UNIVERSITÀ DEGLI STUDI DI TRIESTE

XXVII CICLO DEL DOTTORATO DI RICERCA IN NEUROSCIENZE E SCIENZE COGNITIVE

- INDIRIZZO NEUROBIOLOGIA -

"NEUROTROPHIC FACTORS AND OTHER HUMORAL MEDIATORS IN CHRONIC STRESS"

Settore scientifico-disciplinare: Scienze Biologiche
BIO/06 ANATOMIA COMPARATA E CITOLOGIA

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Ad I.,
perchè "sono cambiamenti,
solo se spaventano".

Di Domenica, Subsonica.

ABSTRACT

Stress was defined as "a specific response of the body to any non-specific demand (stressor) made on it". When the real or perceived stressor is lasting or the response is inappropriate (distress), the physiological short-term changes switch to long-term alterations driving to pathological conditions, including mental illness. According to this "allostatic load" model, primary mediators such as catecholamines and steroid hormones trigger multi-systemic alterations of secondary signaling mediators possibly causing adverse manifestation in different body systems, including nervous and immune ones. Neurotrophins, in particular the Brain-Derived Neurotrophic Factor (BDNF), and immune mediators, like cytokines and chemokines, are possible modulators of the chronic stress response. Therefore, we aimed to assess their circulating levels in healthy persons in conditions of work-related stress. Before that, since measurement of peripheral BDNF is of great interest for psychiatrists but suffers for inconsistencies, possibly due to the lack of consistent procedures for BDNF samples collection and assessment, we aimed to overcome these limitations and defined a standardized methodology for both sample collection and BDNF measurement. Hence, we have selected the serum as the elective body fluid for BDNF quantification and, using sera from 40 healthy adult subjects, we compared the performance of six commercial BDNF ELISA kits. All kits showed 100% of sample recovery and, with one exception, comparable range. However, they exhibited very different inter-assay variations from 5% to 38%. Inter-assay variations, were higher than those declared by the manufacturers with only one exception which also had the best overall performance. Dot-blot analysis revealed that two kits selectively recognize mature BDNF, while the others reacted with both pro-BDNF and mature BDNF. Of note, having defined a standardized procedure which reduces the high variability due to technical, pre-analytical and analytical steps, this study provides the basis to obtain an accurate measure BDNF in human serum, suitable for future clinical applications.

As mentioned, the next step was to assess circulating BDNF and immune mediators in subjects with job-related stress. We therefore measured BDNF and, using the multiplex ELISA technology, other 48 analytes among cytokines, chemokines and growth factors in the sera of 122 healthy subjects (87 female and 35 male). The participants were enrolled among healthcare assistants and evaluated for work-related stress using psychophysical stress and burnout scales extracted from a self-assessed questionnaire standardized for the Italian context (Qu-BO test). Psychophysical stress addressed five items, namely anxiety, emotion (depression-like), gastrointestinal disturbances, cardiac disturbances, ergonomic dysfunction at the workplace and an overall stress state as the average of the five items; burnout state has been measured using three cores adapted from the Maslach Burnout Inventory, specifically emotional exhaustion, depersonalization and personal accomplishment. Higher scores mean higher stress status, except for personal accomplishment. We found that female had higher scores than male for all psychophysical stress items and for the emotional exhaustion core. Then, after correcting for gender and body-mass index (BMI), we attempted to correlate the biological variables with the score scales, using a combination of univariate (partial correlations) and multivariate analysis (partial least square and factor analysis) which took in account correlations among

analytes. As a result, we found no associations between burnout state and biological variables while, on the other hand, we detected negative associations between psychophysical stress items and some markers of the chemokine profile. In particular, we observed that levels of MCP-1/CCL2, CTACK/CCL27, RANTES/CCL5 and Eotaxin/CCL11 negatively correlate, with different grade, with the scores of anxiety, gastric and cardiac problems and ergonomic dysfunction at the workplace. In addition, we observed IL-17, a feature cytokine of the T-helper subtype 17 (Th17) which have been associated to tissue damage also at intestine level, to be positively associated with score in subjects reporting gastric problems ($r=0.280$, $p=0.010$). Intriguingly, we detected also a positive relationship between BDNF and score of ergonomic dysfunction at the workplace ($r=0.284$, $p=0.009$), potentially as a result of compensatory protective mechanisms. Taken together, our results support the hypothesis that chronic stress induces suppression of both cellular and humoral response. However, the consequences are hard to predict since stress is known to have both suppressive and enhancing effects on immune system.
To further explore the potential protective role of BDNF in chronic stress conditions, we moved to an *in-vitro* cellular model of cytotoxic stress, consisting in SK-N-BE human neuroblastoma cell line treated with cisplatin, a chemotherapeutic drug. We found that cisplatin, in particular after 24 of treatment, enhances BDNF production at both transcriptional and protein levels. Of note, it induces also an increment in the transduction rate of BDNF mRNA transcripts. We hypothesized that BDNF translation induction occurs through the activity of Aurora kinase, like what happen for α-Ca^{2+}/calmodulin-dependent protein kinase II (αCaMKII) mRNA, as it is a conserved mechanism from *Xenopus* oocytes to synapses of hippocampal neurons. Therefore, by blocking Aurora kinase activity thanks to the potent inhibitor PHA-680632, we were expecting to detect a reduction in BDNF translation and increased cell mortality as a result of the decreased trophic support. Surprisingly, despite an improved cell death only in combination with cisplatin, we observed an enhanced BDNF translation induction especially from those transcripts containing exon 6, a splice variant of the 5’ untranslated region, which have been already observed to be crucial in cytotoxic stress conditions. In conclusion, our results pointed toward a BDNF translation induction mechanism that is Aurora kinase independent, at least in conditions of cytotoxic stress. Although not investigated, some of these mechanisms may include 5’cap-independent recruitment of the translational machinery. Furthermore, our observations could have important clinical implications in the use of Aurora inhibitors as adjuvant in neuroblastoma treatment as, even if with a cell mortality increase, they facilitate the selection of resistant cells that further enhance BDNF production.

RIASSUNTO

Lo stress è stato definito come "una risposta specifica del corpo a qualsiasi richiesta non specifica (stressor) su di esso". Quando lo stress, reale o percepito, è duraturo o la risposta non è appropriata (distress), i cambiamenti fisiologici di breve termine lasciano spazio ad alterazioni di lungo termine che possono causare condizioni patologiche, tra cui malattie mentali. Secondo questo modello di "carico allostatico", i mediatori primari come le catecolamine e gli ormoni steroidei, innescano alterazioni multi-sistemiche su mediatori secondari che portano a manifestazioni negative in diversi sistemi corporei, inclusi quello nervoso e immunitario. Neurotrofine, in particolare il Brain-Derived Neurotrophic Factor (BDNF), e mediatori del sistema immunitario, come citochine e chemochine, sono possibili modulatori della risposta allo stress cronico. Pertanto, ci siamo posti l'obiettivo di valutare i loro livelli circolanti in soggetti sani in condizioni di stress lavoro-correlato. Prima di ciò, poiché la misurazione del BDNF circolante è di grande interesse per la sfera psichiatrica, ma soffre per inconsistenze probabilmente a causa della mancanza di procedure standard di selezione e valutazione, abbiamo cercato di superare queste limitazioni definendo una metodologia standardizzata sia per la raccolta dei campioni che per la misurazione di BDNF. Per fare ciò, abbiamo selezioniamo il siero come fluido elettivo per la quantificazione di BDNF e, utilizzando sieri di 40 soggetti adulti sani, abbiamo confrontato le prestazioni di sei kit di ELISA commerciali specifici per BDNF. Tutti i kit hanno mostrato la capacità di misurare il BDNF nel 100% dei campioni e, con una sola eccezione, range paragonabili. Tuttavia, essi hanno mostrato variazioni inter-assay molto diverse, dal 5% al 38%. Tali variazioni, erano superiori a quelle dichiarate dai produttori fatta eccezione per un kit, che nel complesso ha mostrato le prestazioni migliori. In aggiunta, l'analisi Dot-blot ha rivelato che due kit hanno riconosciuto selettivamente la forma matura di BDNF, mentre gli altri hanno reagito anche con il suo precursore, il pro-BDNF. Occorre notare che, avendo definito una procedura standardizzata che riduce l'elevata variabilità dovuta ai differenti passaggi tecnici nelle fasi pre-analitiche e analitiche, questo studio fornisce la base per ottenere una misura affidabile del BDNF nel siero umano, adatto per potenziali applicazioni cliniche future. Come accennato, il passo successivo è stato quello di valutare il BDNF e i mediatori immunitari circolanti in soggetti con stress lavoro correlato. Abbiamo quindi misurato il BDNF e, utilizzando la tecnologia ELISA multiplex, altri 48 analiti tra citochine, chemochine e fattori di crescita nel siero di 122 soggetti sani (87 femmine e 35 maschi). I partecipanti sono stati arruolati tra assistenti sanitari e valutati per lo stress lavoro-correlato con scale stress psicofisico e burnout estratte da un questionario di auto-valutazione standardizzato per il contesto italiano (test Qu-BO). Lo stress psicofisico è stato valutato con cinque elementi, vale a dire ansia, disturbi emotivi, gastrointestinali, cardiaci e disfunzioni ergonomiche sul posto di lavoro più uno stato generale di stress stimato come la media delle cinque dimensioni; il burnout è stato misurato con tre elementi adattati dal Maslach Burnout Inventory, nello specifico esaurimento emotivo, depersonalizzazione e realizzazione personale. Alti punteggi di score sono associati a situazioni di stress cronico elevato, ad eccezione della scala di realizzazione personale. Abbiamo individuato che le donne avevano punteggi più elevati rispetto agli uomini per tutti gli elementi di stress psicofisico e per lo stato di esaurimento emotivo. Oltre a ciò, dopo aver corretto per sesso e indice di massa corporea (IMC), abbiamo cercato di

correlare le variabili biologiche con i punteggi delle diverse scale di stress, utilizzando una combinazione di analisi univariate (correlazioni parziali) e multivariate (analisi dei minimi quadrati e fattoriale), per tener conto delle correlazioni tra analiti. Come risultato, non abbiamo trovato alcuna associazione tra le variabili biologiche e lo stato di burnout, mentre, d'altra parte, abbiamo rilevato associazioni negative tra elementi dello stress psicofisico e alcuni marcatori del pattern di chemochine. In particolare, abbiamo osservato che i livelli di MCP-1/CCL2, CTACK/CCL27, RANTES/CCL5 e Eotassina/CCL11 correlano negativamente con diverse scale di stress psicofisico quali ansia, problemi gastrici e cardiaci e disfunzioni ergonomiche sul posto di lavoro. Inoltre, abbiamo osservato che IL-17, una citochina caratteristica del sottotipo T-helper 17 (Th17) e associata a danni a diversi tessuti, incluso quello intestinale, correla positivamente con alti punteggi in soggetti che accusano problemi gastrici (r=0.280, p=0,010). Curiosamente, abbiamo rilevato anche una relazione positiva tra BDNF e il punteggio di disfunzione ergonomica sul posto di lavoro (r=0.284, p=0,009), potenzialmente a causa di meccanismi di compensazione protettivi. Nel loro insieme, i nostri risultati supportano l'ipotesi che lo stress cronico induce una generale soppressione sia risposta immunitaria cellulare e umorale. Tuttavia, le conseguenze sono difficili da prevedere in quanto lo stress è noto per avere un effetto sia soppressivo che stimolante sul sistema immunitario.

Per esplorare ulteriormente il potenziale ruolo protettivo di BDNF in condizioni di stress cronico, a livello cellulare, ci siamo serviti di un modello di stress citotossico *in-vitro*, consistente in una linea di cellule di neuroblastoma umano, le SK-N-BE, trattata con cisplatino, un farmaco chemioterapico. Da ciò abbiamo osservato che il cisplatino, in particolare dopo 24 di trattamento, aumenta la produzione di BDNF sia a livello trascrizionale che proteico. In aggiunta, lo stimolo citotossico dato dal cisplatino aumenta anche il tasso di trasduzione BDNF. Abbiamo ipotizzato che l'induzione della traduzione di BDNF avvenga attraverso l'attività di Aurora chinasi, come avviene, ad esempio, per la chinasi II dipendente da α-Ca^{2+}/calmodulina (αCaMKII), in quanto si tratta di un meccanismo conservato a partire dagli ovociti di Xenopus fino a livello delle sinapsi neuronali di ippocampo. Pertanto, bloccando l'attività di Aurora chinasi grazie al potente inibitore PHA-680.632, ci aspettavamo di osservare una riduzione nella traduzione di BDNF e un aumento della mortalità cellulare come risultato del diminuito supporto trofico. Sorprendentemente, nonostante un aumento della morte cellulare, ma solo in combinazione con cisplatino, abbiamo osservato una maggiore induzione della traduzione di BDNF, soprattutto da quei trascritti contenenti in particolare l'esone 6 di BDNF (una variante di splicinge della porzione 5' non tradotta), il cui ruolo è già stato osservato essere cruciale in condizioni di stress citotossico. In conclusione, i nostri risultati delineano un meccanismo di induzione della traduzione degli mRNA di BDNF potenzialmente indipendente da Aurora chinasi, almeno in condizioni di stress citotossico. Sebbene non dimostrato per BDNF, tali meccanismi potrebbero includere il reclutamento dell'unità trascrizionale con modalità indipendenti dalla presenza del cap al 5' non tradotto degli mRNA. Le suddette osservazioni potrebbero avere importanti implicazioni cliniche nell'uso di inibitori di Aurora come coadiuvanti nel trattamento del neuroblastoma in quanto, nonostante inducano un aumento della mortalità, facilitano la selezione di cellule resistenti che producono una maggiore quantità di BDNF.

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1. INTRODUCTION

1.1 History of stress

From the industrial revolution to nowadays, clinicians have reported an increasing decline in mental health. Since the 19th century, the scientific community argued about three possible major causes: heredity, interior or environmental toxins and, as it was called later, *stress*. The underlying features crossing those centuries are the major and fast changes within the society, as the increased information exchange, spinning market economy and the growing demands over the urban individual. This accelerated exchange-rate potentially brings up the self-feeling of being inadequate or unable to keep up mentally and physically, in line with the challenging environment. A great number of symptoms have been described, as well as many diagnoses have been formulated, to justify the discomfort in a culture filled of competition, achievement and seek of leadership. Lots of hypotheses have been proposed to link the effects of modern society on the susceptible person, spanning from evolution biology to sociology fields. Doctors, who attempt to translate individual's response to threats into medical practice, came out with different theories: from the overstrain or "fatigue problem", in which illness process was physiologically seen as a reduction of mental and body "energy", to "neurasthenia", which essentially bond the chronic fatigue to a reduced nerve energy, intended as a slowed down cerebral and body activity (Arnetz and Ekman 2006, chapter 1, pp. 3-12).

Despite the well known influence of psychological states on physical health, a concrete physiological investigation had to wait until the early 40s of the past century, thanks to the work of Hans Selye (1907-1982) and, later, from other contributors, including Mason, Sterling and Eyer, McEwen, Seeman and Wingfield (Rice 2012, chapter 2, pp. 28).

1.2 Stress definition and models

Selye was a pioneer in addressing the physiological changes occurring during the response to physical or perceived threats and essentially coined the term "*stress*" as we meant it today. At the beginning, he observed that patients suffering for different traumatic or chronic diseases frequently denote common signs or symptoms, like reduced appetite, with loss of weight and muscle strength. He marked this phenomenon as the "syndrome of just being sick". In parallel, he demonstrates that rodents subjected to different noxious stimuli like physical, chemical or emotional (extreme hot or cold, blurring lights, formalin, or perpetual frustration) all exhibited the same pathologic changes including adrenal gland hypertrophy, lymphoid tissue shrinkage and gastrointestinal ulcers. In the long term, these animals were also likely to develop some diseases seen in humans, including hearts attack, kidney problems and rheumatoid arthritis. These observations let him to define the *stress* as "a nonspecific response of the body to any demand made on it" (Selye 1936), and designate *stressor* as the stimulus causing the stress response (although the term *stress* often designates both the stimulus and the response). Following criticisms of being too ambiguous, he later refines the definition of *stress* as "a state manifested by a specific syndrome which consists of all the nonspecifically induced changes within the biological system" (Selye 1976, pp. 64). Therefore, this syndrome seems to reflect an extreme reaction of the body's defence to stressors. At a systemic level the entire stress process was defined as the General Adaptation Syndrome (GAS), including the threat and the individual's reaction to it. The Local Adaptation Syndrome (LAS) was instead referred to the regional response (e.g., localized inflammation where microbes have entered the body). Combining together all these observation and findings, the GAS and LAS are seen as closely coordinated, with the GAS acting as backup (Selye, 1976; Rice 2012, chapter 2, pp.24-25).

1.2.1 General Adaptation Syndrome (GAS)

About GAS, Selye wrote: general "because it was produced only by agents which have a general effect upon large portions of the body," adaptive "because it stimulated defence and, thereby, helped in the acquisition and maintenance of a state of inurement," and syndrome "because its individual manifestations are coordinated and, even partly, dependent upon one another" (Selye, 1976, pp. 38; Rice 2012, chapter 2, pp. 24).

The GAS model brought the airy conception of stress into a suitable manner for the scientific inquiry. This theory recognizes two main actors of the autonomic nervous system, the sympatho-adrenergic-noradrenergic (SAN) and the limbic-hypothalamo-pituitary-adrenal (L-HPA) systems (which will be discussed later); furthermore, it identifies three distinct time phases of the stress response (see figure 1.1):

- Alarm phase: initiate whenever there is a difference between a set state (or "wish state") and the actual state of a variable; is the first response when an insult exceeds the range of "normal" resistance or homeostasis. It corresponds to the *fight-or-flight* reaction described by Cannon (1932) (West, 2010) and is physiologically driven by a general activation of the autonomic nervous system, which causes increased heart rate and respiration, blood pressure, arousal and alertness.
- Resistant stage: is the phase where a full resistance to the stressor has been established and the alarm phase is diminished. Body systems and tissues adaptation occur in order to maintain a high level of functioning despite the presence of the stressor.
- Exhaustion phase: the resistant phase cannot be maintained indefinitely and, if the organism is unable to return to a normal resistance level (pre-alarm phase) or the

stressor is uncontainable, the exhaustion stage occurs. Human resources have been depleted and permanent damage of the body systems are likely to occur.

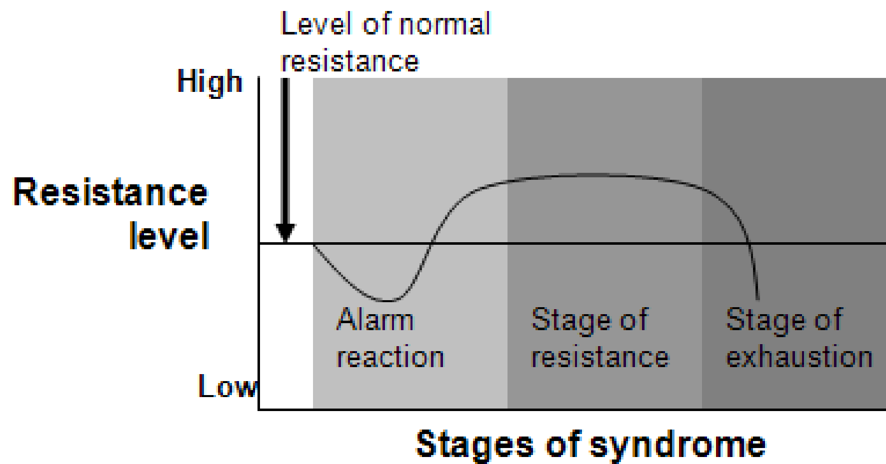


Figure 1.1 Phases of the stress response; from (Carlson, 2007).

The general opinion about stress is usually negative but the stress response is a physiological reaction that is essential for survival and has been maintained throughout the evolution. Stress cannot be avoided because is part of life, but there are both "good" and "bad" stressors. Even winning a lottery or a passionate kiss can evoke a stress response, but of course they are not the same as being stuck at work for an important meeting. Therefore, Selye attempted to discern between healthy stress, denoted as *eustress*, and pathogenic stress, specified as *distress* (Tache, J., & Selye, H. 1985).

1.2.2 Allostatic Load Model (AL)

One of the major criticisms to Selye's work, made by later contributors, is that GAS model does not contemplate perception, cognition and interpretation of the stressor stimulus. The work of Mason (Mason, 1971), Sterling and Eyer (1988), McEwen (McEwen, 1998a, 2000a) and McEwen and Wingfield (McEwen and Wingfield, 2003), overcome this lack

with the formulation of the allostasis and allostatic load theories (Rice 2012, chapter 2, pp. 28). Allostasis refers to the process whereby an organism maintains physiological stability by changing parameters of its internal milieu by matching them appropriately to environmental demands (Sterling and Eyer, 1988). It emphasizes the ability to maintain the equilibrium of the body's parameters over a dynamic range of set-points, rather than static as in homeostasis. Additionally, it reconsiders the central role of the brain in acting as a feedback and views the health as whole brain-body ability to adapt to the contexts (Figure 1.2).

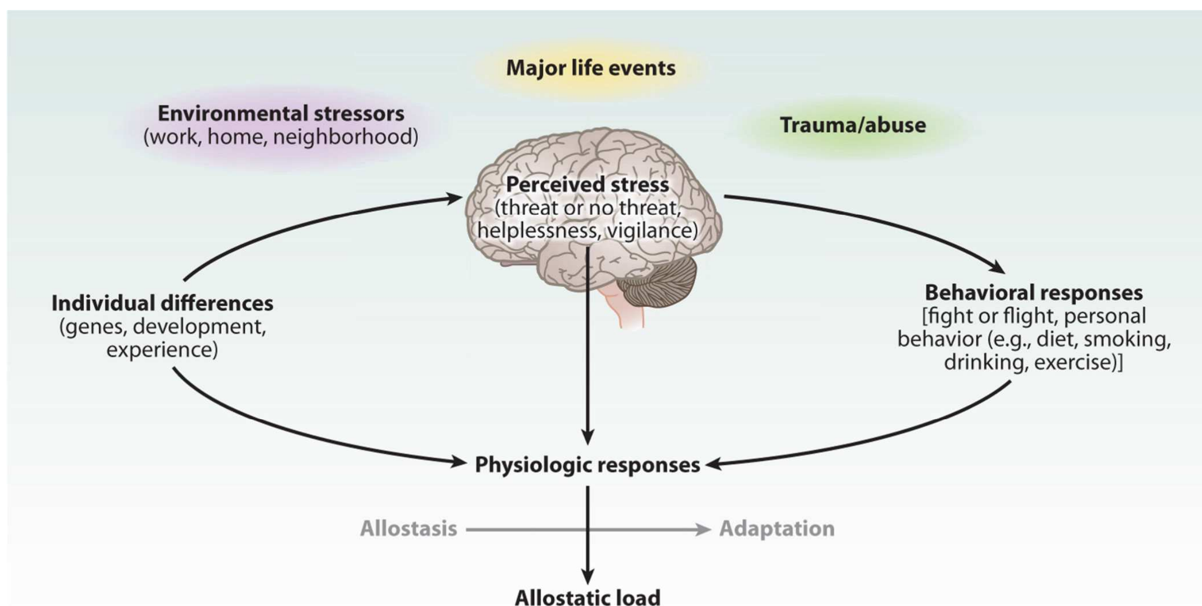


Figure 1.2 The allostic load model; from (McEwen, 1998a; McEwen and Gianaros, 2011).

In the short run, allostasis response is essential for survival and adaptation to adversities; it represents the basis of the *resilience* (Juster et al., 2010; Lupien et al., 2009; McEwen, 2013) of an individual, in analogy with the property of a material to elastically deform upon loading and get back to its original shape when unloaded. However, in the long term, when prolonged stressors chronically induce allostasis responses, it causes the body systems to overload, driving the so called *allostatic load*. This represents the "wear and tear" of the body, which can accelerate disease processes (McEwen and Stellar, 1993). Keeping

parallel with physics, it resembles the break up point of a material. Likewise, as different materials have different resilience properties and break up points, allostasis and allostatic load mechanisms are different for each individual. Indeed diverse constitutional (genetics, development, experience), behavioral (coping and health habits), and historical (trauma/abuse, major life events, stressful environments) factors could influence these processes (McEwen, 1998a, b). Is worthy of note that threat or stressors can be either real or apparent and is their perception and interpretation that triggers allostatic responses. Discernment of what is, or is perceived, stressful and what is not is entirely personal and largely depend on the above factors as well. Despite the heterogeneity in stressors perception and factors that influence allostasis mechanisms, allostatic load model identifies general features in the alteration processes driven by chronic stress (see figure 1.3) (Juster et al., 2010; McEwen, 1998a, 2000a; McEwen and Seeman, 1999):

- Primary mediators: as already seen in GAS model, the first biological systems activated in stress response are the sympathetic–adrenal–medullary (SAM) and the hypothalamic–pituitary–adrenal (HPA) axis. The release of catecholamines and glucocorticoids, respectively, mobilizes energy necessary for fight-or-flight reaction (Sapolsky et al., 2000). While adaptive in acute responses, prolonged secretion of stress hormones and their antagonist, along with other humoral mediators, fails to protect the distressed person and starts to injure its brain and body.
- Secondary outcomes: primary mediators exert direct compensation at cellular levels and, in turns, in different biological systems, causing a shift in their operative ranges in order to maintain functional stability. All these effects compromise the integrity of allostatic mechanisms and cause metabolic, cardiovascular, neuroendocrine and immune parameters to reach sub-clinical levels.

- Tertiary outcomes: the deregulations got in secondary outcomes potentially culminate with an *allostatic overload*, with disorders or disease or even fatal endpoints.

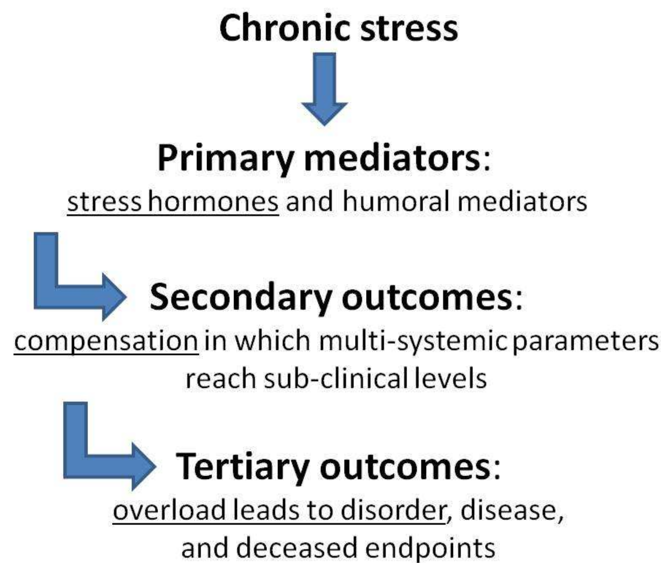


Figure 1.3 Alteraration processes driven by chronic stress, according to the allostatic load model.

An essential aspect of the AL model is that multiple mediators involved in allostasis and allostatic overload mechanisms are interconnected in a complex and non-linear network (Figure 1.4); thereby, the compensation processes over time and, secondly, the steps in the deregulation route happen in a non-linear fashion, so are particularly hard to predict (Juster et al., 2010; McEwen and Gianaros, 2011). Nonetheless, the AL model proposes that study multi-systemic interaction, along with the identification of sub-clinically relevant biomarkers, triangulated with other measurement (genetic, psychological, social assessments, etc.), could help in detect individuals at high risk of tertiary outcomes (McEwen, 2000a).

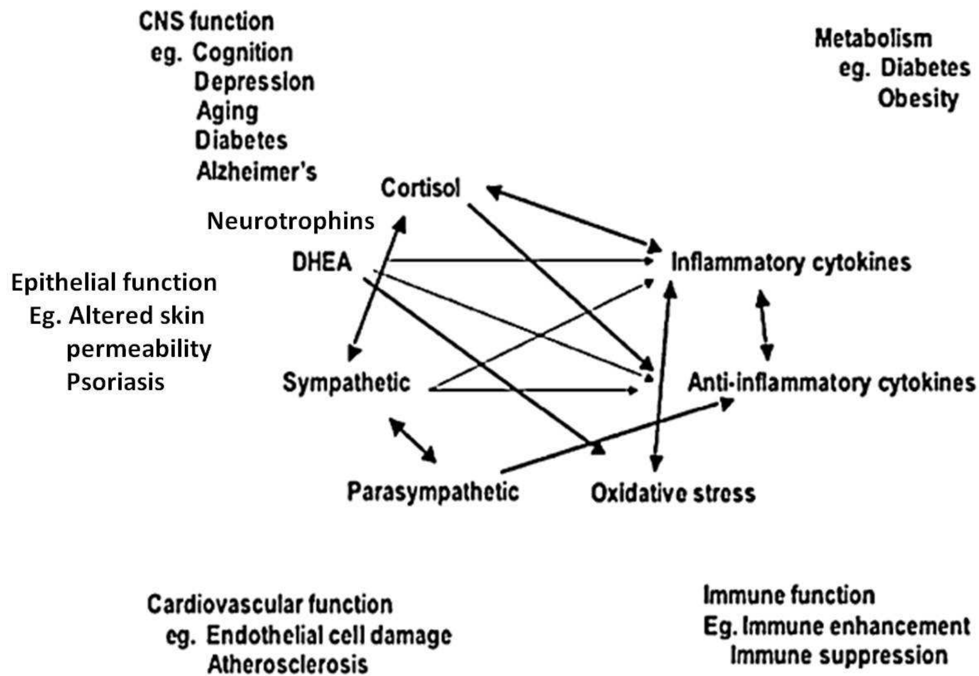


Figure 1.4 Non-linear network of mediators of allostasis involved in the stress response; adapted from (McEwen, 2006).

Numerous studies have been conducted incorporating the AL algorithm in attempting to describe the effects of chronic stress from different life aspects: post traumatic stress disorders (Glover, 2006); stress in neonatal intensive care unit parents (Mackley et al., 2010); recovery from colorectal surgery (Slater, 2010); open heart surgery experiences for patients and their caregivers (Robley et al., 2010); mother of cancer patients (Glover et al., 2008); successful aging (Gruenewald et al., 2006; Karlamangla et al., 2006; Seeman et al., 2004; Seeman et al., 1997b); violence and women's health (Symes et al., 2010).

Also the job stress in relation to allostatic load has been investigated: German school teachers and industrial workers (Bellingrath et al., 2009; Fischer et al., 2009; Schnorpfeil et al., 2003); Dutch telecom managers (Langelaan et al., 2007); Chinese industrial workers (Li et al., 2007, Sun et al., 2007); Swedish public health care workers (von Thiele et al., 2006).

Chronic stress typically account for how adverse work environments contribute to disease trajectories (Taylor et al., 1997). The job-related chronic stress is of increasing interest in both public opinion and scientific community. It has started to being monitored and formally regulated in different American (Occupational Safety & Health Administration directives from U.S. Department of Labor) end European countries, including Italy, as part of a general health framework.

1.3 Job-related stress management: European and Italian policies.

The consequences of the job-related chronic stress have drawn significant attention since the beginning of this century. Almost 22% of the European workers experience job stress (Milczarek et al., 2009), which is the cause of nearly 60% of work absences. Due to health problems, a European worker stays off approximately four and half days per year (Parent-Thirion et al., 2007), with women and dependent workers as the most affected categories. Overall, in 2002, job stress had cost to Europe roughly 20 million of Euros (Milczarek et al., 2009). Also in Italy, a study over the Veneto country in 2005, pointed out that 27% of workers suffers for stress response related symptoms (Mastrangelo et al., 2008).

Many recommendations have been proposed to promote the monitoring of job stress related warning signs. The European Union is committed to promoting the health and safety of workers, so has recognized the work-related stress as an emerging occupational health risk which deserves identification, prevention and management like other work-related risks, in accordance to the principles and methods which underpin EU occupational health and safety policy. In this view, on date 8 October 2004 was signed the *Framework Agreement On Work-Related Stress* (Brussels, 24/02/2011) whose aim are twofold: 1) increase the awareness and understanding of employers, workers and their representatives concerning work-related stress; 2) focalize their attention to

symptoms/signs that could reflect problems due to work-related stress. As reported in the agreement, stress is recognized as a state, accompanied by dysfunction (physical, psychological or social), which results "from individuals feeling unable to bridge a gap with the requirements or expectations placed on them". A subject can cope well with short-term stress, but has greater difficulty to deal with prolonged exposure. It also highlight the fact that different subject cope differently with similar situations; in addition, different reaction to similar situations, at different times in life, can be observed within the same individual. While not necessarily spread over all workplaces, the stress can potentially affect any worker, irrespectively of size of the company, the field of activity and the contract form. As a result, the agreement underlie that "tackling stress at work can lead to greater efficiency and improved occupational health and safety".

In concrete terms, the agreement points out three important elements: 1) identify problems of work related stress; 2) take action to prevent, eliminate or reduce these problems; 3) both employers and workers have responsibilities on this topic. This last point, in particular, fits in the framework directive 89/391 in which "all employers have a legal obligation to protect the occupational safety and health of workers and this responsibility also applies to problems of work-related stress"; on the other hand, "all workers have a general duty to comply with protective measures determined by the employer".

In Italy, the text of the European agreement was implemented, in an almost literal translation, during the revision of the occupational health and safety legislation and included in the Article 28, paragraph 1 of the Legislative Decree of 9/04/2008 n. 81 (Ordinary supplement to n. 108/L of Gazzetta Ufficiale n.101 of 30/04/2008). The text provides indication on how to identifies job-stress risk factors: 1- negative spot events (absenteeism, high turnover, interpersonal conflicts); 2- problems in organization and processes (working time arrangements, degree of autonomy, match between skills and job

requirements, workload); 3- working conditions and environment (exposure to abusive behaviour, noise, heat, dangerous substances, etc.); 4- communication (uncertainty about what is expected at work, employment prospects, or forthcoming change, etc.); 5- subjective factors (emotional and social pressures, feeling unable to cope, perceived lack of support, etc.). All the above indications have been adopted in March 2010 in an operational guide by the National Institute for Occupational Safety and Prevention (ISPESL). In addition to preliminary evaluation (points 1 to 4), the guidelines imply adequate corrective actions and, eventually, a deep evaluation on subjective perception of the risk factors (point 5). The proposed methodology emphasizes the necessity to adopt both subjective and objective techniques to evaluate workers for job-related stress, during the risk assessment process, since integrating as many information as possible would be helpful in the proper management of the stress. A detailed discussion of the possible self-assessed or objective indicators is beyond the scope of the present work, although in later paragraphs will be discussed potential applications of physiological measurements as part of the objective evaluation of work-related chronic stress. At first, however, there will be an overview of the physiology of the stress response, in both allostasis and allostatic load mechanisms.

1.4 Physiology of the stress response

As identified in both General Adaptation Syndrome (GAS) and Allostatic Load (AL) models, the main actors of response to stress are the sympatho-adrenergic-medullary (SAM) and the limbic-hypothalamo-pituitary-adrenal (L-HPA) systems. These systems are interconnected and influence each other activity (Chrousos et al., 1988). Stress biology also includes different brain areas involved in implementation of the appropriate adaptive response. In brief, sensory inputs, triggered by physical or perceived sensations,

communicate information to their specific thalamus area, which act as a relay station and transmit them to sensory cortex and amygdala (Amiragova, 1985; Pezzone et al., 1992). The sensory cortex in turn communicates either directly or via the hippocampus with the lateral amygdala through the perirhinal cortex (Davis and Murphey, 1994; Davis et al., 1994; LeDoux, 1995). Amygdala is a complex structure, made of several nuclei, which receives and integrates signals from cortices, thalamus, locus ceruleus and hippocampus and then retransmit over different brain areas as well. This entire signalling network converges in activating the sympathetic division of the autonomics nervous system (SAS) and in stimulating the paraventricular nucleus of the hypothalamus (PVN) (Petrov et al., 1994; Ziegler and Herman, 2002). SAS activity, through the action of catecholamines (norepinephrine and epinephrine), is responsible for the cardiovascular effects: increased heart rate and force of contraction, dilated blood vessel in skeletal muscles while constricted in gastrointestinal organs and dilated bronchioles, along with other effects over various organs (increased renin secretion, sweat secretion, peristalsis inhibition) (Dunser and Hasibeder, 2009). The PVN is an essential structure because receives various inputs indicative of stress and modulate the activity of the hypothalamus-pituitary-adrenal (HPA) axis (Swanson and Sawchenko, 1983). The HPA response starts from the production of the corticotropin releasing hormone (CRH), by PVN neurons, which is then released into the hypophyseal portal blood and reaches the anterior pituitary gland. CRH then regulate the transcription of the proopiomelanocortin (POMC) gene, a common precursor for the adrenocorticotropin hormone (ACTH), and promotes the release of ACTH in the bloodstream. ACTH, in turns, induces the synthesis and secretion into systemic circulation of glucocorticoids (mainly cortisol in humans, corticosterone in rodents), by the *zona fasciculata* layer of the adrenal cortex (Charmandari et al., 2005; Smith and Vale, 2006).

Glucocorticoids are the final effectors of the HPA axis. Their effects are mediated by two intracellular receptor types: the mineral corticoid receptor (MR) and the glucocorticoid receptor type (GR), which act as ligand-dependent-transcription factors and are distributed throughout the body systems. GRs have lower affinity for corticosterone than MRs but have a wider distribution in different brain areas, including pre-frontal cortex and PVN; MRs are less abundant, but are present especially in some brain structures like the hippocampus. Due to different affinity, GRs are predominantly available for binding during high level of circulating cortisol, so during stressful situations (Finsterwald and Alberini, 2014; Reul et al., 1987). The primary effect of glucocorticoids is to promote conversion of protein and lipid in usable carbohydrates (McEwen, 2000a) and act on the brain to increase appetite and locomotor activity in a food seeking oriented manner (Leibowitz and Hoebel, 1997, chapter 15, pp.313-358); overall, they regulate behaviours which control energy input and expenditure. Of course, glucocorticoids also play a key regulatory role, by switching off the stress response at the end of the stimulus, thus exerting negative feedback at different sites (especially pituitary, hypothalamic and some supra-hypothalamic structures) to inhibit their own release (Figure 1.5).

The primary stress mediators, glucocorticoids and catecholamines, are protective of the individual, in the short run, as they are essential for the reaction to dangerous or life threatening situations (Wamsteeker Cusulin et al., 2013). However during chronic stress situations, repeated activation or altered negative feedback turns them to wield damaging effects (Hyman, 2009; Mizoguchi et al., 2003; van der Laan et al., 2009; Varghese and Brown, 2001). It is a sort of cost that body pays for alertness.

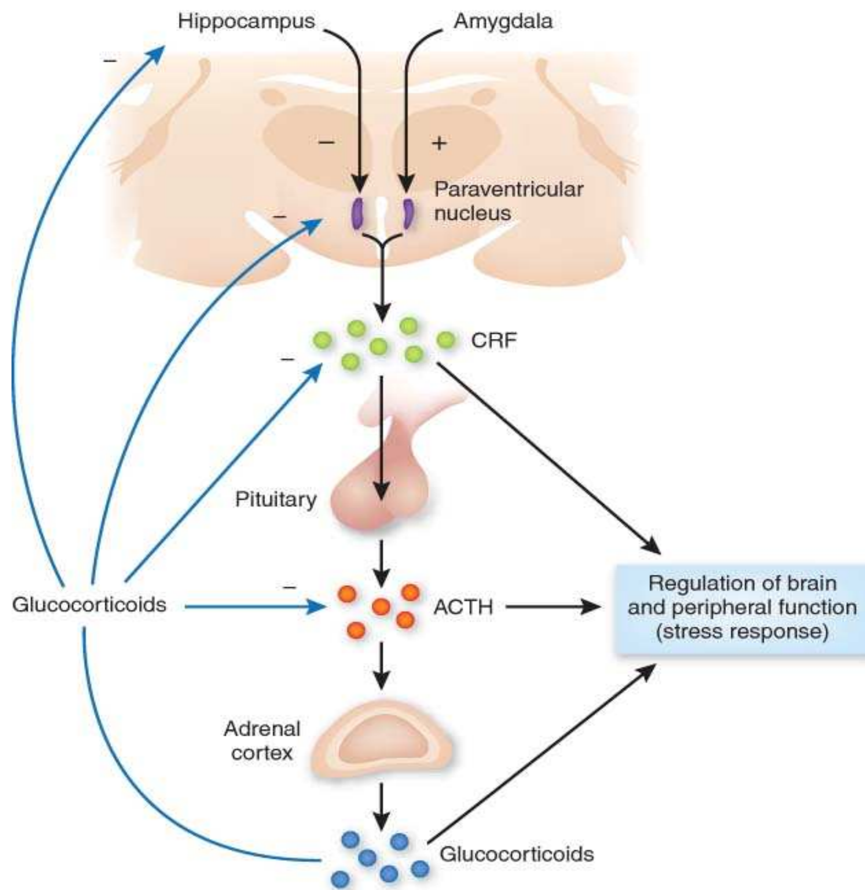


Figure 1.5 The hypothalamus-pituitary-adrenal (HPA) axis in stress response; from (Hyman, 2009).

1.5 Pathophysiology of stress

Normally, an increased activity of the stress response system, after an acute stressor, is followed by a recovery period back to the steady state level. According to the allostatic load model (AL), allostatic overload occurs when the normal response is impaired; this could happen through four types of allostatic load mechanisms (figure 1.6):

- Repeated hits over times: the physiological stress response is challenged many times by new stressors.
- Lack of adaptation: reduced acclimatization to the same kind of stressor.
- Prolonged response: as a result of a delay in switch off the response (impaired negative feedback).

- Inadequate response: insufficient reaction of the stress system that drive compensatory hyperactivity of other mediators.

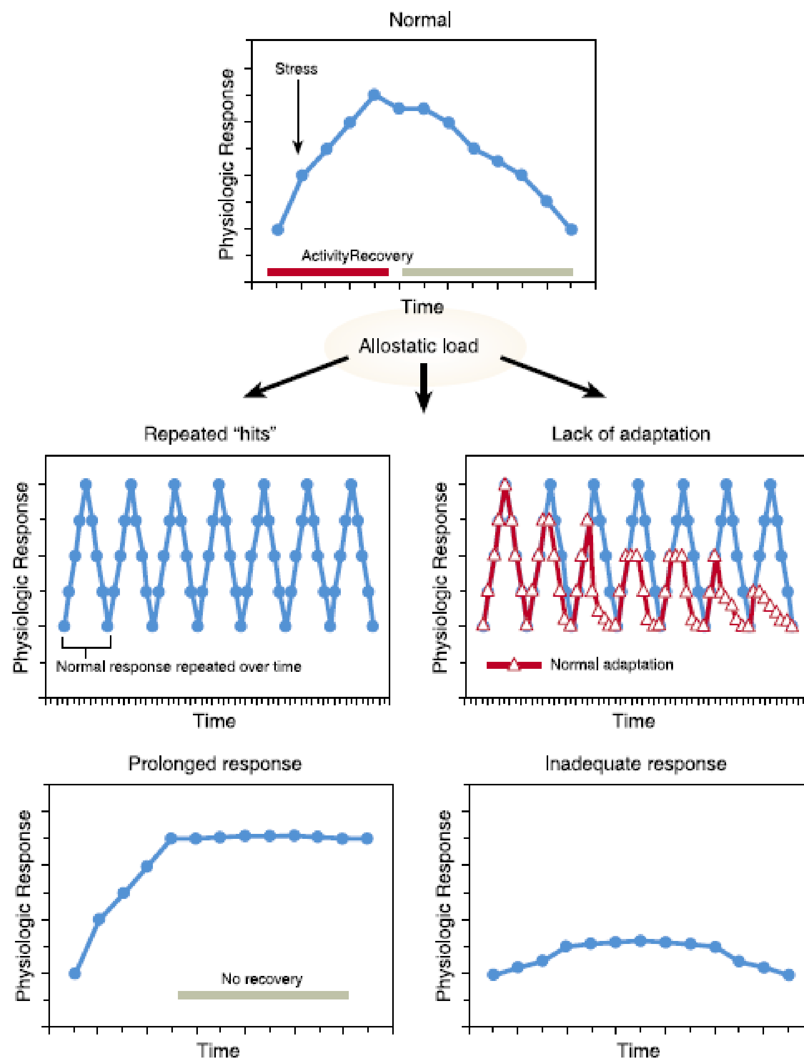


Figure 1.6 Four types of allostatic load .See text for description; from (McEwen, 2007).

Whatever the allostatic load mechanism, repeated call-up of primary mediators during chronic stress causes primary effects throughout the body system (increased blood pressure, heart rate, glucose mobilization, etc.), directly influencing cellular activities. Over time, they can cause compensatory mechanisms in secondary mediators, leading to secondary outcomes (metabolic alterations, brain plasticity, immune hypo- or hyper-activity, etc.). This prodromal stage is the forerunner of possible tertiary outcomes,

consisting in hastened age-related decline or even actual illnesses (cardiovascular disease, depression, cancer, allergic problems, etc.).

The key concept, previously mentioned, is that all mediators are interconnected in a complex network: each mediator system produces biphasic effects and is regulated by other mediators, often in reciprocal fashion, leading to non-linear effects upon many organ systems of the body (McEwen, 1998a, 2007). Primary mediators consist essentially in catecholamines and glucocorticoids and their antagonists, along with cytokines influence. Secondary mediators include, among others, metabolic hormones such as insulin, insulin-like growth factor 1 (IGF-1), leptin and grelin, cholesterol, gonadal hormones, cytokines, as well as neurological mediators like neurotrophins (Lu et al., 2006; McEwen, 2007; Pulford and Ishii, 2001). Although a comprehensive description of mediators is beyond the scope of the Thesis some examples will be further discussed regarding their effect on different body systems, focusing on consequences on brain, immune functions and their interaction.

1.5.1 Cardiovascular effects

As catecholamines increase blood pressure and heart rate, repeated activation of the sympathetic system, due to chronic stress, causes recurrent rise and drop of blood pressure which is a factor risk, along with high cholesterol levels, for the formation of atherosclerotic plaques. At the same time, as glucocorticoids promotes food seeking behaviour, their elevation coupled with a lack of energy expenditure obstruct insulin activity in glucose uptake. As a result, insulin level increases and, in combination with glucocorticoids, promotes the deposition of both body fat and atherosclerotic plaques in the coronary arteries (Brindley and Rolland, 1989; McEwen, 2000a; Vanitallie, 2002). In turn, co-occurrence with smoking, drinking and reduced physical activity greatly enhances the risk of cardiovascular disease, such ischemia and stroke, or myocardial infarction.

1.5.2 Brain effects

Brain is both the mediator and target of the stress response. Is the perception and interpretation of a situation that decides what is stressful or not. Strong emotions impact especially on the activity of pre-frontal cortex, amygdala and hippocampus, which aid to remember the circumstances that turn them in such a powerful manner. The aim is to increase awareness or avoidance for similar situations that may happen in future. However, chronic stress can imbalance this mechanism toward an exaggerated state which lead to anxiety conditions or mental illness (Leuner and Shors, 2013). The reasons of this negative shift are due, at least in part, in changes in neurochemical balance and alteration in dendritic and synaptic structures on neurons within the specified brain regions. Hippocampus, which is one of the most implied structures in the pathophysiology and treatment of mood disorders, is the most flexible and malleable brain structure as it is involved in both memory acquiring and modulation of the SAM and HPA axis. It expresses both glucorticoid receptors (GRs and MRs) and adrenal hormones exert a biphasic effect on excitability of hippocampal neurons in terms of long-term potentiation and primed burst potentiation (Diamond et al., 1992; Pavlides et al., 1995) and show parallel biphasic effects upon memory (McEwen, 2007). The regulation of glutamatergic transmission, by glucocorticoids (Karst et al., 2005), required MRs through a mechanisms that involves glucocorticoid enhancement of extracellular levels of glutamate (Venero and Borrell, 1999), playing an important role in both modulatory and excitotoxic effects of glucocorticoids. In fact, adrenalectomy prevents stress-induced rise of extracellular glutamate (Lowy et al., 1993), while corticosterone infusion recapitulates these effects in absence of stress. According to Sapolsky's glucocorticoid cascade hypothesis, chronic glucocorticoid administration or repeated stress downregulate hippocampal steroid receptors, but not hypothalamic or pituitary receptors (Sapolsky et al., 1986) and display

decreased sensitivity to glucocorticoid fast feedback (Herman et al., 2012; Young and Vazquez, 1996). As a general feature, chronic stress tends to induce dendritic retraction and reduced branching of neurons, as shown in chronic restrain rats. Pyramidal neurons in area CA3 are particularly affected, although pyramidal neurons in area CA1 and granule neurons in the dentate gyrus can be similarly influenced (Magarinos et al., 1996; Vyas et al., 2002; Watanabe et al., 1992). Also changing in spine morphology and spine loss has been observed during chronic stress, although the type and magnitude of these effects seem to vary across hippocampal subregions (McLaughlin et al., 2009; Mucha et al., 2011). Not only excitability and dentritic remodelling are affected, but also neurogenesis and neuron survival are impaired, as both are reduced in chronic stress.

The mechanisms through which stress induces hippocampal changes include a plethora of other mediators, as neither adrenal size nor steroids levels, nor presumed amount of psychosocial stress, per se, determined dentritic remodelling (McEwen, 1999, 2007); (see Figure 1.7). These includes excess of glutamate via NMDA receptors activity (Magarinos and McEwen, 1995), enhanced serotonergic tone (McKittrick et al., 2000) as well as altered inhibitory tone from interneurons and GABA (Cullinan et al., 2008). Metabolic factors involved in glucose regulation further have an impact in hippocampal changes, as also a diet rich in fat impairs hippocampal dependent memory. Furthermore, tissue plasminogen activator (tPA), which is an extracellular protease and signalling molecule that is released with neural activity, is involved in chronic stress-induced loss of spines and NMDA receptor subunits on CA1 neurons (Pawlak et al., 2005). Repeated stress in rodents results in reduced expression of transmembrane glycoprotein M6a, which is associated with a decreased in filopodia number and synaptophysin expression. Interestingly, these effects are prevented by antidepressant treatment with tianeptine (Kuroda and McEwen, 1998). Another important factor is the CRH, which is not only an

inductor of ACTH production, since it is produced also by GABA hippocampal interneurons. While in acute stress low doses enhance memory processes, presumed high level during chronic stress results in spine retraction and loss of synapses (Chen et al., 2012). In addition, the brain-derived neurotrophic factor (BDNF), which is the most abundant neurotrophin in CNS, is known to play a role in dendritic and spine plasticity; its involvement in chronic stress effects will be further anlyzed in the text. Ultimately, the neuronal cytoskeleton undergoes structural reorganization processes, with changes in actin and microtubule architecture concomitantly with alterations in dendrites or spines in the hippocampus (and other regions), induced by stress through intracellular mechanisms. (Jaworski et al., 2009; Penzes and Rafalovich, 2012).

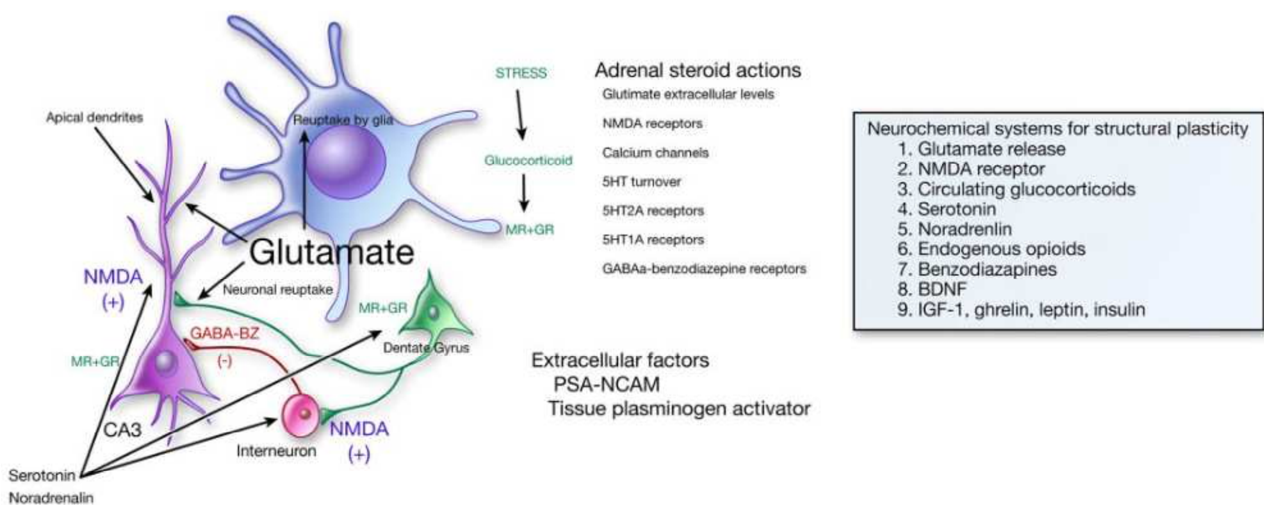


Figure 1.7. Neural cells system involved in stress dendritic remodelling; from (McEwen, 2007).

As demonstrated by chronic restrain paradigm in rats, dentritic atrophy and spine loss occurs also in medial prefrontal cortex (mPFC) in a similar fashion of what observed in hippocampus. However, effects display a high degree of sexual dimorphism: males are more affected to dentritic shrinkage during stress than women (Radley et al., 2006). In addition, chronic stress interacts with aging to influence mPFC plasticity by a structural point of view. While rats of all ages showed stress-induced dendritic retraction, these

effects are reversible after a stress-free recovery period in young rats, but are irreversible in aged rats (Bloss et al., 2010).

The amygdala pyramidal and stellate nuerons, in contrast to the hippocampus and mPFC, exhibit hypertrophy after repeated stress events in a regionally specific manner. In detail, chronic stress enhances dendritic length, branch points, spine number, and spine length on neurons of the BLA without altering these measures in the central nucleus of the amygdala (Vyas et al., 2003). Interestingly, these hypertrophic changes persist even after a period of recovery from stress (Vyas et al., 2004).

Usually hippocampus, amygdala and prefrontal cortex structures are investigated separately. Nevertheless, as they are heavily interconnected and influence each other, their circuitry, as a whole, seems to be impaired during chronic stress, although further investigations are needed on this way. For example, the induction of LTP in the "hippocampus-mPFC pathway" connection is impaired by stress (Cerqueira et al., 2007; Mailliet et al., 2008) as well as in the pathway from the amygdala to the mPFC (Maroun and Richter-Levin, 2003). Moreover, by the reduction of hippocampal structure, stress may diminish activity in hippocampal afferents to the mPFC which could in turn reflect to fewer mPFC dendritic spines (Leuner and Shors, 2013).

The take home message is that chronic stress mediators can induce transitory or lasting changes in neuron morphology and activity (Joels et al., 2007). The types of alterations are region specific and could differentially impact circuitry functions; moreover they may herald anxiety and depression states or other mental illness.

1.5.3 Immune effects

There are two kind of immunity, *natural* and *specific*. The natural immunity is a fast (minutes to hours) and aspecific response against antigens, mediated by complements

proteins, natural killer cells and by cells of the granulocyte/macrophage lineage (including monocyte/macrophages and neutrophils). The typical response mounted by these cells is inflammation, as they get onto the site of infection and release radical oxygen species and other toxic substances that damage both invaders and tissue. Macrophages, in particular, have an important regulatory function in orchestrating the inflammation response, through production of immune mediators, cytokines and chemokines. Differently, the specific immunity is characterized by greater specificity but less speed, because it requires antigen recognition and clonal expansion, so it takes many days until full defence has been mounted. The actors of this response are the three major classes of lymphocytes, namely T-cytotoxic, B cells and T-helper cells. Usually, the first class drives the so called cellular response, which is against intracellular pathogens (for example viruses, but also tumoral cells), while B-cells mount a humoral immunity, producing antibodies against extracellular pathogens. It's important to note that also innate immune actors participate to both cellular and humoral responses. T-helper cells exert important regulatory functions by producing immune mediators, playing at the interface of humoral, cellular and natural immunity (Segerstrom and Miller, 2004). Classically, the T-helper 1 (Th1) and 2 (Th2) sub-populations act by coordinating effector functions of either macrophages and cytotoxic T cells or B cells, respectively (Mosmann and Coffman, 1989). However the situation is much more complex as new kind of T-helper, specifically Th17 and Treg, have been described and display mainly inflammatory and anti-inflammatory properties, respectively (Steinman, 2007).

Nervous and immune systems are known to interact and influence each other activity (Besedovsky and Sorkin, 1977). The huge body of findings describing their relationship have created a new field known as "psychoneuroimmunology". Both systems share common mediators and receptors: immune cells express receptors for catecholamines and

glucocorticoids as well neurons have receptors for immune mediators. These include the aforementioned cytokines and chemokines, which are soluble molecules produced either peripherally by lymphocytes and granulocytes or centrally by microglia and astrocytes and operate within a complex network, acting either synergistically or antagonistically (Reiche et al., 2004). But how chronic stress may impact the immune system? In general, catecholamines increase proinflammatory cytokine production (Bierhaus et al., 2003) while glucocorticoids are known to reduce inflammation (Sapolsky et al., 2000). However there are great exceptions, as glucocorticoids and catecholamines exhibit both inflammatory and anti-inflammatory properties in a dose and cell dependent manner (Dinkel et al., 2003; MacPherson et al., 2005). Also the parasympathetic nervous system may play a regulatory role in this context, since it generally opposes the SA, having potential anti-inflammatory effects (Borovikova et al., 2000). It has been shown that chronic stress can have both immunosuppressive and immunostimulating effects. How this paradox can be explained? The most accepted model hypothesizes a decreased ratio in the Th1/Th2 balance, resulting in an enhanced humoral immunity but suppressed cellular mediated response. A possible mechanism is that primary stress mediators reduce production of interleukin-12 (IL-12) by cells of the innate immunity and naive T helper, limiting the differentiation toward Th1-cells (mainly producers of γ-interferon (IFN-γ) and tumor necrosis factor α and β (TNF-α, TNF-β)). On the other hand, cytokine pattern for Th2 differentiation (including IL-4 and IL-10) seems unaffected by neuroendocrine mediators, thus shifting toward a humoral response (Reiche et al., 2004). Yet, the picture is far from being so simple, as also Th17 and Treg greatly contribute to modulate the effects of Th1 and Th2. The presence of β-transforming growth factor (TGF-β), IL-6 and IL-23, which shares a subunit with IL-12, induce the proliferation of Th17 cells, while the absence of IL-6 pushes toward a Treg phenotype. Th17 and Treg subpopulations appear to antagonize each other, despite they

are both IL-17 producers and its overproduction as been associated to tissue damage. There is also evidence of reciprocal function of Th1 and Th17, as IFN-γ is a Th17 suppressor, despite TNF-α counteracts this effect. Additionally, Th2 cells antagonize Th17 because of IL-4 production (Steinman, 2007).

These observations evidence the high degree of redundancy and pleiotropism of immune mediators, suggesting that is often required more than the action of a single cytokine to influence the fate and function of T helper cells, as well as other immune cells. Being said that, the T-helper unbalance during chronic stress, causes two overall different effects: the suppression of cellular specific immunity has been associated with increased vulnerability to intracellular pathogens (i.e. viruses) and tumour progression, giving the well known roles of T cytotoxic lymphocytes in recognize and suppress infected or cancer cells. On the other hand, the over activation of humoral response has been related to hypersensitivity disease such as allergies, rheumatoid arthritis and gastrointestinal problems, due to activation of B-lymphocytes and other adjuvant cells (eosinophils, mast cells, neutrophils) following recognition of auto-antigens thus driving self-tissues destruction (Segerstrom and Miller, 2004). Also natural-killer (NK) cells and Th17 have been shown to participate to tissue damage, while Treg activity inhibits autoimmunity and protects against tissue injury (Bettelli et al., 2006). The influence on innate immunity is potentially a mixed effect, as innate immune effectors are partners of both cellular and humoral responses (Figure 1.8).

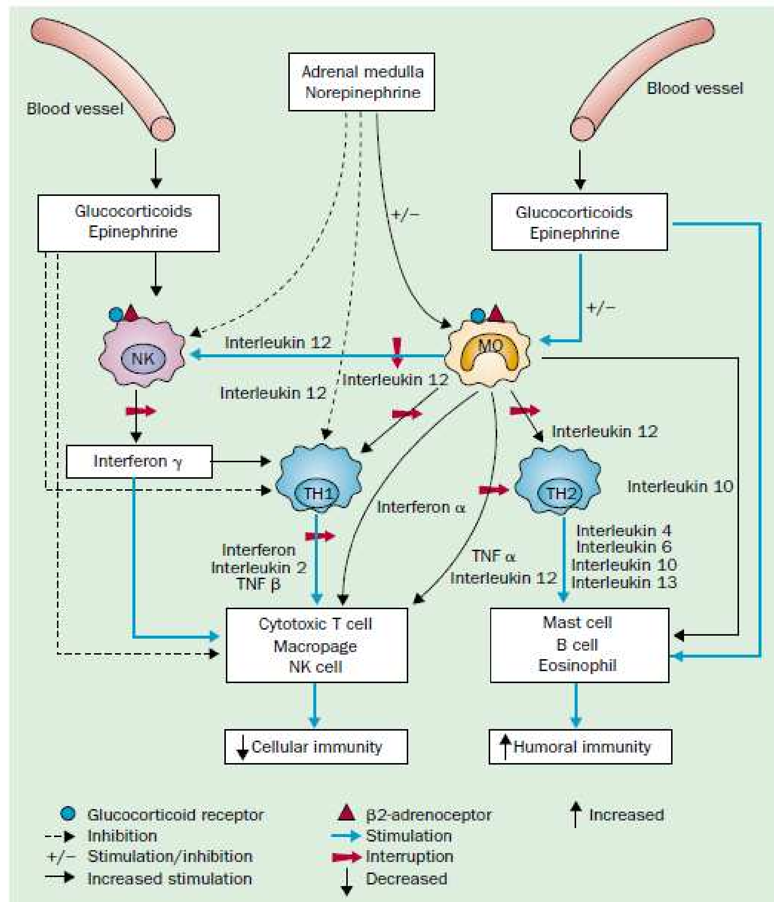


Figure 1.8. Systemic effects of glucorticoids, epinephrine and norepinephrine on the immune system (from Reiche 2004).

The cytokines IL-1, IL-6 and TNF-α, mainly produced by monocytes/macrophages, are classically associated with inflammation as they are major players during the acute phase response. They act locally, promoting migration of leukocytes from the blood stream to the site of infection (TNF and IL-6 promotes the expression of vascular-cellular adhesion molecules (VCAM-1) and intercellular adhesion molecules (ICAM-1), respectively) (Chen et al., 2006a; Yednock et al., 1992), and also centrally, promoting fever, appetite suppression and stimulation of CRH production (thus increasing HPA activity). However, they have a recognized activity of both promoting and inhibiting the inflammation. For example, while anti-TNF therapy has proven efficacy against rheumatoid arthritis and

Crohn disease, treatment with TNF or IL-6 ameliorate the phenotype of animal model with experimental allergic encephalomyelitis (Di Marco et al., 2001; Steinman, 2007).

Beside cytokines, also chemokines, which are a family of smaller protein (8 to 12 kDa), are involved in cellular migration and intercellular communication. Since both ligands and receptors (G-protein coupled) are widely distributed in different brain areas (as hippocampus, hypothalamus and cortex), some authors have started viewing chemokines as a third major system of communication in the brain, along with neurotransmitters and neuropeptides (Adler et al., 2005).

Cytokines and chemokines can influence the central nervous system by two means: 1) they are produced centrally by glia (microglia cell in particular, which are macrophages equivalent) and astrocytes; 2) they are produced peripherally and reach the CNS via blood stream. Usually, they do not cross the blood-brain barrier (BBB), but in some occasion they do, as evidenced by the presence of saturable transport systems, which have been found for IL-1β, IL-6 and TNF-α (Banks, 2005; Gutierrez et al., 1993). Neuroinflammation has been described to sustain disease progression in Alzheimer, Parkinson and Multiple Sclerosis, so has been hypothesized to play a role also in depression (Glass et al., 2010; Nolan et al., 2013; Postal and Appenzeller, 2015), which can be considered one of the ultimate manifestations (tertiary outcome) of chronic psychological stress. Concerning the relationship between psychological stress and inflammation, researchers came out with conflicting results: some reported a broad inflammation status (Hansel et al., 2010; Schmidt et al., 2010), while others found a general immune suppression (Calcagni and Elenkov, 2006; Dhabhar et al., 2012; Kawamura et al., 2001; Lucas et al., 2007). As already discussed, chronic stress can induce both, depending on the type of stressor, its time frame and the psychophysical status of the individual. In addition, concerning the function of cytokines and chemokines, categorization such as “pro-inflammatory” or “anti-

inflammatory" should be taken cautiously as their effect depend on: tissue localization and cell type involved; concomitant presence or absence of other immune modulators (dose-dependence); type of receptor engaged and ultimately its downstream pathways (Dhabhar, 2009). Finally, these apparently contradictory results highlight the complexity of interactions between brain and immune system, either in physiological or allostatic conditions.

As mentioned, the described systems are not the solely involved and affected in the response to chronic stress and all the body systems participate in a coordinated manner. This justify the non-sequential steps cascade that result in allostatic overload and how hard is to define the causalities of events that lead to disease manifestations like cardiovascular incidents, mental illness, hypersensitivity disease and cancers.

The above considerations emphasize the involvement of other physical and psychosocial variables. These, for instance sex, disease state, behaviour and social status, will be briefly discussed in the next paragraph.

1.6 Determinants in chronic stress response and stress management

The brain is both a target and a mediator of the allostatic load occurring in chronic stress. It decides what is stressful and how to cope and the reaction further influences its activity. The degree of stress perception, the type of responses and their effects on body, along with the probability to arise allostatic overload (tertiary outcomes) are individual and largely depend on four broad categories of factors: constitutional, behavioural, historical and socio-environmental. Albeit an exhaustive description is beyond the scope, some of the variables are briefly depicted below, as they worth to be mentioned.

Constitutional:

- Gender: ovarian steroids contribute to a variety of neurochemical processes, inducing gender differences in cognition, emotional and affective styles, pain and psychopathology. MRI studies show activation of different brain areas during stress (Wang et al., 2007). Female have in general a higher risk to develop allostatic overload (Bellingrath et al., 2009; Juster et al., 2010).
- Age: as age increases, the likelihood of tertiary outcomes tends to raise (McEwen, 2000b).
- Genetic factors: the allele T of the angiotensin-1 converting enzyme (ACE, rs4968591) has been related to a higher risk of allostatic load. (Smith et al., 2009). Another example is the short form of serotonin transporter which is associated to higher risk of alcoholism (Herman et al., 2005) and vulnerability in the response to stressful experiences, by developing depressive illness (Caspi et al., 2003). Additionally, having the Val66Met polymorphism of the BDNF gene is associated with reduced hippocampal volume, impaired memory and mood disorders (Baj et al., 2013a; Chen et al., 2006b; Spencer et al., 2010).
- Personality: type A personality traits (highly ambitious, organized and status conscious individuals; Friedman and Schustack 2010) are related to higher risk of coronary heart disease and allostatic overload (Sun et al. 2007).

Behavioural:

- Substance dependence: smoking by an effort-reward imbalance interacts with age lowering bone-marrow derived progenitor, therefore wakening vascular integrity (Fischer et al., 2009; Siegrist et al., 2004). Consequently, smoke is associated to increased risk of cardiovascular disease and cancer. Surprisingly, alcohol intakes

inversely correlate with allostatic load risk (Hu et al., 2007); however, alcohol abuse has a long term negative impact on metabolic and cognitive status (Mukamal et al., 2010).

- Food intake: hypothalamus and hippocampus interaction is involved to control food intake; stress often leads to eat “comfort food” (Dallman et al., 2003) which in turn has been linked to metabolic risk and obesity.
- Sleep: sleep deprivation, which is a frequent result of being “stressed”, has a plethora of consequences on body systems; negatively impacts the neuroendocrine circadian rhythm, increases blood pressure, decreases the parasympathetic tone, and promotes appetite (McEwen, 2006; Spiegel et al., 1999). Reduced sleep has also a negative impact on the immune system, as sleep facilitates T-cell extravasation and redistribution to lymph node and enhances the formation of immunological memory (Besedovsky et al., 2012).
- Physical activity: A sedentary life-style is a major risk factor for many of the diseases of modern life including obesity, diabetes, cardiovascular disease, depression, and dementia. Moderate, but not intense, physical activity improves metabolic and cognitive scores. In this sense, it has an anti-depressant effect and increases hippocampal neurotrophin expression (BDNF in particular) as well as stimulates neurogenesis from the dentate gyrus of the hippocampus (Baj et al., 2012; Krogh et al., 2014; Olson et al., 2006).

<u>Historical:</u>

- Early life experiences: they have effects on human physiology and behaviour. “Bad” experiences negatively influence the ability to cope with future stressful situations. In animal models, poor maternal care leads to a “neophobic” phenotype with

increased emotional and HPA reactivity and less exploration of a novel situation (Lajud and Torner, 2015; McEwen, 2008). It also induces hyppocampal dysfunction and cognitive impairment, which involves also excessive activation of the CRH receptors (Ivy et al., 2010). In humans, the likelihood of poor mental and physical health in adults is increased by childhood experiences in emotionally cold families (Brown et al., 2005; Repetti et al., 2002). Moreover abuse in childhood is a well-known risk factor for depression, posttraumatic stress disorder, idiopathic chronic pain disorders, substance abuse, antisocial behaviour, as well as obesity, diabetes, and cardiovascular disease (Anda et al., 2006; Brown and Heimberg, 2001).

- Trauma: life traumas increase the probability risk for anxiety-related disorders such as post-traumatic stress disorders and are associated with reduced hippocampal volume and HPA axis impairment (Cohen et al., 2006; Gorka et al., 2014).
- Co-morbidities: the concomitant presence of other disturbances such as cardiovascular problems, cancers or dementia, negatively impacts on the ability to react to new or prolonged stressors.

Socio-environmental:

- socioeconomical status (SES): low education and income are collectively associated with increased prevalence of several disease such osteoarthritis, hypertension, as well predict substance abuse and anxiety and mood disorders. Similarly, living in poor conditions (dirty or polluted environment) increases the risk of allostatic load. More in general, SES is an effective predictor of health status (McEwen and Gianaros, 2010; Seeman et al., 2010).
- Ethnicity and spirituality: ethnicity interacts with socioeconomical status as poor Caucasian were less likely than non-poor African Americans to have increased risk

of allostatic overload (Geronimus et al., 2006; Merkin et al., 2009). Positive life outlook, high-self esteem, sense of purpose and religious attendance all contribute to the proper management of stressful situations (Juster et al., 2010).

- Relationships (family, work, social support): interpersonal relationships in all social contexts can bias an individual towards either a positive or negative response in a new situation (Tugade and Fredrickson, 2004).

The described factors not only influence the response to chronic stress but are part of a general framework of healthiness and successful aging. The brain is a malleable structure that is “shaped” by life events, as it changes structurally and functionally in order to adapt to situations. Stressful events do not necessarily damage the brain but can be long-lasting, so proper intervention (when possible) on variables that influence their consequences would be beneficial in stress management but also could ameliorate life quality. Some of the interventions should include government policies that improve community service and personal opportunities and encourage healthy life-styles. More in general social support, in term of having positive relationships with familiars, friends and colleagues in professional context, has beneficial effects on mood and overall mental health (Leskela et al., 2006; Saxena et al., 2006; Silver et al., 2006).

Moreover, changes in personal behaviour and clearing out “bad habits” have shown to give optimal results in term of successful aging and decreased risk of allostatic overload manifestations. Dietary restrictions possibly combined to voluntary moderate exercise have been shown to be beneficial for metabolic, cardiovascular and brain systems. They result in reduced risks of obesity, diabetes, cardiovascular disease and also lowered probability of dementia and depression. In brain these factors, specifically in hippocampus and cortex, have been shown to increase dendritic arborisation, spine density and improved learning in hippocampal-dependent memory tasks (Baj et al., 2012; Cotman and

Berchtold, 2002). Similar results have been obtained following antidepressant drugs treatment, potentially explaining the antidepressant effect of physical exercise. These effects have been related to increase expression of neurotrophins in these areas, in particular BDNF, as it is the major central neurotrophin.

At last, if social and behavioural improvements do not suffice, pharmaceutical treatments may provide useful support. For example, anti-glycolic, anti-cholesterol and beta blockers can be used to control metabolic and cardiovascular problems; similarly, anxio-lytics and antidepressant may control mood manifestations of being “stressed”. Anyway, the usage of any drug must be carefully monitored, because of the non-linearity of allostasis systems: it may not be beneficial for the systems in question or even perturb other systems that interact with it, promoting unwanted side effects.

1.7 Neurotrophins and stress: the role of BDNF

The growth, differentiation and survival of neurons are supported by specific growth factors, known as neurotrophic factors (NT). NTs are four small secreted proteins that play important roles in the development of the nervous system in vertebrates (Chao et al., 2006). In particular the NGF superfamily includes, brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5) and neurotrophin-6 (NT-6). NTs bind with specific affinities to transmembrane-receptors belonging to a small family of tropomyosin-related tyrosine kinases (Trk): TrkA (NGF), TrkB (BDNF and NT-4), and TrkC (NT-3) (Huang and Reichardt, 2003). Trks are receptor tyrosine kinases that dimerize upon ligand binding which converges in their activation and subsequent initiation of several signal transduction cascades. In addition, the pro-NTs form can also bind to the p75 neurotrophin receptor (p75NTR) with an equal affinity, mediating apoptosis or cell cycle arrest. A good control of the development and function of the

neuronal cells is given by the possibility, for the same neurotrophin, to bind different receptors. Among NTs, BDNF is of particular interest because is the most abundant neurotrophin of central nervous system. Like other NTs, is synthesized predominantly by neurons in an activity-dependent manner and released upon neuronal depolarization (Cirulli and Alleva, 2009). The binding between BDNF and TrkB receptor, induces the dimerization and autophosphorilation of the receptor itself which in turn activates three different signaling pathways: the mitogen-activated protein kinase (MAPK), the phosphatidyl-inositol 3-kinase (PI3K) and the phospholipase C (PLC) (Li and Pozzo-Miller, 2014). Each of these pathways influence different BDNF actions: MAPK pathway promotes neuronal differentiation, PI3K pathway is involved into the regulation of transcription favouring survival and growth of neurons, while PLC pathway controls ion channel effects in promoting synaptic plasticity (Dwivedi, 2012; Mattson and Wan, 2008; Yoshii and Constantine-Paton, 2010). For this reason, BDNF has different roles in neuronal cells. During the development of nervous system it contributes to dendritic and axonal growth (Yoshii and Constantine-Paton, 2010), then in mature neurons it is involved in neuronal homeostasis, cell survival and death and synaptic plasticity (spine formation and maturation, long terminal potentiation and long terminal depression) (Poo, 2001). BDNF is also crucial for learning and memory processes (Lu et al., 2008; Yamada et al., 2002).

Due to the described functions, several data suggest that reduced BDNF signalling in the adult brain, as well other NTs, may be implicated in the pathophysiology of the mental consequences of chronic stress, as it is a risk factor for psychiatric disorders, such as depression (Altar, 1999; Duman and Monteggia, 2006; Rothman and Mattson, 2013). BDNF itself does not control mood, but it plays an important functional role in the modulation of networks which ultimately determine how a plastic change influences mood

(Castren et al., 2007; Sen et al., 2008). Stabilization of axonal and dendritic branches and strengthening of synaptic connection are driven by BDNF-TrkB bound in an activity dependent manner, so both BDNF release and TrkB receptor expression must take place, in a coordinated fashion, at the relevant synaptic sites for the optimal synaptic response to occur.

On this basis, these neurotrophic factors play a fundamental role in shaping brain function. Consequently a pathological alteration in NTs concentration or action early during postnatal life could exert long-term effects on synaptic plasticity, for example impairing the ability of the organism to cope with novel/stressful situation, leading to psychopathology (Zubin and Spring, 1977). The importance of (early) developmental influences in the etiology of psychiatric disorders, such as depression, has been recognized, but has been often under-emphasized. Early life stressful events, such as childhood trauma and neglect, are associated with depression and anxiety disorders and sustained changes in the HPA axis (Heim and Nemeroff, 1999, 2001). Numerous data suggest that NT-mediated neuronal plasticity is a critical factor in mood disorders and in their therapy. The synergistic action between alterations in neurotrophin levels, as a result of exposure to stressful events at adulthood and epigenetic changes, caused by early stress, could lead to greater susceptibility for stress-related psychiatric disorders (Cirulli et al., 2009). The negative consequence of chronic stress on mood, memory and cognition, has been associated to a reduction in hippocampal volume, due to dendritic shrinkage and decrease neurogenesis, and they can be account, at least in part, by a reduction in BDNF level or its activity. A consistent number of findings support this hypothesis. In human, smaller hippocampal volumes and memory dysfunction have been associated with a single nucleotide polymorphism (SNP) (Val66Met) of the BDNF gene (Baj et al., 2013a; Cirulli and Alleva, 2009). This finding has been confirmed also in animal models in which the

reduced availability of BDNF, experimentally induced by the Met/Met gene variant, enhances anxiety-related behaviour; interestingly, these are reversed after BDNF treatment. As already introduced, the effect of stress on hippocampal structure and activity depends on the type of stressor, its duration and the time frame when it occurs. Early life stress paradigm in rodents, like maternal deprivation or maternal separation, has been associated with disruptions in later cognitive and emotional functioning of the animal improving potent and long lasting effects on neurotrophin expression levels (Bath et al., 2013; Romeo et al., 2003). In fact, many authors found a general decrease in BDNF mRNA and protein levels and sometimes a decreased expression of TrkB receptors in the hippocampus, effects that are more pronounced when early stressed rodents reach adulthood (Lee et al., 2012; Lippmann et al., 2007). Similar outcomes were obtained also using paradigms of later life stressors such as social defeat or isolation, acute (that induces BDNF increase) or chronic restrain, footshock/unpredictable stress, but results display a higher variability that can be account, in part, as a difficult to disentangle the contribution of pain or the lack of clear controls. Additionally, some groups have failed to find any changes in BDNF levels following repeated restraint stress (Kuroda and McEwen, 1998; Magarinos et al., 2011; Rosenbrock et al., 2005), and others have suggested transient reduction of BDNF levels in the hippocampus (Bath et al., 2013; Lakshminarasimhan and Chattarji, 2012). It should be noted that most of the studies were done on male rats and some of the stress paradigms effect on hippocampal BDNF levels, shows high degree of sexual dimorphism: in particular, maternal deprivation reduces BDNF only in male rats, while repeated (but not acute) footshocks negatively affect BDNF levels only in female. Interestingly, peripheral administration of BDNF in male mice increases hippocampal neurogenesis and reduces anxiety-related behaviour induced by chronic unpredictable stress, novelty-induced hypophagia, forced swim test and elevated

plus maze (Schmidt and Duman, 2010). Hippocampal BDNF reduction was also prevented by antidepressant treatment (Lee and Kim, 2010) and physical exercise (Baj et al., 2012; Papathanassoglou et al., 2014). Overall, data suggest BDNF decrease as a common aspect of chronic stress effects and mood disorders. However, this is not entirely true, at least not for all brain areas. For example, the same chronic immobilization stress insults that cause BDNF reduction and dendritic atrophy in the CA3 region of the hippocampus, cause an increase in BDNF levels, growth of dendrite and spines in basolateral amygdala (BLA); furthermore, unlike CA3 atrophy, BLA hypertrophy persist even after 21 days of stress-free recovery (Lakshminarasimhan and Chattarji, 2012). In addition, increased BDNF in PVN and pituitary gland enhances the expression of CRH and ACTH, respectively, having so a positive feedback on HPA-axis activity. Notably, BDNF-TrkB signalling is essential for fear conditioning in the circuitry connecting PVN and central amygdala (CeL): as a result, selective deletion of either BDNF in the PVN or Trkb in CeL neurons impaired fear conditioning, while infusion of BDNF into the CeL enhances fear learning and elicits unconditioned fear responses.

The open question is how much of the rodent findings can be translated to humans. Reduced hippocampal volume has been found in depressed patients, depending also on the duration of depressive symptoms (Videbech and Ravnkilde, 2004). Concerning BDNF, it can be detected in both plasma and serum, and a close relationship between brain and peripheral BDNF levels has been identified. The main hypothesis that needs to be tested is whether changes in BDNF levels following stress could reflect ‘‘allostatic” processes activated to coordinate brain and body responses to specific external challenges (Cirulli and Alleva, 2009). In a Japanese population of job-related stress workers (Mitoma et al., 2008) was found a negative correlation between serum BDNF and the stress score. Lower serum BDNF was found also in depressed patients, probably as a result of lowered

release from platelet unrelated to platelet reactivity (Karege et al., 2005). In some cases, as found in rodents, serum BDNF level can be reverted by antidepressant treatment (Matrisciano et al., 2009) or moderate physical activity. Also others disorders, including schizophrenia (Carlino et al., 2011; Zhang et al., 2012), bipolar disorder (Machado-Vieira et al., 2007), eating disorders such as bulimia nervosa and anorexia nervosa (Mercader et al., 2007) or Huntington's disease (Ciammola et al., 2007; Zuccato et al., 2011) are characterized by reduced serum and plasma BDNF levels. However, findings are a bit controversial, as some authors find no change or even an increase in BDNF expression in depressed subjects (Pigatto Teche et al., 2013) and no effect on BDNF levels wielded by antidepressant therapy. It should be noted that circulating BDNF is not produced only centrally but also by many cell types, including endocrine and endothelial cells, myocites, adipocytes and immune cells. BDNF exerts immunomodulatory effects as well, as it is involved in T- and B-cell maturation in an autocrine manner. Similarly, BDNF and TrkB receptor are upregulated in activated macrophages (Barouch et al., 2001). Moreover TrkB density negatively correlates with the apoptotic rate of cytotoxic T cells (De Santi et al., 2009). Notably, an elevated concentration of BDNF was found also in atherosclerotic plaques. In addition, there is a plethora of other demographic and behavioural variables (Bus et al., 2011), such as sex, smoking and drinking habits, physical exercise and dietary practice, which can influence the level of circulating BDNF, and account for some of the inconsistencies found in literature. Nevertheless, many of these discrepancies are due to different methodology for collection and analysis of circulating BDNF, since standardized methodologies at both pre-analytical and analytical stages are still lacking. Addressing this topic is mandatory for future studies assessing BDNF in blood.

1.8 Allostatic load measurement: the biomarker potential

The allostatic load theory implies the possibility that studying the alterations in the allostasis mechanisms during chronic stress, and how the parameters change in relation to them, would aid in identify individuals at high risk of disease outcome. Any disease-associated changes that can be reliable measured in tissue or body fluids are referred as *biomarkers* (Drucker and Krapfenbauer, 2013). Many studies applying the allostatic load model have included also the assessment of potential biomarkers as indicator of risky changes in the major physiological systems involved: metabolic, cardiovascular/respiratory, neuroendocrine and immune. For example, metabolic measurements like glucose, total cholesterol, triglycerides and cardiovascular assessment as systolic/diastolic pressures and hear rate are used to determine the risk of metabolic syndrome or cardiovascular disease. Notably, in older-middle aged adult, the prediction of occurrence of cardiovascular problems and, surprisingly cognitive outcomes, is supported by metabolic markers detection, while both metabolic syndrome and neuroendocrine biomarkers accounted for mortality risk and only marginally to physical decline (Juster et al., 2010). Measurement of cytokines like TNF-α, IL-1, IL-6, C-reactive protein (CRP) and coagulation factors are largely used to assess inflammatory status. Neuroendocrine biomarkers include, for example, cortisol, adrenaline and noradrenalin: increased cortisol level accounts most to memory impairment in women (Seeman et al., 1997a; Seeman et al., 1997b), while increased adrenalin correlates to cognitive decline in men (Karlamangla et al., 2005). It appears, however, that primary mediators contribute independently to cardiovascular and metabolic risk factors in predicting cognitive and physical functioning declines (Karlamangla et al., 2002). Furthermore, these studies highlight the advantage of incorporating multi-systemic biomarkers, although specific clusters seem to predict tertiary outcomes differentially.

Due to the non linear network that connects physiological systems together and the non-sequential steps that lead to tertiary outcomes, the associations between diseases and large sets of biomarkers are hugely challenging (Poste, 2011). On one side, the low specificity may provide some insight into the high rates of co-morbidity that exists between many disorders. On the other hand, most of the inconsistencies in biomarker research come from the high variability between individuals, but also due to lack in standardized practice for specimen sampling and analysis. This, in particular, is one of the most important problems in finding specific and reliable biomarkers, as also make difficult to compare studies on similar topics.

The aforementioned difficulties are particularly evident in the context of psychiatry biomarkers. In fact, the availability of biomarkers to support the diagnosis or monitor the efficacy of therapies is a major unmet clinical need in neuropsychiatry. As already stated, the neurotrophin Brain-Derived Neurotrophic Factor (BDNF) is one of the most promising biomarkers in psychiatry; however a definitive clinical validation is still lacking. Meta-analyses and reviews of clinical studies based on the measurement of BDNF in whole blood, serum, or plasma have reported significantly lower BDNF levels in patients with major depression, schizophrenia, bipolar disorders, or autism spectrum disorders (Katoh-Semba et al., 2007). These reviews, however, highlighted severe discrepancies among studies which even report opposed results (increase vs. decrease or no change). On this basis an accurate measurement is a fundamental pre-requisite in order to validate any biomarker for neuropsychiatric diseases (Drucker and Krapfenbauer, 2013).

2. AIMS OF THE PROJECT

The aims of the present Thesis are three-fold:

1) Proper specimen collection, handling and analysis are essential in order to identify any biomarker in a reliable way. This is a predominant problem concerning biological psychiatry, where availability of biomarker is a major unmet clinical need. Brain-Derived Neurotrophic Factor (BDNF) has attracted increasing interest as a potential biomarker to support the diagnosis or monitor the efficacy of therapies in neuropsychiatry. However, a current major limitation to the use of BDNF as clinical biomarker is the poor reproducibility of results. We hypothesized that a major source of inconsistencies among studies could be the variety of methods for sample collection and BDNF analysis. To overcome these limitations, we selected the serum as the elective body fluid for BDNF quantification and defined a standardized method to prepare the samples for BDNF quantification. Then, using serum samples from 40 healthy adult subjects, we compared the performance of six commercial BDNF ELISA kits from the companies: Aviscera-Bioscience, Biosensis, Millipore (ELISA and Luminex®/xMAP® multiplexing technology), Promega and R&D Systems, to identify the most performing one in BDNF assessment.

2) We then analyzed BDNF levels and other 49 distinct cytokines, chemokines and growth factor in the sera of 122 healthy subjects among healthcare assistance; the goal is to find a potential correlation between immune factors and scores assessing psychophysical job-related stressors and burnout.

3) Using a cellular model of cytotoxic stress, given by the SK-N-BE neuroblastoma cells treated with the chemotherapic cisplatin, we address the potential protective role of BDNF.

3. MATERIAL AND METHODS

3.1 AIM 1

3.1.1 Participants and sample collection

Forty healthy subjects (mean age 54±6, range: 41-70 years old; 18/22 Females/Males ratio) participated in this study as normal controls. Subjects were enrolled at the Department of Transfusion Medicine of the Trieste University Hospital and signed written informed consent to participate to the study, according to the recommendations of the declaration of Helsinki and the Italian DL n° 675 of the 31-12-1996. The study was approved by the University Hospital's Ethics Committee (protocol n. 78/2010). Blood samples from healthy control subjects were collected between 9:00 to12:30, in fasting condition and let them clot for 1 hour at room temperature, followed by 1 hour at 4℃. Serum was then separated by centrifugation at 2000 g for 10 minutes at 4℃, aliquoted and stored at -80℃ until use in 0.2 ml tubes strip s (Starstedt, Multiply® µStripPro).

3.1.2 BDNF measurement

Serum levels of total BDNF were measured by using six different ELISA kits, as listed in Table 2: human BDNF ELISA Kit (Cat #: SK00752-01, Aviscera-Bioscience, Santa Clara, CA, USA), BDNF *Rapid*™ ELISA Kit: Human, Mouse, Rat (2 Plates; Cat #: BEK-2211-2P, Biosensis Pty Ltd., SA, Australia), ChemKine™ BDNF Sandwich ELISA (Cat #: CYT306) and Milliplex® Map Human Pituitary Magnetic Bead Panel 2 - Endocrine Multiplex Assay, based on Luminex®/xMAP® technology (Cat #: HPTP2MAG-66K, both from EMD Millipore Corporation, Billerica, MA, USA), BDNF Emax® Immuno-Assay System (Cat #: G7610, Promega Corporation, Madison, WI, USA), Quantikine® human BDNF Immunoassay (Cat

#: DBD00, R&D Systems, Inc., Minneapolis, MN, USA). All samples were assayed in duplicate on each plate, in order to test also the intra-assay variation. A number of two plates per kit were used over two different days in order to assess the inter-assay variation. Protocols were performed according to the manufacturer's instructions. The optical density of each well was measured using an automated microplate reader (GloMax®-Multi Microplate Reader, Promega) or the Bio-Plex®-200 instrument (Bio-Rad) for the multiplexing assay measurements.

3.1.3 Statistical analyses

For each kit, sensitivity and range were estimated based on the standard curves. Sensitivity was stated as equal to the kit declared value if the absorbance of the most diluted standard point was 10% over the background (blank absorbance). Similarly, range was confirmed equal to the declared one if the inclusion of the most concentrated standard point did not reduce the R^2 (linearity of the curve) below the 90%. Because BDNF values were not normally distributed, data are presented as median, 25th-75th percentile and range (see Table 5). Differences in BDNF serum level from the six kits were tested using one-way analysis of variance (ANOVA) followed by the *post hoc* Dunnett test. To evaluate intra-assay variation, the BDNF values from the two different well, for each subject, were mediated and the coefficient of variation (CV) was calculated for all samples. Again, due to the non-normal distribution of the CVs, the median value was considered. Similarly, to assess inter-assay variation, the BDNF concentrations, for each subject, were mediated between the two plates tested and the median CV value was considered. Differences in the BDNF concentrations, as a repeated measure from two independent plates, were assessed using one-way ANOVA for repeated measures. Values of $p<0.05$ were

considered statistically significant. All statistical analysis and graphs were performed using SigmaPlot 11.0 (Systat Sofware, Inc.).

3.1.4 Dot blot

Commercially available human recombinant pro-BDNF (Alomone Labs) and two type of mature-BDNF (Sigma and Alomone Labs) were spotted on a nitrocellulose membrane at the concentration of 10 pg and 1000 pg per lane, respectively. Together, were spotted BSA (1000 pg), as negative control, and the BDNF standards (between 25 pg and 100 pg, depending on the stock concentration) provided by the ELISA kits. Dot-blots were performed according to Abcam guidelines with minor modifications, and all steps were carried out at room temperature. Briefly, once proteins have been spotted, the membrane strips were dried and then non-specific sites were blocked by soaking in 5% BSA in TBS-T (20 mM Tris-HCl, 150 mM NaCl, 0.05% Tween20) for 30 minutes. ELISA's primary antibodies were used at the concentrations suggested by each kit manufacturer in the diluents provided, when possible; Millipore (Chemikine^{TM}) and Promega antibodies were dissolved in BSA/TBS-T (0.1% of BSA in TBS-T). Monoclonal antibody anti-BDNF (Sigma) was used as control at a dilution from stock to 1:1000 in 0.1% BSA in TBS-T. All incubations with primary antibodies were performed for 1 hour and then strips were washed three times in TBS-T for five minutes each. As secondary detector, streptavidine-HRP was used (Pierce Biotechnology; 1:2000 in BSA/TBS-T), except for Promega polyclonal antibody (the anti-IgY-HRP provided with the kit was used) and for Promega and anti-BDNF (Sigma) monoclonal antibodies (the anti-mouse-HRP from Sigma was used, diluted 1:10000 in BSA/TBS-T). R&D System antibody is already HRP-conjugated and did not require further detection by a secondary antibody. Incubations were performed for 30 minutes, followed by three wash steps with TBS-T and then rinsed with TBS. As

reaction substrate, ECL-prime (Amersham) was used and X-ray film exposed in the dark room to display the signals.

3.2 AIM 2

3.2.1 Participants

The study 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 work-related psychophysical stress and burnout state. The questionnaire was completed by 122 subjects who agreed to participate to a clinical interview followed by a blood sample withdrawal. Blood samples were collected and sera isolated as previously described in Aim 1. 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 122 workers, of which 71.3% were women, with a mean age of 45.6 years (*SD* = 9.6). For 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%). All participants gave their written, informed consent, and the study was approved by the local ethic committee according to the recommendations of the declaration of Helsinki.

3.2.2 Work-related stress assessments

Subjects were assessed for psychophysical stress and burnout state determined using five and three items, respectively, taken from the Qu-BO test, an instrument standardized for the Italian context (Falco et al., 2012). The six point response scale ranged from 1

(strongly disagree) to 6 (strongly agree) and is designed to behave as a continuous scale. For psychophysical stress the items assessed are the following: anxiety, emotion (depression-like), gastrointestinal disturbances, cardiac disturbances, ergonomic dysfunction at the workplace. The overall stress score was estimated by averaging the scores of each item. For burnout, the items are the following: emotional exhaustion, depersonalization, personal accomplishment, adapted from the Maslak Burnout Inventory (Maslach et al., 2001). The scores were calculated by summarizing the questions describing each item.

3.2.3 Biochemical assessments

Collected serum was stored at -80℃ until assayed. Multiple analytes were assessed, as listed in Table 1.

	Cytokines	Chemokines	Trophic factors
21-plex (BioRad)	IL-1α; IL-2Rα; IL-3; IL-12 (p40); IL-16; IL-18; IFN-α2; LIF; MIF; SCF; TNF-β; TRAIL/TNFSF10	CTACK/CCL27; GRO-α/CXCL1; MCP-3/CCL7; MIG/CXCL9; SDF-1α/CXCL12;	HGF; M-CSF/CSF1; β-NGF; SCGF-β/CLC11
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; GM-CSF	IL-8/CXCL8; Eotaxin/CCL11; MCP-1/CCL2 (MCAF); IP-10/CXCL10; MIP-1α/CCL3; MIP-1β/CCL4; RANTES/CCL5;	IL-7; basic FGF; G-CSF; PDGF-BB; VEGF
BDNF *Rapid*™ ELISA kit (Biosensis)	/	/	BDNF

Table 1 Biochemical analytes listed by cytokines, chemokines and trophic factors.

Serum BDNF levels were assessed using classical sandwich ELISA, as previously described in Aim 1. 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 duplicates and the same plate was used.

3.2.4 Statistical analyses

Work-related stress scores were used as dependent variables, while biochemical analytes level as independent variables; gender, age and body mass index (BMI) were treated as co-factors. Differences in age and BMI distribution between male and female were assessed performing a Mann-Whitney U-test. Likewise, sex differences in distribution of work-related stress scores were evaluated using Kruskal-Wallis tests.

Many 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. For others, we imputed the half of sensitivity value for each analyte. The influence of the use of drugs on biochemical values was tested performing Kruskal-Wallis tests. Normality distributions of the independent variables were assessed by Shapiro-Wilks tests and visually inspected by Quantile-Quantile (Q-Q) plot analyses. Since most of the variables were not normally distributed we decided to log-transform values in order to approximate normality distributions, thus being able to perform both univariate associations and multivariate analyses.

As univariate analyses, we perform partial correlation analyses controlling for gender and BMI to identify association between dependent variables (scores) and each independent variable (biochemical markers). Since we performed multiple biomarkers assessment on same samples, we chose to not correct for multiple comparisons across the different analytes, since this would enhance the probability of type II errors (Janelidze et al., 2013). Multivariate analyses were 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. Unsupervised analysis of the biochemical markers was also carried out; hence we did a Principal Axis Factoring with 30 iterations and the minimum number of latent factors was extracted based on the Eigen-value cut-off ≥1. Promax rotation (30 iterations) with Kaiser Normalization algorithm was employed to assign variables to latent factors. Correlation values below 0.4 were hidden.

All statistical analyses and graphs were performed by using the SPSS® Statistical Package version 22.0 (IBM®).

3.3 AIM 3

3.3.1 Cell cultures and reagents

Human neuroblastoma cell line SK-N-BE were grown in Dulbecco's Modified Eagle's Medium (DMEM) with stable L-Glutammine, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% decomplemented fetal bovine serum (FBS) at 37°C with 5% CO_2. Differentiation was induced 24 hours after plating by adding 5 µM of 9-cis retinoic acid (Sigma) to cultures and replaced after 48 hours. Differentiated cells (4.5 days) were treated with 5 µg/ml cisplatin (Pfizer) with or without 10 µM of the Aurora kinase inhibitor PHA-680632 (kindly gifted by Fabio Gasparri, Nerviano Medical Science) in serum free medium (SFM).

3.3.2 Real-time PCR for BDNF cds and isoforms quantification

Total RNA was extracted, using TriZol® Reagent (Invitrogen), from differentiated SK-N-BE cells after different hours of cisplatin treatment: one, three, six, twelve and twenty-four hours and a non-treated control. One microgram of total RNA was reverse-transcribed into

cDNA as previously described (Baj and Tongiorgi, 2009) and employed for a quantitative real time PCR (qRT-PCR), performed using the Rotor Gene 6000 instrument according to the manufacturer’s instructions. The PCR reactions were carried out in a final volume of 20µl with 2X Master SYBR Green I Mix (Fluocycle, Euroclone), 0.5 µM of each primer and 1µl of cDNA was added as template. The qRT-PCR was used to evaluate both total and BDNF splice variants expression after cisplatin treatment at the given time points. The primer pairs used along with the PCR conditions are listed in Table 2.

Human exons	**Sequence**	**Product (ex fwd+com rev)**	**PCR conditions (ex fwd+com rev)**
exI fwd	CTTCCAGCATCTGTTGGGGAGACG	205	45X(95℃ 20”,56℃ 20”,72℃45”)
exII fwd	CCAGCGGATTTGTCCGAGGTGG	192-404-487	45X(95℃ 20”,56℃ 20”,72℃45”)
exIII fwd	AGCCCAGTTCCACCAGGTGAG	165	45X(95℃ 20”,56℃ 20”,72℃45”)
exIV fwd	ACCGAAGTCTTCCCCAGAGCAG	219	45X(95℃ 20”,56℃ 20”,72℃45”)
exV fwd	ACCAATAGCCCCCATGCTCTG	227	45X(95℃ 20”,55℃ 20”,72℃45”)
exVI fwd	TGGAGCCAGAATCGGAACCAC	209-227	45X(95℃ 20”,56℃ 20”,72℃45”)
exVII fwd	CCACATCTCTACCCATCCTGC	305	45X(95℃ 20”,56℃ 20”,72℃45”)
exVIII fwd	TGGCATGACTGTGCATCCCAG	253	45X(95℃ 20”,56℃ 20”,72℃45”)
exIXa fwd	ACAATCAGATGGGCCACATG	238	45X(95℃ 20”,56℃ 20”,72℃45”)
com rev	ACGCTCTCCAGAGTCCCATG	/	/
cds fwd	AAACATCCGAGGACAAGGTGGC	230	45X(95℃ 20”,56℃ 20”,72℃45”)
cds rev	GGCACTTGACTACTGAGCATCACC	“	“

Table 2 List of human primers, product size and PCR conditions used for real-time PCR experiments.

3.3.3 ELISA assay for total BDNF detection

BDNF production in differentiated SK-N-BE neuroblastoma cells was assessed using the ELISA BDNF Emax® Immuno-Assay System (Promega Corporation, Madison, WI, USA) at the same time points evaluated in real-time PCR. Briefly, cells in treated and control conditions, as described above, were lysed and 12.5 µg of total protein were loaded on pre-coated wells according to manufacturer’s instructions.

3.3.4 Western blotting

Differentiated SK-N-BE cells were treated, as described above, and lysed on ice in lysis buffer [125 mM Tris-HCl pH 7.5, 2% SDS, protease inhibitor cocktail (Sigma)]. Proteins were resolved into 12% SDS-PAGE, loading equal volumes (20 µl) of the lysates and electroblotted onto nitrocellulose membrane. Membranes were cutted above the 35 KDa marker band and differentially processed. The lower parts were blocked with fat-free 5% milk in TBS-T (50 mmol/L Tris-HCl pH 8.0, 133 mmol/L NaCl, 0.1% (v/v) Tween-20) and then incubated at 4℃ in overnight gentle-shaking w ith anti-BDNF N-20 antibody (1:500 in TBS-T; Santa Cruz). After three wash steps with TBS-T, the membranes were incubated 1 hour at room temperature with anti-rabbit HRP-conjugated antibody (1:2000 in TBS-T; Dako). The upper part of the membranes were blocked with fat-free 5% milk in PBS-T (Phosphate Buffer Saline, 0.1% (v/v) Tween-20), then incubated overnight at 4℃ with anti-αTubulin antibody (1:8000 in blocking solution; Sigma Aldrich); after three washing steps, a further incubation of 1 hour at room temperature with anti-mouse HRP-conjugated antibody (1:10000 in PBS-T; Sigma) was performed. Thereafter, all the membranes were washed three times and developed with ECL Prime (GE Healthcare). Densitometric analyses of the western blots were performed using Quantity One software (Bio-Rad), according to user manual.

3.3.5 Luciferase assay

Luciferase assays, aimed to evaluate the translatability capacity of BDNF isoforms, were performed as described previously (Vaghi et al., 2014). Briefly, plasmid vectors containing 5' UTR exons 1, 2c, 4 or 6 and 3'URT long cloned at the edges of a *Firefly* luciferase (Fluc) reporter were used to test the influence of BDNF UTRs on translatability in

stimulated conditions. Around 3000 SK-N-BE cells per well were seeded in 96-well plate designed for luminescence assays (BD Falcon) and induced to differentiate, as described. Differentiated cells were co-transfected with one of the above construct along with a vector containing a *renilla* (Rluc) reporter as transfection control. Transfection was stopped after 6 hours by replacing media or starting the 24 hour-point cisplatin treatment with/without Aurora kinase inhibitor in SFM. The day after, a 6 hour-point treatment was also performed before carrying out the dual-luciferase assay in an automated Glomax plate reader (both from Promega), according to manufaturer's instructions.

3.3.6 Cell-survival analysis

A colorimetric MTT assay was performed to assess cell survival. 5000 SK-N-BE cells were plated in 96-well plates, maintained, let to differentiate then treated, as described in "Real-Time PCR" paragraph. Viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT from Sigma) following manufacturer's instructions.

3.3.7 Immunofluorescence and microscopy

Immunofluorescence on SK-N-BE cells was performed as follow. After fixation in 4% paraformaldehyde (PFA) in phosphate-buffer-saline (PBS) for 20 minute at room temperature, cells were washed with PBS and permeabilized for 15 minute with PBS-Triton 0.1% (PBS-T), then blocked with PBS-T and 2% BSA. Thereafter, cells were incubated for 2 h at room temperature with primary antibodies diluted 1:50 in blocking solution. After three washes in PBS, cells were incubated 1h at room temperature with secondary antibodies coupled with Alexa 488 or 568, diluted 1:200 in PBS-T, then wash with PBS and stained with Hoechst to mark nuclei. Finally, coverslips were mounted in Mowiol antifade compound (Sigma). The following antibodies were employed: Mouse

monoclonal anti-BDNF (Sigma); Rabbit polyclonal anti-actin (Sigma); anti-mouse Alexa Fluo 488 (Invitrogen); anti-rabbit Alexa Fluo 568 (Invitrogen).

Images were acquired using a Nikon Eclipse E800 epifluorescence microscope with a 20X objective and a Nikon DXM1200 camera, paired with ACT-1 software. Cells were manually counted dividing the image in five subfields and averaging the number over three areas per coverslip. The number of cells is expressed as relative percentage to untreated condition. BDNF spots were counted using ImageJ 1.45b (NIH, Bethesda, USA) and expressed over the cell area in μm^2. Briefly, images were processed for z-stack projections and thresholds were applied to highlight spots and cells area and then analyzed using the Analyze Particle tool. All images were processed in the same manner.

3.3.8 Statistical analyses

All statistical analysis and graphs were performed using SigmaPlot 11.0 (Systat Software, Inc.). One-way ANOVAs followed by Holm-Sidak corrections against control were performed to compare treated groups against controls in order to determine statistical significances, which was set at $p<0.05$.

4. RESULTS

4.1 AIM1- A standardized methodology for human BDNF collection and analysis

As already introduced, BDNF has an attractive potential in aid the diagnosis or monitoring therapies of neuropsychiatry disorders; however, inconsistencies between studies have prevented its clinical use. Our working hypothesis was that the lack of reproducibility described in the literature could be the result of methodological dissimilarities among the different studies at the pre-analytical stage (sample preparation and storage), the analytical stage (analysis execution) or assay-related (intrinsic assay quality). Therefore, we undertook a study aimed at standardizing the methodology to measure BDNF in human serum. Accordingly, we first defined a methodology for sample preparation and storage and then we measured BDNF concentration in the sera from 40 healthy volunteers using 6 different commercial kits and we compared their performance.

4.1.1 Pre-analytical stage

To standardize the pre-analytical stage, we reviewed the current literature on serum BDNF measurement and we evaluated the different methods used for sample preparation and conservation (see table 3). Our approach was to identify similarities among the different studies and define the confidence intervals of the main technical determinants in each step. We first analysed the available literature regarding the patient preparation and the effect of fasting or not fasting blood draw condition in adults and children. El-Gharbawy and colleagues (El-Gharbawy et al., 2006) reported no difference in BDNF levels between fasting and non-fasting children while in adults, non-fasting conditions resulted in attenuated BDNF levels (Bus et al., 2011). Hence, our protocol included blood draw from

adults in fasting conditions. Concerning the effect of the time of blood withdrawal on BDNF levels, data in the literature are controversial. Piccinni (Piccinni et al., 2008) did not observe circadian BDNF variation, neither in men nor in women. On the contrary, Bus and colleagues (Bus et al., 2011; Bus et al., 2012) reported attenuated BDNF serum level from the late morning onward. Giese et al. (Giese et al., 2014) found a reduction trend during the day, which was not statistically significant. However, circadian variation in plasma BDNF level has been described by many authors (Begliuomini et al., 2008; Choi et al., 2011), reporting a peak in the early morning and the lowest concentration by the late evening. Hence, our protocol included blood withdraw between 8 and 12:00. Concerning the clotting time and temperature, there are only few studies taking into account this issue as a possible source of variability in the detection of BDNF serum levels. Katoh-Semba et al. (Katoh-Semba et al., 2007) stated that at 26℃, plasma BDNF starts to be degraded after 30 min, while it reaches a maximum level at 4℃ between 24 h and 42 h after collection. On the other hand, Elfving and colleagues (Elfving et al., 2010) found that BDNF levels in serum were stable for 24 h at room temperature. A recent work from Giese (Giese et al., 2014), established that the mean BDNF concentration is not significantly different between 1 and 5 hours of clotting time at room temperature; moreover, for samples clotted 1 hour at room temperature, followed by 1 hour at 4℃, the results were strictly similar to those obtained after 1 hour at room temperature (Maffioletti et al., 2014). Therefore, in order to have more flexibility during preparation of the sera, our protocol included 1 hour incubation at room temperature, followed by 1 hour at 4℃. Concerning the centrifugation speed, time and temperature, there are no specific data on this topic, however, in the majority of the studies, the centrifugation temperature is either at room temperature or 4℃, while speed and time ranges are between 1000-3500 g and 10-20 minutes, respectively. On this basis, we selected the shortest centrifugation time (10 min)

at an intermediate gravity force (2000 g) and, in order to maximize BDNF conservation, we performed centrifugation at the temperature of 4℃. Recent studies highlighted that the storage temperature of the sample is a critical issue for BDNF degradation. It has recently been demonstrated that samples stored at -20℃ show a significantly lower BDNF concentration already after 6-10 months but, if stored at -80℃, the decline is not significant up to 5 years (Bus et al., 2011; Trajkovska et al., 2007). In this study, aliquots of 50 µl serum samples were stored at -80℃ in thin wa ll 0.2 ml PCR tubes arranged in strips of 8 tubes with attached flat lid (Starstedt, Multiply® µStripPro).

SERUM PREPARATION STEP	INTERVAL OF CONFIDENCE	CHOSEN PROTOCOL	REFERENCES
Patient preparation	- Preferentially fasting condition for adults - Indifferent for children	Adult in fasting conditions	- **Bus BAA,** Psychoneuroendocrinology (2011) 36:228—239 - **El-Gharbawy A H,** The Journal of Clinical Endocrinology & Metabolism (2006) 91(9):3548–3552
Time of blood withdrawal	Data are controversial: little or no circadian variation for serum BDNF – possibly a little decrease of serum BDNF in the afternoon	9 .00 -12.30 a.m.	- **Bus BAA,** The World Journal of Biological Psychiatry (2012) 13: 39–47 - **Bus BAA,** Psychoneuroendocrinology (2011) 36:228—239 - **Begliuomini S,** The Journal of Endocrinology (2008) 197:429–435 - **Piccinni A,** Chronobiol Int. (2008) 25(5):819-26. - **Katoh-Semba R**, Int. J. Devl Neuroscience (2007) 25:367–372 - **Choi S-W**, Psychiatry Research (2011) 186:427–430 - **Giese M**, Journal of Psychiatric Research 59 (2014) 1-7
Clotting time and temperature	30 min to 24h, RT or 1h to 42h, 4℃	1h, RT+ 1h, 4℃	- **Elfving B**, Journal of Neuroscience Methods 187 (2010) 73–77 - **Katoh-Semba R**, Int. J. Devl Neuroscience 25 (2007) 367–372 - **Maffioletti E**, BioTechniques (2014) 57:111-114
Centrifugation time, speed and temperature	No specific studies - 1000 g to 3500 g - 10 min to 20 min - 4℃ or RT	2000g x 10 min at 4℃	Most of the published data
Storage temperature	- 20℃ up to 3-6 months - 80℃ up to 5 years	- 80℃	- **Trajkovska V**, Brain Research Bulletin (2007) 73:143–149 -**Bus BAA,** Psychoneuroendocrinology (2011) 36:228—239

Table 3 Definition of steps protocol for serum preparation to BDNF assessment, based on literature comparison.

4.1.2 Analytical stage

To standardize the analytical stage, we considered five sandwich ELISA assays from different companies (Aviscera-Bioscience, Biosensis, Millipore-Chemikine™, Promega-Emax® and R&D System-Quantikine®) and one multiplexing assay (Millipore-Milliplex®). The main characteristics of the kits and their performance, as declared by the manufacturers, are described in Table 4. BDNF concentration was assessed using the six kits in the sera prepared from 40 healthy blood donors (mean age 54±6 years; 18/22 Females/Males ratio). Analysis of BDNF serum concentration was carried out at controlled room temperature (24°±1°C) and repeated measures we re carried out by the same experimenter using two aliquots of the same sera stored at -80°C and then thawed only once at the time of usage. Whenever a positive control and/or an additional point of the standard curve were requested by the kit, the samples tested were lowered to 38 (Aviscera-Bioscience and Millipore-Milliplex®) or 39 (Biosensis). Sample recovery was 100%, as we always found measurable concentrations of BDNF, irrespectively of the kit used. Distributions of values were not normal and therefore data are shown as box plot with indicated median value and 10th, 25th, 75th, 90th percentile (Figure 4.1). Serum BDNF concentrations resulted to be in the same range for all kits with the exception of Millipore-Chemikine™, which showed a significantly lower median value and distribution range ($p<0.05$, ANOVA on ranks; Figure 4.1, Table 5). Median values and percentiles for the other five kits were comprised between a minimum of 18.2 (14.2-22-2) to a maximum 25.5 (21-30.5) ng/ml (not significant, Table 5).

Company	**Aviscera-Bioscience**	**Biosensis**	**Millipore**	**Millipore**	**Promega**	**R&D System**
KIT Name	**Human BDNF ELISA**	**BDNF *Rapid*™ ELISA**	**Chemikine™**	**Milliplex®**	**BDNF Emax® Immuno-Assay System**	**Quantikine®**
Catalog Number	# SK00752-01	# BEK-2211-2P	# CYT306	# HPTP2MAG-66K	# G7610	# DBD00
Principle of the assay	Sandwich ELISA	Sandwich ELISA	Sandwich ELISA	Luminex®/ xMAP® technology	Sandwich ELISA	Sandwich ELISA
Sensitivity (pg/ml)	5-8	2	7.8	2.5	15.6	20
Range of detection (pg/ml)	23-1500	7.8-500	7.8-500	12 - 50000	7.8-500	62.5-4000
BDNF standard	Human recombinant 1500 pg/vial, 1 vial	Human recombinant 1000 pg/vial, 4 vials	Human recombinant 10000 pg/vial, 2 vials	Mix of BDNF 12500 pg +Prolactin 25000 pg/vial, 1 vial	BDNF standard (not declared type), 1ug/ml, 50 ul	Human recombinant 8000 pg/vial, 3 vials
Coating/capture Antibody	Pre-coated α-BDNF antibody (type not declared)	Pre-coated α-BDNF antibody (mouse monoclonal)	Pre-coated α-BDNF antibody (mouse monoclonal)	Mix of: magnetic beads coated with α-BDNF Ab OR α-Prolactin (types not declared)	Manually coating with α-BDNF Ab (mouse monoclonal) 1000X, 20ul	Pre-coated α-BDNF antibody (mouse monoclonal)
Primary detection antibody	Biotinilated α-BDNF antibody (type not declared); 105 ul/vial, 10X, 1 vial	Biotinilated α-BDNF antibody (maybe mouse monoclonal); 110 ul/vial, 100X, 2 vial	Biotinilated α-BDNF antibody (mouse monoclonal); 25 ul/vial, 1000X, 1 vial	Mix of: Biotinilated α-BDNF antibody AND α-Prolactin (type not declared); 3200 ul/vial, 1X, 1 vial	α-BDNF Ab (chicken polyclonal) 500X, 20ug	α-BDNF antibody (mouse monoclonal)-HRP conjugated 11000 ul/vial, 1X, 1 vial
Type of secondary detection	Streptavidin-HRP conjugate, 60ul/vial, 200X, 1 vial	Streptavidin-HRP conjugate, 110ul/vial, 100X, 2 vial	Streptavidin-HRP conjugate, 50ul/vial, 1000X, 1 vial	Streptavidin-Phycoerythrin conjugate, 5500ul/vial, 1X, 1 vial	Anitibody α-IgY-HRP conjugated, 100ul/vial, 200X, 1 vial	/
Sample dilution (used)	1:40	1:200	1:200	1:10	1:200	1:20
Declared species cross-reactivity	Only human	Human, mouse, rat and others	Human and rat	Only human	Not declared	Only human
Processing time	6-7 hours	3-4 hours	Overnight incubation (16 hours) + 5-6 hours =21-22 hours	Overnight incubation (16 hours) + 3-4 hours =19-20 hours	Overnight incubation (16 hours) + 7-8 hours =23-25 hours	4-5 hours

Table 4 BDNF ELISA Kits. Description and main characteristics of the tested BDNF ELISA kits

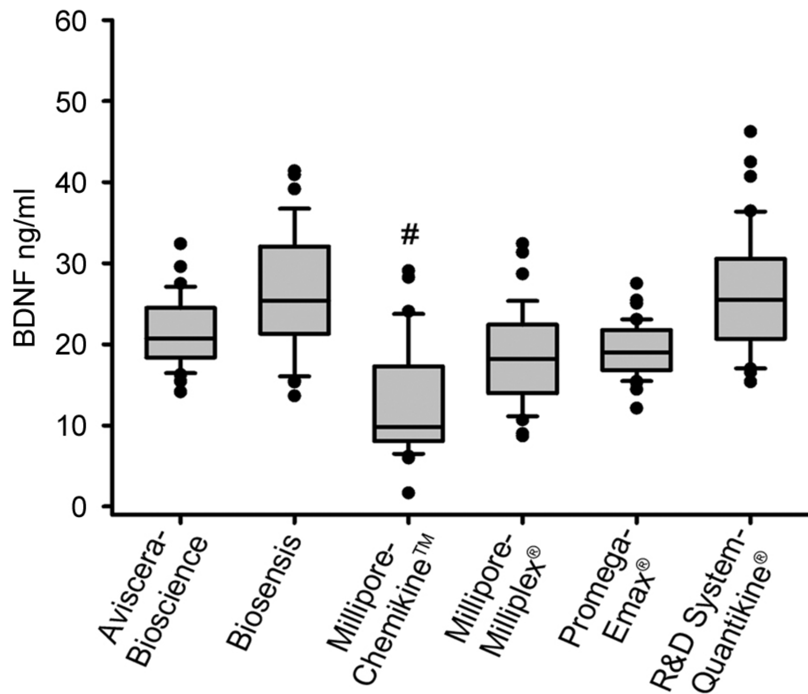


Figure 4.1 BDNF serum levels measured with the indicated ELISA assays. Box plot of serum BDNF concentrations (ng/ml) from healthy volunteers (n=38-40, see Table 5) represented as mean of two independent measures. The upper line of the box marks the 75th percentile, the middle line is the median value and the lower line specifies the 25th percentile. Whiskers above and below the box indicate the 90th and 10th percentiles, respectively. Dots indicate the outlier values within each group. ($^{\#}P<0.05$ using ANOVA on ranks test with respect all tested kits).

Intrinsic assay quality was evaluated by testing the intra-assay and the inter-assay coefficients of variation (CV) for each kit brand (Table 4). Intra-assay CV was assessed by comparing BDNF concentration values measured twice into the same plate, for each subject. Five kits presented values within the declared CV, with the only exception of Millipore-Milliplex® which showed a higher than expected intra-assay CV (13-14% versus <10%).

Number of plates tested: 2	Aviscera-Bioscience (n=38)	Biosensis (n=39)	Millipore Chemikine™ (n=39)	Millipore Milliplex® (n=38)	Promega Emax® (n=40)	R&D System Quantikine® (n=40)
Range ng/ml: min-max	14.1 - 32.4	13.6 - 41.4	1.7 – 29.1	8.7 - 32.4	12.1 – 27.5	15.4 – 46.2
Median ng/ml (25% - 75%)	20.7 (18.5-24.4)	25.4 (21.5-32.0)	9.8 (8.1-17.2)	18.2 (14.2-22.2)	19.0 (16.9-21.7)	25.5 (21.0-30.5)
Declared intra-assay CV	4 – 6 %	2 – 6 %	4 %	< 10 %	2 – 9 %	4 – 6 %
Tested intra-assay CV	2 % - 3 %	1 % - 1 %	2 % - 3%	14 % - 13 %	3 % - 7 %	6 % - 5 %
Declared inter-assay CV	8 - 10 %	5 - 8 %	9 %	< 10 %	< 9 %	8 – 11 %
Tested inter-assay CV	12 %	5 %	38 %	18 %	16 %	20 %

Table 5 BDNF ELISA kits performance. Comparison between declared performances and actual tested performances of the specified BDNF ELISA kits. Median and range values, intra- and inter-assay coefficients of variation (CV) were assessed by measuring BDNF serum level from healthy volunteers (n=38-40). All the kits, except Milliplex®, which is based on multiplex technology (Luminex®/xMAP®), are based on classical sandwich ELISA.

Measurements of BDNF concentrations were repeated on a different day by the same experimenter using two different plates of the same kit and then, inter-assay reproducibility was verified by one-way ANOVA for repeated measures (Figure 4.2). With the only exception of Biosensis, which showed reproducible results, five kits provided significantly different results between plate at Day 1 and plate at Day 2 (Figure 4.2). As a consequence, the inter-assay CV tested showed values higher than the declared one, with the exception of Biosensis (5% tested, versus 5-8% declared). The worst performance was obtained by the Millipore-Chemikine™ kit, with a 38% of inter-assay CV, which is far from the 9% stated by the manufacturer.

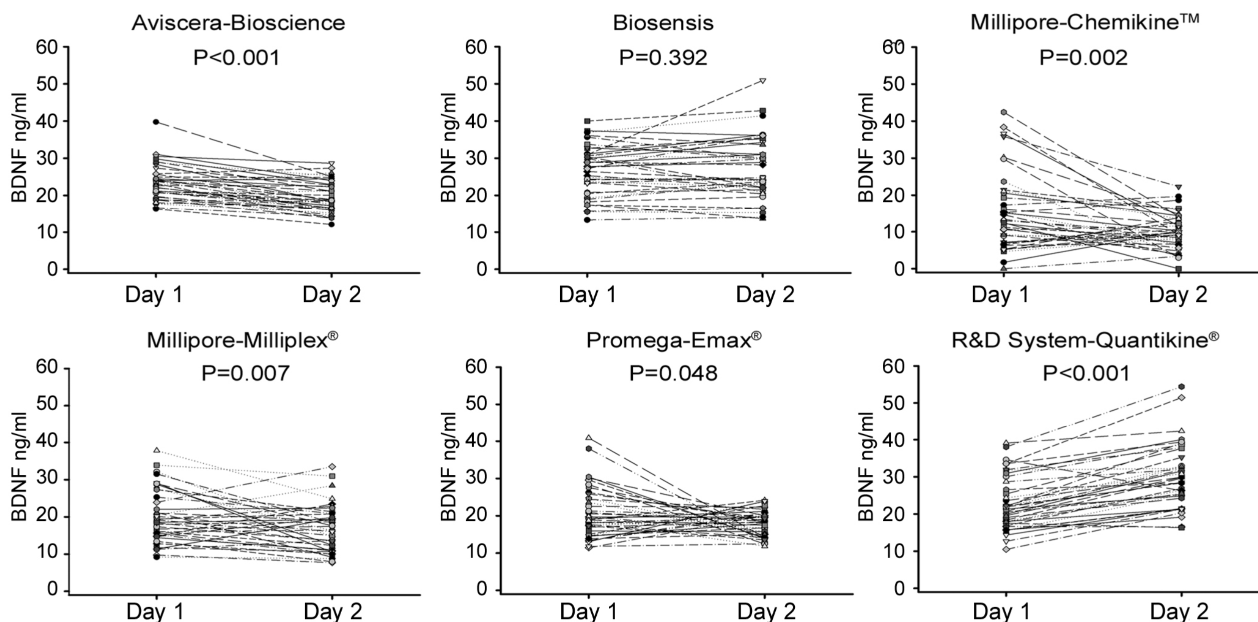


Figure 4.2 Inter-assay variation of the BDNF ELISA kits. Scatter plot showing the BDNF values distribution measured by the same operator on two different days using two plates of the same lot for each brand (Day 1 & Day 2). Each dot represents a BDNF value from one subject and the dashed lines link together two assessments of the same subject. The reproducibility was checked performing one-way ANOVA for repeated measures and the P values are specified. All tested kits except Biosensis showed a significant difference between the repeated measures of the same sera.

To complete the assessment on the assay performance, we verified if the detection antibodies of each kit were able to recognize mature BDNF or proBDNF, or both, in a dot blot assay (Figure 4.3). In the case of the Promega kit, also the capture monoclonal antibody was available (mAb). As a negative control, we spotted on the same membrane strip Bovine Serum Albumin (BSA) and, as a positive control, the BDNF standard provided in each kit (standard-BDNF; Figure 4.3 A). In addition, a monoclonal anti-BDNF antibody commercially available from Sigma was tested on mature BDNF or proBDNF. Results demonstrated similar reactivity of the antibodies against commercial mature BDNF from Alomone Labs or Sigma (1000 pg). Biosensis, Millipore (both kits) and Promega antibodies reacted also with pro-BDNF while those from Aviscera-Bioscience and R&D System-

Quantikine® showed only marginal reactivity to pro-BDNF (Fig. 4.3 A). Of note, Aviscera-Bioscience and R&D System-Quantikine® kits claimed to be specific for mature BDNF and to cross-react by a 13 % with the human pro-BDNF. Since some antibodies resulted to have a very strong reactivity against pro-BDNF, the spotted quantity of pro-BDNF was reduced (1:100) respect to mature BDNF. To better evaluate reactivity against pro-BDNF, the central region of the dot-blot shown in Figure 4.3 A was overexposed (Figure 4.3 B; 10 pg of pro-BDNF). Moreover, Figure 4.3 C shows the reactivity of Promega (pAB), Biosensis and Sigma against the same amount of pro-BDNF and mature-BDNF (100 pg) spotted on the substrate, highlighting a stronger reactivity of these antibodies against pro-BDNF with respect to mature-BDNF.

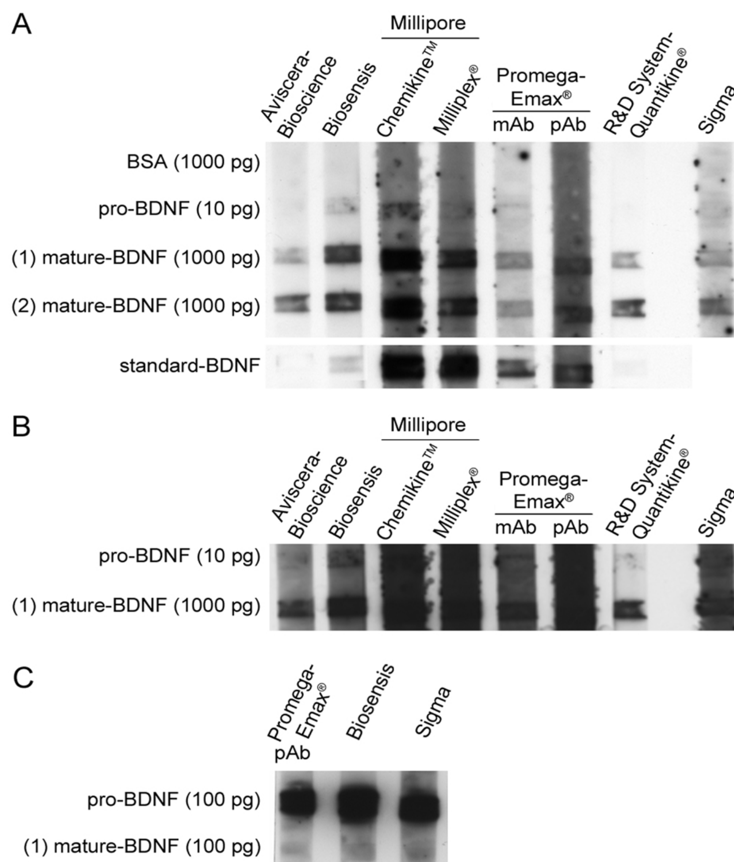


Figure 4.3 Line-blot for qualitative analysis of anti-BDNF antibodies specificity. A) The antibodies from each ELISA kit were tested for specificity against pro-BDNF or mature-BDNF. The BDNF standards blotted were commercial pro-BDNF (Alomone; 10 pg/lane), mature-BDNF (1 and 2 from Alomone and Sigma,

respectively; both 1000 pg/lane) and the standard-BDNF protein included in each kit (Aviscera and Biosensis: 10 pg/lane; Millipore-Chemikine™, Millipore-Milliplex®- and R&D System-Quantikine®: 100 pg/lane; Promega-Emax®: 1000 pg/lane). BSA (1000 pg/lane) was used as a negative control. The mouse monoclonal anti-BDNF antibody, (1:1000; Sigma) was tested as a control. B) Central region of the same blot shown in A, from an overexposed film to better visualize the reactivity against pro-BDNF. C) Reactivity of antibodies from Biosensis, Promega pAb and Sigma on a dot blot in which the same quantity of pro-BNDF and mature BDNF were spotted (100 pg each). Each antibody from the ELISA kits was used at the dilution suggested by the manufacturer's instructions. mAb: Promega monoclonal capture antibody for plate coating. pAb: Promega polyclonal detection antibody.

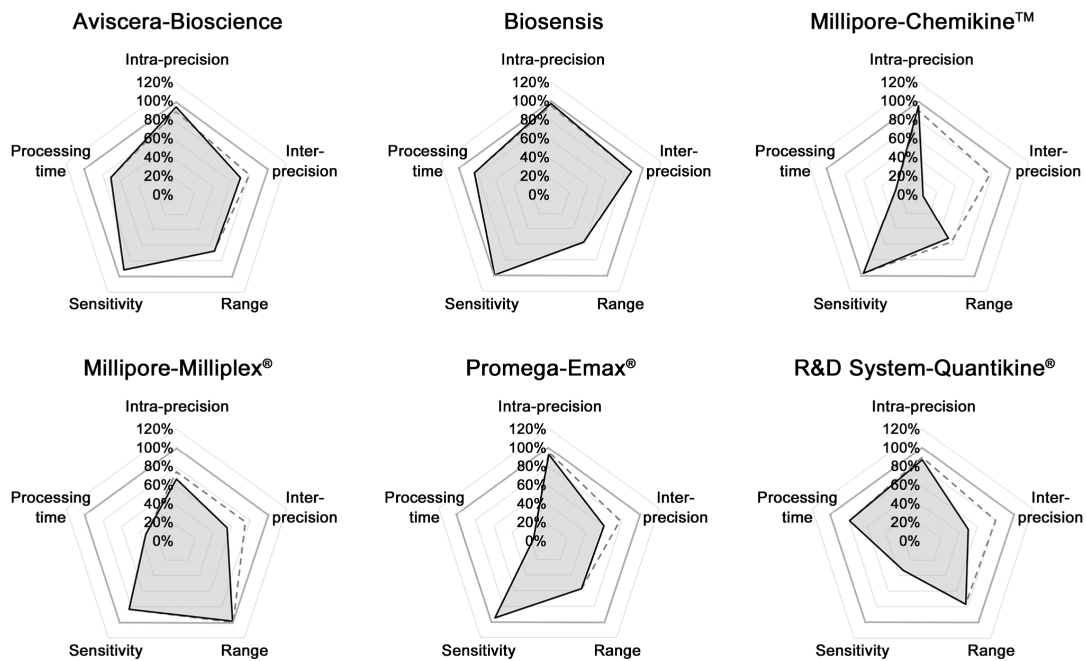


Figure 4.4 Graphic summary of BDNF ELISA kit performances. Polar plot showing the kit performances based on intra-precision (1 - intra assay coefficient of variation), inter-precision (1 -inter assay coefficient of variation), range, sensitivity and processing time. The black solid line indicates the assessed performances, while the dashed line shows those declared by the manufacturers. To obtain a graphical comparison, the best value for each factor among the six kits was set as the reference (100%; bold grey line); the highest the value, the better was the score. For sensitivity, the best value was defined after correction for the dilution factor. Concerning the processing time, values were normalized against an ideal value set as t=0 hour (100%) over 24 hour (0%); the higher the value, the faster the processing. The declared processing times were essentially equal to the effective times.

The performance of the six BDNF kits examined is summarized graphically in Figure 4.4. The largest area corresponds to the most performant assay, taking as 100% the best performance measured among the six kits for five parameters: intra-assay variation, inter-assay variation, detection range, sensitivity and processing time (see figure legend for definitions).

We found that five out of six kits (Aviscera-Bioscience, Millipore-Chemikine™ and Milliplex®, R&D System-Quantikine® and Promega-Emax®) exhibited a high inter-assay variation, because two measurements of the same serum sample performed in two distinct days were significantly different.

4.2 AIM 2- BDNF and immune mediators in job-related chronic stress

4.2.1 Demographic variables

We undertook a study aimed at address a possible relationship between the serum levels of BDNF, other trophic factors, cytokines and chemokines with the scores of job-related psychophysical stress and burnout. As described in materials and methods, 122 subjects among Italian healthcare assistants match the inclusion criteria for this study; demographic values as age and body mass index (BMI), separated by gender, are given in table 6.

		N	Mean	SD	Min/Max	P
Age, years	Female	87	44.92	8.29	25/60	0.434
	Male	35	46.23	10.98	25/67	
BMI, kg/m^2	Female	86	23.03	2.75	17.72/33.46	<0.001
	Male	35	25.75	3.50	17.63/33.56	

Table 6. Age and BMI distributions split by gender. Population sizes (N), average values (mean), standard deviations (SD) and minimum and maximum values (min/max) are given, along with p values (P) marked in bold.

Of note, BMI scores in female were significantly lower than those in male population (Mann-Whitney U test, $z=-4.508$, $p<0.001$).

Psychophysical stress was assessed using five items, namely anxiety, emotion (depression-like), gastrointestinal disturbances, cardiac disturbances, ergonomic dysfunction at the workplace and by an overall "stress" item, defined as the average of the five items. The burnout state was assessed using the three items of emotional exhaustion, depersonalization, and personal accomplishment. The score distributions of both psychophysical stress and burnout state are given in table 7 (A and B respectively). Differences in the number of valid cases are because many subject missed to answer few item questions, so were treated as invalid.

A

Statistics							
		Anxiety	Emotion	Gastric	Cardiac	Ergonomics	Stress
N	Valid	115	115	115	115	115	115
	Missing	7	7	7	7	7	7
Mean		2.41	1.77	2.07	1.58	2.59	2.08
Std. Deviation		1.04	0.80	1.01	0.85	1.13	0.81
Minimum		1.00	1.00	1.00	1.00	1.00	1.00
Maximum		5.40	5.00	6.00	5.00	5.40	5.16

B

Statistics				
		Emotional Exhaustion	Depersonalization	Personal Accomplishment
N	Valid	118	121	122
	Missing	4	1	0
Mean		22.77	9.66	34.52
Std. Deviation		7.30	3.60	4.94
Minimum		9.00	5.00	24.00
Maximum		41.00	22.00	47.00

Table 7. A) Scores distribution of psychophysical stress items B) Scores distribution of burnout state items

The first step was to verify if the demographic variables account for distribution differences among the different items. Indeed, performing a non-parametric Kruskal-Wallis test, we found that female subjects had significantly higher score values than male for all the psychophysical stress items, as shown in table 8. Concerning the burnout state, female display higher score values only for emotional exhaustion but not for depersonalization,

which is higher in male; no differences between male and female in the scores of personal accomplishment.

Psychophysical	Female. N=82; Mean rank	Male. N=33; Mean rank	Chi-Square	df	Asymp. Sig. P
Anxiety	62.86	45.92	6.11	1	**0.013**
Emotion	63.40	44.59	7.56	1	**0.006**
Gastric	62.53	46.74	5.34	1	**0.021**
Cardiac	62.15	47.68	5.01	1	**0.025**
Ergonomics	62.51	46.80	5.24	1	**0.022**
Stress	63.68	43.88	8.30	1	**0.004**
Burnout	Female N; Mean rank	Male N; Mean rank	Chi-Square	df	Asymp. Sig. P
Emotional Exhaustion	83; 64.47	35; 47.71	5.92	1	**0.015**
Depersonalization	87; 56.81	34; 71.72	4.46	1	**0.035**
Personal Accomplishment	87; 59.25	35; 67.09	1.23	1	0.267

Table 8. Results of comparison (Kruskal-Wallis test) between female and male scores of psychophysical and burnout items. Sizes, (N), rank values, chi-square, degrees of freedom (df) and asymptotic p values (Asymp. Sig. P) are given. In bold are highlighted $p<0.05$.

Regarding the confounding effect of BMI, after controlling for gender in a partial correlation analysis, we found that it negatively correlates with anxiety, gastrointestinal disturbance items and the overall stress state (see table 9 for correlation, df and p values). Differently, BMI did not correlate with any of the burnout item, even after gender control.

Moreover, age did not correlate with any of the items, neither from psychophysical nor burnout states, even after controlling for gender or BMI (not shown). Of note, age showed a partial positive correlation with BMI, after controlling for gender (r=226, df=118, p=0.013).

Due to given considerations, the variable “age” has not been considered any further, while corrections for gender and BMI have been performed in all subsequent analysis.

Partial Correlations							
Control Variable: Gender		Anxiety	Emotion	Gastric	Cardiac	Ergonomics	Stress
BMI (kg/m2)	Correlation	-0.230	-0.149	-0.295	-0.144	-0.054	-0.209
	Significance (2-tailed)	**0.014**	0.116	**0.002**	0.129	0.570	**0.026**
	df	111	111	111	111	111	111

Table 9. Results of partial correlation analysis, controlling for gender, between BMI and the psychophysical stress items. Significant p values (p<0.05) are highlighted in bold; df, degrees of freedom.

4.2.2 Biochemical variables

The pre-analytical stages, consisting in serum samples collection and storage, were performed as described in the previous section (AIM 1). For the analytical stage, taking advantage of the multiplex technology, we screened 48 analytes among cytokines, chemokines and trophic factors; BDNF was assessed using the Biosensis kit, which exploits a sandwich ELISA technology and which resulted to be the most reliable among six kits tested (see AIM1, 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): IL-2, IL-15, IL-1α, IL-3, IL-12p40, IFN-α2, LIF, MCP-3/CCL7, M-CSF/CSF1, β-NGF, TNF-β. The distribution values of the remaining analytes (38) are listed in table 10.

Statistics							
Variable ID (pg/ml)	N		Median	Minimum	Maximum	Percentiles	
	Valid	Missing				25 %	75 %
IL-2Rα	122	0	43.44	0.07	163.78	21.38	61.80
IL-16	122	0	61.91	0.13	842.08	37.53	93.70
IL-18	122	0	38.74	3.12	184.56	24.06	53.88
CTACK/CCL27	122	0	510.01	124.57	1335.66	389.95	654.04
GRO-α/CXCL1	122	0	52.62	0.24	253.39	28.56	84.34
HGF	122	0	424.84	66.67	1707.16	276.12	595.51
MIF	122	0	308.09	18.51	7937.97	198.25	438.75
MIG/CXCL9	122	0	426.46	68.47	3821.65	272.02	577.60
SCF	122	0	61.21	0.25	261.82	37.41	95.12
SCGF-β/CLC11	122	0	19584.75	398.76	68535.28	12747.37	28157.71
SDF-1α/CXCL12	122	0	99.24	0.29	438.52	46.54	164.28
TRAIL/TNFSF10	122	0	43.07	0.46	557.24	0.46	69.81

IL-1β	121	1	1.56	0.24	7.57	0.97	1.89
IL-1Rα	121	1	104.04	11.71	2462.88	68.79	150.97
IL-4	121	1	2.79	0.95	5.51	2.19	3.32
IL-5	121	1	1.47	0.02	11.37	0.29	2.37
IL-6	121	1	5.15	0.86	280.59	3.62	7.57
IL-7	121	1	6.81	1.68	33.64	4.75	9.75
IL-8/CXCL8	121	1	15.24	3.26	34.12	12.32	20.04
IL-9	121	1	11.57	3.00	785.62	8.36	19.83
IL-10	121	1	4.25	0.01	277.64	2.26	7.68
IL-12 (p70)	121	1	30.37	0.03	787.62	16.76	50.00
IL-13	121	1	4.92	0.30	40.25	3.19	7.05
IL-17	121	1	47.95	0.22	307.36	17.27	88.08
Eotaxin/CCL11	121	1	139.26	8.71	2109.58	88.17	202.52
FGF basic	121	1	63.88	22.46	277.42	48.61	83.74
G-CSF	121	1	29.28	9.92	140.54	21.86	42.40
GM-CSF	121	1	11.60	0.15	172.01	2.42	20.70
IFN-γ	121	1	147.34	25.32	621.07	96.56	182.18
IP-10/CXCL10	121	1	917.03	302.60	3190.41	662.03	1216.50
MCP-1/CCL2 (MCAF)	121	1	23.85	3.18	82.55	16.41	33.39
MIP-1α/CCL3	121	1	3.89	1.43	15.00	2.94	5.40
PDGF-BB	121	1	4579.39	1504.00	12052.56	3597.62	6303.76
MIP-1β/CCL4	121	1	85.06	23.18	661.33	62.62	103.67
RANTES/CCL5	121	1	16944.41	3746.43	71382.91	12216.15	21775.42
TNF-α	121	1	23.90	7.75	4710.80	19.41	30.13
VEGF	121	1	66.53	13.83	610.35	39.40	112.03
BDNF (ng/ml)	120	2	28.61	11.66	54.02	22.19	34.82

Table 10. Distribution values in pg/ml of circulating cytokines, chemokines and growth factors.

Because 30 persons out of 122 use pharmaceutical drugs, we verified if drug assumption has an impact on the analytes levels. As a result, many of the analytes (21) showed significant different levels between cases who use drugs and who do not (Kruskal-Wallis test; see table 11). In general, drugs had a negative influence on analytes level, with the exception of INF-γ, MIP-1β and RANTES, of which we observed increased median values in drug users.

Variable ID	NO Drugs N; Mean rank	Drugs N; Mean rank	Chi-Square	df	Asymp. Sig. P
IL-2Rα	92; 70.41	30; 34.17	23.86	1	<0.001
IL-16	92; 67.92	30; 41.82	12.37	1	<0.001
IL-18	92; 69.52	30; 36.90	19.25	1	<0.001
CTACK/CCL27	92; 66.08	30; 47.47	6.27	1	0.012
GRO-α/CXCL1	92; 67.49	30; 43.13	10.79	1	0.001
HGF	92; 66.46	30; 46.28	7.37	1	0.007
MIF	92; 68.57	30; 39.82	14.96	1	0.000
MIG/CXCL9	92; 65.16	30; 50.27	4.01	1	0.045
SCF	92; 66.20	30; 47.10	6.61	1	0.010
SDF-1α/CXCL12	92; 65.88	30; 48.08	5.77	1	0.016
TRAIL/TNFSF10	92; 71.45	30; 30.98	30.55	1	<0.001
IL-5	91; 69.46	30; 35.33	21.81	1	<0.001
IL-7	91; 68.25	30; 39.00	15.70	1	<0.001

IL-10	91; 67.16	30; 42.32	11.32	1	0.001
IL-13	91; 65.90	30; 46.13	7.17	1	0.007
IL-17	91; 65.36	30; 47.77	5.68	1	0.017
FGF basic	91; 66.40	30; 44.63	8.69	1	0.003
IFN-γ	91; 55.08	30; 78.97	10.47	1	0.001
MIP-1α/CCL3	91; 66.45	30; 44.47	8.86	1	0.003
MIP-1β/CCL4	91; 55.95	30; 76.33	7.62	1	0.006
RANTES/CCL5	91; 56.11	30; 75.83	7.13	1	0.008

Table 11. Comparison (Kruskal-Wallis test) between distribution levels of analytes in subjects how use drugs and how do not. Sizes, (N), rank values, chi-square, degrees of freedom (df) and asymptotic p values (Asymp. Sig. P) are given. Here are listed only the significantly different ($p<0.05$) comparisons.

There was no difference in gender distribution among drug users (Female/Male, 20/10, p=0.068); moreover there was also no significant difference between the scores of both psychophysical stressors and burnout items in subjects who use drugs and who do not (Kruskal-Wallis test; not shown). Given that and to the high influence of drugs on biological variables, in order to limit this confounder and increase the power of predictors, we decided to exclude all the 30 subjects who use drugs. Distribution of psychophysical and burnout scores of the remaining 92 cases were essentially equal to the previous ones (not shown).

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 (not shown), but Q-Q plot analysis displays a good approximation to normal distribution (see example in Figure 4.5).

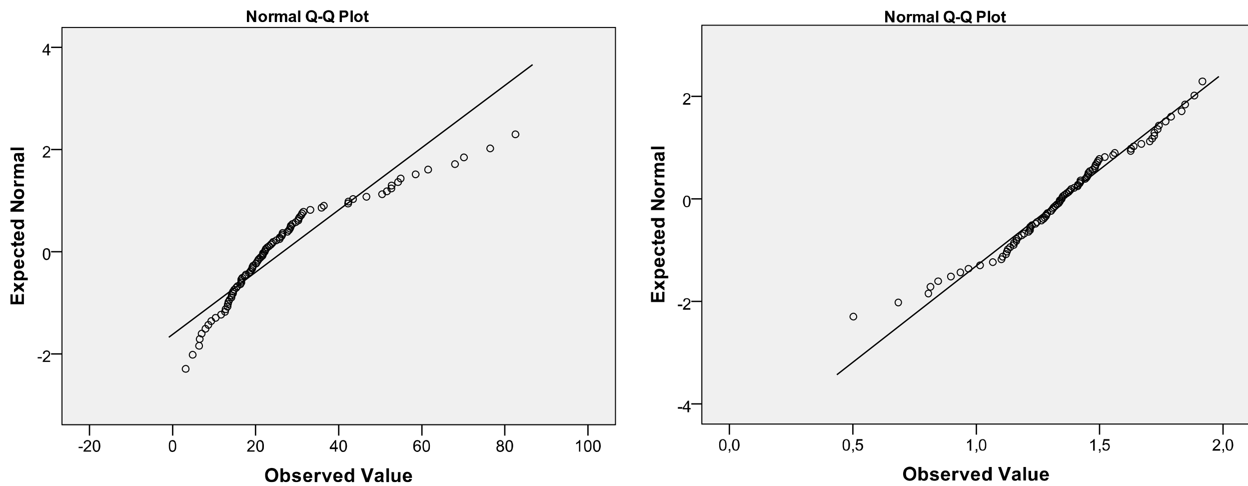


Figure 4.5 Representative Q-Q plot from MCP-1/CCL2 before (left) and after (right) log transformation

The presence of inferred values for those under the lower limit of detection (OOR), introduced a strong bias in the distribution of the following variables, even after log transformation: IL-1β (26%), IL-5 (26%), GM-CSF (24%), IL-2Rα (4%), IL-16 (5%), GRO-α/CXCL1 (6%), SCF (4%), SDF-1α/CXCL12 (7%), TRAIL/ TNFSF10 (14%). An example of this effect is displayed by the frequency distribution graph of IL-1β in figure 4.6.

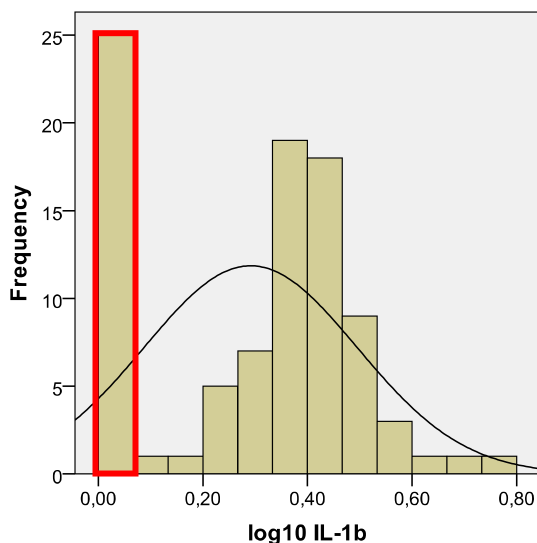


Figure 4.6 Distortion on frequency distribution introduced by inferred OOR values (red box), despite the usage of log transformed values. Black curve indicates the shape of theoretical Gaussian distribution. Here is reported IL-1β as an example, but the effect of the presence of inferred OOR value is similar for other analytes (see text).

Since these OOR cases were randomly distributed among psychophysical stress or burnout scores, we applied a stringent criterion and decided to exclude these variables from further analysis.

4.2.3 Univariate analyses

The transformation of the predictor variables, in order to approximate normal distributions, allow to more reliably correlate independent variables (analytes) and dependent variables (item scores), which are both continuous scale, and permit also to perform subsequent multivariate analysis. As univariate analysis, we performed partial correlations using the log-transformed data, controlling for gender and BMI, in order to detect association between each predictors (29 in total) and the psychophysical stressor or burnout items. Significant associations were found for only 8 independent variables, i.e. CTACK, IL-6, IL-17, Eotaxin, MCP1, Rantes, BDNF and MIF. The table 12 summarizes the significant results:

Variable ID	Anxiety	Gastric	Cardiac	Ergonomics	Stress	Depersonalization
Eotaxin/CCL11 (df=83)	-0.304 (**0.005**)	-0.325 (**0.002**)		-0.240 (**0.027**)	-0.307 (**0.004**)	
CTACK/CCL27 (df=84)	-0.301 (**0.005**)	-0.235 (**0.029**)	-0.272 (**0.011**)		-0.258 (**0.017**)	
MCP-1/CCL2 (df=83)	-0.342 (**0.001**)	-0.214 (**0.049**)			-0.263 (**0.015**)	
RANTES/CCL 5 (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**)					
MIF (df=87)						0.210 (**0.049**)

Table 12. Summary of the significant results from partial correlation analysis, controlling for gender and BMI, between biological variables (independent variables, first column) and scores of the psychophysical stress and burnout items (first row). Correlation values (minus sign indicate a negative correlation) and p values in brackets, highlighted in bold, are given.

For psychophysical stressor items, the majority of the associations are negative, except for IL-17 with the Gastric disturbance and for BDNF with the Ergonomics problems. No associations with any of the predictors were found for the Emotion item. For burnout state, only an association between a slight increase in MIF levels and Depersonalization was found. Results are also graphically summarized as scatter plots in Figure 4.7

A

B

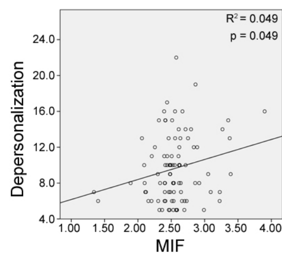


Figure 4.7 Scatter plots showing correlations between the given chemokines (MCP-1/CCL2, Eotaxin/CCL11, CTACK/CCL27, RANTES/CCL5), cytokines (IL-6, IL-17, MIF) and trophic factor (BDNF) and the items of psychophysical stress in panel A and burnout in panel B. Regression lines, R square and p values are displayed.

4.2.4 Multivariate analyses

Multivariate analysis was also performed to explore association between predictors, and partial least square regression procedure (PLS) is well suited for this purpose; in fact is particularly useful when predictor variables are highly correlated or the number of predictors is considerable in respect to the number of cases. It 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 item of the psychophysical stress state as dependent variable and cytokines, chemokines, growth factors and the interaction effect between gender and BMI as independent variables. The graphs in Figure 4.8 show the contribution of information (VIP) of each variable to the item of anxiety, gastric and cardiac disturbances, ergonomic problems and overall stress. Results essentially replicated the findings obtained with the partial correlation analysis, in which the chemokines MCP-1/CCL2, RANTES/CCL5, CTACK/CCL27, Eotaxin/CCL11, the interleukin 17 and the BDNF are the best contributors. Other analytes, which were not identified in partial correlation analysis, shows up with PLS: IL-9 for anxiety and cardiac problems, IL-18 for ergonomic problems and overall stress and MIP-1β/CCL4 for gastric problem and overall stress. Differently, IL-6 was weakly associated with anxiety score as well with any other stress item.

Results for burnout are not displayed since PLS analysis did not replicate the already weak association between depersonalization and MIF. Likewise, no other association showed up, since VIP scores were close to one for all biological variables (not shown).

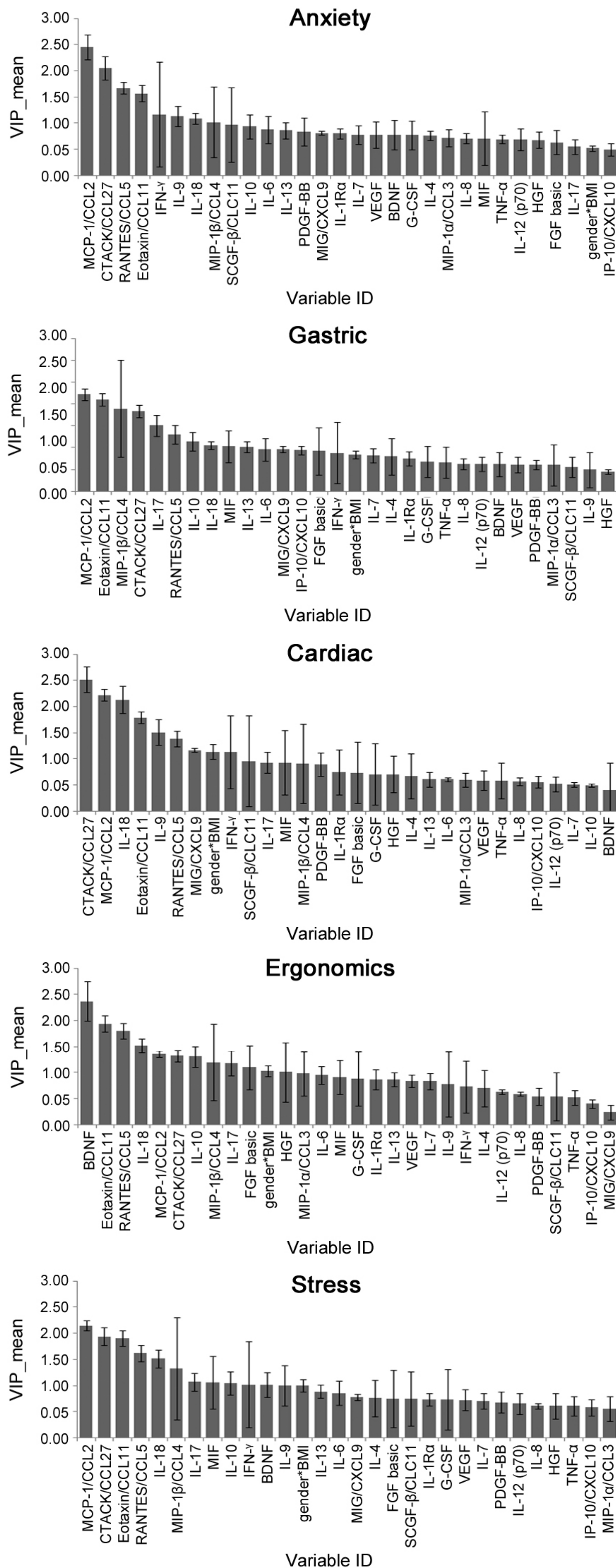


Figure 4.8 Variable Importance to the Projection (VIP) ranking biological markers for their contribution to the association to psychophysical stress items, assessed by PLS discriminant analysis.

In addition to PLS, we performed factor analysis (FA) for unsupervised analysis of the dataset, aimed at identifies underlying variables (latent factors) that explain the correlation within the set of observed variables. Unlike PLS, FA does not extract predictors based on the item scores (dependent variable), that is the reason of the term “unsupervised analysis”, but does not permit the use of categorical variables (i.e. gender). Performing the Principal Axis Factor method (PAF), which is similar to the Principal Component Analysis (PCA) and gives similar results, we identified the presence of 7 different latent factors, which explained 74% of the total variance (see table 13 A). Interestingly, the independent variables, identified by PLS and partial correlations as potential indicators of the several psychophysical stress items, were found to belong to different latent factors (see table 13 B), highlighting *bona fide* their independence (despite used Promax rotation algorithm allowed for some grade of correlation between factors; however, the Varimax rotation algorithm does not permit correlation between factors but gave similar results). There are two exceptions to the above statement: 1) Eotaxin/CCL11 and MCP-1/CCL2 were in the same latent factor and the correlation analysis confirmed that these two variables were significantly associated ($r=0.582$; $df=83$; $p<0.001$). 2) BDNF and BMI were in the same latent factor and they showed a positive correlation ($r=0.303$; $df=83$; $p=0.005$)

A

	Total Variance Explained Initial Eigen values		
Factor	Total	% of Variance	Cumulative %
1	10.929	36.428	36.428
2	3.073	10.243	46.671
3	2.433	8.108	54.779
4	1.838	6.125	60.905
5	1.513	5.043	65.948
6	1.333	4.442	70.390
7	1.137	3.792	74.182

B

Pattern Matrix

Variable ID (log 10 transformed data)	Factor						
	1	2	3	4	5	6	7
IL-4	0.991						
IL-8	0.973						
G-CSF	0.892						
IFN-γ	0.866						
RANTES/CCL5	0.683				0.421		
PDGF-BB	0.458						
MIP-1β/CCL4							
TNF-α							
IL-10		0.997					
IL-12		0.865					
IL-13		0.596					
IL-7		0.515					
VEGF		0.498					
MCP-1/CCL2 (MCAF)			0.867				
IL-1Rα			0.728				
Eotaxin/CCL11			0.637				
IL-6			0.494				
SCGF-β/CLC11							
FGF basic				0.835			
IL-17				0.829			
MIP-1α/CCL3				0.707			
IL-9				0.580			
IL-18					0.698		
CTACK/CCL27					0.688		
HGF					0.624		
MIF					0.506		
MIG/CXCL9						0.792	
IP-10/CXCL10						0.692	
BDNF							0.598
BMI							0.575

Table 13. Results from the unsupervised analysis performed using Principal Axis Factoring as extraction method. A) Number of factor extracted based on Eigen values >1; the percentage of variance explained by each factor and the total cumulative percentage are displayed. B) Matrix table showing the correlation values connecting variables (first column) to each latent factor (head row) obtained using Promax rotation method with Kaiser normalization. The variables of interest are underlined. Values less than 0.4 are hidden.

Taken together, the presented results pointed out a connection between some elements of the chemokine pattern and the features of the chronic psychophysical stress. Interestingly, peripheral BDNF was positively associates with the score of the ergonomic problems at the workplace.

4.3 AIM 3- BDNF expression in cells under cytotoxic stress

To further explore the potential protective role of BDNF in chronic stress conditions, we moved to an *in-vitro* cellular model of cytotoxic stress, consisting in SK-N-BE human neuroblastoma cell line treated with the cisplatin drug. Cisplatin is a non-cell cycle specific alkylating agent forming intrastrand DNA crosslinks and adducts that cause changes in the conformation of the DNA leading to a blockade of DNA replication and activation of signal transduction pathways, including those involving ATR, p53, p73, and MAPK, which culminate in the activation of apoptosis. Other mechanisms of cisplatin cytotoxicity include mitochondrial damage, decreased ATPase activity, and altered cellular transport mechanisms (Siddik, 2003). The choice for this type of model has been driven by different reasons: 1) SK-N-BE is a neural crest, tumour-derived human cell line that is well characterized, easy to culture and express both BDNF protein and TrkB receptor after differentiation induction. 2) Chemotherapeutic drugs, such as cisplatin, are known to give stressful insult to tumour cells, so the effect of prolonged treatment (death or resistance) can be conveniently compared to the effect of chronic stressors at cellular level. 3) Any finding that gives some clues inside the mechanisms of drug-induced cell death or resistance may have a potential clinical impact, since neuroblastoma tumours are responsible for at least 15% of cancer-related deaths in children; moreover, in this tumour,

expression of BDNF and its associated receptor (TrkB) are associated to a more malignant phenotype.

4.3.1 Cisplatin treatment increases BDNF in differentiated SK-N-BE neuroblastoma cells

We previously demonstrate that SK-N-BE neuroblastoma cells differentiated with 9-cis retinoic acid show higher production of BDNF and its receptor, TrkB. Moreover, they are more resistant to cisplatin induce death, with respect undifferentiated cells, while BDNF silencing attenuate this effect (Baj and Tongiorgi, 2009). Giving these observations, we investigated whether cisplatin is able to modulate BDNF expression. SK-N-BE cells were then differentiated for 4.5 days with 9-cis retinoic acid followed by cisplatin treatment (5 μg/ml) in serum-free medium for one, three, six, twelve and twenty-four hours. After RNA extraction and retro-transcription, total BDNF transcripts were quantified through real time PCR (N=3 independent cultures) and we found a significant increment (3 folds, $P<0.001$) in BDNF coding sequence mRNA already after six hours, which is stable up to twelve hours and more pronounced (9 folds, $P<0.001$) after 24 hours of treatment (Figure 4.9 B). BDNF has a complex gene structure (see Figure 4.9 A) characterized by different 5'-untranslated (UTR) exons spliced to a common coding sequence (CDS) linked to a 3'UTR that exists in two forms, one short and one long, due to the presence of two polyadenylation sites. The different BDNF splice variants are transcribed independently and give rise to a total of 22 possible transcripts in rodents, 34 in humans (Aid et al., 2007; Pruunsild et al., 2007; Pruunsild et al., 2011).

As a consequence, the increased expression was not limited to the coding sequence but involve almost all transcript variants (1, 2, 3, 4, 6, 7 and 9a), especially after 24 hours. The exceptions regarded the expression of exons 5 and 8 which was slightly increased,

although not significantly. Additionally, expression of exons 3, 6 and 7 was increased already after 6 hour of cisplatin treatment while, on the contrary, we observed a transient reduction in the expression of exon 4 at this time point (Figure 4.9 C).

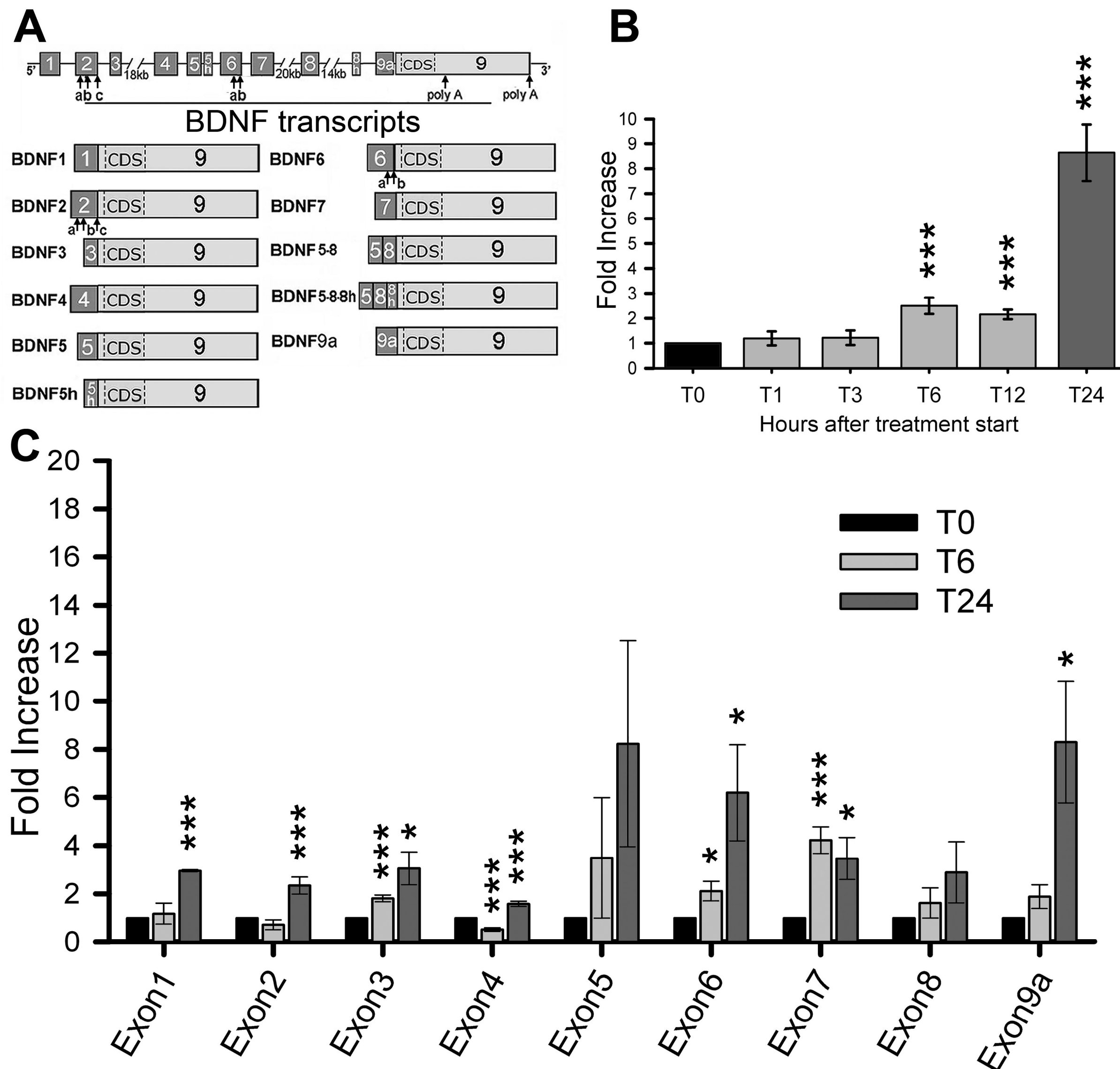


Figure 4.9 A) BDNF human gene structure accordingly to Pruunsild et al. (2007) B) Total BDNF CDS expression in SK-N-BE cells (differentiated 4.5 DIV) and treated in serum free medium (SFM) + Cisplatin (5 μg/ml). RNA extracted at different time points (hour after treatment) (N=3). C) Single BDNF mRNA isoforms modulation at the chosen treatment time points (T0 = control; T6 = 6 hours; T24 = 24 hours) (N ≥ 4). B and C data were collected through real time PCR with SYBR® Green Technology (*P<0.05, **P<0.01; ***P<0.001, one-way ANOVA).

In parallel to the increased mRNA expression, also BDNF protein production was enhanced by cisplatin treatment. Using the ELISA technique, we found that after a slight but significant decrease at 1 hour of treatment, there was a recovery of BDNF to control by 12h while the chronic treatment with cisplatin for 24 hours boosted the BDNF protein levels of almost 20 % over the basal level (untreated control; $P<0.001$, one-way ANOVA; see figure 4.10 A). Similar results were obtained also by western blot analysis. Indeed, there was no significant variation in BDNF level after 6 hour but after 24 hour of treatment there was an almost 30% increase in BDNF protein production ($P<0.001$, one-way ANOVA; Figure 4.10 B). The observed reduction in the tubulin signal was an expected finding which reflects a reduction in cell number due to increased cell death in treated cultures.

Since the different BDNF transcripts are upregulated by cisplatin treatment, we aimed also at determining which of them is more efficiently translated, hence contributing further to BDNF protein production. In a previous study from our laboratory, we developed an in vitro translation assay by which we demonstrated that the different BDNF transcripts display a different ability to translate, basally and after stimulation, and this ability is influenced by the presence of both 5'- and 3'-UTRs (Vaghi et al., 2014). Briefly, we transfected SK-N-BE cells with a construct that bear a Firefly luciferase (FLUC) reporter gene flanked by different 5'-UTR (exons 1, 2c, 4 and 6, alternatively) and the 3'-UTR long; exons 1, 2, 4 and 6, all together, were the most abundant 5'UTR of BDNF transcripts. Simultaneously with each one of these vectors we co-transfected a control reporter, namely Renilla luciferase (RLUC), to normalize for differences in transfection efficiency. After treatments, FLUC bioluminescence activity was recorded and double normalized over the RLUC activity and the untreated condition. As a result from this translatability assay, we observed that after 24 hours of cisplatin treatment all tested transcripts underwent a significant increased translation ($p<0.01$ for exons 1, 2c, 4; see Figure 4.10 C); exon 6 translation

increment, although significant (P<0.05), displayed a high variability, possibly due to the fact that exon 6 is translationally repressed in basal conditions, so its contribution to BDNF protein production is limited, potentially reflecting a tight regulation. Of note, the 3'UTR long alone, does not substantially contribute to the translatability in stimulated conditions (Vaghi et al., 2014).

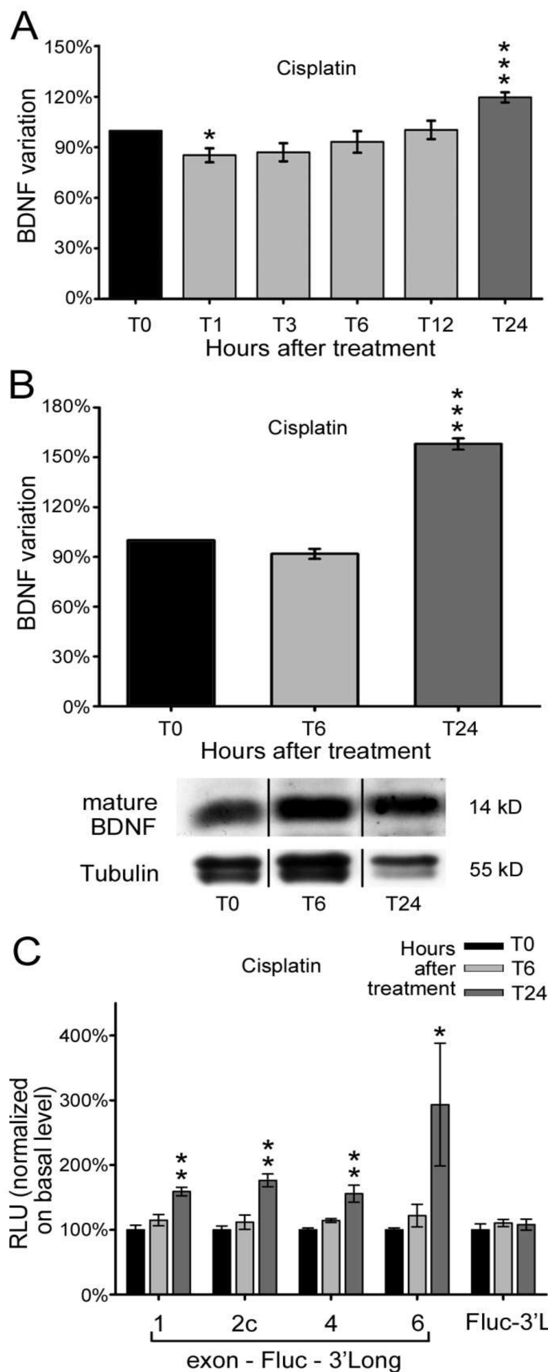


Figure 4.10 A) ELISA detection of total BDNF protein level in 4.5 DIV differentiated SK-N-BE cells after cisplatin treatment, at different time points: untreated control (T0), 1 hour (T1), 3 (T3), 6 (T6), 12 (T12) and 24 (T24) hours (N=4, each in duplicate).

B) Evaluation of mature BDNF protein production through densitometric analysis of western blot, in the same conditions as described in panel A; BDNF signal is normalized to tubulin and given as percentage of variation with respect to control. Lower panel: representative western blot of mature BDNF and tubulin (N=3).

C) Translatability capacity of different BDNF isoforms under cisplatin cytotoxic stress, at 6 and 24 hours time points. The isoforms tested are as follows: exon1-Fluc-3'UTR long, exon2c-Fluc-3'UTR long, exon4-Fluc-3'UTR long and exon6-Fluc-3'UTR long. Values from each isoform are normalized for transfection efficiency over a control vector (Rluc) and given as fold increase on basal condition (T0); N=3, each in duplicate; RLU: relative luciferase unit.

In all panels, data are represented as mean ± s.e.m. *P<0.05; **P<0.01; ***P<0.001, one-way ANOVA.

4.3.2 Aurora kinases inhibitor enhances the cisplatin cytotoxic effect

To further explore the regulation mechanism of BDNF translation, we focused our attention on Aurora proteins, a family of serine/threonine kinases involved in translational regulation of diverse transcripts. The reasons for this choice are threefolds. First, Aurora kinases regulate translation by phosphorylation of cytoplasmic polyadenylation element binding protein (CPEB), which binds to cytoplasmic polyadenylation element (CPE) on mRNA transcripts and promotes translation induction (Groisman et al., 2006; Otto et al., 2009); this mechanism has been described also in dendritic spines, not only in tumuor cells (Huang et al., 2002). Second, all BDNF mRNA transcripts include a CPE element in the 3'-UTR, before the first polyadenylation signal (Oe and Yoneda, 2010). Third, Aurora proteins are important regulators of the cell cycle, since deregulation of their activity can result in mitotic abnormality and genetic instability (Fu et al., 2007); moreover, both the expression level and the kinase activity of Aurora kinases are found to be up-regulated in many human cancers, including neuroblastomas, especially those with N-myc gene amplification (as SK-N-BE cells), which predicts poor prognosis and resistance to therapy (Otto et al., 2009). Given that, inhibition of Aurora activity represents not only a tool to study translational regulation mechanisms, but also an important anticancer therapeutic target.

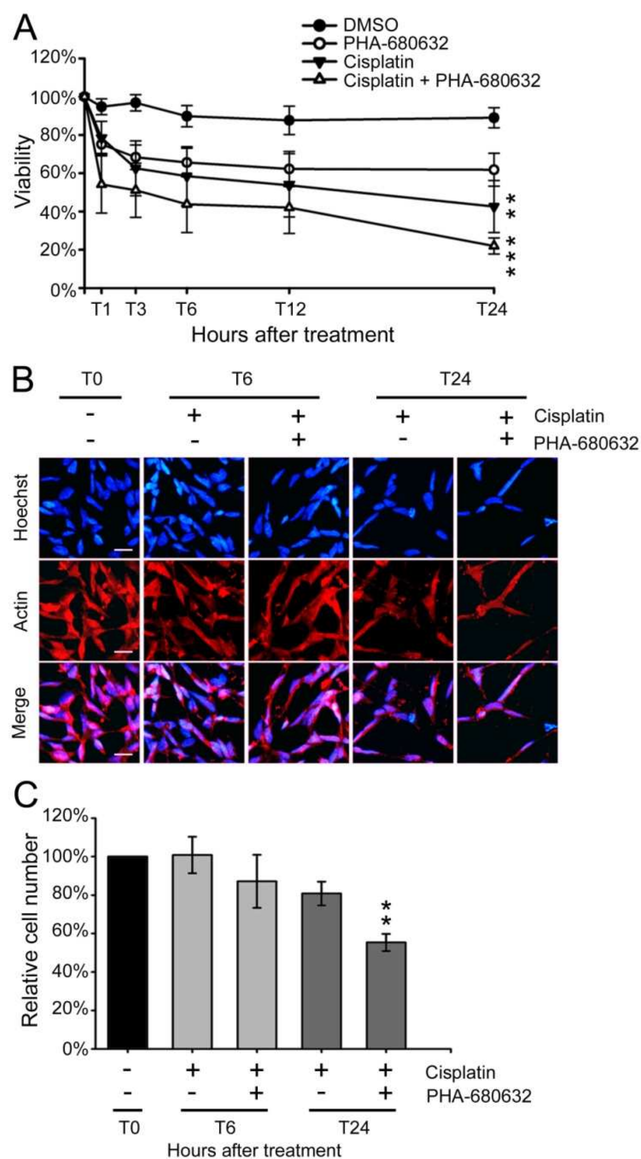
Our hypothesis was that reducing Aurora kinase activity, by a potent inhibitor (PHA-680632) (Soncini et al., 2006), we can enhance the cytotoxic stress effect of the cisplatin also as consequence of reduced BDNF translation. To demonstrate that, we treated SK-N-BE cells with cisplatin and Aurora inhibitor PHA-680632 (10 μM), either alone or in combination, at the same time points described previously. At first, we test the cytotoxic effects through a MTT viability assay and found that combined treatment with cisplatin and Aurora inhibitor significantly reduced cell viability with respect untreated condition, after 24 hours (DMSO; $P<0.001$, one-way ANOVA followed by Holm-Sidak correction). Cisplatin

and PHA-680632 together, at this time point, enhanced cell mortality of about 20% in comparison to cisplatin alone (not significant) and of more than 30% with respect Aurora inhibitor only (P=0.012, one-way ANOVA). Of note, these differences were maintained at all tested time points (Figure 4.11 A). Similar results were obtained also via immunofluorescence experiments in which we stained neuroblastoma cells nuclei with Hoechst and marked actin to point out cell bodies, in order to compare qualitative the effect of cisplatin only and cisplatin+PHA680632 treatments at 6 and 24 hours, with respect untreated condition. We also performed a quantitative assessment by counting cells in the different treatment conditions relative to the untreated control, which was set at 100% (Figure 4.11, panels B and C). A reduction in cell number was obtained, in particular, after 24 hour of combined treatment with cisplatin and Aurora inhibitor (P=0.003; one-way ANOVA).

Figure 4.11 A) Residual viability in SK-N-BE cells treated for different hours with cisplatin and/or PHA-680632 (Aurora kinases inhibitor). All treatment were performed after 4.5 DIV of differentiation and quantified through colorimetric MTT assay. DMSO treatment represent the control group. Data are given as mean ± s.e.m. of percentage viability (N=3, each in triplicate); ***P<0.001; **P=0.006.

B) Immunofluorescence on SK-N-BE cells treated for 6 or 24 hours with cisplatin alone or in combination with PHA-680632 inhibitor. Hoechst was used to highlight nuclei and Actin was marked to emphasize cell bodies; the merge between the two channels is displayed. (N=3; scale bar: 20 μm).

C) Cell count from the immunofluorescence described in panel B. Values are given as mean ± s.e.m. of percentage of cell number with respect basal conditions (T0) (N=3; **P=0.003).



4.3.3 Effect of Aurora inhibition on BDNF protein production

In the following series of experiments, we tested the effect of Aurora kinases inhibition on BDNF protein expression. We were expecting a decrease in BDNF protein levels reflecting a reduction in translatability of mRNA transcripts. Unexpectedly, we found exactly the opposite. In western blot experiments normalized to tubulin, after 24 hours of treatment BDNF signal was more than double with respect to untreated control (P<0.001, one-way ANOVA), while no significant change was observed after 6 hours (Figure 4.12 a). Performing a translation assay, we found that, while translation from exons 1, 2c and 4

was unaffected at both 6 and 24 hours of combined treatment with cisplatin and Aurora inhibitor, translation from exon 6 was increased about 6 folds at 24 hours (but not 6) of treatment ($P<0.001$, one-way ANOVA; see Figure 4.12 B). This finding is consistent with the hypothesis of a tight regulation of the translation of transcripts bearing exon 6, consisting in a repressive state in basal conditions that is released under cytotoxic stimuli. Again, the 3'UTR long sequence alone did not affect translation under cisplatin treatment. The results of an increased production of BDNF protein were confirmed also by immunofluorescence. Using a monoclonal antibody, we were able to identify BDNF spots inside the neuroblastoma cells and we observed a clear enrichment of these spots after 24 hours of treatment with cisplatin in the presence with the PHA-680632 Aurora inhibitor, in comparison to 6 hours or the untreated control (see upper row of panel C, Figure 4.12). Again, actin was marked in red to depict cell bodies; outline of the merged figures (see bottom row of panel C, Figure 4.12) are given, with black BDNF spots and red cell body edges, to highlight the rising number of spots and the fact that they are inside the cell boundaries. Panel D of Figure 4.12 simply displays the quantitative analysis of BDNF spots, which are given as number per micron square. From 0.21±0.04 BDNF spots/μm^2 (mean±S.E.M.) in basal condition, which was essentially stable after 6 hour of treatment either with cisplatin only or combined to PHA-680632, after 24 hours of treatment the number rised to 0.31±0.03 BDNF spots/μm^2 with cisplatin alone ($P=0.039$, one-way ANOVA) up to 0.40±0.02 after treatment with both cisplatin and Aurora inhibitor ($P=0.004$, one-way ANOVA).

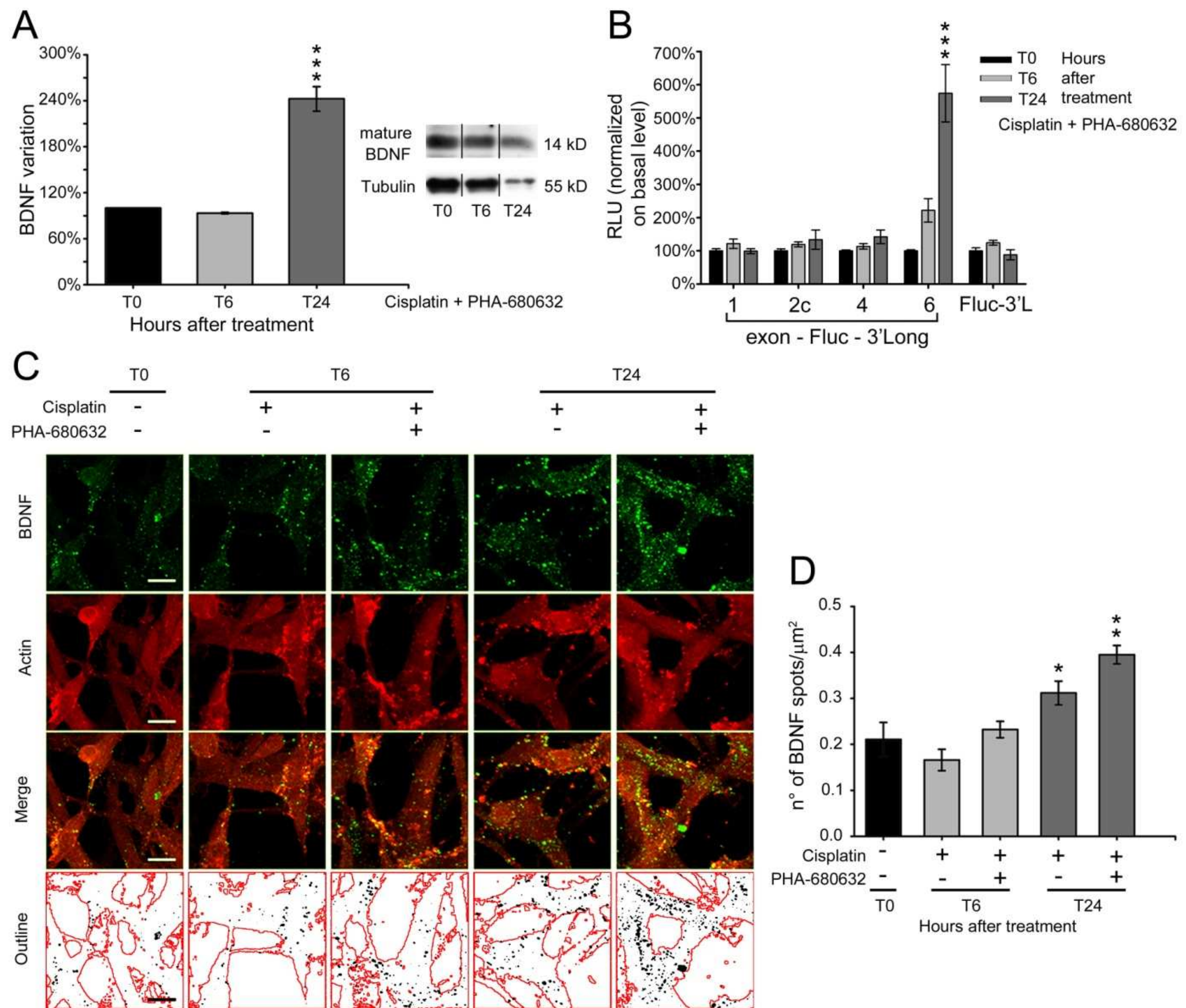


Figure 4.12 A) Evaluation of mature BDNF production, through western blot analysis and relative densitometric analyses, in differentiated SK-N-BE cells after cisplatin + PHA-680632 (Aurora K inhibitor) treatment, at different time points: untreated control (T0), 6 (T6) and 24 (T24) hours (N=3; ***P<0.001).
B) Translatability capacity of different BDNF isoforms in differentiated SK-N-BE treated as described in panel A. The isoforms tested and the normalization procedures are the same as described in figure 2, panel C. RLU: relative luciferase unit (N=3, each in duplicate; ***P<0.001). C) Immunofluorescence on SK-N-BE cells treated with cisplatin ± PHA-680632 at the same time points described in panel A. BDNF spots are highlighted using a mouse mAb and actin is marked using a rabbit pAb to emphasize cell bodies; the merge of the two channel is given as well its outline, where black dot represents the BDNF spots and red lines the edges of the cell bodies. Images are analized after z-project from 11 stacks, performed using Fiji-ImageJ (N=3; scale bar: 10 µm). D) BDNF spot analysis from the immunofluorescence described in panel C. Data are given as mean ± s.e.m. number of spots per µm^2 (N=3; **P=0.004, *P=0.039).

In conclusion, cisplatin induced cytotoxic stress was able to stimulate BDNF production by enhancing both transcription and translation of mRNAs. Additionally, the translation induction was particularly effective for transcripts bearing exon 6 along with the 3'UTR long sequence. Although the combined treatment with PHA-680632 Aurora kinases inhibitor resulted effective in enhancing the cytotoxic effect of cisplatin, we observed an enhancement in BDNF production in a subpopulation of cells that became resistant, thus suggesting induction of an Aurora kinase-independent mechanism for BDNF translation induction under cytotoxic stress conditions.

5. DISCUSSION

5.1 AIM1

Measuring BDNF, as well as other circulating markers, from body fluids in a reliable manner is often an underestimates issue that still undermine the reproducibility of results and make studies on similar topics difficult to compare. In the first part of the present work we propose a well reproducible BDNF quantification method potentially suitable also for clinical applications. To obtain a reduction in the variability of measurements due to technical issues, we defined a procedure to standardize the pre-analytical steps from blood collection to serum isolation and storage. Moreover, we identified the best performing assay for BDNF detection by comparing six widely used commercial kits based on the ELISA technique, the most common way to measure circulating BDNF and, according to our results, one of the main sources of inconsistencies among studies.

A recurrent question among researchers is whether it is more correct to measure BDNF levels in plasma, serum or whole blood. We strongly advocate for the serum as elective body fluid for a reliable detection of circulating BDNF. There are several reasons that support this choice. First, BDNF concentration in serum is about 100 folds higher than plasma levels (Radka et al., 1996). Second, BDNF concentration in plasma is affected by handling of the blood sample because of the presence of platelets, which store BDNF and upon degranulation can secrete it (Fujimura et al., 2002; Karege et al., 2005). Simple shearing forces produced by the needle during blood withdrawal can cause platelet degranulation and even changes in room temperature and timing can produce significant release of BDNF in the plasmatic fraction (Elfving et al., 2010). In addition, it has been shown that because of the release from platelets, BDNF concentration raises progressively within few hours from plasma preparation (Elfving et al., 2010; Radka et al.,

1996). Consequently, the BDNF quantification from plasma is extremely sensitive to preparation procedures and is very difficult to be reproducible among different operators. Secondly, release of BDNF from platelets can be influenced by age, specific disease conditions, or pharmacological treatments, which may be difficult to control (Karege et al., 2005; Lommatzsch et al., 2005). Concerning the whole blood, although measurement of BDNF in serum and whole blood give comparable results, we agree with Elfing and colleagues (Elfving et al., 2010) that, since whole blood must be lysed before the BDNF measurements, the cell lysis step may add additional variability during sample preparation. In conclusion, since BDNF is not produced in megakaryocyte precursor cells but actively picked up by platelets from the circulating BDNF pool (Fujimura et al., 2002; Lommatzsch et al., 1999; Nakahashi et al., 2000), it can be assumed that serum seems reflect the totality of circulating BDNF. Indeed, serum BDNF concentration is almost identical to the amount of BDNF in washed plateled lysates (Fujimura et al., 2002; Lommatzsch et al., 2004). In addition, while BDNF circulate in plasma for less than an hour, platelets circulate as much as 11 days (Kishino et al., 2001; Poduslo and Curran, 1996).

The performance of the six BDNF kits examined is summarized graphically in Figure 4. The largest area corresponds to the most performant assay, taking as 100% the best performance measured among the six kits for five parameters: intra-assay variation, inter-assay variation, detection range, sensitivity and processing time (see figure legend for definitions). We found that five out of six kits (Aviscera-Bioscience, Millipore-Chemikine™ and Milliplex®, R&D System-Quantikine® and Promega-Emax®) exhibited a high inter-assay variation, because two measurements of the same serum sample performed in two distinct days were significantly different. Of note, Promega kit was previously demonstrated to give unreproducible results upon repeated measures on sera from Huntington's disease patients (Zuccato et al., 2011). The only exception was the kit by

Biosensis, which showed the lowest (5%) coefficient of variation (CV) and no significant difference between two independent measurements. In terms of CV, Promega and Aviscera-Bioscience assays showed acceptable values (16% and 12% CV, respectively), although measured inter-assay variations were slightly higher than those declared by the manufacturers. Millipore-Chemikine™ showed the poorest performance in terms of inter-assay variation (38%). Kit descriptions provided by producers indicated a factor 10 difference in assay sensitivity between kits, ranging from 2 pg/ml to 20 pg/ml (mean 9.07 pg/ml) and even greater differences in the range of detection, from the narrowest range of 7.8-500 pg/ml for the Biosensis, Millipore-Chemikine™ and Promega kits, to the broadest range of 12-50,000 pg/ml provided by the Millipore-Milliplex® assay, which uses the Luminex®/xMAP® technology. All kits examined are able to detect BDNF within a restricted concentration range (from 7.8 pg/ml to 4 ng/ml), with the exception of multiplex assay which had a broader range (12 pg/ml to 50 ng/ml). Most studies including ours, report a range of BDNF concentrations between 8-46 ng/ml, with an average around 18-26 ng/ml for healthy Caucasian adults, and generally not beyond a minimum of 3 and a maximum 80 ng/ml when the extreme values are considered (Karege et al., 2005; Trajkovska et al., 2007; Yoshida et al., 2012). Therefore, a dilution step must be applied to process the samples, which may introduce a potential source of additional errors. Nevertheless, the results from our study suggest that all kits with the exception of Millipore-Chemikine™, which appears to systematically underestimate BDNF concentration, are able to perform well in the range of concentrations of serum BDNF found in a healthy Caucasian population.

The sandwich ELISA assays tested here differed also for the type of capture and detection antibodies. In addition, capture antibody are generally pre-coated, with the exception of the Promega kit, which requires preparation by the experimenter. The Millipore-Milliplex® kit

exploits the Luminex®/xMAP® technology, where magnetic microspheres, filled with a dye mixture, are coated with the capture antibody; this technology allows multiplexing and high throughput screening, but requires special equipment. Remarkably, the Biosensis kit is built on a unique strategy, because it uses the same antibody for both capture and detection, based on the fact that BDNF is naturally occurring as a dimer and therefore, once a monomer is captured, the other monomer is available for detection. The Biosensis kit provides also the quickest procedure (results in less than four hours for a full 96 well plate) while the kit by Promega, which is probably the most used, is time consuming (23-25 hours), because it requires an overnight plate coating, another potential source of variability.

Of note, the Promega kit resulted to be the only assay among those tested, for which the species-specificity was not declared, while the other kits were declared to be specific for detection of human BDNF. In addition, Biosensis and Millipore-Chemikine™ also can cross-react with BDNF from rodent species. Concerning the specificity for the different proteolytic forms of BDNF, four kits recognized both pro and mature BDNF forms while Aviscera-Bioscience and R&D System kits showed a remarkable preferential specificity for the mature form of BDNF, as indicated by the manufacturers. The ability of a diagnostic assay to distinguish between BDNF precursor and BDNF mature forms might represent a critical issue in specific clinical applications. Indeed, we recently proposed that altered biosynthesis of the different BDNF proteolytic forms may represent a common hallmark of neurological diseases with cognitive dysfunctions (Carlino et al., 2013; Carlino et al., 2011). This hypothesis is based on studies in animal models and post-mortem human brains, which highlighted that different forms of learning and memory require either the pro-BDNF precursor or the mature BDNF form. In particular, decreased synthesis of mature BDNF, especially in hippocampus and prefrontal cortex, is associated with memory

loss and learning impairment; while decreased production of pro-BDNF in amygdala is associated with dysfunctions in emotional control and finally, alterations of both mature BDNF and pro-BDNF can affect several cognitive functions at the same time (Baj et al., 2013a; Carlino et al., 2013). Of note, specific kits for detection of pro and mature BDNF forms are now becoming available (Riffault et al., 2014; Yoshida et al., 2012). It remains to be determined if the ELISA kits specific for pro-BDNF have sufficient sensitivity to be appropriate for clinical applications.

Besides the technical issues discussed here, several studies investigated socio-demographic determinants and other factors that may affect serum levels of BDNF such as gender, age, Body Mass Index (BMI), urbanicity, smoking status, and alcohol intake. While there are conflicting studies regarding BMI (Bus et al., 2011; Elfving et al., 2012; Lommatzsch et al., 2005; Monteleone et al., 2004; Ziegenhorn et al., 2007), concerning the effect of gender, our findings are in agreement with studies showing no gender difference regarding BDNF serum level (Bus et al., 2011; Lang et al., 2004; Lommatzsch et al., 2005; Ziegenhorn et al., 2007). However, it was also reported that BDNF in whole blood or plasma was higher in women compared to men (Elfving et al., 2010; Trajkovska et al., 2007) and several studies have suggested an interaction, with respect to circulating BDNF, between gender and age (Bus et al., 2011; Bus et al., 2012; Lommatzsch et al., 2005; Suwa et al., 2006; Ziegenhorn et al., 2007). Serum BDNF resulted to be increased in association with Nicotine assumption (Jamal et al., 2014; Suriyaprom et al., 2013) and to be decreased in association with high alcohol intake (>2U/day), but not with moderate alcohol consumption (Bus et al., 2011; Elfving et al., 2012). A final note regards the putative effects of the Val66Met polymorphism in *BDNF* gene on the levels of serum BDNF. The large majority of studies reported no association between Val66Met and serum BDNF in Asiatic, European and American populations (Grande et al., 2014; Jamal et al.,

2014; Katsuki et al., 2012; Suriyaprom et al., 2013; Yoshimura et al., 2011) as also summarized in a recent meta-analysis (Terracciano et al., 2013). It is worth mentioning however, that multiple regression analysis highlighted that the Val66Met polymorphism may affect serum levels of BDNF through a complex interaction with gender and in response to specific treatments such as IFN-α (Elfving et al., 2012; Lotrich et al., 2013) or linked to neurophysiologic response associated with working memory processes (Cerasa et al., 2010). These confounding factors are all to be taken in consideration in future studies aimed at defining a cut-off in serum BDNF concentration useful for diagnostic purposes.

In conclusion, the availability of reliable blood-based biomarkers for diagnosis and therapy monitoring still remains a major unmet medical need in neurology and neuropsychiatry(Kapur et al., 2012; van Beveren and Hoogendijk, 2011). There is a general consensus that BDNF may represent an important measurable biomarker however, the poor reproducibility of BDNF measures has to date prevented its validation for clinical purposes. This study, by providing a methodology which reduces the high variability due to technical, pre-analytical and analytical steps provides the basis to obtain an accurate measure BDNF in human serum, suitable for future clinical applications.

5.2 AIM 2

In the second part of the present work, also taking advantage of the methodology issues addressed for BDNF measurement, we undertook a research aimed at indentify associations between the scores of job-related stress indicators (in a healthcare assistants population) and the levels of circulating BDNF and other 48 biological markers, consisting in trophic factors and immune mediators such as cytokines and chemokines.

Work-related chronic stress is a condition that occurs when a person has, or feels, a high job demand having, however, low job control, thus felling unable to cope with the requests. Another means driving this kind of chronic stress is the alteration in the effort-reward balance (Siegrist et al., 2004), where a worker challenges demanding job but obtain improper remuneration, in term of salary, accomplishment or job perspectives. Job-related chronic stress is an emerging factor in the development of physical and mental illness (such as depression) and its effects can be explained well by the allostatic load model, similarly to what happen for other type of chronic stress. Job stress has high social costs, both in term of wellness (increased illness and injuries) and productivity (lower efficiency, high rate of absenteeism, etc.), thus European policies, since more than a decade, have recognized the work-related stress as an emerging occupational health risk which deserves identification, prevention and management like other work-related risks. The proposed methodology for stress risk assessment emphasizes the necessity to integrate subjective evaluations with objective measurements, to better identify subjects at risk to develop chronic work stress.

Therefore, in our study, we attempt to couple the scores outcome from self-assessed questionnaires for the stress-risk evaluation (subjective assessments), with changes in circulating level of humoral mediators (objective measures), focusing in particular on trophic factors and immune mediators. The perceived stress at work was estimated by two means, using an instrument standardized for the Italian context, the Qu-BO test (Falco et al., 2012): three items by an Italian adaptation of the Maslach Burnout Inventory (Maslach et al., 2001), which evaluate burnout condition, a psychosomatic syndrome characterized by the three core dimensions of emotional exhaustion, feelings of depersonalization, and state of personal accomplishment; five items that evaluate job-related pshychophysical stress in term of increased anxiety, emotional status, gastrointestinal and cardiac

disturbances, along with ergonomic problems at the workplace (headache, hurting neck or shoulders or back pain). Our results highlighted a significant gender difference in the perceived job-stress. This is not surprisingly, since male and female are known to differently respond to stress, both acute and chronic (McEwen, 2007). In particular, we found that women had higher scores for all the items of psychophysical stress and for the dimension of emotional exhaustion in burnout. No sex difference for personal accomplishment was found, while men had higher scores of depersonalization. The putative effect of gender on burnout state remain to be verified, since previous study found either no difference (Moragon et al., 2005), or address burnout state only in female (Grossi et al., 2003) or male (Langelaan et al., 2007), while the majority of the study did not address this topic and applied gender control. Differently, the finding that women are more susceptible to job-related psychosocial stress is in line with evidences showing a higher tendency for female to develop stress-related pathology such as anxiety and depression (Altemus et al., 2014; Young and Altemus, 2004). This condition is potentially due to an estrogen-driven delayed shut off of the HPA axis (Goel et al., 2014; Kajantie and Phillips, 2006). Brief considerations are of worth also for BMI and age. We found no association with burnout dimensions and BMI, in agreement with previous finding by Armon and colleagues (Armon et al., 2008). On the other hand, we observed a negative correlation with the psychophysical items, significant for anxiety, gastric problems and overall stress; however, we considered BMI as a covariate of gender, since it was significantly lower in female than in male and the interaction effect of gender-BMI did not associate with any of the scores in partial least square regression. All subsequent analysis for association with biochemical variables were therefore conducted controlling for both gender and BMI. Despite the well known positive association linking age and higher risk for allostatic load (Fischer et al., 2009), we did not found any correlation between age and both burnout and

psychophysical stress scores. Actually, this is not surprisingly, since the population of healthcare assistant under study comprised middle aged adults, within a comparable age range.

Since chronic stress is known to influence immune functions, we decided to examine its effect on serum level of immune mediators, namely cytokines and chemokines. As a result, we found that higher score of psychophysical stress, but not burnout, were associated with lower serum concentration of some member from the chemokine family, in particular MCP-1/CCL2, RANTES/CCL5, CTACK/CCL27 and Eotaxin/CCL11. Since these chemokines are involved in immune cells migration and communications, 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). Differently, 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 overcommitment show increased production of IL-6 and TNF-α cytokines, which are mainly classified as pro-inflammatory (Bellingrath et al., 2010); they found, however, dampened innate immune defence in term of reduction in natural-killer (NK) cells, which are important players of the humoral immunity. It is important to note that in the mentioned study, subjects were exposed to acute laboratory stressor, consisting in Trier Social Stress test, before performing immune assessments. Similarly, other studies assessing the effects of chronic stress on immune system, before doing immune mediators titration, often performed brief mental tasks on subjects or challenged immune cells with lypopolisaccharid (LPS) *in vitro* (Gibb et al., 2008; Lee et al., 2010) and these are potential confounders that make difficult to

disentangle the effect of acute stress, since is known to induce immune activation (Dhabhar, 2009).

Monocyte Chemoattractant Protein-1 (MCP-1 or chemokine C-C motif Ligand 2, CCL2) is one of the best characterized chemokine, able to attract monocytes and basophils but not neutrophils or eosinophils. Has been categorized mainly as pro-inflammatory and implicated in different stress-related pathologies characterized by monocytic infiltrates, like psoriasis, rheumathoid arthritis or atherosclerosis (Amasyali et al., 2009). However, it augments monocyte anti-tumor activity and exploit also anti-inflammatory effects (Deshmane et al., 2009). Our observation of reduced circulating MIP-1/CCL2 protein in job-related stress dimensions (anxiety and gastric problems) are apparently in contrast with previous finding on psychosocial stress in women made by Asberg and colleagues (2009), as they found increased level of MCP-1 and also trophic factors, namely VEGF and EGF, in stressed workers. However, they included in the study a heterogeneous group of long-term sickleave subjects diagnosed for any affective or stress-related mental disorder (depression, anxiety disorder, stress disorder, burnout syndrome, exhaustion disorder); in addition, they performed parametric analysis on non-transformed, non-normally distributed data, thus increasing type I error probability, specifically the possibility to reject a true null hypothesis.

Of note, MCP-1/CCL2, 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. For example, high MCP-1 reactivity has been found in neurons and plaques of Alzheimer patients (Sokolova et al., 2009), in white-matter hippocampal lesions of multiple sclerosis subjects (Prins et al., 2014; Yao and Tsirka, 2014)and has been shown to compromise the blood-brain-barrier integrity (Yao and Tsirka, 2014). However, maintaining doses of MCP-1have neuroprotective effects,

independently of microglia/macrophages infiltration, on retinal ganglion cells in an experimental model of glaucoma (Chiu et al., 2010). A role of MCP-1 has been suggested also in mental disorders: a negative correlation between peripheral MCP-1 and cognition was found in persons experiencing first psychotic episodes (Martinez-Cengotitabengoa et al., 2012) and higher levels of MCP-1 have been described in major depressive subjects (Young et al., 2014). On the other hand, lower chemokine levels, including MCP-1/CCL2 and Eotaxin/CCL11, were found in cerebrospinal fluid (CSF) of suicide attempters respect to healthy controls (Janelidze et al., 2013).

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

Intriguingly, we found increased interleukin 17 (IL-17) in subject 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; Zhou et al., 2013). 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 IL-17 increased production (Schmidt et al., 2010). Furthermore, in our study, we observed a positive association between serum BDNF and the score of ergonomic problems at the 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, increase BDNF levels have been found in amygdala while reduction has been described in prefrontal cortex and hippocampus, during chronic stress. Our observation of a BDNF increment in chronic stress could be the result of induction of compensatory protective mechanism. Despite the possible connection between BDNF circulating levels and brain levels, however, we cannot draw any conclusion regarding neither the central effect of the peripheral BDNF changes, nor the causality of the alterations in stress conditions. These considerations can be applied also to the observation concerning chemokines and cytokines levels: both acute and chronic stress are known to induce even rapid redistribution of leukocytes from blood and lymphoid streams to compartments and *vice versa* (Dhabhar, 2009), thus we cannot exclude that the observed reduction in circulating chemokine levels occurring in job-related chronic stress are due to compartmentalization of immune cells blood to lymph nodes or other sites such as skin or intestine. In addition, our results should be seen as preliminary given other intrinsic limitations of this study: 1) the study design involved only one single time point, thus the results may suffer for short time course variations. 2) Scores of work-

related stress resulted to be positive skewned, 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 biological marker 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 extract from the same environment. In addition, immune alterations are hard to predict given the enhancing versus suppressive effects of stress on immune function (Dhabhar, 2009).

Despite these limitations, we believe our study adds some valuable insight in immune mediator changes occurring in conditions of work-related chronic stress. The few biological variable identified deserve further investigations as they can be potential objective indicators to be coupled with the subjective assessments of job-related stress. However, replications with larger samples, possibly on a longitudinal study analyzing the effect of intervention aim to reduce stressing situations at work, are warranted. As a future perspective, to identify potential relations between the chemokine reduction and brain effects, in particular on hippocampal pyramidal neurons, we will inhibit chemokine receptors on stressed organotypic hippocampal slice, or primary cultures, extracted from wild type mice, and evaluate the effects on dendrites and spines morphology.

5.3 AIM 3

In the last part of the project, we aimed at investigating how chronic cytotoxic stress impact on the BDNF production at a cellular level, in a stress model consisting of SK-N-BE neuroblastoma cells treated with cisplatin in condition of serum free medium. Of note, this cell line has been previously used as an in vitro stress model to test the effect of corticosteron administration (Sun et al., 2010) or to study oxidative processes in Alzheimer and Parkinson's diseases (Batelli et al., 2008; Gamba et al., 2011). Considering the role of BDNF in neuroblastoma tumours, previos studies suggested that BDNF and its receptor TrkB are involved in an autocrine loop that promote cell survival and resistance to chemotherapy (Baj and Tongiorgi, 2009; Jaboin et al., 2002). Here, we demonstrate that cisplatin treatment and serum deprivation, in particular after 24 hour, are able to increase both total BDNF and single mRNA transcripts of all 5' UTR exons, with the exceptions of exons 5 and 8. These findings overlap with previous observation from our lab by which silencing of exons 4 and 6 result in a significant reduction of SK-N-BE viability under cytotoxic stress conditions (Baj and Tongiorgi, 2009). In addition, we found that transcription enhancement is coupled with a delayed but significant increased in BDNF protein production. Furthermore, cisplatin stress insult also boost translation of a firefly reporter gene embedded within both 5' and 3' UTRs of BDNF, highlighting the involvement of mechanisms that control BDNF translation induction, in particular in condition of cytotoxic stress. In particular, we observed that cisplatin improve translation of all tested transcript, consisting in exons 1, 2c, 4 or 6 along with 3'UTR long sequence. As already known, BDNF comes as a unique protein encoded by a single coding sequence (CDS), so all the complexity in its regulation is due to the presence of multiple untranslated regions, which are alternatively spliced to give rise, in humans, to 34 mRNA transcripts, each of them is independently transcribed (Aid et al., 2007; Pruunsild et al., 2007; Pruunsild et al.,

2011). Has been already demonstrated that different BDNF mRNA variants target different cell compartments, especially in neurons, in an activity dependent manner (Tongiorgi et al., 1997); for example, exons 1, 3, 5, 7, 8 and 9a preferentially segregate to soma, exon 4 to proximal while exons 2 and 6 to distal dendrites (Baj et al., 2013b; Baj et al., 2011). Hence, they display also different ability to translate in response to various compounds (Vaghi et al., 2014).

How cytotoxic stress induced by cisplatin can promote BDNF translation? Cisplatin [cis-diamminedichloro platinum(II)] is a metal compound commonly used in clinic as antitumor drug that induces cell damage mainly by cross-linking genomic DNA and, to a lesser extent, proteins. As known, DNA damage triggers different phenomena: on one side, causes cell death by necrosis (mainly by PARP-induced ATP depletion) (Fuertes et al., 2003) and/or apoptosis (both via intrinsic and extrinsic programmed cell death pathways, leading to final activation of caspase-3-6-7) (Zhou et al., 2002). On the other hand, DNA damage promotes cell cycle arrest, DNA repairing mechanisms and activation of cell survival pathways. These pathways require functional activity of transduction mediators, some of which are shared in the BDNF signaling cascade, like Akt, ERK and mTOR, and are known to control both gene expression and protein translation (Cepeda et al., 2007; Cunha et al., 2010). The translation ability of an mRNA resides in its UTR regions and the presence of a 5' cap, secondary structures, multiple upstream AUGs (uAUGs) and open reading frames (ORFs), internal ribosome entry sites (IRESs) and the binding of trans-acting factors are all relevant for translation regulation. These features can control the synthesis of a protein by affecting stability of the mRNA, its accessibility to ribosomes, circularization and interaction with the translation machinery (Vaghi et al., 2014). Despite that, the mechanisms regulating translation of BDNF variants are still unknown. However, a potential mechanism that has been described for CPE-containing α-Ca^{2+}/calmodulin-

dependent protein kinase II (αCaMKII) mRNA, in both *Xenopus* oocytes and at hippocampal neuron synapses, involve the cytoplasmic polyadenilation-induced translation of dormant mRNAs (Huang et al., 2002). According to this mechanism, the CPE element (UUUUUAU) in the 3'UTR is bound by the CPE binding protein (CPEB) and Maskin that, in quiescent conditions, is associated to the m^7G cap-binding subunit, the elongation factor 4E (eIF4E). This dormant state prevent the association between eIF4E and eIF4G, the initiation starter, and mRNAs are characterized by short poly(A) tails. Upon stimulation, the cascade events cause the phosphorylation of CPEB that recruit the multisubunit cleavage and polyadenilation specificity factor (CPSF) to a downstream element of CPE, the AAUAAA sequence; this event probably attract active poly(A) polymerase (PAP) to the mRNA end. The subsequent polyadenilation causes Maskin to dissociate from eIF4E which is then free to bind eIF4G, thereby recruiting the 40S ribosomal subunit to mRNA and start scanning the sequence for the initiation codon (Pestova et al., 1998). The critical event is the phosphorylation of CPEB on Ser174, which is mediated by Aurora kinase. Aurora proteins are a family of serine/threonine kinases primarily implicated in several mitosis events and cell cycle control (Fu et al., 2007). Of note, tumours with increased Aurora activity are associated with a more malignant phenotype (Maris, 2009). Considering that BDNF transcripts bear the CPE element in 3'UTR, we hypothesised for BDNF a translation induction mechanism mediated by Aurora kinase, similar to those described above for αCaMKII mRNAs. Therefore, blocking Aurora activity by using the PHA-680632 inhibitor (Soncini et al., 2006), we would expect a dual effect: increased cell mortality, due to altered cell cycle control and a decreased BDNF protein production as a result of a reduced translation induction. In contrast, while we observed an increased cell death, although only in combination with cisplatin treatment, we also found unexpectedly, an increase in BDNF protein production, mainly driven by transcripts bearing the exon 6.

Noteworthy, exon 6 transcripts have been reported to be both transcriptionally and translationally repressed in resting conditions, pointing out a tight regulation of translation (Baj and Tongiorgi, 2009; Vaghi et al., 2014). Here we show that exon6 transcripts also play a crucial role in providing a BDNF source under stressing conditions.

Our results are not conclusive, since we did not check for other mechanisms, therefore other study are warranted. Despite we cannot exclude an involvement of Aurora kinase, other mechanisms for translation induction are likely to occur and we can speculate on some of them based on the current literature. For example, BDNF pathway downstream to TrkB receptors was shown to facilitate local translation at synapses by activation of mTOR via PI3K; both mTOR and ERK are able to regulate the assembly of the eIF4E complex, contributing to translation induction (Schratt et al., 2004), so it is conceivable if BDNF contribute to its own production by such mechanism. In addition, other non-canonical ways for translation induction are possible, especially during stressful conditions. Hence, for example, ribosomal shunting provides a way to bypass mRNA secondary structure complexes, which usually causes the ribosome to dissociate in the classical scanning model (Hellen and Sarnow, 2001; Yueh and Schneider, 1996). Other mRNAs, like those for elongation factors and ribosomal proteins, contain a 5' terminal oligo pyrimidine sequence (5' TOP) that acts as a translational regulatory element. Another important means for translation initiation is represent by the internal ribosome entry site (IRES), which are complex secondary structure in 5' non-translated regions that, with the aid of other trans-acting factors, direct the recruitment of ribosomal subunits in a 5'-cap independent manner. IRES-driven mechanism was at first described for picornaviral mRNAs direct translation (Jang et al., 1988) but, after that, many eukaryotic mRNAs have shown to display IRES properties; these include many transcription factors, oncogenes, elongation factors, apoptotic activators and growth factors like FGF2, PDGF-b and VEGF

(Hellen and Sarnow, 2001). It is worth of note that several of these IRES-containing mRNAs encode proteins that are produced as a response to a variety of stress situations, such as inflammation, angiogenesis and cytotoxic drugs (Hellen and Sarnow, 2001; Johannes et al., 1999). Unfortunately, to our knowledge, IRES structure in BDNF mRNAs have not been described so far and deserve further investigations. Another level of complexity is added by the fact that translation and mRNA degradation are in competition with each other but linked by a dynamic process, referred to as the mRNA Cycle, in which mRNP granules known as P-bodies and stress granules plays a pivotal role in control translation (Decker and Parker, 2012).

In conclusion, our results pointed toward a BDNF translation induction mechanism that is Aurora kinese independent, at least in conditions of cytotoxic stress. Furthermore, the observations made could have important clinical implications in the use of Aurora inhibitors as adjuvant in neuroblastoma treatment as, even if increase cell mortality, they facilitate the selection of resistant cells that further enhance BDNF production.

6. CONCLUDING REMARKS

In the present Thesis, we addressed an important topic regarding the inconsistency of assessments of peripheral BDNF protein from body fluids and provide a standardized methodology, at both pre analytical and analytical stages,that we believe would aid to reduce the variability of serum BDNF measurements among studies, making it suitable for potential clinical applications. Secondly, we provide support to the hypothesis that work-related chronic stress causes an imbalance in immune response, with a generalized suppression, as we found reduced circulating chemokine to be associated with high scores of many stress indicators; interestingly, we also observed a positive association with serum BDNF with one of the stress item, suggesting a possible involvement in mechanisms concerning chronic stress protection. Lastly, we gave evidences that BDNF is implicated protection from stressful insults at cellular level, at least in a neuroblastoma cell line treated with the chemotherapeutic drug cisplatin plus an Aurora kinase inhibitor as adjuvant. Further studies will address, *in vitro*, the neuronal effects of the inhibition of chemokines in presence or absence of BDNF trophic support, both in primary and hippocampal slice cultures.

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8. ATTACHMENTS

Published manuscripts

Vaghi V*, Polacchini A*, Baj G, Pinheiro VL, Vicario A, Tongiorgi E. Pharmacological profile of brain-derived neurotrophic factor (BDNF) splice variant translation using a novel drug screening assay: a "quantitative code". J Biol Chem. 2014 Oct 3;289(40):27702-13. doi: 10.1074/jbc.M114.586719. Epub 2014 Jul 29. PubMed PMID: 25074925; PubMed Central PMCID: PMC4183807.
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Ganassi M, Badodi S, Polacchini A, Baruffaldi F, Battini R, Hughes SM, Hinits Y, Molinari S. Distinct functions of alternatively spliced isoforms encoded by zebrafish mef2ca and mef2cb. Biochim Biophys Acta. 2014 Jul;1839(7):559-70. doi: 10.1016/j.bbagrm.2014.05.003. Epub 2014 May 17. PubMed PMID: 24844180; PubMed Central PMCID: PMC4064114.

Submitted manuscript

Polacchini A, Francavilla R, Metelli G, Baj G, Florean M, Mascaretti L G and Tongiorgi E. A standardized method for reproducible measurements of serum BDNF suitable for clinical applications. Scientific Report, NPG Group. (reference number: SREP-15-04914)

Manuscript in preparation

Polacchini A, Albani C, Baj G, Tongiorgi E. Drug resistance in neuroblastoma cells: the role of BDNF synthesis.

9. AKNOWLEDGMENT

Non sarebbe sufficiente questo foglio per esprimere gratitudine verso tutte le persone che mi hanno sostenuto in questo percorso, quindi mi scuso già in anticipo se dimenticherò di ringraziare qualcuno.

Il primo doveroso ringraziamento va al mio supervisore, Enrico, che ha voluto credere in me per questo progetto e che ha saputo darmi i consigli giusti nei momenti di difficoltà.

Grazie anche a Gabriele, Gab, perché mi ha insegnato tanti picccoli e grandi "segreti del mestiere", nonché una buona dose di critica scientifica. Un immenso ringraziamento va chiaramente ad Andrea e Giuliana, con cui non ci annoia mai e riescono a rendere divertente le giornate lavorative (e non). Così come ai ragazzi che nei vari anni anni si sono susseguiti in laboratorio: Tamara "zio mestre", il mitico Alessandro "Pivetta", Elena, Kia, Marco, Saretta, Ruggiero.

Ai miei genitori, che mi hanno sempre sostenuto e consigliato in ogni scelta e ai quali devo tutto.

Ai miei amici, che anche se non ho l'occasione di vedere spesso, so che ci sono e so di poter contare in loro: Richy, Bulfa, Toro, Nello, Bersa e tanti altri che non elenco per motivi di spazio, ma ai quali va ugualmente la mia immensa gratitudine.

Infine, ma non ultima, a Ilenia, che è il motore della mia forza interiore, che mi aiuta ogni giorno, con amore ed estrema pazienza a credere di più in me stesso e senza la quale non sarei mai stato in grado di scrivere queste pagine.