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Lower Plasma Levels of Selective VGF (Non-Acronymic) Peptides in Bipolar Disorder: Comparative Analysis Reveals Distinct Patterns across Mood Disorders and Healthy Controls

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Keywords
AQEE peptide · Bipolar disorder · Depression · NAPP peptide · TLQP peptide · Biomarkers · VGF

Abstract

Introduction: Discriminating bipolar disorder (BD) from major depressive disorder (MDD) remains a challenging clinical task. Identifying specific peripheral biosignatures that can differentiate between BD and MDD would significantly increase diagnostic accuracy. Dysregulated neuroplasticity is implicated in BD and MDD, and psychotropic medications restore specific disrupted processes by increasing neurotrophic signalling. The nerve growth factor inducible *vgf* gene (non-acronymic) encodes a precursor protein named proVGF, which undergoes proteolytic processing to produce several VGF peptides, some of which were suggested to be implicated in mood disorders and have antidepressant effects. Since the presence of VGF peptides in humans has been exclusively investigated in brain and cerebrospinal fluid, we aimed to identify which VGF peptides are present in the plasma and to investigate whether their levels could differentiate BD from MDD as well as responders from non-responders to pharmacological interventions. ***Methods:*** VGF peptides were investigated in plasma from patients diagnosed with MDD ($n = 37$) or BD ($n = 40$ under lithium plus $n = 29$ never exposed to lithium), as well as healthy controls (HC; $n = 36$). ***Results:*** Three VGF peptides (TLQP-11, AQEE-14, and NAPP-19) were identified using spectrometry analysis of plasma from HC. These peptides were then measured in the entire sample using ELISA, which showed significantly lower levels of AQEE and NAPP in BD than in HC and MDD ($p = 5.0 \times 10^{-5}$, $p = 0.001$, respectively). ***Conclusion:*** Our findings suggest that lower plasma levels of NAPP and AQEE are specifically associated with BD, thus possibly representing a diagnostic biomarker in mood disorders.

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Introduction

Mood disorders, including bipolar disorder (BD) and major depressive disorder (MDD), are characterised by longitudinal occurrence of mood episodes, alternating from depression to hypo/mania in BD or exclusively of depressive polarity in MDD. According to the Global Burden of Diseases, MDD and BD are among the leading causes of disease burden worldwide [1]. The combined 12-month prevalence estimate of these two disorders is higher than 9% [2]. Accurate differential diagnosis of MDD and BD remains difficult in clinical practice because, in two-thirds of the patients with BD, the onset is of depressive polarity; episodes of opposite polarity occur later in the course of the illness and might not be recognised, especially if hypomanic [3]. Thus, misdiagnosis between MDD and BD is common, leading to ineffective treatment, which in turn might further worsen long-term outcomes [4]. However, no validated markers, including peripheral biomarkers, can discriminate BD from MDD with accuracy.

Evidence suggests that altered synaptic plasticity might be implicated in mood disorders [5]. Indeed, it has been shown that depression is correlated with neuronal atrophy in limbic and cortical regions responsible for mood and emotion control [6]. Some studies have shown that antidepressant therapy can increase neuroplasticity and reverse neuroanatomical changes observed in depression [7], while lithium, the first-line treatment in BD, has been proven to exert neuroprotection and increase neuroplasticity [8]. This effect seems to be mediated by increased levels of neurotrophic factors, particularly brain-derived neurotrophic factor (BDNF) [7, 8]. Interestingly, the *vgf* gene (non-acronymic) has been shown to be one of the BDNF targets [9], and its encoded polypeptide protein (VGF non-acronymic) has been found to induce antidepressant-like effects in rats through the enhancement of proliferation in the hippocampus [10]. VGF is a neuroprotein precursor of 615 amino acids in humans [11], secreted in different brain regions, including the hippocampus [10]. The non-acronymic name used for VGF originates from the procedures through which it was identified since the clone was selected from plate "V" of a rat pheochromocytoma PC12 cDNA library [12, 13].

The VGF neuroprotein is also named proVGF or VGF precursor because it contains cleavage sites for neuroendocrine prohormone convertases leading to a variety of peptides (named by their 4 N-terminal amino acids and length) some of these with reported bioactivity each with specific tissue expression and modulations, including IEHV [14–16], TLQP [17, 18], NAPP [19, 20], and AQEE peptides [20]. Different modifications of the VGF, its mRNA or even its cleaved peptides have been implicated in pathways connected to mood disorders. For instance, overexpression of the VGF protein in mice causes the opposite effects from *vgf* gene ablation, resulting in pro-depressant or antidepressant behaviour in the dorsal hippocampus or nucleus accumbens, respectively [21]. Another study showed that *vgf* mRNA levels were significantly reduced in human leukocytes from drug-free depressed patients compared to controls, while exposure to antidepressants restored its expression [22]. Furthermore, *vgf* mRNA was found to be downregulated in BD patients in the CA region of the hippocampus and Brodmann's area 9 of the prefrontal cortex [23].

The fact that the proVGF is related to mechanisms involved in mood disorders has stimulated the investigation of its potential role as a blood biomarker for MDD and BD. In this regard, a recent study showed that VGF levels were found to be lower in drug-free MDD and higher in BD patients compared with controls [24]. Similarly, another study showed lower serum VGF levels in MDD patients with an increase following therapy [25, 26] and an inverse link to suicide risk [27]. These results were obtained using ELISA with antibodies against the C-terminus [24] and N-terminus [25–27] of the proVGF-, which were non-specific to any known VGF peptide. Interestingly, two antibodies, anti-TLQP-62 and AQEE-30, have been reported to have antidepressant activity in previous investigations [21] and the peptides used for their production have been well characterised by proteomic analyses in human cells [28] and CSF [29, 30]. However, despite these findings, whether these or other VGF peptides are detectable in peripheral blood has yet to be elucidated. Based on the findings reported above, we hypothesise that VGF peptides might indirectly or directly play a role in mood regulation and that specific VGF peptides might be implicated in BD and MDD as well as in modulating treatment response.

To test this hypothesis we performed a study to characterise all naturally occurring VGF peptides in human plasma from a cohort of healthy controls (HCs) and patients with a diagnosis of mood disorders (BD and MDD) characterised for response to pharmacological treatments.

Material and Methods

Sample

The cohort comprised 69 patients with BD and 37 patients with MDD recruited at the community mental health centre at the Psychiatry Unit of the Department of Medical Science and Public

Health, University of Cagliari and University Hospital Agency of Cagliari, and the Unit of Clinical Pharmacology, University Hospital Agency, Cagliari. The recruitment process was based on the inclusion and exclusion criteria described elsewhere [31]. Briefly, the diagnosis was made according to the Diagnostic and Statistical Manual of Mental Disorders IV (DSM-IV) criteria and Schedule for Affective Disorders and Schizophrenia Lifetime (SADS-L) (BD patients), and Structured Clinical Interview for DSM-IV-TR Axis I Disorders (SCID) (MDD patients). Exclusion criteria comprised the presence of acute infections, diagnosis of eating disorders, post-traumatic stress disorder, substance use disorders, neurological disorders, traumatic brain injury, or severe medical conditions. In the MDD cohort, treatment resistance was defined according to the criteria of Souery et al. [32] based on the clinical course and the assessment of treatment response patterns. The BD cohort included patients treated with lithium (n = 40) and patients who were never exposed to lithium but treated with either valproate or lamotrigine (n = 29). Response to lithium or valproic was characterised using the Retrospective Criteria of Long-Term Treatment Response in Research Subjects with BD scale (or ALDA scale) [33–35]. The scale quantifies the degree of improvement during treatment with a score from 0 to 10, adjusted for potential confounders. Patients with a total score of 7 were considered responders [34, 35]. All BD patients enrolled in the study were under mood-stabilising treatment at the time of recruitment, while 12 out of the 26 BD patients receiving lithium treatment were responders. Among the BD patients treated with valproate, 3 were responders, 21 were non responders, and 4 were not classified. The cohort under study also included 38 non-psychiatric, HC subjects with no personal or familial history of psychiatric disorders in first-degree relatives. HCs were recruited based on the same exclusion criteria of the patients and were administered the Italian version of the SCID-I/NP 26 to rule out the presence of Axis I psychiatric disorders. Patients and controls were all recruited from Sardinia (Italy) and were Caucasian and of Italian origin. Characteristics of the patients and controls are reported in Table 1. Physical activity was defined as any sports activity performed at least once a week; cardio-metabolic disorders included cardiovascular diseases, obesity and diabetes mellitus type 2 while smoking status was defined as smoking regularly. Fasting blood was collected in the morning using tubes containing ethylenediamine tetra-acetic acid (EDTA, 1.5 mg/mL). Blood samples were centrifuged (3,000 *g*, 10 min), and, after addiction to extraction solution (PBS plus 5 μL/mL protease inhibitor cocktail: Sigma P8340), were frozen. At blood drawn, patients with BD and MDD were in euthymic phases with an interval of at least 6 months from the last mood episode meeting diagnostic criteria.

Nano-RP-HPLC-High Resolution ESI-MS/MS Analysis

The characterisation of the VGF peptides was obtained by nano-reverse-phase high-performance liquid chromatography (nano RP-HPLC) with Ultimate 3,000 Nano System HPLC (Dionex-Thermo Fisher Scientific) coupled with a high-resolution mass spectrometer (ESI-HR-MS) LTQ Orbitrap Elite (Thermo Fisher Scientific) on a pooled plasma sample obtained by mixing 2 μL from 5 HC samples (4 males, 1 female, age ± standard deviation 70 ± 8, 60 μg/μL of total protein concentration each). Following the manufacturer's instructions, the pooled plasma sample was then depleted of albumin and IgG using the High Select™ Depletion Spin Columns (Thermo Fisher Scientific, Waltham, MA, USA). After the depletion, the total protein concentration was determined by the bicinchoninic acid assay kit (Sigma-Aldrich/Merck, Darmstadt, Germany), following the manufacturer's instructions. Finally, the sample was dried and resuspended in aqueous formic acid (FA) 0.1% v/v with a final protein concentration of 0.1 μg/μL prior the RP-nanoHPLC-ESI-HR-MS/MS analysis. The injection volume was 10 μL, and the column was a C18 Easy Spray reverse-phase nano-column (250 mm × 75 μm inner diameter I.D., Thermo Fisher Scientific) with 2 μm beads. The elution of proteins and peptides was achieved with aqueous solvent A (0.1% FA) and aqueous solvent B (0.1% FA, 80% Acetonitrile v/v) in 50 min at a flow rate of 0.3 μL/min with the following gradient: 0–3 min at 4% B, 3–30 min 4–80% B, 30–31 min 80–90% B, 31–41 min 90% B, 41–45 min 4% B, 45–50 4% B. The mass spectrometer operated at 1.6 kV in the data-dependent acquisition mode, with the capillary temperature set at 275°C and S-Lens RF level 68.0%. Complete MS experiments were performed in positive ion mode from 350 to 2,000 m/z with a resolution of 60,000 (at 400 m/z). The 10 most intense ions were subjected to Collision Induces Dissociation (CID) or Higher-Energy Collisional Dissociation (HCD) fragmentation by setting 35% of normalised collision energy for 20 ms, isolation width of 2 m/z and activation q of 0.25. Only single-charged ions were excluded from the fragmentation process, and when required, a Full MS window in a range of 100 m/z centred on the ion of interest was set to improve the quality of fragmentation by applying the same parameters described above. The synthetic TLQP, AQEE, and NAPP peptides (from Pepmic Co., Suzhou, China) corresponding to the aminoacidic sequence of TLQP-62 from rats and AQEE-30 and NAPP-19 from humans were analysed (200 fmol) as standard reference under the same analytical conditions. (HR)-MS and MS/MS data were generated by Xcalibur 2.2 SP 1.48 (Thermo Fisher Scientific, CA, USA) and deconvoluted by using the Xtract algorithm available in FreeStyle (version 1.8.63.0, Thermo Fisher Scientific, CA, USA) with the following settings: 44% fit factor, 25% remainder threshold, minimum intensity set to 1, expected intensity error set to 3, and S/N threshold set to 2. The characterisation of TLQP, AQEE, and NAPP peptides in plasma from controls was achieved by comparing the theoretical MS/MS fragmentation generated in silico by the MS-Product tool available at the Protein Prospector website (http://prospector.ucsf.edu/prospector/mshome.htm), setting a fragment tolerance of 300 ppm, ESI-FT-ICR-CID as MS/MS fragmentation instrument, search of b and y.

Fragment ions, neutral losses of H_2O and NH3, concerning the undeconvoluted MS/MS spectra obtained experimentally for each peptide of interest. Furthermore, the Peptide Fragments Annotation tool available in FreeStyle was also used to annotate the same experimental undeconvoluted MS/MS spectra.

VGF Antibody Production and Validation

The NAPP-9 peptide (human VGF $_{485–493}$) (CPC Scientific, San Jose, CA, USA) was conjugated at its N-terminus with keyhole limpet haemocyanin while the TLQP-10 peptide (human VGF $_{554–563}$) was synthesised (CPC Scientific) and conjugated at its C-terminus for immunisations in guinea pigs and rabbit, respectively. Antibodies were raised in rabbits against the AQEE-10 peptide at the proVGF 586–595 position (Affiniti Research Products, Enzo Life Sciences, UK), conjugated to bovine thyroglobulin via an additional cysteine at its C-terminus. All antibodies

Table 1. Diagnostic groups and control subjects

Variables	BD (n = 69)	MDD (n = 37)	HC (n = 38)	Statistics
Gender (M/F)	29/40	12/25	21/15	$\chi^2 = 5.12$; $p = 0.77$
FH (Y/N/U)	35/33/1	20/13/4	0	$\chi^2 = 1.4$; $p = 0.50$
Suicide attempt history (Y/N/U)	17/52	6/30/1	0	$\chi^2 = 0.87$; $p = 0.46$
Smoke (Y/N/U)	33/32/3	18/19	13/23	$\chi^2 = 2.29$; $p = 0.32$
Cardio-metabolic disorders (Y/N)	17/52	10/27	8/28	$\chi^2 = 0.27$; $p = 0.87$
Physical activity (Y/N)	26/40	13/24	25/11	$\chi^2 = 10.88$; $p = 0.004$[a]
BMI (means ± SD)	27.8 ± 6.4	25.3 ± 5.1	23 ± 3.5	$F = 9.23$; $p = 0.0002$[b]
Age (means ± SD)	52.1 ± 9.9	51.2 ± 12.9	43.3 ± 10.7	$F = 8.10$; $p = 0.0004$[c]
Age at disease onset (means ± SD)	27.6 ± 9.9	35.0 ± 13.4	/	$t = -2.96$; $p = 0.004$
Years of illness (means ± SD)	24.5 ± 11.6	16.3 ± 12.7	/	$t = 3.38$; $p = 0.001$
Mood episodes (means ± SD), *N*	16.5 ± 12.7	3.2 ± 2.3	/	$t = 6.99$; $p = 2.86e^{-9}$
Patients under Li	40	1	/	/
Duration of Li treatment (means ± SD), years	16.5 ± 10.3	/	/	/
Duration of VPA treatment (means ± SD), years	18.6 ± 16.9	/	/	/
Duration of AD treatment (means ± SD), years	/	3.1 ± 2.9	/	/
VPA responders/non-responders	3/21	/	/	/
Li responders/non-responders	12/26	/	/	/
Treatment-resistant depression/responders	/	10/27	/	/

BD, bipolar disorder; MDD, major depressive disorder; HC, healthy controls; Li, lithium; VPA, valproic acid; AD, antidepressants; M, males; F, females; FH, family history of psychiatric disorders; Y, yes; N, no; BMI, body mass index; SD, standard deviation; *N*, number; AD, antidepressants. Bold statistics are significant at a p value threshold of 0.05. Significance between [a]BD versus HC and MDD versus HC; [b]BD versus HC; [c]BD versus HC and MDD versus HC.

were affinity purified by incubation with the corresponding immunogen antigen covalently immobilised on a sulfolink coupling resin (Thermo Fisher Scientific) and eluted in buffer glycine-HCl 1 M pH 2.5, after several rinses with phosphate buffer saline (PBS) 0.5 M (online supplementary material (for all online suppl. material, see https://doi.org/10.1159/000540673; Fig. 1). Antibody validation was carried out using either ELISA or Western blot (WB). In ELISA, each VGF antibody was matched to either the appropriate antigen-peptide or other extended peptides (varying for the amino acid counts) in order to assess antibody reactivity. Among the extended forms, we employed NAPP-19 (CPC Scientific; [30]) as well as tVGF peptides with demonstrated biological activity, as the TLQP-21 [36] (CPC Scientific), TLQP-62 (Pepmic Co.) [37], AQEE-30 (Pepmic Co.) [21], and TLQP-62 that were also included in the AQEE assay to examine antibody cross-reactivity. Intra- and inter-assay (CV1 and CV2) and detection limit (DL) were obtained for each assay. Every VGF antibody was examined in WB using PC12 cells (thanks to Prof. R. Possenti), either expressing proVGF or not (PC12+ or PC12–, respectively), as well as using TLQP-62 and AQEE-30 synthetic peptides (since short MW peptides are scarcely retained in WB). For WB analysis, samples were diluted in 2xSDS buffer (Origene) to load 20 µg of each sample and then were heated to 95°C for 5 min and subjected to SDS-PAGE using precast polyacrylamide gradient gel (NuPAGE 4–12% Bis-Tris, Thermo Fisher Scientific) for 20 min at 200 Volt. Internal MW standards (PageRuler Plus prestained protein ladder 10 to 250 KDa, Thermo Scientific) were run in parallel. Samples were transferred onto a polyvinylidene difluoride (PVDF) membrane (Amersham Hybond-P, GE Healthcare) for 1 h at 20 Volt. Blots were blocked by immersion in 50 mM Tris base and 150 mM Sodium Chloride (TBS) containing 0.1% Tween-20 (TBS-T) and 5% bovine serum albumin (BSA) for 1 h at room temperature. Incubation with rabbit anti-AQEE, sheep anti-TLQP and rabbit anti-NAPP primary antibodies (1:500 common dilution in the same blocking buffer) was carried out for 1 night at 4°C. The next day, blots were rinsed 4 times with TBS-T and incubated at room temperature for 1 h with horseradish peroxidase-conjugated goat anti-rabbit or donkey anti-sheep secondary antibodies (Jackson ImmunoResearch) diluted 1:10,000 in TBS-T. After several washing with TBS-T, protein bands were developed using Thermo Scientific Pierce Enhanced chemiluminescence (ECL) WB substrate. The ImageQuant LAS-4000 was used to detect the chemiluminescence. To reveal which peptides, among the others, were identified by the appropriate antibody, we used dot-blot (DB) with the same VGF synthetic peptides used in WB or ELISA. For

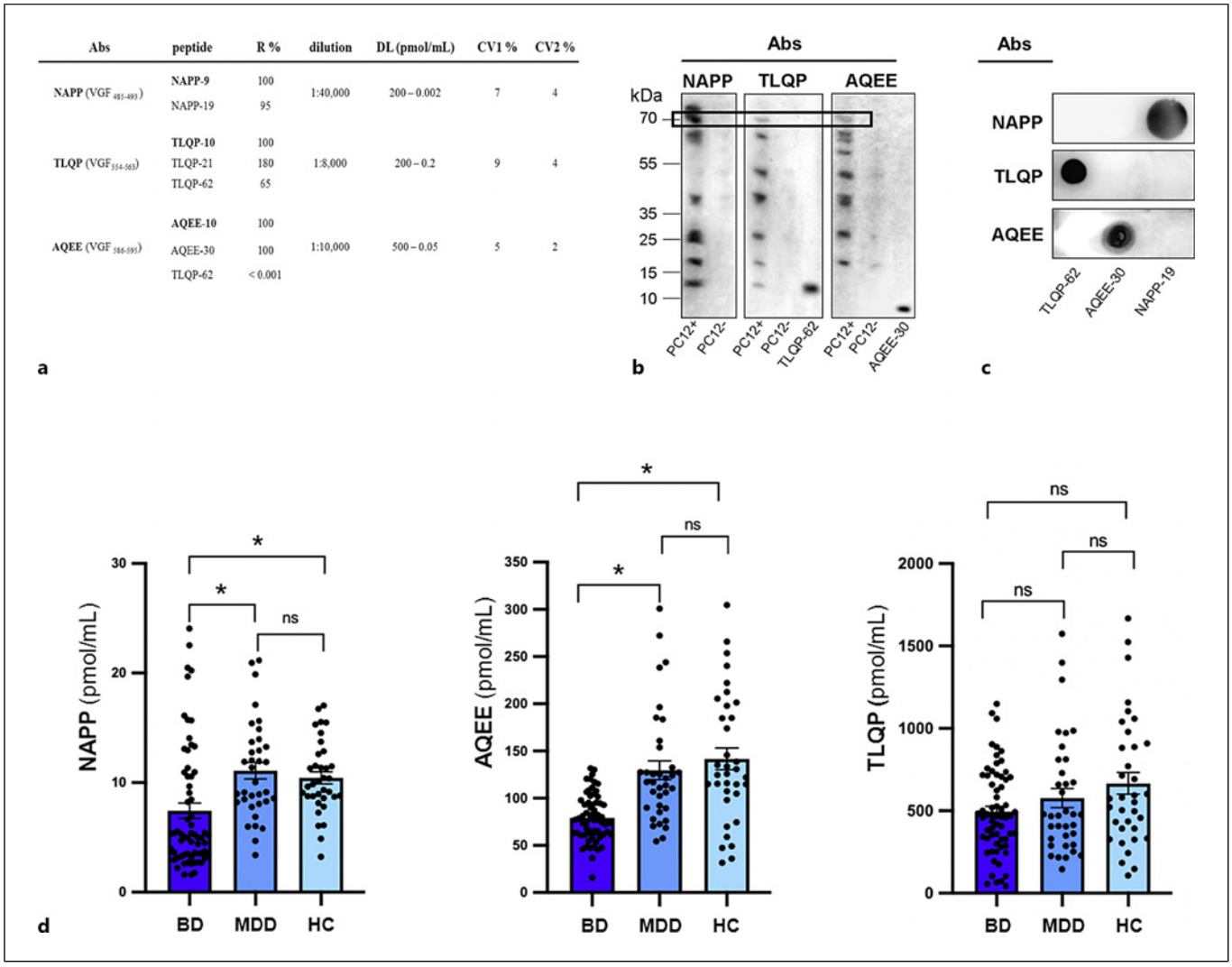

Fig. 1. a Each antibody was tested for reactivity against its corresponding antigen-peptide (in bold, used for immunisation) or longer peptides. The TLQP-62 was also used to test the AQEE antibody, although the antibody's reactivity was rather low. R stands for reactivity, DL for detection limit, and CV1 and CV2 for intra- and inter-assay. **b** Using WB, the 70 kDa proVGF band (inside the black rectangle) was labelled by all VGF antibodies using PC12+ but not with PC12− cells. The AQEE-30 and TLQP-62 synthetic peptides were labelled by their paired antibodies, revealing bands corresponding to the appropriate peptide MW bands. **c** Using DB analysis, each VGF antibody recognised the peptide that matched it but not the others. **d** Graphs showing NAPP, AQEE, and TLQP peptide levels in the studied groups. BD, bipolar disorder, MDD, major depressive disorder; HC, healthy controls; ns, not significant; Abs, antibodies. *$p < 0.05$.

DB, 4 μL of each synthetic peptide was applied on PVDF membranes, where it was let to spread and dry for 30 min at room temperature. Following their blocking, the following steps were carried out in accordance with the WB protocol.

Competitive ELISA

To measure the VGF levels in plasma samples, each VGF peptide was coated on a separate plate (Nunc Thermo Scientific, Milan, Italy) for 3 h at room temperature; then the plates were treated with blocking buffer (PBS containing 90 mL/L normal serum from donkey, 20 nmol/L aprotinin, and 1 g/L EDTA) for 2 h. Primary incubations using the corresponding VGF antibodies diluted in blocking buffer, were carried out in triplicate, including both serial standard synthetic peptide dilutions or plasma samples (1:10). Biotinylated secondary antibodies (1 h, 1:10,000 dilution in blocking buffer; Jackson, West Grove, PA, USA), streptavidin-peroxidase conjugate (30 min, 1:10,000 dilution in PBS; Biospa, Milan, Italy), and tetramethyl benzidine (X-tra Kem-En-Tec, Taastrup, Denmark) were used to reveal the positive labelling. The reaction was stopped with hydrogen chloride (1 mol/L), and the optical density was measured at 450 nm using a multilabel plate reader (Chameleon: Hidex, Turku, Finland).

Statistics

The normality of distribution was tested with Shapiro-Wilk and the presence of outliers with the Grubbs test. Plasma levels of the AQEE, TLQP, and NAPP peptides were not normally distributed (Shapiro-Wilk $p < 0.001$), and as such, nonparametric tests were used for all the analyses. Differences among diagnostic and response groups were tested with the χ^2 test for discrete variables or with a t test for continuous variables (Table 1). The predictive value of the peptides in discriminating diagnostic groups was tested using the Receiver Operating Characteristic (ROC) curve.

The effect of discrete variables on plasma levels of VGF peptides was tested with the Mann-Whitney U test, while the effect of continuous variables was tested with Spearman correlation. Considering none of the discrete variables influenced the peptide levels, we used the Quade nonparametric ANCOVA to test differences in VGF peptide levels among groups accounting for the effect of covariates. Only variables showing different distributions among groups or affecting plasma levels of VGF peptides were included as covariates in the analytical models. The analyses were conducted using IBM SPSS Statistics v. 28 (IBM Corporation, Armonk, NY, USA) and Graphpad Prism V. 9 (GraphPad Software, San Diego).

Results

Qualitative Analysis of the VGF Peptides

The qualitative analysis of the VGF peptides allowed characterisation of three peptides: (i) the AQEE peptide of 14 amino acids in position 585–598, corresponding to the sequence RAQEEAEAEERRLQ (theoretical monoisotopic $[M + 1H]^{+1}$ mass 1,714.85, experimental monoisotopic $[M + 1H]^{+1}$ mass 1,714.89); (ii) the NAPPEPVPPPRAAPAPTHV peptide of 19 amino acids in position 485–503 (theoretical monoisotopic $[M + 1H]^{+1}$ mass 1,915.02, experimental monoisotopic $[M + 1H]^{+1}$ mass 1,914.96); and (iii) the RTLQPPSALRR peptide of 11 amino acids in position 553–563 (theoretical monoisotopic $[M + 1H]^{+1}$ mass 1,294.77, experimental monoisotopic $[M + 1H]^{+1}$ mass 1,294.77). The structural characterisation of these three peptides is reported in the online supplementary materials (online suppl. Fig. 1–4; Tables 1–3).

The synthetic AQEE-30 (theoretical monoisotopic $[M + 1H]^{+1}$ mass 3,706.84 from human) and TLQP-62 (theoretical monoisotopic $[M + 1H]^{+1}$ mass 7,397.75 from rat) peptides were analysed by nano-RP-HPLC-High Resolution ESI-MS/MS, which allows to detect AQEE-30 at the retention time 28.6–29.3 min and TLQP-62 at the retention time 26.0–28.0 min. Based on this reference, we searched for the same two peptides in human plasma by both untargeted and targeted analysis, but were unable to detect them.

VGF Antibody Reactivity

When antibody reactivity was investigated using ELISA (Fig. 1a), the NAPP antibody almost identically recognised the NAPP-9 and -19; the TLQP antibody labelled the TLQP-10, -21, and -62 (with a greater reactivity degree for TLQP-21 and less for TLQP-62); and the anti-AQEE antibody similarly recognised both the short and extended AQEE forms, but not the TLQP-62. For each assay, the values of DL, CV1, and CV2 displayed accurate data. In WB (Fig. 1b), all VGF antibodies, as predicted, labelled the proVGF (70 kDa band) and other smaller bands corresponding to cleaved peptides. The anti-TLQP and AQEE antibodies labelled the TLQP-62 and AQEE-30 synthetic peptides, respectively, with higher affinity when compared to their reactivities against the proVGF. In DB (Fig. 1c), the NAPP antibody labelled the NAPP-19 only, the TLQP antibody selectively identified TLQP-62 while the AQEE antibody exclusively recognised the AQEE-30.

VGF Peptide Levels

As mentioned in the methods section, the levels of the three studied peptides were not normally distributed across the entire sample, as indicated by the Shapiro-Wilk test ($p < 0.05$ for all peptides; online suppl. material Fig. 5). A lack of normal distribution could be expected in this context since the cohort included patients with different diagnoses as well as HCs, thus contributing to heterogeneity and skewed distribution of the molecular variables, especially if these variables are correlated with some of the studied traits but not others (i.e., diagnosis).

The Grubbs test identified 8 outliers: one outlier for AQEE, two outliers for NAPPE, one outlier for LTQP in the MDD group, two outliers for NAPPE, and one outlier for TLQP in the BD group, and one outlier for TLQP in the HC group. These samples were removed from the analyses.

The diagnostic groups differed in several features, as reported in Table 1. TLQP, AQEE, and NAPP peptides were significantly correlated with each other. Age was significantly correlated with AQEE (rho = −0.215, p = 0.012), TLQP (rho = −0.231, p = 0.007), and NAPP (rho = −0.225, p = 0.009), while body mass index (BMI) was correlated with TLQP (rho = −0.273, p = 0.002) and NAPP (rho = −0.216, p = 0.013). Years of illness were only correlated with AQEE (rho = −0.316, p = 0.001) and the number of episodes with AQEE (rho = 0.381, $p < 0.001$) and NAPP (rho = −0.430, $p < 0.001$). The effect of these variables was tested in each model. None of the discrete

Table 2. Results of the pairwise comparisons with Quade's analysis of covariance controlling for age, BMI, and physical activity

Subject groups	NAPPE		AQEE		TLQP	
	t	*p* value	*t*	*p* value	*t*	*p* value
BD versus MDD	−4.592	0.00001	−5.058	$1.0e^{-5}$	−0.455	0.650
BD versus NPC	−3.380	0.001	−4.784	$5.0e^{-5}$	−0.578	0.564
MDD versus HC	1.072	0.286	0.203	0.839	−0.114	0.910

BD, bipolar disorder; MDD, major depressive disorder; HC, healthy controls.

variables influenced plasma levels of any of the peptides. The 2 samples of BD patients (treated with lithium or other mood stabilizers) did not differ for any of the tested variables or levels of AQEE, TLQP, and NAPP. As such, the two samples were combined in the following analyses. As shown in Table 1, the BD and MDD patients differed from the HC group in terms of physical activity and age, while BD patients had significantly higher BMI than both MDD and HC groups. The Quade test was statistically significant for NAPP (covariates: age, BMI and physical activity; model $F = 12.25$, $p = 0.00001$) and for AQEE (model $F = 17.78$, $p = 1.49e^{-7}$), with lower levels in patients with BD compared to those with MDD and controls, while patients with MDD did not differ from controls (Table 2; Fig. 1d). TLQP showed no difference among diagnostic groups (model $F = 0.201$, $p = 0.818$). The ROC curve had a specificity of 0.77 and sensitivity of 0.97 for NAPPE (area under the curve = 0.74, CI: 0.65–0.84), and a specificity of 0.85 and sensitivity of 0.97 for AQEE (area under the curve = 0.80, CI: 0.71–0.89), suggesting a better classification performance of AQEE (ROC curves reported in online suppl. material Fig. 6).

We then compared patients with treatment-resistant depression with responders to antidepressants as well as patients responders to lithium with non-responders showing no significant differences among the studied groups. Response to valproate was not included in the analyses since only 3 patients resulted responders to the treatment. Considering that a small proportion of MDD patients was not under pharmacological treatment at recruitment (5 patients), we also tested whether AQEE was associated with being exposed or not to antidepressants, reporting no significant differences.

Discussion

Through the use of a top-down mass spectrometry analysis, our study was able to show, for the first time, that AQEE-14 and TLQP-11 are present in human blood and also confirmed the presence of NAPP-19 [19]. Interestingly, when compared to HC levels of AQEE and NAPP peptides were significantly lower in patients with BD but not in those with MDD while there was no difference for TLQP. The latter finding does not confirm previous evidence suggesting that TLQP-62 could be involved in depression [21]. However, it is noteworthy that the anti-TLQP antibody can identify a range of TLQP peptides with varying lengths, including TLQP-62. Using HPLC-MS/MS analysis, we were only able to prove the presence of TLQP-11 but not of TLQP-62. Nevertheless, none of the previous proteomic studies identified TLQP-62 or any other TLQP peptide in human plasma. Therefore, based on our findings and the currently available evidence, we may speculate that the TLQP-62 is either not present in human plasma or its levels could be below the sensitivity thresholds of our techniques.

In our study, NAPP and AQEE peptides were both lower in BD patients compared to MDD and HC. Previous works showed that NAPP peptides are present in CSF [30, 31] other than in plasma, and while a previous study reported lower levels of the two peptides in obese people [19], to date, there is no evidence of their involvement in psychiatric diseases. On the other hand, the association of AQEE peptides with mood disorders has been widely reported. Neuronal biological activity has been demonstrated for AQEE-30, affecting the synaptic activity and depressed behaviour in animals [21], while an acute antibody-mediated AQEE peptide sequestration has been shown to have a pro-depressant effect in dorsal hippocampus [21]. Considering that both vgf mRNA and AQEE-11 peptide have been reported to be lower in brain and CFS from BD patients [23, 38] it could be speculated that the differences in AQEE plasma lavels observed in plasma from BD individuals might be correlated with AQEE changes in the brain. Our findings are in contrast with previous studies showing that, compared to controls, serum VGF levels were higher in BD patients and lower MDD patients [24–26]. However, these investigations used different VGF antibodies compared to our study, such as those directed against the C-terminus amino acid sequence, which spans from 591

to 610 [24] or the N-terminus amino acid sequence, which spans from 23 to 174 [25, 26]. Specifically, while it is possible that TLQP-62, NAPP-129, and AQEE-30 were recognised by the antibody employed by Chen et al. [24], the antibodies' specificity for these peptides – as well as for other VGF peptides – was not examined specifically. Instead, we used antibodies specifically designed to be directed against three different VGF peptides, including those with antidepressant activity, and demonstrated their presence in plasma using the spectrometry technique.

Our findings should be viewed in the context of some limitations. While we applied a stringent pipeline to purify and test the antibodies' specificity (for instance, we showed that the anti-AQEE antibody does not react with TLQP-62), the NAPP and AQEE antibodies we used could not distinguish between VGF peptides with similar sequences (for instance, the anti-NAPP antibody could not distinguish between NAPP-19 and NAPP-9, which have the same NAPP sequence but different amino acid counts). This limitation arises from the fact that antibodies, by nature, are unable to differentiate between peptides or proteins that are very similar. Furthermore, because of our incomplete knowledge of proVGF processing mechanisms, we were unable to measure the amount of similar VGF-cleaved peptides with overlapping sequences that would be present in blood. Nevertheless, even considering the possible limitations of our methods, only AQEE-14 and NAPP-19 (and not other smaller or longer AQEE or NAPP peptides) were identified by our analysis of the plasmatic proteome. Hence, we can assume that in ELISA, our antibodies specifically labelled the plasmatic AQEE-14 and NAPP-19. A second limitation of our study was its retrospective design with a single-time point observation, which made not possible to explore temporal changes in VGF peptides levels at different time points, which would have allowed us to test their correlation with longitudinally assessed features, such as pharmacological interventions. The strengths of our study consist in the accurate clinical characterisation of the cohorts of patients for whom information on response to treatment was available, thus allowing us to explore the effect of exposure to treatments at the time of recruitment as well as the correlation between VGF peptides and response or resistance to pharmacological interventions, something that has been scarcely investigated before.

In conclusion, our research shows that the plasma levels of the VGF peptides AQEE-14 and NAPP-19 differ significantly among patients with a different diagnoses of mood disorders, suggesting their potential utility as diagnostic biomarkers. Moreover, we suggest that VGF may not serve as a biomarker of response to lithium. On the other hand, considering the limitations, more research is needed to fully understand the pathophysiological processes that underlie the role of VGF in mental illness and in pharmacological interventions with psychotropic medications. Future studies should focus on stringent identification methods of the different VGF peptides, possibly overcoming the limitations of current methodologies. Moreover, an effort should be made to design prospective longitudinal studies, possibly in risk studies, to clearly explore the clinical implications of VGF in mood disorders research.

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Statement of Ethics

All participants signed informed written consent after a detailed description of the study procedures. The research protocol followed the principles of the Declaration of Helsinki and was approved by the Ethics Committee of the University of Cagliari, Italy (Approval No. 348/FC/2013 and PG/2018/11693).

Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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Author Contributions

Barbara Noli and Elias Manca provided ELISA experiments. Barbara Manconi and Cristina Contini provided proteomic analysis, Cristina Cocco and Alessio Squassina designed the study; Claudia Pisanu, Anna Meloni, Mirko Manchia, Pasquale Paribello, Caterina Chillotti, Raffaella Ardau, and Giovanni Severino provided patients and their clinical and relevant information. All authors reviewed the manuscript.

Data Availability Statement

All data generated or analysed during this study are included in this article and its online supplementary material files. Further enquiries can be directed to the corresponding author.

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