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ORIGINAL ARTICLE

Antibody reactivity in cerebrospinal fluid and serum against the insulin-insulin-like growth factor 2 (INS-IGF2) protein is associated with psychotic symptomatology in patients with schizophrenia or related psychosis.

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Abstract

OBJECTIVES: Evidence has accumulated that an autoimmune-mediated process may underlie development of schizophrenia, and in two recent studies, we found increased antibody reactivity against the insulin receptor-A (INSR-A) and insulin-like growth factor 1 receptor (IGF1R) and their ligands (insulin and insulin-like growth factor 1) in cerebrospinal fluid (CSF) and/ or serum from patients with schizophrenia or related psychosis. The aim of this study was to analyze antibody reactivity in schizophrenia against the insulin-insulin-like growth factor 2 (INS-IGF2) protein, which hypothetically also may be a ligand to INSR-A and IGF1R and involved in the pathogenesis of schizophrenia.

MATERIAL AND METHODS: Patients with schizophrenia or related psychosis and controls were analyzed regarding antibody reactivity against INS-IGF2 in CSF (n = 12/ n = 11) and serum (n = 17/ n = 11), using bead-based antigen arrays of one protein fragment and 24 peptides of this protein. Additionally, the patients were assessed for clinical symptoms with the Positive and Negative Syndrome Scale (PANSS) for schizophrenia.

RESULTS: Significantly higher antibody reactivity against the peptides 11 and 12 was found in patients in partial than full symptom remission. Patients' antibody reactivity against the peptides 5, 11 and 12 correlated positively to their PANSS scores of positive symptoms. Furthermore, significantly higher antibody reactivity against the peptides 2, 3, 10 and 22 was found in patients with, than without, heredity for diabetes mellitus type 1 or 2.

CONCLUSION: The findings in this study pointed that the INS-IGF2 protein may be present in the CNS and involved in the autoimmune-mediated process underlying the development of schizophrenia.

Abbreviations:

CNS	- central nervous system
C-peptide	- connecting-peptide
CSF	- cerebrospinal fluid
His_6ABP	- six histidine albumin binding protein
IGF1	- insulin-like growth factor 1
IGF2	- insulin-like growth factor 2
IGF1R	- insulin-like growth factor 1 receptor
IGF2R	- insulin-like growth factor 2 receptor
IgG	- immunoglobulin G
INS	- insulin
INS-IGF2	- insulin-insulin-like growth factor 2
INSR	- insulin receptor
INSR-A	- insulin receptor-A
INSR-B	- insulin receptor-B
MADs	- median absolute deviations
MFI	- median fluorescent intensity
PANSS	- positive and negative syndrome scale
r_s	- Spearman rank correlation coefficient
SBA	- suspension bead array
vs	- versus

INTRODUCTION

Schizophrenia is a psychotic disorder that affects approximately 0.5 % of the population worldwide (Charlson *et al.* 2018; McGrath *et al.* 2008). In general it is disabling with a chronic course, beginning in late adolescence or early adulthood and continuing throughout life (Freedman, 2003). The literature provides strong evidence for a role of genetic factors in its aetiology (Craddock *et al.* 2005), and of all approximately 295 schizophrenia-associated genetic risk loci identified to date (for review, see Melkersson & Persson, 2023, and to that Lee *et al.* 2023; Li *et al.* 2022; Morita *et al.* 2014; Singh *et al.* 2022), it is the gene region encompassing the major histocompatibility complex on chromosome 6p22.1 playing an important role in the immune system that is the most significant and consistent, followed by genes involved in calcium ion import into cells, those involved in cell membrane depolarization during action potential, and those in synaptic transmission (Hall *et al.* 2020; Pardiňas *et al.* 2018; Ripke *et al.* 2014; Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2022; Sekar *et al.* 2016). However, a substantial proportion of the heritability for schizophrenia is still unknown (Pardiňas *et al.* 2018; Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2022).

Over the years, evidence has also accumulated indicating that schizophrenia is a systemic disorder and not only a brain disease (Flyckt, 2001; Kirkpatrick *et al.* 2014), and that an inflammatory, probably autoimmune-mediated, process in the central nervous system (CNS) and to some extent in peripheral organs, combined with an aberrant immune system, may underlie the development of schizophrenia, at least in a subgroup of patients (Al-Diwani *et al.* 2017; Alves-Peixoto *et al.* 2023; Bejerot *et al.* 2023; Bergink *et al.* 2014; Braun *et al.* 2017; Endres *et al.* 2022; Ermakov *et al.* 2017; Fourrier *et al.* 2019; Horváth & Mirnics, 2014; Hwang *et al.* 2013; Jeppesen & Eriksen Benros, 2019; Johansson *et al.* 2012; Laskaris *et al.* 2016; Mané-Damas *et al.* 2019; Melkersson & Bensing, 2018, 2021, 2023; Miller *et al.* 2011; Mobarrez *et al.* 2013; Momtazmanesh *et al.* 2019; Mongan *et al.* 2020; Müller *et al.* 2000; Schlaaff *et al.* 2020; Schmitt *et al.* 2011; Schwarz *et al.* 2000; Upthegrove *et al.* 2014; van Mierlo *et al.* 2019; Wang *et al.* 2018; Wedervang-Resell & Szabo, 2023; Wetterberg *et al.* 2002). Earlier studies have too reported signs of impaired blood-brain barrier in patients with schizophrenia (Bauer & Kornhuber, 1987; Bechter *et al.* 2010; Dion-Albert *et al.* 2022; Kirch *et al.* 1985, 1992; Melkersson & Bensing, 2018; Müller & Ackenheil, 1995; Stanca *et al.* 2024). The aberrance of the immune system in schizophrenia includes reduced capacity of both the cellular and humoral immune responses, as patients with schizophrenia exhibit both a decreased cutaneous cellular immune response to stimulation with tuberculin or other foreign proteins, and a reduced antibody production after, for example, salmonella vaccination (Melkersson, 2013; Melkersson & Bensing, 2018; Molholm, 1942; Müller *et al.* 1991, 2000; Steiner *et al.* 2010; Özek *et al.* 1971).

Autoantibodies directed against brain tissue, thymocytes, blood cells (i.e. erythrocytes, lymphocytes and thrombocytes), heat shock proteins, nuclear antigen and nerve growth factor have earlier been detected in serum of patients with schizophrenia (for review, see Melkersson & Bensing, 2021). In addition, cases of autoimmune encephalitis, presenting as schizophrenia-like psychosis and caused by autoimmune reaction against the *N*-methyl-D-aspartate receptor and the glutamic acid decarboxylase enzyme have previously been reported (for review, see Melkersson & Bensing, 2021). We recently also found increased antibody reactivity against the insulin receptor-A (INSR-A) and insulin-like growth factor 1 receptor (IGF1R) and their ligands insulin (INS) and insulin-like growth factor 1 (IGF1) in cerebrospinal fluid (CSF) and/ or serum of patients with schizophrenia or related psychosis (Melkersson & Bensing, 2021, 2023).

However, we did not find any differences in antibody reactivities against the insulin-like growth factor 2 (IGF2) or insulin-like growth factor 2 receptor (IGF2R) between patients with schizophrenia or related psychosis and control subjects in that recently-published study (Melkersson & Bensing, 2021). Nevertheless, serum levels of IGF2 have been reported to be lower in schizophrenia patients with first episode- or acute exacerbation of psychosis, and higher in patients with chronic schizophrenia, compared with control subjects (Akanji *et al.* 2007; Chao *et al.* 2020; Fernández-Pereira *et al.* 2022; Yang *et al.* 2020). Insulin-like growth factor 2 is a ligand not only to IGF2R, but also to the INSR and IGF1R (Rui & White, 2004). All these three receptors and their ligands (i.e. INS, IGF1 and IGF2) are present in both the CNS and peripheral organs in humans (Carlsson-Skwirut *et al.* 1986; Dorn *et al.* 1983; Kahn & Saltiel, 2005; McCowen & Smith,

2005; Rui & White, 2004; Sara *et al.* 1982; Werner & LeRoith, 2014), and although the IGF2R binds only IGF2, there is substantial cross-reactivity for the other two receptors; the INSR binds INS, IGF1 and IGF2, and the IGF1R binds IGF1, INS and IGF2, though there are differences in their binding affinities and functions (Dimitriadis *et al.* 2000; Pardo *et al.* 2019; Rui & White, 2004; Werner & LeRoith, 2014). Studies regarding functional differences between INSR-A and insulin receptor-B (INSR-B) have also revealed that INSR-A, in contrast to INSR-B, binds IGF2 with high affinity close to that of INS (Denley *et al.* 2004; Frasca *et al.* 1999), and that activation of INSR-A by IGF2 primarily leads to mitogenic effects, whereas activation of INSR-A by INS primarily leads to metabolic effects, utilizing different intracellular signalling pathways (Frasca *et al.* 1999).

Hypothetically, the newly-identified insulin-insulin-like growth factor 2 (INS-IGF2) protein may be a ligand to INSR-A and IGF1R too, and thereby may play a role in the pathogenesis of schizophrenia. It is 200 amino acid long and consists of the INS signal peptide (amino acid 1-24), the INS ß-chain (amino acid 25-54) and the first 8 amino acids of the connecting-peptide (C-peptide) chain (amino acid 55-62), in addition to 138 amino acids (amino acid 63-200) coded from the intergenic DNA-sequence between the *INS*- and *IGF2* genes (Monk *et al.* 2006). The INS-IGF2 protein has not yet been studied in schizophrenia, but as regards other diseases the involvement of this protein has been reported in metabolic disorders such as diabetes mellitus type 1 and 2, in cancers such as insulinoma, phaeochromocytoma, non-small-cell lung cancer and uterine cervical cancer, and in the autoimmune disorder systemic sclerosis (Følling *et al.* 2021; Gao *et al.* 2019; Johannessen *et al.* 2016; Kanatsuna *et al.* 2013, 2015; Masuda *et al.* 2020; Ng *et al.* 2014; Zhu & Deng, 2024).

Given this background, the aim of this study was to analyze antibody reactivity directed to this newly-identified INS-IGF2 protein in CSF and serum from patients with schizophrenia or related psychosis, and compare to that in CSF and serum from control subjects.

MATERIAL AND METHODS

Ethical approval

The study was approved by The Ethics Committee of Karolinska Institutet and The Regional Ethical Review Board, Stockholm, Sweden, and all patients and control subjects participated after giving informed consent.

Patients and control subjects

Consecutive outpatients at psychiatric polyclinics in the region of Stockholm, Sweden, diagnosed with schizophrenia or schizoaffective disorder according to Diagnostic and statistical manual of mental disorders criteria (American Psychiatric Association, 2013), were invited to participate in this study. Any patients having a substance-related disorder or a physical illness that could influence the evaluation were excluded. In total, 17 patients were included. In addition, 11 individuals diagnosed with non-inflammatory neurological diseases were included as control subjects.

Clinical evaluation and laboratory analyses

The clinical evaluation of the patients was done by a psychiatrist on the day before the taking of CSF and serum specimens by using the Positive and Negative Syndrome Scale (PANSS) for schizophrenia (Kay *et al.* 1987; Von Knorring & Lindström, 1992). The PANSS includes 30 items and each item is accompanied by a complete definition as well as detailed anchoring criteria in a 7-point format ranging from 1 = absent to 7 = extreme. The scale includes 3 subscales – a 7-item subscale for positive symptoms, such as hallucinations,

Tab. 1. Data regarding the one protein fragment and 24 peptides selected

Protein/ Gene[a,b]	Protein fragment ID and position (amino acid sequence)[a]
INS-IGF2	HPRR4270030 117-166 (PVLFIHCPGAAGTAQGLEY RGRRVTTELVWEEVDSS PQPQGSESLPAQPP)
	Peptide number and position (amino acid sequence)[a]
	1. 33-47 (SHLVEALYLVCGERG)
	2. 40-54 (YLVCGERGFFYTPKT)
	3. 48-62 (FFYTPKTRREAEDLQ)
	4. 63-77 (ASALSLSSSTSTWPE)
	5. 64-78 (SALSLSSSTSTWPEG)
	6. 65-79 (ALSLSSSTSTWPEGL)
	7. 66-80 (LSLSSSTSTWPEGLD)
	8. 67-81 (SLSSSTSTWPEGLDA)
	9. 72-86 (TSTWPEGLDATARAP)
	10. 73-87 (STWPEGLDATARAPP)
	11. 74-88 (TWPEGLDATARAPPA)
	12. 82-96 (TARAPPALVVTANIG)
	13. 97-111 (QAGGSSSRQFRQRAL)
	14. 112-126 (GTSDSPVLFIHCPGA)
	15. 117-131 (PVLFIHCPGAAGTAQ)
	16. 132-146 (GLEYRGRRVTTELVW)
	17. 147-161 (EEVDSSPQPQGSESL)
	18. 152-166 (SPQPQGSESLPAQPP)
	19. 167-181 (AQPAPQPEPQQAREP)
	20. 168-182 (QPAPQPEPQQAREPS)
	21. 169-183 (PAPQPEPQQAREPSP)
	22. 170-184 (APQPEPQQAREPSPE)
	23. 171-185 (PQPEPQQAREPSPEV)
	24. 186-200 (SCCGLWPRRPQRSQN)

Abbreviations: A=alanine; C=cysteine; D=aspartic acid; E=glutamic acid; F=phenylalanine; G=glycine; H=histidine; I=isoleucine; INS-IGF2=insulin-insulin-like growth factor 2; K=lysine; L=leucine; M=methionine; N=asparagine; P=proline; Q=glutamine; R=arginine; S=serine; T=threonine; V=valine; W=tryptophan; Y=tyrosine
[a]According to https://www.ncbi.nlm.nih.gov/protein, [b]According to https://www.ncbi.nlm.nih.gov/gene

Tab. 2. Characteristics of patients and control subjects

	Ethnicity, n	Age[a], y	Smoking, n (%)	Diagnosis, n	Heredity for schizophrenia or related psychosis[b], n (%)	Heredity for diabetes mellitus[c], n (%)	Duration of psychotic disorder[a], y	Type of current antipsychotic, n	Treatment time with current antipsychotic[a], y
Patients (n=17; 7 men, 10 women)	Caucasian (n=17)	44 (26-58)	7 (41.2)	Schizophrenia[d] (n=16) Schizoaffective disorder[d] (n=1)	8 (47.1)	type 1: 1 (5.9) type 2: 9 (52.9) both types: 2 (11.8)	17.0 (3.0-35.0)	Haloperidol (n=1) Clozapine (n=3) Olanzapine (n=6) Perphenazine (n=1) Risperidone (n=5) Zuclopentixol (n=1)	5.0 (1.0-14.4)[e]
Control subjects (n=11; 3 men, 8 women)	Caucasian (n=10) Asian (n=1)	44 (28-55)	nda	Non-inflammatory neurological disease[f] (n=11)	nda	nda	na	na	na

Abbreviations: n=number, na=not applicable, nda=no data available, y=year
[a]The data are given as median (min-max)
[b]I.e. patients who had one or more first-, second-, third- or fourth-degree relatives (excluding siblings) with schizophrenia or related psychosis (Melkersson, 2009)
[c]I.e. patients who had one or more first-, second-, third- or fourth-degree relatives (excluding siblings) with diabetes mellitus type 1, type 2 or both types (Melkersson, 2009)
[d]According to Diagnostic and statistical manual of mental disorders criteria (American Psychiatric Association, 2013)
[e]1 missing value
[f]In the foregoing study (Melkersson & Bensing, 2021), one of the 12 primary controls was found to have increased antibody reactivity against the calcium-activated chloride-channel protein fragment ANO2_HPRR3070036 that is identified as an autoimmune target in multiple sclerosis, and was therefore excluded as control

delusions and disorganized thinking, a 7-item subscale for negative symptoms, characterized by deficits in cognitive, affective and social functions, including blunting of affect and passive withdrawal, and a 16-item subscale covering general psychopathology. In addition to these 3 subscales, a bipolar composite subscale can be achieved by subtracting the negative subscale score from the positive subscale score.

The lumbar punctures and takings of blood samples were carried out in the morning after the patients and control subjects had been fasting overnight, and the collected samples were frozen directly and stored in -80°C until analysis. Duplicates of CSF and serum samples from the patients and control subjects were then analyzed for immunoglobulin G (IgG) antibody reactivity by a bead-based antigen array of protein fragments or peptides, as previously described (Ayoglu *et al.* 2013, 2014, 2016; Pin *et al.* 2019). For design of the antigen array, in total one protein fragment- and 24 peptide antigens of the INS-IGF2 protein were selected, which are described in detail in Table 1.

The protein fragment included was produced within the Human Protein Atlas project, using a whole-genome bioinformatics approach based on the principle of no or low protein sequence homology with any other human protein (www.proteinatlas.org). It was 50 amino acid long, and was expressed in *Escherichia coli* with a six histidine albumin binding protein (His_6ABP) tag. The peptides included were designed to complete the protein fragment in the analysis. They were all 15 amino acid long with a biotin and 6-aminohexanoic acid spacer conjugated to their N-terminals, and ordered from GenScript Biotech (The Netherlands).

The protein fragment, four technical controls, and neutravidin (31000, Thermo Scientific) were covalently coupled to colour coded magnetic beads (MagPlex, Luminex Corporation, Austin, TX, USA) using NHS- and EDC based chemistry. The peptides and pure biotin were subsequently coupled to the neutravidin covered beads. All beads were then combined into one pool; the suspension bead array (SBA). The controls consisted of His_6ABP (negative, tag binding control for the protein fragment), buffer (negative, bead binding control), biotin (negative, bead binding control; B4501-5G, Sigma-Aldrich), rabbit anti human IgG (positive, sample loading control; 309-005-082, Jackson Immunoresearch), and Ebstein Barr Nuclear Antigen 1 (semi-positive control; ab138345, abcam). The crude samples were diluted manually: CSF 1:5 in final-assay buffer (5 % BSA in 1xPBS supplemented with 0.05 % Tween-20, 0.16 mg/ml His_6ABP tag and 0.01

Tab. 3. Antibody reactivity in cerebrospinal fluid and serum, expressed as median fluorescent intensity (MFI) for the one protein fragment and as median absolute deviations (MADs) for the 24 peptides of the insulin-insulin-like growth factor 2 (INS-IGF2) protein in patients, compared with controls

Protein/ Gene[a,b]	Protein fragment[a]	Cerebrospinal fluid - MFI[c,d]			Serum - MFI[c,d]		
		Patients (n=12) vs	Controls (n=11)	*p*-value[e]	Patients (n=17) vs	Controls (n=11)	*p*-value[e]
INS-IGF2	HPRR4270030	-5.75 (-15.00-221.50)	-9.00 (-13.00-24.00)	0.16	-3.50 (-57.50-255.50)	-9.00 (-59.50-185.00)	0.30
	Peptide[a]	Cerebrospinal fluid - MADs[c]			Serum - MADs[c]		
		Patients (n=12) vs	Controls (n=11)	*p*-value[e]	Patients (n=17) vs	Controls (n=11)	*p*-value[e]
	1.	0.65 (-0.87-1.66)	-0.10 (-1.59-1.69)	0.64	-0.67 (-2.02-5.41)	-0.08 (-1.04-1.39)	0.57
	2.	-1.02 (-3.03-219.22)	-2.17 (-3.24-25.69)	0.21	-2.28 (-6.44-218.09)	-3.10 (-6.25-63.50)	0.48
	3.	0.17 (-2.31-1.13)	0.18 (-1.51-4.22)	0.48	0.92 (-0.60-3.83)	1.38 (0.13-3.00)	0.45
	4.	0.63 (-0.77-1.88)	1.30 (0.75-2.97)	**0.02**[f]	0.90 (-1.04-5.53)	0.23 (-1.02-1.37)	0.07
	5.	0.02 (-0.83-1.25)	0.62 (-0.29-1.58)	0.12	0.82 (-0.89-1.38)	0.47 (-0.25-2.71)	0.96
	6.	-0.64 (-1.45-1.01)	-0.45 (-0.82-0.39)	0.44	1.27 (-0.29-3.15)	0.92 (-0.60-1.66)	0.06
	7.	0.38 (-1.21-5.78)	0.38 (-0.61-1.24)	0.64	-0.94 (-1.81-4.62)	-0.82 (-2.05-2.77)	0.57
	8.	-0.14 (-1.78-1.08)	0.24 (-0.95-0.91)	0.31	0.15 (-1.19-9.13)	0.85 (-0.46-3.45)	**0.01**[f]
	9.	0.34 (-1.13-1.11)	0.09 (-0.80-0.81)	0.52	-0.09 (-0.78-0.61)	0.42 (-0.37-1.27)	0.10
	10.	0.71 (-1.89-2.03)	0.63 (-0.20-7.27)	0.48	0.03 (-1.19-4.71)	-0.84 (-1.54-14.81)	0.06
	11.	0.50 (-0.80-1.85)	0.22 (-0.72-4.50)	0.52	-0.19 (-0.76-0.87)	0.45 (-0.34-13.29)	**<0.01**[f]
	12.	-2.68 (-5.32-102.23)	-1.94 (-3.93-5.46)	0.15	-0.37 (-1.32-85.56)	0.31 (-0.93-21.21)	0.10
	13.	0.28 (-2.30-3.86)	0.04 (-2.53-11.67)	0.98	-0.45 (-2.27-3.88)	-0.48 (-3.72-23.91)	0.85
	14.	0.34 (-2.09-3.95)	0.05 (-2.22-2.64)	0.60	-0.62 (-1.77-4.71)	-1.19 (-2.83-0.56)	0.05
	15.	0.37 (-1.25-5.17)	0.30 (-1.54-2.65)	0.64	-0.09 (-0.88-7.80)	0.13 (-1.57-2.15)	0.89
	16.	0.25 (-1.23-1.35)	0.38 (-1.69-2.41)	0.78	-0.05 (-2.25-26.13)	-0.25 (-1.44-0.63)	0.81
	17.	-2.41 (-4.13-37.24)	-2.35 (-5.20-6.96)	0.98	0.13 (-1.30-12.22)	0.83 (-1.29-57.90)	0.19
	18.	0.53 (-1.02-2.73)	-0.33 (-1.94-3.50)	0.83	0.31 (-0.70-5.83)	-0.06 (-1.10-3.35)	0.08
	19.	-0.53 (-1.54-15.53)	0.06 (-1.46-11.99)	0.69	-1.09 (-2.12-27.74)	0.56 (-1.76-5.87)	0.93
	20.	-0.34 (-2.06-1.54)	0.01 (-1.49-0.45)	0.64	-0.87 (-1.78-29.44)	-0.44 (-1.18-0.41)	0.85
	21.	0.44 (-0.86-11.70)	0.50 (-0.06-1.48)	0.83	0.63 (-1.49-24.45)	0.84 (-0.70-1.37)	0.93
	22.	-3.37 (-8.24-328.32)	-4.88 (-5.84--0.78)	0.44	-3.26 (-11.13-309.11)	-5.90 (-8.38-55.24)	0.07
	23.	0.64 (-0.64-2.16)	0.31 (-1.13-2.55)	0.56	-0.48 (-2.44-18.90)	-0.71 (-2.31-1.08)	0.40
	24.	1.82 (-3.71-12.13)	-0.69 (-5.29-10.02)	0.12	-0.99 (-6.33-17.74)	-1.51 (-9.51-3.99)	0.22

[a]According to https://www.ncbi.nlm.nih.gov/protein, [b]According to https://www.ncbi.nlm.nih.gov/gene, [c]Expressed as median (min-max), [d]As described in Material and Methods, the MFI value was calculated per sample by subtracting the MFI signal of the His_6ABP tag from the MFI signal of the protein fragment with the His_6ABP tag included, [e]*p*-values <0.05 are written in bold text, [f]No statistically significant difference could be demonstrated between patients and controls regarding the proportion of observations above the presumed limit for increased antibody reactivity (> 3 MADs)

Tab. 4. Scores obtained with the Positive and Negative Syndrome Scale (PANSS) for schizophrenia in the patients studied[a]

	PANSS scores			
	Positive symptoms	**Negative symptoms**	**Positive and negative symptoms combined**	**General psychiatric symptoms**
Median (min-max)	10.0 (7.0-17.0)	9.0 (7.0-12.0)	1.0 (-5.0-9.0)	21.0 (16.0-26.0)
Range[b]	7-49	7-49	-42-42	16-112

[a]n = 16 due to missing PANSS data in one patient
[b]According to Kay et al. 1987

mg/ml neutravidin) and serum 1:30 in pre-assay buffer (5 % BSA in 1xPBS supplemented with 0.05 % Tween-20). The diluted serum was further diluted instrumentally 1:5 in final-assay buffer (i.e. in total diluted 1:150). Then, the diluted samples were incubated at +4°C (CSF 2.5 hours, serum 1 hour) in order for the His_6ABP and neutravidin in the buffer to pre-block any potential antibodies towards these. The diluted samples were then incubated with the SBA; CSF overnight (19 hours) and serum 1 hour. An R-Phycoerythrin conjugated anti-human IgG (H10104, Invitrogen) was then applied for 30 minutes to enable a read out.

To read out, a FlexMap 3D instrument (Luminex Corporation, Austin, TX, USA) was used. The read out consisted of the median fluorescent intensity (MFI) and count of number of beads for each antigen in each sample that the MFI was based on. Quality control was done using both MFI and bead count to exclude any antigens and samples not reaching technical criteria. Since duplicates were included, a correlation check of the duplicates was also performed. Summarized, the quality control was good in all aspects for both the CSF and serum assays.

Statistical methods

The continuous data age, duration of psychotic disorder, treatment time with current antipsychotic and PANSS scores, are given as median and range (min-max), and the categorical data smoking, heredity for schizophrenia or related psychosis and heredity for diabetes mellitus type 1, type 2 or both types, are summarized using frequency counts and percentages. The mean values of the duplicates were calculated and used. In order to adjust for sample specific backgrounds, the MFI values for the peptide antigens were adjusted to the same median per peptide, and subsequently the number of median absolute deviations (MADs) was calculated per sample (Melkersson & Bensing, 2021; Neiman *et al.* 2019). The MFI value for the protein fragment antigen was calculated per sample by subtracting the MFI signal of the His_6ABP tag from the MFI signal of the protein fragment with the His_6ABP tag included. Otherwise, no processing was done for the protein fragment or controls. Comparisons between all patients and control subjects, or between subgroups of patients, were conducted with Mann-Whitney test or Fisher's exact test. To measure the correlation between pairs of variables, Spearman rank correlation coefficient (r_s) was calculated. A *p*-value of less than 0.05 was considered statistically significant. All calculations were done with the statistical program Statistica for Windows 13.5 (TIBCO Software Inc., Palo Alto, CA, USA).

RESULTS

Characteristics of patients and control subjects are given in Table 2. All patients were Caucasians and had a diagnosis of schizophrenia, except one woman who was diagnosed with schizoaffective disorder. Eight (47.1 %) of the patients had heredity for schizophrenia or related psychosis, and twelve (70.6 %) of the patients had heredity for diabetes mellitus type 1, type 2 or both types (Melkersson, 2009, Table 2). None (0.0 %) of the male patients compared to seven (70.0 %) of the female patients were smokers ($p = 0.01$), otherwise no sex differences in characteristics were found among the patients. The patients were on long-term treatment with antipsychotics (Table 2), and were all in a stable state of their psychotic disorder. Male and female patients were also treated with similar antipsychotics, and the only concomitant medications used were benzodiazepine derivatives (n = 3), lithium (n = 1), orphenadrine (n = 1), propiomazine (n = 1), zopiclon (n = 1) and zopiderm (n = 1). The patients were classified according to PANSS scores as partial symptom remitted (n = 8, PANSS scores of positive symptoms > 9 and of positive and negative symptoms combined > 0) or fully symptom remitted (n = 8, PANSS scores of positive symptoms ≤ 9 and of positive and negative symptoms combined ≤ 0). The control subjects were all Caucasians except one woman who was Asian (Table 2), and none had any diagnosis of psychotic disorder; neither did they use any drugs with anti-inflammatory effect that could influence their neurological disease, such as cortisone, interferon or cytostatics.

Median fluorescent intensity or MADs of antibody reactivity in CSF and serum of patients and control subjects against the one protein fragment and 24 peptides of the INS-IGF2 protein investigated are given in Table 3. Median fluorescent intensity ≥ 50, or MADs > 3 were considered as increased levels of antibody reactivity. No significantly higher median-, or frequency of increased levels, of antibody reactivity was found in patients, compared with control subjects.

Tab. 5. Antibody reactivity in cerebrospinal fluid and serum, expressed as median fluorescent intensity (MFI) for the one protein fragment and as median absolute deviations (MADs) for the 24 peptides of the insulin-insulin-like growth factor 2 (INS-IGF2) protein in patients in partial remission (PR), compared with patients in full remission (FR), of symptoms

Protein/ Gene[a,b]	Protein fragment[a]	Cerebrospinal fluid - MFI[c,d,f]			Serum - MFI[c,d,f]		
		Patients/PR (n=5) vs	Patients/FR (n=6)	*p*-value[e]	Patients/PR (n=8) vs	Patients/FR (n=8)	*p*-value[e]
INS-IGF2	HPRR4270030	-7.50 (-12.50-30.00)	-5.75 (-15.00-221.50)	0.85	6.50 (-26.00-154.00)	-9.00 (57.50-255.50)	0.23
	Peptide[a]	**Cerebrospinal fluid - MADs[c,f]**			**Serum - MADs[c,f]**		
		Patients/PR (n=5) vs	**Patients/FR (n=6)**	***p*-value[e]**	**Patients/PR (n=8) vs**	**Patients/FR (n=8)**	***p*-value[e]**
	1.	0.54 (-0.45-1.51)	0.96 (-0.87-1.66)	0.78	0.17 (-1.07-2.07)	-0.72 (-2.02-5.41)	0.49
	2.	-1.87 (-3.03--0.75)	0.63 (-1.89-219.22)	0.06	-2.55 (-6.24-9.15)	12.73 (-6.44-218.09)	0.16
	3.	0.08 (-0.89-1.13)	0.39 (-2.31-0.46)	0.52	1.04 (-0.60-3.83)	1.10 (-0.04-3.64)	0.64
	4.	0.49 (-0.77-1.88)	0.79 (0.00-1.22)	0.78	1.00 (0.10-2.60)	0.67 (-1.04-5.53)	0.79
	5.	0.19 (-0.18-1.10)	-0.22 (-0.83-1.25)	0.17	0.38 (-0.20-1.06)	0.89 (-0.89-1.38)	0.64
	6.	-0.99 (-1.45--0.19)	-0.24 (-1.17-0.51)	0.12	0.90 (-0.29-1.60)	1.53 (0.77-3.15)	**0.02[g]**
	7.	0.88 (-0.59-5.78)	0.16 (-1.15-1.34)	0.65	-0.74 (-1.46-4.62)	-1.13 (-1.81--0.57)	0.13
	8.	-0.14 (-0.36-0.84)	-0.01 (-1.78-1.08)	0.93	-0.31 (-1.19-0.34)	0.53 (-0.56-9.13)	**0.02[g]**
	9.	0.35 (-0.72-0.65)	0.38 (-0.13-1.11)	0.41	-0.03 (-0.56-0.39)	-0.21 (-0.78-0.61)	0.56
	10.	1.74 (-0.16-2.03)	0.35 (-1.89-1.08)	0.12	0.46 (-0.94-4.71)	0.01 (-1.19-1.66)	0.37
	11.	0.70 (0.10-1.85)	0.16 (-0.73-0.94)	0.24	0.11 (-0.32-0.87)	-0.53 (-0.76-0.63)	**0.01**
	12.	-2.17 (-2.73-102.23)	-3.91 (-5.32--2.64)	**0.01**	-0.06 (-0.74-85.56)	-0.79 (-1.32-0.32)	**0.03**
	13.	2.49 (-1.76-3.86)	-0.25 (-2.30-2.20)	0.24	-0.28 (-2.15-1.60)	-0.67 (-2.27-3.88)	0.87
	14.	-0.65 (-2.09-0.92)	1.34 (-1.28-3.95)	0.06	-1.00 (-1.63-1.43)	-0.30 (-1.77-4.71)	0.32
	15.	0.20 (-0.36-1.85)	0.85 (-1.25-5.17)	0.65	-0.21 (-0.88-1.41)	-0.08 (-0.55-7.80)	0.56
	16.	0.79 (0.35-1.10)	-0.22 (-0.66-1.35)	0.08	0.18 (-1.26-26.13)	-0.14 (-2.25-4.75)	0.56
	17.	-1.89 (-2.47-9.61)	-3.23 (-4.13--2.28)	0.06	0.42 (-1.02-9.44)	-0.03 (-1.30-1.31)	0.37
	18.	-0.59 (-1.02-0.62)	0.63 (-0.86-2.73)	0.08	0.41 (-0.70-5.33)	0.23 (-0.02-5.83)	0.79
	19.	-0.54 (-0.72-15.53)	-0.63 (-1.54-9.59)	0.41	-0.69 (-2.12-19.93)	-0.84 (-2.12-27.74)	0.96
	20.	-0.72 (-1.46-1.54)	-0.19 (-2.06-0.18)	0.78	-0.82 (-1.46-7.68)	-0.63 (-1.78-29.44)	0.96
	21.	0.22 (-0.22-11.70)	0.99 (-0.86-2.52)	0.52	0.19 (-1.49-9.39)	1.03 (-0.30-24.45)	0.16
	22.	-3.45 (-6.31-328.32)	-4.19 (-8.24-119.62)	0.93	-2.52 (-11.13-115.14)	-3.44 (-10.55-137.88)	0.96
	23.	1.41 (0.36-2.10)	-0.28 (-0.64-0.83)	0.06	-0.78 (-1.74-3.68)	-0.95 (-2.44-1.19)	0.27
	24.	0.47 (-2.47-9.66)	5.38 (-3.71-12.13)	0.65	-1.19 (-6.33-3.42)	-0.20 (-4.15-17.74)	0.43

[a]According to https://www.ncbi.nlm.nih.gov/protein, [b]According to https://www.ncbi.nlm.nih.gov/gene, [c]Expressed as median (min-max), [d]As described in Material and Methods, the MFI value was calculated per sample by subtracting the MFI signal of the His_6ABP tag from the MFI signal of the protein fragment with the His_6ABP tag included, [e]*p*-values <0.05 are written in bold text with significantly higher antibody reactivity in patients in partial remission, compared with patients in full remission underlined, [f]n=11 or 16 due to missing PANSS data in one patient, [g]No statistically significant difference could be demonstrated between patients in partial remission and patients in full remission regarding the proportion of observations above the presumed limit for increased antibody reactivity (> 3 MADs)

Tab. 6. Relationships found between patients' antibody reactivity against peptides of the insulin-insulin-like growth factor 2 (INS-IGF2) protein in cerebrospinal fluid and serum and their Positive and Negative Syndrome Scale (PANSS) scores for schizophrenia

Cerebrospinal fluid (n=11)[a]	Serum (n=16)[a]
Correlations between antibody reactivity against INS-IGF2 peptides and PANSS scores of positive symptoms	
peptide 5: $r_s = 0.71$, $p = 0.02$ peptide 12: $r_s = 0.85$, $p < 0.01$	peptide 11: $r_s = 0.52$, $p = 0.04$ peptide 12: $r_s = 0.54$, $p = 0.03$
peptide 2: $r_s = -0.67$, $p = 0.02$ peptide 14: $r_s = -0.87$, $p < 0.01$	peptide 8: $r_s = -0.55$, $p = 0.03$
Correlations between antibody reactivity against INS-IGF2 peptides and PANSS scores of positive and negative symptoms combined	
peptide 5: $r_s = 0.71$, $p = 0.02$ peptide 12: $r_s = 0.85$, $p < 0.01$	peptide 11: $r_s = 0.52$, $p = 0.04$
peptide 14: $r_s = -0.79$, $p < 0.01$	peptide 6: $r_s = -0.52$, $p = 0.04$ peptide 8: $r_s = -0.64$, $p < 0.01$
Correlations between antibody reactivity against INS-IGF2 peptides and PANSS scores of general psychiatric symptoms	
peptide 10: $r_s = 0.83$, $p < 0.01$ peptide 12: $r_s = 0.67$, $p = 0.02$ peptide 17: $r_s = 0.65$, $p = 0.03$ peptide 23: $r_s = 0.74$, $p = 0.01$	peptide 12: $r_s = 0.59$, $p = 0.02$
peptide 2: $r_s = -0.83$, $p < 0.01$ peptide 24: $r_s = -0.70$, $p = 0.02$	

Abbreviations: n=number, r_s=Spearman rank correlation coefficient
[a]n=11 or 16 due to missing PANSS data in one patient

The median (with a min-max range) of PANSS scores in patients (n = 16) are given in Table 4. Subgroup analysis of patients showed that the median (but not the frequency of increased levels) of antibody reactivity against the peptides no 12 in CSF and no's 11 and 12 in serum was significantly higher in patients in partial than full symptom remission ($p = 0.01$, $p = 0.01$ and $p = 0.03$, respectively) (Table 5, Figure 1 a-b). In addition, patients' antibody reactivity against the peptides no's 5 and 12 in CSF and no's 11 and/ or 12 in serum correlated positively, and that against the peptides no's 2 and/ or 14 in CSF and no's 6 and/ or 8 in serum correlated inversely, to their PANSS scores of positive symptoms, or positive and negative symptoms combined (Table 6). For PANSS scores of general psychiatric symptoms, positive correlations with patients' antibody reactivity against the peptides no's 10, 12, 17 and 23 in CSF and no 12 in serum, and inverse correlations with that against the peptides no's 2 and 24 in CSF, were found (Table 6). However, no significant correlations were found between patients' antibody reactivity against the protein fragment or the other peptides of the INS-IGF2 protein and PANSS scores.

Further, subgroup analysis of patients did not show significantly higher median-, or frequency of increased levels, of antibody reactivity against the protein fragment or peptides of INS-IGF2 in patients with (n = 8) than without (n = 9) heredity for schizophrenia or related psychosis. An association was found between sex and smoking ($p = 0.01$), but not between heredity for schizophrenia or related psychosis and sex, or smoking. Moreover, the median of antibody reactivity against the peptides no's 2 and 3 in serum was significantly higher in patients with than without heredity for diabetes mellitus type 1 ($p = 0.03$ and $p < 0.01$, respectively) (Figure 2 a-b), and that against peptides no 22 in CSF and no 10 in serum was significantly higher in patients with than without heredity for diabetes mellitus type 2 ($p = 0.04$ and $p = 0.02$, respectively) (Figure 3 a-b). The frequencies of increased levels of antibody reactivity against the peptides no's 2 and 3 in serum were also significantly higher in patients with than without heredity for diabetes mellitus type 1 (3/3 (100.0 %) vs 3/14 (21.4 %), $p = 0.03$ and 2/3 (66.7 %) vs 0/14 (0.0 %), $p = 0.02$, respectively), but no significantly higher frequencies of increased levels of antibody reactivity were found against the protein fragment or the other peptides of INS-IGF2 in CSF or serum. No associations were found between heredity for diabetes mellitus type 1 and type 2, or between heredity for diabetes mellitus type 1 or type 2 and sex, smoking, or heredity for schizophrenia or related psychosis.

DISCUSSION

In this study, higher antibody reactivity in CSF and/ or serum against the INS-IGF2 peptides no's 11 and 12 was found in patients in partial than full symptom remission, there the most pronounced antibody reactivity in CSF and serum noted was against the INS-IGF2 peptide no 12 (amino acid 82-96). It was also found that patients' antibody reactivity in CSF

and/ or serum against the INS-IGF2 peptides no's 5, 11 and 12 correlated positively, and that against the INS-IGF2 peptides no's 2, 6, 8 and 14 correlated inversely, to their PANSS scores of positive symptoms, or positive and negative symptoms combined. These findings indicate that the INS-IGF2 protein may be present in both the CNS and peripherally and involved in the autoimmune-mediated process underlying the development of schizophrenia, and that the INS-IGF2 peptides being positively associated with psychotic symptomatology in patients with schizophrenia or related psychosis are located in the 138 amino acid sequence of INS-IGF2. To compare, we found in earlier studies both that antibody reactivity in serum against the INS peptide no 2 (= INS-IGF2 peptide no 2), which is located in the INS ß-chain, was inversely correlated to PANSS scores of negative symptoms, and that serum concentrations of INS autoantibodies tended to be inversely correlated to PANSS scores of positive, or positive and negative symptoms combined, in patients with schizophrenia or related psychosis (Melkersson & Bensing, 2021, 2023).

It was too found in this study no higher antibody reactivity against the protein fragment and peptides of INS-IGF2 in patients with than without heredity for schizophrenia or related psychosis. This finding is in line with earlier genetic studies on the INSR and its ligand INS in schizophrenia, showing no associations between gene variants in the *INS* gene and heredity for schizophrenia or related psychosis, but between gene variants in the *INSR* gene and heredity for schizophrenia or related psychosis (Melkersson, 2018; Melkersson & Persson, 2022, 2023).

Of interest is also the findings of higher antibody reactivity against the INS-IGF2 peptides no's 2 and 3 in serum in patients with than without heredity for diabetes mellitus type 1, and against the INS-IGF2 peptides no 22 in CSF and no 10 in serum in patients with than without heredity for diabetes mellitus type 2. The peptides no's 2 and 3 are located in the INS ß-chain and C-peptide part of INS-IGF2, and the peptides no's 10 and 22 are located in the 138 amino acid sequence of INS-IGF2. Consequently, peptide no 10 overlaps the peptides that were found being positively associated with psychotic symptomatology in the patients (i.e. no's 5, 11 and 12), while the other three of these INS-IGF2 peptides (i.e. no's 2, 3 and 22) are located in other parts of the amino acid sequence of INS-IGF2. To compare, a link has also been reported earlier between diabetes mellitus type 1 and schizophrenia in autoimmune-, genetic- and register studies (Eaton *et al.* 2006; Finney, 1989; Juvonen *et al.* 2007; Melkersson, 2020; Melkersson & Bensing, 2021, 2023; Melkersson & Persson, 2022, 2023; Melkersson & Wernroth, 2019), and between diabetes mellitus type 2 and schizophrenia in genetic- and register studies (Melkersson, 2020; Melkersson & Persson, 2023). Regarding the INS-IGF2 protein, it has not been studied earlier in patients with schizophrenia, but in patients with diabetes mellitus type 1, INS-IGF2 autoantibodies in serum have been reported to be more common than in controls (Kanatsuna *et al.* 2013, 2015), and in patients with diabetes mellitus type 2, the *INS-IGF2* gene has displayed lower levels of expression in pancreatic islets, compared with pancreatic islets from normal donors, as well as the SNP rs3842770

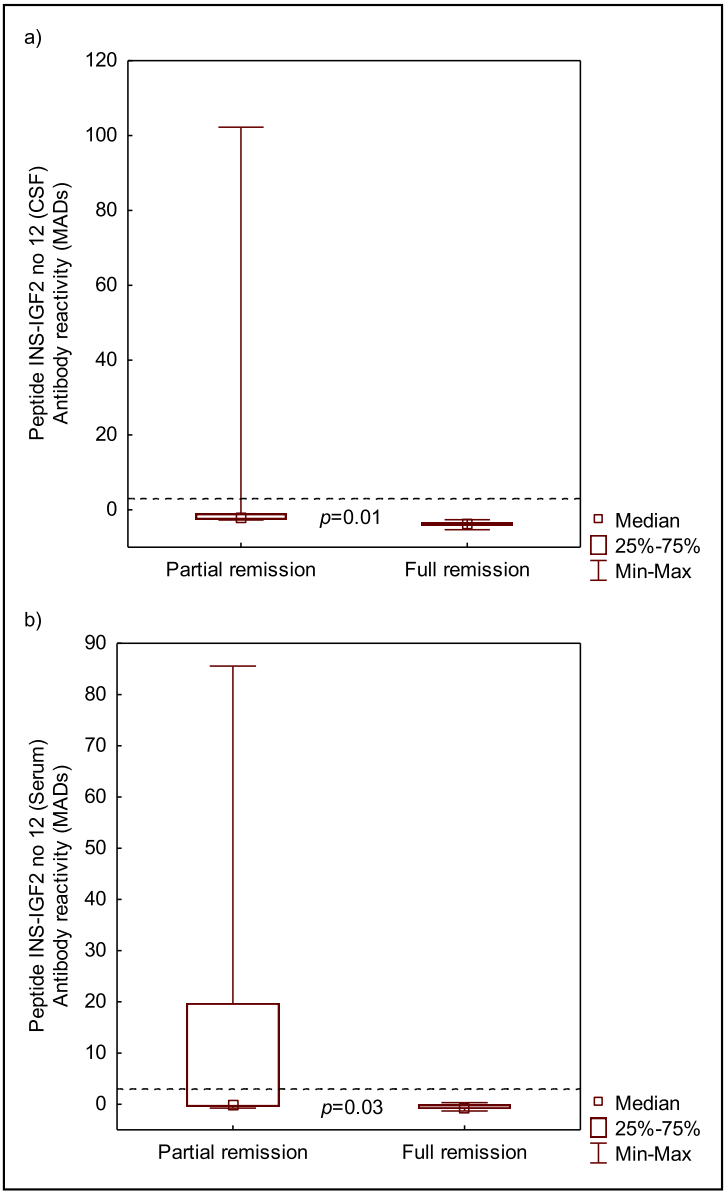

Fig. 1a-b. Antibody reactivity, expressed as median absolute deviations (MADs), against the insulin-insulin-like growth factor 2 (INS-IGF2) peptide no 12 in cerebrospinal fluid (CSF) and serum was significantly higher in patients in partial (n_{csf} = 5, n_{serum} = 8) than full (n_{csf} = 6, n_{serum} = 8) symptom remission. (---) indicates MADs > 3 which were considered as increased levels.

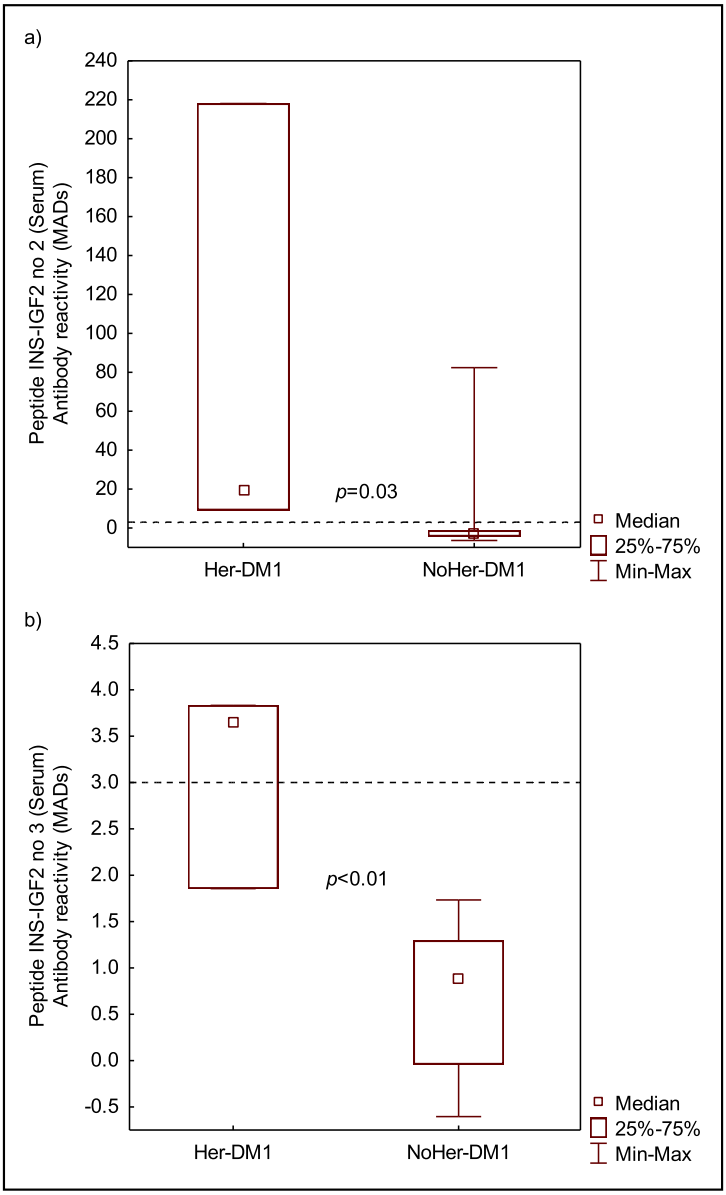

Fig. 2a-b. Antibody reactivity, expressed as median absolute deviations (MADs), against the insulin-insulin-like growth factor 2 (INS-IGF2) peptides no's 2 and 3 in serum was significantly higher in patients with (n = 3) than without (n = 14) heredity for diabetes mellitus type 1 (Her-DM1). (---) indicates MADs > 3 which were considered as increased levels.

in intron 2-3 in the *INS-IGF2* gene has shown association (Kanatsuna *et al.* 2013; Ng *et al.* 2014).

The IGF2 protein, that is a ligand to both the IGF2R, INSR and IGF1R (Rui & White, 2004), has been reported to be lower in serum of schizophrenia patients with first episode- or acute exacerbation of psychosis, compared with control subjects (Chao *et al.* 2020; Fernández-Pereira *et al.* 2022; Yang *et al.* 2020). Serum levels of IGF2 have also been found to be inversely correlated to negative and cognitive symptoms in patients with schizophrenia, to increase with antipsychotic treatment and declining of psychotic symptomatology, and to be higher in patients with chronic schizophrenia, compared with control subjects (Akanji *et al.* 2007; Chao *et al.* 2020; Fernández-Pereira *et al.* 2022; Yang *et al.* 2020). Further, the *IGF2* gene (located downstream of the *INS* gene on chromosome 11p15.5; Hampton *et al.* 1989; Jansen *et al.* 1985; Rotwein, 1991) has been found in an RNA sequencing study to be the top down-regulated gene in post-mortem brains of patients with schizophrenia (Fromer *et al.* 2016). Thus, these earlier findings indicate some involvement of IGF2 in the schizophrenia process. However, the newly-identified INS-IGF2 protein, that has a somewhat misleading name, is coded from a part of the *INS* gene (i.e. the INS signal peptide, INS ß-chain and the first 8 amino acids of the C-peptide chain) and an intergenic DNA-sequence between the *INS*- and *IGF2* genes (i.e. the 138 amino acid sequence). Hence, it is neither coded from the *IGF2* gene, nor it is identical with the IGF2 protein. This may explain why we did not find any increased antibody reactivity against the IGF2 protein in patients with schizophrenia or related psychosis, compared with control subjects, in our previous study (Melkersson & Bensing, 2021), although in this study I found higher antibody reactivity against the INS-IGF2 protein in patients with schizophrenia or related psychosis in partial than full symptom remission.

The findings in this study are in line with a) our previously-described hypothesis that impaired cellular signalling via the INSR-A, and probably also via the IGF1R, may underlie known abnormalities in the CNS and peripheral organs in schizophrenia (Melkersson & Persson, 2011, 2012; Melkersson *et al.* 2011), b) results in our foregoing studies, indicating that an autoimmune-mediated process underlies the development of a core group of schizophrenia cases and that the INSR-A and IGF1R and their ligands INS and IGF1 may constitute main antigen targets (Melkersson & Bensing, 2021, 2023), and c) are also supported by several studies by others, reporting sign of a hypometabolic state in brains, INSR deficits and decreased IGF1R-, IGF1- and IGFBP2 mRNA expression in post-mortem brains, INS signalling abnormalities, altered IGF2 signalling, and an association between an *IRS-2* SNP and auditory hallucinations, in patients with schizophrenia (de Bartolomeis *et al.* 2023; Huang *et al.* 2006; Kapogiannis *et al.* 2019; Kim *et al.* 2013; van Beveren *et al.* 2014; Weissleder *et al.* 2021; Wu *et al.* 2013; Yang *et al.* 2020; Zhao *et al.* 2006).

The strength of this study includes the use of a methodological and technical antigen array for antibody profiling (Ayoglu *et al.* 2016) that gives a possibility to study protein fragment- and peptide antigens of a specific protein of

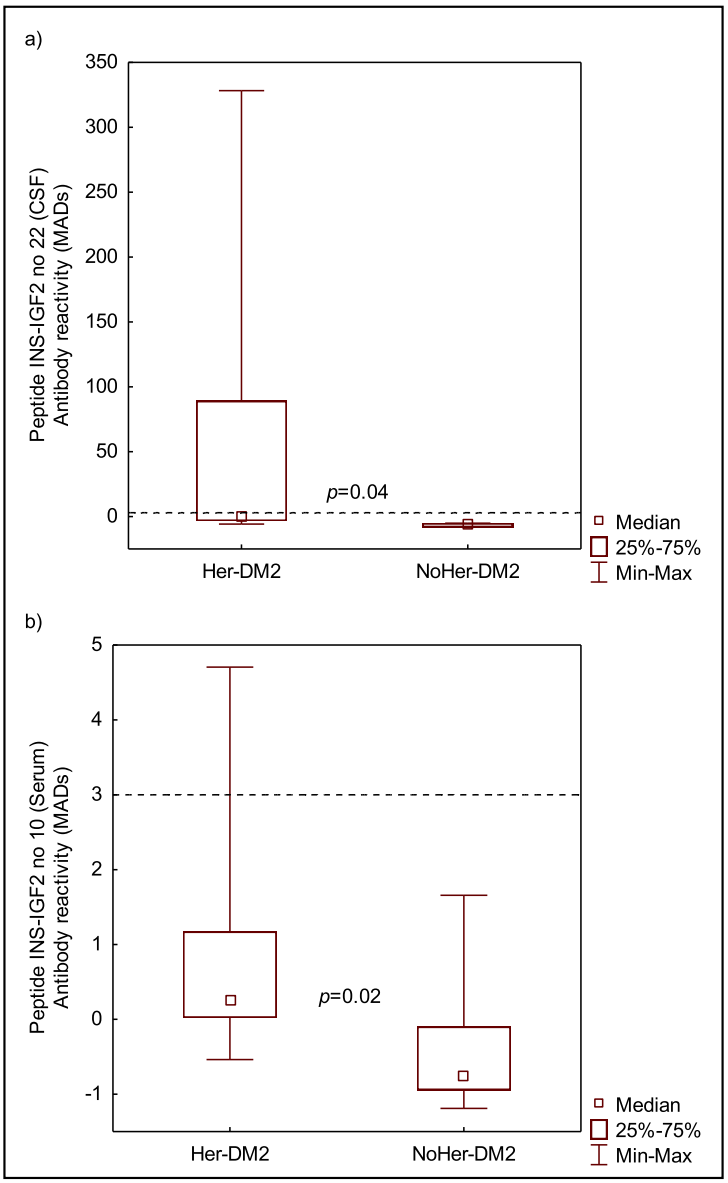

Fig. 3a-b. Antibody reactivity, expressed as median absolute deviations (MADs), against the insulin-insulin-like growth factor 2 (INS-IGF2) peptides no 22 in cerebrospinal fluid (CSF) and no 10 in serum was significantly higher in patients with (n_{csf} = 9, n_{serum} = 11) than without (n_{csf} = 3, n_{serum} = 6) heredity for diabetes mellitus type 2 (Her-DM2). (---) indicates MADs > 3 which were considered as increased levels.

potential interest for, as in this case, schizophrenia. A further strength includes the narrow diagnostic selection of, in the main, only patients with a diagnosis of schizophrenia, allowing investigation of a diagnostically homogeneous patient group. The main limitation of the study, on the other hand, consists of the fact that the number of patients and control subjects in the study was small, which means that until the present results are replicated in independent investigations, they have to be regarded as preliminary and be interpreted with caution. The limitations also include that the patients were on antipsychotic drug treatment and were not acutely psychotic, which may have moderated the antibody reactivities observed (Ezeoke *et al.* 2013; Jernbom Falk *et al.* 2021; Ponsford *et al.* 2019), and that the control group did not comprise healthy individuals. However, it seems unlikely that the control group selected, in which all control subjects were diagnosed with solely non-inflammatory neurological diseases and did not have any diagnosis of psychotic disorder, can have confounded the results.

In summary, this study shows higher antibody reactivity in CSF and/ or serum against INS-IGF2 peptides no's 11 and 12 in patients with schizophrenia or related psychosis in partial than full symptom remission. It also shows that patients' antibody reactivity against INS-IGF2 peptides no's 5, 11 and 12 correlated positively to their PANSS scores of positive symptoms, or positive and negative symptoms combined. These findings indicate that the INS-IGF2 protein, which hypothetically is a ligand to INSR-A and IGF1R, may be present in both the CNS and peripherally and involved in the autoimmune-mediated process underlying the development of schizophrenia.

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