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Eeden, W.A. van; Filali, E. el; Hemert, A.M. van; Carlier, I.V.E.; Penninx, B.W.J.H.; Lamers, F.; ... ; Giltay, E.J.

Full-length Article

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Wessel A. van Eeden [a,1,*], Ebtisam El Filali [a,b,1], Albert M. van Hemert [a], Ingrid V.E. Carlier [a], Brenda W.J.H. Penninx [c], Femke Lamers [c], Robert Schoevers [d], Erik J. Giltay [a]

[a] *Department of Psychiatry, Leiden University Medical Center, Leiden, The Netherlands*
[b] *Department of Mood disorders, PsyQ, Parnassia Group, The Hague, The Netherlands*
[c] *Department of Psychiatry, Amsterdam Public Health Research Institute and Amsterdam Neuroscience, Amsterdam UMC, Vrije Universiteit, and GGZ inGeest, Amsterdam, The Netherlands*
[d] *Department of Psychiatry, University Medical Center Groningen, Groningen, The Netherlands*

A B S T R A C T

A cross-sectional relationship between low-grade inflammation –characterized by increased blood levels of C-reactive protein (CRP) and pro-inflammatory cytokines– and anxiety has been reported, but the potential longitudinal relationship has been less well studied. We aimed to examine whether basal and lipopolysaccharide (LPS-)induced levels of inflammatory markers are associated with anxiety symptom severity over the course of nine years.

We tested the association between basal and LPS-induced inflammatory markers with anxiety symptoms (measured with the Beck's Anxiety Inventory; BAI, Fear Questionnaire; FQ and Penn's State Worry Questionnaire; PSWQ) at 5 assessment waves over a period up nine years. We used multivariate-adjusted mixed models in up to 2867 participants of the Netherlands Study of Depression and Anxiety (NESDA).

At baseline, 43.6% of the participants had a current anxiety disorder, of which social phobia (18.5%) was most prevalent. Our results demonstrated that baseline inflammatory markers were significantly associated with several outcomes of anxiety at baseline over nine subsequent years. BAI subscale of somatic (arousal) symptoms of anxiety, and FQ subscale of agoraphobia demonstrated the strongest effects with standardized beta-coefficients of up to 0.14. The associations were attenuated by 25%-30% after adjusting for the presence of (comorbid) major depressive disorder (MDD), but remained statistically significant.

In conclusion, we found that participants with high levels of inflammatory markers have on average high levels of anxiety consisting of physical arousal and agoraphobia, which tended to persist over a period of nine years, albeit with small effect sizes. These associations were partly driven by co-morbid depression.

1. Introduction

Anxiety is regarded as a psychobiological state or reaction that, amongst others, consists of unpleasant subjective feelings of tension, nervousness and worry, often accompanied by physiological manifestations such as increased heart rate and blood pressure, and irregularity of breathing (Pitsavos et al., 2006). Earlier studies have suggested that inflammation could be involved in the pathophysiology of anxiety (Costello, Gould, Abrol, & Howard, 2019; Naude, Roest, Stein, de Jonge, & Doornbos, 2018; Renna, O'Toole, Spaeth, Lekander, & Mennin, 2018;

Salim, Chugh, & Asghar, 2012; Vogelzangs, Beekman, de Jonge, & Penninx, 2013). There are many pathways which may underlie this link. In laboratory conditions, anxiety can be induced by an external stressor (Trier social stress test), resulting in the characteristic physiological changes, as well as the biochemical response of cortisol and catecholamines release (Foley & Kirschbaum, 2010). Interestingly, this also activated inflammatory pathways in peripheral mononuclear cells through the transcription factor-kB (NF-kB), leading to increased levels of circulating pro-inflammatory cytokines such as interleukin-6 (Bierhaus et al., 2003; Pace et al., 2006). Similarly, chronic psychosocial

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distress, which goes hand in hand with symptoms of anxiety (Leonard, 2005), has been linked to dysregulation of the hypothal-amic–pituityadrenal axis, which has been shown to impact immune regulation (de Kloet et al., 2006; Michopoulos, Powers, Gillespie, Ressler, & Jovanovic, 2017). In reverse, following administration of the cytokine interferon alpha (IFN-α), significant anxiety as well as depressive symptoms may arise (Capuron et al., 2002; Roest, Martens, de Jonge, & Denollet, 2010). These symptoms could be prevented when patients were pretreated with selective serotonin reuptake inhibitors (SSRIs) before the start of IFN-α administration, indicating that these inflammation-related symptoms may in part be mediated through serotonin (Musselman et al., 2001).

There is increasing evidence for higher circulating concentrations of acute-phase proteins and pro-inflammatory cytokines in anxiety patients versus healthy subjects. Specifically C-reactive protein (CRP) as well as the pro-inflammatory cytokine interleukin-6 (IL-6) appear to have been repeatedly associated with symptoms and disorders of anxiety, such as panic disorders (Belem da Silva et al., 2017), generalized anxiety disorders (Khandaker, Zammit, Lewis, & Jones, 2016), agoraphobia (Glaus et al., 2018; Wagner et al., 2015) and anxiety symptoms in general (Liukkonen et al., 2011; Pitsavos et al., 2006). However, other studies did not find significant associations or even found reduced levels of inflammatory markers in subjects with anxiety symptoms (Baune et al., 2012; Song, Zhou, Guan, & Wang, 2007; Vogelzangs et al., 2013). Almost all previous studies had cross-sectional designs. One large longitudinal study that included 3,113 participants from the general population found that anxiety disorders, of which particularly agoraphobia, were associated with a steeper increase in CRP over time (not with IL-6 and Tumor Necrosis Factor-alpha; TNF-a), but baseline inflammatory markers did not predict anxiety disorders the other way around during up to 5.5 years of follow-up (Glaus et al., 2018).

Lipopolysaccharide (LPS) stimulated cytokine levels may better reflect physiological immune system functioning *in vivo* than basal levels of inflammation markers (van den Biggelaar et al., 2007). After *ex vivo* exposure of whole blood samples to LPS (the cell membrane of Gram-negative bacteria that strongly induce immunological responses), a wide array of pro- and anti-inflammatory cytokines are released that can be measured in the supernatant (van der Linden, Huizinga, Stoeken, Sturk, & Westendorp, 1998; van Exel et al., 2009; Westendorp, Langermans, Huizinga, Verweij, & Sturk, 1997). Whereas basal serum levels of inflammatory mediators generally show low values with high variability between and within persons over time (partly due to circadian rhythmicity), LPS-stimulated cytokine levels may have less of these drawbacks (Üçeyler, Häuser, & Sommer, 2011).

Previous cross-sectional analyses from the NESDA cohort, that we used, have shown that basal inflammatory markers (Duivis, Vogelzangs, Kupper, de Jonge, & Penninx, 2013; Vogelzangs et al., 2013), as well as LPS-induced inflammatory markers (Gaspersz et al., 2017; Vogelzangs, de Jonge, Smit, Bahn, & Penninx, 2016), were positively associated with anxiety and major depressive disorders at baseline (MDD). Vogelzangs et al. (2016) showed that LPS-stimulated inflammation was associated with increased odds of anxiety disorders, whereas Gaspersz et al. (2017) found that LPS-induced inflammatory markers were especially elevated among MDD patients with the DSM-5 'anxious distress'-specifier. Although several analyses within the NESDA cohort have focused on the prospective relationship of inflammation and depression, the longitudinal relation with anxiety symptoms has not been analysed (Lamers et al., 2019; van Eeden et al., 2020). Prospective studies regarding anxiety symptom severity remain scarce.

The aim of the present study is to examine whether basal as well as LPS-induced inflammatory markers determined at baseline are associated with the course of anxiety symptoms in the large Netherlands Study of Depression and Anxiety (NESDA) cohort. For this purpose, we chose three often-used self-reported measures of anxiety symptoms as outcome variables. Together this gives a broad spectrum of anxiety symptom-atology containing subjective and somatic experienced anxiety,

avoidance and worry. We hypothesize that markers of (low-grade) inflammation are associated with elevated levels of anxiety over the course of nine years, measured at baseline and up to five following time-points. In order to study whether the relationship with anxiety was independent of that with depression, we adjusted for the presence of MDD in a sensitivity analysis.

2. Materials and methods

2.1. Study sample and procedure

We evaluated baseline and follow-up data from participants from the Netherlands Study of Depression and Anxiety (NESDA) cohort. A detailed description of the NESDA design and sampling procedures have been published elsewhere (Penninx et al., 2008); its aim was to investigate the course and consequences of depressive and anxiety disorders. The first wave (baseline) started in 2004 and ended in September 2007, and the 6th wave of measurement at 9-year follow-up finished in October 2016. The baseline measurement (n = 2,981) consisted of demographic and personal characteristics, a standardized diagnostic psychiatric interview, medical assessment (e.g. BMI, blood sampling, etc.), and self-report questionnaires. The 1-year follow-up consisted of self-report questionnaires and was completed by 2,445 participants (82.0%). Face-to-face follow-up assessments with standardized diagnostic psychiatric interviewing and self-report questionnaires were conducted at 2 years (n = 2,596, 87.1%), 4 years (n = 2,402, %), 6 years (n = 2,256, 75.7%) and 9 years post-baseline (n = 2,069, 69.4% of the baseline sample).

This cohort was recruited from the community (n = 564, 18.9%), general practice (n = 1,610, 54.0%), and secondary mental healthcare (n = 807, 27.1%; Penninx et al., 2008). Basal serum levels of inflammation were collected from 2,867 of 2,981 participants (96.2%). LPS induction in blood was only assessed during the last year of baseline data collection, due to logistical reasons. As a consequence, inflammatory markers after in vitro LPS induction of whole blood samples was therefore available for the subgroup of 1,229 out of 2,981 participants (41.2%). A general inclusion criterion was an age of 18 through 65 years. Only two exclusion criteria existed: 1) a primary clinical diagnosis of a psychiatric disorder not subject of NESDA which will largely affect course trajectories, including a psychotic disorder, obsessive compulsive disorder, bipolar disorder, or severe addiction disorder; and 2) not being fluent in Dutch, since language problems would harm the validity and reliability of collected data (Penninx et al., 2008). The study protocol was approved centrally by the Ethical Review Board of the VU University Medical Centre and subsequently by local review boards of each participating center. After full verbal and written information about the study, written informed consent was obtained from all participants at the start of baseline assessment (Penninx et al., 2008).

2.2. Measures

2.2.1. Demographics and clinical features

The Composite International Diagnostic Interview (CIDI WHO version 2.1) was used to assess the presence of depressive- and anxiety disorders according to the Diagnostic and Statistical Manual of Mental Disorders – Fourth Edition (DSM-IV) at baseline, after 2-, 4-, 6- and 9 years. These included dysthymia, MDD, social phobia, panic disorder, agoraphobia, generalized anxiety disorder, and lifetime anxiety disorder. The CIDI is a fully standardized diagnostic interview with validated psychometric characteristics (Penninx et al., 2008; Wittchen, 1994).

Baseline demographic variables included gender, age, ethnicity (yes/no from north European heritage), level of education (i.e., elementary or less; general intermediate or secondary education; college or university), BMI, illness prior to interview, chronic somatic diseases, and anti-inflammatory medication. BMI was calculated by dividing weight (kg) by squared height (m^2). Patients were asked about illness (e.g., a mild

cold or fever) prior to interview. A wide variety of diseases were assessed through a self-report questionnaire, asking for the presence of 20 common chronic diseases including asthma, chronic bronchitis or pulmonary emphysema, heart diseases or infarct, diabetes, stroke or CVA, arthritis or arthrosis, rheumatic complaints, tumor and/or metastasis, stomach or intestinal disorders, liver disease or liver cirrhosis, epilepsy, thyroid gland disease, or another chronic disease for which the patient receives treatment. A count was made of the chronic diseases for which a person reported receiving treatment. More details regarding this variable can be found elsewhere (Gerrits, van Oppen, van Marwijk, van der Horst, & Penninx, 2013). Anti-inflammatory medication use (ATC codes M01A, M01B, A07EB, A07EC) was based on inspection of medication containers (further referred to as anti-inflammatory medication).

2.2.2. Basal and LPS-induced inflammatory markers

Inflammatory markers C-reactive protein (CRP), IL-6 and TNF-α were determined from fasting morning blood plasma at baseline. After an overnight fast, 50 ml blood was drawn which was immediately transferred to a local laboratory and kept frozen at −80 °C. High-sensitivity plasma levels of CRP were measured in duplicate by an in-house high-sensitivity enzyme linked immunosorbent assay (ELISA) based on purified protein and polyclonal anti-CRP antibodies (Dako, Glostrup, Denmark). The lower detection limit of CRP is 0.1 mg/l and the sensitivity is 0.05 mg/l. Intra- and interassay coefficients of variation were 5% and 10%, respectively. Plasma IL-6 levels were measured in duplicate by a high-sensitivity ELISA (PeliKine CompactTM ELISA, Sanquin, Amsterdam, the Netherlands). The lower detection limit of IL-6 is 0.35 pg/ml and the sensitivity is 0.10 pg/ml. Intra- and interassay coefficients of variation were 8% and 12%, respectively. Plasma TNF-α levels were assayed in duplicate using a high-sensitivity solid phase ELISA (Quantikine HS Human TNF-α Immunoassay, R&D systems, Minneapolis, MN, USA). The lower detection limit of TNF-α is 0.10 pg/ml and the sensitivity is 0.11 pg/ml. Intra- and interassay coefficients of variation were 10% and 15%, respectively. As done before (van Eeden et al., 2020), we created an overall basal inflammation index, as we assumed that high inflammatory marker levels in multiple markers are the best indication of general low-grade inflammation. The basal inflammation index consisted out of the mean value of all 3 $\log_e$-transformed (due to their positively skewed distributions) and standardized markers.

The innate immune response of 12 cytokines and inflammatory markers was assessed in blood that was *ex vivo* stimulated with LPS at basline. Serial venous whole blood samples were obtained at baseline in a 7-ml heparin-coated tube (Greiner Bio-one, Monroe, NC, USA). Between 10 and 60 min after blood draw, 2.5 ml of blood was transferred into a PAXgene tube (Qiagen, Valencia, CA, USA). Remaining blood (4.5 ml) was stimulated by addition of LPS (10 ng ml^{-1} blood; Escherichia coli, Sigma, St. Louis, MO, USA), as done by others (van Exel et al., 2009). LPS-stimulated samples were laid flat and incubated at a slow rotation for 5–6 h at 37 °C. A 2.5-ml sample of this LPS-stimulated blood was transferred into a PAXgene tube. This LPS procedure was carried out at four laboratories (Amsterdam, Leiden, Groningen, Heerenveen). Remaining plasma (±0.5 ml) was kept frozen at − 80 °C for later analysis.

Levels of interferon (IFN)-γ, IL-2, IL-6, IL-8, IL-10, IL-18, monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein (MIP)-1α, MIP-1β, matrix metalloproteinase-2 (MMP2), and TNF-α were assayed simultaneously for all available samples, using a multi-analyte profile (Human CytokineMAP A v 1.0; Myriad RBM, Austin, TX, USA). This commercial platform adheres to stringent guidelines of quality control and has Clinical Laboratory Improvement Amendments (CLIA) approval, which means that the platform is validated and calibrated on a continuous basis. Cytokines were $\log_e$-transformed to normalize their positively skewed distributions.

In order to reduce the number of statistical tests and because we did not have specific hypotheses about individual inflammation markers, we used exploratory factor analysis (EFA) with Principal Axis Factoring and Oblimin rotation to examine dimensionality of the 12 inflammatory markers, that yielded two LPS-induced inflammation indexes, as previously described (Bandalos & Finney, 2018; van Eeden et al., 2020). The two LPS-induced inflammation indexes are further referred to as LPS-induced inflammation index-1 and LPS-induced inflammation index-2. Markers IFN-γ, IL-10, IL-2, IL-6, MMP-2, TNF-α, and TNF-β loaded on LPS-induced inflammation index-1 with factor loadings between 0.41 and 0.88 and a raw alpha of 0.86. IL-8, IL-18, MCP-1, MIP-1α, and MIP-1β loaded on LPS-induced inflammation index-2 with factor loadings between 0.34 and 0.94 and a raw alpha of 0.89. Together with the basal inflammation index, these indexes were considered the main independent variables of interest.

2.2.3. Anxiety symptoms

The Beck's Anxiety inventory ((BAI; Beck, Steer, & Carbin, 1988), the Fear Questionnaire (FQ; Marks & Mathews, 1979), and the Penn State Worry Questionnaire (PSWC; Meyer, Miller, Metzger, & Borkovec, 1990), as well as its subscale scores, were used as the outcome measures for severity of anxiety symptoms over time. These measures capture different aspects of, but is not exclusive for, anxiety disorders such as symptoms of arousal (BAI), avoidance (FQ), and worry (PWSQ). These constructs are common in panic disorders, common phobias, and generalized anxiety disorder among others.

The BAI is a self-report questionnaire which assesses common symptoms of anxiety such as fear of dying, fear of losing control and nervousness (Beck et al., 1988). It consists of 21 equally weighted items, rated on a 4-point scale, ranging from 0 (not at all) to 3 (severely, "I could barely stand it"). The BAI is scored by summing the ratings for all of the 21 symptoms to obtain a total score that can range from 0 to 63. It contains a Somatic subscale (14 items) and a subjective subscale (7 items), representing physical- and cognitive symptoms of anxiety (Kabacoff, Segal, Hersen, & Van Hasselt, 1997). The reliability and validity of the BAI are well-established (Beck et al., 1988; Steer, Rissmiller, Ranieri, & Beck, 1993). Research has showed adequate reliability estimates for the BAI in a sample of psychiatric inpatients (α = 0.92) and high school adolescents (α = 0.88; Osman et al., 2002). In our study, the Cronbach's alpha coefficient was α = 0.93 at baseline.

The 15-item Fear Questionnaire (FQ) is a self-report instrument that assesses the level of avoidance in relation to common phobias, including social phobia (five items), agoraphobia (five items), and hematophobia/traumatophobia (five items; Marks & Mathews, 1979). It consists of 15 equally weighted items, rated on a 9-point scale, ranging from 0 ("Would not avoid it") to 8 ("Always avoid it"). The sum-score ranges from 0 through 120. Three phobia subscales of five items can be derived, a blood phobia subscale, a social phobia subscale, and a agoraphobia subscale. The psychometric properties of the FQ has been researched in multiple studies among both non-clinical populations (Gillis, Haaga, & Ford, 1995) and patients with an anxiety disorder (Mavissakalian, 1986; Oei, Moylan, & Evans, 1991). These studies conclude that the psychometric properties of the FQ are sufficient with moderate to high Cronbach's alpha coefficients per subscale, ranging from α = 0.71 to α = 0.83 (Mavissakalian, 1986; Oei et al., 1991). In our study, the Cronbach's alpha coefficient was α = 0.88 at baseline.

The Penn State Worry Questionnaire (PSWQ) is also a self-report questionnaire which consists of 16 equally weighted items rated on a 5-point scale (1–5) with 1 meaning "not at all typical of me" to 5 "very typical of me". The total score ranges from 16 to 80. This 16-item instrument emerged from factor analysis of a large number of items, and was found to possess high internal consistency and good test–retest reliability (Meyer et al., 1990). The psychometric properties of the PSWQ were considered satisfactory in a community sample (van Rijsoort, Emmelkamp, & Vervaeke, 1999) and a sample of anxiety patients (Brown, Antony, & Barlow, 1992). Cronbach's alpha coefficients of 0.94 were found in a community sample (van Rijsoort et al., 1999), and ranging from 0.86 to 0.93 in a clinical sample (Brown et al., 1992). In

our study, the Cronbach's alpha coefficient was α = 0.96 at baseline.

2.3. Statistical analysis

A multivariate linear mixed model was used with BAI, FQ, PSWQ total- and subscale scores at baseline, and after 1, 2, 4, 6, and 9 years as outcome variables and baseline inflammatory indexes as the main independent variables. PSWQ was not assessed at 1 year. Because of the heterogeneity of our sample (both healthy participants as well as anxiety patients at baseline), random intercepts and slopes were added, as they resulted in a significantly better fit compared to model without random effects, as tested with −2LL ratio tests. Adding an interaction between a continuous modelled time variable and inflammatory markers resulted in a minimal increase of model fit and was therefore not included in the main analyses, but instead was added as a sensitivity analysis of which the results were included in the supplementary material. This resulted in mixed models which assessed whether participants with elevated level

of inflammation were more likely to have higher symptom-levels of anxiety at baseline and throughout a follow-up period of up to nine years. Models were adjusted for baseline variables of gender, age, reported sickness prior to interview, the use of anti-inflammatory medication, and BMI.

Analyses were done separately for each of the three inflammatory index scores as main independent variables and as exploratory analysis for each of the individual markers. In sensitivity analyses, we repeated the analysis in which we adjusted for the presence of (comorbid) MDD (about 35.4% of the total sample) as a dichotomous variable. Moreover, in a sensitivity analysis we repeated the analyses in a subsample of participants who met DSM-IV criteria for an anxiety disorder (see supplementary material Fig. 1). For the main analyses with the index scores, we adjusted the outcomes of the inflammation indexes for multiple testing with the Bonferroni-correction which resulted in p-values regarded as being significant at p = 0.001 (Bland & Altman, 1995). In order to yield beta-coefficients, that can be compared among different

Table 1
Sociodemographic and clinical characteristics. T1 (year 1; n = 2388) included only self-report measures, it was therefore included in the study but not included in the Table 1. Anti-inflammatory medication included ATC codes M01A, M01B, A07EB, A07EC. Chronic somatic diseases included: asthma, chronic bronchitis or pulmonary emphysema, heart diseases or infarct, diabetes, stroke or CVA, arthritis or arthrosis, rheumatic complaints, tumor and/or metastasis, stomach or intestinal disorders, liver disease or liver cirrhosis, epilepsy, thyroid gland disease, or another chronic disease for which the patient receives treatment. Tumor necrosis factor = TNF. Interleukin = IL. C-reactive protein = CRP. Interferon-ɣ = IFN-ɣ. Higher monocyte chemoattractant protein-1 = MCP-1. Macrophage inflammatory protein = MIP. Matrix metallopeptidase-2 = MMP-2.

A. Sociodemographic and clinical characteristics		
	Whole sample n = 2867	LPS-induced sample n = 1227
Age in years (mean, SD)	41.9 (13.0)	42.8 (12.7)
Female (%)	66.5	65.6
North European ethnicity (%)	94.9	94.8
BMI (mean, SD)	25.6 (5.0)	25.67 (5.0)
Smoking status (%)		
Never smoker	28.0	29.0
Former smoker	33.6	34.2
Current smoker	38.4	36.8
Education level (%)		
Elementary or lower	6.5	6.4
Secondary education	58.2	56.7
College or university	35.4	36.9
Sickness prior to interview (%)	27.9	30.1
Chronic somatic disease, yes (%)	40.4	44.3
Anti-inflammatory med., yes (%)	4.9	3.1
Inflammatory markers (median, IQR)		
TNF-α (pg/ml)	0.80 (0.50)	
IL-6 (pg/ml)	0.80 (0.76)	
CRP (mg/L)	1.22 (2.48)	
Inflammatory markers after LPS induction (median, IQR)		
IFN-ɣ (pg/ml)		10.2 (7.44)
IL-10 (pg/ml)		205.5 (281.75)
IL-18 (pg/ml)		249.0 (104.0)
IL-2 (pg/ml)		9.07 (6.17)
IL-6 (ng/ml)		25,800 (17875)
IL-8 (ng/ml)		10,400 (8500)
MCP-1 (ng/ml)		1510 (1270)
MIP-1α (ng/ml)		17,800 (12975)
MIP-1β (ng/ml)		234,000 (146500)
MMP-2 (pg/ml)		73.0 (20.40)

B. Sociodemographic and clinical characteristics										
	Whole sample					LPS-induced sample				
	n = 2867	n = 2529	n = 2338	n = 2195	n = 2014	n = 1227	n = 1051	n = 955	n = 893	n = 818
	Baseline	T2	T4	T6	T9	Baseline	T2	T4	T6	T9
MDD, yes (%)	27.1	13.8	11.3	9.7	9.8	28.8	15.2	10.1	8.4	9.2
Dysthymia, yes (%)	9.3	8.3	6.1	6.1	4.3	10.4	8.2	6.4	6.1	4.0
Anxiety disorder, yes (%)	43.6	27.5	22.7	19.8	19.5	44.4	27.9	20.3	20.9	19.4
Social phobia, yes (%)	18.5	17.5	17.5	16.7	16.6	20.4	19.8	17.9	17.3	17.3
Panic disorder, yes (%)	17.0	15.9	15.7	14.7	14.9	17.3	16.5	14.6	14.5	13.3
Agoraphobia, yes (%)	17.1	16.0	155.6	14.7	14.8	17.0	16.1	13.6	13.0	13.1
General anxiety disorder, yes (%)	13.3	12.2	12.0.1	11.8	11.8	14.5	13.5	12.3	12.2	11.3
Comorbid mood and anxiety disorder (%)	19.9	10.1	8.0	6.7	6.4	21.4	10.8	8.3	6.8	5.9
No current anxiety or mood disorder (%)	47.9	66.2	71.7	74.7	75.6	46.9	70.4	66.5	68.8	66.7

tests, all outcome and independent variables were standardized (i.e., z-scores). For all analyses, we used RStudio (R version 3.6.3; R Foundation for Statistical Computing, Vienna, Austria, 2016. URL: https://www.R-project.org/), with main packages 'lme4′ (version 1.20.1), and 'emmeans' (version 1.4.6).

3. Results

3.1. Sociodemographic and clinical characteristics at baseline

The demographics of the study sample are shown in Table 1. Our study sample was 66.5% female (n = 1,930), and the age ranged from 18 through 64 years at baseline (mean 41.9 years; SD 13.0; see also Table 1A). As demonstrated in Table 1B, at baseline a total of 1,299 (43.6%) of the participants had an anxiety disorder in the month prior to the baseline wave, of which social phobia (18.5%) was most common. There were also 27.1% patients with (comorbid) MDD (n = 796). Of the total sample, 47.9% did not have a mood or anxiety diagnosis (n = 1,368) of whom 54.2% never had had a psychiatric diagnosis before (n = 742). As a considerable percentage of the sample was recruited from

general practice and secondary mental healthcare, percentages of patients meeting DSM criteria for anxiety or mood disorders were the highest at baseline and decreased at later follow-ups, most likely due to symptoms naturally resolving over time and by means of treatment, as well as due to regression to the mean effects.

3.2. Basal inflammation

The associations between basal inflammation index score in relation to anxiety symptom severity over the course of 9 years are shown in Fig. 1 and Fig. 2 (first column). Basal level of inflammation was significantly positively associated to BAI total score (β = 0.057, p = < 0.001) and its somatic subscale (β = 0.070, p = <0.001). This translates as a 0.057 SD increase of (BAI) anxiety severity with each SD increase of the basal inflammation index. Basal inflammation was also significantly associated to the FQ agoraphobia subscale (β = 0.074, p < 0.001). Additionally, significant associations were found for the FQ total score (β = 0.048, p = 0.008), although this was no longer significant after adjusting for multiple testing. Similar effects were found when only a subsample of participants who met DSM-IV criteria for an anxiety
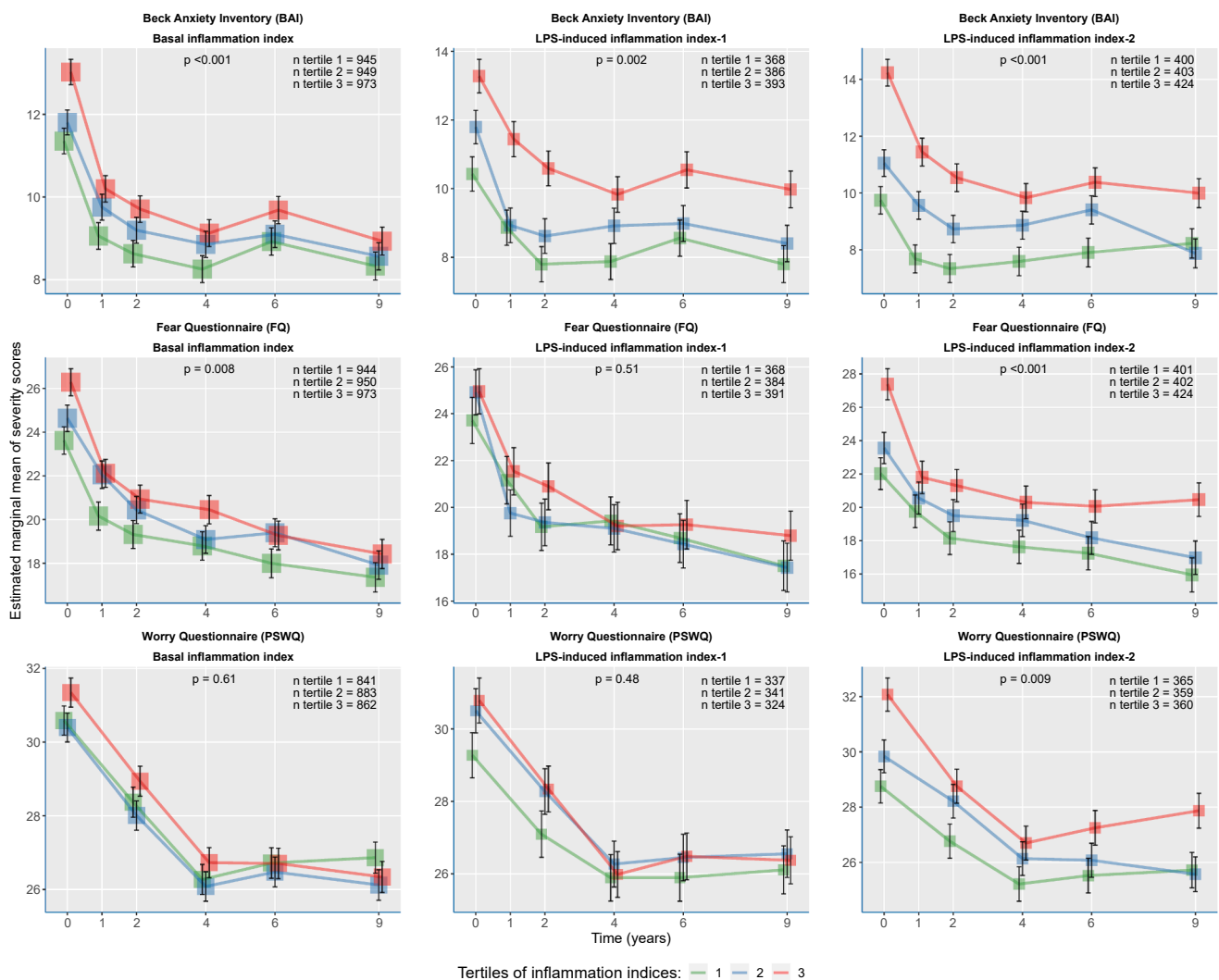

Fig. 1. Tertiles of the basal inflammation index, LPS-induced inflammation index-1, and LPS-induced inflammation index-2 related to BAI, FQ, and PSWQ total scores over the course of 9 years. Inflammation indexes are divided into tertiles of equal proportions of the sample distribution at baseline (1. lowest inflammatory markers: 0.0–0.33; 2. middle: 0.33–0.66; 3. highest: 0.66–1.0). Sample sizes for each tertile at baseline are presented in the graphs. Sample sizes can vary due to missing individual variables of inflammatory markers and anxiety totals scores. Y-axis represents estimated marginal mean values of total scores adjusted for gender, age, reported sickness prior to interview, the use of anti-inflammatory medication, and BMI. Error bars represent standard errors.
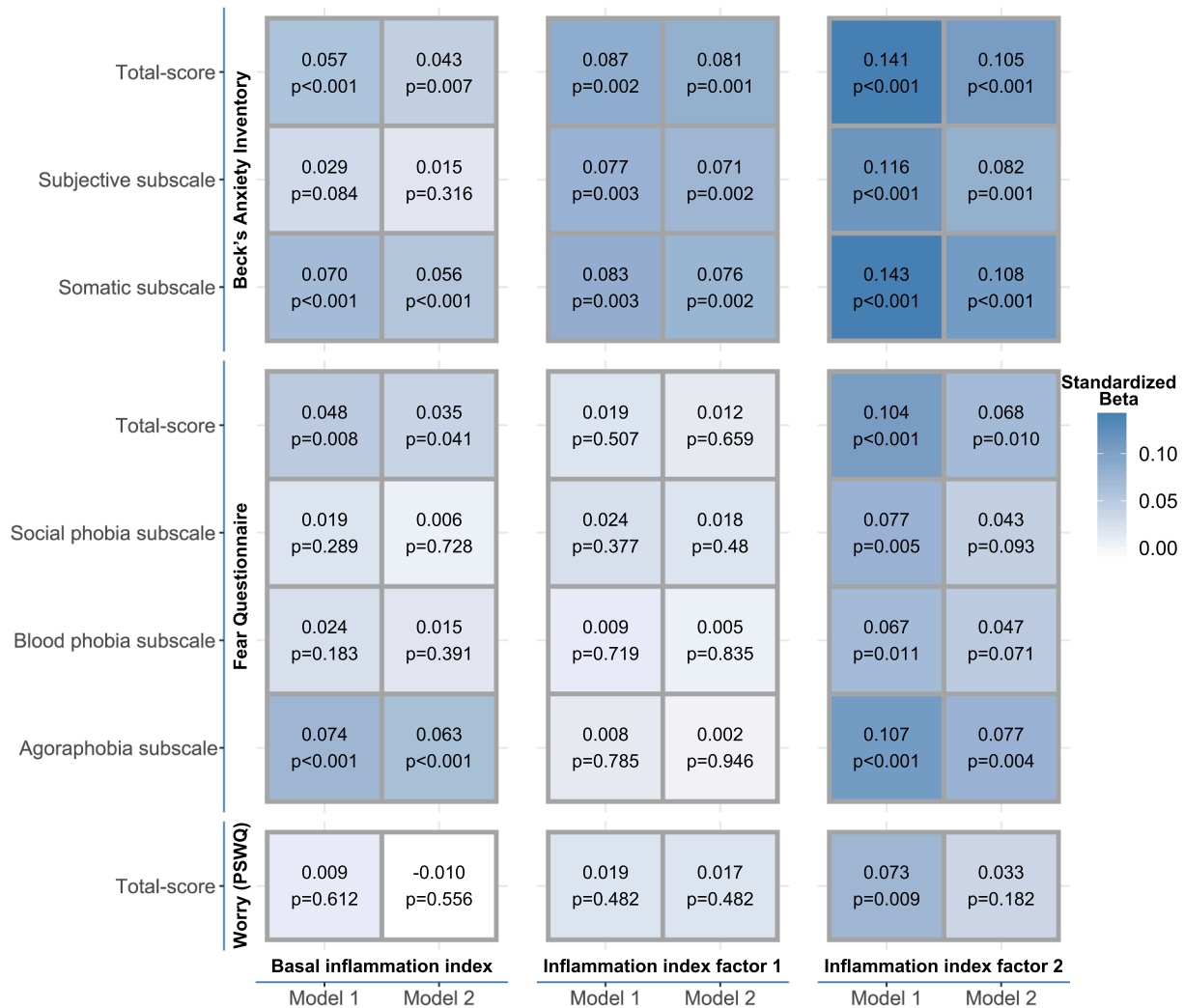
Fig. 2. Standardized beta coefficients of the association between basal and LPS-induced inflammation indexes and anxiety symptoms. Linear mixed models fitted with repeated measures, which were assessed up to six times over 9 years of follow-up. P-values remain statistically significant after adjusting for multiple testing at p = 0.001. Model 1 standardized beta coefficients for basal inflammation index and LPS-induced inflammation index-1 and -2 were adjusted for baseline variables of gender, age, sickness prior to interview, the use of anti-inflammatory medication, and BMI. Model 2 beta-coefficients were additionally adjusted for the presence of MDD.

disorder were included (see supplementary material Fig. 1). Significant associations were present at baseline and tended to persist over the course of nine years, as shown in Fig. 1. This was further confirmed by small effect sizes of the interaction terms with time (with a maximum β = -0.006 ;p = 0.009), which was not statistically significant when adjusted for multiple testing (Supplementary material Table 1). No significant associations were found between basal inflammation and the BAI subjective subscale (β = 0.029, p = 0.084), the FQ social phobia subscale (β = 0.019, p = 0.2882), and the PSWQ scale (β = 0.009, p = 0.610).

After adjustment for the presence of MDD, we found that the effect estimates of basal inflammation with anxiety severity were attenuated by 25–30%, but remained statistically significant. When assessing the individual inflammatory markers of the basal index score, we found that TNF-α, IL-6, and CRP were related to anxiety with roughly equal effect sizes, although no longer statistically significant (see Fig. 3).

3.3. LPS-induced inflammation

The associations between LPS-induced inflammation index – 1 in relation to anxiety over the course of 9 years are shown in Fig. 1 and

Fig. 2 (middle column – index 1; last column index 2). LPS-induced inflammation index – 1 was significantly positively associated to the BAI total score (β = 0.087, p = 0.002), its somatic subscale (β = 0.083, p = 0.003) and subjective subscale (β = 0.077, p = 0.003). However, none of these associations with the BAI remained significant (p's > 0.001) after adjustment for multiple testing. LPS-induced inflammation index – 1 was not significantly associated to the FQ and PSWQ (sub)scales. When we adjusted these analyses for the presence of MDD (comorbidity), we found that the (lack of) association of LPS-induced inflammation index – 1 remained roughly similar. When assessing the individual components of biomarkers of LPS-induced inflammation index – 1, we found that there were significant positive associations between TNF-β, IL-2, IL-6 and MMP-2 and BAI total score, BAI somatic subscale, and BAI subjective subscale (see Fig. 3).

Contrary to LPS-induced inflammation index – 1, LPS-induced inflammation index – 2 demonstrated significant associations with all BAI, FQ, and PWSQ (sub) scales. Standardized beta's ranged from β = 0.067, p = 0.011 (for FQ blood phobia) to β = 0.1, p < 0.001 (for BAI somatic subscale). When adjusting for multiple testing, associations remained statistically significant for the BAI (sub) scales, and the FQ total score and agoraphobia subscale. Similar effects were found when
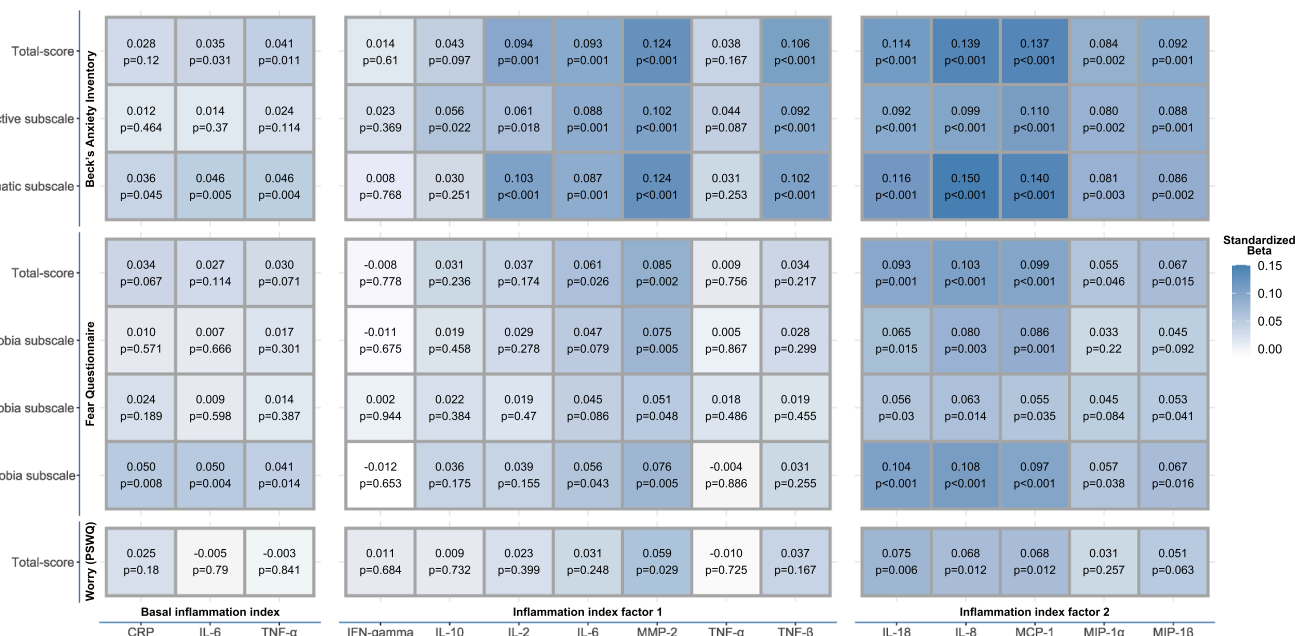
Fig. 3. Standardized beta coefficients of the association between individual basal serum, and LPS-induced inflammatory markers and anxiety symptoms. Linear mixed models fitted with repeated measures, which were assessed up to six times over 9 years of follow-up. Standardized beta coefficients for basal inflammation index and LPS-induced inflammation index -1 and -2 were adjusted for baseline variables of gender, age, sickness prior to interview, the use of anti-inflammatory medication and BMI.

only a subsample of participants who met DSM-IV criteria for an anxiety disorder were included (see supplementary material Fig. 1). As is demonstrated in Fig. 1, these statistical associations were strongest at baseline, but persisted over time. We found a significant negative interaction term of up to β = -0.014 (p = < 0.001), between a continuous modelled time variable and LPS-induced inflammation index – 2 (Supplementary material Table 1). This suggests that the relationship with baseline LPS-induced inflammation index – 2 tended to attenuate somewhat over time, although to a small degree.

Similar to basal inflammation, the association between LPS-induced inflammation index – 2 and the anxiety (sub) scales were attenuated by approximately 30%, when adjusted for the presence of MDD (comorbidity). When assessing the individual biomarkers that LPS-induced inflammation index – 2 consisted of, we found that all 5 markers were significantly related to these anxiety scales. However, the estimated associations of IL-8, IL-18, and MCP-1 were substantially stronger compared to those of MIP-1α and MIP-1β (see Fig. 3).

4. Discussion

Our study is the first to examine the relationship between basal as well as LPS-induced inflammatory markers with longitudinal measures of anxiety symptom severity over a period of up to nine years. Our results demonstrated that participants with elevated inflammatory markers at baseline had on average higher levels of anxiety at baseline, which persisted during the course of nine years follow-up. However, the effect sizes of these associations were small. Inflammatory markers were especially associated with somatic symptoms of anxiety (e.g., sensations of physical arousal), and symptoms of agoraphobia.

Thus far, most prospective studies examining the relationship between inflammation and anxiety used basal inflammatory markers such as CRP, TNF-α and IL-6 (Baune et al., 2012; Copeland, Shanahan, Worthman, Angold, & Costello, 2012; Glaus et al., 2018; Wagner et al., 2015). We found stronger association for LPS-induced inflammatory markers index −2 with anxiety compared to the basal inflammatory index, which were assessed through distinct methods (ELISA versus multiplex). Earlier studies have shown that basal circulating levels of

inflammatory markers (assessed by using Elisa method) are typically low and show a high degree of intra-individual variability (van den Biggelaar et al., 2007). The expression of inflammatory markers in response to ex vivo stimulation of LPS (using multiplex method) mimics the natural environment more closely and induces an inflammatory reaction reflecting the innate production of inflammatory markers (van der Linden et al., 1998; van Exel et al., 2009). Our results underline the idea that basal inflammation levels and stimulated levels are a reflection of two different aspects of the immune system. LPS-induced inflammatory markers may show less (within person) variability compared to basal inflammatory markers serum level (Üçeyler et al., 2011). That being said, LPS-induced inflammatory index -1 demonstrated smaller effect sizes than LPS-induced inflammatory index-2, suggesting that LPS-induced inflammatory index -2 is made up of cytokines that may better reflect the innate immune response that is associated with anxious mood states than markers from index-1. Previously, similar results were found for this index score in relation to the course of symptoms of depression (van Eeden et al., 2020). Within LPS-induced inflammatory index -2, especially MCP-1, IL-8 and IL-18 demonstrated strong associations with anxiety. Cytokines are believed to play an important role in immune homeostasis and can display heterogenic, pleiotropic and overlapping functional properties as is illustrated by their ability to act in both a pro- and an anti-inflammatory manner in complex interactions with one another (Jones & Jenkins, 2018). It appears that pro-inflammatory markers (e.g., IL-8 and IL-18) may contribute more than anti-inflammatory markers (e.g., IL-10; as shown in Fig. 3), but such findings need to be replicated as our prospective results contrast with those by Vogelzangs et al., (2016) showing that both pro- and anti-inflammatory markers were positively associated with anxiety and depression in a cross-sectional analysis.

According to the "pathogen-host defence theory" (PATHOS-D), across evolutionary time, heavy pathogen load induced significant pressure on human survival (Raison & Miller, 2017). This has led to adaptations which shaped interactions between the immune system and the brain, resulting in a set of behaviors such as anhedonia and fatigue (commonly referred to as *sickness behavior*), but also anxiety arousal and alarm (Raison & Miller, 2017). First, due to these processes, modern

humans may have inherited a genomic bias towards inflammation, because this response - and the symptoms it promotes - enhanced protective behaviors, host survival, and reproduction in the highly pathogenic environment in which humans evolved (Miller & Raison, 2016). Second, stress perception by the brain may serve as an early warning signal to activate the immune system in preparation of subsequent wounding (Dhabhar, 2009; Miller & Raison, 2016), in which case symptoms of anxiety would lead to an increase of inflammatory markers. Finally, our findings could also be explained in light of the "sickness behavior theory" (Dantzer, O'Connor, Freund, Johnson, & Kelley, 2008; Rosenblat, Cha, Mansur, & McIntyre, 2014), which is part of the PATHOS-D theory. The sickness behavior theory postulates that crosstalk between several inflammatory pathways and neurocircuits of the hypothalamic–pituitary–adrenal (HPA) axis could lead to sickness behavior—a set of motivational and behavioral changes including both somatic symptoms (low energy, malaise, etc.) and reward sensitivity related symptoms (anhedonia, and withdrawal; Maes et al., 2012; Miller & Raison, 2016; Shattuck & Muehlenbein, 2015). We found relative strong associations with agoraphobia, which supports this idea.

Alongside the PATHOS-D and sickness behavior theories (Dhabhar, 2009; Miller & Raison, 2016), the associations found between inflammation and anxiety symptoms, in particular the arousal anxiety symptoms may be explained by activation of the HPA axis. Replicated studies have demonstrated that following acute stress, cytokines such as IL-6 and TNF-α activate the HPA-axis, increasing levels of corticotrophin releasing hormone (CRH), adrenocorticotropic hormone (ACTH) and cortisol (Beishuizen & Thijs, 2003). Repeated activation of the inflammatory system due to chronic stress has been shown to disproportionally increase HPA-axis activity compared to the usual response (Grinevich et al., 2001), which in turn have been shown to induce mood and anxiety symptoms (Leonard & Myint, 2009). Although our results may partially be explained by HPA-axis activation this seems not to be the most important mechanism as IL-6 and TNF-α were relatively weakly related to anxiety.

An additional finding of the present study was that a substantial part of this association was driven by MDD comorbidity, as the strength of the relationship between inflammatory markers and anxiety symptomatology attenuate by about 25% to 30% when adjusted for the presence of MDD. Although comorbid MDD may also be an indicator for overall severity, the findings of this study seem to replicate that the link found between anxiety and inflammation is partly driven by depression (Baune et al., 2012; Copeland et al., 2012; Glaus et al., 2018; van Eeden et al., 2020; Wagner et al., 2015).

Considering the positive association of several inflammatory markers with anxiety, opportunities may arise for developing treatment options. Several *meta*-analyses have found predominantly positive effects of anti-inflammatory medication (NSAIDs, fatty acids, statins and cytokine inhibitors amongst others) on depression (Bai et al., 2020; Husain et al., 2019; Köhler-Forsberg et al., 2019; Köhler et al., 2014; Yatham, Yatham, Ravindran, & Sullivan, 2019). Anti-inflammatory treatment may result in a decrease of depressive symptoms, but likely only for a subset of patients with chronic low-grade inflammation (van Eeden et al., 2020). For example, there is some evidence for efficacy of add-on treatment with minocycline for treatment resistant depression, but only among those with low-grade inflammation defined as CRP ≥ 3 mg/L (Nettis et al., 2021). Perhaps anti-inflammatory drugs can also be used for treating some patients with anxiety, especially those with elevated (LPS-induced) inflammatory markers and who suffer from somatic anxiety symptoms or agoraphobia. It could be promising to devise strategies to identify such a subgroup of patients with anxiety disorders that may benefit from a (personalized) treatment with anti-inflammatory drugs.

Our study has several strengths. With a substantial sample size, we analyzed individual symptom domains of anxiety over a follow-up period of nine years. A wide array of inflammatory markers was assessed at baseline, including more costly and laborious LPS-induced markers. Moreover, we had a heterogenic sample containing patients with anxiety disorders as well as healthy controls recruited from multiple settings and with only few exclusion criteria, making this sample easier to generalize to other populations.

A number of limitations of our study need to be discussed. Firstly, we found no strong effects of interaction terms with time, but rather that baseline associations persisted over a long follow-up period. Therefore, our findings cannot disentangle the relationships in time, whether inflammation predated anxiety or vice versa. Moreover, an earlier study demonstrated that comorbid depressive and anxiety disorders and higher symptom severity were associated to attrition, which could have been a potential bias in our analyses. However, we do not expect large confounding effects with regard to our findings, when doing a sensitivity analysis with a subset of complete cases (n = 1713), the relationships between basal inflammation index and the BAI total score did somewhat increase in effect size, and remained statistically significant (β = 0.057; p = 0.011). Second, we focused on a dimensional approach of anxiety symptoms based on self-report severity scales, which differs from clinician-rated categorical DSM diagnoses of anxiety disorders. Anxiety DSM-diagnoses can be viewed as discrete categorical syndromes imposed on a continuum of anxiety symptoms of varying severity and duration. Future research could assess whether inflammatory markers are also related to onset and remission of diagnoses over several years. Moreover, NESDA focussed on depression and anxiety and patients with other diagnoses have not been invited for the NESDA project. Although clinically overt diagnoses, such as bipolar disorder and severe PTSD were excluded, our sample was not diagnostically homogeneous. Future research with homogenic samples and clinician-rated DSM criteria are needed. Third, a large proportion of our sample had a prevalent chronic somatic condition, although detailed information of the nature of these conditions was lacking. We choose not to adjust our analyses for somatic comorbidity, because the consequent pro-inflammatory state could be part of the causal pathway between inflammation and anxiety. However, when we adjusted the effects of basal inflammatory markers on BAI total score for the presence of a chronic somatic disorder (yes/no), the effect was only slightly reduced and remained significant (β = 0.049, p = 0.004). Future research should examine if inflammation is a mediating factor for the relationship between many chronic somatic diseases and anxiety (Costello, Gould, Abrol, & Howard, 2019; Renna, O'Toole, Spaeth, Lekander, & Mennin, 2018). Fourth, due to logistical reasons, LPS-stimulated markers were only added to the study, after the inclusion was well underway, resulting in a smaller sample of 1,229 participants. Fortunately, the sample size was still reasonably large and was not substantially different with regard to baseline characteristics. Fifth, as more LPS-induced markers compared to basal serum markers were assessed, the results could be biased toward identifying relationships with one methods over the other. Sixth, the two LPS indued inflammatory indexes were calculated based on data driven methods (Factor analysis; Bandalos & Finney, 2018), as was done in our earlier research (van Eeden et al., 2020). An alternative option would have been grouping of these individual markers based on underlying pro- and anti-inflammatory properties. Finally, our inflammatory markers were based on a single blood sample only. Sequential day-to-day measures of inflammatory markers would have increased the precision of the markers.

In conclusion, we found that participants with high levels of inflammatory markers have on average high levels of somatic symptoms of anxiety (arousal) and agoraphobia, which tended to persist over a period of nine years, albeit with small effect sizes. These associations were partly driven by co-morbid depression. These findings suggest that some of these patients could benefit from anti-inflammatory agents. Future studies are needed to develop strategies in order to select these patients and to test treatment effectiveness. The small effect sizes found in this study suggest that a large impact on group level may not be feasible.

Declaration of Competing Interest

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements and financial disclosures

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbi.2021.09.001.

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