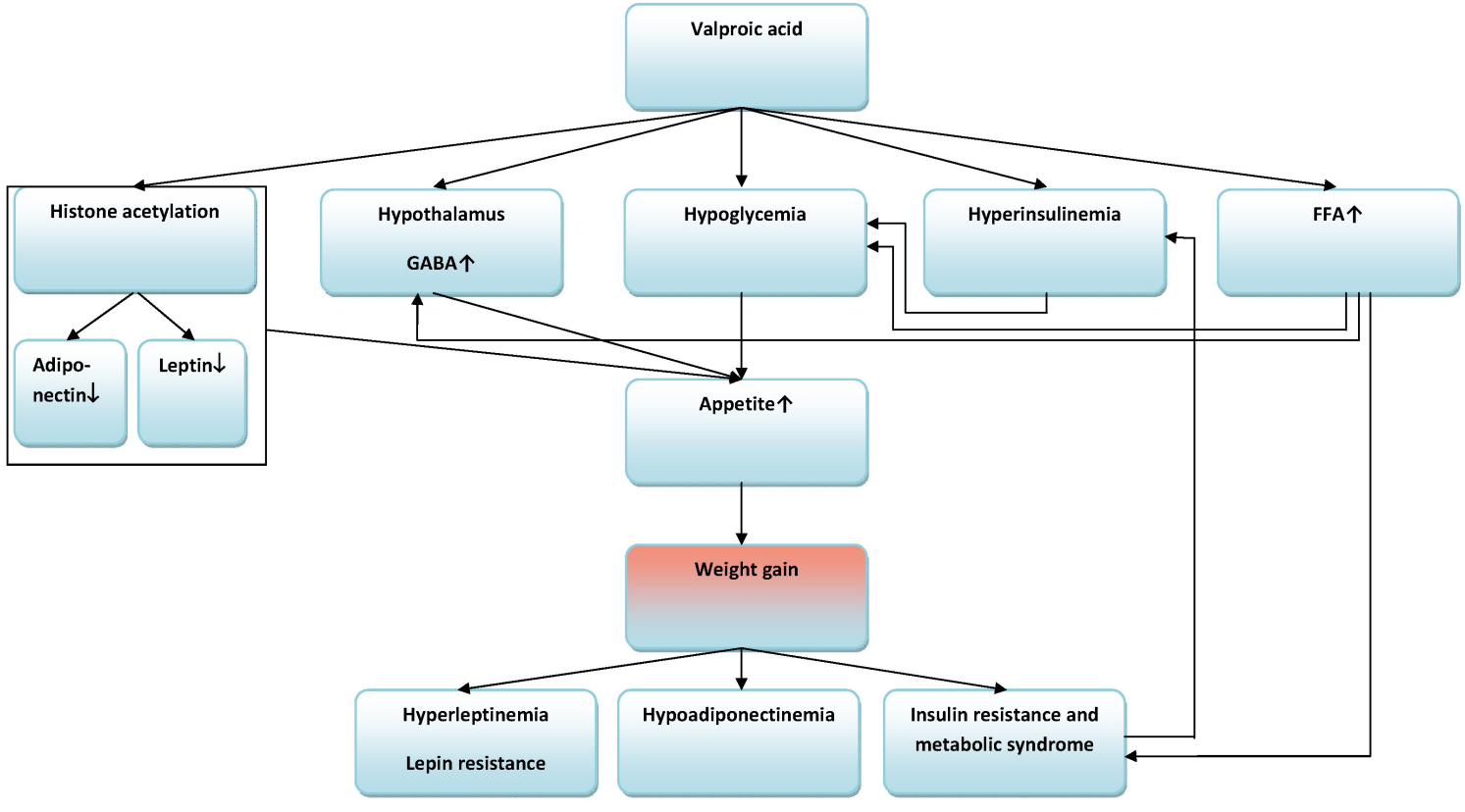

Figure 3. Possible mechanisms of valproate effect on weight. GABA, γ-aminobutyric acid; FFA, free fatty acid.

5. Valproate is a branched-chain fatty acid. Thus, VPA administration could lead to increased plasma levels of free fatty acids (FFAs), which are key modulators of endogenous glucose production (Luef et al., 2002b). Elevated FFA levels play an important role in IR development. Dietary intake and plasma levels determine the fatty acid content in cell membranes, and higher levels of membrane-saturated fatty acids could impair the action of insulin (Verrotti et al., 2009). VPA may inhibit β-oxidation of fatty acids in mitochondria, thereby shifting substrate use from fats to carbohydrates and decreasing glucose concentrations (Demir and Aysun, 2000). The increased level of FFAs may directly influence the hypothalamus, which regulates food intake and whole-body glucose handling (Schwartz et al., 2000). Intracerebroventricular infusion of the long-chain fatty acid oleic acid in rats caused rapid reductions in food intake and inhibited endogenous glucose production (Obici et al., 2002).

2.5. Metabolic syndrome in patients with epilepsy

MS is a constellation of metabolic risk factors, which include central obesity, atherogenic dyslipidemia, hypertension, and elevated blood glucose levels associated with IR (Reaven, 1988). As it is associated with an approximately twofold increased risk of cardiovascular and cerebrovascular diseases (Isomaa et al., 2001; Lakka et al., 2002), MS represents a major economic burden and public health problem (Fu et al., 2007; Wang et al., 2010).

The overall mortality rate among people with epilepsy is two to three times higher than that of the general population (Nevalainen et al., 2014). Epilepsy is associated with a higher risk of cardiovascular diseases (Gaitatzis et al., 2004; Janszky et al., 2009), which undoubtedly contributes to the increased mortality rate. In people with epilepsy, a sedentary lifestyle and AED use contribute to obesity and MS (Ben-Menachem, 2007; Steinhoff et al., 1996). In addition, the comorbidity profile differs between patients with epilepsy and healthy subjects, as patients more frequently have psychiatric disorders or brain damage-related symptoms, which could influence the risk of MS.

Numerous studies have explored the effects of different anticonvulsants on specific metabolic and anthropometric parameters. However, only a few studies, with conflicting results, have examined the presence of MS in patients with epilepsy. Some of these studies focused only on specific populations, such as females (Kim and Lee, 2007) or children (Dhir et al., 2015; Verrotti et al., 2010), whereas others studied different metabolic side effects of AEDs, such that the occurrence of MS was assessed only indirectly (Luef et al., 2009; Pylvanen et al., 2006b). Verrotti *et al*. (Verrotti et al., 2010) showed that the presence of MS in VPA-treated children and adolescents who became obese did not differ from MS prevalence in otherwise healthy overweight subjects. Pylvänen *et al*. reported similar frequencies of MS between VPA-treated adults and control subjects (Pylvanen et al., 2006b). A study evaluating the prevalence of MS among Chinese

adult obese patients with epilepsy treated with VPA showed a tendency toward a higher risk of MS compared to obese control subjects, although this difference was not statistically significant (Fang et al., 2012). On the other hand, Kim and Lee reported that females with epilepsy on VPA monotherapy more frequently have MS compared to females treated with CBZ, LTG, or TPM (Kim and Lee, 2007). In a recent study from India the use of VPA was associated with significant risk of MS in patients with epilepsy, attending outpatient clinic (Nair et al., 2016). Furthermore, NAFLD, which is the hepatic manifestation of MS, occurred more frequently in patients on VPA treatment compared to patients on CBZ or LTG monotherapy (Luef et al., 2009).

In people with epilepsy who are indicated for AED treatment, physicians frequently choose between different anticonvulsants. Information on the potential relationship between different AEDs and MS risk could help physicians in this decision-making process. As is apparent, current knowledge about this topic is insufficient and inconsistent.

3. AIMS OF THE STUDY

1. To evaluate the prevalence of MS and its components in VPA-treated patients with epilepsy in Estonia compared to the general population (Paper I).
2. To compare the risk of MS and evaluate related factors for MS among people with epilepsy treated with VPA or CBZ (Paper II).
3. To characterize the effect of acute IV VPA treatment on the blood levels of glucose, insulin, and C-peptide after first VPA exposure in naive patients with newly diagnosed epilepsy (Paper III).
4. To analyze the effects of chronic VPA treatment on the peripheral blood gene expression profile induced by 3 months of treatment in patients with newly diagnosed epilepsy (Paper IV).

4. SUBJECTS AND METHODS

All the studies included in this thesis have been approved by Ethics Review Committee on Human Research of the University of Tartu. An informed consent has been obtained from all study participants.

4.1. Study design

Papers I and II
The study design was cross-sectional population-based study including patients with epilepsy treated with VPA, CBZ and control subjects from southern Estonia.

Papers III and IV
Case-crossover study including patients with newly diagnosed epilepsy, who were indicated for VPA therapy.

4.2. Prevalence and risk factors of metabolic syndrome in patients treated with valproate and carbamazepine (Papers I and II)

4.2.1. Subjects of the studies

The study was carried out in the Departments of Neurology and Internal Medicine, Tartu University Hospital, Estonia.

Using the prescription database of the Estonian Health Insurance Fund, patients with epilepsy from six southern Estonian counties (including the city of Tartu and Tartu County) who were prescribed VPA and patients with epilepsy from the city of Tartu and Tartu County who were prescribed CBZ were identified. Exclusion criteria for patients with epilepsy were as follows: age ≤ 18 years; VPA or CBZ monotherapy for < 3 months; polytherapy with other AEDs; pregnancy. As physical inactivity is a major risk factor of MS, and as the aim of the study was to evaluate possible relationships between AEDs, epilepsy syndromes, and MS risk, patients with severe physical or mental disabilities related to severe brain damage were not included. Specifically, patients who lived at nursing homes or who were dependent at or outside the home were excluded. Study participants should be able to engage in normal occupational and social activities, despite minor physical or mental deficits. Patients with endocrine disturbances, such as diabetes or thyroid dysfunction, were not excluded.

The data was collected between 1 January and 31 December 2012 for VPA-treated patients as well as between 1 December 2014 and 1 September 2015 for patients treated with CBZ.

A total of 384 (206 men, 178 women) and 484 (292 men, 192 women) patients with epilepsy diagnoses who had received VPA or CBZ treatment, respectively, were initially identified. Applying the data collection methods, 118 VPA-treated patients (63 men, 55 women) and 95 CBZ-treated patients (55 men, 40 women) were included in the final study sample. From the initial drug prescription database, 17 (4.4%) VPA-treated subjects and 138 (28.5%) CBZ-treated patients were not included in the final sample due to severe physical or mental disability related to brain damage caused by severe head injury, cerebrovascular disease, progressive brain neoplasm, pre- or perinatal risk factors, central nervous system infection, or other factors (Figure 4). Clinical characteristics of the patients are summarized in Table 2.

The control group comprised subjects who participated in a population-based cross-sectional multicenter study of MS prevalence conducted in southern Estonia between November 2008 and May 2009 (Eglit et al., 2012). Control subjects were adults who were randomly selected from four general practices. An invitation letter about the study was sent to each participant representing control group. The total response rate was 53.2% (493 control subjects). The control subjects were representative of the general Estonian population in terms of age and gender. Distributions of the ethnic groups and the socioeconomic status of inhabitants are similar among the various counties in southern Estonia.

4.2.2. Collection of anthropometric and laboratory data

All patients with epilepsy were contacted by phone and those who met the inclusion criteria were invited to the outpatient clinic of the Department of Neurology at Tartu University Hospital. The author of this thesis interviewed and clinically examined all patients. The medical histories of participants were documented during the evaluation meeting. Concomitant diseases of relevance to the study, including known endocrinopathies, lipid metabolism disorders, and vascular diseases, were noted. Blood pressure, waist circumference, weight, and height were measured. Body mass index (BMI) was calculated as the weight (kg) divided by height squared (m^2). Blood samples were obtained in the morning (between 08:00 and 11:00) after an overnight fast (≥10 h) for the analysis of serum insulin, C-peptide, high-density lipoprotein cholesterol (HDL-C), triglyceride (TG), and fasting blood glucose concentrations. Patients with unmeasurable serum VPA or CBZ levels were excluded because of poor compliance.

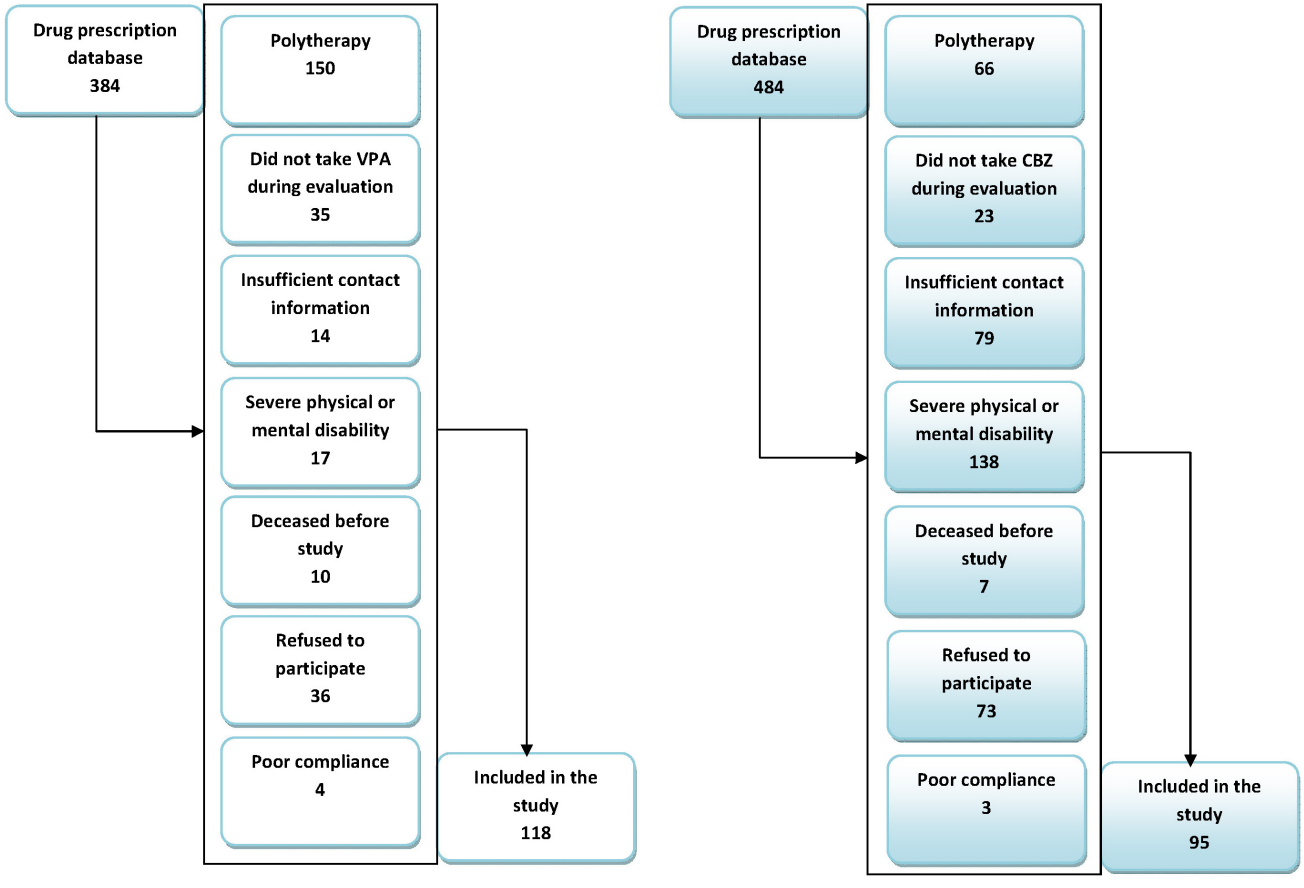

Figure 4. Flowcharts for the inclusion of valproate-treated (white boxes) and carbamazepine-treated (blue boxes) patients with epilepsy.

Table 2. Clinical characteristics of patients with epilepsy treated with valproate or carbamazepine.

	Valproate-treated patients			Carbamazepine-treated patients		
	Male	Female	All	Male	Female	All
No. of patients	63 (53%)	55 (47%)	118 (100%)	55 (58%)	40 (42%)	95 (100%)
Age (years)	36.1±15.3[a]	37.1±16.0[b]	36.6±16.3[c]	52.7±14.6	58.0±14.7	54.9±14.8
Patients with seizures during last year	24 (20%)	30 (25%)	54 (46%)	24 (25%)	12 (13%)	36 (38%)
Etiology:						
Gen	33 (28%)[a]	36 (31%)[b]	69 (58%)[c]	3 (3%)	1 (1%)	4 (4%)
Str/met	15 (13%)[a]	5 (4%)[b]	20 (17%)[c]	35 (37%)	24 (25%)	59 (62%)
Unk	16 (14%)	13 (11%)	29 (25%)	17 (18%)	15 (16%)	32 (34%)
Daily VPA or CBZ dose (mg/d)	1027±487	894±386	964±446	600±333	610±242	604±297
Serum VPA concentration (µg/ml)	58.6±29.2	63.7±32.4	61.0±30.7	n.a.	n.a.	n.a.
Serum CBZ concentration (µg/ml)	n.a.	n.a.	n.a.	6.1±2.2	6.3±2.2	6.2±2.2

Values are expressed as means (%) ± standard deviations, except for the number of patients and epilepsy etiology.
No, number; Gen, genetic; Str/met, structural/metabolic; Unk, unknown; VPA, valproate; CBZ, carbamazepine; n.a., not applicable.
[a] $P < 0.0001$ when compared with CBZ-treated males; [b] $P < 0.0001$ when compared with CBZ-treated females; [c] $P < 0.0001$ when compared with CBZ-treated patients.

During the face-to-face interviews and the review of previous medical history, the patients' epilepsy diagnoses were re-evaluated, and the date of the last seizure was recorded. Epileptic syndromes were classified based on the recently proposed International League against Epilepsy classification (Berg et al., 2010). The date of initiation, duration, and current dosage of VPA or CBZ treatment were recorded. Patients were asked whether they had noticed any change in body weight after initiation of VPA or CBZ treatment. The final regression analysis, comparing MS risk factors of patients treated with VPA or CBZ, included concomitant conditions as a covariate. Concomitant conditions included psychiatric comorbidities treated with antidepressants and/or antipsychotics, and thyroid dysfunction. The regular use of medications for the treatment of arterial hypertension, dyslipidemia, or previously diagnosed diabetes was not considered as an independent covariate in the regression analysis, because the use of these medications is included in the definition of MS.

4.2.3. Assays

Plasma glucose levels were measured by the hexokinase method. HDL-C and TG concentrations were measured by an enzymatic colorimetric assay. VPA and CBZ concentrations were measured by fluorescence polarization assay (COBAS INTEGRA 800 Plus Analyzer; Roche, Basel, Switzerland). In control subjects, plasma insulin concentrations were measured by a chemiluminescent assay (Immulite 2000 Analyzer; Siemens Healthcare Diagnostics, Deerfield, IL, USA), whereas in patients, they were measured by an electrochemiluminescent assay (COBAS 6000 Analyzer; Roche). To compare plasma insulin corrected concentrations between patients and controls, the difference between the two measurement assays was calculated according to the Clinical and Laboratory Standards Institute's guidelines (Wayne, 2013). Plasma C-peptide concentrations were measured only in patients by an electrochemiluminescent assay (COBAS 6000 Analyzer; Roche).

4.2.4. Definition of metabolic syndrome

MS was diagnosed based on the presence of at least three of the following National Cholesterol Education Program Adult Treatment Panel III criteria (Grundy et al., 2005): waist circumference $\geq$ 102 cm in men and $\geq$ 88 cm in women, BP $\geq$ 130/85 mmHg or antihypertensive medication use, fasting glucose concentration $\geq$ 5.6 mmol/L or previously diagnosed diabetes, TG concentration $\geq$ 1.7 mmol/L or lipid-regulating medication use, and HDL-C concentration $<$ 1.03 mmol/L in men and $<$ 1.3 mmol/L in women or drug treatment for reduced HDL-C. IR was estimated by the homeostasis model assessment-estimated insulin resistance (HOMA-IR) index, calculated with the following equation: fasting glucose (mmol/L) $\times$ fasting insulin (mU/L) / 22.5. Patients with BMIs $\geq$ 25 kg/m^2 were categorized as overweight.

4.2.5. Statistical analysis

Descriptive analytical methods, such as the calculation of means, standard deviations (SDs), medians, and interquartile ranges (IQRs), were used for continuous variables, depending on the distribution.

Logistic regression model for the risk of MS and its components in patients treated with VPA compared to control subjects and with CBZ compared to control subjects was adjusted by age and sex. Age- and sex-adjusted linear regression models were used to compare anthropometric and laboratory data between VPA-treated patients and control subjects, as well as between CBZ-treated patients and controls. Linear regression models adjusted by age, sex, epilepsy etiology, seizure frequency, treatment duration and concomitant diseases were used to compare anthropometric and laboratory data between VPA- and CBZ-treated patients.

Multiple logistic regression analysis was performed to identify factors associated with MS development in VPA- and CBZ-treated patients. The final multiple logistic regression model, which compared the risk for MS between VPA- and CBZ-treated patients was adjusted for age, sex, epilepsy etiology, seizure occurrence in the last year, treatment duration, and concomitant diseases.

A receiver operating characteristic (ROC) analysis was conducted to evaluate whether BMI and HOMA-IR had similar predictive abilities for MS in VPA-treated patients and control subjects.

The prevalence of MS in VPA-treated patients was calculated by the indirect method of standardization, considering the age and sex distribution of the control group as the standard. Odds ratios (ORs) and 95% confidence intervals (95% CIs) are reported. The R software package (The R Foundation for Statistical Computing; version 2.15.1) was used for statistical analyses. Differences with $p < 0.05$ were considered statistically significant.

4.3. The effect of acute intravenous valproate treatment on the glucose, insulin and C-peptide metabolism (Paper III)

4.3.1. Subject selection

Sixteen consecutive adult patients from the outpatient clinic of the Tartu University Hospital were recruited between March 2011 and March 2015. These individuals were newly diagnosed with epilepsy and were indicated for VPA therapy. Patients were excluded if they were treated with any medication, had a history of diabetes, a cardiovascular, hepatic, renal, oncologic or progressive neurological disease that could impact glucose or insulin metabolism, evidence of progressive lesions on computed tomography or magnetic resonance imaging. Main subject characteristics including age and epileptic syndrome are summarized in Table 3. Seizures and epileptic syndromes were classified based on the criteria proposed by the ILAE (Berg et al., 2010).

4.3.2. Study design

The study protocol assumed two visits for each patient. These visits began at 8 a.m. following an overnight fast. During the first visit, the oral glucose tolerance test (OGTT) was performed using a 75 g glucose load. Plasma glucose, serum insulin, and C-peptide levels were measured in the fasting state, as well as one and two hours postprandially. At the second visit, patients received VPA (Orfiril, Desitin) administered IV during 20 minutes at a load of 900–1800 mg diluted in 100 ml 0.9% NaCl. Directly following VPA infusion, the OGTT was performed. Serum VPA concentrations were measured directly following VPA infusion, as well as one and two hours postprandially. Cardiovascular parameters, such as heart rate and blood pressure, as well as signs of irritation at the injection site were closely monitored. For every single patient, the number of days between the two visits varied from 1 to 7 days. The second OGTT was performed as fast as possible after first OGTT in order to diminish the potential for additional confounding factors to affect the results. A deadline of 7 days between visits was established by the study protocol.

4.3.3. Assays

Plasma glucose levels were measured using the hexokinase method. VPA concentrations were measured by fluorescence polarization assay (COBAS INTEGRA 800 Plus Analyzer; Roche, Basel, Switzerland). Plasma insulin and C-peptide concentrations were measured using an electrochemiluminescent assay (COBAS 6000 Analyzer; Roche, Basel, Switzerland).

Table 3. Clinical and demographic characteristics of the patients (Paper III).

	Number of patients	Age (years)	Epilepsy etiology		BMI (kg/m^2)
			Gen	Str/met	
Male	6	22 ± 3.5	6	0	26.9 ± 5.8
Female	10	28.5 ± 9.1	8	2	22.9 ± 2.9
All	16	26 ± 8.0	14	2	24.4 ± 4.5

Values are expressed as means ± SD; Gen, genetic; Str/met, structural/metabolic; BMI, body mass index

4.3.4. Statistical analysis

The data are presented as mean ± standard deviation (SD). Statistical significance was evaluated by paired t-test. P values <0.05 were considered to be statistically significant. The R software package (The R Foundation for Statistical Computing; version 2.15.1) was used for statistical analyses.

4.4. The effect of chronic valproate treatment on peripheral blood gene expression (Paper IV)

4.4.1. Subjects

Nine otherwise healthy subjects with newly diagnosed epilepsy and for whom VPA treatment was indicated were enrolled. Patients were excluded if there was a history or evidence of noncompliance with medical regimens; treatment with any medication other than VPA, including hormonal contraception; evidence of a cardiovascular, hepatic, renal, oncologic, or progressive neurological disease that could have an impact on blood RNA expression; evidence of progressive lesions on computed tomography or magnetic resonance imaging; or if the patient was pregnant.

The main subject characteristics, including epileptic syndrome and VPA dose, are summarized in Table 4. Before and 3 months after the start of VPA treatment, the weight and height were measured, and the BMI was calculated.

Table 4. Clinical and demographic characteristics of patients (Paper IV).

Sex	Age (years)	Epilepsy etiology	Daily VPA dose (mg/d)	Daily VPA dose (mg/kg)	BMI before treatment (kg/m^2)	BMI after treatment (kg/m^2)
F	38	Gen	600	12.5	21.3	21.8
F	38	Str/met	900	15	21.8	21.8
M	21	Gen	900	8.3	34.5	34.5
M	21	Gen	600	6.9	24.1	25.5
M	18	Gen	600	9.7	20.0	23.6
F	21	Gen	600	12.8	17.7	18.8
F	33	Gen	600	11.8	20.2	21.0
F	18	Gen	600	9.8	19.5	19.8
F	32	Str/met	1200	15.8	24.8	24.5

Gen, genetic; Str/met, structural/metabolic; VPA, valproate; BMI, body mass index.

4.4.2. Sample collection and RNA preparation

Blood samples were collected during two visits, before and around 3 months after the start of treatment with VPA. The daily VPA dose was 600 to 1200 mg (mean dose: 11.4 ± 2.8 mg/kg). Blood samples were collected into Tempus tubes (Applied Biosystems, Foster City, USA). Blood was frozen and stored until further processing. RNA was extracted from whole blood with an RNA extraction kit, in accordance with the manufacturer's protocol (Applied Biosystems).

4.4.3. Statistical analysis

Differential gene expression was analyzed by using the EdgeR package with non-normalized raw counts after the quality control of samples. EdgeR is a very flexible tool for RNA sequencing data analysis, which uses model-based scale normalization, dispersion estimates, negative binomial model fitting, and testing procedures to determine the differential expression of genes (McCarthy et al., 2012; Robinson et al., 2010). As our sample contains paired samples (pre- and post-VPA), a paired testing approach was used. General linear modeling was applied, with subjects being added to the contrast matrix. The general linear modeling likelihood ratio test was applied to compare pre- and post-VPA results. False discovery rate adjustment was used for multiple testing correction (Storey and Tibshirani, 2003). The threshold for statistical significance was an adjusted false discovery rate of 0.1.

4.4.4. Functional analysis of differentially expressed genes

Functional network analysis is used to identify the biological functions that are most significantly related to the molecules in a network. To define the functional networks of differentially expressed genes, the data were analyzed by using the Ingenuity Pathway Analysis (Ingenuity Systems, *www.ingenuity.com*), which calculates a significance (network) score for each network. This score indicates the likelihood that the assembly of a set of focus genes in a network could be explained by random chance alone (e.g., a score of 2 indicates that there is a 1 in 100 chance that the focus genes are together in a network due to random chance). A data set containing the Affymetrix probe-set identifiers and their corresponding fold change ($\log_2$) values was uploaded into the Ingenuity Pathway Analysis software. Each gene identifier was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base to identify molecules whose expression was significantly differentially regulated. These focus genes (or Network Eligible molecules) were overlaid onto a global molecular network developed from information contained in the Ingenuity Pathways Knowledge Base. Networks of these focus genes were then algorithmically generated on the basis of their connectivity.

A network is a graphical representation of the molecular relationships between genes or gene products (represented as nodes). The biological relationship between two nodes is represented as an edge (line). All edges are supported by at least one reference from the literature, or from canonical information stored in the Ingenuity Pathways Knowledge Base.

5. RESULTS

5.1. Clinical characteristics of patients and control subjects

Median ages of patients treated with VPA and CBZ and control subjects were 32 years (IQR, 24–45 years), 56 years (IQR, 43–66 years) and 47 years (IQR, 35–59 years), respectively. In the final sample, most of the VPA-treated patients had a genetic epilepsy etiology ($P < 0.0001$), whereas CBZ-treated patients more frequently had a structural or metabolic cause of epilepsy ($P < 0.0001$) (Table 2). Twenty-five (11.7%) patients with epilepsy had psychiatric comorbidities that were treated with antidepressants and/or antipsychotics. Five (2.3%) patients had been diagnosed previously with thyroid dysfunction. Nine (4.2%) patients had been previously diagnosed with diabetes, three of them were treated with VPA and six patients were treated with CBZ.

5.2. Prevalence of metabolic syndrome and it's components in valproate- and carbamazepine-treated patients with epilepsy

The crude prevalence of MS in adult patients with epilepsy who received VPA monotherapy was 20.3% (95% CI, 13.7–28.9%), with a prevalence of 22.2% (95% CI, 13.1–34.8%) in men and 18.2% (95% CI, 9.5–31.4%) in women. The prevalence of MS in VPA-treated patients, weighted by the age and sex distribution of the control cohort, was 25.8% (95% CI, 18.4–34.8%). The prevalence of MS in the control group was 27.9% (95% CI, 24.0–32.1%). In the final multiple logistic regression, after adjustment for age and sex, the risk of MS in VPA-treated patients was not increased (OR = 1.00; 95% CI, 0.59–1.68; $P = 0.998$). However, a larger proportion of VPA-treated patients had abnormal diastolic BP (≥85 mmHg) compared with control subjects (OR = 1.86; CI 1.15–3.03; $P = 0.011$; Table 5). The risk of MS in CBZ-treated patients compared to controls was also not increased (OR = 1.23; CI 0.76–1.99; $P = 0.407$). Patients treated with CBZ had increased risk of high blood pressure, and high fasting blood glucose concentration. However, the occurrence of abnormally low HDL-C in these patients was significantly lower than in controls (Table 5).

5.3. Comparison of anthropometric and metabolic parameters in valproate-treated patients and controls

BMI did not differ between VPA-treated patients and control subjects ($P = 0.295$); however, VPA-treated patients had higher fasting serum insulin levels compared to control subjects ($P < 0.0001$). The HOMA-IR index was higher in patients than in control subjects ($P = 0.0001$). These differences

persisted when only normal-weight subjects from both cohorts were compared (P = 0.010 for insulin and P = 0.031 for HOMA-IR). Fasting plasma glucose concentrations tended to be lower in patients than in control subjects, although this difference was not significant (P = 0.169). The HDL-C and TG levels were similar in patients and control subjects (Table 6).

Table 5. Logistic regression model for metabolic syndrome and its components in patients treated with valproate compared to control subjects and with carbamazepine compared to control subjects

Component	VPA-treated patients OR	95% CI	P	CBZ-treated patients OR	95% CI	P
Waist circumference	0.75	0.47–1.22	0.248	0.79	0.49–1.27	0.328
men, >102 cm	0.89	0.45–1.75	0.90	0.90	0.47–1.72	0.756
women, >88 cm	0.64	0.32–1.26	0.67	0.67	0.33–1.36	0.267
HDL-C	0.95	0.57–1.59	0.851	0.13	0.04–0.43	0.001
men, <1.03 mmol/L	1.31	0.63–2.69	0.20	0.20	0.05–0.88	0.033
women, <1.30 mmol/L	0.76	0.36–1.63	0.07	0.07	0.01–0.59	0.014
Triglycerides ≥ 1.7 mmol/L	1.34	0.80–2.24	0.267	1.18	0.69–2.03	0.539
Fasting glucose ≥ 5.6 mmol/L	0.78	0.46–1.31	0.352	1.86	1.15–2.99	0.011
Systolic blood pressure ≥ 130 mmHg	1.54	0.91–2.60	0.112	1.80	1.06–3.04	0.029
Diastolic blood pressure ≥ 85 mmHg	1.86	1.15–3.03	0.011	2.16	1.33–3.51	0.002
Metabolic syndrome	1.00	0.59–1.68	0.998	1.23	0.76–1.99	0.407

Odds ratios (ORs) for metabolic syndrome and its components in valproate-treated, as well as in carbamazepine-treated patient cohort compared to controls and adjusted for age and sex.
VPA, valproate; CBZ, carbamazepine; HDL-C, high-density lipoprotein cholesterol; CI, confidence interval.

Table 6. Anthropometric and laboratory data in valproate-treated patients and control subjects

	Patients on VPA treatment N=118	Control subjects N=493	P
BMI (kg/m^2)	26.38±5.34	28.18±6.16	0.295
Fasting glucose (mmol/L)	5.18±0.78	5.43±0.82	0.169
Fasting insulin (mU/L)	9.10±8.51	6.35±5.52	<0.0001
HOMA-IR	2.21±2.01	1.60±1.4	<0.001
HDL-C (mmol/L)	1.51±0.49	1.53±0.45	0.581
Triglycerides (mmol/L)	1.34±0.91	1.31±0.72	0.375

Data presented as means ± standard deviations.
BMI, body mass index; HOMA-IR, homeostasis model assessment of insulin resistance; VPA, valproate.
P values were calculated after comparison of parameters between cohorts using multiple linear regression model adjusted for age and sex.

5.4. HOMA-IR predictive ability for metabolic syndrome occurrence in valproate-, carbamazepine-treated patients and controls

ROC analysis showed that BMI and HOMA-IR similarly predicted MS occurrence in control subjects, with areas under the ROC curve (AUCs) of 0.847 and 0.848 (P = 0.97), respectively (Figure 5). The predictive ability of HOMA-IR was lower than that of BMI in VPA-treated patients, with AUCs of 0.808 and 0.897 (P = 0.05), respectively (Figure 6). In CBZ-treated patients the difference of predictive ability of HOMA-IR and BMI was statistically not significant (P = 0.36) (Figure 7).

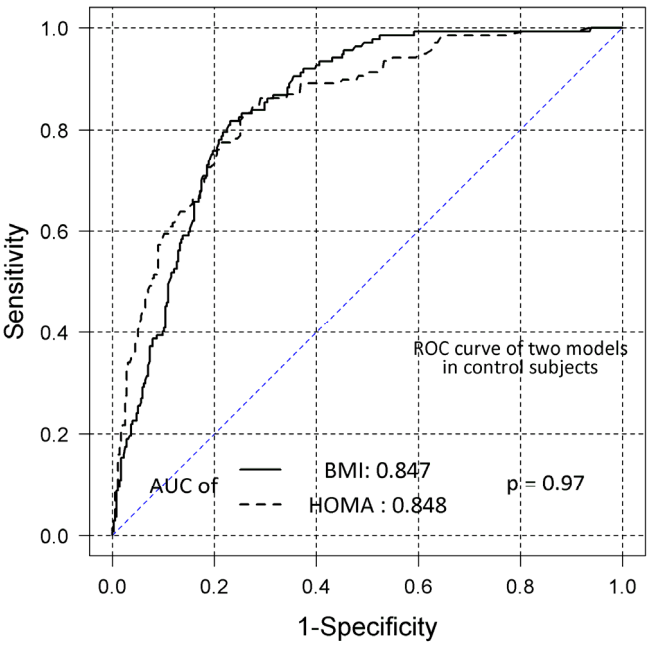

Figure 5. Receiver operating characteristic (ROC) curves of the homeostasis model assessment-estimated insulin resistance (HOMA-IR) and body mass index (BMI) values in control subjects. BMI and HOMA-IR curves were obtained by logistic regression to determine the probability of metabolic syndrome (MS) in control subjects. AUC, area under the curve.

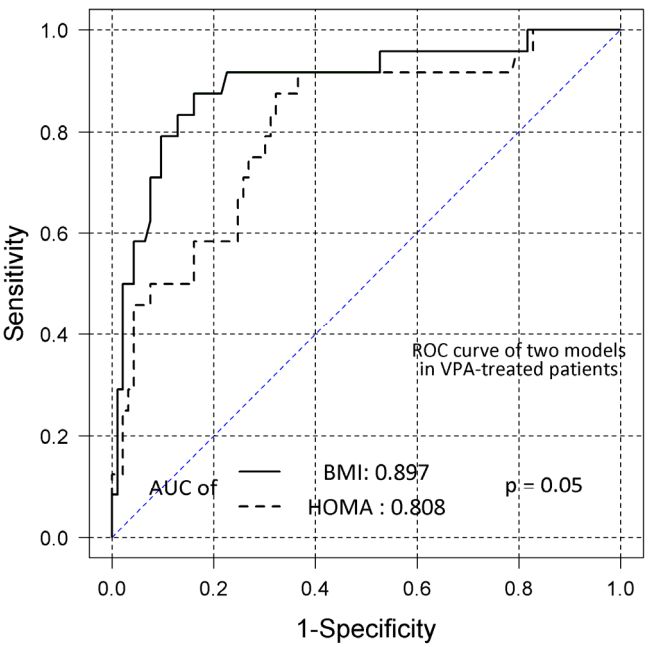

Figure 6. Receiver operating characteristic (ROC) curves of the homeostasis model assessment-estimated insulin resistance (HOMA-IR) and body mass index (BMI) values in valproate (VPA)-treated patients. BMI and HOMA-IR curves were obtained by logistic regression to determine the probability of metabolic syndrome (MS) in patients treated with VPA. AUC, area under the curve.

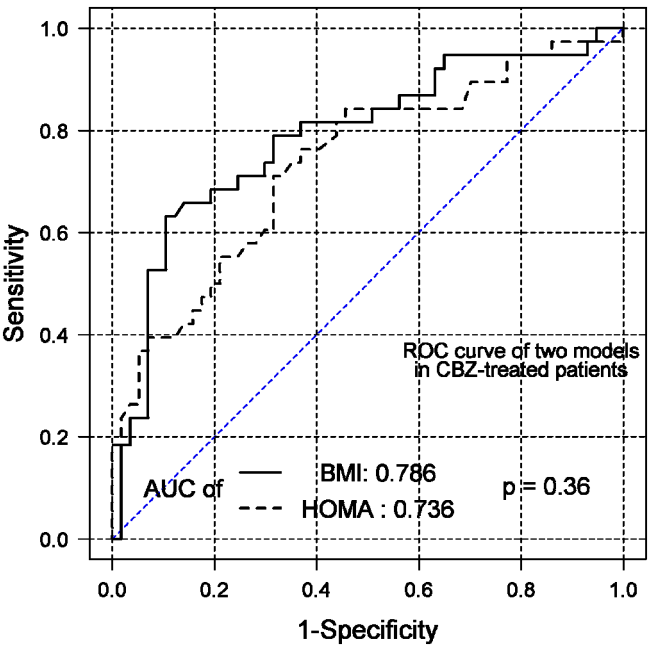

Figure 7. Receiver operating characteristic (ROC) curves of the homeostasis model assessment-estimated insulin resistance (HOMA-IR) and body mass index (BMI) values in carbamazepine (CBZ)-treated patients. BMI and HOMA-IR curves were obtained by logistic regression to determine the probability of metabolic syndrome (MS) in patients treated with CBZ. AUC, area under the curve.

5.5. Comparison of risk factors for metabolic syndrome in epilepsy patients treated with valproate or carbamazepine

Stepwise logistic regression analysis revealed no significant correlation between MS development in patients with epilepsy and clinical characteristics, such as sex, epilepsy etiology, treatment with VPA or CBZ, seizure occurrence in the last year, HOMA-IR, and concomitant diseases. One of the factors to be significantly correlated with the occurrence of MS in people with epilepsy was the patient's age, which showed a positive correlation (OR = 1.03; 95% CI, 1.01–1.06; P = 0.007). There was no correlation between the daily dosage of medication and the risk of MS in patients on CBZ treatment (P = 0.948). In VPA-treated patients, a trend toward a positive correlation between drug dose and risk of MS was observed. However, this correlation did not reach statistical significance (OR = 1.11; 95% CI, 0.99 – 1.24; P = 0.075). Longer durations of VPA treatment tended to increase the risk of MS (OR = 1.01; 95% CI, 1.0–1.02; P < 0.01), however the duration of CBZ-treatment was not correlated to the MS risk (P = 0.941). In the final multiple logistic regression, after adjustment for age, the risk of MS in CBZ-versus VPA-treated patients was not increased (OR = 1.30; 95% CI, 0.65–2.62; P = 0.459). After adjustment of this model for other parameters, including sex, epilepsy etiology, seizure occurrence in the last year, treatment duration, and concomitant diseases, this difference was even less pronounced (OR = 0.99; 95% CI, 0.43–2.26; P = 0.979). Females treated with

VPA tended to have a higher risk of MS (OR = 1.48; 95% CI, 0.50–4.41; $P = 0.485$) compared to males (OR = 0.74; 95% CI, 0.28–1.96; $P = 0.551$), although this difference was not statistically significant (Table 7).

Regarding individual components of MS, a lower proportion of CBZ-treated patients had abnormally low HDL-C levels (OR = 0.10; 95% CI, 0.02–0.42; $P = 0.002$). This effect was more pronounced in women (OR = 0.02; 95% CI, 0.001–0.22; $P = 0.002$) compared to men (OR = 0.47; 95% CI, 0.07–2.99; $P = 0.425$). A higher proportion of VPA-treated females had waist circumference values exceeding 88 cm (OR = 5.51; 95% CI, 1.05–29.01; $P = 0.044$). A lower proportion of VPA-treated patients had abnormally high FBG concentrations (OR = 0.30; 95% CI, 0.13–0.69; $P = 0.004$, Table 7). Overall, patients on VPA treatment had lower concentrations of HDL-C ($P < 0.0001$) and tended to have lower concentrations of fasting glucose ($P = 0.055$, Table 8).

Table 7. Logistic regression model for metabolic syndrome and its components in patients treated with carbamazepine compared to valproic acid.

Component	OR	95% CI	*P*
Waist circumference	0.61	0.26–1.44	0.262
men, >102 cm	0.86	0.28–2.61	0.788
women, >88 cm	0.18	0.03–0.96	0.044
HDL-C	0.10	0.02–0.42	0.002
men, <1.03 mmol/L	0.47	0.07–2.99	0.425
women, <1.30 mmol/L	0.02	0.001–0.22	0.002
Triglycerides ≥ 1.7 mmol/L	0.78	0.33–1.87	0.577
Fasting glucose ≥ 5.6 mmol/L	3.39	1.46–7.85	0.004
Systolic blood pressure ≥ 130 mmHg	1.19	0.50–2.86	0.694
Diastolic blood pressure ≥ 85 mmHg	1.13	0.51–2.47	0.768
Metabolic syndrome	0.99	0.43–2.26	0.979

Odds ratios (ORs) for metabolic syndrome and its components in carbamazepine- compared to the valproate-treated patients adjusted for age, gender, epilepsy etiology, seizure frequency, treatment duration, and concomitant diseases.
HDL-C, high-density lipoprotein cholesterol; CI, confidence interval.

Table 8. Comparison of anthropometric parameters and laboratory data in the treatment groups

	VPA-treated patients	CBZ-treated patients	*P*
BMI (kg/m^2)	26.38±5.34	28.91±5.88	0.996
Waist circumference (cm)	87.41±16.16	96.04±16.27	0.729
Fasting glucose (mmol/L)	5.18±0.78	5.75±0.87	0.055
Fasting insulin (mU/L)	10.23±8.88	11.18±6.75	0.956
C-peptide (nmol/L)	0.79±0.38	0.84±0.32	0.407
HOMA-IR	2.48±2.40	2.90±1.90	0.729
HDL-C (mmol/L)	1.51±0.49	1.89±0.63	<0.0001
Triglycerides (mmol/L)	1.34±0.91	1.49±1.55	0.632

Data are presented as means ± standard deviations.
VPA, valproate; CBZ, carbamazepine; BMI, body mass index; HOMA-IR, homeostasis model assessment of insulin resistance; HDL-C, high-density lipoprotein cholesterol.
P values were calculated after comparison of parameters between cohorts using multiple linear regression model adjusted for age, gender, epilepsy etiology, seizure frequency, treatment duration, and concomitant diseases.

5.6. The effect of acute intravenous valproate treatment on the glucose, insulin and C-peptide blood levels

Plasma glucose levels on the 120 minute of OGTT session following infusion of VPA were significantly lower than those measured during OGTT without VPA treatment (4.28 ± 0.94 mmol/l vs. 4.75 ± 1.09 mmol/l respectively, P = 0.038). However, the concentrations of insulin and C-peptide did not differ significantly between the two measurements (Table 9). Overall, the administration of IV VPA was well tolerated in the patients, with no significant changes observed in heart rate or blood pressure. Of the 16 patients evaluated, three complained of moderate paresthesia or pain at the site of the injection.

Table 9. Oral glucose tolerance test in patients before the start of VPA treatment and immediately after the first intravenous VPA dose.

	Basal OGTT	OGTT after IV VPA	P value
Glucose (mmol/L)			
Fasting	5.15 ± 0.46	5.06 ± 0.45	0.381
60 min pp	5.71 ± 1.25	6.01 ± 1.75	0.385
120 min pp	4.75 ± 1.09	4.28 ± 0.94	0.038
Serum insulin (mU/L)			
Fasting	9.64 ± 6.69	13.66 ± 21.53	0.425
60 min pp	51.79 ± 29.82	67.53 ± 50.98	0.135
120 min pp	26.84 ± 29.14	25.80 ± 28.88	0.851
Serum C-peptide (nmol/L)			
Fasting	0.71 ± 0.27	0.79 ± 0.51	0.382
60 min pp	2.21 ± 0.68	2.63 ± 1.20	0.060
120 min pp	1.52 ± 0.85	1.53 ± 0.79	0.897
VPA (µg/ml), fasting	n.a	112.1 ± 25.7	n.a
VPA (µg/ml), 60 min pp	n.a	86.0 ± 20.9	n.a
VPA (µg/ml), 120 min pp	n.a	77.2 ± 19.3	n.a

Values are expressed as means ± SD; OGTT, oral glucose tolerance test; VPA, valproate; pp, postprandial.

5.7. The effect of chronic valproate treatment on peripheral blood gene expression

Gene expression profiles of blood RNA samples isolated from patients with epilepsy before and three months after the start of VPA treatment slightly differed. Eleven of the 23,099 analyzed genes showed statistically significant differential expression with multiple testing-adjusted false discovery rate values less than 0.1 (Table 10). Relative differences in the expression signal (fold change or log fold change) between two time points were generally low. After initiation of VPA treatment, only 2 genes were up-regulated more than 1.1 fold, and no genes were down-regulated more than 1.1 fold, compared to the status before VPA treatment.

Table 10. Most significantly up- or down-regulated genes after exposure to valproate.

Gene symbol	Log(FC)	*P*	FDR	Gene name
TPT1	–0.14	2.00×10^{-8}	0.0004611	Tumor protein, translationally controlled 1
HBG2	0.86	3.16×10^{-7}	0.0036545	Hemoglobin, gamma G
ARAP3	0.51	6.32×10^{-7}	0.0037739	ArfGAP with RhoGAP domain, ankyrin repeat and PH domain 3
RBM43	–0.58	6.54×10^{-7}	0.0037739	RNA-binding motif protein 43
ALAS2	0.72	9.99×10^{-7}	0.0046131	Aminolevulinate, delta-, synthase 2
MOSC1	0.58	4.61×10^{-6}	0.0177665	Mitochondrial amidoxime reducing component 1
KLHDC8B	0.68	1.08×10^{-5}	0.0356676	Kelch domain containing 8B
SNORD89	–0.31	1.73×10^{-5}	0.0498153	Small nucleolar RNA, C/D box 89
PELI3	0.80	2.14×10^{-5}	0.0550179	Pellino E3 ubiquitin protein ligase family member 3
TMEM150C	2.09	3.84×10^{-5}	0.0887540	Transmembrane protein 150C
C10orf25	1.12	4.55×10^{-5}	0.0954488	Chromosome 10 open reading frame 25

FDR, false discovery rate.

Functional annotation of expression profiles was subsequently applied to identify functional changes in the context of genetic networks. A dataset containing 11 genes with significant differential expression between before and after initiation of VPA treatment was uploaded into the Ingenuity Pathway Analysis software program. Only one genetic network was identified (enrichment score = 30), which included genes related to cardiovascular system development and function, cell morphology, as well as hematological system development and function (Table 11, Figure 8).

Table 11. Network of genes significantly changed after treatment with valproic acid.

Molecules in network	Score	Focus molecules	Top functions
ABCB6, ACO1, ALAS2, ARAP3, BNIP3L, C10orf25, ETV6, GRB2, GSTP1, HBB, Hbb-b1, HBE1, HBG2, HBZ, IRAK2, IREB2, KLF1, KLHDC8B, MARC1, miR-3677-3p (miRNAs w/seed UCGUGGG), miR-4651 (and other miRNAs w/seed GGGGUGG), MTA1, NR2C1, PELI3, PER1, PER2, RBM43, RCOR1, SMAD5, SP1, SUCLA2, TMEM150C, TPT1, UBC, UBE2V1	30	10	Cardiovascular system development and function, Cell morphology, Hematological system development and function

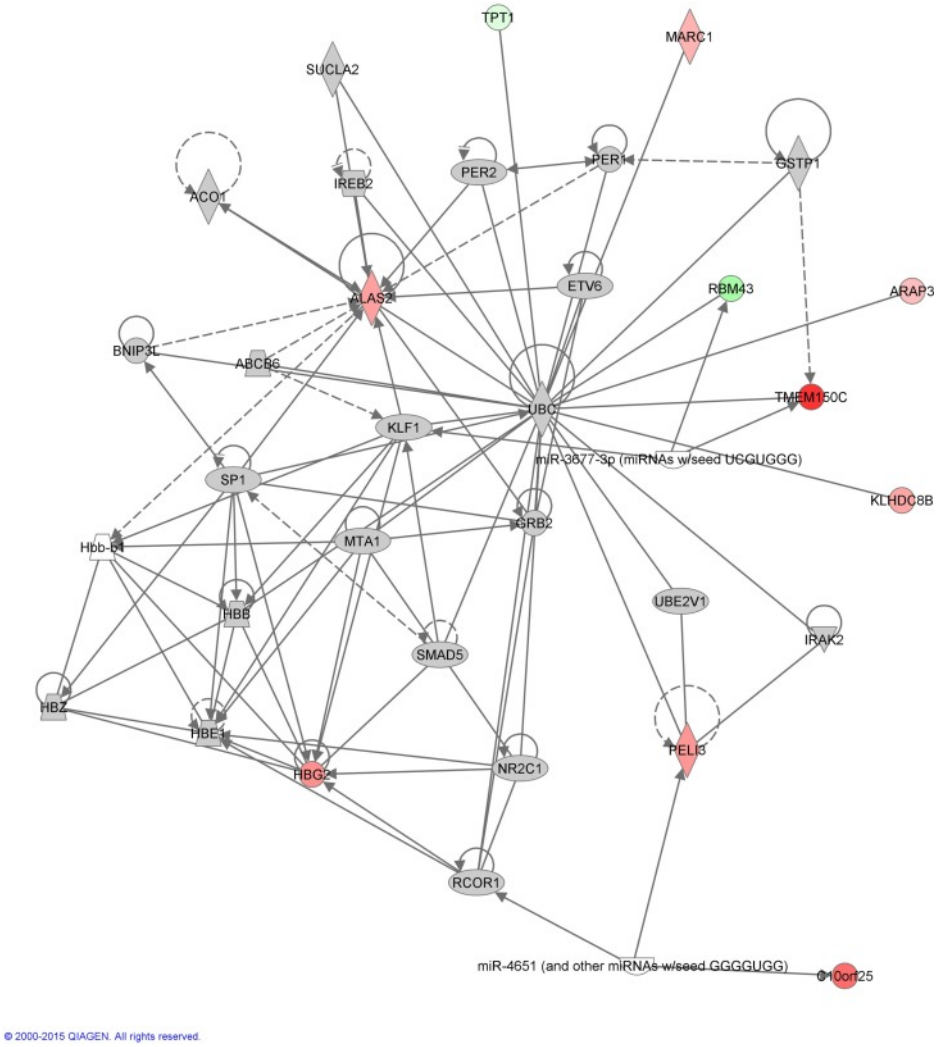

Figure 8. Annotation enrichment analysis.

A network including the gene functions "cardiovascular system development and function," "cell morphology," and "hematological system development and function" was significantly enriched in patients with epilepsy after initiation of treatment with valproic acid (score = –log(*p*-value) = 30). Red nodes designate upregulated genes. Numbers indicate the $\log_2$ fold change (0 is equal expression). Uncolored nodes are genes in this network that were on our list of differentially expressed genes.

6. DISCUSSION

6.1. Comparison of the metabolic syndrome risk in valproate-treated patients with epilepsy and the general population

For the first time, we demonstrated that the prevalence of MS in adult patients with epilepsy who received VPA treatment was 25.8%, which did not differ from the results of a population-based study (Eglit et al., 2012). Because subjects in both cohorts were drawn from the same population over the same extended time period, probably differences in genetic background and nutritional habits were minimal, which makes the results even more reliable.

The tendency of patients with epilepsy on long-term VPA treatment to be overweight is generally recognized in the medical community (Stephen et al., 2001). However, most studies examining weight gain and abdominal obesity during VPA treatment have been incident studies (Biton et al., 2003; Biton et al., 2001; Dinesen et al., 1984; Prabhakar et al., 2007; Privitera et al., 2003), that have used selected control subjects for comparison (only males or females) (de Vries et al., 2007; Isojarvi et al., 1996; Morrell et al., 2003; Prabhakar et al., 2007; Pylvanen et al., 2003) or have not used controls at all (Dinesen et al., 1984; El-Khatib et al., 2007; Prabhakar et al., 2007). Our results show that BMI is not higher in these patients than in the general population. Our study design is one of the main reasons for these results, as patients whose VPA treatment was discontinued due to side effects were not included in our cohort. This design likely resulted in negative selection bias against VPA-related side effects, such as weight gain. Nevertheless, the study reflects the true clinical practice of medical VPA usage and the management of VPA-related side effects in patients with epilepsy. Several studies reported that VPA-treated patients exhibited hyperinsulinemia and a tendency toward lower plasma glucose levels (reviewed by (Verrotti et al., 2009)), findings that were confirmed by our results. The observation of these changes in both obese and normal-weight patients suggests that hyperinsulinemia is the leading factor for weight gain and MS development during VPA treatment (Pylvanen et al., 2006a).

Lipid metabolism findings in VPA-treated patients have been inconsistent. Some studies have associated VPA treatment with decreased HDL-C (Pylvanen et al., 2006b) and increased TG (Luef et al., 2002a; Pylvanen et al., 2006b; Stephen et al., 2001) levels, whereas others have found no effect of VPA treatment on lipid metabolism (Fang et al., 2012; Pylvanen et al., 2003). One possible explanation is that VPA has no direct influence on TG or HDL-C metabolism, in contrast to its effect on insulin. Thus, the unfavorable changes in lipid levels probably occur during the development of MS, the prevalence of which was not increased among VPA-treated patients in our study. The diversity of previously reported data on changes in lipid levels during VPA treatment is probably related to differences in the subject selection methods. Specifically, the lipid

profiles are probably more unfavorable in cohorts with higher proportions of VPA-treated patients with MS.

Previous studies have paid little attention to the important side effects of prolonged VPA treatment, such as a tendency towards elevated BP (Fang et al., 2012; Pylvanen et al., 2006b). This characteristic can be explained by the increased serum insulin levels in VPA-treated patients, which can lead to elevated sympathetic activity (Gallagher et al., 2010). The increased proportion of VPA-treated patients with high BP, in the absence of differences in the prevalence of other MS components, suggests that the tendency towards hypertension is a direct effect of VPA treatment, probably due to hyperinsulinemia. Although MS did not occur more frequently in our VPA-treated patients, the risks of cardiovascular and cerebrovascular diseases may be increased, given than hypertension is the leading cause of these conditions.

The pathophysiology of MS remains unclear, but many of its features are associated with IR, which is typically defined as decreased sensitivity or responsiveness to the metabolic action of insulin (Chiang et al., 2011). The gold standard method for assessment of IR is the hyperinsulinemic-euglycemic clamp technique originally developed by DeFronzo et al. (DeFronzo et al., 1979). As this method is complex and unsuitable for epidemiological investigations, several surrogate indices, such as the HOMA-IR index, have been developed.

Previous studies using the HOMA-IR index have reported the presence of IR in patients receiving VPA treatment (Fang et al., 2012; Luef et al., 2002a; Pylvanen et al., 2003; Verrotti et al., 2011b; Verrotti et al., 2009). However, an increased HOMA-IR index in these patients most likely reflects hyperinsulinemia, which can occur in obese and normal-weight patients. Pylvänen et al. (Pylvanen et al., 2006a) first raised this issue and suggested that VPA interferes with insulin degradation in the liver, resulting in higher insulin concentrations in the peripheral circulation. In this case, an increased HOMA value probably does not reflect reduced sensitivity to insulin. Indeed, we found significantly higher HOMA indices in VPA-treated patients than in control subjects, although the prevalence of MS was similar in both cohorts. ROC analyses of the predictive abilities of BMI and HOMA-IR for MS suggested that HOMA-IR was an inferior predictor of MS in VPA-treated patients compared to the general population. These results allow us to speculate that previous studies applying the HOMA-IR index in VPA-treated patients have probably overestimated the occurrence of IR.

6.2. Comparison of risk factors for metabolic syndrome in epilepsy patients treated with valproate or carbamazepine

The primary aim of this part of study was to determine whether treatment with either of the two most frequently used AEDs is correlated with the risk of MS and its components in patients with epilepsy. We identified all patients from

selected southern Estonia counties who were prescribed with VPA or CBZ. The final sample included 213 persons with epilepsy treated with VPA or CBZ monotherapy. The risk of MS appeared to be similar in both groups. However, due to the limitations of the cross-sectional study design, we did not have data on body weight or MS prevalence before the start of AED treatment. Moreover, many patients had been diagnosed with epilepsy for decades, and we were unable to collect data from all patients concerning their previously used AEDs and AED dosages, because old medical documentation was missing. Therefore, whether treatment with VPA or CBZ directly influences the risk of MS could not be ascertained. Females on VPA treatment had a higher risk of MS compared to males, although this difference was not statistically significant. Similarly, Kim and Lee reported that women with epilepsy on VPA monotherapy more frequently suffer from MS compared to women treated with CBZ, LTG, or TPM (Kim and Lee, 2007). In our study, two MS components (increased waist circumference and decreased HDL-C concentration) were more frequently observed in VPA-treated females compared to males.

In the initial study population, a significantly higher number of CBZ- compared to VPA-treated patients had severe physical or mental disability due to brain damage and were excluded from the final sample. This difference between patients treated with different AEDs can be explained by the fact that CBZ is the first-line AED for focal seizures, which are frequently caused by focal brain damage of different etiologies (Schmidt and Schachter, 2014). Although the etiology of epilepsy differed between the treatment groups, we did not find a significant correlation between the risk of MS and epilepsy etiology.

A very consistent finding across different populations is that the prevalence of MS is highly age-dependent (Eckel et al., 2005). For example, in an Estonian population, the prevalence of MS was determined to be 19% in the 20- to 44-year-old age group compared to 45% in the 61- to 74-year-old age group (Eglit et al., 2012). In our sample, age was the only significant risk factor for MS in patients with epilepsy. This result is probably a reflection of the global trend.

Our study showed that the presence of MS was associated with higher VPA doses, with borderline statistical significance ($P = 0.075$), and was not associated with CBZ doses. Consistent with our result, Fang et al. reported a similar association between higher VPA doses and MS ($P = 0.049$) (Fang et al., 2012). Another study in children reported a significant correlation between the daily administered VPA dose and insulin resistance, which is one of the main signs of MS (Masuccio et al., 2010). A long duration of VPA therapy tended to increase the risk of MS, which is consistent with previous reports showing a positive correlation between VPA treatment duration and significant weight gain (Verrotti et al., 2011b).

We found no difference in BMI between VPA- and CBZ-treated subjects, which could be explained by the similar effect of both drugs on weight and by the prevalence design of our study. Patients whose VPA or CBZ treatment was discontinued due to side effects were not included in our cohort.

The real pathogenic mechanism underlying VPA-induced weight gain is unknown, although many authors believe that IR may play a central role. It is not clear, however, whether this IR is a result of weight gain, or whether VPA treatment itself can directly cause hyperinsulinemia and IR (Belcastro et al., 2013). Increasing evidence suggests that VPA treatment is associated with lower blood glucose concentration, a finding that is not consistent with the reduced insulin sensitivity hypothesis (Demir and Aysun, 2000; Pylvanen et al., 2006a; Terasmaa et al., 2011). In our study, patients on VPA treatment had lower fasting blood glucose concentrations, with borderline significance. These findings argue against the presence of increased IR among these patients. One possible mechanism to explain the weight gain in VPA-treated patients could be that the VPA-mediated decrease in glucose levels stimulates the appetite, leading to obesity and, probably, increased IR (El-Khatib et al., 2007; Farinelli et al., 2015).

Patients treated with CBZ had significantly higher HDL-C concentrations. This effect was especially pronounced in women. TG levels also tended to be higher in this patient group, although without reaching the level of statistical significance. The lipid-increasing effect of CBZ has been described in the medical literature, as recently reviewed by Katsiki et al. (Katsiki et al., 2014) and Vyas et al (Vyas et al., 2015). CBZ is a well-known enhancer of the hepatic cytochrome P450 enzyme system, which is extensively involved in the synthesis and metabolism of cholesterol. In contrast, VPA is an enzyme inhibitor and exerts the opposite action from CBZ (Nebert and Russell, 2002). CBZ monotherapy has been associated with increases in total cholesterol, low-density lipoprotein cholesterol, HDL-C, and TG concentrations (Katsiki et al., 2014; Luef et al., 2002a). Interestingly, the observed gender effect of CBZ on HDL-C was also reported by others (Sudhop et al., 1999).

Although the enzyme-inducing effect of CBZ undoubtedly causes dyslipidemia in patients treated with this drug, the increased concentrations of some lipid fractions, especially HDL-C, could potentially have a protective effect against MS. Indeed, in our study, the presence of a pathologically low plasma HDL-C concentration was significantly less frequent in CBZ-treated patients. This trend probably decreased the overall prevalence of MS in this group. However, in populations of patients who use lipid metabolism-modifying drugs, such as CBZ, the MS diagnostic criteria should be applied with caution, considering the direct effects of such drugs on these criteria.

6.3. The effect of acute intravenous valproate-treatment on the glucose, insulin and C-peptide metabolism

In the third part of the study, to evaluate the effect of VPA on glucose and insulin metabolism in naive patients with newly diagnosed epilepsy several parameters were measured. Our findings show, for the first time, that VPA administration appears to decrease glucose levels during OGTT directly following the initial VPA exposure. This is consistent with previous studies on

animal models, which have suggested that VPA may influence blood glucose concentration. Turnbull et al. showed that VPA causes a moderate fall in blood glucose levels following either oral or intraperitoneal administration in Wistar rats (Turnbull et al., 1985). Recently, Terasmaa et al. also showed that acute VPA administration lowers blood glucose levels in streptozocin-induced type 1 diabetic mice, in the absence of insulin (Terasmaa et al., 2011). In our study, decreased glucose levels during OGTT following VPA administration occurred without significant changes in insulin and C-peptide concentrations, consistent with the idea that an insulin-independent mechanism may be involved. Evidence for the central regulation of glucose homeostasis has been accumulating from both animal and human studies. Hypothalamic ATP-sensitive potassium (K_{ATP}) channels have been shown to play a significant role in the central regulation of glucose homeostasis; activation of K_{ATP} channels within the medial hypothalamus is sufficient to decrease blood glucose levels via the suppression of glucose production (Pocai et al., 2005). Recently, it has been shown in vitro, that exposure to VPA modulates the activity of K_{ATP} channels (Manaka et al., 2014). Further investigations are needed to explore the possible relationship between K_{ATP} channel activity and the metabolic changes that result from VPA treatment.

Another possibility is that VPA itself has a direct effect upon the control of endogenous glucose production by the central nervous system. VPA is a branched chain fatty acid and thus, with VPA administration, plasma levels of FFAs, which are key modulators of endogenous glucose production, could increase (Luef et al., 2002b). The hypothalamus has been shown to sense circulating levels of nutrients, which include fatty acids, and to accordingly regulate food intake and whole-body glucose handling (Schwartz et al., 2000). For example, intracerebroventricular infusion of the long-chain fatty acid, oleic acid, in rats caused rapid reductions in food intake and inhibits endogenous glucose production (Obici et al., 2002).

Investigators in the field have debated whether metabolic changes that occur during VPA treatment are directly caused by VPA, or are simply the consequence of weight gain, which is a frequent side effect of the drug (Belcastro et al., 2013). Our findings are consistent with previous reports (Luef et al., 2003; Pylvanen et al., 2006a) that support the first idea, namely, a direct impact of VPA upon glucose metabolism. In this case, decrease in blood glucose level that results from VPA treatment may stimulate appetite, thereby increasing food intake that results in weight gain. Correspondingly, in a study conducted by El-Khatib et al., VPA treatment was associated with a high frequency of carbohydrate cravings, especially in women with epilepsy, and this led to weight gain (El-Khatib et al., 2007).

VPA is a potent inhibitor of HDAC enzymes; VPA treatment promotes histone acetylation, leading to the relaxation of the chromatin and facilitating transcriptional activation. HDAC inhibitors were recently shown to be promising for the treatment of diabetes mellitus. Administration of these compounds can promote pancreatic β-cell development, proliferation, differentiation and

function, and ameliorate microvascular complications in later stages of the disease (Christensen et al., 2011). The acute moderate hypoglycemic effect of IV VPA that was observed in the current study is likely too rapid to be mediated via HDAC inhibition. This effect, however, could serve as motivation for future preclinical and clinical trials to test the utility of HDAC inhibitors, and especially VPA, as a potential novel therapy for diabetes. In fact, VPA has been used for years in diabetes patients to treat neuropathic pain. According to the guidelines for the treatment of diabetic peripheral neuropathy, developed by the American Academy of Neurology, VPA may be effective and should be considered for the treatment of diabetic polyneuropathy (Bril et al., 2011). The results of our study suggest that VPA could be used more widely in selected patients with diabetes.

6.4. The effect of chronic valproate-treatment on peripheral blood gene expression

In the last part of study, we examined VPA-induced changes in gene expression in the peripheral blood of patients with newly diagnosed epilepsy who were treated with therapeutic doses of the drug.

More than a decade ago, VPA was identified as a potent promotor of histone acetylation. Histone acetylation leads to relaxation of the nucleosome structure and transcriptional activation (Gottlicher et al., 2001). In theory, HDAC inhibition could directly or indirectly affect between 2% and 5% of all genes (Van Lint et al., 1996). In mouse embryonic stem cells exposed to VPA, the expression levels of 2.4% of genes were altered by more than 1.5 fold (Boudadi et al., 2013). This property of VPA is believed to have an impact on embryogenesis (Phiel et al., 2001), causing increased rates of major congenital malformations among children exposed to this drug *in utero* (Tomson and Battino, 2012). Only one study has compared the genomic expression patterns in peripheral blood between epilepsy patients treated with VPA or without any drug. Expression levels of 461 genes were altered in VPA-treated compared to drug-free epilepsy patients; however, no information was provided regarding the VPA doses that were used (Tang et al., 2004).

Although we did not measure VPA-induced HDAC inhibition, the finding that a surprisingly small number of genes were differently expressed before and after the start of VPA treatment suggests that HDAC inhibition in our patients was very low or even absent. *In vitro*, HDAC inhibition is achieved at concentrations of 0.3 to 1.0 mM, corresponding to the serum drug concentration in patients treated with a daily dose of 20 to 30 mg/kg (Gottlicher et al., 2001). According to the guidelines of epilepsy treatment in adults (Schmidt and Schachter, 2014), we initiated VPA treatment in our patients at a minimal effective dose, with the mean daily dose of 11.4 ± 2.8 mg/kg. The lack of the HDAC inhibition in our study is probably related to the relatively low daily VPA dose that was used. Indeed, the risk of major congenital malformations in patients treated with VPA increases with doses above 700 mg/d and is

pronounced with doses above 1500 mg/d (Tomson and Battino, 2012), which are clearly higher than the doses used in our study (600–1200 mg/d).

During the evaluation period of about 3 months, no patient reported any epileptic seizures. However, the small sample size and short evaluation period prohibit us from making conclusions about whether seizure freedom was related to the VPA treatment or to the natural course of disease. Most patients had a presumed genetic etiology for their epilepsy, which usually suggests a self-limiting course. The anticonvulsive effect of VPA is mediated mainly through the reduced degradation, increased synthesis, decreased turnover, and, therefore, reduced activity of gamma amino butyrate, and is probably not related to the alteration of gene expression (Chateauvieux et al., 2010). We could speculate that the anticonvulsive effect of VPA in our study was achieved without significant general activation of transcriptional activity.

After exposure to VPA, we observed changes in the expression levels of several genes (TPT1, ARAP3, and KLHDC8B) that are reported to be important in cancer-related pathways (Kobayashi et al., 2014; Krem et al., 2012; Yagi et al., 2011). Furthermore, the expression levels of two genes related to mitochondrial function, MOSC1 and ALAS2, were significantly changed. The product of MOSC1 localizes to the outer mitochondrial membrane and contributes to the regulation of nitric oxide synthesis (Klein et al., 2012). ALAS2 is an erythroid-specific, mitochondrially located enzyme that participates in heme biosynthetic pathways (Fujiwara and Harigae, 2013). Among the antiepileptic drugs, VPA has the highest potential to induce mitochondrial toxicity and could be even fatal in patients with some mitochondrial diseases. Use of VPA could be associated with the inhibition of respiratory chain complexes, impaired structural organization, and altered potential of the inner mitochondrial membrane (Nanau and Neuman, 2013; Pourahmad et al., 2012). Further investigations are needed to determine whether the differentially expressed genes identified in our study play roles in VPA-related mitochondrial toxicity.

6.5. Strengths and limitations of the study

Strengths of the two first parts of the study include the population-based design and the relatively large cohort size compared to other published studies investigating MS risk in patients with epilepsy.

The major limitation of the first part of the study was that patients with epilepsy on VPA treatment were compared to subjects without epilepsy. In people with epilepsy, a sedentary lifestyle and decreased level of physical activity due to epilepsy itself are related to the risk of obesity and metabolic disturbances (Ben-Menachem, 2007; Steinhoff et al., 1996). In addition, the comorbidity profile differs between patients with epilepsy and healthy subjects, as described above.

To overcome these limitations, only patients with epilepsy treated with different anticonvulsants were studied in the second part of this research.

Nevertheless, the heterogeneity of patients treated with VPA or CBZ was a major confounding factor in the second part. One of the most important determinants of MS, patient age, differed between the cohorts. VPA-treated patients were younger than CBZ-treated patients. VPA is used mainly for the treatment of generalized epilepsies, which usually manifest in children and young adults. For the same reason, the epilepsy etiology was different between the two groups, which could have an effect on MS risk. Moreover, our study may have underestimated the effect of the epileptic syndrome on MS risk. It was difficult to differentiate the potential effect of VPA from the effect of generalized epilepsy on MS risk, as almost no patients with generalized epilepsy were treated with CBZ. Physical activity level and diet, as important MS risk factors, were not assessed, constituting another limitation. Lack of information on patient weight and MS status before treatment, as well as partially missing data on previously used AEDs, prevented us from making conclusions about the causal relationships of the studied AEDs and MS. In addition, a substantial proportion of patients from the initial drug prescription database refused to participate or had incorrect or missing contact information. Considering these limitations, the results of this study should be interpreted carefully. Given that the study design focused on prevalence, the findings cannot be used to indicate whether VPA or CBZ causes weight gain. Nevertheless, we observed that patients with epilepsy who received VPA or CBZ treatment were not more overweight than other people in our population.

One of the limitations of the third part of the study was the relatively small sample size. However, the paired design of the study allowed us to minimize the influence of external factors and discover significant changes. Another limitation was that we used IV administration of relatively high doses of VPA, an approach that is not common in clinical practice. In addition, blood glucose levels were not measured after prolonged use of VPA. Consequently, no data are available regarding the long-term effects of VPA treatment on glucose metabolism. Future studies should include a wider range of ages, including children, and should be designed to evaluate the long-term effects of administering lower doses of VPA orally on glucose metabolism.

The relatively small sample size of patients was also a limitation of the fourth part of the study. The relatively low VPA dose received by study participants leaves open the possibility that the expressions of many genes were not influenced. On the other hand, the results probably reflect the gene expression profile in a real clinical practice, with relatively low doses of VPA. Another important limitation was the absence of relevant clinical problems in our patients, besides epilepsy. It was not possible for us to correlate changes in gene expression with the potential side effects of the drug.

6.6. Practical implications and future perspectives

The current study does not show increased general risk of MS in patients treated with VPA, CBZ, or control subjects. Due to sex differences, from a practical point of view, these results suggest that in females with a higher risk of cardiovascular diseases, AEDs other than VPA could be considered first. Treatment with either VPA or CBZ in males probably does not correlate with different MS risk in the future. The association of MS with higher VPA doses suggests that the lowest effective dose should be used, to minimize the possibility of MS in VPA-treated patients.

The general opinion in the medical community is that patients with epilepsy who receive long-term VPA treatment gain weight. However, due to the paucity of well-controlled prospective trials, the real incidence and magnitude of this problem are unknown (Verrotti et al., 2011b). Recently published meta-analyses have confirmed that, in general, overweight and obesity are associated with higher all-cause mortality (Global, 2016). The overall mortality rate among people with epilepsy already is increased due to epilepsy-related comorbidities and sudden unexpected death syndrome (Moshe et al., 2015). Since VPA is used by millions of people worldwide, double-blind, prospective, controlled clinical trials are needed to determine the real incidence of weight increase in these patients.

The direct hypoglycemic effect of VPA has remained largely unnoticed in the medical literature, but it could be one of the main molecular mechanisms of weight gain in VPA-treated patients. It would be interesting to examine whether VPA has long-term effects on glucose metabolism. For example, would a lower glucose concentration during the OGTT after acute IV administration of VPA lead to a higher risk of future weight gain? If so, then the results of OGTT could serve as a biomarker for predicting the risk of an important side effect in patients with newly diagnosed epilepsy in whom VPA treatment is planned. Which genetic factors are important for mediating the effects of VPA on glucose metabolism? Further preclinical and clinical studies are needed to investigate the exact mechanism of the hypoglycemic effect of VPA and to test the utility of HDAC inhibitors, especially VPA, as potential novel therapies for diabetes.

Regarding the part of the study examining VPA-induced gene expression, the results suggest that the lowest effective dose of VPA should be used, to avoid side effects of HDAC inhibition. Future studies should explore whether the use of higher VPA blood concentrations (e.g., VPA doses > 1500 mg/d) significantly changes the gene expression profile. In this case, due to ethical issues, a different study design should be used, probably comparing gene expression in independent samples, such as patients treated with high VPA doses compared to patients treated with other antiepileptic drugs or healthy subjects.

7. CONCLUSIONS

1. The prevalence of MS in adult patients with epilepsy who received VPA monotherapy was 25.8%, which was not different from the prevalence of MS in the Estonian population (OR = 1.00; 95% CI, 0.59–1.68). However, VPA-treated patients had higher serum insulin concentrations, independent of body mass index. This finding suggests that hyperinsulinemia is not a consequence of obesity, but probably a direct effect of VPA treatment. HOMA-IR had a lower predictive ability for MS in patients who received VPA treatment compared to the general population, probably suggesting that this index is not suitable to assess IR in VPA-treated patients.
2. The general risk of MS was similar between adult patients with epilepsy who were treated with VPA or CBZ monotherapy. However, the distribution of MS components in these patients was different. Patients on CBZ treatment less frequently had decreased HDL-C levels, whereas patients on VPA treatment less frequently had increased blood glucose levels. Females on VPA treatment could be at higher risk of MS than males.
3. VPA administration decreased glucose levels during OGTT directly following the initial VPA exposure in patients with newly diagnosed epilepsy. This effect occurred without significant changes in insulin and C-peptide concentrations, suggesting that an insulin-independent mechanism may be involved. Glucose-lowering effect of VPA may stimulate appetite, which could result in weight gain.
4. At low therapeutic dosages, VPA modulated the expression of a surprisingly small number of genes. Therefore, to avoid potential side effects of HDAC inhibition, epilepsy treatment with the lowest effective dose of VPA is recommended.

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9. SUMMARY IN ESTONIAN

Valproaadi metaboolsed mõjud epilepsiaga patsientidel akuutse ja kroonilise ravi puhul

Valproaat (VPA) on maailmas laialt kasutatav epileptiliste hoogude kontrollimiseks mõeldud preparaat, mida erinevatel hinnangutel iga päev kasutab rohkem kui üks miljon inimest (Farinelli et al., 2015; Perucca, 2002). Selle ravimi antikonvulsiivse toime avastas 1962. aastal prantsuse teadlane Pierre Eymard (Chateauvieux et al., 2010). Vaatamata sellele, et viimase 50 aasta jooksul on sünteesitud rohkem kui kümme uut antikonvulsanti, on VPA esmavaliku preparaadiks paljude epilepsia vormide puhul (Perucca, 2002). VPA-d kasutatakse ka bipolaarsele häirete ravis, diabeetilise polüneuropaatiaga kaasuva valu ravis, migreeni profülaktikas (Bril et al., 2011; Nanau and Neuman, 2013). Hiljuti hakati seda ravimit kasutama ka teatud pahaloomuliste kasvajate ravis (Weller et al., 2011). Kuna tegemist on pikaajalist ravi vajavate krooniliste haigustega, on väga oluline tagada kasutatavate preparaatide ohutus ja vastuvõetav kõrvaltoimete profiil kestval kasutamisel. VPA sagedaste kõrvaltoimete hulka kuuluvad posturaalne treemor, juuste väljalangemine, hüperammoneemia, gastrointestinaalsed ja hematoloogilised kõrvaltoimed, maksatoksilisus (Perucca, 2002). Antud ravim mõjutab ka embrüogeneesi, põhjustades kaasasündinud anomaaliaid ja käitumishäireid lastel, kelle emad kasutasid VPA-d raseduse ajal (Tomson and Battino, 2012). Kõige sagedasemaks VPA kõrvaltoimeks peetakse siiski kehakaalu tõusu. Selle probleemi levimus pole täpselt teada, erinevatel andmetel võib kehakaalu tõus esineda kuni pooltel VPA-d tarvitavatel haigetel. Ei ole ka täpselt teada VPA-st tingitud kaalutõusu molekulaarsed mehhanismid (Verrotti et al., 2009). Kehakaalu tõusuga võivad sageli kaasneda metaboolsed ja endokriinsed häired, mida hästi kirjeldab metaboolse sündroomi kontseptsioon (Reaven, 1988).

Metaboolne sündroom on riskifaktorite (rasvumine, glükoosiregulatsiooni häired, hüpertensioon ja düslipideemia) kogum, mille puhul esineb kõrgem risk haigestuda 2. tüüpi diabeeti ja südameveresoonkonna haigustesse (Eckel et al., 2005). Seoses istuva eluviisi ja antikonvulsantide kõrvaltoimetega võib epilepsiaga patsientidel olla kõrgem metaboolse sündroomi risk (Ben-Menachem, 2007; Steinhoff et al., 1996). Üsna vähe on tehtud uuringuid, mis kirjeldaksid metaboolse sündroomi levimust ja riskifaktoreid nendel patsientidel. Osa autoreid väidavad, et VPA-d tarvitavatel patsientidel on metaboolse sündroomi risk tõusnud, teised aga seda seost ei leia (Kim and Lee, 2007; Verrotti et al., 2010). Enamus nendest uuringutest on fokuseerunud spetsiifilistele populatsioonidele nagu lapsed või naised ja populatsiooni-põhiseid uuringuid sellel teemal ei ole läbi viidud.

Uuringu eesmärgid

1. Hinnata metaboolse sündroomi ja selle komponentide levimust VPA-d tarvitavatel epilepsiaga inimestel ja võrrelda seda üldpopulatsiooniga.
2. Võrrelda metaboolse sündroomi esinemissagedust ja riskitegureid VPA-d ja karbamasepiini (CBZ) tarvitavatel epilepsiaga patsientidel.
3. Iseloomustada boolusena veeni manustatava VPA mõju glükoosi, insuliini ja C-peptiidi vere kontsentratsioonile esmaselt diagnoositud epilepsiaga patsientidel vahetult pärast esmast ekspositsiooni.
4. Hinnata VPA toimet geeniekspressioonile perifeerses veres kolme kuu jooksul VPA ravi saanutel esmaselt diagnoositud epilepsiaga patsientidel.

Uuritavad ja meetodid

Uuringu kiitis heaks Tartu Ülikooli Inimuuringute Eetikakomitee ja kõik uuritavad allkirjastasid kirjaliku informeeritud nõusoleku uuringus osalemiseks. Uuringu esimese ja teise eesmärgi täitmiseks on identifitseeritud kõik patsiendid kuues Lõuna-Eesti maakonnas, kes ostsid apteegist VPA-d ning Tartu linna ja maakonna patsiendid, kes ostsid apteegist CBZ-d epilepsia näidustusel. Selleks kasutati Eesti Haigekassa väljakirjutatud retseptiravimite andmebaasi. Patsientidega võeti ühendust ja kutsuti uuringuvisiidile. Välistamiskriteeriumideks olid: vanus ≤ 18 aastat, VPA- või CBZ- monoteraapia kestusega < 3 kuud, polüteraapia teiste antikonvulsantidega, rasedus. Uuringusse ei haaratud patsiente, kellel oli seoses ajukahjustusega tõsine füüsiline või vaimne puue. Siia kuulusid kõik patsiendid, kes viibisid uuringu ajal hooldekodudes või vajasid kõrvalisikute abi oma igapäevastes toimingutes. Endokriinsete häiretega patsiendid ei olnud välistatud. VPA-d tarvitavate patsientide andmete kogumine toimus perioodil 01.01.2012-31.12.2012 ja CBZ-d tarvitavate patsientide andmete kogumine perioodil 01.12.2014–1.09.2015. Esialgses andmebaasis oli 384 (206 meest ja 178 naist) VPA-d tarvitavat ja 484 (292 meest ja 192 naist) CBZ-d tarvitavat patsienti. Pärast väljaarvamise kriteeriumide kasutamist on jäänud 118 (63 meest ja 55 naist) VPA-d tarvitavat ja 95 (55 meest ja 40 naist) CBZ-d tarvitavat patsienti. Uuring toimus Tartu Ülikooli Närvikliinikus. Kõik uuringusse arvatud patsiendid kutsuti visiidile. Iga patsiendi kohta täideti protokoll, mis sisaldas isikuandmeid, epilepsia ja muude haiguste anamneesi. Epileptilised sündroomid olid klassifitseeritud Rahvusvahelise Epilepsiavastase Liiga juhtnööride alusel (Berg et al., 2010). Fikseeriti vastava antikonvulsandi annus ja ravi kestus. Patsientidel mõõdeti vererõhk, vööümbermõõt, pikkus ja kaal ning määrati paastuplasmast glükoosi, HDL-kolesterooli, triglütseriidide, insuliini, C-peptiidi ja VPA või CBZ plasmakontsentratsioon.

Metaboolse sündroomi hindamiseks kasutati Rahvusvahelise Kolesterooli Õppeprogrammi Täiskasvanute III Ravipaneeli (NCEP ATP III) kriteeriume. Kontrollgruppi kuulusid isikud, kes osalesid Lõuna-Eesti rahvastikupõhises läbilõikeuuringus aastatel 2008–2009 (Eglit et al., 2012). Kontrollisikuid valiti juhuslikult nelja Lõuna-Eesti perearsti nimistutest ning kutsuti uuringust osa

võtma kirja teel, vastamismäär oli 53% (493 isikut). Kontrollgrupi struktuur vastas Eesti rahvastiku vanuselisele ja soolisele struktuurile.

Uuringu kolmanda eesmärgi täitmiseks pakuti patsientidele, kellel oli esmaselt diagnoositud epilepsia ja näidustatud antikonvulsantravi alustamine valproaadiga, saada esimene VPA annust intravenoosselt (n = 16). Selle uuringu osa protokoll eeldas kahte visiiti. Esimese visiidi ajal tehti patsientidele glükoositaluvuse proov. Plasma glükoosi-, insuliini- ja C-peptiidi sisaldust määrati enne testi algust ning 1 ja 2 tundi pärast 75 grammi glükoosilahuse joomist. Teise visiidi ajal, mis toimus mitte rohkem kui 7 päeva pärast, korrati glükoositaluvuse proovi, kuid vahetult enne glükoosilahuse joomist manustati veenisiseselt 900–1800 mg VPA (Orfiril, Desitin), mis oli lahustatud 0.9% NaCl 100 ml-s. Selle protseduuri ajal jälgiti pulssi, vererõhku ja punktsiooni koha ärrituse nähtusid.

Uuringu neljanda eesmärgi täitmiseks esmaselt diagnoositud epilepsiaga patsientidel on võetud vereanalüüs RNA eraldamiseks ja geenieksprессiooni analüüsimiseks enne ravi ja 3 kuud pärast VPA monoteraapia algust (n = 9). Keskmine VPA päevaannus oli 11.4 ± 2.8 mg/kg. Geenieksprессiooni erinevust hinnati Affymetrix GeneChip analüsaatoriga.

Peamised tulemused ja arutelu

Metaboolse sündroomi levimus VPA-d tarvitavatel epilepsiaga patsientidel

Metaboolse sündroomi kaalutud levimus VPA-monoteraapial olevatel patsientidel on 25.8% (95% CI, 18.4–34.8%), mis oluliselt ei erine selle sündroomi levimusest Eesti üldpopulatsioonis 27.9% (95% CI, 24.0–32.1%). Logistilise regressiooni analüüs samuti näitas, et metaboolse sündroomi esinemise riskisuhe VPA-d tarvitavatel patsientidel ja kontrollisikutel on 1.00; 95% CI, 0.59–1.68; P = 0.998. Ka kehamassi indeksi näitajad ei erinenud oluliselt kahe kohordi vahel. Samas, sõltumata kehamassi indeksist, oli patsientidel, kes tarvitasid VPA-d, kõrgem paastu insuliini kontsentratsioon veres. Tulemus viitab sellele, et hüperinsulineemia VPA-d tarvitavatel patsientidel ei ole kehakaalu tõusu tagajärg, vaid hoopis üks selle põhjustest. Võrreldes kontrollisikutega, oli HOMA-IR indeksil madalam ennustatav väärtus metaboolse sündroomi esinemise prognoosimisel VPA-d tarvitavatel patsientidel. Tõenäoliselt tähendab see, et antud indeks ei kajasta korrektselt insuliini resistentsuse esinemist nendel patsientidel, kuna VPA mõjutab otseselt insuliini metabolismi.

Metaboolse sündroomi esinemise ja selle riskifaktorite võrdlus VPA-d ja CBZ-d tarvitavatel epilepsiaga patsientidel

Üks faktoritest, mis oli positiivselt seotud metaboolse sündroomi esinemise riskiga VPA-d või CBZ-d tarvitavatel epilepsiaga patsientidel, oli vanus (OR = 1.03; 95% CI, 1.01–1.06; *P* = 0.007). See kajastab ilmselt paljude populatsioonide üldist trendi – kõrgema vanusega metaboolse sündroomi risk tõuseb. Pikem VPA kasutamine soodustas samuti metaboolse sündroomi riski (OR = 1.01; 95% CI, 1.0–1.02; P < 0.01). Ka VPA annus oli tendentsina seotud meta-

boolse sündroomi esinemise riskiga, piiripealse statistilise olulisusega (OR = 1.11; 95% CI, 0.99 – 1.24; P = 0.075). Kuigi üldine metaboolse sündroomi esinemise risk VPA-d ja CBZ-d tarvitavatel patsientidel oli sarnane, oli selle sündroomi üksikute parameetrite jaotus erinev: VPA-d tarvitavatel patsientidel esines sagedamini madal HDL-C ja harvemini kõrge glükoosi kontsentratsioon veres. VPA-d tarvitavatel naistel esinesid sagedamini kaks metaboolse sündroomi komponenti: suurenenud vöö ümbermõõt ja langenud HDL-C kontsentratsioon veres. Seega, antikonvulsant-ravi planeerimisel esmaselt diagnoositud epilepsiaga patsientidel tuleb soovitada metaboolsete kõrvaltoimete tõenäosuse vähendamiseks minimaalset toimivat VPA annust. Naistel, kellel juba enne ravi algust esinesid metaboolsed häired, ei ole VPA kasutamine ekvivalentse alternatiivi olemasolul soovitav.

Akuutse veenisisese VPA manustamise efekt glükoosi, insuliini ja C-peptiidi metabolismile

Veenisisese VPA manustamine oli üldiselt hästi talutav. Pärast veenisisese VPA manustamist, glükoositaluvuse testi 120 minutil, langes patsientidel plasma glükoosi kontsentratsioon. Samas, insuliini ja C-peptiidi plasma kontsentratsioonid oluliselt ei muutunud. Akuutse veenisisese VPA manustamise glükoosi langetavat efekti inimestel tuvastasime esmakordselt, varem seda kirjeldatud ei ole. Plasma glükoosisisalduse languse täpne molekulaarne mehhanism ei ole hetkel selge. Samas võib VPA otsene hüpogükeemiline toime ja sellega kaasnev võimalik söögiisu suurenemine seletada kaalu muutusi VPA-d tarvitavatel patsientidel. Töö tulemusena tekkis küsimus, kas on võimalik kasutada VPA-d diabeedi ravis arvestades selle ravimi glükoosi langetavat efekti.

Kroonilise VPA-ravi efekt geenikspressioonile perifeerses veres

Analüüsitud geenidest (23099) leiti ainult 11 geeni ekspressiooni muutus (võrdlus enne uuringu algust ja 3 kuud hiljem). Analüütilist tarkvara (Ingenuity Pathway Analysis) kasutades, leiti, et aktiveeritud olid südame-veresoonkonna ja hematoloogilise süsteemi arengu ja funktsiooniga ning rakude morfoloogiaga seotud geneetilised rajad. Kuigi loomkatsetes on näidatud, et VPA võib mõjutada kuni 5% geenide ekspressiooni, on madal aktivatsiooni tase meie uuringus ilmselt seotud suhteliselt madalate kasutatud VPA annustega (600–1200 mg päevas). VPA tugevat teratogeenset efekti seostatakse histoonide deatsetülaasi inhibeeriva toimega, mille tõttu võib paljude loote arengus osalevate geenide transkriptsioon olla häiritud. Antud töö tulemused lubavad väita, et selle kõrvaltoime tõenäosuse vähendamiseks on soovitav kasutada võimalikult väikest VPA-annust.

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