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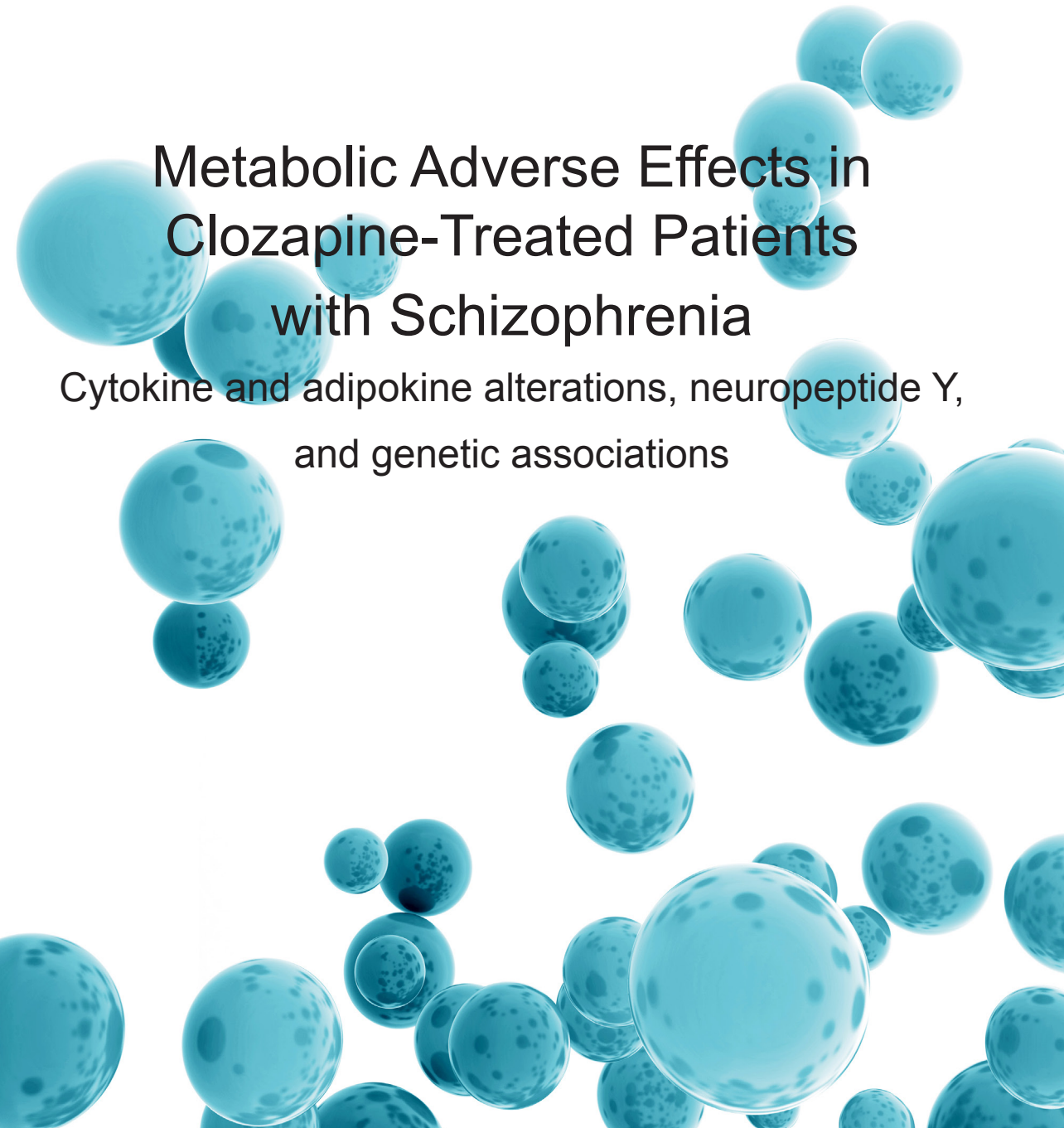
Metabolic Adverse Effects in Clozapine-Treated Patients with Schizophrenia

Cytokine and adipokine alterations, neuropeptide Y, and genetic associations



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ACADEMIC DISSERTATION
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UNIVERSITY OF TAMPERE

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To Tuula-Mari, Jaakko, Emma and Eerika

Abstract

Objective: Patients with schizophrenia are characterized by high prevalence of obesity, smoking, substance use, cardiovascular disease, and suicidality with an approximately twice the standardized mortality ratio. Cardiovascular disease is a major cause of excess deaths in this patient group. Weight gain is a common side effect of many atypical antipsychotics, with metabolic consequences and comorbidity, social stigmatization and nonadherence. Clozapine is an atypical antipsychotic drug, with unique effects on treatment resistant schizophrenia. Clozapine has been consistently associated with weight gain and a high prevalence of metabolic syndrome. The mechanisms behind obesity and metabolic comorbidity in patients with schizophrenia are not fully understood, whether they are associated with medication, common genetic background, or schizophrenia-related defect in satiety regulation. There is a need for biomarkers for antipsychotic-induced weight gain, sufficiently sensitive and specific to account for metabolic syndrome and disturbed food intake behaviors. Both schizophrenia, obesity and metabolic syndrome are all associated with a chronic low-grade inflammatory state with abnormalities in several inflammatory cytokines and adipokines. The results so far from studies on cytokine and adipokine alterations and their associations with obesity or metabolic syndrome and schizophrenia are inconsistent, with substantial heterogeneity between populations and medications. Likewise, in terms of genetic association studies, the study samples have been limited, heterogeneous and have yielded inconclusive results.

In this study markers of obesity and metabolic comorbidity, inflammatory markers and genetic associations were investigated in a sample of patients with treatment resistant schizophrenia on clozapine treatment.

Materials and methods: 190 patients with schizophrenia on clozapine treatment completed a questionnaire eliciting, among others, estimated weight and height, trend in weight change (marked increase, slight increase, no change, decrease), weight gain in kilograms during clozapine treatment, and smoking. Information on past medical and psychiatric history and duration of clozapine treatment was collected from patient records. Blood samples were taken for the

analysis of serum clozapine concentration, glucose, insulin, HDL-cholesterol, triglycerides, cytokines, and adipokines (IL-6, IL-1Ra, TNF-α, hs-CRP, leptin, adiponectin, resistin, adipsin), and neuropeptide Y, and for the genetic association study of SNPs *LEP* rs7799039, *ADIPOQ* rs1501299, *HTR2C* rs1414334, and *NPY* and arcuate nucleus NPY neuron receptor genes (21 genes and 215 SNPs). Two historical control samples (n=903, n=502) were available for reference of clinical markers and a sample of blood donors (n=395) for the genotype analysis of *LEP*, *ADIPOQ* and *HTR2C* genes.

Results: The presence of metabolic risk factors and morbidity was substantial, including overweight/obesity, smoking, hypertriglyceridemia, low HDL-cholesterol, high HOMA-IR, low adiponectin levels, elevated hs-CRP levels and elevated IL-1Ra levels. As expected, gender differences were found in many of the cytokines and adipokines studied. Weight gain during clozapine treatment explained the obesity of the present patients. Women with weight gain had the highest levels of leptin. Weight gain among male patients was associated with low adiponectin levels. Elevated IL-1Ra was a sensitive marker of metabolic comorbidity in this patient population. Among female patients high IL-6 levels were associated with obesity, and with levels of leptin. Adipsin levels were linked to levels of leptin in both genders. Levels of resistin were associated with levels of IL-1Ra, and trend-like with hs-CRP and TNF-α, and with low levels of HDL-cholesterol in male patients. Levels of NPY were also associated with resistin. The levels of resistin were higher among smokers than non-smokers, and correlated with IL-1Ra and hs-CRP among smokers.

Conclusion: There are cytokine and adipokine alterations in patients with treatment resistant schizophrenia on clozapine treatment. Some of these were gender dependent and related to risk of metabolic comorbidity. Levels of leptin, weight gain during clozapine treatment, and inflammatory markers related to metabolic comorbidity (IL-6, IL-1Ra) showed a marked interaction, especially among female patients. In male patients low adiponectin level was a more specific marker of metabolic comorbidity and clozapine-induced weight gain. As a biomarker of systemic inflammation resistin may have a role as a marker of cardiovascular comorbidity, especially among male patients. The results of the present study do not support a major role of genetic polymorphisms *LEP* rs7799039, *ADIPOQ* rs1501299 and *HTR2C* rs1414334 in the regulation of serum leptin and adiponectin levels or weight gain. Serum NPY level does not seem to be a potential biomarker for antipsychotic-induced weight gain.

Moreover, in genes encoding arcuate nucleus NPY neuron receptors serum NPY level alterations were not associated with the polymorphisms studied.

In clinical practice, excess cardiovascular morbidity and mortality associated with schizophrenia are a concern. Further effort should be invested in the prevention and treatment of metabolic abnormalities and smoking cessation among these patients.

Tiivistelmä

Tavoitteet: Ylipaino, tupakointi, päihdekäyttö, sydän- ja verisuonisairaudet sekä itsemurhavaara ovat tavanomaista yleisempiä skitsofreniaa sairastavilla henkilöillä, mukaan lukien noin kaksinkertainen ikävakioitu kuolleisuus. Sydän- ja verisuonisairaudet ovat keskeinen ylikuolleisuuden aiheuttaja tässä potilasryhmässä. Painon nousu on usean ns. toisen polven, tai epätyypillisen, psykoosilääkkeen yleinen haittavaikutus, mikä johtaa aineenvaihdunnan häiriöihin, liitännäissairauksiin, sosiaaliseen leimautumiseen ja heikentyneeseen hoitomyöntyvyyteen. Klotsapiini on epätyypillinen psykoosilääke, jolla on erityinen asema hoitoresistentin skitsofrenian hoidossa. Toisaalta klotsapiiniin liittyy huomattavasti kohonnut riski painon nousuun ja metaboliseen oireyhtymään. Tarkat mekanismit skitsofreniaan liittyvän ylipainon ja metabolisen oheissairastavuuden taustalla ovat epäselviä. Ne saattavat liittyä sekä lääkehoitoon että yhteiseen geneettiseen taustaan näiden häiriöiden välillä, tai skitsofreniaan liittyvään häiriöön kylläisyyden säätelyssä. Kliinisessä työssä tarvittaisiin biomarkkereita, jotka tunnistaisivat psyykoosilääkehoitoon liittyvää painonnousualttiutta, riskiä metaboliseen oireyhtymään ja häiriöitä syömiskäyttäytymisessä. Sekä skitsofreniaan, että lihavuuteen ja metaboliseen oireyhtymään liittyy krooninen matala-asteinen inflammaatio ja poikkeavuuksia useissa inflammatorisissa sytokiineissa ja adipokiineissa. Toistaiseksi tutkimukset sytokiinien ja adipokiinien vaihtelusta ja assosioitumisesta skitsofreniaan ja lihavuuteen sekä metaboliseen oireyhtymään ovat olleet heterogeenisiä ja tulokset vakiintumattomia. Samoin geneettisten assosiaatiotutkimusten otokset ovat olleet kooltaan rajallisia, aineistoiltaan heterogeenisiä ja tuloksiltaan vaihtelevia. Tässä tutkimuksessa tarkasteltiin hoitoresistenttiä skitsofreniaa sairastavien klotsapiinihoitoa käyttävien potilaiden obesiteettiin ja metaboliseen oheissairastavuuteen liittyvien kliinisten mittarien ja näihin yhteydessä olevien inflammatoristen biomarkkerien sekä geneettisten assosiaatioden yhteyttä.

Aineisto ja menetelmät: 190:stä skitsofreniaa sairastavasta klotsapiinilääkehoitoa käyttävästä potilaasta on täytetty tutkimuslomake, johon on kerätty tiedot mm. pituudesta ja painosta, arvioidusta painon muutoksesta

(merkittävä nousu, lievä nousu, ei muutosta, painon lasku), arvioidusta painon noususta kilogrammoina klotsapiinihoidon aikana, sekä tiedot tupakoinnista. Tiedot aiemmasta hoitohistoriasta ja klotsapiinihoidon kestosta on kerätty sairauskertomusasiakirjoista. Potilailta on otettu verinäytteet, joista on määritetty seerumin klotsapiinipitoisuus, sekä seerumin glukoosi, insuliini, HDL-kolesteroli, triglyseridit, sekä sytokiini- ja adipokiini- (IL-6, IL-1Ra, TNF-α, hs-CRP, leptiini, adiponektiini, resistiini, adipsiini) pitoisuudet ja neuropeptidi Y-pitoisuus. Verinäytteistä on tehty lisäksi genotyyppimääritykset assosiaatiotutkimuksia varten SNP:stä LEP rs7799039, ADIPOQ rs1501299, HTR2C rs1414334, sekä NPY- ja NPY-reseptorigeeneistä ja nucleus arcuatuksen NPY neuronien reseptoreita koodaavista geeneistä (21 geeniä ja 215 SNP:ä). Vertailuaineistona oli käytettävissä kliinisten markkerien osalta kaksi (n=903, n=502) historiallista kontrolliaineistoa ja LEP, ADIPOQ ja HTR2C genotyyppien osalta verenluovuttajien aineisto (n=395).

Tulokset: Metabolisia riskitekijöitä esiintyi tutkimusryhmässä runsaasti, mukaan lukien ylipaino/lihavuus, tupakointi, korkea triglyseriditaso ja matala HDL-kolesterolitaso, korkea insuliiniresistenssi-indeksi (HOMA-IR), matala adiponektiinitaso, sekä kohonneet hs-CRP- ja IL-1Ra tasot. Odotetusti usean sytokiinin ja adipokiinin tasoissa esiintyi sukupuolittaista vaihtelua. Klotsapiinihoidon aikainen painonnousu selitti potilaiden ylipainoa. Korkeimmat leptiinitasot olivat naisilla, joiden paino noussut. Sitä vastoin matala adiponektiinitaso liittyi miesten painonnousuun. Korkea IL-1Ra oli herkkä metabolisen oheissairastavuuden markkeri tässä potilasjoukossa. Naispotilailla IL-6 taso oli yhteydessä obesiteettiin ja leptiinipitoisuuteen. Adipsiinipitoisuudella oli yhteys leptiinipitoisuuteen molemmilla sukupuolilla. Resistiinipitoisuus oli yhteydessä IL-1Ra pitoisuuteen sekä viitteellisesti myös hs-CRP- ja TNFα-pitoisuuteen, ja miehillä matalaan HDL-kolesterolitasoon. NPY-pitoisuus oli myös yhteydessä resistiinipitoisuuden kanssa. Resistiinipitoisuudet olivat korkeammat tupakoitsijoilla, kuin tupakoimattomilla. Tupakoitsijoilla resistiinipitoisuus oli yhteydessä IL-1Ra- ja hs-CRP-pitoisuuksien kanssa.

Johtopäätökset: Hoitoresistenttiä skitsofreniaa sairastavilla klotsapiinia käyttävillä potilailla esiintyy sytokiinien ja adipokiinien vaihtelua. Tämä vaihtelu on osittain sukupuolittaista ja yhteydessä metaboliseen oheissairastavuuteen. Seerumin leptiinipitoisuus, painon nousu klotsapiinihoidon aikana, ja metaboliseen oheissairastavuuteen liittyvät inflammatoriset markkerit (IL-6, IL-1Ra) olivat keskenään merkittävässä

yhteydessä erityisesti naispotilailla. Matala adiponektiinitaso oli spesifisempi metabolisen oheissairastavuuden ja klotsapiiniin liittyvän painon nousun markkeri miespotilailla. Resistiinillä yleisen systeemisen inflammation markkerina saattaa olla merkitystä sydän- ja verisuonisairastavuuden riskin osoittajana erityisesti miespotilailla. Ajankohtaisen tutkimuksen tulokset eivät tue geneettisten vaihteluiden LEP rs7799039, ADIPOQ rs1501299 ja HTR2C rs1414334 osuutta seerumin leptiini- ja adiponektiinipitoisuuksien tai psykoosilääkehoitoon liittyvän painon nousun säätelyssä. Myöskään seerumin NPY-pitoisuus ei vaikuttaisi olevan käyttökelpoinen psykoosilääkehoidon painovaikutuksen osoittaja. Seerumin NPY-pitoisuuden vaihtelu ei näytä liittyvän tiettyjen nucleus arcuatuksen NPY neuronien reseptoreita koodaavien geenien SNP:en muunteluun.

Sydän- ja verisuonisairauksiin liittyvä oheissairastavuus ja ylikuolleisuus ovat erityinen huolen aihe skitsofreniapotilaiden hoidossa. Hoidollisia voimavaroja ja toimenpiteitä tulee painokkaasti kohdentaa metabolisten poikkeavuuksien ehkäisyyn ja hoitoon sekä tupakoinnin lopettamiseen.

Contents

List of original studies

The present thesis is based on the following original studies, referred to in the text by the Roman numerals I-IV.

I Klemettilä, J.-P., Kampman, O., Seppälä, N., Viikki, M., Hämäläinen, M., Moilanen, E., Leinonen, E., 2014. Cytokine and adipokine alterations in patients with schizophrenia treated with clozapine. Psychiatry Res 218(3):277-283.

II Klemettilä, J.-P., Kampman, O., Seppälä, N., Viikki, M., Hämäläinen, M., Moilanen, E., Mononen, N., Lehtimäki, T., Leinonen, E., 2015. Association study of the HTR2C, leptin and adiponectin genes and serum marker analyses in clozapine treated long-term patients with schizophrenia. Eur Psychiatry 30(2):296-302.

III Klemettilä, J.-P., Kampman, O., Seppälä, N., Viikki, M., Hämäläinen, M., Moilanen, E., Leinonen, E., 2016. Resistin as an inflammatory marker in patients with schizophrenia treated with clozapine. Nord J Psychiatry (Epub ahead of print).

IV Klemettilä, J.-P., Kampman, O., Solismaa, A., Lyytikäinen, L.-P., Seppälä, N., Viikki, M., Hämäläinen, M., Moilanen, E., Mononen, N., Lehtimäki, T., Leinonen, E., 2017. Association study of arcuate nucleus neuropeptide Y neuron receptor gene variation and serum NPY levels in clozapine treated patients with schizophrenia. Eur Psychiatry 40:13-19 (Epub ahead of print).

Abbreviations

5-HT	5-hydroxytryptamine (serotonin)
5-HT_{1A}	5-hydroxytryptamine (serotonin) 1A receptor
5-HT_{1B}	5-hydroxytryptamine (serotonin) 1B receptor
5-HT_{2A}	5-hydroxytryptamine (serotonin) 2A receptor
5-HT_{2C}	5-hydroxytryptamine (serotonin) 2C receptor
5-HT_{6}	5-hydroxytryptamine (serotonin) 6 receptor
5-HT_{7}	5-hydroxytryptamine (serotonin) 7 receptor
ADIPOQ	adiponectin gene
AdipoR1	adiponectin receptor 1
AdipoR2	adiponectin receptor 2
ADIPOR1	adiponectin receptor 1 gene
ADIPOR2	adiponectin receptor 2 gene
ADRA2A	adrenoreceptor alpha 2A gene
AIC	Akaike information criteria
AIWG	antipsychotic-induced weight gain
ANOVA	analysis of variance
ANCOVA	analysis of covariance
ARC	arcuate nucleus
ASTN2	astrotactin 2 gene
ATF7IP2	activating transcription factor 7 interacting protein 2 gene
BDNF	brain-derived neurotrophic factor
BDNF	brain-derived neurotrophic factor gene
BMI	body mass index
CNR1	cannabinoid receptor 1 gene
CATIE	Clinical Antipsychotic Trials of Intervention Effectiveness
CBT	cognitive behavioral therapy
CCKBR	cholecystokinin B receptor gene
CRHR1	corticotropin-releasing factor receptor 1gene

CHRNA4	α4β2 nicotinic acetylcholine receptor α4 subcomponent gene
CHRNA7	α7 nicotinic acetylcholine receptor gene
CHRNB2	α4β2 nicotinic acetylcholine receptor β2 subcomponent gene
COPD	chronic obstructive pulmonary disease
COX2	cyclooxygenase-2
Cpz	chlorpromazine
CRP	C-reactive protein
CSF	cerebrospinal fluid
DNA	deoxyribonucleic acid
DRD2	dopamine receptor 2 gene
DRD3	dopamine receptor 3 gene
DSM	The Diagnostic and Statistical Manual of Mental Disorders
ECG	electrocardiogram
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme linked immunosorbent assay
EPS	extrapyramidal side effects
FDR	false discovery rate
GABA	gamma-amino-butyric-acid
GCG	glucagon-like peptide-1 gene
GHRL	ghrelin gene
GHSR	ghrelin receptor gene
GLM	general linear univariate model
GLP-1	glucagon-like peptide-1
GLP1R	glucagon-like peptide-1 receptor gene
GNB3	G-protein receptor β3-subunit gene
GR	glucocorticoid receptor
GRIN1	N-methyl-D-aspartate receptor 1 (NMDAR1) gene
GRS	genetic risk score
H_1	histamine 1 receptor
HCRTR1	hypocretin receptor 1 gene
Hcy	homocysteine
HDL	high-density lipoprotein

HOMA-IR	Insulin resistance index assessed by homeostasis model assessment
HSV-1	herpes simplex virus-1
hs-CRP	high-sensitivity C-reactive protein
HTR1B	5-hydroxytryptamine (serotonin) 1B receptor gene
HTR2A	5-hydroxytryptamine (serotonin) 2A receptor gene
HTR2C	5-hydroxytryptamine (serotonin) 2C receptor gene
HTR3A	5-hydroxytryptamine (serotonin) 3A receptor gene
ICD-10	The International Statistical Classification of Diseases and Related Health Problems, Tenth Revision
ID	personal identity number
IFN-γ	interferon-gamma
IL-1	interleukin-1
IL-1β	interleukin-1 beta
IL-1B	interleukin-1 beta gene
IL-1Ra	interleukin-1 receptor antagonist
IL-2	interleukin-2
IL-6	interleukin-6
IL-8	interleukin-8
IL-10	interleukin-10
IL-12	interleukin-12
IL-15	interleukin-15
IL-16	interleukin-16
IL-17	interleukin-17
IL-18	interleukin-18
IL-25	interleukin-25
iNOS	nitric oxide synthase
INSIG2	insulin-induced gene 2
INSR	insulin receptor gene
IQ	intelligence quotient
IQR	interquartile range
LD	linkage disequilibrium
LDL	low-density lipoprotein
LEP	leptin gene
LEPR	leptin receptor gene

MC3R	melanocortin 3 receptor
MC3R	melanocortin 3 receptor gene
MC4R	melanocortin 4 recepto
MC4R	melanocortin 4 receptor gene
MDS	multidimensional scaling
MEIS2	Meis homebox 2 gene
MetS	metabolic syndrome
MHC	major histocompatibility complex
MIF	migration inhibitory factor
mRNA	messenger ribonucleic acid
MTHFR	methylenetetrahydrofolate reductase gene
NCEP: ATPIII	the National Cholesterol Education Program Adult Treatment Panel III
NDMC	N-desmethylclozapine
NFκB	nuclear factor kappa B
NHANES	National Health and Examination Survey
NMDAr	N-methyl-D-asparate receptor
NMDAR1	N-methyl-D-aspartate receptor 1
NPY	neuropeptide Y
NPY	neuropeptide Y gene
NPY1R	neuropeptide Y receptor 1
NPY2R	neuropeptide Y receptor 2
NPY5R	neuropeptide Y receptor 5
NR3C1	glucocorticoid receptor (GR) gene
OX1R	orexin receptor 1 gene
PANSS	Positive and Negative Syndrome Scale
PET	positron emission tomography
PMBC	peripheral mononuclear blood cells
PMC	PubMed Central®
PP	pancreatic polypeptide
PPARG	peroxisome proliferator-activated receptor gamma gene
PPARγ	peroxisome proliferator-activated receptor gamma
PYY	peptide tyrosine-tyrosine
RBP4	retinol binding protein 4
RNF144A	ring finger protein 144A gene

rs	reference SNP
rTMS	repetitive transcranial magnetic stimulation
sCD40L	soluble CD40 ligand
SD	standard deviation
SERMs	selective estrogen receptor modulators
sIL-2R	soluble interleukin-2 receptor
SLC2A2	glucose transporter 2 (GLUT2) gene
SNAP25	synaptosomal-associated protein 25kDA gene
SNP	single-nucleotide polymorphism
SOX5	sex determining region Y-box 5
sTNF-R1	soluble tumor necrosis factor receptor 1
TGF-β	transforming growth factor-beta
TNF-α	tumor necrosis factor-alpha
Trigly	Triglycerides
VIF	variance inflation factor
VNTRs	variable number tandem repeats
WHO	World Health Organization

1 Introduction

Schizophrenia is a neuropsychiatric disorder with substantial heterogeneity in etiology, symptoms, course and outcome. The disorder is generally diagnosed according to positive psychotic symptoms, usually associated with impaired social functioning. Other characteristic features are negative symptoms and cognitive impairment. Functional decline can often be seen years before the onset of psychotic symptoms in adolescence or early adulthood. Patients with schizophrenia are characterized by higher prevalence of obesity, smoking, substance use, cardiovascular disease, and suicidality with approximate doubling of the standardized mortality ratio (Tandon et al., 2009). Cardiovascular disease is a major cause of excess deaths in this patient group (Osby et al., 2000; Kilbourne et al., 2009; Talaslahti et al., 2012).

Weight gain is a common side effect of atypical antipsychotics, which leads to metabolic consequences and comorbidity, social stigmatization, and nonadherence (Newcomer, 2005). Metabolic abnormalities are already apparent in first-episode patients, and become even more common as the disorder progresses (De Hert et al., 2006; Beary et al., 2012). A recent meta-analysis reported an overall 32.5% prevalence of metabolic syndrome among patients with schizophrenia (Mitchell et al., 2013). In a Finnish general population survey the prevalence of metabolic syndrome was 36.2% among subjects with schizophrenia and 30.1% among subjects without psychotic disorder (Suvisaari et al., 2007).

Clozapine is an atypical antipsychotic drug for treatment resistant schizophrenia. It has been shown to ameliorate positive symptoms in a large proportion of patients in this group, and to reduce the risk for suicide and decrease overall mortality (Kane et al., 1988; Tiihonen et al., 2009; Meltzer, 2013). On the other hand, clozapine is consistently associated with weight gain (Newcomer, 2005) and a high prevalence of metabolic syndrome (Mitchell et al., 2013). Moreover, there is some evidence that insulin homeostasis and lipid profiles in clozapine-treated obese schizophrenia patients are different from those with non-psychiatric obesity (Wu et al., 2008).

Although the weight gained varies markedly individually, there are no tools available to predict the personalized risk of weight gain associated with antipsychotic treatment (Shams and Müller, 2014). The mechanism of antipsychotic-induced weight gain is largely unclear, but the serotonin receptor 5-HT_{2C}- and histamine receptor H_1-blocking activity of antipsychotic agents has been associated with increased risk of metabolic side effects (Lett et al., 2012). Findings from twin and sibling studies and genetic association studies, especially concerning serotonin receptor *HTR2C* and leptin genes, suggest that genetic factors do indeed play a key role in predisposing to drug-induced weight gain (Lett et al., 2012). Both schizophrenia, obesity, and metabolic syndrome are all associated with a chronic low-grade inflammatory state with abnormalities in several inflammatory cytokines and adipokines (Potvin et al., 2008; Miller et al., 2011a; Harwood, 2012), with adipose tissue a major source of many of them (Raucci et al., 2013).

The results so far from studies on cytokine and adipokine alterations and their associations with obesity or metabolic syndrome and schizophrenia are inconsistent, with substantial heterogeneity between studies. Likewise, in terms of genetic association studies, the study samples have been limited and heterogeneous with inconclusive results. In this study markers of obesity, metabolic comorbidity, inflammatory markers, and genetic background were investigated in a sample of patients with treatment resistant schizophrenia on clozapine treatment.

2 Review of the Literature

2.1 Schizophrenia

2.1.1 Epidemiology of schizophrenia

Schizophrenia is characterized by diverse set of signs and symptoms including characteristic distortions of thinking and perception, cognitive impairments, motor abnormalities, avolition and apathy, difficulties in communication, and restricted affective expression. These abnormalities are generally classified into positive, negative, cognitive, disorganization, mood, and motor symptom dimensions, with psychopathology differentially expressed across patients and through the course of the illness (Tandon et al., 2009).

The incidence of schizophrenia is 15.2 per 100,000 persons per year, and according a systematic review by Saha et al. the median (10%, 90% quantiles) prevalence for point is 4.6 (1.9, 10.0) and for lifetime 4.0 (1.6, 12.1) per 1,000 persons (Saha et al., 2005). The median incidence rate ratio male:female is 1.4 (0.9, 2.4). The incidence rate and prevalence estimates are higher among migrants versus native-born people. The incidence is higher among urban than mixed urban-rural settings. Prevalence estimates are higher in developed countries than in less-developed economies (McGrath et al., 2008). In a Danish nationwide study the cumulative incidence (95% CI) for schizophrenia and related disorders at 50 years of age was males/females 3.06% (3.01-3.12)/ 2.43% (2.39-2.48) (Pedersen et al., 2014). In the Lundby Study in Sweden the period prevalences in a 50-year follow-up period were for non-affective psychosis 2.25% and for schizophrenia 1.43% and the life time prevalences were 1.38% and 0.84% respectively (Bogren et al., 2009). In a Finnish general population survey the lifetime prevalences were 0.87% for schizophrenia, 0.32% for schizoaffective disorder and 0.07% for schizophreniform disorder (Perälä et al., 2007).

In addition to the higher incidence rate among men there are also other gender differences in course and outcome of schizophrenia. The illness begins

on average four years earlier, in the early twenties in men, while in women there is a second incidence peak between 45 and 54 years of age. The illness outcome tends also to be more favorable in women than in men until age 45, when this second incidence peak among women may be asssociated with more severe psychopathology and poorer treatment response (Hayes et al., 2012).

2.1.2 Different aspects of etiology

Schizophrenia is associated with high heritability, estimated at 80% (Owen et al., 2016). According to a recent report by the Schizophrenia Working Group of the Psychiatric Genomics Consortium, at least 108 independent genomic loci are associated with schizophrenia susceptibility. Notable associations include genetic variance in dopamine D_2 receptor gene *DRD2*, genes involved in glutamatergic neurotransmission, synaptic function and plasticity, and genes encoding calcium channels and other neuronal ion channels, and genes associated with neurodevelopment. Independent of genes expressed in brain, associations were increased among genes expressed in tissues with immune functions, thereby strengthening the hypothesis of immune dysregulation in schizophrenia (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014), with the most significant associations found with variants in the major histocompatibility complex (MHC) region (Owen et al., 2016). The MHC region, which is located in chromosome 6, encodes HLA molecules and many other immune (such as TNF and complement) molecules, but some schizophrenia susceptibility associated nonimmune genes have also been found in the the MHC region (Debnath et al., 2013; Corvin and Morris, 2014). Recently variation in alleles at the complement component 4 (C4) genes has been identified to explain in part the association between MHC region and schizophrenia (Sekar et al., 2016).

The neurodevelopmental hypothesis of schizophrenia postulates, that the disorder emerges from abnormalities that occur during the pre- and postnatal development of the central nervous system. The heterogeneity of the disorder may be the result of differencies in etiology and gene- environment interactions existing in early brain development and during postnatal brain maturation (Notaras et al., 2015; Owen et al., 2016). Schizophrenia has been suggested to relate to at least three interacting pathophysiological mechanisms, dopaminergic

dysregulation, disturbed glutamatergic neurotransmission, and increased proinflammatory status of the brain (Kahn and Sommer, 2014).

The dopaminergic dysfunction is characterized by presynaptic increased dopamine synthesis and release in striatum, which is already seen in the prodromal state of the illness. N-methyl-D-aspartate receptor (NMDAr)/ glutamate system has been hypothesized to constitute a major deficit in schizophrenia. Dysfunction of NMDAr leads to less effective inhibition of glutamatergic neurons by gamma-amino-butyric-acid (GABA)-ergic interneurons. This leads to excessive firing of dopamine neurons in the mesolimbic pathway. NMDAr is also involved in synaptic plasticity, which is associated with the development of cognitive functions (Kahn and Sommer, 2014). It has been suggested that this glutamatergic dysfunction is related to dysfunction of NMDAr sensitive parvalbumin-positive interneurons in the cerebral cortex and hippocampus. Inflammatory processes and oxidative stress affect synaptic pruning, the balance between inhibitory and excitatory neurons and signal transmission (Owen et al., 2016).

In all, schizophrenia is associated with alterations in inflammatory markers, exposure to infection *in utero* and childhood, pregnancy and birth complications, structural brain changes, cognitive deficits, developmental motor delays, minor physical anomalies, neurological soft signs and sensory changes. Moreover, childhood adversities, immigration, urbanicity and premorbid cannabis use has been associated with the risk for schizophrenia (Matheson et al., 2014).

2.1.2.1 Prenatal inflammation

An inflammatory insult *in utero* can lead to an enhanced expression of proinflammatory cytokines, which interferes with neonatal white matter development during the second trimester of pregnancy. High maternal levels of TNF-α and interleukin-8 (IL-8) during pregnancy have been associated with increased risk of schizophrenia in offspring (Smyth and Lawrie, 2013). Various prenatal infections have been associated with schizophrenia susceptibility, e.g. herpes simplex virus (HSV-1), cytomegalovirus, influenza and toxoplasma gondii (Smyth and Lawrie, 2013; Réus et al., 2015). Maternal infection may have a negative impact on neurodevelopmental processes, which may lead to a thinning of the cortical areas, reduced gray and white matter and accelerated gray matter loss, predisposing to neuropsychological and neuropathological

deficits associated with schizophrenia (Smyth and Lawrie, 2013). It has been suggested that immune response through cytokines could mediate the effects of prenatal infection. Inflammatory activation leads to astrocyte atrophy, neuronal apoptosis and an increased gene expression of proinflammatory cytokines observed in patients with schizophrenia. Microglial activation and an increase in microglial cells in the brain of patients with schizophrenia have been reported in post-mortem studies (Réus et al., 2015). Moreover, elevated microglial activity measured with positron emission tomography (PET) imaging has been reported in both patients with schizophrenia and also among ultra-high-risk individuals as being associated with the severity of subclinical symptoms (Bloomfield et al., 2016). Activated microglia produce proinflammatory cytokines and high levels of glutamate. Increased proinflammatory status may also cause or worsen NMDAr hypoactivation. Inflammation has also been associated with dopamine dysregulation, while animal studies have shown increased mesolimbic dopamine neuron activation in offspring with prenatal inflammation (Kahn and Sommer, 2014). In a recent Finnish study elevated maternal CRP level was associated with schizophrenia in offspring (Canetta et al., 2014). However, recently no differences in inflammatory markers were found at the time of birth between those individuals who later develop schizophrenia and those who do not, although susceptible individuals may have deficient immune responses to chronic maternal infectious diseases (Gardner et al., 2013; Blomström et al., 2015; Nielsen et al., 2015).

2.1.2.2 Aspects of autoimmune etiology

An autoimmune etiology in schizophrenia has been supported according to findings of either higher or lower than expected prevalences of several autoimmune disorders in patients with schizophrenia. The most consistent of these is the negative association between schizophrenia and rheumatoid arthritis. A Danish national register-based study found higher prevalences of thyreotoxicosis, celiac disease, acquired hemolytic anemia, interstitial cystitis, and Sjögren's syndrome among both patients with schizophrenia prior to schizophrenia onset and in patients' parents, compared to matched controls (Eaton et al., 2006). An autoimmune etiology has also been suggested according to reports of NMDAr autoantibodies found in approximately 5-10% of patients with schizophrenia. Antibody subtypes have seemed to differ from those in NMDAr encephalitis (Smyth and Lawrie, 2013; Steiner et al., 2013). However,

in a recent large study with 1,325 healthy controls and 1,081 patients with schizophrenia, an overall 10.5% seroprevalence of NMDAr antibodies was detected in both patients and controls with a common genetic variant and influenza A or B infection as predisposing factors. Seropositive patients with history of neurotrauma or birth complications had more neurological abnormalities than seronegative patients with comparable history (Hammer et al., 2014).

2.1.2.3 Inflammation in the course of illness

According to two recent meta-analyses of cytokine alterations in schizophrenia, elevated levels of interleukin-1 receptor antagonist (IL-1Ra), soluble interleukin-2 receptor (sIL-2R) and interleukin-6 (IL-6) have been associated with schizophrenia (Potvin et al., 2008). It also seems, that alterations vary according to disease state. Interleukin-1β, IL-6 and transforming growth factor-beta (TGF-β) seem to be increased during the first-episode and acute relapse (no data on IL-1β in acute relapse) and to normalize with antipsychotic treatment. Instead, interleukin-12 (IL-12), interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α) and sIL-2R appear to remain elevated and sIL-2R even increases in acute exacerbations during antipsychotic treatment. IL-6 levels seem not to differ between controls and outpatients with stable medication, or patients with treatment resistant psychosis (Miller et al., 2011a). However, most of the studies included in the meta-analysis did not control for potential confounding factors such as BMI and smoking (Miller et al., 2011a).

Recent case-control studies have yielded more evidence of inflammatory responses in schizophrenia. Increased homocysteine (Hcy) and decreased prostaglandin 15-deoxy-PGJ_2 plasma levels, as well as increased expression of nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX2), and decreased expression of inhibitory subunit IκBα of nuclear factor kappa B (NFκB) and peroxisome proliferator activated receptor gamma (PPARγ) in peripheral mononuclear blood cells (PMBC) have been reported in recently diagnosed patients (Garcia-Bueno et al., 2013). Levels of IL-1Ra, IL-10 and IL-15 have been reported to be increased in antipsychotic-naïve first-episode patients of which IL-10 was associated with treatment response (De Witte et al., 2014). Increased serum levels of IL-1Ra, soluble tumor necrosis factor receptor 1 (sTNF-R1) and soluble CD40 ligand (sCD40L) have been reported to be negatively associated with cognitive abilities in both, schizophrenia and bipolar

disorder. Whether this inflammatory process is a cause or consequence of pathological mechanisms leading to cognitive impairment is uncertain (Hope et al., 2015). It is notable that of the large number of cytokines/chemokines, the studies are focused mainly on the most common ones which may bias the results.

In order to identify molecular subclasses of schizophrenia Schwarz et al. analyzed 23 immune molecules and 30 growth factors/ hormones known to be altered in patients with paranoid schizophrenia, in acutely ill antipsychotic-naïve patients, and in matched controls. According to moleculer profiles, patients could be separated into two subgroups with abnormalities predominantly in immune molecules (more frequent changes in macrophage, migration inhibitory factor (MIF), IL-8, IL-1Ra, IL-18, and IL-16) or in growth factors and hormones (higher frequency of changes in prolactin, resistin, testosterone, insulin, platelet-derived growth factor, leptin, and angiotensinogen), with lower frequency or no changes of these molecules in the other subgroup. The authors discuss the possibilities of different therapeutic strategies targeting either the immune- and inflammation-related pathways or the underlying metabolic dysfunction (Schwarz et al., 2014).

2.1.2.4 Other risk factors for schizophrenia

The strongest risk factor for schizophrenia is a family history of psychosis. The risk is 10-fold among first-degree relatives. Many of the biological and psychosocial early risk factors for schizophrenia, such as obstetric complications, high birth weight, high maternal age, maternal depressed mood during pregnancy, being an unwanted pregnancy, and urban birth place, have been found to be more prevalent in patients with a history of parental psychosis. Moreover, high maternal BMI, low birth weight, grand multiparity, high maternal education (in those with no parental psychosis) and high paternal age have been found to increase the risk for schizophrenia (Miller et al., 2011b; Keskinen et al., 2013). In a very recent review Davis et al. discussed the risk-vulnerability model and the environmental vulnerability factors in schizophrenia. People who have ever used cannabis have an up to 40% greater risk of psychosis and the schizophrenia risk seem to be dose dependent. Cannabis use in adoloscence may induce first episode psychosis to susceptible individuals at younger age. Individuals with childhood trauma are at a three-fold risk of psychosis later in life. Childhood trauma seem to be associated with

more severe positive symptoms and non-remission. Decreased brain-derived neurotrophic factor (BDNF) serum levels are associated schizophrenia. Exposure to trauma also affects BDNF expression in the brain. Brain volume reductions are associated with both schizophrenia and childhood trauma. Moreover, social defeat has been associated with increased risk of psychosis. Maternal malnutrition may increase the risk of schizophrenia in the offspring possibly associated with deficiency in vitamin D, folate or iron, or maternal unhealthy dietary pattern with metabolic consequences. Smoking is associated with schizophrenia vulnerability and with earlier age of onset, but the mechanism and direction of this association is inconclusive. The relation between intelligence quotient (IQ) and schizophrenia susceptibility is likewise inconclusive. However, low IQ seem to be a risk factor for schizophrenia, but the relation is non-linear, while some evidence of high IQ as a risk factor has also been presented. Dysfunctional social cognition, especially deficits in emotion recognition, are already present in the early phases of illness, and may be associated with social impairment and stress in social relations (Davis et al., 2016).

2.1.2.5 Estrogen hypothesis

Various aspects of gender differences in schizophrenia are associated with hormonal factors. There are diffrences in course and outcome between genders, and especially in female patients in association to age. Moreover, findings of twenty-fold increased risk of first-episode or relapse of psychosis during the postpartum period, improvement of psychosis during pregnancy, and fluctuation of symptoms according to menstrual cycle have supported the evidence for this so-called “estrogen protection hypothesis” (Hayes et al., 2012).

Animal models have yielded evidence of the antipsychotic properties of estrogen with interactions of estrogen with both dopaminergic, serotonergic and glutamatergic systems. Estrogen moreover has several neuroprotective functions (Hayes et al., 2012). Variance in estrogen receptor alpha gene has been associated with schizophrenia risk (Lee et al., 2013). The effect of antipsychotics on biosynthesis and regulation of fatty acids and cholesterol has been found to be similar to that of selective estrogen receptor modulators (SERMs) tamoxifen, raloxifene, and clomiphene, thereby suggesting that the estrogen pathway is involved in the antipsychotic effect (Polymeropoulos et al., 2009). Moreover, clinical studies have suggested antimanic effect of tamoxifen

and antipsychotic effect of estradiol and raloxifene (Polymeropoulos et al., 2009; Hayes et al., 2012).

2.1.3 Symptoms and diagnostic criteria

The current criteria for the diagnosis of schizophrenia according to the World Health Organization's International Statistical Classification of Diseases and Related Health Problems, Tenth Revision (ICD-10)—Section V, Mental and Behavioral Disorders, are presented in Table 1. (World Health Organization, 1992).

Table 1. Diagnostic criteria of schizophrenia according to ICD-10.

At least one very clear symptom of the following: -Thought echo, thought insertion or withdrawal, and thought broadcasting -Delusions of control, influence, or passivity, clearly referred to body or limb movements or specific thoughts, actions, or sensations; delusional perception -Hallucinatory voices giving a running commentary on the patient's behaviour, or discussing the patient among themselves, or other types of hallucinatory voices coming from some part of the body -Persistent delusions of other kinds that are culturally inappropriate and completely impossible, such as religious or political identity, or superhuman powers and abilities (e.g. being able to control the weather, or being in communication with aliens from another world)	Or at least two of the following symptoms: -Persistent hallucinations in any modality, when accompanied either by fleeting or half-formed delusions without clear affective content, or by persistent over-valued ideas, or when occurring every day for weeks or months on end -Catatonic behaviour, such as excitement, posturing, or waxy flexibility, negativism, mutism, and stupor -Breaks or interpolations in the train of thought, resulting in incoherence or irrelevant speech, or neologisms -Negative symptoms such as marked apathy, paucity of speech, and blunting or incongruity of emotional responses, usually resulting in social withdrawal and lowering of social performance; it must be clear that these are not due to depression or to neuroleptic medication -A significant and consistent change in the overall quality of some aspects of personal behaviour, manifest as loss of interest, aimlessness, idleness, a self-absorbed attitude, and social withdrawal	Symptons present for most of the time during a period of 1 month or more Diagnostic subtypes: F20.0 Paranoid schizophrenia F20.1 Hebephrenic schizophrenia F20.2 Catatonic schizophrenia F20.3 Undifferentiated schizophrenia F20.4 Post-schizophrenic depression F20.5 Residual schizophrenia F20.6 Simple schizophrenia F20.8 Other schizophrenia F20.9 Schizophrenia, unspecified Pattern of course: F20.x0 Continuous F20.x1 Episodic with progressive deficit F20.x2 Episodic with stable deficit F20.x3 Episodic remittent F20.x4 Incomplete remission F20.x5 Complete remission F20.x8 Other F20.x9 Course uncertain, period of observation too short

The Diagnostic and Statistical Manual of Mental Disorders (DSM) criteria published by the American Psychiatric Association is widely used in psychiatric research. The latest edition, DSM-5, was published in 2013. In DSM-5 the somewhat unreliable clinical subtypes of schizophrenia have been omitted in order to emphasize the heterogeneity of the disorder, likewise special treatment of bizarre delusions and Schneiderian "First-rank" hallucinations. The other main differences between ICD-10 and DSM-5 are the minimum six months duration of illness and functional impairment included in the DSM-5 criteria (Tandon et al., 2013).

2.1.4 Treatment of schizophrenia

A stepwise treatment protocol is suggested from the early initial prodromal phase to late high-risk prodromal states. Cognitive behavioral therapy (CBT), omega-3 fatty acids (and some other neuroprotective and anti-inflammatory substances, such as glycine, low-dose-lithium, n-acetylcysteine, estrogen, aspirin possibly in the future), and symptomatic drug treatment with antidepressant medication may be beneficial and delay or even prevent the onset of psychosis. There is some evidence that a low dose of atypical antipsychotic medication would be favorable for symptom reduction and psychosis prevention in late initial prodromal states (Klosterkötter et al., 2011; Kahn and Sommer, 2014).

The integrated treatment of schizophrenia consists of various psycho-social and biological interventions, which are modified according to the course of the individual patient's illness, including assertive community treatment, psychoeducational family treatment, social skills training, and antipsychotic medication (Rosenbaum et al., 2006). Cognitive behavioral therapy and cognitive training programs are beneficial as part of the treatment (Wykes et al., 2011). Moreover, there is evidence for the benefits of adjunctive treatments, such as repetitive transcranial magnetic stimulation (rTMS) for auditory hallucinations and negative symptoms, antidepressants for negative symptoms, estrogen for positive and negative symptoms in women, and *Ginkgo biloba* for positive symptoms (Matheson et al., 2014). Physical exercise improves mood and self-esteem, but may also reduce psychotic and negative symptoms. Physical exercise also affects gene expression in anti-inflammatory pathway and decreases inflammation parameters. The benefits of exercise are also associated

with the prevention of the metabolic side-effects of antipsychotic medication (Kahn and Sommer, 2014).

2.1.4.1 Antipsychotic medication

Antipsychotic medication reduces the symptoms of schizophrenia and prevents relapses compared to placebo (Matheson et al., 2014). The antipsychotic effect of first-generation, or typical, antipsychotics (phenothiazines, thioxanthenes and butyrophenones) is linked to dopamine D_2 receptor antagonism especially in mesolimbic dopamine pathway. On the other hand, the extrapyramidal, cognitive, and metabolic side effects of typical antipsychotics are linked to D_2 antagonism in nigrostriatal, mesocortical, and tuberoinfundibular dopamine pathways (O'Connor and O'Shea, 2015). Most of the second-generation or atypical antipsychotics (clozapine, aripiprazole, asenapine, lurasidone, olanzapine, paliperidone, quetiapine, risperidone, sertindole, and ziprasidone) have serotonergic actions, especially blockade of serotonin 5-HT_{2A} receptor and stimulation of serotonin 5-HT_{1A} receptor, and weaker dopamine D_2 affinity compared to 5-HT_{2A} affinity. Some of the atypical antipsychotics are also 5-HT_{2C}, 5-HT_6 and 5-HT_7 antagonists. The effects on 5-HT receptors are associated with lower risk of extrapyramidal side effects (EPS), lack of prolactin elevation (except risperidone and paliperidone), antipsychotic action and cognitive improvement in patients with schizophrenia. The D_2/D_3 antagonist amisulpride has no other serotonergic effects but 5-HT_7 antagonism, which may be behind its effects on cognitive functioning (Meltzer and Massey, 2011).

Antipsychotic agents alter the expression of numerous genes associated with lipid and fatty acid biosynthesis (Foley and Mackinnon, 2014). This common effect of antipsychotics suggests a role of altered lipid homeostasis in the pathogenesis of schizophrenia. Accordingly, this altered lipid homeostasis associated with schizophrenia may support the use of omega-3 fatty acids in these patients (Polymeropoulos et al., 2009).

First-episode patients respond to antipsychotic medication reasonably well. However, treatment response seems to diminish as the number of relapses increases. It also seems that patients who respond poorly to the first antipsychotic medication most probably do not have a reasonable response to the second antipsychotic medication either. In the future medications with non-dopaminergic mechanisms of action may have potential in these nonresponders.

So far these glutamatergic and anti-inflammatory medications are mainly experimental (Kahn and Sommer, 2014).

According to a Finnish nationwide cohort study of antipsychotics after first hospitalization for schizophrenia, 57.8% of patients were rehospitalized because of relapse during a mean follow-up period of two years. Use of any antipsychotic medication was associated with lower risk of rehospitalization and lower mortality compared with no use of medication. Clozapine, olanzapine, and depot medications were associated with more favourable outcomes (Tiihonen et al., 2011).

2.1.4.2 Treatment-resistant schizophrenia

Thirty per cent of patients with schizophrenia suffer from persistent psychotic symptoms although treated with adequate antipsychotic medication (Samara et al., 2016). There is some, although limited, evidence that treatment-resistant schizophrenia differs from non-treatment-resistant schizophrenia both genetically and in relation to brain structure and functioning and cognitive deficits, suggesting differing etiologies (Seppälä et al., 2016). However, there is no standardized definition for treatment resistance in schizophrenia. Commonly used criteria are non-response to two different antipsychotic agents (at least one atypical) taken in adequate doses for at least 2-8 weeks. The strict so-called "Kane criteria" were introduced in 1988, when the superiority of clozapine in treatment-resistant schizophrenia was demonstrated. In this study patients had to be non-responders to at least three periods of antipsychotic treatment from at least two different classes with notably higher dosages than preferred today of over 1000mg/day cpz-equivalents for at least six weeks and without any period of good functioning during the five years preceding the study. The patients also had to be non-responders to haloperidol up to 60mg/day before randomization to clozapine or chlorpromazine groups. Today in clinical practice it is recommended that the treatment procedure should be carefully re-evaluated if the patient does not respond adequately to the first antipsychotic agent (Dold and Leucht, 2014). Clozapine has the strongest evidence of efficacy for treatment-resistant schizophrenia despite some controversy according to a recent meta-analysis by Samara et al. (Dold and Leucht, 2014; Kane and Correll, 2016; Samara et al., 2016). However, due to its risk profile clozapine should be used only after treatment with at least two different antipsychotic agents with adequate dose and duration (Dold and Leucht, 2014).

2.1.5 Clozapine

The clozapine molecule was synthesized in 1959. After clinical testing of its antipsychotic effect with fewer EPS it was introduced in some Western countries in the early 1960s (Meltzer, 1992). In 1975 eight patients died from infectious diseases after agranulosytosis during clozapine treatment in Finland (Idänpää-Heikkilä et al., 1975). This led to the withdrawal of clozapine from general clinical use. However, limited use was permitted due to relapses and non-responsiveness to other antipsychotics after clozapine discontinuation. After several clinical trials showing the antipsychotic efficacy and low risk of EPS and tardive dyskinesia, and no prolactin increase, clozapine was once again approved for clinical use in 1990 (Meltzer, 1992).

2.1.5.1 Use in schizophrenia

Clozapine is an atypical antipsychotic drug with unique efficacy in treatment resistant schizophrenia. Among treatment-resistant patients 30% respond within six weeks (Kane et al., 1988) and another 30-40% respond within six months (Meltzer, 2013). It has been shown to ameliorate positive symptoms in a large proportion of patients in this group, decrease aggression, reduce substance abuse, and reduce the risk for suicide and decrease overall mortality (Kane et al., 1988; Tiihonen et al., 2009; Meltzer, 2013). However, the side-effect profile of clozapine reduces its applicability. The risk of agranulosytosis at a prevalence of 0.8% requires regular white blood cell count, and absolute neutrophil count monitoring during treatment. Other notable adverse effects are fever and eosinophilia at initiation, sedation, metabolic side-effects, seizures, myocarditis, hypersalivation, urinary incontinence, sweating, constipation, and ileus (Meltzer, 2013).

2.1.5.2 Mechanisms of action

Clozapine has a broad receptor affinity profile, with strongest antagonist affinity for serotonin 5-HT_{2A} and 5-HT_{2C}, histaminergic H_1, and α_1 receptors, moderate antagonist affinity for dopamine D_4, adrenergic α_2, and $GABA_A$ receptors, and weak antagonist affinity for dopamine D_2 receptor. Clozapine is also a muscarinic cholinergic M_1, M_2, and M_3 receptor antagonist. Moreover, it has

agonist properties, which are more potent for serotonin 5-HT_{1A}, and NMDA receptors, and weaker for dopamine D_1 and muscarinic cholinergic M_4 receptors. It also has partial agonist/ competitive antagonist affinity for muscarinic cholinergic receptors M_1-M_5 (O´Connor and O´Shea, 2015). The mechanism of action of clozapine is believed to be based on its more potent blockade of serotonin 5-HT_{2A} than of dopamine D_2 receptor, as well as on other non-D_2 dopamin receptor-mediated actions. An atypical antipsychotic is simply described as one that produces minimal extrapyramidal side effects (EPS) at clinically effective doses. This low EPS potential is related to 5-HT_{2C} antagonism and other serotonin receptor effects. Partial 5-HT_{1A} receptor agonism of clozapine and other atypical antipsychotics is absent from typical antipsychotics. Clozapine is also a potent antagonist of 5-HT_6 and 5-HT_7 receptors. Animal models suggest that these serotonergic actions would have benefits against cognitive impairments (Meltzer, 2013). Antagonist affinity for excitatory α_1 and inhibitory α_2 adrenoreceptors has also been suggested to be associated with cognitive improvements. Weak agonist affinity for NMDA receptor and antagonist affinity for $GABA_A$ receptor may to some extent also be associated with the therapeutic profile of clozapine (O´Connor and O´Shea, 2015). Accordingly, it is also well known that clozapine has immunomodulatory effects (Pollmächer et al., 1996; Maes et al., 1997; Monteleone et al., 1997; Chen et al., 2013). There is some evidence that clinical response to clozapine varies genetically. According to a recent meta-analysis three polymorphisms in serotonin receptor genes *HTR2A* (rs6313, rs6314) and *HTR3A* (rs1062613) have been reported to be associated with clozapine response (Gressier et al., 2016).

It is noteworthy that the active metabolite of clozapine, N-desmethylclozapine (NDMC), is a muscarinic M_1 agonist. NDMC is also a weaker D_4 antagonist than clozapine. These features of NDMC may be associated with positive cognitive effects, especially on working memory. M_1 receptor agonism activates hippocampal NMDA receptors. Moreover, animal models have shown that both clozapine and NDMC increase the release of acethylcholine. Acethylcholine enhances the effect of NDMC and opposes the effect of clozapine on muscarinic receptors, and also stimulates α7 and α4β2 nicotinic receptors. These actions of acethylcholine on nicotinic receptors are also associated with pro-cognitive effects. Plasma concentrations of NDMC may vary from 20% to 150% of clozapine concentrations. A high NDMC/clozapine ratio seems to be associated with working memory, which deteriorates during the first six weeks of clozapine treatment, but recovers to

baseline within six months. Other cognitive dimensions, such as verbal fluency, declarative memory, speed of processing, and attention, already start to improve during the first six weeks of treatment and this improvement continues for up to six months (Meltzer, 2015; Rajji et al., 2015).

2.1.5.3 Pharmacokinetics of clozapine

There is a marked variation in plasma clozapine concentrations at a stable dose between and within patients. This variation is associated with several factors, such as adherence, smoking, hepatic metabolism, gastric absorption, drug interactions, age, and sex. The maximum concentration after oral administration is reached within four hours and clozapine half-life is approximately 12 hours. Approximately 95% of the drug binds to plasma proteins. The compound undergoes first-pass metabolism in the liver. The main serum metabolite (20-30% of metabolites) NDMC (or norclozapine) is formed through CYP1A2 (70% of clozapine metabolism) and CYP3A4 enzymes in the liver. CYP2D6 and 2C19 also play some role. N-oxide-clozapine (10% of metabolites) is catalyzed by flavin mono-oxygenase 3 (FMO3) in association with CYP3A4. CYP1A2 activity is influenced by environmental factors such as cigarette and cannabis smoking (inductors), caffeine (CYP1A2 substrate), infections and other inflammatory states, and drugs (carbamazepine and phenytoin inductors, omeprazole, theophylline, modafinil, fluvoxamine inhibitors), and by genetic variability (Koponen et al., 1996; Seppälä et al., 1999; Darling and Huthwaite, 2011; Krivoy et al., 2015; Hefner et al., 2016). The strongest evidence of genetic variance in clozapine concentration is associated with transmembrane transporter *ABCB1* gene as a candidate for the regulation of clozapine absorption. Moreover, there is some evidence that estrogen regulates CYP3A4 gene expression (Krivoy et al., 2015).

2.1.5.4 Metabolic side effects of clozapine

Of the antipsychotic agents clozapine and olanzapine are most often associated with weight gain (Newcomer, 2005; Leucht et al., 2013), thereby increasing the risk of type 2 diabetes mellitus and dyslipidemia. According to a recent meta-analysis the highest rates of metabolic syndrome among patients with schizophrenia were found in those prescribed clozapine (51.9%) compared to

olanzapine (28.2%), risperidone (27.9%) and unmedicated (20.2%) patients (Mitchell et al., 2013). Waist circumference has been found to be the best predictor of insulin resistance in clozapine-treated patients (Henderson et al., 2009). However, case reports also suggest that substantial weight gain or obesity may not be a factor in up to 25% of cases of new-onset diabetes occurring during antipsychotic treatment. Accordingly, some studies support the hypothesis that clozapine may have direct effects on glucose regulation independent of adiposity (Newcomer, 2005). In a five-year naturalistic study patients treated with clozapine had significant weight gain until approximately month 46 after clozapine initiation, and increase in serum triglycerides and total cholesterol. Weight gain correlated with increases in levels of triglycerides and cholesterol. Clozapine dose did not correlate with weight change, but tended to be associated with change in triglyceride levels. Thirty-six point six per cent of patients had diabetes mellitus during follow-up. Weight gain or clozapine dose were not statistically significant risk factors for diabetes mellitus in that study (Henderson et al., 2000). However, there is evidence of an association between clozapine concentration and metabolic adverse effects (Simon et al., 2009).

Weight gain is in part due to histamine H_1 and serotonin 5-HT_{2C} receptor antagonism (Meltzer, 2013), and 5-HT_{2A}, M_3 and α_1 receptors also seem to be involved in clozapine-induced weight gain (O´Connor and O´Shea, 2015). There is some evidence that insulin homeostasis, with higher fasting insulin, lower insulin sensitivity and higher HOMA-IR and lipid profiles with higher triglycerides are different in clozapine-treated obese schizophrenia patients from an obese control sample (Wu et al., 2008). Clozapine may impair insulin secretion in pancreatic β-cells via muscarinic M_3-antagonism. Antagonism of hypothalamic 5-HT_{2C}-receptors may moreover dysregulate glucose homeostasis (Reynolds and Kirk, 2010). Clozapine influences the biosynthesis and regulation of fatty acids and cholesterol as well (Fernø et al., 2009; Canfrán-Duque et al., 2013). Inhibition of brown adipogenesis may be one possible mechanism to explain weight gain induced by clozapine (Oh et al., 2012).

Adjunctive treatment strategies for clozapine-induced weight gain have been discussed in recent meta-analysis. Metformin improves glycemic control and was shown to promote weight loss in both diabetic and non-diabetic subjects. However, especially concerning the elderly, there is a rare association with lactic acidosis, and accumulation of Alzheimer's disease related beta-amyloid associated with metformin (Choi, 2015; Whitney et al., 2015). Aripiprazole augmentation may be beneficial in weight reduction, although clozapine-

aripiprazole combination may induce side effects such as nausea, anxiety and akathisia (Choi, 2015; Whitney et al., 2015). Topiramate appears to be beneficial in weight reduction, but the effect on psychiatric symptomatology is contradictory (Choi, 2015; Whitney et al., 2015). Orlistat has been suggested to be effective for clozapine treated men in weight reduction (Whitney et al., 2015). Lithium may also have some benefits for weight loss (Whitney et al., 2015). The research on non-pharmacological interventions is limited. However, interventions with both, nutritional counseling, diet modification, and physical activity, have yielded some promising results (Daumit et al., 2013; Bruins et al., 2014; Whitney et al., 2015).

2.2 Obesity and metabolic syndrome

Obesity is a lifestyle related variable which, along with sedentary lifestyle, has an adverse effect on insulin-mediated glucose tolerance, and leads to increasing risk of syndromes associated with insulin resistance (Reaven, 2005). Insulin resistance is a pathological situation leading to the metabolic and hemodynamic abnormalities known as the metabolic syndrome. The metabolic syndrome (MetS) is a cluster of factors indicating increased risk for type 2 diabetes mellitus and coronary artery disease. The main features of this condition include dyslipidemia (elevated triglyceride and low HDL cholesterol levels), hypertension, glucose intolerance or type 2 diabetes, abdominal obesity, hypercoagulability and defects in the fibrinolytic system, hyperandrogenism, non-alcoholic fatty liver, sleep apnea, and an increased incidence of coronary heart disease (Ascaso et al., 2003; Kassi et al., 2011).

2.2.1 Epidemiology

According to the National Health and Examination Survey (NHANES) 2003-2006 in the United States, approximately 34% of people studied met the National Cholesterol Education Program Adult Treatment Panel III (NCEP: ATPIII) revised criteria for metabolic syndrome (Ervin, 2009). In a Finnish general population sample, the FINRISK cohort, the metabolic syndrome was present in 38.8% of men and 22.2% of the women according to the WHO criteria. The high prevalence among men was closely associated with abdominal obesity (Ilanne-Parikka et al., 2004). Metabolic syndrome is associated with a

1.5-3 times increase in cardiovascular disease and cardiac disease mortality (Kassi et al., 2011) and a fivefold increase in risk for type 2 diabetes mellitus (Alberti et al., 2009).

2.2.2 Definition of metabolic syndrome

Various criteria for metabolic syndrome have been proposed by different organizations over the years (WHO in 1998, the National Cholesterol Education Program Adult Treatment Panel III in 2001, the International Diabetes Federation in 2005, the American Heart Association/National Heart, Lung, and Blood Institute in 2005). A joint interim statement by the International Diabetes Federation Task Force on Epidemiology and Prevention, the National Heart, Lung, and Blood Institute, the American Heart Association, the World Heart Federation, the International Atherosclerosis Society, and the International Association for the Study of Obesity, in order to harmonize the criteria for the clinical diagnosis of the metabolic syndrome was issued in 2009 (Table 2.) (Alberti et al., 2009).

Table 2. Harmonized criteria of metabolic syndrome (Alberti et al. 2009).

Any 3 of the 5 risk factors:	
1. Elevated waist circumference	Population- and country-spesific definitions, in Finland: men ≥ 94cm and women ≥ 80 cm
2. Elevated triglycerides	≥ 1.7 mmol/l (drug treatment for elevated triglycerides is an alternate indicator)
3. Reduced HDL- cholesterol	< 1.0 mmol/l in males, and 1.3 mmol/l in females (drug treatment for reduced HDL-C is an alternate indicator)
4. Elevated blood pressure	systolic ≥ 130 and/or diastolic ≥ 85 mmHg (antihypertensive drug treatment is an alternate indicator)
5. Elevated fasting glucose	≥ 5.6 mmol/l (drug treatment of elevated glucose is an alternate indicator)

2.2.3 Metabolic comorbidity in schizophrenia

Cardiovascular risk factors such as type 2 diabetes mellitus, dyslipidemia, hypertension, smoking, and obesity are more prevalent in patients with schizophrenia than in general population (Hennekens et al., 2005). Schizophrenia is associated with a marked increase in mortality in every age group (Saha et al., 2007; Talaslahti et al., 2012) and cardiovascular disease is a major cause of excess deaths in these patients (Osby et al., 2000; Kilbourne et al., 2009; Talaslahti et al., 2012). The average lifestyle of patients with schizophrenia, with lack of regular physical activity, poor diet, substance use, and high rates of smoking, increases the risk of cardiovascular disease (De Hert et al., 2009). According to a recent review, 56% of patients with treatment-resistant schizophrenia smoke and according to a recent population-based cohort study from the United States, among patients with schizophrenia tobacco-related conditions accounted for 53% of total deaths (Callaghan et al., 2014; Kennedy et al., 2014). Metabolic abnormalities are already present in first-episode patients, and become even more common as the disorder progresses (De Hert et al., 2006; Beary et al., 2012). A recent meta-analysis reported an overall 32.5% prevalence of metabolic syndrome among patients with schizophrenia. The most predictive factors of full metabolic syndrome in these patients seem to be waist circumference and illness duration over 7.8 years (Mitchell et al., 2013). In the CATIE study sample the male patients were 138% (OR 2.38; 95% CI 1.78-3.18) and female patients 251% (OR 3.51; 95% CI 2.19-5.62) more likely to have metabolic syndrome than the NHANES sample counterparts (McEvoy et al., 2005). In a Finnish general population survey the prevalence of metabolic syndrome was 36.2% among subjects with schizophrenia and 30.1% among subjects without psychotic disorder (Suvisaari et al., 2007). According to a recent Finnish study, 58.7% of the patients with schizophrenia had metabolic syndrome. Clozapine treatment doubled the risk (Eskelinen et al., 2015).

2.2.3.1 Antipsychotic-induced weight gain

Antipsychotic agents may have a direct negative effect on some of the cardiovascular and metabolic syndrome related risk factors, but most of them can be explained by their tendency to induce weight gain (De Hert et al., 2009). Weight gain has been observed in up to 30% of patients treated with second-generation antipsychotics, although the variation between antipsychotic agents

is considerable (Brandl et al., 2014). Affinity for muscarinic M_3 receptor seems to be among the main predictors for an antipsychotic to induce weight gain, and also type 2 diabetes (Potvin et al., 2015). Antipsychotic-naïve patients and those with lower baseline BMI are more likely to gain weight during antipsychotic treatment (Bak et al., 2014). In a Finnish study of first-episode patients even 81.8% of subjects had clinically significant weight increase at 12-month follow-up (Keinänen et al., 2015). In order to identify peripheral molecular alterations predisposing to AIWG, Schwarz et al. recently studied the associations between weight change and concentrations of 191 molecules during six weeks of antipsychotic treatment in 77 patients with paranoid schizophrenia. The baseline serum levels of 10 molecules before treatment initiation were associated with weight gain during treatment (negative correlation: apolipoprotein C III, apolipoprotein H, epidermal growth factor, follicle stimulating hormone, IL-25, interleukin-6 receptor, matrix metalloproteinase I, placenta growth factor and thyroid stimulating hormone, and positive correlation: IL-18). Change in serum levels of four molecules during treatment correlated with weight change (alpha fetoprotein, apolipoprotein AIV, prolactin and tenascin C). Changes in leptin levels were associated with weight change, but this was no longer significant after baseline BMI and leptin levels were considered as covariates (Schwarz et al., 2015).

2.2.3.2 Monitoring of metabolic adverse effects

Guidelines for monitoring metabolic parameters in patients taking antipsychotic medication have been published in several countries in recent years. However, screening practices are inconsistent and monitoring of patients is inadequate. It is also well known that people with psychiatric diagnoses receive poorer quality of medical care (Mitchell et al., 2012). According to the Finnish Current Care Schizophrenia guidelines height, weight, waist circumference, body mass index, blood pressure, fasting plasma cholesterol, triglycerides and glucose, and ECG should be included in the medical examination of a first-episode patient. Lifestyle variables, such as dietary pattern, physical activity and smoking, and familiar cardiovascular risk factors should be evaluated. Weight and waist circumference should be measured on a regular basis. Plasma cholesterol, triglycerides, and glucose should be tested three months after initiation of a new antipsychotic medication and yearly after that. ECG monitoring is

recommended after changes in antipsychotic medication (Salokangas et al., 2013).

2.3 Cytokines, adipokines, and neuropeptide Y in schizophrenia and metabolic syndrome

Cytokines are proteins involved in the regulation of immunologic and inflammatory responses in physiologic and pathologic processes (Steinke and Borish, 2006). Adipose tissue is a source of several inflammatory cytokines and adipokines (Raucci et al., 2013). Abnormalities in these including tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), interleukin-1 receptor antagonist (IL-1Ra), interleukin-6 (IL-6), C-reactive protein (CRP), leptin and adiponectin, and a chronic low-grade inflammatory state are associated with both schizophrenia and obesity, or metabolic syndrome (Potvin et al., 2008; Miller et al., 2011a; Harwood, 2012). Accordingly, higher white blood cell count has been associated with a risk of metabolic syndrome and more severe psychiatric symptomatology in patients with schizophrenia (Fan et al., 2010). Inflammatory cytokine alterations associated with metabolic syndrome and schizophrenia are discussed in more detail below. Neuropeptide Y (NPY) plays an important role as a central regulator of energy homeostasis and appetite (Brown and Clegg, 2010). Plasma NPY levels have been found to be associated with several features of metabolic syndrome. NPY may also be involved in the mechanisms underlying antipsychotic-induced weight gain (Fitzgerald et al., 2003; Reynolds et al., 2006).

2.3.1 Leptin

Leptin is a hormone secreted mostly by adipocytes (Raucci et al., 2013), which plays an important role in appetite regulation (Elias and Hofflich, 2008). Leptin also modulates immunity and inflammation (Fantuzzi et al., 2005). Obesity is associated with high leptin levels and leptin resistance (Raucci et al., 2013). Obese patients are insensitive to the anorectic actions of leptin and continue to maintain high levels of body fat (Harwood, 2012). The circulating leptin correlates better with subcutaneous fat than with total body fat, and leptin levels are generally higher in women. Estrogen and testosterone modulate leptin synthesis and secretion. Leptin levels are inversely correlated with testosterone.

Testosterone exposure inhibits leptin expression in fat cells. Estradiol increases adipose tissue leptin mRNA levels and increases hypothalamic leptin sensitivity (Shi et al., 2009).

Clozapine treatment has been associated with an increase in serum leptin level (Panariello et al., 2012). This increase seems already to begin during the first two weeks after initiation of clozapine (Sentissi et al., 2008). A role of leptin in mediating antipsychotic-induced weight gain but also a therapeutic effect of antipsychotic medication in schizophrenia has been speculated (Venkatasubramanian et al., 2010). However, the increase of serum leptin during antipsychotic treatment appears to be a result of weight gain rather than a direct impact of these drugs on leptin physiology (Jin et al., 2008; Risselada et al., 2012), although second-generation antipsychotics may also directly affect adipocytokine dysregulation (Sugai et al., 2012). The evidence linking leptin increase to changes in other metabolic markers during antipsychotic treatment is likewise contradictory (Jin et al., 2008). A gender-specific effect of schizophrenia on leptin regulation, with a stronger effect in female patients, has been suggested, but the results are inconclusive (Matsuda et al., 2005; Wang et al., 2007; Beumer et al., 2012).

2.3.2 Adiponectin

Concentration of circulating adiponectin is negatively correlated with BMI and is decreased in obese subjects, in patients with type 2 diabetes or with cardiovascular disease (Raucci et al., 2013). Levels of adiponectin decline prior to a decrease in insulin sensitivity. Decreased adiponectin levels may serve as predictors for the future development of metabolic syndrome (Harwood, 2012) and type 2 diabetes (Salomaa et al., 2010). Increased TNF-α of obese subjects may downregulate adiponectin production. Likewise the anti-inflammatory activities of adiponectin extend to the inhibition of IL-6 production, reduced production and activity of TNF-α, and induction of the anti-inflammatory cytokines IL-10 and IL-1Ra (Fantuzzi, 2005). Thus adiponectin has direct anti-inflammatory and anti-atherosclerotic effects (Harwood, 2012). Adiponectin levels are generally higher in women than in men. This could partly explain the better insulin sensitivity of females. Low adiponectin levels in women are even more closely related to the risk of metabolic syndrome than in men (Santaniemi et al., 2006). Obese men in particular seem to have lowered adiponectin levels (Plaisance et al., 2009). Contradictory findings to these, showing no association

between plasma adiponectin and BMI or gender, have also been reported, and it seems that low plasma levels of adiponectin are more closely related to insulin resistance or type 2 diabetes than to adiposity as such (Kuo and Halpern, 2011).

Some discrepancies exist in reports concerning the association between adiponectin levels, schizophrenia, and antipsychotic medications. Most studies report lowered levels of adiponectin (Matsuda et al., 2005; Hanssens et al., 2008; Jin et al., 2008; Oriot et al., 2008; Chen et al., 2011), but reports of no change (Jin et al., 2008; Fernandez-Egea et al., 2009), or increased adiponectin levels (Beumer et al., 2012) have also been published. However, both olanzapine and clozapine have been associated with lowered adiponectin levels in several studies (Togo et al., 2004; Richards et al., 2006; Hanssens et al., 2008; Sugai et al., 2012; Wampers et al., 2012). Accordingly, hypoadiponectinemia has been suggested to be a potential biomarker of the metabolic syndrome in clozapine-treated patients with schizophrenia (Bai et al., 2007). In schizophrenia as well as in obesity there is an imbalance between adiponectin and pro-inflammatory cytokines TNF-α and IL-6 favoring the latter ones (Leonard et al., 2012). According to a recent meta-analysis, it seems that schizophrenia in itself is not associated with lowered levels of adiponectin, but treatment with second-generation antipsychotics, especially clozapine and olanzapine, does associate with it (Bartoli et al., 2015b).

2.3.3 Interleukin-6

Interleukin-6 (IL-6) is a cytokine produced by several cells, such as fibroblasts, endothelial cells, monocytes, and adipocytes (Raucci et al., 2013), having both pro- and anti-inflammatory effects (Eder et al., 2009). Circulating levels and adipose tissue production of IL-6 are increased in obesity (Raucci et al., 2013). Visceral white adipose tissue produces higher levels of IL-6 than subcutaneous white adipose tissue (Fantuzzi, 2005). These levels are also increased in subjects with insulin resistance, diabetes, and metabolic syndrome (Marques-Vidal et al., 2013). IL-6 promotes insulin resistance by affecting the insulin signaling in adipose tissue. There is also a direct correlation between the concentrations of IL-6 and systemic CRP (Gustafson, 2010). Moreover, IL-6 increases leptin secretion and reduces adiponectin secretion (Harwood, 2012).

Increased concentrations of IL-6 are among the consistently reported cytokine alterations in schizophrenia (Leonard et al., 2012). This increase may already be present in first-episode patients and in patients with acute relapse,

and seems to normalize after antipsychotic treatment. It seems that IL-6 levels do not differ from those of controls in outpatients with stable medication, nor in patients with treatment-resistant psychosis (Miller et al., 2011a). Fernandez-Egea et al. reported that newly diagnosed antipsychotic-naïve patients with schizophrenia had higher prevalence of abnormal glucose tolerance or diabetes and higher IL-6 concentrations, even when confounding factors, such as BMI, gender, age, medication, cortisol concentration, and smoking were considered (Fernandez-Egea et al., 2009). However, in a study by Beumer et al. a significant rise in the serum level of IL-6 was reported in patients with schizophrenia, but no further effects of metabolic syndrome on IL-6 levels were found (Beumer et al., 2012). Clozapine treatment has been associated with elevated IL-6 levels in several studies (Pollmächer et al., 1996; Maes et al., 1997; Schmitt et al., 2005; Kluge et al., 2009; Löffler et al., 2010a; Røge et al., 2012), but findings of lowered levels (Lu et al., 2004; Sugino et al., 2009), or no effect have been reported (Himmerich et al., 2011; Røge et al., 2012).

2.3.4 Interleukin-1 receptor antagonist

Interleukin-1 receptor antagonist (IL-1Ra) is produced in response to several inflammatory stimuli, including IL-1β and IL-6, and is elevated in a variety of infections and inflammatory diseases, such as sepsis, chronic polyarthritis, Crohn's disease and ulcerative colitis (Potvin et al., 2008; Palomo et al., 2015). IL-1Ra is a sensitive marker of cytokine response in the pre-diabetic state (Ruotsalainen et al., 2006) and predicts the progression of metabolic syndrome to diabetes mellitus independently of C-reactive protein and other risk factors (Luotola et al., 2011). Moreover, serum IL-1Ra levels are markedly increased in obese subjects (Fantuzzi, 2005).

IL-1Ra gene (*IL1RN*) polymorphisms have been associated with susceptibility to schizophrenia (Kim et al., 2004). Levels of IL-1Ra have also been reported to be increased in patients with schizophrenia, both in unmedicated patients and in acute relapse, suggesting that these alterations are not related to antipsychotic medication alone (Potvin et al., 2008; Miller et al., 2011a). Clozapine treatment has been reported to be associated with no effect (Pollmächer et al., 1996) or increase in IL-1Ra levels (Maes et al., 1997). In a recent study by Witte et al. elevated serum levels of IL-1Ra in first-episode antipsychotic-naïve patients with schizophrenia declined after six weeks of treatment with either olanzapine or risperidone (Witte et al., 2014).

2.3.5 Tumor necrosis factor-alpha

Tumor necrosis factor-alpha (TNF-α) is an immunomodulatory and pro-inflammatory cytokine (Harwood, 2012). It is overexpressed in the adipose tissue of obese individuals, and circulating levels of TNF-α are also elevated in obese subjects, falling after weight loss (Raucci et al., 2013). Subjects with diabetes, metabolic syndrome and increased insulin resistance present with increased levels of TNF-α (Marques-Vidal et al., 2012). Plasma concentration of TNF-α has been associated with degrees of early atherosclerosis in a healthy population of middle-aged men (Raucci et al., 2013). TNF-α increases leptin secretion and decreases adiponectin secretion (Harwood, 2012). However, in a data analysis by Kuo and Halpern no correlation was found between BMI and plasma levels of TNF-α in healthy adults when metabolic abnormalities, such as diabetes and cardiovascular disease, were excluded (Kuo and Halpern, 2011).

A meta-analysis by Potvin et al. showed a heterogeneity in studies on associations between TNF-α and schizophrenia, with no significant effect sizes (Potvin et al., 2008). The results of recent studies on associations between TNF-α and schizophrenia are likewise inconsistent, but it seems that TNF-α levels are increased in the acute phases, and normalize in the non-acute state of the illness (Coelho et al., 2008; O´Brien et al., 2008; Drexhage et al., 2010; Francesconi et al., 2011; Kunz et al., 2011; Miller et al., 2011a; Pedrini et al., 2012; Di Nicola et al., 2013). Beumer et al. found a significant rise in TNF-α in patients with schizophrenia compared to the healthy controls with no further effect of metabolic syndrome. Smoking was associated with lowered TNF-α levels in female patients (Beumer et al., 2012). A history of childhood trauma has been associated with higher TNF-α levels in first-episode patients, suggesting a long-lasting chronic activation of the immune system (Di Nicola et al., 2013). TNF-α levels have been reported to both increase and decrease during clozapine treatment. The sample sizes in the studies, however, have been small with possible confounding factors (Pollmächer et al., 1996; Monteleone et al., 1997; Ajami et al., 2014). Animal models have reported a suppressive effect of clozapine on serum TNF-α levels (Sugino et al., 2009). It seems that TNF-α gene polymorphisms (-308A/G (rs1800629), -238A/G (rs361525), -857T/C (rs1799724), -863T/C (rs1800630), -1031C/T (rs1799964)) are not associated with schizophrenia susceptibility at least in Caucasian populations (Lee and Song, 2015).

2.3.6 C-reactive protein

C-reactive protein (CRP) is an acute-phase protein, which is synthesized and released primarily by hepatocytes (Shen and Ordovas, 2009). Concentrations of CRP under 10mg/l are called low-grade inflammation and values above that are considered as clinically significant inflammatory state. Production of CRP is induced by proinflammatory cytokines IL-1, IL-6 and IL-17 (Eklund, 2009). High-sensitivity CRP (hs-CRP) assessment makes it possible to measure lower concentrations of CRP more precisely than the standard CRP assessment. Increased hs-CRP (high risk hs-CRP≥3mg/l) is associated with multiple risk factors for cardiovascular diseases, including obesity, insulin resistance, and hypertension, and has been demonstrated to have predictive value for risk of metabolic syndrome and cardiovascular mortality (Shen and Ordovas, 2009; Parrinello et al., 2015). CRP concentrations have been found to be higher in women than in men, whereas IL-6 and TNF-α concentrations have been reported to be lower in women than men (McConnell et al., 2002; Cartier et al., 2009). Higher CRP concentrations found in women appear to be due to their greater accumulation of subcutaneous fat than observed in men and estrogen's increasing effect on CRP concentrations (Cartier et al., 2009). The gender difference in the association between hs-CRP and obesity seems to almost disappear after adjustment for leptin (Rossi et al., 2012).

According to a recent meta-analysis by Miller et al. the prevalence of an elevated CRP level (>5mg/l) in patients with schizophrenia and related disorders was 28%, a significant difference from that of the controls (Miller et al., 2014). It seems that levels of CRP are elevated in patients with schizophrenia independent of antipsychotic medication, and are associated with the severity of positive symptoms, but not with negative symptoms according to a most recent meta-analysis (Fernandes et al., 2016). Moreover, elevated serum levels of CRP in schizophrenia have been reported to be associated with the severity of cognitive impairment (Dickerson et al., 2007). Associations between elevated CRP and the severity of the psychopathology on the Positive and Negative Syndrome Scale (PANSS) negative symptom score have been reported (Fan et al., 2007; Fawzi et al., 2011). Patients with non-affective psychosis and metabolic syndrome have been reported to have higher hs-CRP levels than patients without metabolic syndrome (Miller et al., 2013). During treatment with second-generation antipsychotics, elevated hs-CRP was associated with high BMI and high glucose levels in patients with psychotic disorders (Dieset et

al., 2012). In the CATIE-study patients taking quetiapine or olanzapine had the highest median levels for CRP at three months. Eighteen-month repeated measures analysis of CRP confirmed the higher values for those olanzapine patients with low baseline CRP (Meyer et al., 2009). As a part of transient acute-phase response, hs-CRP increases within one week after initiation of clozapine treatment, but the elevation seems to be temporary and disappears after one year of treatment (Löffler et al., 2010b).

2.3.7 Resistin

Resistin is an adipokine associated with several acute and chronic inflammatory states (Kunnari et al., 2009; Kontunen et al., 2011; Schwartz and Lazar, 2011; Koskinen et al., 2014; Laurikka et al., 2014; Scotece et al., 2014). Several pro-inflammatory cytokines, including IL-1, IL-6, and TNF-α, enhance the transcription of the resistin gene. On the other hand, resistin promotes the expression of TNF-α and IL-6 by human mononuclear cells. Resistin also directly opposes the anti-inflammatory effects of adiponectin on vascular endothelial cells (Raucci et al., 2013). The association between levels of resistin and hs-CRP is inconclusive with findings of positive correlation or no correlation between levels of resistin and hs-CRP (Kunnari et al., 2006; Esbah et al., 2011; Malo et al., 2011). An association between resistin and IL-1Ra has also been reported (Forsblad d'Elia et al., 2008). Circulating levels of resistin do not usually differ between genders, although some findings have suggested higher levels in women (Kunnari et al., 2006; Chen et al., 2009; Harwood, 2012; Cabrera de León et al., 2014).

The functional role of resistin is somewhat unclear (Kwon and Pessin, 2013). The evidence linking serum resistin levels to obesity and diabetes is inconsistent (Harwood, 2012; Lee and Kim, 2012). However, high plasma resistin concentration has been associated with metabolic syndrome and its components (central obesity, low HDL cholesterol, high triglycerides) and markers of insulin resistance (fasting insulin, HOMA-IR) (Malo et al., 2011). This association between resistin and insulin resistance may be gender dependent, with a stronger association in men (Kawamoto et al., 2012). Moreover, resistin appears to have a role in coronary artery disease (Schwartz and Lazar, 2011). Elevated level of resistin is associated with increased risk of heart failure (Frankel et al., 2009), This association is possibly stronger among women than men (Cabrera de León et al., 2014). High serum resistin level has also been reported to be a

risk factor for cardiovascular disease and all-cause mortality in patients with type 2 diabetes mellitus (Menzaghi et al., 2013).

Resistin seems to be a disease activity marker of pulmonary inflammation in smokers, asthma, and chronic obstructive pulmonary disease (Al Mutairi et al., 2011; Esbah et al., 2011; Leivo-Korpela et al., 2011; Gürsoy et al., 2012). Cigarette smoke activates macrophages to secrete proinflammatory cytokines TNF-α, IL-1β and IL-6 (Barnes, 2008). Nicotine, in turn, reduces the production of the same proinflammatory cytokines (Arnson et al., 2010). All in all, associations between smoking and cytokine alterations have so far been inconclusive (Clendenen et al., 2011; Shiels et al., 2014).

The role of resistin in antipsychotic treatment or schizophrenia has been little studied (Birkás Kováts et al., 2005; Perez-Iglesias et al., 2008; Oh et al., 2012; Kawabe et al., 2015). In the study by Birkás Kováts et al. patients taking second-generation antipsychotics had significantly higher resistin levels than healthy controls. Resistin levels correlated positively with levels of TNF-α, insulin and HOMA-IR (Birkás Kováts et al., 2005). The study by Perez-Iglesias et al. showed no changes in levels of resistin nor associations between levels of resistin and weight gain after one year of antipsychotic treatment with haloperidol, olanzapine or risperidone (Perez-Iglesias et al., 2008). Clozapine has been found to inhibit resistin mRNA expression in mouse brown adipocytes (Oh et al., 2012). The applicability of that finding in human cells is not clear, especially considering that monocytes and macrophages are the major sources of resistin in man (Kwon and Pessin, 2013).

2.3.8 Adipsin

Adipsin is an adipokine expressed by adipocytes and monocytes-macrophages (White et al., 1992; Fantuzzi, 2005). In obese subjects adipsin levels are either elevated or unchanged (Napolitano et al., 1994; Fantuzzi, 2005). In a study by Derosa et al. levels of adipsin correlated positively with BMI and HOMA-IR in healthy obese vs. non-obese subjects (Derosa et al., 2013). Adipsin may play a role in fat cell metabolism and/or energy homeostasis (Raucci et al., 2013). Adipsin plasma level may predict changes in abdominal subcutaneous fat during times of increased energy intake. Genetic variation at the adipsin locus may play a role in individual response to food intake inducing weight gain, increase of subcutaneous fat, and increase in plasma levels of leptin (Ukkola et al., 2003).

2.3.9 Neuropeptide Y

Neuropeptide Y (NPY) is a 36-amino acid neuropeptide belonging to the pancreatic polypeptide family, which includes peptide tyrosine–tyrosine (PYY) and pancreatic polypeptide (PP) (Bi et al., 2012). NPY is a key regulator of energy homeostasis and appetite in hypothalamus. Arcuate nucleus (ARC) NPY neurons are activated by signals indicating reduced energy availability, such as decreased levels of circulating glucose, leptin or insulin, which increase NPY release (Brown and Clegg, 2010). The arcuate nucleus of the hypothalamus is an integrating center of energy homeostasis. ARC NPY neurons are the main source of hypothalamic NPY (Mercer et al., 2011). Animal studies suggest an association between ARC NPY overexpression and obesity, and elevated serum NPY levels (Sousa-Ferreira et al., 2011). NPY synthesis and release in ARC NPY neurons is regulated by several peripheral hormones and central hypothalamic peptides. Among the receptors expressed in ARC NPY neurons are insulin receptor, leptin receptor, ghrelin receptor, adiponectin receptors AdipoR1 and AdipoR2, orexin receptor 1, serotonin receptor 5-HT_{1B}, NPY receptor Y2 and melanocortin receptors MC3R and MC4R (Mercer et al., 2011; Kageyama et al., 2012; Sobrino Crespo et al., 2014).

Neuropeptide Y binds in hypothalamus at the receptors NPY1R, NPY2R and NPY5R. NPY1R and NPY5R are expressed in several hypothalamic nuclei mediating the orexigenic effects of NPY. NPY2R is an autoreceptor mainly expressed in ARC. NPY2R also mediates the anorexigenic effects of peripheral PYY (Mercer et al., 2011; Sohn et al., 2013; Zhang et al., 2014). NPY1R is most directly involved in food intake and energy expenditure (Zhang et al., 2014). Moreover, NPY1R mediates anti-inflammatory actions against obesity associated inflammation and insulin resistance in immune cells (Farzi et al., 2015). In a rodent model NPY5R has been found to mediate obesity and adipose tissue insulin resistance induced by NPY overexpression in paraventricular nucleus (Long et al., 2015).

Plasma NPY levels have been found to be associated with several features of metabolic syndrome, obesity (Baranowska et al., 2005; Baltazi et al., 2011; Sitticharoon et al., 2013), lipid status (Nyström et al., 1996), type 2 diabetes mellitus (Milewicz et al., 2000b; Ilhan et al., 2010), and hypertension (Solt et al., 1990; Baltazi et al., 2011). NPY has also been found to mediate stress-induced abdominal obesity and metabolic syndrome (Kuo et al., 2007). Moreover, NPY plasma levels have been found to be elevated in patients with

bulimia nervosa independent of BMI (Baranowska et al., 2001), and to increase in response to ghrelin stimulation (Coiro et al., 2006). The association between obesity and NPY levels is somewhat inconclusive, with some reports of a converse correlation (Milewicz et al., 2000a; Orbetzova et al., 2012).

NPY acts in the stimulation of food intake, and regulation of hypothalamic-pituitary-gonadal (HPG) hormone axis function. Leptin and serotonin (5-hydroxytryptamine, 5-HT) both inhibit hypothalamic NPY production. Atypical antipsychotics could produce elevated NPY production through 5-HT receptor antagonism resulting in weight gain (Fitzgerald et al., 2003; Reynolds et al., 2006). Clozapine has been found to produce an increase in NPY immunoreactivity in rat ARC, but haloperidol not (Kirk et al., 2006). Olanzapine exposure has also been found to upregulate NPY in rat ARC (Fernø et al., 2011). However, in a study by Raposo et al. NPY serum levels correlated negatively with levels of olanzapine among male patients treated with olanzapine or haloperidol (Raposo et al., 2011). In a Turkish sample of first-episode patients the NPY levels of patients increased after olanzapine treatment, but risperidone treatment had no effect. Interestingly, the NPY levels of patients were lower than the levels of the control group both pre- and post-treatment. However, the BMI of the patients was also somewhat lower, and they had reported loss of appetite before entering the study (Ak et al., 2013; Yanik et al., 2013). In a recent study by Wysokinski NPY levels were not associated with clozapine treatment, obesity or metabolic parameters in patients with schizophrenia compared with healthy controls, although there was a gender difference among clozapine-treated patients, with higher NPY levels in female patients (Wysokinski, 2015).

There is also some evidence, that NPY level alterations may be associated with schizophrenia risk, with findings of lowered CSF NPY-like immunoreactivity, reduced brain NPY concentrations, and reduced brain NPY mRNA expression (Inoue et al., 2009). Findings of differing regional alterations between atypical (clozapine and olanzapine) and typical (haloperidol) antipsychotics in rat brain NPY mRNA expression may reflect the differences between these drugs in their effects in treating positive and negative symptoms of schizophrenia (Huang et al., 2006). NPY levels in CSF have also been reported to correlate with social competence and may predict some aspects of longitudinal outcome in patients with schizophrenia, possibly reflecting susceptibility to stress (Stålberg et al., 2014). NPY is also involved in coping

with stress exposure, anxiety, and post-traumatic adaptation (Reichmann and Holzer, 2016).

2.3.10 Obesity-related alterations in adipokine secretion and development of insulin resistance

An overview of the mechanisms leading to insulin resistance and metabolic syndrome is presented in Figure 1. Overnutrition leads to expansion of adipose tissue, increased size and number of adipocytes, and macrophage infiltration. These taken together lead to increased free fatty acid release, dysregulated adipokine secretion from adipocytes, and increased release of inflammatory cytokines from macrophages. Dysregulated secretion of these adipokines has adverse effects on various tissues, which further increase food intake and reduce energy expenditure, leading to the development of systemic insulin resistance and associated metabolic consequences (Harwood, 2012).

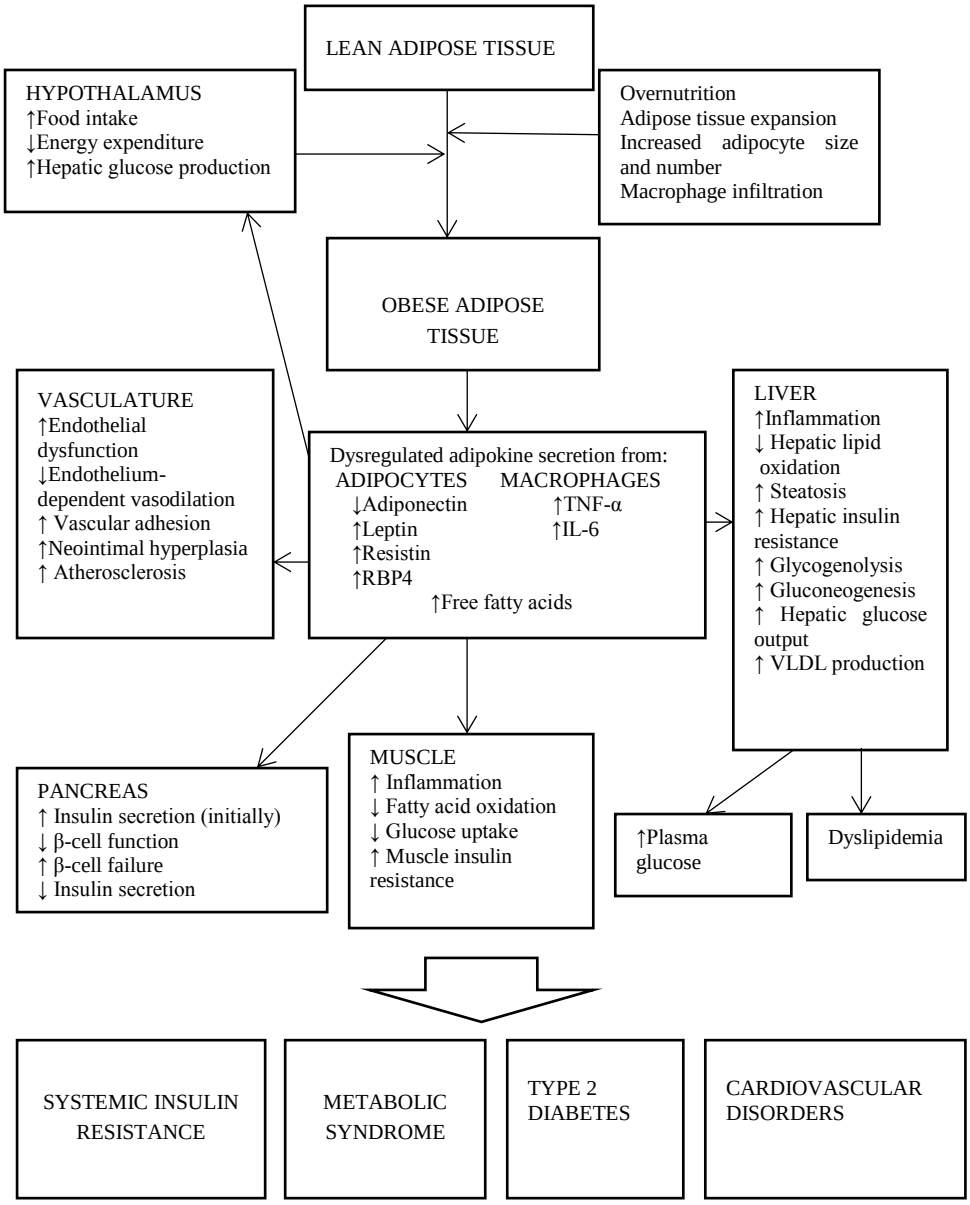

Figure 1. Obesity-related alterations in adipokine secretion and development of insulin resistance (Modified from Harwood, 2012).

2.4 Genetics of antipsychotic-induced weight gain

2.4.1 Genetic polymorphism

DNA has a double-helix structure consisting of four nucleotides (adenine, guanine, thymine and cytosine). The combination and order of these four nucleotides code for the functions of genes. The human genome has about 3 billion pairs of these nucleotides, of which more than 99% is identical between individuals. The individual sequence variations in less than 1% of the genome are called polymorphisms. In genetic studies two kinds of polymorphisms are commonly used, variable number tandem repeats (VNTRs), also known as microsatellites, and single-nucleotide polymorphisms (SNPs). SNPs are single-nucleotide substitutions in a particular position of a DNA sequence. Of all SNPs discovered 1-2% are functional, resulting in amino acid change (Kendler and Eaves, 2005).

Genetic variation research has developed from studies of single polymorphisms to genome-wide association studies (GWAS). GWAS enables us to study the possible associations between a disease and the majority of common variants, while genotypes at several hundred thousand variant sites and associated linkage disequilibrium (LD) are available to test (1000 Genomes Project Consortium et al., 2010). Recent technological developments make it possible to sequence the whole genome/exome for the analysis of unknown causative mutations (Kato, 2015).

Impact of genetic variation on antipsychotic-induced weight gain has been widely studied during the recent years. Several genes have been found to associate with AIWG. The strongest associations have been found with leptin gene *LEP* -2548A/G (rs7799039) and serotonin receptor 5-HT_{2C} gene *HTR2C* -759C/T (rs3813929) polymorphisms (MacNeil and Müller, 2016).

2.4.2 Leptin and leptin receptor gene

According to a recent meta-analysis, polymorphisms in *LEP* (rs2167270, rs7799039) and *LEPR* (rs1137101, rs1137100, rs8179183, rs62589000) gene are not associated with obesity susceptibility in general population (Yu et al., 2012). However, several original studies have suggested that variations in the leptin gene, especially SNP *LEP* -2548A/G (rs7799039) may have an impact on

weight during antipsychotic treatment (Mou et al., 2008; Zhang et al., 2003; Arranz et al., 2011; Kuo et al., 2011; Wu et al., 2011; Brandl et al., 2012; Kang et al., 2014). Some negative results have also been reported (Gregoor et al., 2009; Fernández et al., 2010; Opgen-Rhein et al., 2010; Gregoor et al., 2011). *LEP* rs1137101 has also been reported to be associated with AIWG (Shams and Müller, 2014). Leptin receptor *LEPR* gene polymorphism Q223R (rs1137101) has been associated with risk of obesity in women with a psychotic disorder on antipsychotic medication (Gregoor et al., 2011).

2.4.3 Serotonin receptor HTR2C gene

Serotonin receptor *HTR2C* -759C/T (rs3813929) polymorphism has been linked with antipsychotic-induced weight gain in several studies (Miller et al., 2005; Arranz et al., 2011; Opgen-Rhein et al., 2010; Sicard et al., 2010; Wu et al., 2011), but negative findings have also been reported (Houston et al., 2012). C-allele of the -759C/T polymorphism and G-allele of the *LEP* -2548A/G polymorphism together may be associated with obesity and metabolic syndrome in patients on atypical antipsychotics (Yevtushenko et al., 2008; Gregoor et al., 2010). Some studies have found no associations between *HTR2C* -759C/T polymorphism and metabolic syndrome in patients taking antipsychotic medication (Mulder et al., 2007a; Risselada et al., 2012).

HTR2C rs1414334 C-allele carriership has been associated with an increased risk of metabolic syndrome in patients taking antipsychotics, especially clozapine and risperidone (Mulder et al., 2009; Risselada et al., 2012). However, the association between rs1414334 polymorphism and obesity is still inconclusive (Mulder et al., 2007a; Mulder et al., 2007b; Mulder et al., 2009; Risselada et al., 2012).

The homozygote haplotype A (-759C (rs3813929, -697G (rs518147), 23Cys) has been found to be more common in clozapine treatment related obesity (Gunes et al., 2009) and seems to be a high-risk haplotype for AIWG (Sicard et al., 2010). Three *HTR2C* SNPs in strong linkage disequilibrium, rs6318, rs2497538 and rs1414334, have been found to be associated with greater weight gain in women but not in men in olanzapine-treated patients with mood disorder (Houston et al., 2012). *HTR2C* rs498177 has been found to be associated with metabolic syndrome in female patients with schizophrenia on atypical antipsychotic medication (olanzapine, clozapine, risperidone), whereas haplotype A-C of rs521018-rs498177 decreased the risk of metabolic syndrome

in female patients (Bai et al., 2011). Rs498207 has been reported to be associated with antipsychotic-induced weight gain as well (Opgen-Rhein et al., 2010).

2.4.4 Adiponectin gene

ADIPOQ gene locus has been reported to be the only major gene for plasma adiponectin (Heid et al., 2010). Adiponectin gene *ADIPOQ* +276G/T (rs1501299) polymorphism has been suggested to be associated with the risk of obesity (Siitonen et al., 2011; Yu et al., 2012) and cardiovascular diseases (Leu et al., 2011; Gui et al., 2012; Zhang et al., 2012). Associations between antipsychotic-related changes in weight and *ADIPOQ* gene have also been reported (Jassim et al., 2011). The G-allele of +276G/T polymorphism may be a risk allele for antipsychotic-induced weight gain (Li et al., 2009; Wu et al., 2011).

2.4.5 Neuropeptide Y gene

Studies on NPY and NPY receptor gene polymorphisms have yielded some findings regarding genetic susceptibility to antipsychotic-induced weight gain (Lett et al., 2012; Shams and Müller, 2014). Tiwari et al. found associations between antipsychotic-induced weight gain and *NPY* polymorphisms rs16147, rs5573 and rs5574 in patients of European ancestry treated mostly with clozapine or olanzapine (Tiwari et al., 2013). SNP rs16147 (also named C399T, C485T and C454T) has also been found to be associated with NPY plasma levels (Shah et al., 2009), serum leptin levels and body fat distribution in women (Mutschler et al., 2013), anxiety and depressive symptoms possibly associated with prefrontal *NPY* gene expression (Sommer et al., 2010), ischemic stroke (Kim et al., 2010), and tobacco dependence (Mutschler et al., 2012). The association with obesity susceptibility is somewhat inconclusive (Yeung et al., 2011; Olza et al., 2013; Zain et al., 2015), with possible interaction with dietary fat (Lin et al., 2015). *NPY* gene polymorphism rs16147 may not be linked to schizophrenia susceptibility (Itokawa et al., 2003; Wang et al., 2005; Lindberg et al., 2006; Inoue et al., 2009).

NPY polymorphism rs16139 (also called T1128C and Leu7Pro) has been found to be associated in animal models with alterations in the synthesis and

secretion of NPY (Mitchell et al., 2008). Higher serum levels of total and LDL-cholesterol in obese subjects (Karvonen et al., 1998), coronary artery atherosclerosis (Ilveskoski et al., 2008), insulin resistance and risk for type 2 diabetes mellitus (Ukkola and Kesäniemi, 2007), plasma levels of inflammatory molecules (E-selectin, insulin, amyloid P, CRP) and NPY in diabetes patients (Jaakkola et al., 2010), and metabolic syndrome (Masoudi-Kazemabad et al., 2013) have likewise been reported to be associated with rs16139. The association between rs16139 and risk of obesity is inconclusive (Aberle et al., 2008; Pesonen, 2008; Bhaskar et al., 2010; Yeung et al., 2011, Zain et al., 2015).

Findings of some other *NPY* gene polymorphisms have also been reported in population based studies. Rs17149106 and rs5574 have been reported to be associated with susceptibility to obesity (Yeung et al., 2011; Zain et al., 2015). Rs16131 has been suggested to be associated with risk of obesity, and with features of metabolic syndrome (Olza et al., 2013). Rs 9785023 (also called -1258G/A and Ser50Ser) has been reported to be associated with alcohol consumption (Francès et al., 2011).

2.4.6 Neuropeptide Y receptor genes

NPY1R gene has been associated with obesity and features of metabolic syndrome (Marrades et al., 2010; Li et al., 2014). Various polymorphisms in *NPY2R* gene (rs11099992, rs17304901, rs2234759, rs10212868, rs12641982, rs17376826, rs6857715, rs1047214, rs6857530, rs12649641, rs2342676) have been associated with weight in several studies (Lavebratt et al., 2006; Torekov et al., 2006; Campbell et al., 2007; Siddiq et al., 2007; Zhang et al., 2009; Friedlander et al., 2010; Takiguchi et al., 2010; Hunt et al., 2011). However, Wang et al. found no evidence for an association between seven common variants of *NPY2R* gene and early onset obesity in German children (Wang et al., 2007). *NPY2R* gene has also been associated with type 2 diabetes in men (Campbell et al., 2007). Some studies have suggested *NPY5R* gene associations with dyslipidemia (rs11100493, rs12501691, P1, rs11100494, rs12512687) (Coletta et al., 2007) and susceptibility to obesity (P1, P2, rs12512687 (P3), Y5R1c52) (Jenkinson et al., 2000; Li et al., 2014).

2.4.7 Other genes associated with antipsychotic-induced weight gain

There are some other replicated genetic polymorphisms associated with AIWG including synaptosomal-associated protein 25kDA (*SNAP25*) gene (Ddell, Mnll, Tail), adrenoreceptor alpha 2A (*ADRA2A*) gene (-1291C/G) and G-protein receptor β3-subunit (*GNB3*) gene (-825C/T) (Brandl et al., 2014). Moreover, associations indicating effect on AIWG has been reported on dopamine receptor D_2 (*DRD2*) gene (rs4436578, rs6277, rs1079598, rs1800497) (Hong et al., 2010; Müller et al., 2012), insulin-induced gene 2 (*INSIG2*) (rs17587100, rs10490624, rs17047764) (Le Hellard et al., 2009), cholecystokinin B receptor (*CCKBR*) gene (rs2929183, microsatellite CTn) (Tiwari et al., 2010), dopamine D_3 receptor (*DRD3*) gene (Ser9Gly), methylenetetrahydrofolate reductase (*MTHFR*) gene (rs1801133, rs1801131), serotonin transporter (*SLC6A4*) gene (S/L alleles), neurexin 3 (*NRXN3*) gene (rs11624704, rs7154021), cannabinoid receptor 1 (*CNR1*) gene (rs806378, rs1049353), nuclear-encoded mitochondrial genes (rs6435326, rs1053517, rs1801318), Glucagon-like peptide-1 (GLP-1) gene (*GCG*) (rs13429709), and GLP-1 receptor (*GLP1R*) gene (rs3799707, rs4714210, rs2268639). Recent reports have reported an effect in the region of melanocortin 4 receptor (*MC4R*) gene (rs17782313, rs11872992 rs489693, rs8087522) on the development of obesity and antipsychotic-induced weight gain (Shams and Müller, 2014). Most recently polymorphisms in *IL-1B* (rs1143634) and brain-derived neurotrophic factor (*BDNF*) (Val66Met) genes have been associated with AIWG (Bonaccorso et al., 2015; Fonseka et al., 2015). However, according to an association study of 60 candidate genes with 233 SNPs by Ryu et al., none of the SNPs showed a statistically significant association with BMI or appetite change after eight weeks of antipsychotic treatment. Ghrelin (*GHRL*) gene polymorphism showed trend-like association with weight gain and appetite (Ryu et al., 2016). Recent genome-wide association studies have reported also some other, although not yet replicated, AIWG associated polymorphisms: Meis homeobox 2 (*MEIS2*), protein kinase, cyclic adenosine monophosphate-dependent, regulatory, type II, beta (*PRKAR2B*), G protein-coupled receptor 98 (*GPR98*), formin homology 2 domain containing 3 (*FHOD3*), ring finger protein 144A (*RNF144A*), astrotactin 2 (*ASTN2*), sex determining region Y-box 5 (SOX5), and activating transcription factor 7 interacting protein 2 (*ATF7IP2*) genes (Brandl et al., 2014).

3 Aims of the Study

There is a need for biomarkers for antipsychotic-induced weight gain, which would be sensitive and specific enough for metabolic syndrome and disturbed food intake behaviours. It is currently unknown to what extent obesity and metabolic comorbidity in patients with schizophrenia are associated with medication, genetic background, or schizophrenia-related defect in satiety regulation.

The specific aims of the study were:

1. To investigate inflammatory cytokine and adipokine (IL-6, IL-1Ra, TNF-α, hs-CRP, adiponectin, leptin, adipsin, resistin) serum level alterations in relation to components of metabolic syndrome (weight gain, BMI, insulin resistance, lipid profile) and associations between these inflammatory markers in clozapine treated patients with treatment resistant schizophrenia (Studies I, II and III).
2. To explore the associations of serum levels of these markers among smokers and non-smokers in this patient group (Study III).
3. To test the possible role of serum neuropeptide Y level alterations as a marker of weight gain in association with metabolic and inflammatory markers in patients with schizophrenia on clozapine treatment (Study IV).
4. To test the associations of some SNPs of *LEP*, *ADIPOQ* and *HTR2C* genes on levels of leptin and adiponectin and weight gain (Study II), and to explore whether polymorphisms in *NPY* gene, NPY receptor genes and genes encoding ARC NPY neuron receptors are associated with NPY serum levels in this patient group (Study IV).

4 Materials and methods

4.1 Patients and data collection

The study was conducted in three hospital districts in Western Finland (Satakunta, Pirkanmaa, and Seinäjoki). The patients were recruited at secondary in and outpatient clinics and from sheltered accommodation units between April 2008 and January 2010. The inclusion criteria for the study were: 1. Stabilized clozapine medication, and 2. Clinical diagnosis of F2 group according to ICD-10. The exclusion criteria were organic brain disease or other neurological disease. The diagnosis was set by experienced psychiatrists in clinical settings. Of the 256 patients screened, 19 declined. All patients completed a questionnaire eliciting, among others, estimated weight and height, trend in weight change (marked increase, slight increase, no change, decrease), weight gain in kilograms during clozapine treatment (in cases of marked increase) and smoking (not smoking, not smoking daily, smoking daily n cigarettes). The body mass index (BMI) was calculated by the physicians collecting the data using formula weight(kg)/(height(m))^2. Information on past medical history and duration of clozapine treatment was collected from patient records. The study was approved by the ethics committees of Satakunta and Pirkanmaa Hospital Districts, and these approvals applied to all three sites. All patients gave informed consent on entry to the study. The blood samples were taken during a routine laboratory visit related to the clozapine treatment. Of the 237 patients entering the study, blood samples for laboratory analysis of the metabolic markers were available from 190 patients (Figure 2.).

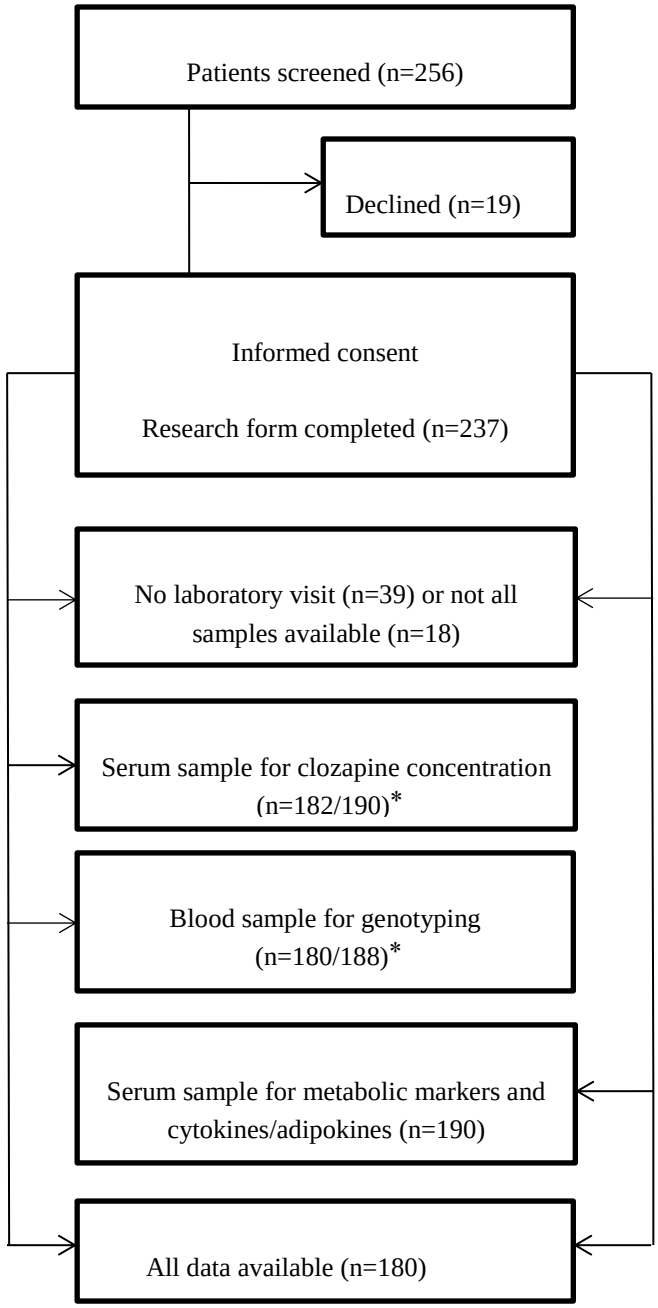

Figure 2. Patient screening (Modified from Seppälä, 2014).

*Number of samples available from the patients included in the present study/ total number of samples.

4.2 Controls

Historical control samples from two Finnish population based studies were used as references for clinical markers and cytokine/ adipokine levels in Study I. The samples were a subsample of the Finnish Health 2000 survey, n=502, with mean age 58 years (Malo et al., 2011), and a population based sample from one Finnish town, n=903 (403 men and 500 women), with mean age 46 years, and subjects with hs-CRP>10mg/l excluded (Ahonen et al., 2012). For the genotype analysis in Study II, the control population consisted of healthy Finnish blood donors, n=395 (215 men, 180 women) with mean age 45 years. Blood donors complete a health questionnaire and are also interviewed by a nurse to elicit their possible medications and chronic illnesses every time they donate blood (Hänninen et al., 2008).

4.3 Laboratory and clinical methods

For the laboratory analysis venous blood samples were collected after over-night fasting. Serum was separated and stored at -80 °C until analyzed. Glucose, insulin, HDL-cholesterol, and triglycerides were measured with Cobas c6000 e601 (Roche Diagnostics Ltd, Rotkreuz, Switzerland) with detection limits of 0.11 mmol/l, 0.2 µU/ml, 0.08 mmol/l and 0.1 mmol/l respectively. Levels of adipsin, adiponectin, resistin, leptin, IL-6, IL-1Ra, and TNF-α were measured by enzyme linked immunosorbent assay (ELISA) with the following reagents: DuoSet® ELISA (R&D Systems Europe Ltd, Abingdon, UK) for adipsin, adiponectin, resistin and leptin, Ready-SET-GO!® ELISA (eBioscience Inc., San Diego, CA, USA) for IL-6, and Quantikine® for IL-1Ra and Quantikine® HS for TNF-α (R&D Systems). Detection limits for adipsin, adiponectin, resistin, leptin, and IL-1Ra were 15.6 pg/ml, and for IL-6 and TNF-α 0.78 and 0.5 pg/ml, respectively. Hs-CRP was measured using Tinaquant C-reactive protein (latex) high sensitive assay (Roche Diagnostics) with detection limits 0.05-10 mg/l. Peptides were first extracted from serum samples using C18 SEP-COLUMNs, and NPY levels in the reconstituted samples were measured by ELISA according to instructions from Phoenix Pharmaceuticals Inc. (Burlingame, CA, USA) with detection limit of 10 pg/ml and interassay coefficient of variation of 8.8%.

Undetectable IL-6 levels (41 cases) were considered to be 0 pg/ml and included in the statistical analysis. Missing serum cytokine values (NPY, n=10; IL-6 and IL-1Ra, n=3; TNF-α, n=2) due to limited amount of serum were excluded from the analysis.

Insulin resistance index assessed by homeostasis model assessment (HOMA-IR) was calculated using the formula insulin (mU/l) x glucose (mmol/l)/22.5 (Matthews et al., 1985).

The individual criteria for metabolic syndrome (glucose ≥5.6 mmol/l, HDL-cholesterol <1.0 mmol/l males/ 1.3 mmol/l females, triglycerides ≥1.7 mmol/l) were defined according to the definition by the International Diabetes Federation (IDF) Task Force on Epidemiology and Prevention, the National Heart, Lung and Blood Institute, the American Heart Association, the World Heart Federation, the International Atherosclerosis Society and Association for the Study of Obesity (Alberti et al., 2009).

4.4 Gene selection (Studies II and IV)

Three SNPs previously reported to be associated with antipsychotic-induced weight gain or metabolic syndrome, *LEP* -2548A/G (rs7799039), *ADIPOQ* +276G/T (rs1501299) and *HTR2C* rs1414334 were selected for the association study on serum levels of leptin and adiponectin, and weight gain (II).

For the association analysis of NPY (IV), genes encoding NPY and NPY receptors NPY1R, NPY2R and NPY5R were included. The receptors expressed in arcuate nucleus NPY neurons were selected according to recent reviews (Mercer et al., 2011; Kageyama et al., 2012; Sobrino Crespo et al., 2014). The gene selection was supplemented with a PMC search for papers published between January 1, 2011 and November 30, 2015. All ARC NPY neuron receptors referred to in these publications were included. The PMC search phrases used were: “arcuate” or “ARC” and “npy neuron” and “receptor” and “feeding” and “food intake” and “appetite” and publication date from 01.01.2011 to 30.11.2015. This yielded 847 publications. One hundred and sixteen papers including information on possible target genes were selected for thorough reading according to title and abstract. The receptors and corresponding genes selected (n=21) were ghrelin receptor *GHSR*, leptin receptor *LEPR*, insulin receptor *INSR*, adiponectin receptors *ADIPOR1* and *ADIPOR2*, orexin/hypocretin receptor 1 *OX1R/HCRTR1*, serotonin receptor

HTR1B, melanocortin receptors *MC3R* and *MC4R*, α7 nicotinic acetylcholine receptor *CHRNA7*, α4β2 nicotinic acetylcholine receptor subcomponents *CHRNA4* and *CHRNB2*, glucocorticoid receptor (GR) *NR3C1*, N-methyl-D-aspartate receptor 1 (NMDAR1) *GRIN1*, corticotropin-releasing factor receptor 1 *CRHR1*, and peroxisome proliferator-activated receptor gamma *PPARG*. Moreover, glucose transporter (GLUT2) gene *SLC2A2* was included in the association study, as the effects of glucose on ARC NPY neurons are mediated through Glut-2-dependent glucose-sensitive neurons.

4.5 DNA extraction and genotyping

For DNA extraction, 9.0 ml EDTA-whole blood was taken from the participants and stored in a freezer at -20 °C. Genomic DNA was extracted from peripheral blood leukocytes using QIAamp®DNA Blood Minikit and automated biorobot M48 extraction (Qiagen, Hilden, Germany). Genotyping of rs1501299, rs1414334 and rs7799039 was performed using Taqman®SNP Genotyping Assays (assays; C___7497299_10; C___7455701_10 and C___1328079_10 respectively) and ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). For the analysis of NPY neuron genes the samples were genotyped using Illumina Infinium HumanCoreExome-12 DNA Analysis Beadchip version 1.0., according to the manufacturer's recommendation at Helmholtz Zentrum, Munich, Germany. The following quality control filters were applied: GenCall score < 0.15, GenTrain score < 0.20, sample and SNP call rate < 0.95, Hardy-Weinberg equilibrium p-value < 10^{-6}, excess heterozygosity, cryptic relatedness (pi-hat > 0.2), gender check and MDS outliers. After quality control, 180 samples and 531,983 SNPs were available. SNPs with a minor allele frequency of less than 0.01 were excluded from the study.

4.6 Statistical analysis

Distributions of each cytokine or adipokine marker variable were checked manually to control for possible technical errors in laboratory assays. In continuous variables, absolute values of standard deviation ≤+2 skewness were considered normal distributions, and the highest individual values, if needed, were omitted as outliers. Depending on the variable, 0-7 outlier cases were

omitted from the data. This procedure also controlled for multivariate outliers. The statistical significance of correlations between markers was set at p=0.003-0.005, depending on the analysis run, using the Bonferroni correction by dividing the significance level of p=0.05 by the number of markers analyzed. In all other statistical analyses the level of significance was set at $p<0.05$ (I-IV).

Comparison of means between genders, between patients and controls, and between subgroups were analyzed with two-tailed Student's or Welch's t-tests (I-IV). Due to the asymmetric distribution, logarithmic transformation was used for levels of HDL, triglycerides, and HOMA-IR in Study II. Effect size was determined as Cohen's d for leptin and adiponectin levels between different subgroups (II). One-way ANOVA was used to test the association between *HTR2C* rs1414334 genotype and BMI, and levels of leptin and adiponectin (II). Mann-Whitney U test was used to test the associations between *HTR2C* GG-genotype or C-carriers, and leptin, adiponectin, adipsin, and BMI levels in both genders separately (II), and for the comparison of hs-CRP levels between smokers and non-smokers (III).

Pearson's chi-square ($\chi 2$) test was used to compare numbers of genotypes and allele frequencies between patients and controls (II), and to compare the number of smokers and non-smokers between male and female patients (III).

Pearson's correlation was used to analyze the associations between clinical markers and cytokines/adipokines (I-IV). Due to asymmetric distributions of levels of NPY, triglycerides, HOMA-IR, IL-1Ra, TNF-α and hs-CRP, the logarithmic transformation of these markers was used in the bivariate analysis of correlations (IV), and in the bivariate analysis of correlations between levels of resistin and IL-1Ra and hs-CRP (III). Partial correlations were calculated for both genders separately, controlling the confounding factors age and smoking (I).

Logistic regression analyses with backward stepwise method were used to test the effects of cytokines/adipokines as predictors for single metabolic syndrome criteria according to Alberti et al. (2009), and controlling for potential confounding factors gender, age and smoking status in these models (I).

In the power calculations adiponectin, leptin, and BMI differences of ±0.84, ±16.2, and ±3.14 respectively, between *HTR2C* rs1414334 C-carriers or GG-genotype, ±0.68, ±13.1, and ±2.55 between *ADIPOQ* rs1501299 T-carriers or GG-genotype, and ±0.87, ±16.8, and ±3.26 between *LEP* rs7799039 genotypes GG, AG or AA were discernible with a probability of 0.8 (II).

General linear univariate model (GLM, ANCOVA) was used to analyze the explanatory factors (weight increase/no increase, gender, and SNPs *LEP* rs7799039 and *ADIPOQ* rs1501299) and a covariate (BMI) for leptin and adiponectin levels (II). BMI as a covariate and gender and rs1414334 genotype as factors were used to explain triglyceride levels (II). GLM was used to analyze the explanatory factors (regular smoking, IL-1Ra level, hs-CRP level) for levels of resistin. Several other factors (age, gender, BMI, clozapine dose, clozapine concentration, IL-6 level, triglyceride level, number of cigarettes/day) were also tested in this model (III). GLM was used to analyze the effects of explanatory factors for levels of neuropeptide Y. The best-fitting model comprised resistin level, insulin level, BMI, age and gender as explanatory factors. Several other factors and covariates (leptin, adiponectin, HOMA-IR, IL-1Ra, hs-CRP, IL-6, HDL-cholesterol, triglycerides) were explored as explanatory variables (IV). Data analysis was carried out using SPSS/Win software (version 19.0, SPSS inc., Chicago, IL, USA), GraphPad QuickCalcs (GraphPad Software Inc., La Jolla, CA, USA), and Power and Sample Size Calculator (Dupont and Plummer, 1998).

GLMs were used to analyze the effects of selected explanatory factors gender, SNPs encoded separately with additive, dominant and recessive coding and covariates (triglycerides, HDL-cholesterol, HOMA-IR, weight change and age) for NPY serum level (IV). The model used an inverse normal transformation of the NPY serum level to form a symmetrical distribution. The significance levels of explaining variables in the GLMs were adjusted using the false discovery rate (FDR) method for multiple testing, which accounts for the number of SNPs. Additive, dominant and recessive models were coded as follows. Where a polymorphism contained two alleles, for example G and g, in the additive model serum level of NPY is thought to rise cumulatively according to the number of G alleles, whereas in the dominant model serum level of NPY is thought to be associated with GG and Gg genotypes, and in the recessive model serum level of NPY is thought to be associated with gg genotype (IV). LD (linkage disequilibrium) and haploblocks were defined using the confidence intervals method (Gabriel et al. 2002). The statistical significance level was set at $p<0.05$.

Statistical analyses were performed using R (version 3.1.2, 2014, The R Foundation for Statistical Computing), SPSS (version 22, IBM inc.), gPLINK (version 2.050), and PLINK (version 1.07). Haploview (version 4.2) was used for LD plot illustrations (IV).

4.6.1 Genetic risk score

Genetic risk scores (GRSs) were calculated for each individual as a weighted sum of the risk alleles in relation to serum level of NPY (IV). First, all SNPs that had an unadjusted level of significance, $p<0.05$, for serum level of NPY were selected from the GLM. These selected SNPs were inserted into the same model as other covariates and analyzed using Akaike information criteria (AIC) (Akaike 1981). To avoid overfitting, only tag SNPs among SNPs with high linkage disequilibrium (LD) were selected. SNPs remaining in the model, as indicated by AIC, were used in the GRS. For all SNPs analyzed in the study, a linkage disequilibrium (LD) test was performed with 1 Mbp window and a threshold of $r^2=0.2$. The model returned by AIC was analyzed with stepwise VIF to examine collinearity between the SNPs in the GRS. For each SNP, the corresponding B-estimate value from the GLM was used as the adjusting weight for the individual effects of a SNP in the GRS. This score was used as an explanatory variable in the final GLM, together with triglycerides, HDL-cholesterol, HOMA-IR, weight change, and age (IV).

Linear combination of genetic effects estimated from univariate models may over-fit data and cause inflated type I errors. To avoid this type of error, an estimation of the null distribution of the GRS test statistics is critical (Hu et al., 2013). Therefore a permutation test was performed by sampling patient ID numbers from the phenotype data, resulting in SNP genotypes that were independent of study phenotype (serum level of NPY) (IV). The permutation was conducted 500 times with the sampled data, using the method described above. The GRS was calculated for each sampled data set and an association analysis with GLM was performed. If the p-value derived from the actual data analysis was smaller than 5 % of the p-values derived from the permutated data, then the result was considered statistically significant (IV).

5 Results

Of the 190 patients, 109 (57.4%) were male and 81 (42.6%) were female. Mean age of the patients was 42.92 years (range 20-67 years). The average time from the first hospital admission was 17.67 years (range 2-42 years). One hundred and eleven patients (58.4%) had been on clozapine treatment for more than five years, 60 patients (31.6%) from one to five years and four patients (2.1%) from three to twelve months. Those fifteen patients with no data on treatment duration available were recruited from long-term care units. They had all been on clozapine treatment for more than one year. One hundred and twenty-one patients (63.7%) were on clozapine monotherapy, and 69 patients (36.3%) had a combination therapy with other antipsychotics. The mean clozapine dose was 403 mg/day (range 100-800mg/day). The mean BMI was 29.92 kg/m^2. One hundred and one patients (53.2%) were regular smokers (smoking daily) with median 20 cigarettes per day. Smoking status did not differ between genders and was not associated with BMI.

5.1 Cytokine and adipokine alterations (Study I)

The levels of metabolic markers and cytokines/adipokines are presented in Table 3. BMI and levels of adiponectin, triglycerides, and HDL-cholesterol differed between genders among the patient population. The mean (SD) BMI was 28.99 (5.81) vs. 31.18 (6.87) kg/m^2 ($p=0.025$) in males vs. females. The mean serum adiponectin, males vs. females, was 2.91 (1.39) vs. 3.76 (1.84) µg/ml ($p=0.001$) and HDL-cholesterol 1.06 (0.29) vs. 1.28 (0.36) mmol/l ($p<0.001$) respectively. Triglyceride levels in males 2.17 (1.17) mmol/l were higher than in females 1.67 (0.78) mmol/l ($p=0.001$). Correlations between BMI, lipids, and levels of cytokines/adipokines were calculated separately for both genders (Table 4). Low adiponectin levels were associated with high triglycerides and low HDL-cholesterol levels in both genders and with high BMI and high IL-1Ra levels in males. High IL-1Ra levels were associated with high BMI and high triglycerides in both genders, and with high HOMA-IR in

the whole patient sample. Likewise high IL-1Ra levels were associated with low adiponectin levels in males and high levels of IL-6 and hs-CRP in females. IL-6 levels correlated positively with BMI and IL-1Ra in females and with hs-CRP levels in males. Hs-CRP levels correlated positively with BMI and levels of IL-1Ra in females and with levels of IL-6 in males.

Table 3. Levels of metabolic markers and cytokines/adipokines, and comparisons* between patients and historical controls.

Marker	Patients	Controls	P Value
Age	43 (11), n=190	46 (6), n=903	
BMI kg/m²,men	29.0 (5.8), n=105	26.7 (3.7), n=403	<0.001
BMI kg/m², women	31.2 (6.9), n=77	26.3 (4.9), n=500	<0.001
HDL mmol/L, men	1.1 (0.3), n=108	1.3 (0.3), n=403	<0.001
HDL mmol/L, women	1.3 (0.4), n=82	1.5 (0.3), n=500	<0.001
Trigly mmol/L, men	2.2 (1.2), n=108	1.7 (1.3), n=403	<0.001
Trigly mmol/L, women	1.7 (0.8), n=81	1.2 (0.6), n=500	<0.001
Glucose mmol/L, men	6.0 (1.7), n=108	5.9 (0.6), n=403	0.17
Glucose mmol/L, women	5.8 (1.7), n=80	5.6 (0.5), n=500	0.07
Adiponectin µg/ml, men	2.91 (1.39), n=108	4.87 (2.70), n=403	<0.001
Adiponectin µg/ml, women	3.76 (1.84), n=77	7.87 (4.36), n=500	<0.001
IL-1Ra pg/ml, men	470.58 (246.42), n=102	172.27 (127.70), n=403	<0.001
IL-1Ra pg/ml, women	534.85 (293.83), n=77	191.49 (152.58), n=500	<0.001
hs-CRP mg/L, men	3.58 (5.28), n=106	1.33 (1.48), n=403	<0.001
hs-CRP mg/L, women	4.71 (5.04), n=82	1.48 (1.45), n=500	<0.001
Age	42.9 (41.4-44.5), n=190	57.9 (57.2-58.6), n=502	
HOMA-IR, all °	4.7 (10.0), n=190	1.6 (1.6), n=502	
IL-6 pg/ml, all °	2.2 (2.1), n=187	1.6 (1.3), n=502	

*T-test

Data are means (SD) or medians (interquartile range)°.

Control data from Ahonen et al., 2012, except control data of HOMA-IR and IL-6 as medians and IQR, and age as mean (95% confidence interval) from Malo et al., 2011.

Table 4. Correlations° between metabolic markers and levels of IL-1Ra, IL-6, hs-CRP, and adiponectin (* p≤0.001).

Marker	IL-1Ra	IL-6	hs-CRP	Adiponectin
BMI, men	0.45*	0.12	0.16	-0.42*
BMI, women	0.48*	0.45*	0.37*	-0.26
HOMA-IR, all patients	0.24*	0.05	0.079	-0.17
HDL, men	-0.27	-0.14	-0.02	0.49*
HDL, women	0.25	0.08	0.02	0.51*
Trigly, men	0.39*	0.17	-0.04	-0.49*
Trigly, women	0.36*	0.30	0.16	-0.53*
IL-1Ra, men	1	0.11	0.04	-0.37*
IL-1Ra, women	1	0.40*	0.37*	-0.25
IL-6, men	0.11	1	0.42*	0.02
IL-6, women	0.40*	1	0.27	-0.13
hs-CRP, men	0.04	0.42*	1	0.06
hs-CRP, women	0.37*	0.27	1	0.04
Adiponectin, men	-0.37*	0.02	0.06	1
Adiponectin, women	-0.25	-0.13	0.04	1

°Pearson's correlation

After controlling for the effects of confounding factors age and smoking status, adiponectin correlated positively with HDL-cholesterol (males r=0.56, p<0.001, females r=0.49, p<0.001) and negatively with triglyceride levels (males r=-0.35, p=0.002, females r=-0.61, p<0.001). Instead, the correlations between adiponectin and IL-1Ra levels, as well as between adiponectin levels and BMI among male patients disappeared. IL-1Ra correlated positively with BMI (males r=0.45, p<0.001, females r=0.53, p<0.001), among male patients negatively with HDL-cholesterol (r=-0.32, p=0.006) and among female patients positively with hs-CRP (r=0.39, p=0.002). IL-6 levels correlated positively with BMI among female patients (r=0.40, p=0.001). Hs-CRP levels correlated positively with BMI (males r=0.41, p<0.001, females r=0.35, p=0.004).

The results of the logistic regression concerning the associations between cytokines/adipokines and single metabolic syndrome criteria (glucose ≥5.6 mmol/l, HDL-cholesterol <1.0 mmol/l males/ 1.3 mmol/l females, triglycerides ≥1.7 mmol/l) after potential confounding factors gender, age and smoking are presented in Table 5.

Table 5. Associations between cytokines/adipokines and single metabolic syndrome criteria* according to three logistic regression models.

Target variable	Correctly classified (total)	Explaining variable°	P	OR	95% CI
Glucose ≥5.6mmol/L	69.0%	Age	0.003	1.05	1.02-1.08
		Adiponectin	0.004	0.73	0.58-0.91
		IL-1Ra	0.03	1.00	1.000-1.002
		IL-6	0.11	0.91	0.81-1.02
HDL-Cholesterol <1.0(m) or <1.3(f) mmol/L	64.7%	Adiponectin	<0.001	0.65	0.52-0.80
Triglycerides ≥1.7mmol/L	71.2%	Female gender	0.09	0.55	0.28-1.1
		Adiponectin	<0.001	0.55	0.42-0.72
		IL-1Ra	0.05	1.00	1.000-1.002

*Alberti et al., 2009

° Explaining variables at first step: gender, age, smoking status, levels of serum adiponectin, IL-1Ra, IL-6, and hs-CRP.

5.2 Associations between HTR2C, leptin and adiponectin genes and corresponding serum levels (Study II)

The mean BMI (SD) males (n=109) vs. females (n=81) was 29.00 (5.79) vs. 31.20 (6.91) kg/m², p=0.021 (Data error in Study I). One hundred and one (55.8%) patients (48 men and 53 women) reported weight gain during clozapine treatment. Eighty (44.2%) patients (54 men and 26 women) reported their weight either unchanged or decreased. The mean BMI of those who had reported weight gain was higher than that of those reporting no weight gain. Among female patients this difference in the mean (SD) was 33.26 (7.04) vs. 27.36 (5.15) kg/m^2, p<0.001. Among male patients the mean BMI was 31.11 (6.14) vs. 27.59 (4.75) kg/m^2, p=0.002, between those patients who reported weight gain and those who did not.

Leptin levels were higher in female patients, mean (SD), male vs. female, 13.47 (16.13) vs. 47.86 (37.11) ng/ml, p<0.001, Cohen's d=1.27. Women with weight gain had higher leptin levels than women with no weight gain, 58.81 (38.58) vs. 27.64 (23.67) ng/ml, p<0.001, Cohen's d=0.90. Among male patients the corresponding difference in leptin levels was 17.66 (20.90) vs. 10.11 (9.97) ng/ml, p=0.026, Cohen's d=0.47. The levels of leptin also correlated with (males, females) BMI (r=0.68, p<0.001, r=0.65, p<0.001), levels

of adipsin (r=0.39, p<0.001, r=0.44, p<0.001), and levels of IL-1Ra (r=0.50, p<0.001, r=0.53, p<0.001). In female patients leptin also correlated with levels of IL-6 (r=0.47, p<0.001), and with weight gain in kilograms among those reporting a marked weight increase (r= 0.47, p=0.006, n=33). Neither of those were found among male patients. In male patients leptin correlated with levels of triglycerides (r=0.34, p<0.001), but not in female patients.

Adiponectin levels were lower in men with weight gain than in men without weight gain, mean (SD) 2.56 (1.04) vs. 3.09 (1.48) µg/ml, p= 0.037, Cohen's d=0.41. Among women there was no such difference in levels of adiponectin between those who gained weight and those who did not.

Adipsin levels, mean (SD), were among males 1.27 (0.30) µg/ml and among females 1.30 (0.31) µg/ml. Levels of adipsin correlated with levels of IL-1Ra (r=0.23, p=0.002) and IL-6 (r=0.21, p=0.004) in the total patient population. In both genders adipsin levels correlated with levels of leptin (males r=0.39, p<0.001, females r=0.44, p<0.001). In male patients adipsin levels correlated with IL-1Ra (r=0.28, p=0.004), and in female patients with IL-6 (r=0.40, p<0.001). After Bonferroni correction, there was a trend for an association between levels of adipsin and BMI among female patients (r=0.30, p=0.008).

The univariate analysis of variance (GLM) for explanatory factors of leptin and adiponectin levels is presented in Table 6. In GLM the complete model explained 62.1% of the variance in leptin levels. BMI explained 36.2%, gender 29.1%, and gender and weight gain together 3.3% of the variance in leptin levels. Gender dependent leptin levels in association with BMI adjusted weight change are presented in Figure 3. Of the variance in adiponectin levels the complete model explained 27.5%, BMI explained 7.8%, gender 8.7%, and gender and weight gain together 4.3%.

Table 6. Two ANCOVA models using serum levels of adiponectin (1) and leptin (2) as target variables and gender, weight change and *ADIPOQ* rs 1501299 (1) or *LEP* rs7799039 (2) genotype as factors and BMI as covariate.

	Adiponectin serum level			Leptin serum level		
	ηp2 °	p	power	ηp2 °	p	power
Complete model	0.275	<0.001	1	0.621	<0.001	1
Explanatory variables						
BMI	0.078	<0.001	0.94	0.362	<0.001	1
Gender	0.087	<0.001	0.96	0.291	<0.001	1
Weight change*	0.006	0.36	0.15	0.024	0.06	0.47
Gender x Weight change	0.043	0.01	0.73	0.033	0.02	0.62
ADIPOQ genotype	0.010	0.48	0.17			
LEP genotype				0.016	0.30	0.26

*weight increase/no weight increase

° partial eta squared, explanatory proportion

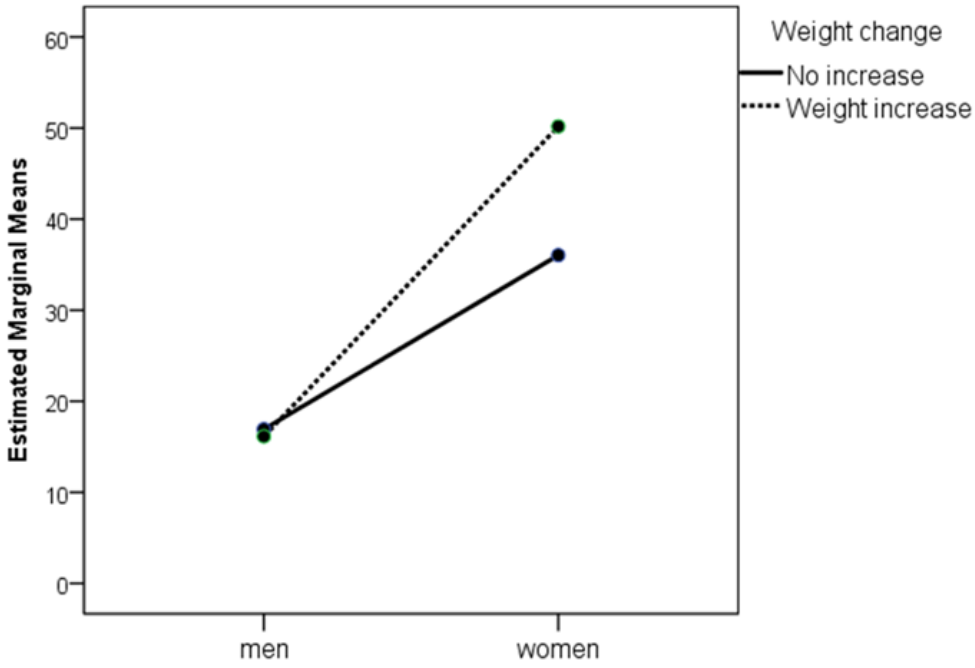

Figure 3. Gender dependent serum leptin level (ng/ml) alterations associated with BMI adjusted weight change according to the GLM model (Table 6.).

Adiponectin, leptin and adipsin serum levels, and BMI between *LEP* rs7799039, *ADIPOQ* rs1501299 and *HTR2C* rs1414334 genotypes are presented in Table 7. *ADIPOQ* rs1501299 genotype frequencies did not differ between patients and healthy controls (n=395). Frequency of *HTR2C* polymorphism rs1414334 CC genotype was 9.6% among patients compared with 3.7% among controls (p=0.013). BMI, weight increase, HOMA-IR and levels of adiponectin, leptin and IL-1Ra were not associated with rs1414334 genotype. Instead, rs1414334 GG genotype was associated with higher levels of triglycerides (p=0.001) and lower levels of HDL cholesterol (p=0.048). In GLM the complete model explained 21.7% of the triglyceride levels, (p<0.001). BMI explained 14.4% (p<0.001), gender 3.4% (p=0.016) and *HTR2C* rs1414334 genotype 3.4% (p=0.017).

Table 7. Serum adipsin, adiponectin and leptin levels, and body mass index, mean (SD) or median (IQR), between *LEP* rs7799039, *ADIPOQ* rs1501299 and *HTR2C* rs1414334 genotypes.

		S-adipsin µg/ml	S-adiponectin µg/ml	S-leptin ng/ml	BMI kg/m^2
LEP	GG (n=40)	1.31 (0.36)	3.55 (1.87)	28.41 (32.38)	29.3 (6.4)
	AG (n=86)	1.26 (0.28)	3.06 (1.36)	26.95 (31.89)	29.2 (5.6)
	AA (n=53)	1.31 (0.31)	3.29 (1.82)	29.36 (34.22)	31.2 (7.4)
ADIPOQ	GG (n=89)	1.25 (0.29)	3.12 (1.40)	27.71 (30.46)	30.2 (6.1)
	T-carrier (n=91)	1.31 (0.33)	3.37 (1.83)	28.41 (34.46)	29.5 (6.6)
HTR2C					
Men	GG* (n=92)	1.27 (0.36)	2.54 (1.41)	8.53 (12.21)	[1]27.6 (7.6)
	C-carrier* (n=15)	1.11 (0.29)	2.67 (2.78)	7.40 (8.27)	26.6 (5.4)
Women	GG* (n=51)	1.22 (0.4)	3.50 (2.17)	41.56 (42.83)	30.5 (9.5)
	C-carrier* (n=22)	1.34 (0.38)	3.21 (2.10)	42.33 (53.11)	29.8 (9.4)
All	GG (n=143)	1.28 (0.30)	[2]3.14 (1.54)	26.48 (30.68)	30.0 (6.3)
	C-carrier (n=37)	1.29 (0.34)	3.67 (1.91)	34.20 (38.43)	29.0 (6.9)

[1] p=0.08 between genotypes (Mann-Whitney U-test)

[2] p=0.08 between genotypes (t-test)

All other comparisons between genotypes p>0.10

*Median, interquartile range (IQR)

5.3 Resistin as an inflammatory marker (Study III)

The mean (SD) serum level of resistin among patients was 27.06 (9.24) ng/ml. Age, gender, BMI, clozapine dose, clozapine concentration, HOMA-IR, or levels of triglycerides were not associated with resistin levels. Resistin serum

levels correlated in the whole patient group with levels of IL-1Ra (r=0.41, p<0.001), and in male patients negatively with HDL-cholesterol (r=-0.29, p=0.003). Levels of resistin, after Bonferroni correction, also tended to correlate in the whole patient group with hs-CRP (r=0.20, p=0.005) and TNF-α (r=0.15, p=0.037). The comparisons of serum resistin levels between subgroups according to age, gender, BMI, smoking, and clozapine treatment are presented in Table 8. The correlations between serum resistin level and other metabolic and inflammatory markers are given in Table 9.

Table 8. Serum resistin levels between subgroups°.

Subgroup		n	Resistin mean (ng/ml)	SD
Age	20-43 years	95	27.49	9.31
	44-65 years	94	26.74	9.17
Gender	Men	109	26.81	9.29
	Women	81	27.39	9.21
BMI (kg/m^2)	<30	104	26.83	9.06
	≥30	78	27.26	9.80
Regular smokers*		101	29.39	10.07
Non-smokers		87	24.54	7.43
Clozapine	monotherapy	121	27.58	9.38
	combination	69	26.13	8.97
Cloz.+norcloz	<2.2	88	26.42	8.02
concentration	≥2.2	94	26.71	9.54
Clozapine	<5 years	64	27.36	9.42
duration	≥5 years	111	26.76	9.34

°T-test

* p<0.001. All other group comparisons p>0.05.

Table 9. Correlations (r) between levels of serum resistin and other metabolic and inflammatory markers.

Resistin	r all (n=190)	r men (n=109)	r women (n=81)
HOMA-IR	0.09°		
HDL-cholesterol*		-0.29,p=0.003	-0.15°
log10 Trigly*		0.07°	0.09°
Adipsin	0.09°		
Adiponectin*		-0.10°	-0.14°
Leptin*		-0.01°	0.10°
IL-1Ra	0.41, p<0.001 (n=179)		
log IL-1Ra	0.35, p<0.001 (n=186)		
IL-6	0.09° (n=187)		
TNF-α	0.15, p=0.037 (n=186)		
log TNF-α	0.17, p=0.023 (n=186)		
hs-CRP	0.20, p=0.005 (n=188)	0.17, p=0.085 (n=107)	0.25, p=0.027
log hs-CRP	0.21, p=0.005 (n=188)		

*Correlations calculated separately for both genders due to differed levels between genders.
° p>0.05

The mean (SD) levels of resistin between smokers and non-smokers in all patients were 29.39 (10.07) vs. 24.54 (7.43) ng/ml (p<0.001), among smoking vs. non-smoking male patients 29.65 (9.87) vs. 23.51 (7.29) ng/ml (p=0.001), and among female patients 29.00 (10.46) vs. 25.75 (7.51) ng/ml (p=0.11), respectively. Among smokers serum resistin level correlated positively with levels of IL-1Ra (r=0.47, p<0.001) and hs-CRP (r=0.35, p<0.001), Figure 4. Among non-smokers no associations were found between levels of resistin and the other cytokines/adipokines studied. In GLM, with explanatory factors smoking, IL-1Ra and hs-CRP, the complete model explained 16.6% (p<0.001), interaction of smoking and hs-CRP explained 13.7% (p<0.001), and IL-1Ra 2.7% (p=0.026) of the variance of resistin levels.

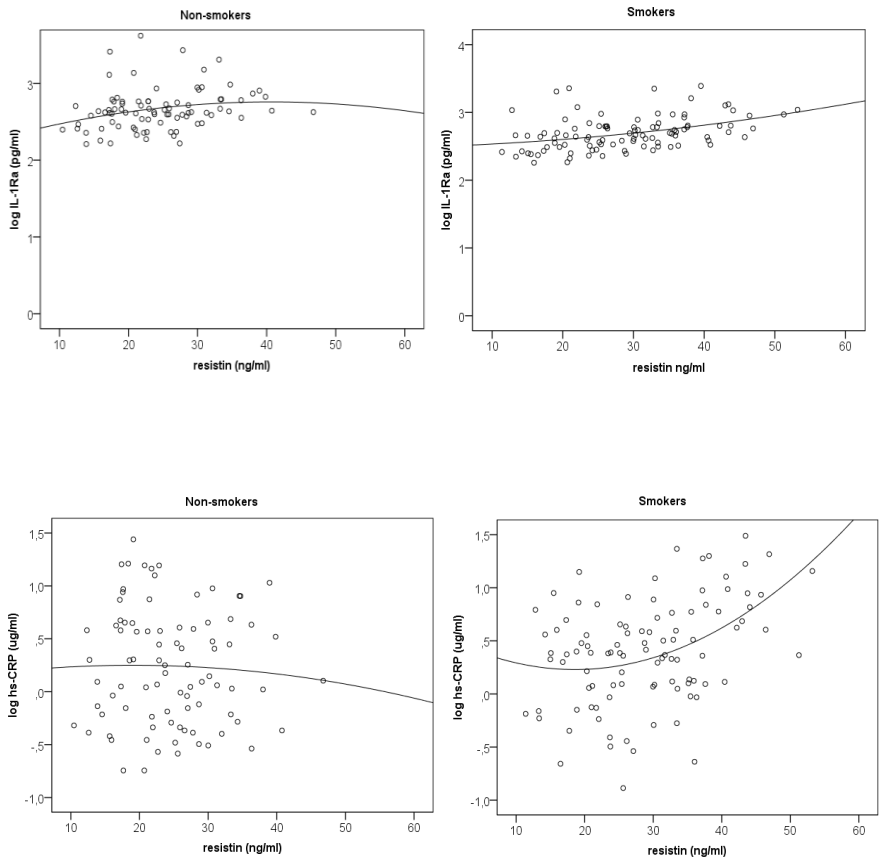

Figure 4. Scatters between serum IL-1Ra and resistin (upper row) and hs-CRP and resistin (lower row) in non-smokers and smokers.

Smoking was also associated in all patients with higher levels of hs-CRP. The mean (SD) levels of hs-CRP among smokers and non-smokers were 4.48 (5.50) and 3.64 (4.85) mg/l (p=0.041, Mann-Whitney U-test). Among male patients smoking was also associated with higher levels of IL-6, mean (SD) smokers vs. non-smokers 3.04 (2.69) vs. 2.10 (2.07) pg/ml (p=0.046).

5.4 Associations between serum neuropeptide Y levels, arcuate nucleus NPY neuron receptor gene variation and weight gain (Study IV)

Serum NPY level in the patient sample was, median (IQR), 111.00 (105.50) pg/ml. Of these 180 patients 104 (57.8%) were male and 76 (42.2%) were female. Mean age of these patients was 43 years.

The serum NPY levels correlated with levels of resistin ($r=0.31$, $p<0.001$) and age ($r=0.22$, $p=0.003$). On the contrary, serum NPY levels did not correlate with BMI, weight change, smoking, clozapine concentration, clozapine dose, antipsychotic medication in chlorpromazine-equivalents, nor serum levels of adipsin, adiponectin, leptin, glucose, insulin, HDL-cholesterol, triglycerides, IL-1Ra, IL-6, TNF-α, hs-CRP, or HOMA-IR either.

In GLM the best-fitting model explained 18.0% ($p<0.001$) of the variance of serum NPY levels. Explanatory factors in this model were age, serum resistin level, serum insulin level, BMI and gender. Resistin level explained 9.4% ($p<0.001$), age 7.0% ($p=0.001$) and insulin level 2.4% ($p=0.044$).

According to LD analysis only one of the SNPs in the GRS was in over 0.2 LD compared with all other SNPs in the study: *CHRNA7* rs1909884 and *CHRNA7* kgp10538347 are in 0.23 LD. This can be considered low. All predictor variables had a VIF lower than the threshold, which was set at 10, meaning there is no considerable collinearity between the SNPs or other variables.

In the GLM analysis with gender, triglycerides, HDL-cholesterol, HOMA-IR, weight change and age as confounding factors and covariates, none of the SNPs tested from the following genes *NPY*, *NPY1R*, *NPY2R*, *NPY5R*, *GHSR*, *LEPR*, *INSR*, *ADIPOR1*, *ADIPOR2*, *OX1R/HCRTR1*, *HTR1B*, *MC3R*, *MC4R*, *CHRNA7*, *CHRNA4*, *CHRNB2*, *NR3C1*, *GRIN1*, *CRHR1*, *PPARG* and *SLC2A2* (Total number of SNPs 215) had a significant effect on serum NPY concentrations after adjustment. The SNPs with unadjusted $p<0.05$ are presented in Table 10. The genetic risk score (GRS_{NPY}) analysis found twelve SNPs with $p<0.05$ (Table 10.). In the further GLM analysis with aforementioned confounding factors and covariates, the GRS_{NPY} showed a highly significant association with NPY concentrations ($p<2x10^{-16}$). However, after validation of the score with 500 permutations the effect of GRS_{NPY} on serum NPY concentrations remained nonsignificant ($p=0.078$).

Table 10. SNPs with unadjusted p<0.05.

Gene	SNP	Coding	Estimate (B)	Unadjusted p
*CHRNA7**	kgp10538347	Recessive	1.11	0.002
*INSR**	rs3786681	Recessive	-0.50	0.003
*NR3C1**	rs4912916	Additive	-0.51	0.009
*NR3C1**	rs246430	Dominant	0.34	0.009
NR3C1	rs246430	Additive	0.68	0.009
CHRNA7	rs1909884	Additive	0.31	0.012
CHRNA7	rs904951	Dominant	0.23	0.012
*CHRNA7**	exm2260416	Additive	0.38	0.013
*LEPR**	rs10749753	Dominant	0.22	0.014
*NPY**	rs16131	Additive	0.43	0.014
CHRNA7	rs11071503	Additive	0.40	0.014
*NR3C1**	rs325260	Dominant	0.20	0.014
NR3C1	rs325260	Additive	0.32	0.015
LEPR	rs6697315	Recessive	-0.24	0.016
*CHRNA7**	kgp9391258	Recessive	-0.34	0.017
CHRNA7	exm2260416	Dominant	0.21	0.020
CHRNA7	rs11071503	Dominant	0.22	0.020
NR3C1	rs4912916	Dominant	-0.24	0.021
CHRNA7	rs904951	Additive	0.30	0.022
NR3C1	rs4912916	Recessive	-1.10	0.025
CHRNA7	rs11637923	Additive	0.45	0.028
NPY	rs16131	Dominant	0.20	0.029
*CHRNA7**	rs1909884	Dominant	0.18	0.030
*INSR**	newrs1799816	Dominant	-0.54	0.030
INSR	newrs1799816	Additive	-1.07	0.030
CHRNA7	kgp10538347	Additive	0.39	0.030
NR3C1	rs17100500	Additive	-0.25	0.035
CRHR1	exm1330732	Additive	-0.78	0.039
NPY	rs16131	Recessive	1.00	0.043
LEPR	rs9436748	Dominant	0.17	0.043
NPY	rs16478	Recessive	-0.30	0.044
*NR3C1**	rs1445873	Recessive	0.50	0.044
NR3C1	rs11948121	Additive	-0.23	0.048
NR3C1	rs17100500	Recessive	-0.24	0.048

*SNPs included in the GRS according to significance level p<0.05 in the GLM model

6 Discussion

6.1 Cytokine and adipokine alterations in patients with schizophrenia treated with clozapine (Studies I, II and III)

The presence of metabolic risk factors and morbidity among the present patients with schizophrenia on clozapine treatment was considerable. These risk factors, with corresponding prevalences, included overweight/obesity (BMI ≥30 kg/m^2: 43%), smoking (53%), hypertriglyceridemia (triglycerides ≥1.7 mmol/l: 52%), low HDL-cholesterol (HDL <1.0/1.3 mmol/l: 54%), hyperglycemia (glucose ≥5.6 mmol/l: 47%), high HOMA-IR, low adiponectin levels, elevated hs-CRP levels (hs-CRP ≥5 mg/l: 23%), and elevated IL-1Ra levels. According to a meta-analysis by Mitchell et al. the corresponding prevalences among patients with schizophrenia in general were: overweight 44%, smoking 54%, hypertriglyceridemia 39%, low HDL-cholesterol 43%, and hyperglycemia 19% (Mitchell et al., 2013). Historical control samples from two published Finnish population based studies were used as references in the present study (Malo et al., 2011; Ahonen et al., 2012). Gender differences were found in many of the cytokines and adipokines studied as expected.

Weight gain during clozapine treatment explained most of the obesity of the present patients. Women with weight gain had the highest leptin levels and the difference in these between female patients with or without weight gain was noteworthy. Among the present male patients weight gain was associated with low adiponectin levels. According to earlier findings adiponectin levels have been found to correlate negatively with visceral adiposity (typical for male obesity) to a greater extent than with subcutaneous adiposity (Gustafson, 2010). It has been suggested that the regulation of adiponectin in the visceral adipose tissue of men is more sensitive to catecholamine-stimulated lipolysis with an association of higher nonesterified fatty acids and lower adiponectin levels in obese men only (Plaisance et al., 2009).

Elevated IL-1Ra level was a sensitive marker of metabolic comorbidity in the present patient population. IL-1Ra level associated with insulin resistance, obesity, and lipid status. This elevation of IL-1Ra levels may be due in part to

schizophrenia itself and partly to associated factors such as smoking, other living habits, and medication (Potvin et al., 2008). In the Finnish Health 2000 study sample elevated IL-1Ra levels were not associated with schizophrenia after controlling for confounding factors (Suvisaari et al., 2011).

The levels of leptin were associated with IL-1Ra levels in both genders, with levels of triglycerides in male patients, and with IL-6 levels in female patients. These results are in line with findings from a Swedish general population sample. In that sample leptin was associated with factors for metabolic syndrome in men whereas in women leptin was associated with inflammatory factors. These findings suggest different regulatory mechanisms and effects of leptin in men and women (Andreasson et al., 2012). In a study by Beumer et al. (2012) the effect of schizophrenia on leptin levels was reported to be noticeable in females only. An earlier study by Wang et al. (2007) suggests also a possible gender-specific leptin dysregulation in antipsychotic-naïve patients with schizophrenia. A meta-analysis of longitudinal studies by Potvin et al. (2015) found no gender difference in antipsychotic-induced leptin changes, but only a few studies included in the meta-analysis had performed analyses for each sex separately. A more recent meta-analysis of cross-sectional studies by Stubbs et al. (2016) reported elevated levels of leptin in patients with schizophrenia compared to controls with higher levels in female and multi-episode patients. The present findings support a gender-dependent leptin dysregulation among these patients. Estrogen regulates body weight by controlling food intake, energy expenditure, and body adiposity. Estrogen deficiency is associated with leptin resistance and an increase of visceral adiposity (Shi et al., 2009). There is also an interaction between estrogen and several other orexigenic (NPY, ghrelin, MCH) and anorexigenic (insulin, serotonin, cholecystokinin) neuropeptides (Brown and Clegg, 2010; Asarian and Geary, 2013). It has been suggested that serotonin 5-HT_{2C} receptor mediates the anorectic effects of leptin. Blocking this receptor may interrupt the inhibitory effect of leptin on NPY neurons. Clozapine is a high-affinity 5-HT_{2C} receptor antagonist (Kirk et al., 2006). Additionally estrogen deficiency may also have some role in the pathophysiology of schizophrenia (Hayes et al., 2010; Lee et al., 2013). Estrogen pathway has been suggested to be also involved in the therapeutic effect of antipsychotics (Polymeropuolos et al., 2009).

In the present study female patients' high IL-6 levels were associated with both obesity and levels of leptin, which was not the case in male patients. IL-6 has been found to increase leptin secretion, which is associated with obesity in

the general population (Harwood, 2012). In general population circulating levels of IL-6 are associated especially with visceral adiposity (Gustafson, 2010). It can be assumed that the visceral fat component of weight gain would be particularly increased in the present female patients. However, data on patients' waist circumference was not available. In general, excess adiposity in the central visceral region of the body (usually typical for male obesity) correlates with increased risk for and mortality from metabolic disorders (Shi et al., 2009). Based on studies on healthy adults it seems that there is only a very limited obesity-related increase in IL-6 levels in women (Kuo and Halpern, 2011). Thus the obesity related inflammation in the present female patients may be related either to the gender specific effects of schizophrenia itself or to clozapine treatment. However, the basic mechanisms behind the gender differences in IL-6 levels remain unclear. In all, the IL-6 levels of the present patients were close to the values reported in general populations. This more likely indicates either that the present patients with schizophrenia were on a stable state of illness (Miller et al., 2011a) or a possible suppressing effect of long-term clozapine treatment on IL-6 levels (Lu et al., 2004; Sugino et al., 2009).

In general population studies the associations between hs-CRP and obesity have been more apparent in women than in men (Rossi et al., 2012) and appear to be related to pronounced accumulation of subcutaneous fat especially in women (Cartier et al., 2009). These gender differences in the association between hs-CRP and adiposity may be partially mediated by the secretion of leptin (Rossi et al., 2012). In a recent study by Joseph et al. (2015) higher hs-CRP levels in patients with schizophrenia or schizoaffective disorder compared to matched controls were associated with female gender, more severe negative symptoms, greater medical comorbidity, and worse metabolic risk factors such as BMI, glucose and hemoglobin A1C. In the present patients, however, hs-CRP was associated with BMI also among male patients after controlling for age and smoking status.

Adiponectin levels were low in the present patient group. This replicates the findings of Hanssens et al. (2008). In the present study, the multivariate analyses (Study I) suggested that low adiponectin levels associated with the development of metabolic syndrome, hypertriglyceridemia, low HDL-cholesterol and high glucose levels, after potential confounding factors age, gender and smoking were taken into account. Among male patients low adiponectin levels were also associated with obesity and high IL-1Ra levels. This concurs with a general population study where obese men had lower

adiponectin levels than lean men or lean and obese women (Plaisance et al., 2009). However, according to an earlier report based on studies on healthy (no metabolic syndrome) lean to obese adults of both genders, no correlations between plasma adiponectin and BMI or gender difference in plasma adiponectin levels were found (Kuo and Halpern, 2011). Accordingly, in the present study the associations between low adiponectin levels, and obesity and high IL-1Ra levels among male patients disappeared after controlling for age and smoking status. In a study by Matsuda et al. (2005) among non-diabetic patients with schizophrenia plasma adiponectin correlated negatively with BMI and with HOMA-IR in men, but not in women. In a recent study by Sapra et al. (2016) decreased serum adiponectin levels in male patients with schizophrenia were associated with insulin resistance compared to age and BMI matched healthy male controls. No such association with HOMA-IR among male patients was found in the present study. Accordingly, weight gain and truncal fat accumulation have been reported to be associated with decline of adiponectin, especially in male patients with schizophrenia on atypical antipsychotic treatment and free of MetS when entering the study (Oriot et al., 2008). The present results suggest that hypoadiponectinemia may be a potential biomarker of metabolic syndrome in this population, especially in male patients. However, the role of adiponectin as a predictor of nascent metabolic syndrome could not be investigated with this kind of study design.

A study by Wampers et al. reported adiponectin increase in risperidone-treated patients and a decrease in olanzapine treated patients independent of BMI and MetS. The association between olanzapine treatment and adiponectin decrease would suggest a specific effect of olanzapine on adipose tissue (Wampers et al., 2012). In addition, association between olanzapine treatment and lowered adiponectin levels have been reported in several studies (Togo et al., 2004; Richards et al., 2006; Sugai et al., 2012; Wampers et al., 2012; Bartoli et al., 2015b). Effect of clozapine treatment on serum adiponectin levels seems to be similar to that of olanzapine treatment (Bartoli et al., 2015a). The present results concerning adiponectin levels are in line with some earlier studies suggesting a suppressing effect of clozapine on adipose tissue (Bai et al., 2007; Hanssens et al., 2008; Oh et al., 2012).

Adipsin levels were linked to levels of leptin in both genders. Moreover, among female patients adipsin levels were associated with levels of IL-6. Levels of IL-6 were associated with obesity among the present female patients, but not among males. Adipsin levels tended to be associated with BMI in female

patients. However, no gender difference in mean levels of adipsin was found. This may indicate that adipsin would play a role in gender dependent response to food intake, and as a predictor of changes in fat distribution. Whether adipsin and its genetic variation plays a role as a predictor of obesity related inflammation needs to be studied further.

Levels of resistin were associated with levels of IL-1Ra in the whole patient population. Trend-like associations were also found between resistin and hs-CRP and TNF-α. This supports resistin's role as an inflammatory marker. In male patients high resistin levels were associated with low levels of HDL-cholesterol. An association between high levels of resistin and low HDL-cholesterol has also been reported in general population (Gupta et al., 2011; Cabrera de León et al., 2014). This finding may reflect the dietary pattern of the present male patients. Low fruit and vegetable content in diet is associated with low-grade inflammation (Barbaresko et al., 2013). Contrary to an earlier study on patients treated with atypical antipsychotics (Birkás Kováts et al., 2005), no association between levels of resistin and HOMA-IR was found in the present patients. Moreover, there were no associations between levels of resistin and other adipokines as previously associated with clozapine treatment and metabolic abnormalities in people with schizophrenia (Bartoli et al., 2015a, Bartoli et al., 2015b, Potvin et al., 2015).

6.2 Smoking associated inflammation in patients with schizophrenia (Study III)

The present smokers had higher levels of resistin than non-smokers, especially among male patients. Among smokers levels of resistin were also associated with higher IL-1Ra and hs-CRP levels. Among present smoking men levels of IL-6 were also higher than among non-smoking men. Patients with COPD and asthma have higher levels of resistin than healthy controls, and resistin levels of smokers are similar to those of patients with inflammatory obstructive airway disease (Al Mutairi et al., 2011). Accordingly, the prevalence of smoking has been found to be higher in those subjects with the highest levels of resistin, and resistin levels have been found to be higher in smoking men than in non-smoking men, but the levels were not associated with CRP (Esbah et al., 2011; Cabrera de León et al., 2014).

Smoking, obesity, physical inactivity, airway inflammation and obstruction, and adipose tissue and inflammatory marker activation are all related to systemic inflammation phenomena (Clini et al., 2013). Among the present male patients smoking was also associated with higher levels of IL-6. In the general population, smoking seems to be associated with visceral fat accumulation (Nakanishi et al., 2014) and insulin resistance (Chiolero et al., 2008). Visceral adipose tissue produces more IL-6 and resistin than does subcutaneous adipose tissue (Harwood, 2012). Excess central visceral adiposity is associated with increased risk and mortality from metabolic disorders (Shi et al., 2009). Among patients with schizophrenia, too, smoking has been associated with metabolic syndrome (Yevtushenko et al., 2008).

In the study by Zhang et al. levels of IL-6, and also levels of IL-2, were lower among smoking than among non-smoking male patients with schizophrenia. Smokers had also fewer positive symptoms, and smoking a greater number of cigarettes correlated with fewer negative symptoms. The authors suggested nicotine-induced suppression of inflammatory responses in schizophrenia (Zhang et al., 2008). However, the association between smoking and IL-6 levels was the opposite in the present male patients. There may be some confounding factors explaining difference between these results, such as variations in body weight and fat distribution, psychiatric status and genetic factors. The present patients were more obese than the patients in the study by Zhang et al., who were also chronically hospitalized, and less than half of them were on clozapine treatment. Moreover, the patients were of different ethnic origin (Zhang et al., 2008). The clozapine concentrations in the present patients did not differ between smokers and non-smokers, although smokers had higher clozapine doses (Seppälä et al., 2014). Nor were clozapine concentrations associated with levels of resistin in the present patients.

6.3 Neuropeptide Y in clozapine induced weight gain (Study IV)

The NPY serum levels of the present patients were associated with their levels of resistin. Resistin is considered to be a mediator in several obesity related inflammatory states, insulin resistance, atherosclerosis, and carcinogenesis. It has also been suggested that resistin is associated specifically with the initial stages of metabolic syndrome (Codoñer-Franch and Alonso-Iglesias, 2015). Animal studies have shown associations between centrally infused resistin and

hypothalamic expression of NPY (Singhal et al., 2007; Vázquez et al., 2008; Cifani et al., 2009). Neuropeptide Y, like resistin, is associated with insulin resistance (Macia et al., 2012; Güneş and Bukan, 2015). Both of these have several immunomodulatory functions (Farzi et al., 2015). Serum insulin level appeared to be also an explanatory variable of serum NPY level in the present GLM analysis. This concurs with earlier reports of associations between plasma NPY levels and insulin levels in patients with type 2 diabetes mellitus (Ilhan et al., 2010). This association between circulating NPY and insulin may be explained by the effect of insulin on hypothalamus, or by increased NPY secretion from adipocytes by insulin stimulation (Kos et al., 2007; Ilhan et al., 2010). An association between serum NPY levels and age like that among the present patients has been reported also in earlier studies (Solt et al., 1990).

Serum neuropeptide Y level was not associated with obesity or weight gain in the present patients. Likewise, in a recent study by Wysokiński (2015) no associations were found between serum NPY and clozapine treatment, or obesity and metabolic parameters. The serum NPY levels of the present patients were not associated with levels of leptin, either. This concurs with the findings of Dötsch et al. (1997) suggesting no associations between human serum and cerebrospinal fluid levels of NPY and leptin. Elevated level of leptin is commonly known to be associated with obesity. Serum leptin levels were also associated with BMI and weight gain in the present patients. Associations between elevated plasma leptin and NPY levels and weight gain after smoking cessation, and weight gain in children during valproate treatment have been reported (Hussain et al., 2012; Tokgoz et al., 2012). However, the results of the present study do not support the role of serum NPY level as a marker of antipsychotic-induced weight gain.

6.4 Some genetic contributions of weight gain in patients with schizophrenia treated with clozapine (Studies II and IV)

Leptin gene *LEP* rs7799039 genotype was not associated with serum levels of leptin in the present patient group. This concurs with some earlier findings suggesting that this polymorphism in *LEP* gene is not associated with obesity susceptibility (Yu et al., 2012) or antipsychotic-induced weight gain (Gregoor et al., 2009; Opgen-Rhein et al., 2010; Gregoor et al., 2011).

The present results do not suggest a major role of adiponectin gene *ADIPOQ* rs1501299 polymorphism in serum adiponectin alterations or clozapine induced weight gain. Earlier studies on associations between *ADIPOQ* rs1501299 polymorphism and serum adiponectin levels have been controversial, with results suggesting a minor T-allele association with either lowered, elevated or unchanged levels of adiponectin (Mousavinasab et al., 2006; Siitonen et al., 2011; Gui et al., 2012; Ramya et al., 2013; Tong et al., 2013). This suggests heterogeneity across different populations. In a Finnish diabetes study sample no association was found between *ADIPOQ* rs1501299 and serum adiponectin levels (Siitonen et al., 2011).

In some earlier reports, and also in the present study no association was found between the C-allele of serotonin receptor *HTR2C* polymorphism rs1414334 and obesity (Risselada et al., 2012). Neither was there any association between *HTR2C* rs1414334 polymorphism and HOMA-IR, or levels of IL-1Ra, which are considered as markers of metabolic comorbidity (Saltevo et al., 2007; Suvisaari et al., 2011). In contrast to some earlier findings (Risselada et al., 2012), the GG genotype was associated with lower HDL-cholesterol levels and higher triglyceride levels among the present patients. The CC genotype was more common among the present patients than in the healthy control population. However, in fact the minor C allele frequency of the controls was less than expected. No more detailed explanation for this could be sought in this setting. All in all, due to a low minor allele frequency of this SNP, the results must be interpreted with caution. *HTR2C* polymorphism -759C/T (rs3813929), which has been previously considered as a most obvious candidate regarding antipsychotic-induced weight gain, was not analyzed in this study due to earlier unpublished results. These have suggested no associations with BMI or weight gain in this patient group, and no differences in genotype frequencies between patients and controls (Viikki et al., in preparation).

The genetic polymorphisms of *NPY* gene and NPY neuron receptor genes explored (n=21) did not explain the serum neuropeptide Y concentrations in this patient group (after statistical correction of p-values). This suggests that serum NPY levels are not associated with these genes, which are regulating central NPY expression and energy homeostasis. However, the neuronal circuits of the hypothalamus are complex. Therefore it can be speculated that other centrally expressed genes may also be involved in peripheral NPY regulation. In the GLM model the explanatory variables for the genetic associations were selected according to common knowledge of the factors associated with metabolic

syndrome. The HumanCoreExome Beadchip covers the essential functional SNPs itself or by linkage disequilibrium. However, it is obvious that serum NPY phenotype is multi-factorial, thus larger sample sizes may be needed to detect the effects of single genetic variants.

6.5 Strengths and limitations

To the best of knowledge, this is the largest study so far of cytokine alterations in clozapine treated patients with schizophrenia. The sample sizes in earlier studies have been mostly some tens of patients (Miller et al., 2011a; Røge et al., 2012; Bartoli et al., 2015b; Stubbs et al., 2016). Moreover, no previous data exists on serum levels of adipsin in patients with schizophrenia. This is also the largest study so far to combine genetic polymorphisms *LEP* rs7799039, *ADIPOQ* rs1501299 and *HTR2C* rs1414334 and the corresponding cytokine/adipokine levels with weight gain, and serum levels and genetic associations of neuropeptide Y in patients with schizophrenia on clozapine treatment. The study sample can be considered representative of this group of clozapine-treated patients with long-lasting schizophrenia, although there may be some selection bias assuming that the most non-compliant patients declined to participate or did not adequately attend the laboratory (Seppälä, 2014). Moreover, over 90% of the present patients had been on clozapine for at least one year. Potential confounding factors, such as gender, age, smoking and ethnic origin were taken into consideration in the analyses. The gene selection for the association study for NPY neuron receptor genes was based on a comprehensive literature search. Genomic data was analyzed using adjusted multiple testing with permutation tests to avoid random significance in the GRS analysis. To study all the most likely ways for biological traits of gene functionality and phenotype to be inherited, it seemed necessary to investigate three models of inheritance, recessive, dominant and additive. FDR (False Discovery Rate) was used in the adjustment of statistical significance. FDR also takes account of LD between SNPs in the correction of p-values in multiple testing. As the sample size was relatively small for a genetic association study, a less strict multiple test correction method (FDR) was used to find a sufficient number of variants to construct a genetic risk score. After the LD calculation and the stepwise VIF collinearity analysis, the final SNP set consisted of the most significant independent variants of the all studied SNPs.

However, this study was cross-sectional, the clinical data was collected retrospectively, and only a historical control population, with differing methods of cytokine/adipokine analysis and differed BMI levels, was available as a reference. Thus it was not feasible to conduct a case-control analysis with matched controls, and the reliability of comparisons between patients and controls was problematic. Moreover, levels of resistin and neuropeptide Y are not comparable between different studies due to the non-standardized nature of the assays. Also, the smoking status of the historical controls was not available. Whether these cytokine alterations are due to clozapine treatment, treatment with other antipsychotics prior to clozapine, or to the schizophrenia process itself remains unanswered in this kind of study procedure. Moreover, the severity of the patients' symptoms was not evaluated. There may also be some unavailable confounding factors (alcohol, substance use, chronic inflammatory disease, anti-inflammatory medications) in the patient group, which may have influenced the inflammatory markers. Combination therapy with other antipsychotics with different side-effects and receptor profiles in one third of the patients may also be a confounding factor, nor can the possibility of an acute infection in some patient be excluded. The weight and height of the patients were registered according to reported estimate. However, according to general population surveys there is a correlation between measured and self-reported values, and the use of self-reported estimates appears acceptable. Weight seems to be underestimated and height overestimated leading to underestimation of BMI (Niedhammer et al., 2000; Paccaud et al., 2001; Yoong et al., 2013). Due to a lack of data on blood pressure and waist circumference, it was not possible to reliably identify patients with full metabolic syndrome or central obesity. Therefore only single metabolic syndrome criteria were used in the analyses. Duration of illness or duration of clozapine treatment could not be used as confounding factors in logistic regression model due to a limited sample. Moreover, there may be other peripheral factors affecting serum NPY levels which could not be studied in this patient sample, for example the patients' cardiovascular status and adipose tissue distribution.

In the genetic association studies of *LEP*, *ADIPOQ* and *HTR2C* genes only single SNPs were studied and linkage disequilibrium with some other previously studied SNPs cannot be excluded. Moreover, the sample size for a genetic association study was limited, although in the study II the group differences at effect size ≥0.5 in the target variables were discernible according to the power calculations. In study IV there may have been some other

functional ARC NPY neuron receptor genes which were not picked up with the search method used. However, given the present findings and the current literature it is likely that these genes play only a minor role.

7 Summary and conclusions

Antipsychotic-induced weight gain is associated with obesity related morbidity and mortality, stigmatization, and non-adherence. Adverse effects of antipsychotics vary between individuals and agents, and there are no clinically reliable markers so far available in clinical practice to predict weight gain and metabolic consequences. However, it seems that there can be some alterations in serum markers already prior to treatment in patients who develop weight gain during antipsychotic treatment. Genetic influence on antipsychotic-induced weight gain has been studied widely in recent years. Genome wide association studies have found associations in several genes, which need to be replicated. This study strengthens the awareness of clozapine treatment related metabolic adverse effects. Studies on predictive biomarkers of AIWG and metabolic syndrome with prospective, longitudinal design from the initiation of clozapine treatment are needed in the future.

In conclusion of this thesis;

1. There are partly gender dependent cytokine and adipokine alterations in patients with treatment resistant schizophrenia on clozapine treatment. These alterations are related to a risk of metabolic comorbidity. Levels of leptin, weight gain during clozapine treatment, and inflammatory markers related to metabolic comorbidity (IL-6, IL-1Ra) showed a marked interaction, especially among female patients with schizophrenia treated with clozapine. In male patients low adiponectin level was a more specific marker of metabolic comorbidity and clozapine-induced weight gain. As a biomarker of systemic inflammation resistin may play a role as a marker of cardiovascular comorbidity, especially among male patients. Genetic and other biological mechanisms of these alterations need to be further investigated with a longitudinal study design.

2. Smoking as a cardiovascular risk factor was associated with inflammatory markers (resistin, hs-CRP, and in males IL-6) among the present patients.

3. Serum NPY level does not seem to be a feasible biomarker of antipsychotic-induced weight gain. In order to clarify the mechanisms and directions of associations in serum NPY alterations, and the relations between central and peripheral regulation of neuropeptide Y, longitudinal studies are needed.

4. The results of the present study do not support a major role of *LEP* rs7799039, *ADIPOQ* rs1501299 and *HTR2C* rs1414334 polymorphisms in the regulation of serum leptin and adiponectin levels or weight gain. Serum NPY level alterations are not associated with polymorphisms in genes encoding arcuate nucleus NPY neuron receptors. However, it can not be excluded that the genes now studied in association with serum NPY levels may still be involved in appetite and weight regulation.

In clinical practice the excess cardiovascular morbidity and mortality associated with schizophrenia are of great concern. The clinical guidelines for the assessment and treatment of antipsychotic medication associated metabolic adverse effects should become an integral part of treatment. Further effort should be invested in the prevention and treatment of metabolic abnormalities and smoking cessation among these patients.

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Cytokine and adipokine alterations in patients with schizophrenia treated with clozapine

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ABSTRACT

Metabolic syndrome is associated with both schizophrenia and antipsychotic medication, especially clozapine, with alterations in inflammatory cytokines and adipokines. However, the data in this field is heterogeneous and the sample sizes of the patients are limited. In this study we assessed the serum levels of cytokines/adipokines IL-6, IL-1Ra, hs-CRP and adiponectin, and components of metabolic syndrome in 190 patients with treatment resistant schizophrenia treated with clozapine. Substantial metabolic comorbidity was found in this patient group; overweight/obesity, smoking, hypertriglyceridemia, low HDL-cholesterol, high HOMA-IR, low adiponectin levels, elevated hs-CRP levels and elevated IL-1Ra levels. Elevated IL-1Ra levels are associated with insulin resistance, obesity and hypertriglyceridemia. Low adiponectin levels were associated with hypertriglyceridemia, low HDL cholesterol and high glucose, and in male patients also with obesity and high IL-1Ra levels. After controlling for confounding factors age and smoking, levels of IL-1Ra and hs-CRP associated with obesity, and the levels of IL-6 associated with obesity in female patients. We conclude that there are partly gender dependent cytokine and adipokine alterations in patients with schizophrenia on clozapine treatment associated with metabolic comorbidity. The genetic background of these cytokine alterations needs to be further investigated.

1. Introduction

Cardiovascular disease is a major cause of excess deaths in patients with schizophrenia (Osby et al., 2000; Kilbourne et al., 2009). Metabolic abnormalities are present already in first-episode patients, and become even more common as the disorder progresses (De Hert et al., 2006; Beary et al., 2012). A recent meta-analysis reported an overall 32.5% prevalence of metabolic syndrome (MetS) among patients with schizophrenia (Mitchell et al., 2013). In a Finnish general population survey the prevalence of metabolic syndrome was 36.2% among subjects with schizophrenia and 30.1% among subjects without psychotic disorder (Suvisaari et al., 2007).

Clozapine is an atypical antipsychotic drug for treatment resistant schizophrenia. It has been shown to ameliorate positive symptoms in a large proportion of patients in this group, reduces the risk for suicide and decreases overall mortality (Kane et al., 1988; Tiihonen et al., 2011; Meltzer, 2013). Of all antipsychotic agents clozapine and olanzapine are most often associated with weight gain (Newcomer, 2005), thereby increasing the risk of diabetes mellitus and dyslipidemia. There is some evidence that insulin homeostasis and lipid profiles in clozapine-treated obese schizophrenia patients are different from those in non-psychiatric obesity (Wu et al., 2008). According to a recent meta-analysis the highest rates of MetS among patients with schizophrenia were found in those prescribed clozapine (51.9%) (Mitchell et al., 2013). Case reports suggest that substantial weight gain or obesity may not be a factor in up to 25% of cases of new-onset diabetes occurring during antipsychotic treatment (Newcomer, 2005). Accordingly, some studies support the hypothesis that clozapine may have a direct effect on glucose regulation independent of adiposity (Newcomer, 2005).

Cytokines are involved in the regulation of immunologic and inflammatory responses in physiologic and pathologic conditions

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(Steinke and Borish, 2006). Adipose tissue is a major source of several inflammatory cytokines and adipokines (Raucci et al., 2013). Abnormalities in these including the tumor necrosis factor alpha (TNF-α), interleukin 1 (IL-1), interleukin 1 receptor antagonist (IL-1Ra), interleukin 6 (IL-6), high sensitivity C-reactive protein (hs-CRP), leptin and adiponectin, and a chronic low-grade inflammatory state are associated with both schizophrenia and obesity, or MetS (Potvin et al., 2008; Miller et al., 2011; Harwood, 2012). It is also well known that clozapine has immunomodulatory effects (Pollmächer et al., 1996; Maes et al., 1997; Monteleone et al., 1997).

IL-1Ra is a sensitive marker of cytokine response in the pre-diabetic state (Ruotsalainen et al., 2006) and predicts the progression of MetS to clinically incident diabetes independently of CRP and other risk factors (Luotola et al., 2011). Serum IL-1Ra levels are markedly increased in obese subjects (Fantuzzi, 2005) and has also been reported to be increased in patients with schizophrenia (Potvin et al., 2008). Most probably these alterations are not related to antipsychotic medication alone (Potvin et al., 2008). Clozapine treatment has been reported to associate both with no effect (Pollmächer et al., 1996), and increase in IL-1Ra levels (Maes et al., 1997).

Increased concentrations of IL-6 are also among consistently reported cytokine alterations in schizophrenia (Leonard et al., 2012). This increase may already be present in first-episode patients and in patients with acute relapse. However, also no difference in IL-6 levels compared with controls has been found in outpatients with stable medication and in patients with treatment resistant psychosis (Miller et al., 2011). Circulating levels and adipose tissue production of IL-6 are increased in obesity (Raucci et al., 2013). These levels are also increased in subjects with diabetes, metabolic syndrome and increased insulin resistance (Marques-Vidal et al., 2013). IL-6 increases leptin secretion and reduces adiponectin secretion (Harwood, 2012). Fernandez-Egea et al. (2009) reported that newly diagnosed antipsychotic-naive patients with schizophrenia had higher prevalence of abnormal glucose tolerance or diabetes and higher IL-6 concentrations, which could not be attributed to confounding factors. However, in a recent study a significant rise in the serum level of IL-6 was reported in patients with schizophrenia, but no further effects of MetS were found (Beumer et al., 2012). Clozapine treatment is associated with elevated IL-6 levels in several studies (Pollmächer et al., 1996; Maes et al., 1997; Schmitt et al., 2005; Kluge et al., 2009; Loffler et al., 2010a; Røge et al., 2012), but also findings of lowered levels (Lu et al., 2004; Sugino et al., 2009), or no effects are reported (Himmerich et al., 2011; Røge et al., 2012).

Increased hs-CRP is associated with multiple risk factors for cardiovascular diseases, including obesity, insulin resistance, and hypertension, and has been demonstrated to have predictive value for risk of MetS (Shen and Ordovas, 2009). As a part of transient acute-phase response, hs-CRP increases soon after initiation of clozapine treatment, but the elevation seems to be temporary and disappears after one year of treatment (Loffler et al., 2010b). Patients with non-affective psychosis and MetS are reported to have higher hs-CRP levels than patients without metabolic syndrome (Miller et al., 2013). During treatment with second generation antipsychotics, elevated hs-CRP was associated with high BMI and high glucose levels in patients with psychotic disorders (Dieset et al., 2012). According to a recent meta-analysis, the prevalence of an elevated CRP level in patients with schizophrenia and related disorders was 28% (Miller et al., 2014).

Circulating adiponectin is negatively correlated with BMI and is decreased in obese subjects, in patients with type 2 diabetes or with cardiovascular disease (Raucci et al., 2013). Adiponectin levels decline prior to a decrease in whole-body insulin sensitivity and decreased adiponectin levels may serve as predictors for future development of MetS (Harwood, 2012) and risk of type 2 diabetes (Salomaa et al., 2010). The anti-inflammatory activities of adiponectin extend to inhibition of IL-6 production accompanied by induction of the anti-inflammatory cytokines IL-10 and IL-1Ra (Fantuzzi, 2005). Thus, adiponectin has direct anti-atherosclerotic and anti-inflammatory effects (Harwood, 2012). Adiponectin levels are generally higher in women than in men, which could partly explain the better insulin sensitivity of females. Low adiponectin levels in women are even more related to the probability of MetS than in men (Santaniemi et al., 2006). In schizophrenia as well as in obesity there is an imbalance between adiponectin and pro-inflammatory cytokines TNF-alpha and IL-6 are in favor of the latter cytokines (Leonard et al., 2012). There is some discrepancy in reports concerning the relation of adiponectin levels and schizophrenia, and antipsychotic medications. Most studies report lowered levels of adiponectin (Matsuda et al., 2005; Hanssens et al., 2008; Jin et al., 2008; Oriot et al., 2008; Chen et al., 2011), but also reports of no change (Jin et al., 2008; Fernandez-Egea et al., 2009), or increased adiponectin levels (Beumer et al., 2012) have been published. However, both olanzapine and clozapine have been associated with lowered adiponectin levels in several studies (Togo et al., 2004; Richards et al., 2006; Hanssens et al., 2008; Sugai et al., 2012; Wampers et al., 2012). Accordingly, hypoadiponectinemia has been reported to be a potential biomarker of the metabolic syndrome in patients taking clozapine for schizophrenia (Bai et al., 2007). Obesity-induced changes in adipokine secretion, and the development of systemic insulin resistance, metabolic syndrome, type 2 diabetes mellitus, and cardiovascular disorders, are presented in Fig. 1.

The results so far on cytokine and adipokine alterations and their associations with MetS and schizophrenia (especially with treatment resistance) are inconsistent, and most of the studies have not been controlled for potential confounding factors (Miller et al., 2011). The aim of the present study was to investigate cytokine and adipokine (IL-6, IL-1Ra, hs-CRP, adiponectin) serum level alterations in relation to components of metabolic syndrome (BMI, insulin resistance, lipid profile) in 190 clozapine treated patients with treatment resistant schizophrenia.

2. Materials and methods

2.1. Patients

The study was conducted in three hospital districts in Western Finland (Satakunta, Pirkanmaa and Seinäjoki Hospital Districts). The patients were recruited at secondary in and outpatient clinics and from sheltered accommodation units. The inclusion criteria for the study were (1) stabilized clozapine medication, and (2) clinical diagnosis of F2 group according to ICD-10. The exclusion criteria were organic brain disease or other neurological disorders. The diagnosis was set by experienced psychiatrists in clinical settings. All patients were on clozapine treatment. They completed a questionnaire eliciting, among others, weight and height, possible weight gain during clozapine treatment, and smoking. The body mass index was calculated by the physicians collecting the data. Information on past medical history and duration of clozapine treatment was collected from patient records. The study was approved by the local ethics committee. All patients gave informed consent on entry to the study. The blood samples were taken during a routine laboratory visit related to the clozapine treatment.

2.2. Laboratory and clinical methods

For the laboratory analysis, 9.0 ml EDTA-whole blood fasting morning sample was taken from the participants and stored in a freezer at −20 °C. Glucose, insulin, HDL-cholesterol, and triglycerides were measured with Cobas c6000 e601 (Roche Diagnostics) with detection limits of 0.11 mmol/L, 0.2 μU/mL, 0.08 mmol/L and 0.1 mmol/L respectively. Levels of adiponectin, IL-6 and IL-1Ra were measured using enzyme linked immunosorbent assay (ELISA) using the following reagents: DuoSet® ELISA (R&D Systems Europe Ltd., Abingdon, UK) for adiponectin, Ready-SET-GO!® ELISA (eBioscience Inc., San Diego, CA, USA) for IL-6, and Quantikine® (R&D Systems Europe Ltd., Abingdon, UK) for IL-1Ra. Detection limit for IL-6 was 0.78 pg/ml. Hs-CRP was measured using Tina-quant C-reactive protein (latex) high sensitive assay (Roche Diagnostics) with detection limits 0.05–10 mg/L.

Undetectable IL-6 levels (41 cases) were considered to be 0 pg/ml and included in the statistical analysis. Missing serum cytokine values due to limited amount of

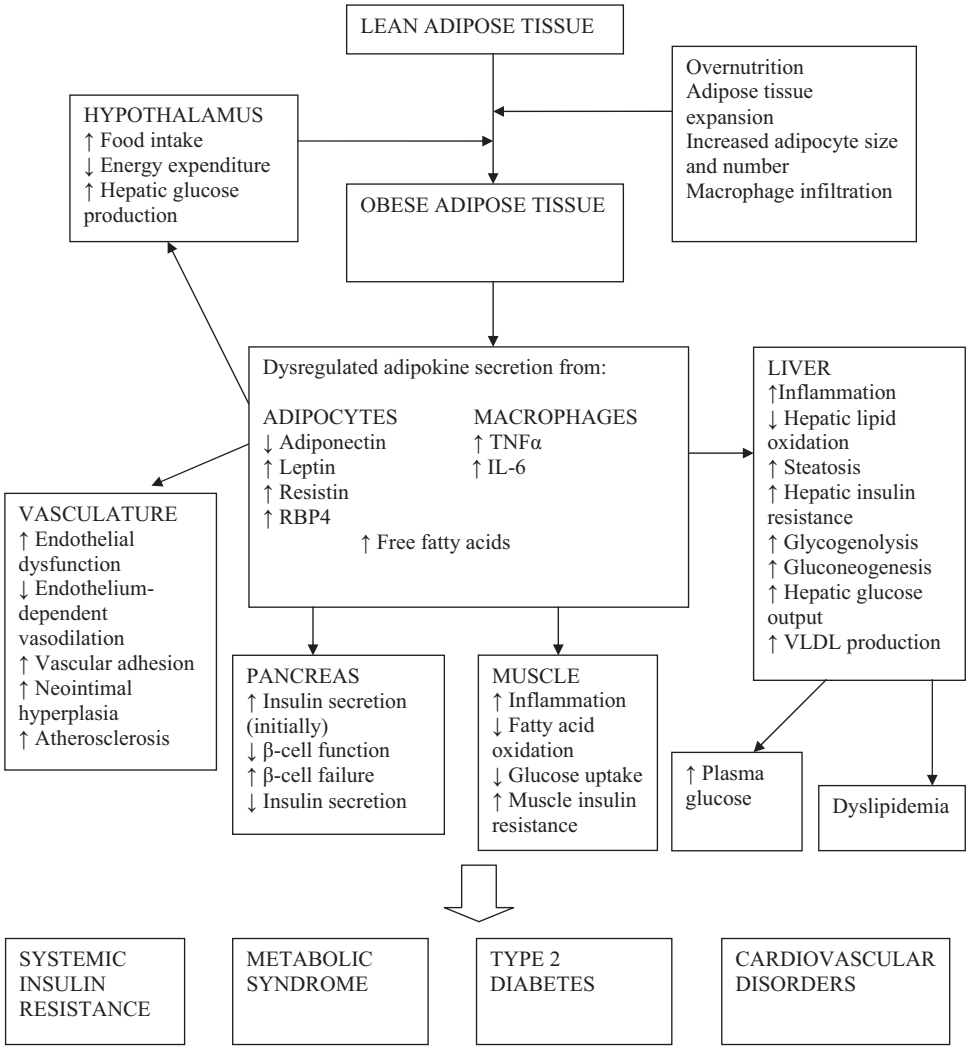

Fig. 1. Obesity-related changes in adipokine secretion and development of systemic insulin resistance. Overnutrition leads to adipose tissue expansion, increased adipocyte size and number, and macrophage infiltration that, together lead to increased free fatty acid release, dysregulated adipokine secretion from adipocytes, and increased release of inflammatory cytokines from macrophages. Dysregulated secretion of these adipokines elicits adverse effects on numerous tissues, that further increase food intake and reduce energy expenditure and lead to the development of systemic insulin resistance (Reproduced from: Harwood, 2012).

serum were excluded from the analysis. Insulin resistance index assessed by homeostasis model assessment (HOMA-IR) was calculated using the formula insulin (mU/l) × glucose (mmol/l)/22.5 (Matthews et al., 1985).

The single criteria for metabolic syndrome (glucose ≥ 5.6 mmol/l, HDL-cholesterol < 1.0 mmol/l males/1.3 mmol/l females, Trigly ≥ 1.7 mmol/l) were defined according to the definition by the IDF Task Force on Epidemiology and Prevention, National Heart, Lung and Blood Institute, American Heart Association, World Heart Federation, International Atherosclerosis Society and Association for the Study of Obesity (Alberti et al., 2009).

2.3. Statistical analysis

Comparison of means between genders and between patients and controls were analyzed with *t*-tests. Pearson's correlation was used to analyze the associations between markers. For the statistical analysis we omitted, depending on the variable, 0–7 outlier cases from the data. In continuous variables, absolute values for skewness ≤ 2 were considered normal distributions, and the highest individual values, if needed, were omitted as outliers. This procedure is also controlled for multivariate outliers. Partial correlations were calculated for both genders separately with controlling the confounding factors age and smoking. The statistical significance of correlations between markers was set at $p=0.004$ using the Bonferroni correction by dividing the significance level of $p=0.05$ by the number of markers analyzed. In all other statistical analyses the level of significance was $p=0.05$. Logistic regression analyses with backward stepwise method were used to test the effects of cytokines/adipokines as predictors for single metabolic syndrome criteria according to Alberti et al. (2009), and controlling for potential confounding factors gender, age and smoking status in these models. Data analysis was carried out using SPSS/Win software (version 19.0, SPSS inc., Chicago, IL, USA) and GraphPad QuickCalcs (GraphPad Software Inc., La Jolla, CA, USA).

3. Results

Of the 190 patients of Finnish origin, 108 (56.8%) were male and 82 (43.2%) were female. Mean age was 42.92 years (range 20–67 years). The average time from the first hospital admission was 17.67 years (2–42 years). One hundred and eleven patients (58.4%) had been on clozapine treatment for more than five years, 60 patients (31.6%) from one to five years and four patients (2.1%) from three to twelve months. Fifteen patients with no exact data on starting date of clozapine available were recruited from long-term care units, and they all had been on clozapine treatment for more than one year. One hundred and twenty-one patients (63.7%) were on clozapine monotherapy, and 69 (36.3%) had a combination therapy with other antipsychotics. The mean clozapine dose was 403.43 mg/day (100–800 mg/day). The mean BMI was 29.92 kg/m^2. One hundred and one patients (53.2%) were regular smokers, with a median of 20 cigarettes per day.

The levels of metabolic markers and cytokine/adipokine levels are presented in Table 1. Among the patient population BMI, levels

Table 1
Metabolic markers and cytokine and adipokine levels of patients, and comparisons between patients and historical control samples.

Marker	Patients	Controls	*P* value
Age	42.9 (11.0), $n=190$	46 (6), $n=903$	<0.001
BMI kg/m², men	28.99 (5.81), $n=105$	26.7 (3.7), $n=403$	
BMI kg/m², women	31.18 (6.87), $n=77$	26.3 (4.9), $n=500$	<0.001
HDL mmol/L, men	1.06 (0.29), $n=108$	1.3 (0.3), $n=403$	<0.001
HDL mmol/L, women	1.28 (0.36), $n=82$	1.5 (0.3), $n=500$	<0.001
Trigly mmol/L, men	2.17 (1.17), $n=108$	1.7 (1.3), $n=403$	<0.001
Trigly mmol/L, women	1.67 (0.78), $n=81$	1.2 (0.6), $n=500$	<0.001
Glucose mmol/L, men	6.04 (1.69), $n=108$	5.9 (0.6), $n=403$	0.17
Glucose mmol/L, women	5.77 (1.70), $n=80$	5.6 (0.5), $n=500$	0.07
Adiponectin μg/ml, men	2.91 (1.39), $n=108$	4.87 (2.70), $n=403$	<0.001
Adiponectin μg/ml, women	3.76 (1.84), $n=82$	7.87 (4.36), $n=500$	<0.001
IL-1Ra pg/ml, men	470.58 (246.42), $n=102$	172.27 (127.70), $n=403$	<0.001
IL-1Ra pg/ml, women	534.85 (293.83), $n=77$	191.49 (152.58), $n=500$	<0.001
hs-CRP mg/L, men	3.58 (5.28), $n=106$	1.33 (1.48), $n=403$	<0.001
hs-CRP mg/L, women	4.71 (5.04), $n=82$	1.48 (1.45), $n=500$	<0.001
Age	42.9 (41.4–44.5), $n=190$	57.9 (57.2–58.6), $n=502$	
HOMA-IR, all[a]	4.65 (9.95), $n=190$	1.6 (1.6), $n=502$	
IL-6 pg/ml, all[a]	2.22 (2.13), $n=187$	1.6 (1.3), $n=502$	

Data are means (S.D.) or medians (interquartile range).

[a] Control data taken from Ahonen et al. (2012), except: the published control data of HOMA-IR and IL-6 were available as medians and IQR, and age as mean (95% confidence interval), and were taken from Malo et al. (2011). Metabolic Syndr Relat Disord, 9:206, Table 2, Resistin tertile 3 (Malo et al., 2011).

Table 2
Correlations between IL-1Ra, IL-6, hs-CRP, adiponectin and metabolic markers in the patient sample.

Marker	IL-1Ra	IL-6	hs-CRP	Adiponectin
BMI, men	0.45*	0.12	0.16	−0.42*
BMI, women	0.48*	0.45*	0.37*	−0.26
HOMA-IR, all patients	0.24*	0.05	0.079	−0.17
HDL, men	−0.27	−0.14	−0.018	0.49*
HDL, women	0.25	0.078	0.015	0.51*
Trigly, men	0.39*	0.17	−0.04	−0.49*
Trigly, women	0.36*	0.30	0.16	−0.53*
IL-1Ra, men	1	0.11	0.042	−0.37*
IL-1Ra, women	1	0.40*	0.37*	−0.25
IL-6, men	0.11	1	0.42*	0.019
IL-6, women	0.40*	1	0.27	−0.13
hs-CRP, men	0.042	0.42*	1	0.061
hs-CRP, women	0.37*	0.27	1	0.042
Adiponectin, men	−0.37*	0.019	0.061	1
Adiponectin, women	−0.25	−0.13	0.042	1

* $p \leq 0.001$.

of adiponectin, triglycerides and HDL-cholesterol differed between genders. BMI (S.D.) was 28.99 (5.81) vs. 31.18 (6.87) kg/m² in males vs. females ($p=0.025$), adiponectin 2.91 (1.39) vs. 3.76 (1.84) μg/ml ($p=0.001$) and HDL-cholesterol 1.06 (0.29) vs. 1.28 (0.36) mmol/l ($p<0.001$) respectively. Triglyceride levels in males 2.17 mmol/l (1.17) were higher than in females 1.67 mmol/l (0.78) ($p=0.001$). Correlations between BMI, lipids, and cytokine/adipokine levels were calculated separately for both genders (Table 2). Low adiponectin levels were associated with high triglycerides and low HDL levels in both genders and with high BMI and high IL-1Ra in males. High IL-1Ra levels were associated with high BMI and high triglycerides in both genders and with high HOMA-IR in the whole patient sample as well as with low adiponectin levels in males and high IL-6 and hs-CRP levels in females. IL-6 levels were associated with BMI and IL-1Ra in females and with hs-CRP levels in males. Hs-CRP levels were associated with BMI and IL-1Ra levels in females and with IL-6 levels in males.

After controlling for effects of confounding factors age and smoking status, low adiponectin correlated with low HDL-cholesterol (males $r=0.56$, $p<0.001$, females $r=0.49$, $p<0.001$) and high triglyceride levels (males $r=-0.35$, $p=0.002$, females $r=-0.61$, $p<0.001$). Instead, the correlations between levels of adiponectin, and IL-1Ra as well as BMI among male patients disappeared. IL-1Ra correlated with BMI (males $r=0.45$, $p<0.001$, females $r=0.53$, $p<0.001$), among male patients with low HDL-cholesterol ($r=-0.32$, $p=0.006$) and among female patients with hs-CRP ($r=0.39$, $p=0.002$). IL-6 levels correlated with BMI among female patients ($r=0.40$, $p=0.001$). Hs-CRP correlated with BMI (males $r=0.41$, $p<0.001$, females $r=0.35$, $p=0.004$).

The results of logistic regression concerning the associations between cytokines/adipokines and single MetS criteria (glucose ≥ 5.6 mmol/l, HDL-cholesterol <1.0 mmol/l males/ 1.3 mmol/l females, Trigly ≥ 1.7 mmol/l) after potential confounding factors gender, age and smoking are given in Table 3.

4. Discussion

The presence of metabolic risk factors and morbidity among patients with schizophrenia on clozapine treatment was substantial, including overweight/obesity, smoking, hypertriglyceridemia, low HDL-cholesterol, high HOMA-IR, low adiponectin levels, elevated hs-CRP levels and elevated IL-1Ra levels. Historical

Table 3
Results of three logistic regression models with single metabolic syndrome criteria (Alberti et al., 2009) as target variables. All models were estimated with the backward stepwise method using significance limits of 0.05–0.10 for entering and removing the explaining variables. Gender, age, smoking status, serum adiponectin, IL-1Ra, IL-6, and hs-CRP levels were used as explaining variables at the first step.

Target variable	Correctly classified (total)	Explaining variable	*P*	OR	95% CI
Glucose ≥ 5.6 mmol/L	69.0%	Age	0.003	1.048	1.016–1.082
		Adiponectin	0.004	0.727	0.583–0.905
		IL-1Ra	0.030	1.001	1.000–1.002
		IL-6	0.105	0.908	0.808–1.020
HDL-Cholesterol < 1.0 (m) or < 1.3 (f)mmol/L	64.7%	Adiponectin	< 0.001	0.646	0.521–0.802
Triglycerides ≥ 1.7 mmol/L	71.2%	Female gender	0.089	0.549	0.276–1.095
		Adiponectin	< 0.001	0.549	0.417–0.721
		IL-1Ra	0.047	1.001	1.000–1.002

control samples from two Finnish population based studies were used as reference (Malo et al. 2011, Ahonen et al. 2012). As expected, gender differences were found in many of the cytokines and adipokines studied.

Elevated IL-1Ra was a sensitive marker of metabolic comorbidity in this patient population with marked associations with insulin resistance, obesity, and lipid status. This elevation may in part be due to schizophrenia itself and partly to associated factors such as smoking and other living habits and medication (Potvin et al., 2008).

In women high IL-6 levels were associated with obesity, which was not the case in men. Data from studies on healthy adults have suggested only a very limited obesity-related increase in IL-6 levels in women (Kuo and Halpern, 2011). Thus obesity related inflammation in present female patients may be related either to gender specific effects of schizophrenia or clozapine treatment. In normal population circulating levels of IL-6 are associated with visceral adiposity (Gustafson, 2010). It can be presumed that the visceral fat component of weight gain was particularly increased in the present female patients. In general, excess adiposity in the central visceral region of the body (usually typical for male obesity) is correlated with increased risk and mortality from metabolic disorders (Shi et al., 2009).

IL-6 has been found to increase leptin secretion, which has further been associated with obesity among normal population (Harwood, 2012). In a study by Beumer et al. (2012) the effect of schizophrenia on leptin levels was reported to be noticeable only in females. This is in line with one earlier study suggesting possible gender-specific leptin dysregulation in antipsychotic-naïve schizophrenic patients (Wang et al., 2007). However, the basic mechanisms of gender differences in IL-6 remain unclear. In all, the IL-6 levels of the present patients were close to the values reported in general populations. This more likely indicates either a stable state of schizophrenic illness in the present patients (Miller et al.,, 2011) or a possible suppressing effect of long-term clozapine treatment on IL-6 levels (Lu et al., 2004; Sugino et al., 2009).

In general population studies the correlations between hs-CRP and obesity have been more evident in women than men (Rossi et al., 2012) and appear to relate to pronounced accumulation of subcutaneous fat especially in women (Cartier et al., 2009). These gender differences in the relationship between hs-CRP and adiposity may be partially mediated by the secretion of leptin (Rossi et al., 2012). In the present patients hs-CRP associated with BMI also among male patients after controlling for age and smoking status.

Adiponectin levels were low in the present patient group, which replicates the findings of Hanssens et al. (2008) suggesting a suppressing effect of clozapine treatment on adiponectin levels. In the present study, the multivariate analyses suggested, that low adiponectin levels predicted the condition of metabolic syndrome, with hypertriglyceridemia, low HDL-cholesterol and high glucose levels after potential confounding factors age, gender and smoking were taken into account. Among male patients low adiponectin levels also associated with obesity and high IL-1Ra levels. This concurs with a finding in a general population study where obese men had lower adiponectin levels than lean men or lean and obese women (Plaisance et al., 2009). However, in the earlier report based on studies on healthy (non-MetS) lean to obese adults of both genders, no correlations between plasma adiponectin and BMI or gender difference in plasma adiponectin levels were found (Kuo and Halpern, 2011). Accordingly, in the present study the associations between levels of adiponectin, and obesity and IL-1Ra levels among male patients disappeared after controlling for age and smoking status. In a study by Matsuda et al. (2005) plasma adiponectin correlated negatively with BMI and with HOMA-IR in men, but not in women among non-diabetic patients with schizophrenia. No such association with HOMA-IR was found in the present study. Accordingly, weight gain and truncal fat accumulation have been reported to be associated with decline of adiponectin in patients with schizophrenia on atypical antipsychotic treatment and free of MetS when entering the study (Oriot et al., 2008). The study by Fan et al. (2010) suggested that high white blood cell account may be a useful marker to predict an increased risk for metabolic syndrome in patients with schizophrenia. The present results suggest, that also hypoadiponectinemia is a potential biomarker of metabolic syndrome in this population, especially in male patients. However, the role of adiponectin as a predictor of future MetS could not be investigated with this study design.

A recent study by Wampers et al. (2012) reported adiponectin increase in risperidone-treated patients and decrease in olanzapine treated patients independent of BMI and MetS. The latter would suggest a specific effect of olanzapine on adipose tissue (Wampers et al., 2012). In addition, negative correlations between olanzapine treatment and adiponectin levels have been reported in several studies (Togo et al., 2004; Richards et al., 2006; Sugai et al., 2012; Wampers et al., 2012). The present results with adiponectin levels are in line with those of some earlier studies suggesting a suppressing effect of clozapine on adipose tissue (Bai et al., 2007; Hanssens et al., 2008; Oh et al., 2012).

As far as we know, this is the largest study so far of cytokine alterations in clozapine treated patients with schizophrenia (Pollmächer et al., 1996; Maes et al., 1997; Monteleone et al., 1997; Lu et al., 2004; Schmitt et al., 2005; Kluge et al., 2009; Sugino et al., 2009; Loffler et al., 2010a; Himmerich et al., 2011; Røge et al., 2012). Moreover, over 90% of the present patients had been on clozapine for at least one year. Potential confounding factors, such as gender, age, smoking and race have been taken into consideration. However, this study was cross-sectional, the clinical data was collected retrospectively, and only a historical control population, with differing methods of cytokine/adipokine analysis and differed BMI levels, was available as a reference. Due to this, a case-control analysis with matched controls was not

possible to conduct, and the reliability of comparisons between patients and controls was problematic. Also, the smoking status of controls was not available. Whether these cytokine alterations are due to clozapine treatment, treatment prior to clozapine, or schizophrenia process itself remain unanswered in this kind of study procedure. There may also be some unavailable confounding factors (alcohol, substance use and chronic inflammatory disease) in the patient group, which might influence inflammatory markers. Also a possibility of an acute infection in some patient cannot be excluded. Due to lack of data on blood pressure and waist circumference, it was not possible to identify reliably patients with full metabolic syndrome. Therefore, only single metabolic syndrome criteria were used in analyses. Duration of illness or duration of clozapine treatment was not possible to use as confounding factors in logistic regression model due to a limited sample. However, this population can be considered quite solid and homogenous, the group of patients with long-lasting schizophrenia on clozapine treatment.

In conclusion, there are partly gender dependent cytokine and adipokine alterations in patients with treatment resistant schizophrenia on clozapine treatment. These alterations are related to high risk of metabolic comorbidity. Genetic and other biological mechanisms of these alterations need to be further investigated. In clinical practice, excess cardiovascular morbidity and mortality associated with schizophrenia is of great concern, especially among female patients.

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Original article

Association study of the HTR2C, leptin and adiponectin genes and serum marker analyses in clozapine treated long-term patients with schizophrenia

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Weight gain

ABSTRACT

Clozapine treatment is associated with weight gain and cardio-metabolic consequences among patients with schizophrenia. Polymorphisms of leptin, serotonin receptor HTR2 C and adiponectin genes have been associated with antipsychotic-induced weight gain and metabolic comorbidity. However, the results of the studies so far are inconclusive. The aim of the present study was first to test for a possible role of serum leptin and adiponectin levels as a marker of weight gain in association with inflammatory cytokines/adipokines (IL-6, IL-1Ra, hs-CRP and adipsin), and second to study associations between SNPs LEP rs7799039 (-2548 A/G), ADIPOQ rs1501299 and HTR2 C rs1414334 and weight gain and levels of leptin and adiponectin, in 190 patients with schizophrenia on clozapine treatment, with retrospectively assessed weight change and cross-sectionally measured cytokine levels. A strong association was found between serum levels of leptin and weight gain and cytokines/adipokines related to metabolic comorbidity, especially among female patients (in women leptin vs. weight gain, IL-6 and IL-1Ra, $P < 0.001$; in men leptin vs. weight gain, $P = 0.026$, leptin vs. IL-1Ra, $P < 0.001$). In male patients low adiponectin level was a more specific marker of clozapine-induced weight gain ($P = 0.037$). The results of the present study do not support a major role of SNPs LEP rs7799039, ADIPOQ rs1501299 and HTR2 C rs1414334 in the regulation of weight gain or association of serum levels of leptin and adiponectin and corresponding studied SNPs in patients with schizophrenia on clozapine treatment.

1. Introduction

Cardiovascular risk factors such as type 2 diabetes mellitus, dyslipidemia, hypertension, smoking and obesity are more common in patients with schizophrenia than in general population [48]. Antipsychotic agents may have a direct negative impact on some of these cardio-metabolic risk factors, but most of them can be explained by their tendency to induce weight gain [10]. Weight gain is observed in up to 30% of patients treated with second generation antipsychotics [7]. Of all antipsychotics clozapine and olanzapine are associated with the greatest risk of weight gain [45]. Adverse effects vary individually, and are influenced by both, clinical and genetic factors [7]. However, there are so far no reliable clinical predictors or markers for the selection of personalized antipsychotic treatment [7,66].

Obesity is associated with high leptin levels and leptin resistance in general population [53]. This hormone plays an important role in appetite regulation [13]. Obese patients are insensitive to the anorectic actions of leptin and continue to maintain high levels of body fat [23]. Leptin also modulates immunity and inflammation [14]. The circulating leptin correlates better with subcutaneous fat than with total body fat, and leptin levels are generally higher in women. Estrogen and testosterone modulate leptin synthesis and secretion [61].

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Clozapine treatment is associated with an increase in serum leptin level [49]. It seems already to increase during the first two weeks after initiation of clozapine [60]. A role of leptin in mediating antipsychotic-induced weight gain but also therapeutic effect in schizophrenia has been speculated [71]. However, the increase of leptin during antipsychotic treatment appears to be a result of weight gain rather than a direct impact of these drugs on leptin physiology [29,56]. The evidence linking leptin increase to changes in other metabolic parameters during antipsychotic treatment is likewise contradictory [29]. Second-generation antipsychotics may also affect adipocytokines [64]. Inhibition of brown adipogenesis may be one possible mechanism to explain weight gain induced by clozapine [46]. A gender-specific effect of schizophrenia on leptin regulation has been suggested, but the results are inconclusive [5,37,73].

According to a recent meta-analysis, polymorphisms in *LEP* gene are not associated with obesity susceptibility in general population [77]. However, several original studies have suggested that variations in the genes encoding leptin, especially SNP LEP-2548A/G (rs7799039) may have an influence on weight during antipsychotic treatment [2,6,32,39,75,79]. Some negative results have also been reported [18,19,47].

Several studies have linked the serotonin receptor 5-HT2 C -759 C/T (rs3813929) polymorphism with antipsychotic-induced weight gain [2,38,47,62,75], but also a negative finding has been reported [26]. C-allele of the -759 C/T polymorphism and G-allele of the LEP -2548A/G polymorphism together may be determinants of obesity and metabolic syndrome in patients on atypical antipsychotics [17,76]. Some studies have found no associations between HTR2 C -759 C/T polymorphism and metabolic syndrome (MetS) in patients taking antipsychotic medication [42,56]. However, carriership of the C-allele of HTR2 C polymorphism rs1414334 has been associated with an increased risk of metabolic syndrome in patients taking antipsychotics, especially clozapine and risperidone [41,56]. As a whole, the association between rs1414334 polymorphism and obesity is still inconclusive [41–43,56].

Obesity, type 2 diabetes and cardiovascular diseases are associated with decreased adiponectin levels [53]. Adiponectin levels are generally higher in women than in men [59], and obese men in particular seem to have lowered adiponectin levels [50]. Contradictory findings to these, showing no association between plasma adiponectin and BMI or gender, have also been reported [33]. Most studies have suggested that serum adiponectin levels do not differ between patients with schizophrenia and healthy controls, and likewise do not vary according to antipsychotic treatment [29]. Matsuda et al. found negative associations between plasma adiponectin and BMI and HOMA-IR in non-diabetic male patients with schizophrenia [37]. Interestingly, Beumer et al. reported high adiponectin levels in the serum of patients with schizophrenia without MetS, while patients with MetS had even higher serum levels of adiponectin [5]. In a study by Hanssens et al. among patients with schizophrenia, those on clozapine and olanzapine treatment had the lowest adiponectin levels [22]. A negative association between olanzapine treatment and adiponectin levels has been reported in several studies [55,64,68,72].

ADIPOQ gene locus has been shown to be the only major gene for plasma adiponectin [25]. Adiponectin gene ADIPOQ +276G/T (rs1501299) polymorphism has been suggested to be associated with the risk of obesity [63,77] and cardiovascular diseases [20,35,78]. Associations between antipsychotic-related changes in BMI and *ADIPOQ* gene have also been reported [28]. The G-allele of +276G/T polymorphism may be the risk allele for weight gain [36,75].

Adipsin is an endocrine factor expressed by adipocytes and monocytes-macrophages [14,74]. Adipsin levels are either elevated or unchanged in obese human subjects [14,44]. In a recent study by Derosa et al. higher levels of adipsin were associated with higher BMI and HOMA-IR in healthy obese vs. non-obese subjects [11]. Adipsin may play a role in fat cell metabolism and/or systematic energy balance [53]. Adipsin plasma level could be a predictor of changes in abdominal subcutaneous fat during times of increased energy intake. Genetic variation at the adipsin locus may have a role on individual differences in response to food intake inducing weight gain, increase of subcutaneous fat and increase in plasma levels of leptin [70].

The aim of the present study was:

- to test the possible role of serum leptin and adiponectin levels in patients with schizophrenia on clozapine treatment as a marker of weight gain in association with inflammatory cytokines;
- to test the associations of some SNPs of LEP, ADIPOQ and 5-HT2 C on levels of leptin and adiponectin and weight gain during clozapine treatment.

2. Materials and methods

2.1. Patients

The study was conducted in three hospital districts in Western Finland (Satakunta, Pirkanmaa and Seinäjoki Hospital Districts). The patients were recruited at secondary in and outpatient clinics and from sheltered accommodation units. The inclusion criteria for the study were: 1. Stabilized clozapine medication, and 2. Clinical diagnosis of F2 group according to ICD-10. The exclusion criteria were organic brain disease or other neurological disorders. The diagnosis was set by experienced psychiatrists in clinical settings. All patients completed a questionnaire eliciting, among others, estimated weight and height, trend in weight change (marked increase, slight increase, no change, decrease), weight gain in kilograms during clozapine treatment and smoking. The body mass index was calculated by the physicians collecting the data. Information on past medical history and duration of clozapine treatment was collected from patient records. The study was approved by the ethics committee of Tampere University Hospital, and this approval applied to all three sites. All patients gave informed consent on entry to the study. The blood samples were taken during a routine laboratory visit related to the clozapine treatment.

2.2. Controls

For the genotype analysis, the control population consisted of 395 healthy Finnish blood donors. Blood donors complete a health questionnaire and are also interviewed by a nurse to elicit their possible medications and chronic illnesses every time they donate blood.

2.3. Laboratory and clinical methods

For the laboratory analysis venous blood sample was collected after over-night fasting. Serum was separated and stored at –80 °C until analyzed. Glucose, insulin, HDL cholesterol, and triglycerides were measured with Cobas c6000 e601 (Roche Diagnostics) with detection limits of 0.11 mmol/L, 0.2 μU/mL, 0.08 mmol/L and 0.1 mmol/L respectively. Levels of adipsin, adiponectin, leptin, IL-6 and IL-1Ra were measured by enzyme linked immunosorbent assay (ELISA) using following the reagents: DuoSet® ELISA (R&D Systems Europe Ltd, Abingdon, UK) for adipsin, adiponectin and leptin, Ready-SET-GO!® ELISA (eBioscience Inc., San Diego, CA, USA) for IL-6, and Quantikine® for IL-1Ra (R&D Systems). The

detection limits for adipsin, adiponectin, leptin, and IL-1Ra were 15.6 pg/mL, and for IL-6 0.78 pg/mL. Hs-CRP was measured using Tinaquant C-reactive protein (latex) high sensitive assay (Roche Diagnostics) with detection limits 0.05–10 mg/L. The descriptive analysis of HOMA-IR, HDL cholesterol, triglycerides, adiponectin, IL-6, IL-1Ra and hs-CRP levels in this sample has been reported earlier [31].

Undetectable IL-6 levels (41 cases) were considered to be 0 pg/mL and included in the statistical analysis. Missing serum cytokine values (IL-6 and IL-1Ra, $n = 3$) due to limited amount of serum were excluded from the analysis.

2.4. DNA extraction and genotyping

For DNA extraction, 9.0 mL EDTA-whole blood was taken from the participants and stored in a freezer at −20 °C. Genomic DNA was extracted from peripheral blood leukocytes using QIAamp®DNA Blood Minikit and automated biorobot M48 extraction (Qiagen, Hilden, Germany). Genotyping of rs1501299, rs1414334 and rs7799039 was performed using Taqman®SNP Genotyping Assays (assays; C___7497299_10; C___7455701_10 and C___1328079_10 respectively) and ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA).

2.5. Statistical analysis

Comparison of means between subgroups was analyzed with two-tailed *t*-tests. Pearson's correlation was used to analyze the associations between clinical markers. For the statistical analysis, depending on the variable, 0–7 outlier cases from the data were omitted. In the power calculations adiponectin, leptin, and BMI differences of ± 0.84, ± 16.2, and ± 3.14 respectively, between HTR2 C rs1414334 C-carriers or GG-genotype, ± 0.68, ± 13.1, and ± 2.55 between ADIPOQ rs1501299 T-carriers or GG-genotype, and ± 0.87, ± 16.8, and ± 3.26 between LEP rs7799039 genotypes GG, AG or AA were discernible with a probability of 0.8. The statistical significance for correlations between BMI, HOMA-IR, lipids and cytokines/adipokines was set at $P = 0.005$ using correction for multiple testing by dividing the significance level of $P = 0.05$ by the number of markers analyzed. In all other statistical analyses the level of significance was $P < 0.05$. The correlations for weight gain in kilograms were calculated separately for both genders in the subgroups of patients reporting marked increase in weight. Effect size was determined as Cohen's d for leptin and adiponectin levels between different subgroups. Pearson's chi-square (χ^2) test was used to compare numbers of genotypes and allele frequencies between patients and controls. General linear univariate model (GLM) was used for analyzing the explanatory factors (weight increase/no increase, gender and SNPs LEP rs7799039 and ADIPOQ rs1501299) and a covariate (BMI) for leptin and adiponectin levels. One-way ANOVA was used to test the association between HTR2 C rs1414334 genotype and BMI, and levels of leptin and adiponectin, and t-test for the associations between GG-genotype or C-carriers, and BMI and levels of leptin, adiponectin, adipsin, IL-1Ra, HDL, triglycerides and HOMA-IR. Due to the asymmetric distribution, logarithmic transformation was used for levels of HDL, triglycerides, and HOMA-IR. Associations between HTR2 C GG-genotype or C-carriers, and leptin, adiponectin, adipsin, and BMI levels were tested for both genders separately with a non-parametric test (Mann-Whitney U-test). One-way ANOVA was used to test the associations between LEP rs7799039 genotype, and levels of leptin, adiponectin, adipsin, and BMI, and t-test was used for the corresponding associations with ADIPOQ rs1501299 GG-genotype or T-carriers. In the GLM model for triglyceride levels as a target variable, BMI was used as a covariate and gender and rs1414334 genotype were used as factors. Data analysis was carried out using SPSS/Win software (version 19.0, SPSS inc., Chicago, IL, USA) and Power and Sample Size Calculator [12].

3. Results

A descriptive analysis of the patient population has been reported earlier [31]. Of the 190 patients of Finnish origin, 109 (57.4%) were male and 81 (42.6%) were female. Mean age was 42.92 years (range 20–67 years). The average time from the first hospital admission was 17.67 years (2–42 years). One hundred and eleven patients (58.4%) had been on clozapine treatment for more than five years, 60 patients (31.6%) from one to five years and four patients (2.1%) from three to twelve months. Those fifteen patients with no data on treatment duration available were recruited from long-term care units, and had all been on clozapine treatment for more than one year. One hundred and twenty-one patients (63.7%) were on clozapine monotherapy, and 69 (36.3%) had a combination therapy with other antipsychotics. The mean clozapine dose was 403 mg/day (100–800 mg/day). One hundred and one patients (53.2%) were regular smokers, median 20 cigarettes per day.

The mean BMI (SD) males vs. females was 29.00 (5.79) vs. 31.20 (6.91) kg/m^2, $P = 0.021$. One hundred and one (55.8%) patients (48 men and 53 women) reported weight gain during clozapine treatment and 80 (44.2%) patients (54 men and 26 women) reported their weight either unchanged or decreased. The mean BMI of those who had reported weight gain was significantly higher than the mean BMI of those not reporting weight gain. Among female patients this difference in mean (SD) was 33.26 (7.04) vs. 27.36 (5.15) kg/m^2, $P < 0.001$, and among male patients 31.11 (6.14) vs. 27.59 (4.75) kg/m^2, $P = 0.002$.

Leptin levels were higher in female patients, male vs. female, mean (SD), 13.47 (16.13) vs. 47.86 (37.11) ng/mL, $P < 0.001$, Cohen's d = 1.27. Women who had gained weight had higher leptin levels than women who had not, 58.81 (38.58) vs. 27.64 (23.67)ng/mL, $P < 0.001$, Cohen's d = 0.90. In males the corresponding difference in leptin levels was 17.66 (20.90) vs. 10.11 (9.97)ng/mL, $P = 0.026$, Cohen's d = 0.47. The levels of leptin also correlated in the total population with (males, females) BMI ($r = 0.68$, $P < 0.001$, $r = 0.65$, $P < 0.001$), and levels of adipsin ($r = 0.39$, $P < 0.001$, $r = 0.44$, $P < 0.001$) and IL-1Ra ($r = 0.50$, $P < 0.001$, $r = 0.53$, $P < 0.001$). In female patients leptin also correlated with IL-6 ($r = 0.47$, $P < 0.001$), and with weight gain in kilograms among those reporting marked weight increase ($r = 0.47$, $P = 0.006$, $n = 33$), but neither were found among males. In male patients leptin correlated with levels of triglycerides ($r = 0.34$, $P < 0.001$), but not in females.

Men with weight gain had lower levels of adiponectin than men without weight gain, mean (SD) 2.56 (1.04) vs. 3.09 (1.48)μg/mL, $P = 0.037$, Cohen's d = 0.41. Among women there was no difference in adiponectin levels between those who gained weight and those who did not, 3.52 (1.72) vs. 4.32 (2.08)μg/mL, $P = 0.076$, Cohen's d = 0.43.

Adipsin levels, mean (SD), among males and females were 1.27 (0.30) μg/mL and 1.30 (0.31) μg/mL. Levels of adipsin correlated with IL-1Ra ($r = 0.23$, $P = 0.002$) and IL-6 ($r = 0.21$, $P = 0.004$) in total patient population. In both genders adipsin correlated with levels of leptin (males $r = 0.39$, $P < 0.001$, females $r = 0.44$, $P < 0.001$). In male patients adipsin correlated with IL-1Ra ($r = 0.28$, $P = 0.004$), and in female patients with IL-6 ($r = 0.40$, $P < 0.001$). There also tended to be an association between BMI and levels of adipsin among female patients ($r = 0.30$, $P = 0.008$).

The univariate analysis of variance (GLM) for explanatory factors of leptin and adiponectin levels is presented in Table 1. In GLM the complete model explained 62.1% of the variance in leptin levels, $P < 0.001$. BMI explained 36.2% ($P < 0.001$), gender 29.1%

Table 1
Results of the two ANCOVA models in which adiponectin (1) and leptin (2) serum levels were used as target variables and gender, weight change and adiponectin rs 1501299 (1) or leptin rs7799039 (2) genotype as factors and BMI as covariate.

	Adiponectin serum level			Leptin serum level		
	ηp^2	*P*	Power	ηp^2	*P*	Power
Complete model	0.275	< 0.001	1	0.621	< 0.001	1
Explanatory variables						
BMI	0.078	< 0.001	0.943	0.362	< 0.001	1
Gender	0.087	< 0.001	0.964	0.291	< 0.001	1
Weight change[a]	0.006	0.36	0.151	0.024	0.059	0.472
Gender × weight change	0.043	0.010	0.732	0.033	0.024	0.620
ADIPOQ genotype	0.010	0.484	0.172			
LEP genotype				0.016	0.301	0.261

BMI: body mass index.
[a] Weight increase/no weight increase.

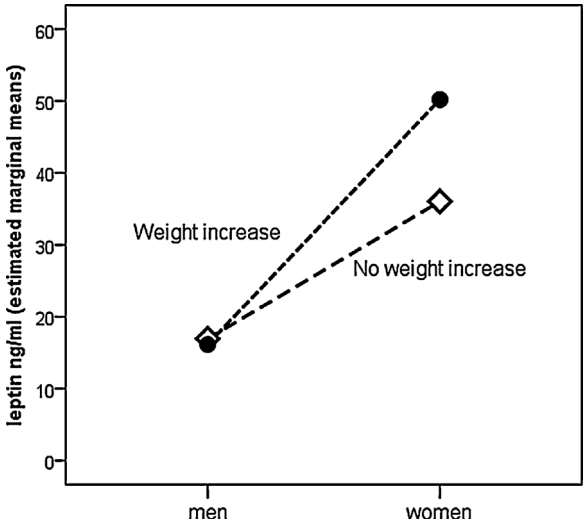

Fig. 1. Estimated marginal means of leptin serum levels (ng/mL) according to GLM model (Table 1) between men and women with weight increase or no weight increase.

($P < 0.001$), and gender and weight gain together 3.3% ($P = 0.024$) of the variance in leptin levels. Gender-dependent leptin level alterations in association with BMI adjusted weight change are presented in Fig. 1. The complete model explained 27.5% of the variance in adiponectin levels, $P < 0.001$. BMI explained 7.8% ($P < 0.001$), gender 8.7% ($P < 0.001$), and gender and weight gain together 4.3% ($P = 0.01$).

Levels of serum adipsin, adiponectin and leptin, and BMI between LEP rs7799039, ADIPOQ rs1501299 and HTR2 C rs1414334 genotypes are presented in Table 2. There were no differences in genotype frequencies of ADIPOQ rs1501299 between patients and controls. In HTR2 C polymorphism rs1414334 the frequency of CC genotype was 9.6% among patients compared with 3.7% among controls ($P = 0.013$). BMI, weight increase, HOMA-IR and levels of adiponectin, leptin and IL-1Ra were not associated with rs1414334 genotype. Instead, GG-genotype was associated with higher levels of triglycerides ($P = 0.001$) and lower levels of HDL cholesterol ($P = 0.048$). In GLM the complete model explained 21.7% of the triglyceride levels, $P < 0.001$. BMI explained 14.4% ($P < 0.001$), gender 3.4% ($P = 0.016$) and HTR2 C rs1414334 genotype 3.4% ($P = 0.017$).

4. Discussion

To the best of our knowledge, this is the largest study so far combining genetic polymorphisms LEP rs7799039, ADIPOQ rs1501299 and HTR2 C rs1414334 and corresponding cytokine/adipokine levels with weight gain in patients with schizophrenia on clozapine treatment. Moreover, no previous data exists on levels of adipsin in patients with schizophrenia.

Weight gain during clozapine treatment explained the obesity of the present patients. Women with weight gain had highest levels of leptin and the difference in leptin levels between female patients with or without weight gain was remarkable. This finding suggests a gender-dependent leptin dysregulation among these patients [5,57,73]. Neuropeptide Y (NPY) mediates the effects of leptin on the control of food intake and regulates HPG axis function [16,54]. Clozapine influences the expression of NPY in hypothalamus [27,30,67]. Estrogen regulates body weight by controlling food intake, body adiposity, and energy expenditure. Estrogen deficiency is associated with leptin resistance and an increase in

Table 2
Levels of serum adipsin, adiponectin and leptin, and body mass index, mean (SD) or median (IQR), between LEP rs7799039, ADIPOQ rs1501299 and HTR2 C rs1414334 genotypes.

	S-adipsin (μg/mL)	S-adiponectin (μg/mL)	S-leptin (ng/mL)	BMI (kg/m^2)
LEP				
GG ($n = 40$)	1.31 (0.36)	3.55 (1.87)	28.41 (32.38)	29.31 (6.41)
AG ($n = 86$)	1.26 (0.28)	3.06 (1.36)	26.95 (31.89)	29.21 (5.61)
AA ($n = 53$)	1.31 (0.31)	3.29 (1.82)	29.36 (34.22)	31.22 (7.38)
ADIPOQ				
GG ($n = 89$)	1.25 (0.29)	3.12 (1.40)	27.71 (30.46)	30.15 (6.11)
T-carrier ($n = 91$)	1.31 (0.33)	3.37 (1.83)	28.41 (34.46)	29.51 (6.65)
HTR2C				
Men				
GG[a] ($n = 92$)	1.27 (0.36)	2.54 (1.41)	8.53 (12.21)	27.59 (7.55)*
C-carrier[a] ($n = 15$)	1.11 (0.29)	2.67 (2.78)	7.40 (8.27)	26.60 (5.38)
Women				
GG[a] ($n = 51$)	1.22 (0.4)	3.50 (2.17)	41.56 (42.83)	30.50 (9.47)
C-carrier[a] ($n = 22$)	1.34 (0.38)	3.21 (2.10)	42.33 (53.11)	29.80 (9.39)
All				
GG ($n = 143$)	1.28 (0.30)	3.14 (1.54)**	26.48 (30.68)	30.04 (6.26)
C-carrier ($n = 37$)	1.29 (0.34)	3.67 (1.91)	34.20 (38.43)	28.96 (6.86)

In all other comparisons between genotypes $P > 0.10$. BMI: body mass index.
[a] Median, interquartile range (IQR).
* $P = 0.08$ between genotypes (Mann-Whitney *U*-test).
** $P = 0.08$ between genotypes (*t* test).

visceral adiposity [61]. Estrogen may mediate its anorectic effects by decreasing NPY expression or release [8]. There is also an interaction between estrogen and serotonergic transmission [3,8]. It has been suggested that 5-HT2 C receptor mediates the anorectic effects of leptin, and blocking this receptor may interrupt the inhibitory effect of leptin on NPY neurons. Clozapine is a high-affinity 5-HT2 C antagonist [30]. Estrogen deficiency may also have some role in the pathophysiology of schizophrenia [24,34] and estrogen pathway might be involved in the therapeutic effect of antipsychotics [51]. Clozapine also directly influences the biosynthesis and regulation of fatty acids and cholesterol [9,15].

The levels of leptin were associated with IL-1Ra in both genders, with triglycerides in male patients and with IL-6 in female patients. These findings are in line with findings from a Swedish general population sample, where leptin was associated with factors for metabolic syndrome in men, while in women leptin was associated with inflammatory factors, suggesting different regulatory mechanisms and effects of leptin in men and women [1].

LEP rs7799039 genotype was not associated with levels of leptin in the present patient group. This concurs with those earlier findings suggesting that this LEP polymorphism is not associated with obesity susceptibility [77] or antipsychotic-induced weight gain [18,19,47].

Weight gain among the present male patients was associated with low adiponectin levels. This concurs with findings according to which adiponectin levels correlated negatively with visceral adiposity (typical for male obesity) to a greater extent than with subcutaneous adiposity [21]. It is suggested that the regulation of adiponectin in visceral adipose tissue of men is more sensitive to catecholamine-stimulated lipolysis with an association of higher nonesterified fatty acids and lower adiponectin levels in obese men only [50]. In an earlier report [4], and also according to our earlier results [31], hypoadiponectinemia has been associated with metabolic syndrome especially among male patients with schizophrenia on clozapine treatment.

The present results do not support a major role of ADIPOQ rs1501299 polymorphism in serum adiponectin alterations or clozapine-induced weight gain. Earlier reports of an association between ADIPOQ rs1501299 polymorphism and serum adiponectin levels have been controversial with results suggesting a minor T-allele association with either lowered, elevated or unchanged levels of adiponectin [20,40,52,63,69], which suggests heterogeneity across different populations. In a Finnish diabetes study sample no association was found between rs1501299 and serum adiponectin levels [63].

As far as we know, levels of adipsin have not been studied previously among patients with schizophrenia. Adipsin levels were linked with levels of leptin in both genders, and among female patients with levels of IL-6. According to our earlier report, levels of IL-6 were associated with obesity among the present female patients, but not among males [31]. In the present study, adipsin levels tended to be associated with BMI in female patients, but no gender difference in mean levels of adipsin was found. This may indicate a role of adipsin in gender-dependent response to food intake, and as a predictor of changes in fat distribution. The role of adipsin and its genetic variation as a predictor of obesity related inflammation needs to be studied further.

As in some earlier reports, in the present study no association was found between the C-allele of HTR2 C polymorphism rs1414334 and obesity [56]. Neither was there any association between rs1414334 polymorphism and HOMA-IR, or levels of IL-1Ra as markers of metabolic comorbidity [58,65]. In contrast to some earlier findings [56], among the present patients the GG-genotype was associated with higher triglyceride levels and lower HDL cholesterol levels. The CC genotype was more common in the present patient population than in the control population, but in fact the minor C-allele frequency of the controls was less than expected. No more detailed explanation for this could be sought in this setting. In all, the results must be interpreted with caution due to a low minor allele frequency of this SNP. HTR2 C polymorphism -759 C/T (rs3813929), although a most obvious candidate regarding antipsychotic-induced weight gain, was not analyzed in this study due to our earlier results with no associations with BMI or weight gain in this patient group, and no differences in genotype frequencies between patients and controls.

There are certain limitations in the study. Only single SNPs were studied and linkage disequilibrium with some other previously studied SNPs can not be excluded. Moreover, the sample size for genetic association study was limited, although group differences at effect size $\geq$0.5 in the target variables were discernible according to the power calculations. The study was cross-sectional, the clinical data was collected retrospectively, and a healthy control population for the adipokine levels would have enhanced the reliability of the results. Combination therapy with other antipsychotics with different side-effect and receptor profiles in one third of the patients may also be a confounding factor. However, this sample can be considered homogenous and representative of the group of patients with long-term schizophrenia on clozapine treatment.

Obesity related morbidity and mortality, stigmatization, and non-adherence are associated with antipsychotic-induced weight gain. Adverse effects of antipsychotics vary between individuals and there are no clinically reliable predictors so far available in clinical practice. However, it seems that there are alterations in serum markers already prior to treatment in patients who develop weight gain during antipsychotic treatment. In a recent study by Schwarz et al. concentrations of 191 molecules were studied longitudinally in 77 patients with schizophrenia. The serum levels of 10 molecules before treatment initiation were associated with weight gain during treatment [66]. Genetic influence on antipsychotic-induced weight gain has been studied widely in recent years. Genome-wide association studies have found associations with antipsychotic-induced weight gain also in several other genes not discussed here, and these studies need to be replicated. Recent data indicates a strong effect in the region of MC4R (melanocortin 4 receptor) gene on the development of obesity and antipsychotic-induced weight gain [7].

In conclusion, levels of leptin, weight gain during clozapine treatment and inflammatory markers related to metabolic comorbidity (IL-6, IL-1Ra) showed a marked interaction, especially among female patients with schizophrenia treated with clozapine. In male patients low adiponectin level was a more specific marker of clozapine-induced weight gain. Longitudinal studies are needed to clarify the mechanisms and directions of interactions. The results of the present study do not support a major role of LEP rs7799039, ADIPOQ rs1501299 and HTR2 C rs1414334 polymorphisms in the regulation of serum leptin and adiponectin levels or weight gain.

Disclosure of interest

J.-P.K. has consulted for Otsuka and received support from Bristol-Myers Squibb, Eli Lilly, Lundbeck and Janssen-Cilag to participate in international congresses. O.K. has consulted for Medivir; received speaker's fees from Janssen-Cilag and Astra-Zeneca and received support from Otsuka to participate in an international congress. N.S. has received support from Bristol-Myers Squibb and Janssen-Cilag to participate in international congresses. M.V. has consulted for Astra Zeneca, GlaxoSmithKline, Eli Lilly and Servier; conducted clinical trials for Bristol-Myers

Squibb, Eli Lilly, Lundbeck, Sanofi-Aventis and Servier; received support from Bristol-Myers Squibb, GlaxoSmithKline, Eli Lilly, Lundbeck and Servier to participate in international congresses; and received a research grant from GlaxoSmithKline, Lundbeck Foundation and Pfizer. E.L. has worked as a lecturer or chairman in symposia sponsored by pharmaceutical companies Astra-Zeneca, Eli Lilly, Lundbeck and Servier; served on the national advisory board of Servier and received support from Astra-Zeneca, Lundbeck, Otsuka and Servier to participate in international congresses.

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Resistin as an inflammatory marker in patients with schizophrenia treated with clozapine

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Abstract

Background: Schizophrenia is associated with excess cardiovascular comorbidity and mortality related to lifestyle factors, such as lack of physical activity, poor diet and smoking. The prevalence of metabolic syndrome is increased among patients with schizophrenia, with the highest rates among patients on clozapine treatment. Smoking, obesity, physical inactivity, airway inflammation and obstruction, and adipose tissue and inflammatory marker activation are related in systemic inflammation. Low-grade inflammation is also associated with schizophrenia. Adipokine resistin is a biomarker involving several acute and chronic inflammatory states. However, the inflammatory role of resistin is so far inconclusive and studies in schizophrenia are scanty.

Aims: The aim of the present study was to explore the role of serum resistin as an inflammatory marker in patients with schizophrenia on clozapine treatment.

Methods: Associations between serum levels of resistin and some other selected cytokines/adipokines (adiponectin, leptin, adipsin, IL-6, IL-1Ra, TNF-α, hs-CRP) and metabolic markers in 190 patients with schizophrenia on clozapine treatment were studied using a cross-sectional study design.

Results: Among male patients especially, smokers had higher levels of resistin than non-smokers, and among smokers resistin levels were associated with IL-1Ra and hs-CRP levels. In whole patient group levels of resistin associated with levels of IL-1Ra, and among male patients with low HDL-cholesterol.

Conclusions: Resistin is a biomarker of systemic inflammation associated with smoking among patients with schizophrenia on clozapine treatment. Resistin might have a role as a marker of cardiovascular comorbidity.

Keywords: cytokine; smoking; IL-1Ra; hs-CRP; IL-6

1. Background

Schizophrenia is associated with a marked increase in mortality (1). Cardiovascular disease accounts for the greatest number of early deaths. The average lifestyle of patients with schizophrenia increases their risk of cardiovascular disease: sedentary lifestyle, lack of regular physical activity, poor diet, substance use and high rates of smoking (2). According to a recent review, 56% of patients with treatment resistant schizophrenia are smokers and according to a recent population-based cohort study from the United States, tobacco-related conditions accounted for 53% of total deaths among patients with schizophrenia (3,4). Increased prevalence of metabolic syndrome is a well-established finding among patients with schizophrenia (5) with the highest rates among patients on clozapine treatment (6), although the overall mortality rate is lowest among clozapine treated patients (7). According to a recent Finnish study, 58.7% of the patients with schizophrenia had metabolic syndrome, and clozapine treatment doupled the risk (8). A chronic low-grade inflammation with alterations in several cytokines is associated with both schizophrenia (9) and obesity/metabolic syndrome (10).

Adipokine resistin is associated with several acute and chronic inflammatory states (11,12,13,14,15,16). Transcription of the resistin gene is enhanced by several pro-inflammatory cytokines, including interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α). Resistin promotes the expression of TNF-α and IL-6 by human mononuclear cells. Resistin also directly counters the anti-inflammatory effects of adiponectin on vascular endothelial cells (17). The association between levels of resistin and high-sensitivity C-reactive protein (hs-CRP) is inconclusive (18,19,20). An association between resistin and interleukin-1 receptor antagonist (IL-1Ra) has also been reported (21). Circulating levels of resistin do not differ between genders, although there are some findings suggesting higher levels in women (10,18,22,23).

The functional role of resistin is not clear (24). There is inconsistency in the evidence linking levels of serum resistin to obesity and diabetes (10,25). High plasma resistin concentration has been associated with metabolic syndrome and its components and markers of insulin resistance (20). This association between resistin and insulin resistance may be gender dependent, with a stronger association in men (26). Resistin appears to have a role in coronary artery disease (13). Elevated level of resistin is associated with increased risk of heart failure (27), possibly more strongly among women (23). High serum resistin is also reported to be a risk factor for cardiovascular disease and all-cause mortality in diabetic patients (28).

Resistin seems to be a disease activity marker of pulmonary inflammation in smokers, asthma, and COPD (19,29,30,31). Cigarette smoke activates macrophages to secrete proinflammatory cytokines TNF-α, IL-1beta and IL-6 (32). On the other hand, nicotine reduces the production of the same

proinflammatory cytokines (33). In all, associations between smoking and cytokine alterations have so far been inconclusive (34).

Associations between resistin and antipsychotic treatment or schizophrenia have been little studied (35,36,37,38). In the study by Birkás Kováts et al., the levels of resistin were significantly higher in patients with second generation antipsychotics than in healthy controls, and correlated positively with levels of TNF-α, insulin and homeostasis model assessment-estimated insulin resistance (HOMA-IR) index (35). The study by Perez-Iglesias et al., showed no changes in levels of resistin nor associations between levels of resistin and weight gain after one year of antipsychotic treatment (36). Clozapine has also been found to inhibit mRNA expression of resistin in mouse brown adipocytes (37). However, the applicability of that finding in human cells is not clear, especially considering that monocytes and macrophages are the major sources of resistin in man (24).

2. Aims

Although schizophrenia and obesity/metabolic syndrome are associated with inflammatory state mediated by cytokines, the role of cytokine alterations is still inconclusive. Therefore the aim of the present study was first to study the role of serum resistin level as a marker of inflammation in association with some other inflammatory cytokines/adipokines (IL-6, IL-1Ra, TNF-α, hs-CRP, leptin, adiponectin, adipsin), weight, lipid status and insulin resistance in patients with schizophrenia on clozapine treatment. The second aim was to explore the associations of serum levels of these markers among smokers and non-smokers in this patient group.

3. Materials and Methods

3.1 Patients

The study was conducted in three hospital districts in Western Finland (Satakunta, Pirkanmaa, and Seinäjoki Hospital Districts). The patients were recruited at secondary in and outpatient clinics and from sheltered accommodation units between April 2008 and January 2010. The inclusion criteria for the study were: 1. Stabilized clozapine medication, and 2. Clinical diagnosis of F2 group according to ICD-10. The exclusion criteria were organic brain disease or other neurological disorders. The diagnosis was set by experienced psychiatrists in clinical settings. Of the 256 patients screened, nineteen declined. All patients completed a questionnaire eliciting, among others, weight and height, trend in weight change (marked increase, slight increase, no change, decrease) and weight gain in kilograms during clozapine treatment, and smoking. The body mass index was

calculated by the physicians collecting the data. Information on past medical history and duration of clozapine treatment was collected from patient records. Of the 237 patients entering the study, blood sample for the laboratory analysis was available from 190 patients.

A descriptive analysis of the patient population has been reported comprehensively earlier (39). Of the 190 patients, 109 (57.4%) were male and 81 (42.6%) were female. Mean age was 42.92 years (range 20-67 years). The average time from the first hospital admission was 17.67 years (2-42 years). One hundred and eleven patients (58.4%) had been on clozapine treatment for more than five years, 60 patients (31.6%) from one to five years and four patients (2.1%) from three to twelve months. Those fifteen patients with no data on treatment duration available were recruited from long-term care units, and had all been on clozapine treatment at least more than one year. One hundred and twenty-one patients (63.7%) were on clozapine monotherapy, and 69 (36.3%) had a combination therapy with other antipsychotics. The mean clozapine dose was 403 mg/day (100-800mg/day). The mean BMI was 29.92 kg/m^2. One hundred and one patients (53.2%) were regular smokers, median 20 cigarettes per day. Smoking did not differ between genders, and it was not associated with BMI.

3.2 Ethics, consent and permissions

The study was approved by the local ethics committee, and this approval applied to all three sites. All patients gave informed consent on entry to the study. The blood samples were taken during a routine laboratory visit related to the clozapine treatment.

3.3 Laboratory and Clinical Methods

For the laboratory analysis venous blood sample was collected after over-night fasting. Serum was separated and stored at -80 °C until analyzed. Glucose, insulin, HDL-cholesterol, and triglycerides were measured with Cobas c6000 e601 (Roche Diagnostics) with detection limits of 0.11 mmol/l, 0.2 µU/ml, 0.08 mmol/l and 0.1 mmol/l respectively. Levels of adipsin, adiponectin, resistin, leptin, IL-6, IL-1Ra, and TNF-α were measured by enzyme linked immunosorbent assay (ELISA) with the following reagents: DuoSet® ELISA (R&D Systems Europe Ltd, Abingdon, UK) for adipsin, adiponectin, resistin and leptin, Ready-SET-GO!® ELISA (eBioscience Inc., San Diego, CA, USA) for IL-6, and Quantikine® for IL-1Ra and Quantikine® HS for TNF-α (R&D Systems). Detection limits for adipsin, adiponectin, resistin, leptin, and IL-1Ra were 15.6 pg/ml, and for IL-6 and TNF-α 0.78 and 0.5 pg/ml, respectively. Hs-CRP was measured using Tinaquant C-reactive protein (latex) high sensitive assay (Roche Diagnostics) with detection limits 0.05-10 mg/l. The descriptive

analysis of HOMA-IR, HDL-cholesterol, triglycerides, adipsin, adiponectin, leptin, IL-6, IL-1Ra and hs-CRP levels in this sample has been reported earlier (39,40).

Undetectable IL-6 levels (41 cases) were considered to be 0 pg/ml and included in the statistical analysis. Missing serum cytokine values (IL-6 and IL-1Ra, n=3; TNF-α, n=2) due to limited amount of serum were excluded from the analysis.

3.4 Statistical Analysis

Comparison of means between subgroups was analyzed with two-tailed t-tests, except for the comparison of hs-CRP levels between smokers and non-smokers where Mann-Whitney U-test was used due to asymmetric distribution. Pearson's correlation was used to analyze the associations between clinical markers. For the statistical analysis, depending on the variable, 0-7 outlier cases in the data were omitted. Distributions of each variable were checked manually to control for possible technical errors in laboratory assays. The statistical significance of correlations between BMI, HOMA-IR, lipids and cytokines/adipokines was set at $p=0.004$ using the Bonferroni correction by dividing the significance level of $p=0.05$ by the number of markers analyzed. In all other statistical analyses the level of significance was $p<0.05$. Pearson's chi-square ($\chi 2$) test was used to compare the number of smokers and non-smokers between male and female patients. General linear univariate model (GLM) was used for analyzing the explanatory factors for levels of resistin. The best-fitting model comprised regular smoking, IL-1Ra level and hs-CRP level as explanatory factors. Several other factors and covariates (age, gender, BMI, clozapine dose, clozapine concentration, IL-6 level, triglyceride level, number of cigarettes/day) were used in this model as explanatory variables. Due to the asymmetric distribution of levels of IL-1Ra and hs-CRP, logarithmic transformation was used in the bivariate analysis of correlations between levels of resistin and IL-1Ra and hs-CRP. The data analysis was carried out using SPSS/Win software (version 19.0, SPSS inc., Chicago, IL, USA).

4. Results

The mean (SD) serum level of resistin among patients was 27.06 (9.24) ng/ml. Age, gender, BMI, clozapine dose, clozapine concentration, HOMA-IR, or levels of triglycerides were not associated with levels of resistin. The serum levels of resistin correlated in the whole patient group with levels of IL-1Ra ($r=0.41$, $p<0.001$), and in male patients negatively with HDL-cholesterol ($r=-0.29$, $p=0.003$). Levels of resistin also tended to correlate in the whole patient group with hs-CRP ($r=0.20$, $p=0.005$) and TNF-α ($r=0.15$, $p=0.037$). The comparisons of serum resistin levels between subgroups according to age, gender, BMI, smoking and clozapine treatment are presented in Table

1. The correlations between serum resistin level and other metabolic and inflammatory markers are presented in Table 2.

Table 1.

Table 2.

The mean (SD) levels of resistin in smokers vs. non-smokers in all patients were 29.39 (10.07) vs. 24.54 (7.43) ng/ml ($p<0.001$), among male patients 29.65 (9.87) vs. 23.51 (7.29) ng/ml ($p=0.001$), and among female patients 29.00 (10.46) vs. 25.75 (7.51) ng/ml ($p=0.11$), respectively. Among smokers levels of resistin correlated with levels of IL-1Ra ($r=0.47$, $p<0.001$) and hs-CRP ($r=0.35$, $p<0.001$), Figure 1. Among non-smokers there were no associations between levels of resistin and the other cytokines/adipokines studied. In GLM the complete model with explanatory factors smoking, IL-1Ra and hs-CRP explained 16.6% of the variance of resistin levels ($p<0.001$). Interaction of smoking and hs-CRP explained 13.7% ($p<0.001$) and IL-1Ra 2.7% ($p=0.026$).

Figure 1.

Smoking was also associated in the whole patient group with higher levels of hs-CRP. The mean (SD) levels of hs-CRP among smokers vs. non-smokers were 4.48 (5.50) vs. 3.64 (4.85) mg/l ($p=0.041$, Mann-Whitney U-test). Among male patients smoking was also associated with higher levels of IL-6, mean (SD) 3.04 (2.69) vs. 2.10 (2.07) pg/ml ($p=0.046$).

5. Discussion

Levels of resistin were associated with levels of IL-1Ra in the whole patient group. Resistin had also trend like correlation after Bonferroni correction with hs-CRP and TNF-α. This supports the role of resistin as an inflammatory marker. The levels of resistin were associated with low levels of HDL-cholesterol in male patients. An association between high levels of resistin and low HDL-cholesterol has also been reported in general population (23,41). This finding may reflect the dietary pattern of the present male patients and its association with low-grade inflammation (42). Contrary to one earlier study on patients taking atypical antipsychotics (35), no association between levels of resistin and HOMA-IR was found in the present patients. Moreover, there were no associations between levels of resistin and other adipokines, that has been associated with clozapine treatment and metabolic abnormalities in people with schizophrenia (43, 44, 45).

Smokers had higher levels of resistin than non-smokers, especially among male patients. Among smokers levels of resistin were also associated with higher levels of IL-1Ra and hs-CRP. The present smoking men also had higher levels of IL-6 than non-smoking men. In samples from general population has been found that patients with COPD and asthma had higher levels of resistin than healthy controls, and resistin levels of smokers were similar to the resistin levels of patients with inflammatory obstructive airway disease (29). Accordingly, the prevalence of smoking has been found to be higher in those subjects with the highest levels of resistin, and resistin levels have been reported to be higher in smoking men than non-smoking men, but were not associated with CRP (19,23).

Among present male patients smoking was associated with higher levels of IL-6. In general population smoking seems to be associated with visceral fat accumulation (46) and insulin resistance (47). Smoking, obesity, physical inactivity, airway inflammation and obstruction, and adipose tissue and inflammatory marker activation are all related in systemic inflammation phenomena (48). Visceral adipose tissue produces more IL-6 and resistin than subcutaneous adipose tissue (10,49). Excess central visceral adiposity (typical for male obesity) is associated with mortality related to increased metabolic disorders (50). Smoking has also been associated with metabolic syndrome among patients with schizophrenia (51).

In the study by Zhang et al., levels of IL-6 were lower among smoking than non-smoking male patients with schizophrenia. Smokers had also decreased IL-2 levels, fewer positive symptoms, and smoking greater number of cigarettes correlated with fewer negative symptoms. The authors suggested nicotine-induced suppression of inflammatory abnormalities in schizophrenia (52). However, the association between smoking and IL-6 levels was the opposite in the present male patients. There may be some confounding factors explaining this difference between these studies. Weight and fat distribution could be different, duration and severity of psychiatric illness and actual clinical psychiatric status and treatments as well as genetic factors may vary. Indeed, the present patients were more obese than the patients in the study by Zhang et al. Moreover, the patients in the study by Zhang et al. were chronically hospitalized, and less than half of them were on clozapine. Also, the patients were of different ethnic origin making comparison difficult (52). Clozapine concentrations in the present patients did not differ between smokers and non-smokers, although smokers had higher clozapine doses (53). As well, levels of resistin did not associate with clozapine concentrations in the present patients. Overall, clozapine is involved in cytokine alterations in several ways. It has direct immunomodulatory effects, treatment response and symptom reduction may affect cytokine levels, and clozapine related metabolic consequences are as such associated with low-grade inflammation.

To the best of our knowledge, this is the largest study so far on resistin in patients with schizophrenia treated with clozapine when studying the contribution of resistin. The study sample can be considered representative in this group of patients. Several concomitant factors were analyzed in GLM, and thus their possible confounding effects were excluded.

There are also certain limitations in the study. The study was cross-sectional and the clinical data was collected retrospectively. Moreover, a healthy control population for resistin levels would have enhanced the value of the information. Levels of resistin are not comparable between different studies due to non-standardized nature of the assays. Data on waist circumference for the present patients was not available for the assessment of central obesity. Hs-CRP turned out to be not such a sensitive marker in this patient population, while levels of hs-CRP were relatively low in most of the patients. Moreover, there may be some confounding factors with no data available in the study group, such as alcohol and substance abuse or some chronic inflammatory diseases, which may have influenced inflammatory markers. The possibility of an acute infection in some patient can likewise not be excluded. Soluble urokinase-type plasminogen activator receptor (SuPAR), another marker of inflammatory activity, which has been associated with mortality, metabolic syndrome and smoking, has been recently found to associate with schizophrenia (54). Association between resistin and SuPAR should be studied in the future.

In conclusion, serum levels of resistin are associated with smoking among patients with schizophrenia on clozapine treatment. Resistin is a biomarker of systemic inflammation, which might have a role as a marker of cardiovascular comorbidity. Further effort should be invested in smoking reduction and cessation among these patients.

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Disclosure of interest

J-P.K. has consulted for Otsuka and received support from Bristol-Myers Squibb, Eli Lilly, Lundbeck and Janssen-Cilag to participate in international congresses.

O.K. has consulted for Medivir; received speaker´s fees from Janssen-Cilag and received support from Otsuka and Lundbeck to participate in international congresses.

N.S. has received support from Bristol-Myers Squibb and Janssen-Cilag to participate in international congresses.

M.V. has consulted for Astra Zeneca, GlaxoSmithKline, Eli Lilly and Servier; conducted clinical trials for Bristol-Myers Squibb, Eli Lilly, Lundbeck, Sanofi-Aventis and Servier; received support from Bristol-Myers Squibb, GlaxoSmithKline, Eli Lilly, Lundbeck and Servier to participate in international congresses; and received a research grant from GlaxoSmithKline, Lundbeck and Pfizer.

E.L. has worked as a lecturer or chairman in symposia sponsored by pharmaceutical companies Astra-Zeneca, Eli Lilly, Lundbeck and Servier; served on the national advisory board of Servier and received support from Astra-Zeneca, Lundbeck, Otsuka and Servier to participate in international congresses.

Table 1. Comparisons of serum resistin levels between subgroups

	n	Resistin mean (ng/ml)	SD
Age 20-43 years	95	27.49	9.31
44-65 years	94	26.74	9.17
Gender Men	109	26.81	9.29
Women	81	27.39	9.21
BMI (kg/m^2) <30	104	26.83	9.06
≥30	78	27.26	9.80
Regular smokers*	101	29.39	10.07
Non-smokers	87	24.54	7.43
Clozapine monotherapy	121	27.58	9.38
combination	69	26.13	8.97
Cloz.+norcloz conc.<2.2	88	26.42	8.02
≥2.2	94	26.71	9.54
Cloz. duration <5 years	64	27.36	9.42
≥5 years	111	26.76	9.34

All other group comparisons $p>0.05$, except $p<0.001$* between regular smokers and non-smokers

Table 2. Correlations (r) between serum resistin levels and other metabolic and inflammatory markers

Resistin	All n=190	Men n=109	Women n=81
HOMA-IR	0.09*		
HDL-cholesterol**		-0.29,p=0.003	-0.15*
log10 Trigly**		0.07*	0.09*
Adipsin	0.09*		
Adiponectin**		-0.10*	-0.14*
Leptin**		-0.01*	0.10*
IL-1Ra	0.41, p<0.001,n=179		
log IL-1Ra	0.35, p<0.001,n=186		
IL-6	0.09*, n=187		
TNF-α	0.15, p=0.037, n=186		
log TNF-α	0.17, p=0.023,n=186		
hs-CRP	0.20, p=0.005,n=188	0.17, p=0.085, n=107	0.25, p=0.027. n=81
log hs-CRP	0.21, p=0.005,n=188		

*All other correlations p>0.05. **Correlations calculated separately for both genders due to gender difference in levels of HDL-cholesterol, triglycerides, leptin and adiponectin (39, 40).

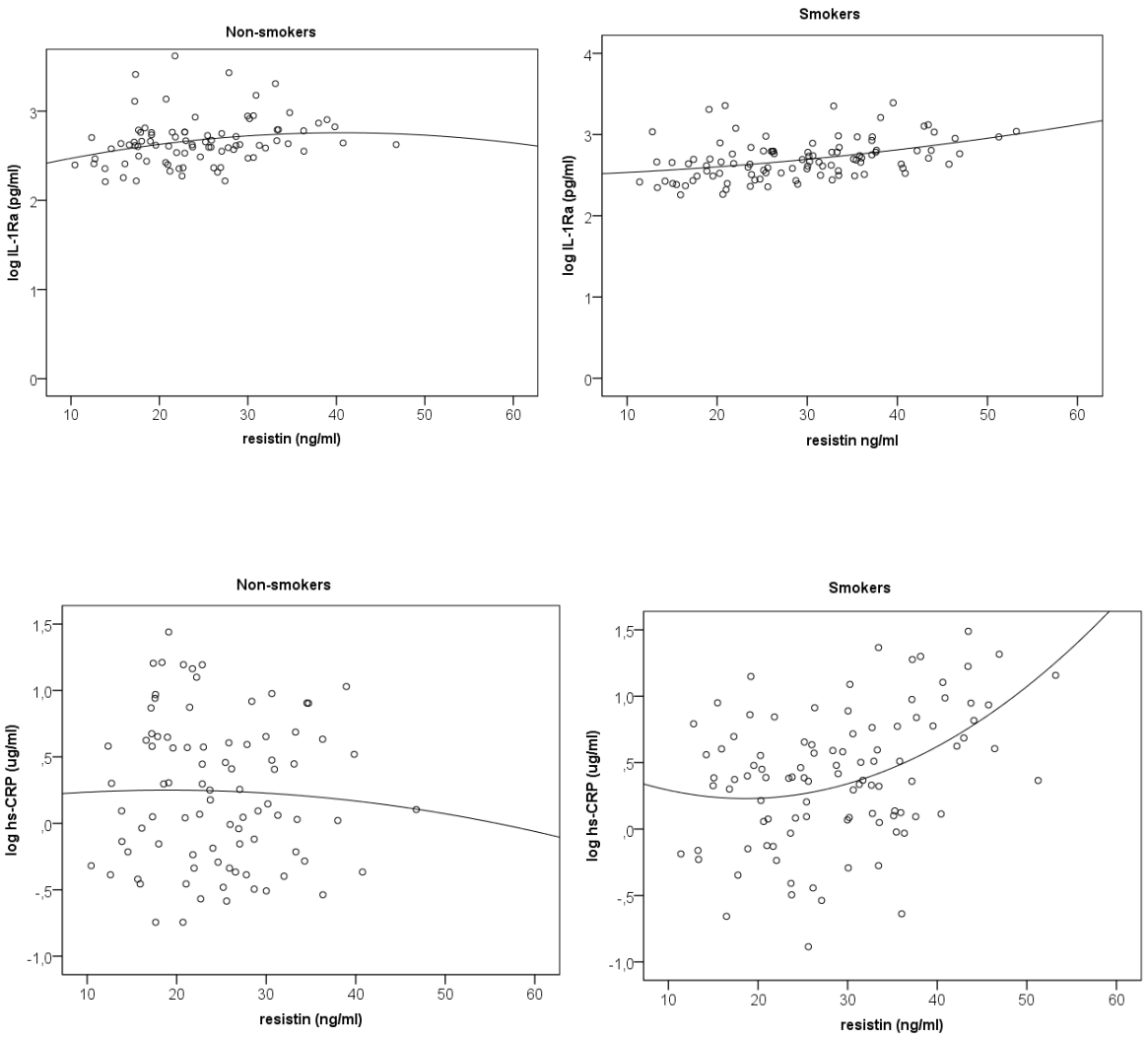

Figure 1. Scatters between serum IL-1Ra and resistin levels (upper row) and hs-CRP and resistin levels (lower row) in non-smokers' and smokers' subgroups.