Neurobiology of Stress 8 (2018) 82–91

Contents lists available at ScienceDirect

Neurobiology of Stress

journal homepage: www.elsevier.com/locate/ynstr

Distinct CCL2, CCL5, CCL11, CCL27, IL-17, IL-6, BDNF serum profiles correlate to different job-stress outcomes

Alessio Polacchini[a], Damiano Girardi[b], Alessandra Falco[b], Nunzia Zanotta[c], Manola Comar[c,d], Nicola Alberto De Carlo[b], Enrico Tongiorgi[a,*]

[a] Department of Life Sciences, University of Trieste, Via Giorgieri 5, 34127 Trieste, Italy
[b] FISPPA Section of Applied Psychology, University of Padova, Via Venezia, 14, 35131, Padua, Italy
[c] Institute for Maternal and Child Health-IRCCS, Burlo Garofolo, Trieste, Italy
[d] Department of Medicine, Surgery and Health Sciences, University of Trieste, Ospedale di Cattinara, Strada di Fiume 447, 34149 Trieste, Italy

ARTICLE INFO

Keywords:
Chronic stress
Psychophysical strain
Biomarkers, chemokines
Interleukines, neurotrophic factors
Growth factors
Multiplex immunoassay
Serum proteomic

ABSTRACT

Chronic psychosocial stress at workplace is an important factor in the development of physical and mental illness. Objective biological measures of chronic stress are still lacking, but inflammatory response and growth factors are increasingly considered as potential stress biomarkers. Therefore, we investigated the relationship between psychophysical strain and serum levels of 48 chemokines, cytokines and growth factors measured using a multiplex immunoassay, and serum brain-derived neurotrophic factor (BDNF) measured by ELISA. Severity of psychophysical strain was scored in 115 healthy hospital workers using specific scales for anxiety, depression-like emotion, gastrointestinal or cardiac disturbances, and ergonomic dysfunction. Multivariate analysis revealed that higher anxiety scale scores were correlated with lower serum chemokine C-C motif ligand-2 (CCL2/MCP-1), chemokine C-C motif ligand-5 (CCL5/RANTES), chemokine C-C motif ligand-27 (CCL27/CTACK), chemokine C-C motif ligand-11 (CCL11/Eotaxin) and interleukin-6 (IL-6); gastrointestinal disturbances correlated with increased levels of interleukin-17 (IL-17) and reduced CCL11/Eotaxin, CCL27/CTACK and CCL2/MCP-1; while cardiac dysfunctions associate only to reduced CCL27/CTACK, and ergonomic dysfunction correlated with increased BDNF and reduced CCL11/Eotaxin and CCL5/RANTES. Thus, these 7 serum factors may provide a distinct signature for each different stress-related psychophysical outcome giving indications on individual vulnerabilities.

1. Introduction

Real or perceived threats at job (i.e. stressors) can activate a network of behavioral, psychological and physical reactions in the individual (i.e. psychophysical strain), collectively known as work-related stress (Girardi et al., 2015). This chronic stress condition is a process that occurs when a person has or feels a high job demand having however low job control, thus feeling unable to cope with the requests (de Jonge et al., 2010). Another means driving this kind of chronic stress is the alteration in the effort-reward balance (Siegrist et al., 2004), in terms of salary, accomplishment or job perspectives in front of demanding tasks. Work-related chronic stress is an emerging factor in the development of physical and mental illness (such as depression) and many people experience physical and psychological symptoms related to stress (Ganster and Rosen, 2013; Nixon et al., 2011). Accordingly, psychophysical strain is associated with increased sickness absences and reduced job performance (Falco et al., 2013a, 2013b).

The pathophysiological events triggered by prolonged exposure to stress in humans are not completely characterized, but they involve the hypothalamus-pituitary-adrenal (HPA) axis involving stress hormones such as corticotropin-releasing hormone (CRH), adrenocorticotropic hormone (ACTH) and glucocorticoids, the sympathetic-adrenal-medullary (SAM) axis as well as the endocrine system (Chandola et al., 2010; Dhabhar, 2014; Juster et al., 2010). Besides the above mentioned mechanisms, the recognition that immune alteration is a primary pathophysiological mechanism in chronic stress is one of the major scientific insights of the decade (Reiche et al., 2004; Schmidt et al., 2010; Segerstrom and Miller, 2004; Dhabhar, 2014; Siegrist and Li, 2017). Previous studies showed that sympathetic system stimulate inflammatory cytokine production (McEwen et al., 2015) which in turn augment glucocorticoid production, that has usually anti-inflammatory effects, but it can be also pro-inflammatory, in some cases (Munhoz et al., 2010). Inflammation has been hypothesized to be a biomarker of chronic psychophysiological stress, with interleukins 6 (IL-6),

* Corresponding author.
E-mail address: tongi@units.it (E. Tongiorgi).

https://doi.org/10.1016/j.ynstr.2018.02.002
Received 8 July 2017; Received in revised form 16 January 2018; Accepted 14 February 2018
Available online 16 February 2018

interferons (IFNs) and tumor necrosis factors (TNFs) as the most investigated candidates (Hansel et al., 2010). In addition, other studies reported a general immune suppression (Calcagni and Elenkov, 2006; Dhabhar et al., 2012), highlighting the complexity of interactions between brain and immune system. Chronic stress is known to increase susceptibility to infection and cancer, due to immune suppression, but it may also exacerbate allergic or autoimmune conditions involving a shift in cytokine balance from type-1 to type-2 response, meaning a decrease in cellular immunity in favour of a humoral immunity (Dhabhar, 2014; Glaser et al., 2001). Moreover, the stress response includes alterations in the levels of growth factors and neurotrophins, like the brain-derived neurotrophic factor (BDNF) (Bath et al., 2013; Mitoma et al., 2008). Indeed, decreased BDNF levels are associated with stress-related reduction in dendritic branching and spine density of CA3 neurons (Davidson and McEwen, 2012).

Psychophysical strain is usually evaluated by the worker himself (i.e., through self-report questionnaires) or by occupational physicians with the aid of ad-hoc questionnaires, which allow quantitative assessment of specific dimensions of psychophysical strain (Falco et al., 2013a). However, in addition to the valuable function of these questionnaires, it would be very useful to include in the assessment also objective biological measures, which are still lacking. In this study, we tested the hypothesis that psychophysical strain may affect blood levels of cytokines, chemokines and growth factors, including BDNF and that these relationships might be stronger in females than in males (Evolahti et al., 2006). From a psychological standpoint, gender may influence in several ways the stress process. For example, previous research has shown that women, compared to men, may perceive work situations as more stressful, engage more frequently in less adaptive coping strategies (e.g., emotion-focused), and also experience higher levels of psychophysical strain (Day and Livingstone, 2003; Eaton and Bradley, 2008; Tytherleigh et al., 2007). From a methodological point of view, we examined the relationship between the severity of different psychophysical strain and blood levels of cytokines, chemokines and growth factors in healthy volunteers working at a hospital. To this aim, we have taken benefit of proteomic analysis using a multiplex immunoassay built on magnetic beads for the quantitation of multiple analytes within a single assay (Zanin et al., 2012). Using this technique, we assessed a panel of 48 chemokines, cytokines and growth factors, aiming to test the hypothesis that serum concentrations of pro-inflammatory cytokines and growth factors are upregulated, and immune defense related chemokines and growth factors are decreased in relation to prolonged psychosocial stress (Segerstrom and Miller, 2004). In addition, we measured the neurotrophin BDNF with an ELISA assay, which we recently validated to be the most reliable among six commercial assays (Polacchini et al., 2015), and we hypothesized that it could be decreased in relation to chronic work-related stress.

2. Material and methods

2.1. Participants

The study, conducted as part of larger project aimed at assessing work-related stress risk, was performed examining a sample of workers in an Italian healthcare organization. Workers were informed beforehand by management and participated voluntarily to the study. Workers completed a self-report questionnaire aimed at determining psychophysical strain. Before the questionnaire was administered, the purpose of the present study was explained by a member of the research team, who emphasized that participants should report only psychophysical symptoms attributable to work-related stressful situations (i.e., not imputable to other stressful life events, such as, for example, providing care for a family member or financial difficulties). The questionnaire was completed by 115 subjects who agreed to participate to a clinical interview followed by a blood sample withdrawal. Then, blood samples were collected and sera isolated as previously described (Polacchini et al., 2015). Subjects reporting mood and anxiety disorders, neuroendocrine diseases, non-pharmaceutical drug abuse or dependence, according to DSM-IV-TR criteria, were excluded from the enrollment. Therefore, the study sample comprised 115 workers, of which 71.3% were women. Regarding the work position, 16.7% were managers-doctors, 63.3% were doctors or head nurses, 20% were nurses. Most respondents had a permanent contract (95.9%) and were working in the hospital for more than 8 years. All participants gave their written, informed consent, and the study was approved by the local ethics committee according to the recommendations of the Declaration of Helsinki.

2.2. Work-related stress assessment

Subjects were assessed for psychophysical strain using five scales taken from the Q-BO test, an instrument standardized for the Italian context (De Carlo et al., 2008; Falco et al., 2012). The first part of the questionnaire asked respondents to indicate how often, over the past six months, the psychophysical symptoms (see list below) attributable to work-related stressful events, had appeared or exacerbated. The six psychophysical strain dimensions assessed were: anxiety, emotion (depression-like), gastrointestinal disturbances, cardiac disturbances, ergonomic dysfunction at the workplace. The six point response scale ranged from 1 (never) to 6 (every day). The overall strain score was estimated by averaging the scores of each subscale. Both the self-report version of the questionnaire (the one adopted in this study, see Trifiletti et al., 2013) and the form developed for the administration by the occupational physician (Falco et al., 2013b) showed good psychometric properties. Additionally, Cronbach's alpha in the present study was 0.80 for the anxiety subscale, 0.80 for emotion (depression-like) symptoms, 0.78 for gastrointestinal disturbances, 0.66 for the cardiac disturbances, and 0.80 for ergonomic dysfunction.

The Q-Bo test consists of various scales, reformulated and reduced in the most recent release to about 200 items (from 350 of the original version) - thanks to an experiment conducted on over 30,000 workers - and with a delivery time of about 1 h. The protocol can be adapted to the different needs of organizations in the various public and private contexts both for modules and for specific constructs as well as social and personal information. In particular, sets of scales integrated with each other are available in function of the different types and sizes of the organizations object of survey. The main test configurations are directed specifically to those areas: Education/Training, Manufacturing, metal working industries, Public Administrations and Public Services, Health Sector, Financial Services/Insurance, Social Services. For the different sectors, in a benchmarking perspective, specific reference samples are available (normative values). The scales were developed based on the most extensive international literature, on the needs created by the Italian legislation and the requirements of European and global organizations working in the field of health and safety at work. Thanks to the adoption of a systemic approach, the multidimensional model is used to detect a broad spectrum of work, organizational and individual factors representing potential sources of risk. In particular, by means of the proposed dimensions, the model aims at assessing behavioural, physiological and psychological stress/strain (with particular reference to burnout). These dimensions are in fact designed in order to take into account factors, such as the quantitative and cognitive workload, the degree of control/autonomy, the social rewards in terms of support from the organization, the professional growth, the organizational equity and justice, and the various forms of conflict including conflicts work/life, person-role, with colleagues, between groups/departments of the same organization, with superiors and co-workers, changes, no clear definition of responsibilities. These elements can have negative consequences for the health of the worker and can cause at the same time a deterioration of corporate performance. Another section of the test is dedicated to the detection of psychological and physical symptoms (such as irritability,

depression, feelings of ineffectiveness, gastrointestinal problems, insomnia, cardiac arrhythmia) and behavioural symptoms (such as abuse of alcohol, drugs, smoking) and any other discomfort, symptoms and diseases that can be associated with the risk of work-related stress and therefore constitute additional sources/consequences of the risk. The scales taken from the Qu-Bo test assess symptoms that are known to be stress-related on an epidemiological basis (see for example Nixon et al., 2011). Moreover, several standardized self-reported questionnaires in the literature assess similar symptoms or dimensions/subscales (e.g., the Physical Symptoms Inventory, Spector and Jex, 1998; the Physical Health Questionnaire, Schat et al., 2005). With respect to gastrointestinal and cardiac symptoms, examples of item are "Stomach/abdomen cramps or pain" and "heart pounding without any apparent reason", respectively.

The Q-Bo test also investigates some individual characteristics and resources such as resilience, optimism, sense of efficacy, coping strategy (i.e. mode of behaviour to cope with the difficulties), negative affectivity, tendency to over-commitment and social desirability, in order to assess the validity and reliability of answers to several questions. The test also evaluates the critical variables for the organization such as security measures, air conditioning, lighting, cleanliness and hygiene, pleasantness of the working place.

2.3. Biochemical assessments

Blood samples were collected in a resting and fasting state between 9.00 am and 12.00 pm in anticoagulant-free tubes and let them to clot for 1 h at room temperature, then resting for an additional hour of at 4 °C. Serum was collected by centrifugation at 2000 × g for 10 min at 4 °C, aliquot and stored at −80 °C until assayed. Multiple analytes were assessed, as listed in Table 1. Serum BDNF levels were as previously described (Polacchini et al., 2015), using the BDNF Rapid™ ELISA kit (Biosensis), based on sandwich technology. Cytokines, chemokines and growth factors were measured taking advantage of the multiplex ELISA technology, employing the Bio-Plex Pro Human xMAP Assay 21-plex and 27-plex (Bio-Rad Laboratories Srl, Milan, Italy) read on a Bio-Plex 200 instrument, according to manufacturer's instructions. Reliability of cytokines measurements with this methodology was previously validated (Zanin et al., 2012). All samples were assayed in duplicate in adjacent wells.

2.4. Statistical analyses

To test for possible differences in age and body-mass index (BMI) distributions between female and male population considered in this study, we used the *t*-test for independent samples. To test sex differences in distribution of psychophysical strain scores we used a Mann-Whitney *U* test. In addition, to verify the possible confounding effect of BMI, we performed a partial correlation analysis with the scores of psychophysical strain, after controlling for gender. When biochemical variables had sample values below the lower limit of detection (Out-Of-Range values or OOR); when OOR values were over the 30% of the total sample, the analytes were excluded. When performing univariate analysis, for remaining OOR values we imputed the half of the declared sensitivity value for each analyte, while for Partial Least Square regression analysis all variables having OOR were excluded in order to avoid any distortion in the normalized distribution. The influence of the use of drugs on biochemical variables values was tested performing a Mann-Whitney *U* test. Normality distributions of the independent variables were assessed by Shapiro-Wilks tests and visually inspected by Quantile-Quantile (Q-Q) plot analyses *(not shown)*. Since most of the biochemical variables were not normally distributed we applied log-transformation in order to approximate normality distributions, thus being able to perform both univariate associations and multivariate analysis, with the aim to investigate correlations between biochemical variations and differences in the scores of psychophysical strain. As univariate analyses, we perform partial correlation analyses controlling for gender and BMI to identify association between dependent variables (psychophysical strain scores) and each independent variable (biochemical markers). Since we performed multiple factors assessment on same samples, we chose to not correct for multiple comparisons across the different analytes, because this would enhance the probability of type II errors (Janelidze et al., 2013). A multivariate analysis was also carried out to take in account for potential correlations within the independent variables. Thus, we performed Partial-Least Square discriminant analysis (PLS) and Variable Importance to the Projection (VIP) scores were estimated, for each independent variable, as the average over three latent factors extracted. VIP scores are estimate of the importance of each variable to the model and values equal to or greater than one can be considered prominent in the given model.

3. Results

3.1. Demographic variables

We investigated a possible relationship between the serum levels of cytokines and chemokines, BDNF and other growth factors (listed in Table 1), with the scores of psychophysical strain in 115 healthcare assistants matching the inclusion criteria for this study. Demographic values (age and BMI) are given in Table 2. Of note, BMI scores in female were significantly lower than those in male population (*t*-test, $t = -4.53$, $p < 0.001$). Psychophysical strain was assessed using five subscales, namely anxiety, emotion (depression-like), gastrointestinal disturbances, cardiac disturbances, ergonomic dysfunction at the workplace and by an overall psychophysical strain score, defined as the average of the five subscales (Table 3). In the initial section of the

Table 1
List of biochemical variables.

	Cytokines	Chemokines	Growth factors
21-plex (BioRad)	IL-1α; IL-2Rα; IL-3; IL-12 (p40); IL-16; IL-18; IFN-α2; LIF; MIF; SCF; TNF-β; TNFSF10/TRAIL	CCL27/CTACK; CXCL1/GRO-α; CCL7/MCP-3; CXCL9/MIG; CXCL12/SDF-1α;	HGF; CSF1/M-CSF; β-NGF; CLEC11A/SCGF-β
27-plex (BioRad)	IL-1β; IL-1Ra; IL-2; IL-4; IL-5; IL-6; IL-9; IL-10; IL-12 (p70); IL-13; IL-15; IL-17; IFN-γ; TNF-α; CSF2/GM-CSF	CXCL8/IL-8; CCL11/Eotaxin; CCL2/MCP-1; CXCL10/IP-10; CCL3/MIP-1α; CCL4/MIP-1β; CCL5/RANTES;	IL-7; basic FGF; CSF3/G-CSF; PDGF-BB; VEGF
BDNF *Rapid*™ ELISA kit (Biosensis)	/	/	BDNF

Biochemical analytes listed by cytokines, chemokines and growth factors. IL = interleukin; LIF = leukemia inhibitory factor; MIF = macrophage migration inhibitory factor; SCF = stem cell factor; TNF = tumor necrosis factor; TNFSF = tumor necrosis factor superfamily member; TRAIL = TNF-related apoptosis-inducing ligand; IFN = interferon; CSF = colony stimulating factor; GM-CSF = granulocyte macrophage colony stimulating factor; CCL = chemokine C-C motif ligand; CTACK = cutaneous T-cell-attracting chemokine; CXCL = chemokine C-X-C motif ligand; GRO = melanoma growth stimulating activity; MCP = monocyte chemoattractant protein; MIG = monokine induced by gamma interferon; SDF = stromal cell-derived factor; Eotaxin = eosinophil chemotactic protein; IP = interferon gamma-induced protein; MIP = macrophage inflammatory protein; RANTES = regulated on activation, normal T cell expressed and secreted; HGF = hepatocyte growth factor; M-CSF = macrophage colony-stimulating factor; NGF = nerve growth factor; CLEC11 = C-type lectin domain family 11; SCGF = stem cell growth factor; FGF = fibroblast growth factor; G-CSF = granulocyte-colony stimulating factor; PDGF-BB = Platelet-derived growth factor subunit B; VEGF = vascular endothelial growth factor; BDNF = brain-derived neurotrophic factor.

Table 2
Demographics of the whole sample population (N = 115).

		N	Mean	SD	Min/Max	P
Age, years	Female	82	44.90	8.34	25/60	0.830
	Male	33	45.30	10.52	25/61	
BMI, kg/m^2	Female	81	22.99	2.79	17.72/33.46	< 0.001
	Male	33	25.67	3.46	17.63/33.56	

		N	Mean	SD	Min/Max	P
Age, years	Female	65	43.82	8.28	25/60	0.508
	Male	24	42.42	10.16	25/61	
BMI, kg/m^2	Female	64	22.86	2.89	17.72/33.46	0.005
	Male	24	24.90	3.10	17.63/30.32	

Age and BMI distributions split by gender. Number of subjects (N), average values (mean), standard deviations (SD) and minimum and maximum values (min/max) are given, along with P values (P) as the result of distribution comparison between male and female, for both age and BMI. Upper table displays whole population (N = 115); lower table the drug-free sample population (N = 89).

questionnaire respondents were asked to indicate how often, over the past six months, the psychophysical symptoms (see list above) attributable to work-related stressful events, had appeared or exacerbated and to discriminate job versus other non-work related stressors. The first step was to verify if the demographic variables (gender, BMI and age) could account for distribution differences of the scores among the different dimensions of psychophysical strain. Indeed, performing a non-parametric Mann-Whitney U–test to assess differences in scores distribution between gender, we found that female subjects had significantly higher score values than males for all the psychophysical strain dimensions (Table 4). Regarding the possible confounding effect of BMI on psychophysical strain scores, after controlling for gender in a partial correlation analysis, we found that it negatively correlates with anxiety, gastrointestinal disturbance and the overall strain score in both total (N = 115) and drug-free (N = 89) sample populations (see Supplementary Table S1). However, since age did not correlate with any of the psychophysical strain score, even after controlling for gender or BMI *(not shown)*, it was not considered as a possible confounder. For all subsequent analysis we performed corrections for gender and BMI.

3.2. Biochemical variables

The pre-analytical stages, consisting in serum samples collection and storage, were performed as described previously (Polacchini et al., 2015). For the analytical stage, taking advantage of the multiplex technology, we screened 48 analytes among cytokines, chemokines and growth factors; BDNF was assessed using the Biosensis kit, which exploits a classical sandwich ELISA technology (see Table 1, Materials and Methods). The following analytes (11) were excluded from the dataset because more than 30% of samples were below the lower limit of detection (Out-Of-Range or OOR values): interleukins 2, 15, 1-α, 3, 12p40 (IL-2, IL-15, IL-1α, IL-3, IL-12p40), interferon α2 (IFN-α2), leukemia inhibitory factor (LIF), chemokine C-C motif ligand 7 (CCL7/MCP-3), colony stimulating factor 1 (CSF1/M-CSF), beta nerve-growth factor (β-NGF) and tumor necrosis factor-β (TNF-β). Except for cases showing value below the limit of detection (OOR cases), none of the other cases show significant upward outliers: log transformed, standardized values (centered on mean) were all below three standard deviations. The distribution values of the remaining analytes (38) are listed in Supplementary Tables S3 and S4. Because 26 persons out of 115 were using pharmaceutical drugs, we verified if drug assumption had an impact on the analyte levels. Drugs taken by subjects are the most common in Western-country population, specifically: anti-hypertensive/heart protectors (42%), anti-lipemic (32%), thyroid stimulators (levotiroxin, 21%), non-steroid anti-inflammatory drugs (16%), anti-migraine (11%) – the sum is not 100% because some subjects are taking more than one drug. We tested their effects on cytokines levels comparing the median values between the two populations of drug takers and non-takers. As a result, 21 analytes showed significantly different levels between cases who used drugs and who did not (Mann-Whitney *U* test; see Supplementary Table S2). In general, pharmaceutical drugs had a negative influence on analytes level, with the exception of interferon-γ (INF-γ) and chemokines C-C motif ligand 4 and 5 (CCL4/MIP-1β and RANTES, respectively), for which we observed increased median values in drug users.

There was no difference in gender distribution among pharmaceutical drug users (Female/Male, 17/9, $\chi^2 = 2.46$, P = 0.117) and there was also no significant difference between the scores of psychophysical strain in subjects who use drugs and who do not (Mann-Whitney *U* test; not shown). Given these results, in order to limit this confounder and increase the power of predictors, we decided to exclude all the 26 subjects who used drugs. Distributions of psychophysical strain scores of the remaining 89 cases were basically equal to the previous ones (Tables 3 and 4 and S1). After that, since most of the biological variables were not normally distributed, we log transformed all independent variables (including BMI), attempting to achieve normal distribution. Shapiro-Wilks test for normality not always gave non-significant results, but Q-Q plot analysis displays a good approximation to normal distribution *(not shown)*. The following correlation analyses were performed also on non-transformed data (*not shown*) giving similar results, leading us to use the transformed data in order to perform multivariate analysis as well.

3.3. Univariate analysis

As univariate analysis, we performed partial correlations using the log-transformed biochemical data, controlling for gender and BMI, in order to detect association between each predictors (29 in total) and the dimensions of psychophysical strain. Significant associations were

Table 3
Psychosocial strain scores distribution in the whole (N = 115) and drug-free (N = 89) sample population.

	Anxiety	Emotion	Gastric	Cardiac	Ergonomics	Overall strain
N total population	115	115	115	115	115	115
Mean/SD	2.41/1.04	1.77/0.80	2.07/1.01	1.58/0.85	2.59/1.13	2.08/0.81
Min/Max	1.00/5.40	1.00/5.00	1.00/6.00	1.00/5.00	1.00/5.40	1.00/5.16

	Anxiety	Emotion	Gastric	Cardiac	Ergonomics	Overall strain
N Valid (Missing)	89 (33)	89 (33)	89 (33)	89 (33)	89 (33)	89 (33)
Mean/SD	2.38/1.02	1.79/0.83	2.01/1.06	1.55/0.84	2.65/1.14	2.10/0.82
Min/Max	1.00/5.40	1.00/5.00	1.00/6.00	1.00/5.00	1.00/5.40	1.00/5.16

Scores distribution of psychophysical strain in the whole sample population (N = 115) and after exclusion of pharmaceutical drug users and subjects missing strain scores (N = 89).

Table 4
Gender difference in psychophysical strain scores in the whole (N = 115) and drug-free (N = 89) sample population.

Psychophysical strain total	Anxiety	Emotion	Gastric	Cardiac	Ergonomics	Overall strain
Female. N = 82; Mean rank	62.86	63.40	62.53	62.15	62.51	63.68
Male. N = 33; Mean rank	45.92	44.59	46.74	47.68	46.80	43.88
Z	−2.47	−2.75	−2.31	−2.24	−2.29	−2.88
Asymp. Sig. P (2-tailed)	**0.013**	**0.006**	**0.021**	**0.025**	**0.022**	**0.004**
Psychophysical strain valid	**Anxiety**	**Emotion**	**Gastric**	**Cardiac**	**Ergonomics**	**Overall strain**
Female. N = 65; Mean rank	46.73	48.06	47.17	46.58	46.17	47.24
Male. N = 24; Mean rank	40.31	36.71	39.13	40.71	41.83	38.94
Z	−1.04	−1.85	−1.31	−1.02	−0.70	−1.35
Asymp. Sig. P (2-tailed)	0.296	0.065	0.190	0.310	0.481	0.179

Results of comparison (Mann-Whitney *U* test) between female and male scores of psychophysical strain in the total sample population (N = 115) and after exclusion of pharmaceutical drug users and subjects missing the strain scores (N = 89). Sizes, (N), rank values, Z scores and asymptotic P values (Asymp. Sig. P, 2-tailed) are given. In bold are highlighted $P < 0.05$.

Table 5
Significant correlation between strain scores and biochemical variables.

Variable ID	Anxiety	Gastric	Cardiac	Ergonomics	Overall strain
CCL11/Eotaxin (df = 83)	−0.304 (**0.005**)	−0.325 (**0.002**)		−0.240 (**0.027**)	−0.307 (**0.004**)
CCL27/CTACK (df = 84)	−0.301 (**0.005**)	−0.235 (**0.029**)	−0.272 (**0.011**)		−0.258 (**0.017**)
CCL2/MCP-1 (df = 83)	−0.342 (**0.001**)	−0.214 (**0.049**)			−0.263 (**0.015**)
CCL5/RANTES (df = 83)	−0.320 (**0.003**)			−0.221 (**0.042**)	−0.274 (**0.011**)
BDNF (df = 82)				0.284 (**0.009**)	
IL-17 (df = 83)		0.280 (**0.010**)			
IL-6 (df = 83)	−0.223 (**0.040**)				

Summary of the significant results from partial correlation analysis, controlling for gender and BMI, between biological variables (independent variables, first column) and scores of psychophysical strain (first row). Correlation values (minus sign indicate a negative correlation) and P values in brackets, highlighted in bold, are given. CCL = chemokine C-C motif ligand; CTACK = cutaneous T-cell-attracting chemokine; MCP = monocyte chemoattractant protein; RANTES = regulated on activation, normal T cell expressed and secreted; BDNF = brain-derived neurotrophic factor; IL = interleukin.

found for a total of seven of the independent variables. Specifically, for anxiety the chemokines C-C motif ligand 2, 5, 11 and 27 (CCL2/MCP-1, CCL5/RANTES, CCL11/Eotaxin, CTACK/CCL27) show a negative Pearson's product moment of respectively −0.342, −0.320, −0.304 and −0.301 and the interleukin 6 (IL-6) of −0.223. Similarly, for the gastric disturbance, the partial correlation of CCL2/MCP-1, CCL11/Eotaxin and CTACK/CCL27 was negative and with a Pearson's product moment of respectively −0.214, −0.325, −0.235, while for interleukin 17 (IL-17) the association was positive (0.280). For the cardiac item, only the chemokine CTACK/CCL27 was negatively associated (−0.272), while for ergonomics problem, a negative partial correlation was found for CCL5/RANTES and CCL11/Eotaxin (−0.221 and −0.240 respectively) and intriguingly, a positive correlation with BDNF (0.284). For the overall strain, results were similar to those found for the anxiety dimension. In fact, it showed a partial negative correlation with CCL2/MCP-1, CCL5/RANTES, CCL11/Eotaxin, CTACK/CCL27, with a Pearson's product moment of respectively −0.263, −0.274, −0.307 and −0.258, but no association with IL-6. All α errors were below 0.05 as reported in detail in Table 5; for a graphical representation of the partial correlations see Fig. 1. No association with any of the predictors was found for the Depression-like subscale. To evaluate the chance that multiple comparisons could increase type I errors, we performed correction of the p-value according to the Benjamini-Hochberg (BH) procedure which estimates the false-discovery rate (FDR) based on ranked ascending p-values. Correction was done on the number of test performed n = 38, according to the formula pj = n/j*pj, where p is the ranked p-value and j is its percentile rank (Noble, 2009). Only CCL2/MCP-1 retains a significant association with anxiety. However, when considering n = 7, factors associated with anxiety, gastric and overall strain (indicated as stress) subitems were significant. Corrected p-values are given in Supplementary Table S5.

Finally, to determine the contribution of each of the identified markers to the different pshychophysical strain subscales, we analyzed the effect size for the calculated correlation factor r, reported as absolute value. On the basis of the Cohen's d values, we found a medium effect size for CCL2/MCP-1, CCL5/RANTES, CCL11/Eotaxin, and CCL27/CTACK on anxiety, for CCL11/Eotaxin on gastric problems and stress in general (overall strain), while the effect of the other factors was small (Supplementary Table S6).

3.4. Multivariate analysis

Multivariate analysis was also performed to explore association between predictors. More specifically, the partial least square regression procedure (PLS) was identified as the best suited method for this purpose because it is particularly useful when predictor variables are highly correlated or the number of predictors is considerable in respect to the number of cases. This method extracts, at first, several latent factors that explain as much of the covariance as possible between dependent and independent variables and then computes a series of score (Variable Importance to the Projection or VIP) that help to identify predictors that best contribute to the association with the dependent variable. As done before with partial correlation analysis, for PLS we used each subscale of psychophysical strain as dependent variable and cytokines, chemokines, growth factors and the interaction effect between gender and BMI as independent variables. In this analysis, we further eliminated the variables whose OOR were substituted with fixed values and therefore could introduce a bias in the distribution: interleukins 1β, 5, 16, 2 receptor-α (IL-1β, IL-5, IL-16, IL-2Rα, respectively), colony stimulating factor 2 (CSF2/GM-CSF), stem cell factor (SCF), chemokines C-X-C motif ligand 1 and 12 (CXCL1/GRO-α and CXCL12/SDF-1α), and tumor necrosis factor super-family member 10 (TNFSF10/TRAIL). In addition, these variables did not correlate with any of the pshychophysical strain subscales in the univariate analysis. The graphs in Fig. 2 show the contribution of information (VIP) of each variable to anxiety, gastric and cardiac disturbances,

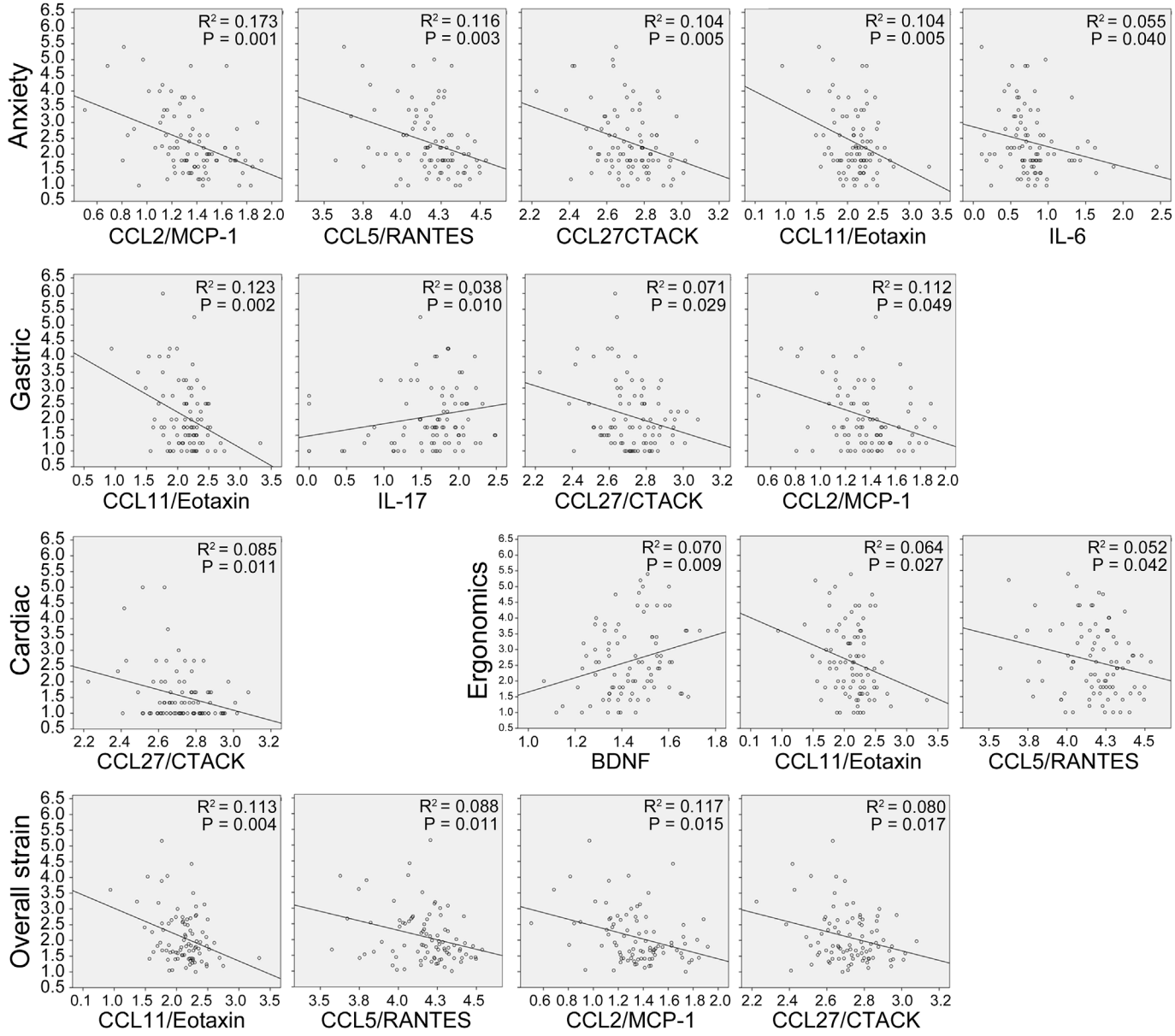

Fig. 1. Scatter plots showing correlations between the given chemokines (CCL2/MCP-1, CCL11/Eotaxin, CCL27/CTACK, CCL5/RANTES), cytokines (IL-6, IL-17) and growth factor (BDNF) and the items of psychophysical strain. Regression lines, R square and P values are displayed. CCL = chemokine C-C motif ligand; CTACK = cutaneous T-cell-attracting chemokine; MCP = monocyte chemoattractant protein; RANTES = regulated on activation, normal T cell expressed and secreted; BDNF = brain-derived neurotrophic factor; IL = interleukin.

ergonomic problems and overall strain. Results essentially replicated the findings obtained with the partial correlation analysis, in which the chemokines CCL2/MCP-1, CCL5/RANTES, CCL27/CTACK, CCL11/Eotaxin, the interleukin IL-6 and IL-17 and the neurotrophin BDNF are the best contributors, since their score is above one. Interestingly, other analytes that were not identified in the partial correlation analysis, showed up with PLS: interleukin 9 (IL-9) for anxiety and cardiac problems, interleukin 18 (IL-18) for ergonomic problems and overall stress and chemokine C-C motif ligand 4 (CCL4/MIP-1β) for gastric problem and overall strain. In contrast, IL-6 resulted weakly associated with anxiety score as well as with the other strain dimensions. Taken together, these results pointed out a clear connection between some elements of the chemokine pattern and the features of psychophysical strain. Interestingly, BDNF was positively associates with the score of the ergonomic problems at the workplace.

4. Discussion

In this study, we investigated the relationship between the scores obtained from self-assessed questionnaires for the stress-risk evaluation (subjective assessments) and changes in circulating level of humoral mediators (objective measures). Serum levels of 48 chemokines, cytokines and growth factors were measured using a multiplex immunoassay, and serum brain-derived neurotrophic factor (BDNF) by ELISA. The perceived stress at work was estimated in 115 healthy hospital workers, using an instrument standardized for the Italian context, the Qu-BO test (De Carlo et al., 2008; Falco et al., 2012): five subscales were administered that evaluate pshychophysical strain in term of anxiety, emotional status, gastrointestinal and cardiac disturbances, along with ergonomic problems at the workplace (headache, hurting neck or shoulders or back pain). Higher anxiety scale scores

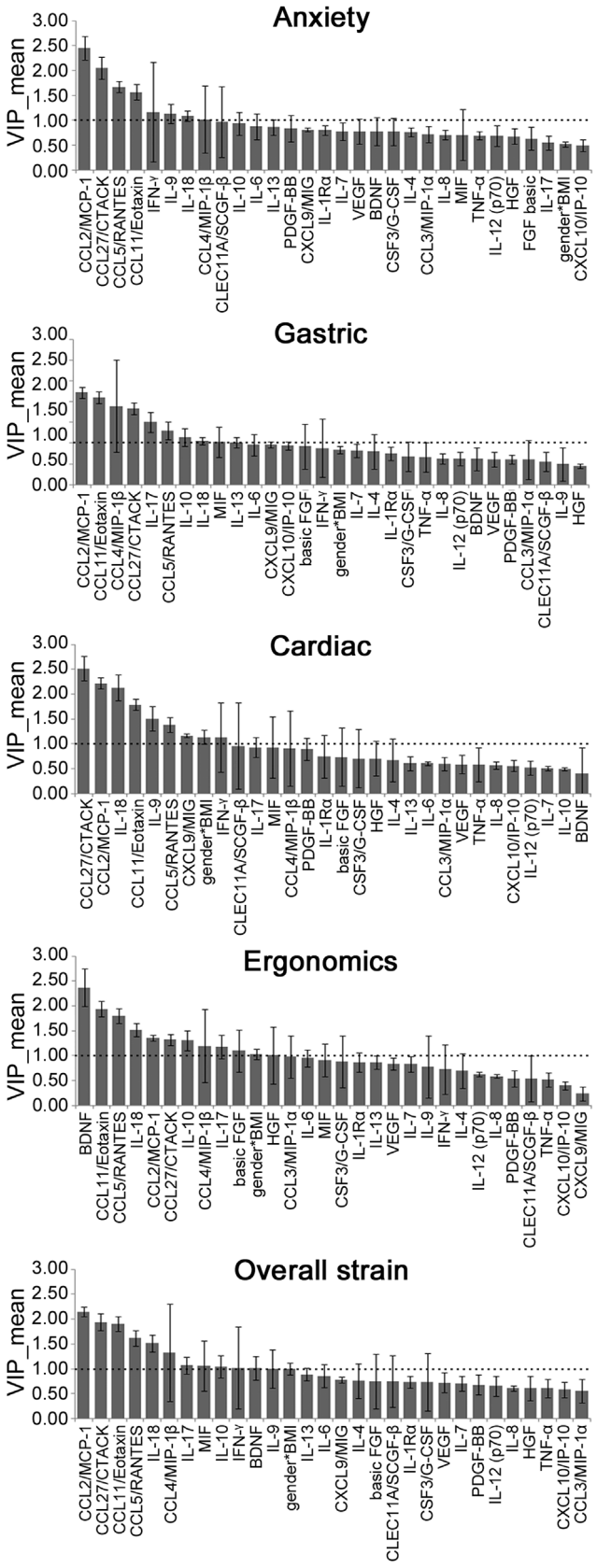

Fig. 2. Variable Importance to the Projection (VIP) ranking biological markers for their contribution to the association to psychophysical strain, assessed by Partial Least Square (PLS) discriminant analysis. Analytes with VIP values equal or above one (dashed line as reference) are the best contributors to a given model. IL = interleukin; LIF = leukemia inhibitory factor; MIF = macrophage migration inhibitory factor; SCF = stem cell factor; TNF = tumor necrosis factor; TNFSF = tumor necrosis factor superfamily member; TRAIL = TNF-related apoptosis-inducing ligand; IFN = interferon; CSF = colony stimulating factor; GM-CSF = granulocyte macrophage colony stimulating factor; CCL = chemokine C-C motif ligand; CTACK = cutaneous T-cell-attracting chemokine; CXCL = chemokine C-X-C motif ligand; GRO = melanoma growth stimulating activity; MCP = monocyte chemoattractant protein; MIG = monokine induced by gamma interferon; SDF = stromal cell-derived factor; Eotaxin = eosinophil chemotactic protein; IP = interferon gamma-induced protein; MIP = macrophage inflammatory protein; RANTES = regulated on activation, normal T cell expressed and secreted; HGF = hepatocyte growth factor; M-CSF = macrophage colony-stimulating factor; NGF = nerve growth factor; CLEC11 = C-type lectin domain family 11; SCGF = stem cell growth factor; FGF = fibroblast growth factor; G-CSF = granulocyte-colony stimulating factor; PDGF-BB = Platelet-derived growth factor subunit B; VEGF = vascular endothelial growth factor; BDNF = brain-derived neurotrophic factor.

were correlated with lower serum chemokine C-C motif ligand-2 (CCL2/MCP-1), chemokine C-C motif ligand-5 (CCL5/RANTES), chemokine C-C motif ligand-27 (CCL27/CTACK), chemokine C-C motif ligand-11 (CCL11/Eotaxin) and interleukin-6 (IL-6); gastrointestinal disturbances correlated with increased levels of interleukin-17 (IL-17) and reduced CCL11/Eotaxin, CCL27/CTACK and CCL2/MCP-1; while cardiac dysfunctions associate only to reduced CCL27/CTACK, and ergonomic dysfunction correlated with increased BDNF and reduced CCL11/Eotaxin and CCL5/RANTES. We propose that these 7 serum factors may provide a distinct signature for each different stress-related psychophysical outcome giving indications on individual vulnerabilities.

Our results highlighted a significant gender difference in the perceived job-stress. This is not surprisingly, since males and females are known to differently respond to stress, both acute and chronic (McEwen, 2012; McEwen et al., 2015). In particular, we found that women had higher scores for all the subscales of psychophysical strain. The finding that women are more susceptible to job-related psychosocial stress is in line with previous evidence showing a higher tendency for female to develop stress-related pathology such as anxiety and depression (Altemus et al., 2014). This condition is potentially due to an estrogen-driven delayed shut off of the HPA axis (Goel et al., 2014).

The Qu-Bo Test is a self-report instrument aimed at determining perceived characteristics of the work environment (i.e., job stressors) that may elicit a stress response in the individual. Similarly, the Qu-Bo Test assesses perceived psychological and physical symptoms due to work-related stress (i.e., psychophysical strain). Other instruments, such as the V.I.S. method (see for example Falco et al., 2013b), investigate observer ratings (e.g., supervisor's rating) of job stressors, as well as physician's rating of psychophysical strain. Accordingly, the aim of this study was to investigate the association between “perceived” psychophysical strain and serum levels of possible biomarkers of work-related stress. Therefore, it is possible that female workers face higher levels of job stressors (e.g., work-family conflict) and, consequently, they experience higher levels of psychophysical strain, as we observed in this study.

Concerning BMI, we observed a negative correlation with psychophysical strain, significant for anxiety, gastric problems and overall strain, therefore, all subsequent analysis for associations with biochemical variables were conducted controlling for both gender and BMI.

After controlling for BMI and gender, we found that higher scores of each psychophysical strain subscale were associated with lower serum concentration of some member of the chemokine family, in particular

CCL2/MCP-1, CCL5/RANTES, CCL27/CTACK and CCL11/Eotaxin. Since these chemokines are involved in immune cells migration and communication, our findings support the hypothesis that chronic stress induces suppression of both cellular and humoral response (Segerstrom and Miller, 2004). Interestingly, a previous study on German industrial workers showed that higher scores of effort-reward imbalance (Siegrist et al., 2004) predict lower bone-marrow derived progenitor cells (Fischer et al., 2009). Other authors suggested an increased pro-inflammatory state in job-related chronic stress (Asberg et al., 2009; Bellingrath et al., 2010). As an example, healthy school teachers with high effort–reward-imbalance and over-commitment show increased production of IL-6 and TNF-α cytokines, which are mainly classified as pro-inflammatory (Bellingrath et al., 2010). Significantly, they also found in agreement with our results, dampened innate immune defense in term of reduction in natural-killer (NK) cells, which are important players of the humoral immunity (Bellingrath et al., 2010). Chemokine C-C motif ligand 2 (CCL2/MCP-1), one of the best characterized chemokines, is able to attract monocytes and basophils but not neutrophils or eosinophils. CCL2/MCP-1 has been categorized mainly as pro-inflammatory and implicated in different stress-related pathologies characterized by monocytic infiltrates, like psoriasis, rheumatoid arthritis or atherosclerosis (Amasyali et al., 2009). However, it augments monocyte anti-tumor activity and can exert also anti-inflammatory effects (Deshmane et al., 2009). Our observation of reduced circulating CCL2/MCP-1 associated to anxiety and gastric problems, is apparently in contrast with a previous study on psychosocial stress in women reporting increased levels of CCL2/MCP-1, vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF) in stressed workers (Asberg et al., 2009). However, the study included a heterogeneous group of long-term sick-leave subjects diagnosed for any affective or stress-related mental disorder (depression, anxiety disorder, stress disorder, burnout syndrome, exhaustion disorder). Of note, CCL2/MCP-1, as well as other chemokines, can be produced centrally by microglia and astrocytes, and also neurons, and plays important roles in both physiological and pathological brain conditions. CCL2/MCP-1 can compromise the blood-brain-barrier integrity (Yao and Tsirka, 2014), but it also has neuroprotective effects (Chiu et al., 2010).

We observed negative associations between CCL27/CTACK and the scores of anxiety, gastric and cardiac problems as well as overall strain dimension. Chemokine C-C motif ligand 27 (CCL27/CTACK) is a chemotactic factor mediating the homing of lymphocytes to cutaneous sites. Interestingly, high anxious mice showed lowered CCL27/CTACK, associated with an increased skin cancer burden (Dhabhar et al., 2012). We also found negative associations between the scores of anxiety and ergonomic problems at the workplace and the chemokines CCL5/RANTES and CCL11/Eotaxin. Lower chemokine levels, including CCL2/MCP-1 and CCL11/Eotaxin, were found in cerebrospinal fluid (CSF) of suicide attempters respect to healthy controls (Janelidze et al., 2013), and increased CCL5/RANTES was found in schizophrenic patients but not in major-depressive subjects (Domenici et al., 2010). Both CCL27/CTACK and CCL5/RANTES are considered mainly pro-inflammatory chemokines and are involved in the recruitment and function of eosinophils. However, at the moment, the literature concerning job-related stress is not sufficient to support or disprove our findings.

Concerning the correlation between interleukins and psychophysical strain, previous studies showed that chronic stress is associated with increased circulating levels of pro-inflammatory cytokines IL1b, IL-6, INF-γ and TNF-α (Bellingrath et al., 2010; Hansel et al., 2010; Hodes et al., 2016) but studies in work-related stress have reported conflicting results. For example, Asberg and colleagues found no association between interleukins, INF-γ, TNF-α and psychosocial stress in women (Asberg et al., 2009). Here, we found a partial negative correlation between IL-6 and anxiety scores. IL-6 acts locally to promote migration of leukocytes from the blood stream to the site of infection, but also centrally, promoting fever, appetite suppression and stimulation of HPA axis. IL-6 has a recognized activity of both promoting and inhibiting inflammation (Scheller et al., 2011). A study on a large adult cohort found that persons with a current anxiety disorder, in particular women suffering of social phobia, had lower levels of IL-6 (Vogelzangs et al., 2013). Interestingly, although IL-6 is predominantly classified as a pro-inflammatory cytokine, it can also trigger anti-inflammatory properties through the so-called 'classical IL-6 signaling' occurring on a subset of T cells, hepatocytes, megakaryocytes, neutrophils and monocytes through activation of the IL-6 receptor and the gp130 signal transducer (Hodes et al., 2016). Thus, a reduction in classical IL-6 signaling may be involved in reduced immune response, in agreement with our findings for chemokines.

Intriguingly, we found increased interleukin 17 (IL-17) in subjects reporting gastric problems. IL-17 is a feature cytokine of the T-helper 17, which are important players in the Th1/Th2 balance regulation and have a role in autoimmunity disease. IL-17 is associated with tissue damage in brain, joints, heart, lungs and intestines in experimental models (Steinman, 2007). Alterations in Th17 function have been suggested also for depressive disorders (Beurel et al., 2013; Schmidt et al., 2010). Interestingly, in a model of chronic psychosocial stress, stressed mice developed spontaneous colitis and gut inflammation and the effects were mediated by Th17 activity and increased IL-17 levels (Schmidt et al., 2010).

In our previous study (Girardi et al., 2015) we reported that IL-12, a pro-inflammatory cytokine implicated in cellular immunity, was positively associated with interpersonal conflict at work (ICW) while we did not find here a significant association with psychosocial stress. Other studies have previously found opposite results for IL-12 in rats, leaving unresolved the issue of regulation of this interleukin in stress. According to transactional models of stress, the relationship between stressors and chronic mental and physical strain is still mediated by coping (Deary et al., 1996), so this leaves room for possible different results between studies. Of note, both this and our previous study are cross-sectional, so it is difficult to know whether the stressor comes before the strain or *vice versa* and which are the corresponding levels of the different serum factors analyzed. Although, the results of IL-17 obtained here are quite in line with those of Girardi and colleagues, the dimensions investigated in the two studies are quite different, which leaves room for differences in results obtained.

In this study, we observed a positive association between serum BDNF and the score of ergonomic problems at workplace. A previous study on Japanese subjects with job-related stress pointed out a reduction trend in circulating BDNF (Mitoma et al., 2008). Differently, another study found that chronically perceived stress increases serum BDNF levels (Feder et al., 2009). Of note, BDNF levels are highly dynamic in response to stress and also vary across different brain regions (Gray et al., 2013); for example, during chronic stress, increased BDNF levels have been found in amygdala while reduction has been described in prefrontal cortex and hippocampus (Davidson and McEwen, 2012). Our findings are in agreement with a previous study showing that patients with chronic musculoskeletal pain associated with Myofascial Pain Syndrome or Fibromyalgia presented increased serum BDNF levels (Caumo et al., 2016). Intriguingly, in these patients, increased serum BDNF levels correlated with higher motor cortex excitability, possibly leading to a disinhibition in the activity of the motor cortex and the descending pain inhibitory system (Caumo et al., 2016). Our results represent a first indication that a similar mechanism may operate also in chronic job-related stress.

We ought to underline some intrinsic limitations of this study such as: 1) the study design involved only one single time point, thus the results may suffer for time course variations. 2) Scores of work-related stress resulted to be positive skewed, meaning our study population have mild to low chronic stress; therefore results need to be replicated in a more stressed population. 3) The sample size was relatively small compared to the number of serum factors considered, thus increasing the possibility of over-fitting problems. 4) We did not control for other primary mediators such as metabolic indicators (blood pressure,

cholesterol) or urinary excretion of cortisol, adrenaline and noradrenaline, thus limiting the possibility to identify an allostatic load index. 5) Lastly, we did not check for historical and socio-economical statuses which are confounders that potentially influence the response to stress; however, our population was relatively homogenous in that sense, since was extracted from the same environment. In addition, immune alterations are hard to predict given the enhancing versus suppressive effects of stress on immune function (Dhabhar, 2014). Replications with larger samples, possibly on a longitudinal study analyzing the effect of interventions to reduce stressing situations at work may resolve these limitations. Nevertheless, we believe our study adds some valuable insight in immune mediator changes occurring in conditions of work-related chronic stress.

4.1. Conclusions

The seven serum factors CCL27/CTACK, CCL11/Eotaxin, CCL2/MCP-1, CCL5/RANTES, IL-17, IL-6 and BDNF that we found associated in a specific combination to the different psychophysical outcomes in work–related chronic stress, are new potential objective indicators to be coupled with the subjective assessments of psychophysical strain. As evidenced in this discussion, these seven factors are not part of a single stress-related mechanism, but are rather associated to different psychological and physical outcomes of stress, and therefore are likely to reflect different pathological mechanisms. Our results suggest reduced immune defence as a potential hallmark of mild job-related stress. Moreover, given that the pathological response to chronic stressful conditions is largely linked to individual characteristics, the possibility to have a distinct signature for the different stress-related psychophysical outcomes may provide a valuable tool to identify individual vulnerabilities in different body districts and prevent the development of more severe diseases.

Author contribution

E.T., N.A.D.C. and A.F. designed the experiments. A.F. and D.G. performed psychophysical assessment. M.C. and N.Z. conducted biochemical measurements. A.P. and D.G. analyzed data and performed statistical analysis. A.P. produced tables and figures. A.P. and E.T. wrote the manuscript. D.G., A.F., N.A.D.C. and M.C. revised the text.

Funding source

The study was founded by Fondazione Cassa di Risparmio di Trieste (E.T.).

Conflict of interest

None.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ynstr.2018.02.002.

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