GENEESKUNDE

master in de biomedische wetenschappen: milieu en gezondheid

2010
2011

Masterproef

Gene expression analysis to monitor stress experienced by humans in spaceflight analogues

Promotor :
Prof. dr. Patrick DE BOEVER
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Masterproef voorgedragen tot het bekomen van de graad van master in de biomedische wetenschappen , afstudeerrichting milieu en gezondheid

Maastricht University

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UNIVERSITEIT VAN DE TOEKOMST

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Preface

I want to thank Prof. dr. Greet Schoeters to give me the opportunity to carry out my senior practical training in the division Environmental health and risk at VITO. Also, I want to thank my promoters Prof. dr. ir. Patrick de Boever and Prof. dr. Jaco Vangronsveld for the possibility they gave me to make interesting contacts with other research groups and the chance to learn many new techniques. Also I want to thank you for listening to my ideas, the independence you have given me to start my own experiments and the good advice you recommended in reading my thesis. My senior practical training was a very good learning experience.

Further I also want to thank a few important persons who helped me during my internship. Lab assistant Karen Hollanders and PhD student Britt Wens, I want to thank for the support when I had some trouble with an experiment and for learning me the new techniques involved in my senior practical training. Also many thanks to Jelle Sterckx, student at KHK Geel, for being so motivated to help me in the lab to process all samples. For one specific experiment, namely cell characterization, I want to thank Daniëlla Ooms, to learn me to make a cytospin and to show me how to use the nucleocounter.
Beside them, I want to thank all students and staff members who participated to my experiments. Also I want to thank them for the nice time, friendship and help during my senior practical training.

Finally, I want to thank my parents and brother for the interest in my project. They have been very patient in stressful periods and supportive all the time.

List of abbreviations

ACTH	Adrenocorticotropic hormone
ACT-B	Actin beta
B2M	Beta 2 microglobulin
BDNF	Brain derived neurotrophic factor
cDNA	Complement deoxyribonucleic acid
cRNA	Complement ribonucleic acid
CNS	Central nervous system
CRH	Corticotrophine-releasing hormone
CRP	C reactive protein
Cy3	Cyanine 3
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate
DTT	1,4-Dithiothreitol
EBV	Epstein-Barr virus
EEG	Electroencephalogram
ESA	European Space agency
GABARAP	Gamma-aminobutyric acid receptor-associated protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCR	Galactic cosmic rays
GEx	Gene expression
GO	Gene Ontology
HIV	Human immunodeficiency virus
HPA axis	Hypothalamic pituitary adrenal axis
HPRT1	hypoxanthine phosphoribosyltransferase 1
HTR2A	5-hydroxytryptamine (serotonin) receptor 2A.
IBMP	Institute for biomedical problems
IFNα	Interferon alpha
IL-1	Interleukin 1
IL-6	Interleukin 6
IL-10	Interleukin 10
IL-1α	Interleukin 1 alpha
IL-1β	Interleukin 1 beta
IOM	Institute of Medicine
IPA	Ingenuity pathway analysis
ISS	International Space Station
KEGG	Kyoto Encyclopedia
MAOA	Mono amine oxidase A
MIR	Russian space station
NASA	National aeronautics space administration
NGF	Nerve growth factor
NOS2	Nitric oxide synthase 2
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RDD	DNA digest buffer
POLR2A	Polymerase (RNA) 2 (DNA directed) polypeptide A
qPCR	Quantitative polymerase chain reaction
RLT	RNA lyses buffer
RN18S1	18S ribosomal 1
RNase	Ribonuclease
RPE	Wash buffer
RT-PCR	Reverse transcription polymerase chain reaction
RW1	Wash buffer
SAS	Space adaptation syndrome
SPE	Solar particle events
TNFα	Tumor necrosis factor alpha
TPH1	Tryptophan hydroxylase 1
US	United States
USSR	Union of Soviet socialist republics
UV	Ultraviolet
VITO	The Flemish Institute of Technological Research

Abstract

Space agencies are faced with important health issues that will be experienced during long-duration missions. The late effects of radiation, loss of bone mineral density, and behavioural adaptation have been identified as being crucial for ensuring the astronaut's health. The bioastronautics roadmap mentions psychosocial adaptation, neurobehavioral problems, inadequate cognitive capabilities, and circadian rhythm problems as important issues during flight. Ground-based activities are used for studying these space related issues and to prepare the astronauts as much as possible for the physical and mental demands of long-duration missions.

This study used spaceflight analogues to investigate biological effects induced by stress. We have focused our analysis on gene expression measurements. Blood and saliva samples were collected during the DLR parabolic flight campaign of November 2010 in Bordeaux and a 2009-2010 winter-over campaign at the Antarctic Station Concordia. RNA was extracted from these samples and a set of genes related to immune functioning [IL-1α, IL-1β, IL-6, CRP, TNF and IFN] and neurotransmitter processing [BDNF, TPH1, MAOA, NOS2, HTR2α and GABARAP] was analyzed using real-time PCR. Significant gene expression changes were observed in the first month of isolation compared to the baseline (before departure). Decreased expression was obtained for brain-derived neurotrophic factor (BDNF) ($p<0.001$) and tryptophan hydroxylase 1 (TPH1) ($p<0.001$). Gamma-aminobutyric acid receptor-associated protein (GABARAP) ($p<0.01$) and tumor necrosis factor (TNF) ($p<0.05$) both showed increased expression. These changes may be related to the adjustment to the Antarctic environment. When the fifth month of isolation was compared with baseline, the expression of tumor necrosis factor (TNF) ($p<0.05$) was upregulated and those of interleukin 1β (IL-1β) ($p<0.05$) was downregulated. This suggests that inflammatory genes are influenced by confinement. The RNA extracted from saliva samples, which were collected with QIAgen technology, was of insufficient quality for real-time PCR analysis.

The parabolic flight conditions resulted in a downregulation of interleukin 1β (IL-1β) ($p<0.01$) and tryptophan hydroxylase 1 (TPH1) ($p<0.001$) compared to preflight conditions, while interleukin 6 (IL-6) ($p<0.05$) and gamma-aminobutyric acid receptor-associated protein (GABARAP) ($p<0.001$) were upregulated in blood. Saliva samples, collected using Oragene RNA technology, were compatible with real-time PCR. However, the data were too preliminary to draw conclusions. Subsequently, we explored the value of saliva for non-invasive monitoring. An average of 628 ng/µL [range 45 – 2932 ng/µL] total RNA of normal quality (RIN = 6.8 ± 1.3) was obtained from 1 mL unstimulated whole saliva. Saliva gene expression analysis via real-time PCR and microarray technology appeared to be feasible. A core gene set of 3854 genes was found in unstimulated whole saliva of ten healthy subjects. 885 genes had more than 90% expression in saliva of all individuals.

In conclusion, our pilot studies demonstrated expression differences for genes related to immune functioning and neurotransmission. The relevance of these changes are studied in the project that is implemented at Concordia during the winter-over campaign 2010-2011. The potential of saliva for non-invasive monitoring via gene expression analysis was also revealed. Our new saliva sampling method is currently used in the winter-over campaign at Concordia.

Samenvatting

Ruimtevaart agentschappen worden geconfronteerd met tal van gezondheidsproblemen tijdens een langdurige missie. Het verlies van bot densiteit, radiatieziekte en de moeilijke aanpassing aan de omgeving zijn de belangrijkste gevolgen voor de gezondheid van de astronaut. Deze omstandigheden veroorzaken stress die complicaties kunnen geven in het prestatievermogen en gemoedstoestand van een persoon, als ook slaapritmestoornissen veroorzaken. Grond gebaseerde ruimtevaart analogen worden gebruikt om astronauten zo goed mogelijk voor te bereiden aan de mogelijke fysische en mentale gevolgen ervaren tijdens een missie.

Deze studie gebruikte ruimtevaart analogen om biologische effecten veroorzaakt door stress the onderzoeken. Hierbij werd gebruikt gemaakt van genexpressie metingen. Bloed en speeksel stalen werden verzameld tijdens de DLR paraboolvlucht campagne van November 2010 in Bordeaux en de overwinteringcampagne in het Antarctica station Concordia. RNA was geïsoleerd en een set van genen gerelateerd met het immuunsysteem [IL-1α, IL-1β, IL-6, CRP, TNF en IFN-α] en neuronale transmissie [BDNF, TPH1, MAOA, NOS2, HTR2α en GABARAP] was geanalyseerd met real-time PCR. Significante genexpressie veranderingen waren geobserveerd in de eerste maand van isolatie in Concordia vergeleken met de meetlijn (voor vertrek). Verlaagde expressie was verkregen voor brain-derived neurotrophic factor (BDNF) ($p<0.001$) en tryptophan hydroxylase I (TPH1) ($p<0.001$). Gamma-aminobutyric acid receptor-associated protein (GABARAP) ($p<0.01$) en tumor necrosis factor (TNF) ($p<0.05$) toonden een verhoogde expressie. Dit kan te wijten zijn aan de aanpassing tot de omgeving in Antarctica. In de vergelijking van de vijfde maand in isolatie met de meetlijn was de expressie van tumor necrosis factor (TNF) ($p<0.05$) verhoogd en de expressie van interleukin β1 (IL-1β) ($p<0.05$) verlaagd. Het RNA geïsoleerd van de speeksel stalen, die verzameld waren met QIAgen technologie, was van onvoldoende kwaliteit voor real-time PCR analyse.

De paraboolvlucht condities resulteerde in een verlaagde expressie van interleukine 1β (IL-1β) ($p<0.01$) en tryptophan hydroxylase 1 (TPH1) ($p<0.001$) in vergelijking met condities voor de vlucht, terwijl voor interleukin 6 (IL-6) ($p<0.05$) en gamma-aminobutyric acid receptor-associated protein (GABARAP) ($p<0.001$) een verhoogde expressie werd aangetoond in bloed. Speeksel stalen, gecollecteerd volgens Oragene RNA technologie, waren compatibel met real-time PCR. Doch, verkregen data was te beperkt voor conclusies te trekken. Vervolgens hebben wij de mogelijkheid van speeksel voor niet-invasieve monitoring verkend. Een gemiddelde van 628 ng/µL [draagwijdte 45 – 2932 ng/µL] totaal RNA van gemiddelde kwaliteit (RIN = 6.8 ± 1.3) was verkregen van 1 mL niet gestimuleerd speeksel. Speeksel genexpressie analyse toonde aan dat real-time PCR en microarray kan uitgevoerd worden. Een universele genen set van 3854 genen werd gevonden in niet gestimuleerd speeksel van 10 gezonde individuen. 885 genen hadden een expressie hoger dan 90% in speeksel van alle individuen.

Onze pilote studies hebben expressie veranderingen aangetoond van genen die gerelateerd zijn met immuunsysteem functies en neuronale transmissie. Onze resultaten worden toegepast in het project dat uitgevoerd wordt in Concordia tijdens de overwinteringcampagne 2010-2011. Deze studie heeft ook de mogelijkheid van speeksel onthuld voor niet-invasieve monitoring via genexpressie analyse. Onze nieuwe speeksel staalname methode wordt op dit moment gebruikt in de overwinteringcampagne in Concordia.

1. Introduction

1.1 Humans in space

The first man in space was the Russian cosmonaut Yuri Gagarin. On the 12th of April 1961, he stayed 108 minutes in space. During the next years, the amount of time spent in space increased steadily to 14 days at the end of 1965. These pioneering missions were the basis for the Apollo program in 1966 to explore the Moon with six successful manned lunar landings. In 1973, the Skylab (US) program and Salyut (USSR) program established missions that lasted up to 84 days in space, which was the start for long duration space exploration. The space shuttle was the first reusable space craft which was used to carry out scientific experiments, to launch large satellites and to construct permanent bases in space such as the Mir-station and the ISS-station. With the advent of long-duration space missions such as a one year ISS mission, a 1-month long stay on lunar surface and a 30-month journey to Mars, some events pose an obstacle to the health of astronauts. These include changes in the physical forces on and within the body caused by a reduced weight of the body's components, the modifications in the levels and types of radiation and the psychosocial changes induced by the long-term confinement in a space mission [1]. The bioastronautics roadmap (http://humanresearchroadmap.nasa.gov) identified a number of risks in five crosscutting areas: Human health and countermeasures, Autonomous medical care, Radiation health, Advanced human support technologies and Behavioural health and cognitive performance of humans. The health issues that can influence behaviour and cognitive performance include alterations in the spacecraft internal environmental conditions, the interpersonal dynamics and psychological factors that limit performance of humans during a space mission.

1.2 Physiological changes during spaceflight

1.2.1 Space adaptation syndrome

The Space adaptation syndrome (SAS) is caused by the fact that the human body lacks the ability to distinguish direction is a significant problem for human spaceflight. SAS causes symptoms as nausea, disorientation and headache which is also referred to as 'space motion sickness'. While the major symptoms of SAS abate within 72 hours of exposure to zero-gravity, some components of neurovestibular adaptation require months in flight and re-adaptation upon return to Earth can also require a lot of time. In addition to the main issues of SAS, oculomotor deficits and post-flight postural instability have been reported in a number of astronauts. This can pose operational risks in human spaceflight [2]

1.2.2 Bone mineralisation and muscle loss

Unloading of bones and muscles in zero-gravity results in a 1.2% mass reduction per month. The mass reduction can be explained by the reduced absorption of calcium and Vitamin D. On earth, the human body absorbs daily 400-500 mg of calcium During space flight, that amount drops to 200-250 mg [3]. The loss of calcium occurs in all bones but primarily in the weight-bearing bones like the hips, spine, ankles and upper femur [4]. Another problem is the time needed to restore the calcium and bone levels after return. This can take up to three months with an increased risk of fracture and the additional possibility of

improper healing [5]. Vitamin D levels, which promote the proper use of calcium also decrease in zero gravity. Because UV radiation from the sun is the major source of vitamin D, it is believed that the reduced exposure to the sun causes the decreased vitamin D levels in the human body [6].

1.2.3 Circadian Rhythm

The circadian rhythm system regulates the changes in alertness, performance and sleep rhythm. This system is controlled by a circadian pacemaker, an endogenous biological clock, which is situated in the nucleus suprachiasmaticus. The adrencorticotropic hormone (ACTH), a stress hormone, also influences the circadian rhythm system. The deviation of the circadian rhythm is associated with sleeplessness, disturbed sleep rhythm and depression [7].

1.2.4 Cardiovascular alterations and changes in control systems

Cardiovascular alterations represent serious issues for astronauts both in- and post-flight [8]. The zero gravity environment cause the human cardiovascular system to undergo adaptive changes in both function and structure. The astronauts experience orthostatic intolerance in the form of hypotension upon return. This limits or impairs the ability to function properly immediately after landing. The astronauts are unable to stand up and in even more severe cases extended care for days to weeks are required. The factors involved in orthostatic intolerance are the structural and functional adaptations of the heart and the changes in lymphatic, neural and hormonal systems. Long duration space flight may increase the risk of heart rhythm disturbances. Electrolyte changes, neural and hormonal alterations, and alterations of cardiac myocytes due to prolonged exposure to zero- or low-gravity lead to changes in heart conduction and repolarization, predisposing astronauts to heart rhythm disturbances. Long term spaceflight may result in a significant reduction in cardiac mass due to changes in myocyte quantity and composition [9].

1.2.5 Radiation exposure

The possible effects of radiation on health, including radiation sickness (nausea, weakness, hair loss, skin burns or diminished organ function) and more severe effects such as cancer and neurological disorders are of major concern. The effects are caused by exposure to galactic cosmic rays (GCR) and solar particle events (SPE). The GCR and the SPE both contain significant numbers of high-energy protons, capable of large penetration and important nuclear interactions. The GCR also contain highly ionizing heavy ions, which can … the shielding and penetrate in tissue. A small fraction of SPE can produce large doses leading to early radiation sickness or death if there is no adequate protection [10].

1.3 Psychosocial issues and interpersonal changes

The impact of psychological and interpersonal factors on human behaviour and performance was minimal during the early years of human space exploration. Missions were relatively short and mostly operated by one organisation. The crew members were of same gender and nationality. But then space missions became longer and crew members became multinational [11-12]. The bioastronautics roadmap identified four risk factors with high priority for a 1-year ISS mission and a 2.5-year Mars mission in relation to behavioural health i.e.

performance failure due to problems with psychosocial adaptation, neurobehavioral problems, inadequate cognitive capabilities, and circadian rhythm problems (http://humanresearchroadmap.nasa.gov). These risk factors are influenced by adjustment difficulties caused by confinement for up to three years with the same small group of people and separation from one's customary social and physical environment. The limited communication with Earth, including a delay of up to 24 minutes in bi-directional communications and the loss of privacy due to habitability constraints influence human performance. Language, culture, gender, differences in work role and stress due to equipment failure will pose a burden on crew communication and effectiveness. These challenges have the potential to change neuro-endocrine, cardiovascular and immune responses and to erode cognitive performance. These stress factors can disrupt basic regulatory physiology, such as appetite and sleep and lead to neurological impairment with anxiety and depression. Different preventative and real-time countermeasures are being considered and developed to ensure that astronaut neurobehavioral health is maintained and that crew psychosocial functioning is optimized. For example, exercise is being used as a countermeasure for physical deconditioning [13].

1.4 Psychosocial factors influence mood and performance in spaceflight

There has been an increased awareness that psychosocial factors are important to the success of missions. A first factor is interpersonal tension, which has been related to personality, gender, and career motivation. For example, during the 211-day Salyut 7 mission a female cosmonaut experienced problems due to gender stereotyping. Upon arrival, she was asked to prepare the meals for the male crew members already on board [14]. A second factor concerns leadership role and cultural differences. In long-term space missions leadership status can decline or leaders can handle the wrong role at the wrong time. This can lead to confusion which can have an effect on crew performance. This has been recognized in studies of the individual and interpersonal problems that occurred during the Shuttle-Mir space program and other long-duration Russian/Soviet missions. In two on orbit studies conducted by Kanas and colleagues, the changes in mood, cultural differences, displacement of tension and leadership roles were examined by using questionnaires [14-15]. The questionnaires included items of the profile of mood states [16] and two measurement scales: Group environmental scale [17] and Work environmental scale [18]. Results showed displacement of negative emotions to outside personnel and other and the role of the leader was related to crewmember cohesion. There was also evidence for national (e.g. American versus Russian), occupational (e.g. crew member versus mission control) and organizational (NASA versus Russian space agency) cultural differences. The cultural differences influence humans in the way they cope with stress and this effect increases when the crew is multicultural. Some characteristics such as the expression of emotions, are familiar in some cultures but unusual in others. Further, the mental health can differ among cultural groups. On board the ISS station, 17 crew members participated a study to observe cultural differences and mental health. Data showed an association between depression and anxiety in American participants and an association of depression with fatigue in Russian subjects. The findings also suggested that anger was related to depression for both Russians and Americans [19]. Language, can also influence crews cohesion negatively. These stresses not only relate to national and dialect differences

but also to technical and slang characteristics of space communication. For example, a Russian cosmonaut experienced feelings of tension and discomfort when a French cosmonaut was sent up to Salyut 7. In contrast, he felt more comfortable with Russian visitors [20]. A number of American space shuttle astronauts who have flown with an international crewmember endorsed language issues [21].

1.5 Space analogues for studying behavioural health and performance in spaceflight

Nearly all of the confinement studies to simulate the psychological effects experienced during long-term spaceflight are performed on Earth in laboratory or special environments. They focus on psychological and behavioural characteristics of individuals and groups in relation to performance and stress reactions. These ground-based activities are utilized to make sure that our astronauts are prepared as much as possible in the future for the physical and mental demands of long-duration exploration missions, and development of countermeasures against any adverse effects of such a mission.

1.5.1 Parabolic flight

In a parabolic flight, there is approximately 20% of free flight, during which zero-gravity is achieved. The period of weightlessness is followed by equal periods of hyper gravity as the aircraft enters and exits the parabolic manoeuvre. Multiple parabolic manoeuvres are carried out during a flight resulting in repeated cycles of normal gravity (1 g), microgravity (0 g) and hyper gravity (1.8 g) (**Figure 1.1**). A number of parabolic flight experiments have shown that processes within the human central nervous system are affected by weightlessness. The acute stress response in parabolic flight participants is characterized by alterations in stress hormone concentrations (ACTH, cortisol, epinephrine and prolactine) which are related to changes in mood and performance [22-23].

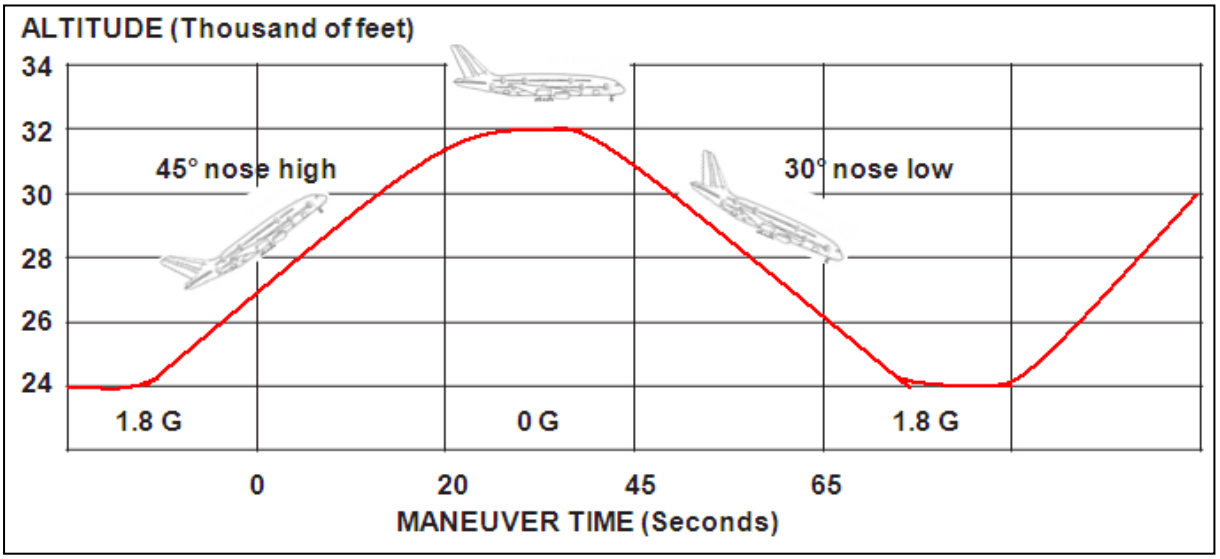


Figure 1.1: Trajectory flown during parabolic flight. The aircraft starts by accelerating to gain velocity before pulling up to convert horizontal velocity into vertical velocity. During the pull-up the *g* level increases. When a sufficient upward velocity is achieved, the pilots "push-over" and reduce power so that the aircraft and occupants fall together. At the end of the parabola the pilots pull up and the *g* level increases again. The cycle is then repeated.

1.5.2 Mars-500 study

The Mars-500 study is an experiment of the European space agency (ESA) in cooperation with the International institute for biomedical problems (IBMP) to gather data, knowledge and experience to help prepare for a real mission to Mars. Across a time frame of 520 days, six crew members (three Russian, two European and one Chinese) are sealed in an isolation chamber to mimic the journey to Mars. One part of the isolation chamber simulates the spacecraft that would transport them on their journey to and from Mars and another part simulates the landing module that would transfer them to and from the Martian surface (**Figure 1.2**). Once sealed into the chamber, the participants only have personal contact with each other and voice contact via a simulated control centre with a 20-minute delay as it occurs in real spaceflight. The crew eats an identical diet to that used in the ISS and they are free to take certain personal items with them. During the isolation period several elements of the Mars mission will be imitated, travelling to Mars, orbiting the planet, landing and return to Earth. The crew will be responsible for monitoring and maintaining health and psychological states of themselves and each other, controlling and maintaining systems, controlling resource consumption, carry out cleaning, as well as fulfilling scientific investigations.

Figure 1.2: Layout of the isolation chamber Mars 500

To prepare for this long duration study, a 105-day isolation study was performed in 2009 to gather information about the facilities and the candidates. ESA hypothesized that exercise would be able to prevent and counteract mood changes during isolation. Electrocortical data (EEG) and a self report on current psychological and physical state were recorded several times prior to and after exercise during the isolation period. Results showed a positive effect of exercise on mood and electrocortical activity. Correlation analysis revealed significant relation between changes in perceived physical state, motivation, psychological state and electrocortical activity, suggesting that confinement is accompanied by behavioural changes such as reduced activity and that exercise counteracts psycho-physiological deconditioning during isolation [24].

1.5.3 Devon Island

NASA assessed comprehensively the immune status of field team members participating in the Haughton-Mars Project on Devon Island in the high Canadian Arctic. The purpose of the study was to evaluate the effect of mission-associated stressors on the human immune system. The immunophenotype analysis was performed by peripheral leukocyte distribution and the different levels of activated T cells, plasma cortisol, EBV viral antibody and intracellular cytokine profiles. Results indicated immune function changes that were also observed in astronauts in space [25].

1.5.4 Antarctic base studies

Many research has been carried out in Antarctic bases in relation to behavioural health and performance similar to those observed in spaceflight. The influence of isolation and confinement on social support and depressed mood was examined in two studies: one study at McMurdo Station in Antarctica and one study at the Amundson-Scott South Pole station. In the first study, the availability of social support and satisfaction were examined by using the social support questionnaire. Depressive symptoms were measured by an index of 18 symptoms (for example loss of energy, fatigue, loss of interest). Mood was examined by completing the profile of mood states. Both studies showed a serious decline in the level of satisfaction with support obtained, as well as a significant increase in depressed mood. High levels of tension-anxiety, depression and anger preceded an increase in advice seeking, but high levels of advice seeking also preceded an increase in tension-anxiety and depression. The results suggest a significant erosion of social support under conditions of prolonged isolation and confinement, leading to an increase in depressed mood [26].

Depressed mood caused by prolonged isolation and confinement is related to changes in cortisol levels. A study on personnel overwintering at two British Antarctic stations examined the diurnal rhythm of saliva cortisol and its association to adaptation, performance and health. Mood was analyzed using questionnaires including standardized measures of subjective health complaints, positive and negative affect, Burnam screen for depression and performance. Results indicated high levels of cortisol immediately after arrival which was correlated positively with base commander's evaluation of performance. During midwinter, the Burnam scale indicated a relation of depression with sleep problems in 58% cases but in general no indication was found that over-wintering led to any disturbance in the diurnal rhythm of cortisol in British Antarctic personnel [27].

Confinement is a stressful condition and a major determinant of immune reactivity. Tingate et al. observed this effect in Australian National Antarctic research expedition populations exposed to prolonged periods of isolation in the Antarctic. Alterations of T cell function were monitored during a 9-month isolation period. The alterations were mediated by changes in inflammatory cytokine production of TNF-a and multiple interleukins such as IL-1, IL-6 and IL-10 [28].

1.6 Biological processes involved in stress response

Environmental stressors (for example, confinement, interpersonal factors and reduced privacy) influence different biological processes (hormone, neurotransmitter, growth factor and cytokine) that may be important for the behavioural health, performance, appetite and sleep rhythm of humans. These biological changes are part of the adaptive process to achieve stability or homeostasis. The process is called allostasis and is essential in order to maintain internal viability. The adaptation within the nervous, endocrine and immune systems, triggered by seasonal changes, confinement and unpredictable events, are a response to stress. Long-term exposure to stress creates an allostatic overload in which neurohormones, neurotransmitters, neuropeptides and neurotrophins stimulate a series of adaptation responses. These typically include behavioural, cardiovascular, metabolic, endocrine and immunological changes (immunosuppression as well as inflammation) (**Figure 1.3**).

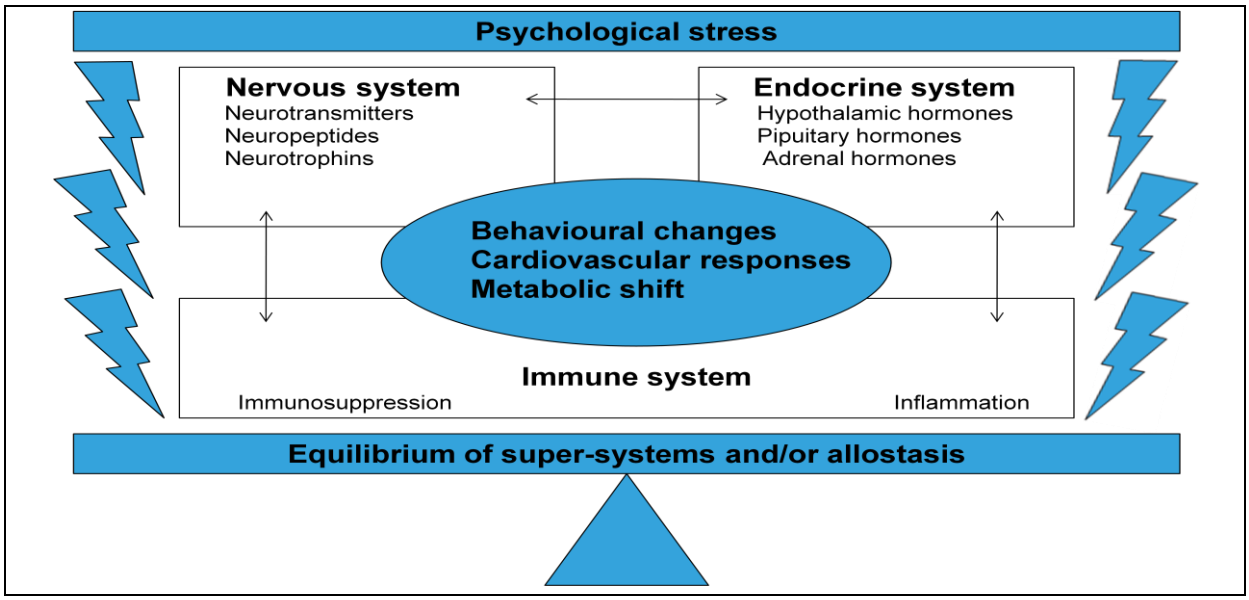


Figure 1.3: Principles of stress response. The activation and eventual downregulation of the nervous, endocrine and immune systems ensures equilibrated stress response patterns that results in allostasis. Allostatic overload causes upregulation of immune responses and diseases [28]

Cytokines and inflammatory mediators such as IL-6, TNF-α, IL-1α, IL-1β and IFN-α1 can signal the brain, thus, influencing behaviour and other complex body reactions which can lead to changes in mood en performance. For example, IL-6 influences the activity of the HPA-axis. When stimulated, the hypothalamus releases the corticotrophine-releasing hormone (CRH). In response, the pituitary gland secretes the adrenocorticotrophic hormone (ACTH), which stimulates the secretion of cortisol [29-30]. The secreted cortisol initiates a series of metabolic effects which act as a feedback system for the negative influence of stress on the hypothalamus and pituitary. The activation of these stress hormones are associated with altered central nervous system function, sleep disturbances, and altered circadian rhythms [31-32]. Proinflammatory cytokines such as IL-6 can also induce sickness behaviour and depressive symptoms. When IL-6 is activated, it stimulates CRP. Higher levels of CRP are associated with cardiac risk. This suggests that CRP and IL-6 should be positively associated with the incidence of mood disorders such as depression [33-34].

Serotonin, a monoamine neurotransmitter, is a well-known contributor to the feelings of a well-being. It is also called the happiness hormone despite not being a hormone. Serotonin has already been associated with psychiatric disorders. It plays numerous roles in appetite, sleep, memory and learning, mood, cardiovascular function and endocrine functions. Tryptophan hydroxylase (TPH) is a rate-limiting enzyme in the biosynthesis of serotonin and therefore a crucial step in serotonin functioning [35]. HTR2a, a serotonin receptor, is also involved in serotonin activation and mono-amine oxidase A (MAOA) has a vital role in the degradation of serotonin. Due to their central role in serotonin synthesis and degradation, genes encoding for TPH and HTR2a are proposed as candidate genes for the incidence of depression [36].

Nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) are also associated with psychiatric disorders. These factors are neurotrophins involved in the development and maintenance of specific populations of neuronal cells. Recently, the NGF and BDNF have been related to physiological homeostasis and behavioural coping. Altered levels of neurotrophins have been associated to several psychiatric disorders, in which particularly BDNF plays a major role in the onset and progression of pathologies such as schizophrenia and depression [37-38].

Gamma-Amino Butyric acid receptor associated protein (GABARAP) responds to GABA, an amino acid which acts as a neurotransmitter in the central nervous system. It inhibits nerve transmission in the brain, calming nervous activity. Without GABA, nerve cells fire too often and too easily. Clinical data indicate that decreased GABA function accompanies depressed or manic mood states. Low GABA function is proposed to be an inherited biological marker of vulnerability for development of mood disorders. Environmental factors, including stress and excessive alcohol use, may increase GABA, causing symptoms of depression or mania [39].

Nitric oxide synthase 2 (NOS2) is an enzyme that generates nitric oxide. The neuronal expression of this enzyme can be induced by proinflammatory cytokines, chronic stress and trauma. Increased expression of NOS2 mRNA was observed in hippocampal neurons, which is the region that appears to play a role in the mechanism of depression. Therefore, gene encoding for NOS2 can be a candidate gene for mood disorders [40].

1.7 Hypothesis and objectives

The hypothesis of this study is to observe the effect of stress on biological processes in spaceflight analogues by means of gene expression profiling in saliva and blood.

The first aim examines if biological effects can be observed by means of gene expression analysis in spaceflight analogues. The first pilot study is embedded in the DLR parabolic flight campaign in November 2010. The overall aim of this campaign is to observe the coherence between brain cortical function and neuro-cognitive performance during changed gravity conditions. During a parabolic flight sequence, acute stress can have an impact on biological processes in the human body. This study will observe the effects of acute stress induced by zero-gravity on biological processes by means of gene expression analysis. The second pilot study is embedded in a project called BEACON or 'Assessment of biomarkers of **Be**havioural **A**daptation and health during isolated stay in **Con**cordia'. The overall aim of this project is to profile the behavioural adaptation and health of healthy volunteers confined in the Antarctic station Concordia. This station is used by the European Space Agency (ESA) as a similar environment for studying specific aspects of space missions. This study evaluates in a pilot study the biological processes that chronic stress induces in a confined environment. It is expected that changes in gene expression profiling, caused by isolation, will be observed and may have an effect on mood and mental fitness (cognitive performance). These effects can be identified at a very early stage.

The second aim of this research is to observe the feasibility of saliva as non-invasive biological fluid for monitoring disease/health. In the past 10 years, the use of saliva as a diagnostic fluid has been successfully applied in diagnostics and in predicting populations at risk of a variety of conditions (for example: HIV and Hepatitis). Recently, the research group of Wong determined that diagnostic human mRNA is present in the saliva of healthy and unhealthy people. Molecular analysis of saliva is a promising research avenue because of the ease with which saliva samples can be collected and the potential of gene expression profiling to identify the health/disease status at an early stage.

This study provides a first insight in the use of saliva for non-invasive monitoring of health via gene expression analysis. The two pilot studies will offer more understanding in the downstream processes and mechanisms that are induced by stress experienced by humans in spaceflight analogues.

2. Materials & Methods

2.1 Molecular characterization of saliva in healthy individuals

2.1.1 Participants

Saliva samples were obtained from 18 healthy donors from the Unit Environmental Health and Risk at the Flemish Institute of Technological Research (VITO) and students from Hasselt University. The study population was composed of eight healthy males and ten healthy females with a mean age of 23 (range 20 - 29). The study carried out on different days from November 2010 through May 2011.

2.1.2 Study design

The subjects were randomly divided in five small experiments (**Figure 2.1**). In the first experiment, two sampling methods were compared. Three female subjects collected saliva with stimulation (sugar) and without stimulation at two different time points (Wednesday and Friday). In the second experiment, degradation of RNA and the volume of saliva needed to extract qualitatively good RNA was examined. Five participants, (4 females and 1 male) each provided three saliva samples without stimulation at one time point. One saliva sample was collected as described by manufacturer's protocol by DNA Genotek. One saliva sample had a reduced volume (250 µL) of saliva and the other sample had a delay of 30 minutes before adding the RNA stabilisation buffer. The third experiment studied the integrity of unstimulated salivary RNA in three female subjects at three time points (Wednesday, Thursday and Friday). The feasibility of saliva for gene expression analysis via qPCR was tested in a fourth experiment. For this experiment, an unstimulated saliva sample was collected from ten individuals at one time point. The fifth experiment examined the feasibility of saliva for microarray profiling via Agilent technology and to determine the differences between low input and high input Cy3 labeling.

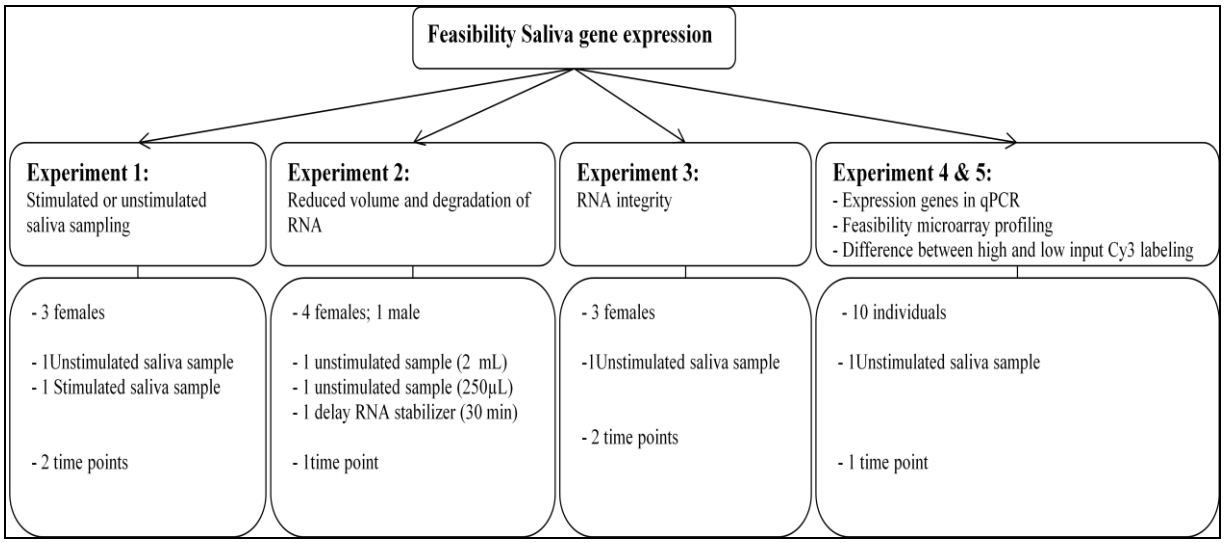


Figure 2.1: Study design of sample collection for molecular characterization of saliva.

2.1.3 Saliva sample collection

Unstimulated or stimulated saliva samples (2 mL) (**Figure 2.2**) were collected with Oragene RNA kit (DNA Genotek) in the morning. Subjects were asked to refrain from eating, drinking, smoking or oral hygiene procedures for at least one hour prior to saliva collection. Before collection, the subjects were asked to rinse their mouth three times with water. Saliva samples were incubated at 50 °C for one hour , aliquoted (1mL) in microcentrifuge tubes and stored at -20 °C.

Figure 2.2: Oragene RNA kit vessel

2.1.4 RNA extraction and quality control

Total RNA was isolated from 1000 µL of whole saliva sample using the standard Qiagen RNeasy Micro kit with minor modifications to the manufacturer's protocol. Two aliquots of sample (500 µL) were incubated at 90 °C for 15 minutes and cooled to room temperature. The aliquots were neutralized with Neutralizer solution ($1/25^{th}$ volume), incubated on ice and centrifuged at maximum speed (>13000g) for 3 minutes. The clear supernatant was transferred into a fresh microcentrifuge tube and two volumes of cold 95% ethanol was added. After incubation at -20°C for 30 minutes and centrifugation at maximum speed (>13000g) for 3 minutes, the supernatant was discarded and pellet was dissolved in 350 µL RLT buffer. The mixture was vortexed and 350µL of 70% ethanol was added. Both aliquots were transferred to a RNeasy MinElute spin column and centrifuged at 12000g for 30 seconds. Different wash steps and on-column DNase treatment was done for each sample during RNA extraction. Total RNA was eluted in 14 µL RNase Free water. The quantity of total RNA was assessed with Nanodrop 1000 spectrophotometer and quality of total RNA was measured on the Agilent Bioanalyzer 2100 using the Agilent RNA 6000 Nano/Pico kit.

2.1.5 Gene Expression analysis by Q-PCR in saliva

5'-Exonuclease assays were carried out using Lightcycler Roche 480 and Primetime qPCR gene expression assays (Integrated DNA technologies). An 0.5 - 1 µg aliquot of the isolated salivary RNA was reverse-transcribed into cDNA by means of Transcriptor First Strand cDNA Synthesis kit (Roche) using a Veriti 96 well Thermal cycler (Applied Biosystems). Incubation occurred for 10 minutes at 65 °C and PCR conditions were set to 10 minutes at 25 °C, 30 minutes at 55 °C, 5 minutes at 85 °C and hold on 4 °C. 5 µL of the resulting cDNA (4 ng) was used for PCR amplification with Lightcycler 480 probe master mix and primer-probe

assays for target genes and housekeeping genes (**paragraph 2.3**). All reactions were carried out in duplicate or triplicate and real-time PCR programme was set to one cycle of pre-incubation at 95°C for ten minutes followed by 45 cycles of amplification (10 seconds at 95°C, 30 seconds at 62°C and 1 second at 72°C) and one cycle of cooling at 40°C for ten seconds.

2.1.6 One colour 4x44K whole genome V2 Microarray (Agilent)

A concentration of 200 ng of total RNA in a final volume of 1.5 µL was used for labeling amplification according to the Low input Quick Amp Labeling kit (Agilent). Hybridizations were carried out with the Tecan 4800 Hybridization station using 1.65 µg of Cy3-labeled linearly, amplified cRNA. Microarrays were hybridized overnight, washed and dried according to manufacturer's protocol and scanned using DNA Microarray scanner with surescan high resolution technology (Agilent). The image files were converted to a data file with Feature extraction v10.7 software using GE1_107_sep09 standard protocol and analyzed with Genespring 11.5.1 (Agilent). This protocol has been repeated with a higher input of total RNA (1 µg) in a final volume of 8.3 µL in more individuals (n=10) using another Cy3 labeling kit (High input Quick Amp Labeling kit from Agilent).

The genes with the highest expression values according to Genespring 11.5.1 (Agilent) were categorized on the basis of their known roles in biological processes and molecular functions through the use of IPA (Ingenuity® Systems, www.ingenuity.com), Gene Ontology (GO) enrichment analysis, Kyoto Encyclopedia of Genes and Genomes (KEGG) and REACTOME via ClueGO, Cytoscape (www.cytoscape.org).

IPA Functional Analysis identified the biological functions and/or diseases that were most significant to the data set. Molecules from the dataset that met the p-value cut-off of 0.05 and were associated with biological functions and/or diseases in the Ingenuity Knowledge Base were considered for the analysis. Right-tailed Fisher's exact test was used to calculate a p-value determining the probability that each biological function and/or disease assigned to that data set is due to chance alone. Canonical pathways analysis identified the pathways from the IPA library of canonical pathways that were most significant to the data set. Molecules from the data set that met the p-value cutoff of 0.05 and were associated with a canonical pathway in the Ingenuity Knowledge Base were considered for the analysis. The significance of the association between the data set and the canonical pathway was measured in 2 ways: 1) A ratio of the number of molecules from the data set that map to the pathway divided by the total number of molecules that map to the canonical pathway is displayed. 2) Fisher's exact test was used to calculate a p-value determining the probability that the association between the genes in the dataset and the canonical pathway is explained by chance alone.

GO, KEGG and REACTOME were carried out with ClueGO, an open-source Java tool that extracts the non-redundant biological information for large clusters of genes. GO describes gene products in terms of their associated biological processes, cellular components and molecular functions. KEGG, a collection of online databases with genomes, enzymatic pathways and biological chemicals, records networks of molecular interactions in the cells and REACTOME is a database of human pathways including DNA replication, transcription,

translation, the cell cycle, metabolism and signalling cascades. The terms are structured in a hierarchical relationship (parent-child). Due to the complexity of the hierarchical structure (directed acyclic graph), the terms can be in several different levels. Criteria such as organism, type of analysis, identifier type and statistics were included for the analysis. GO levels started at level 3 to level 8 and kappa score was set to 0.6. The significance of each term/group was calculated with hypergeometric test and p-value correction with Bonferroni.

2.2 Molecular characterization of blood in healthy individuals

2.2.1 Blood sample collection

Blood samples (about 3 mL) were collected in Tempus tubes. These tubes contained 6 mL of RNA stabilisation buffer (Applied Biosystems, Ambion). When blood was collected, the Tempus tubes were mixed for 10 seconds to homogenize the content and stored at -20°C.

2.2.2 RNA extraction from blood

Total RNA from blood samples were isolated according to Ribopure^{TM} blood kit (Ambion). A frozen blood sample was thawed to room temperature. The cap from the Tempus Tubes was removed and the contents of the tube were poured into a 50 mL tube. 1x Phosphate buffered saline was added to the sample to bring the total volume to 12 mL and mixed vigorously for 30 seconds on a vortex mixer. The sample was centrifuged at 3000 g for 30 minutes at 4°C with a brake at the end. The supernatant was carefully discarded from the tube and RNA pellet (transparent and invisible) stayed behind. To ensure remaining drops were removed, the tube was left inverted on absorbent paper for one or two minutes and the rim was cleaned with absorbent paper. Resuspension solution (400 µL) was added to the bottom of the tube and mixed briefly to resuspend the RNA pellet. Subsequently, 100 µL of wash solution 1 and the resuspended RNA were transferred onto a filter and centrifuged for 30 seconds at 16000 g. Flow-through was discarded and 500 µL wash solution 1 was added to the filter. After centrifugation for 30 seconds at 16000 g, the flow-through was discarded and 500 µL wash solution 2 was applied onto the filter. The last step was repeated and to ensure all solvents were removed from the filter, the membrane was dried by centrifugation for one minute at 16000 g. The filter was transferred to a new microcentrifuge tube and 100 µL Elution solution was applied onto the filter. The microcentrifuge tube was incubated in a heating block at 70°C for 2 minutes and after that centrifuged for 30 seconds at 16000 g. The RNA solution was transferred back onto the filter and centrifuged for 2 minutes at 16.000 g. Subsequently, 90 µL of the RNA eluate was transferred to a new microcentrifuge tube without disturbing pelleted particles at the bottom of the tube.

2.2.3 Gene expression analysis by Q-PCR in blood

5'-Exonuclease assays were performed using Lightcycler Roche 480 and Primetime qPCR gene expression assays (Integrated DNA technologies). 0,6 – 0,7 µg of total RNA was used as starting material for the reverse transcription using the First strand transcriptor cDNA synthesis kit (Roche). cDNA samples were amplified using Lightcycler 480 probe master mix and the commercial gene expression assays with primers and probes for five housekeeping genes and twelve selected genes from literature (**See paragraph 2.3**). Real-time PCR

programme was set to one cycle of pre-incubation at 95°C for ten minutes followed by 45 cycles of amplification (10 seconds at 95°C, 30 seconds at 62°C and 1 second at 72°C) and one cycle of cooling at 40°C for ten seconds. All analysis were run in duplicate.

2.3 Selection of housekeeping genes and genes of interest

Genes of interest were selected from literature **(Paragraph 1.6)**. Proinflammatory cytokines and inflammatory mediators were selected based on the relationship of these molecules with neurotransmission, immune functioning and psychiatric disorders such as depression. Standard available PrimeTime™ std qPCR assays were ordered by IDT according to refseq numbers (**Table 2.2**). To measure integrity of RNA, three RT positive control assays targeting three different positions on the same transcript (three different primer sets and three different probes) were selected. The assays were ordered by Roche according to assay ID.

Table 2.2: Selected genes

Gene	Refseq number	PrimeTime™ Std qPCR Assay
Interleukin 6	NM_000600	Hs.PT.42.3074634
Tumor necrosis factor α	NM_000594	Hs.PT.42.4895449.g
C reactive protein	NM_000567	Hs.PT.42.3341389.g
Interleukin 1α	NM_000575	Hs.PT.42.3819002
Interleukin 1β	NM_000576	Hs.PT.42.2155977
Interferon α1	NM_024013	Hs.PT.42.3184790.g
Brain-derived neurotrophic factor	NM_001143805	Hs.PT.42.1139585.g
Gamma-aminobutyric acid receptor-associated protein	NM_007278	Hs.PT.42.2542712
Mono amine oxidase A	NM_000240	Hs.PT.42.2503798
Tryptophan hydroxylase 1	NM_004179	Hs.PT.42.455878
Nitric oxide synthase 2	NM_000625	Hs.PT.42.3031133.g
5-hydroxytryptamine (serotonin) receptor 2A	NM_000621	Hs.PT.42.158162.g
18S ribosomal 1	NR_003286	Hs.PT.42.122532.g
Glyceraldehyde 3-phosphate dehydrogenase	NM_002046	Hs.PT.42.1164609
Beta 2 microglobulin	NM_004048	Hs.PT.42.2752530
Hypoxanthine phosphoribosyltransferase 1	NM_000194	Hs.PT.42.3476197
Actin B	NM_001101	Hs.PT.42.227970.g
RT+ (5' end): Polymerase (RNA) 2 (DNA directed) polypeptide A	NM_000937	102978
RT+ (middle): Polymerase (RNA) 2 (DNA directed) polypeptide A	NM_000937	102127
RT+ (3’ end): Polymerase (RNA) 2 (DNA directed) polypeptide A	NM_000937	102977

The housekeeping genes (RN18S1, GAPDH, B2M, HPRT1 and ACT-B) used here were preselected because of their constitutive, non-regulated stable expression over a wide spectrum of tissues. The preselected housekeeping genes are not suitable for every kind of tissue and therefore need to be analyzed prior to use. The detection of suitable reference genes was carried out with qPCR. The most stable genes were determined by examining the expression profiles and intensities of the preselected housekeeping genes in Genex Enterprise 5.3.2 software by using geNorm (http://www.multid.se). Technical repeats of raw Cq values

were averaged and converted into relative quantities for analysis with geNorm, where the relative quantity for each gene was compared to the control group. The program selects from a panel of candidate reference genes a combination of multiple stable genes for normalization. The gene expression stability (M) value is based on the combined estimate of intra- and intergroup expression variations of the genes studied and takes the PCR efficiency into account. The threshold for considering a gene stable is M-value ≤ 0.5 [41].

2.4 Pilot study: Parabolic flight

The study was approved by the ethics committee of the German sport University in Cologne. Nine volunteers of good general health were recruited for the DLR parabolic flight campaign in November 2010. The study population was composed of six men and three women. Volunteers were subjected to 30 parabolic flight manoeuvres of each 60 seconds. During each manoeuvre, the volunteers underwent 20 seconds of zero gravity. After 5 manoeuvres, there was a rest period of a few minutes. Blood samples (about 3 mL) were collected in Tempus tubes one day before flight (BF) and immediately after flight (AF). Unstimulated saliva samples were collected according to Oragene RNA protocol (DNA Genotek) one day before flight and after each period of ten cycles (**Figure 2.2**). RNA was isolated from all samples and cDNA synthesis was carried out as described in previous sections. The effect of acute stress was measured in preselected genes (paragraph 2.3) via real-time PCR.

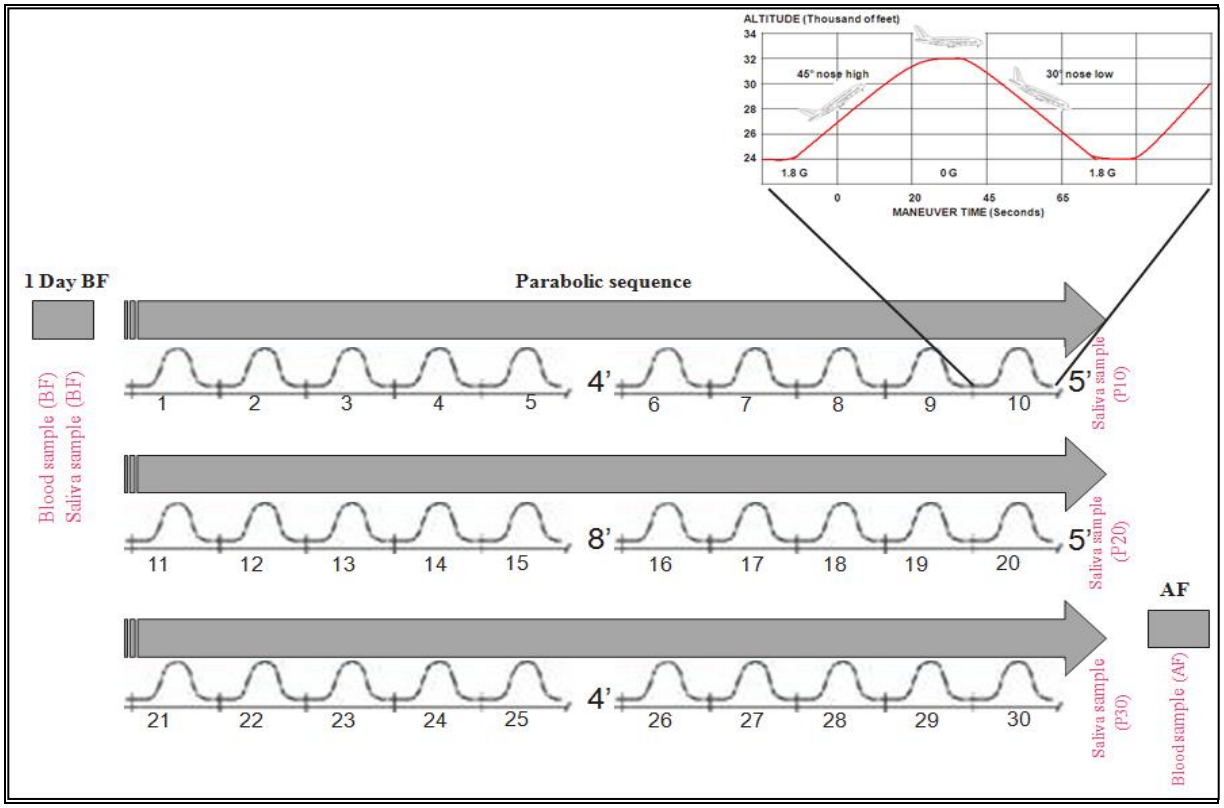


Figure 2.2: Study design of the parabolic flight sequence during the DLR parabolic flight campaign in November 2010

2.5 Pilot study: BEACON

BEACON or 'Assessment of biomarkers of **Be**havioural **A**daptation and health during isolated stay in **Con**cordia' is a research project from ESA in collaboration with VITO, the French polar institute (Institut Paul Emile Victor, IPEV) and the Italian Antarctic Programme (Consorzio per l'Attuazione del Programma Nazionale di Ricerche in Antartide, PNRA S.C.r.l.). The Antarctic station Concordia is used as a similar environment for studying specific aspects of space missions (**Figure 2.3**). This station is a research facility that was built 3,200 m above sea level at a location called Dome C on the Antarctic Plateau. Dome C is one of the coldest places on Earth. Temperatures hardly rise above −25°C in summer and can fall below −80°C in winter with a recent record –84.6°C in 2010. The annual average air temperature is −54.5°C and air pressure is 645 hPa. Humidity is low and it is also very dry, with very little precipitation throughout the year.

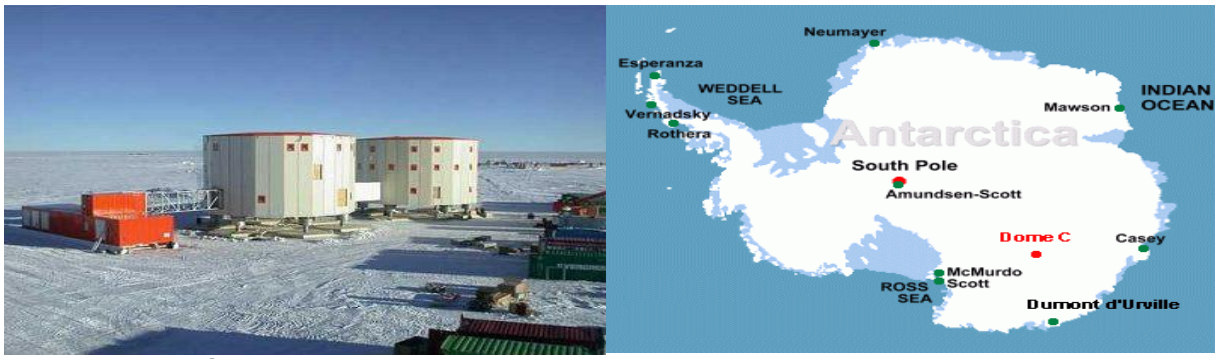


Figure 2.3: Concordia station at Dome C in Antarctica

Thirteen healthy volunteers were confined in the Antarctic station Concordia for ten months during the Antarctic winter (from January 2010 until November 2010). Two volunteers did not participate to the study. One volunteer only gave a baseline sample and one volunteer was excluded from the pilot study due to illness at baseline sample collection. The study population was composed of nine men. Health status, cognitive performance and mood were determined in a pre-departure meeting by using RAVEN intelligence test and MMPI personality test. Intelligence and personality can be possible confounders in the study.

Table 2.3: Description and time point sampling

test	predeparture	Months at Concordia									
		1	2	3	4	5	6	7	8	9	10
RAVEN	■										
MMPI	■										
PPS						■		■			
SCL-90							■				
NES		■	■	■	■	■	■	■	■	■	■
COWAT		■								■	
KSSFC			■							■	
blood GEx	■	■	■	■	■	■	■	■	■	■	■
saliva GEx	■	■	■	■	■	■	■	■	■	■	■
blood protein		■		■		■		■		■	
saliva protein		■		■		■		■		■	
urine		■		■		■		■		■	

During confinement, the participants were subjected to a psychological evaluation. Intelligence, personality, stress evaluation and mental health were observed using a neurobehavioral evaluation system (NES), perceived stress scale-development of a cold (PPS), SCL-90 symptoms checklist and Ketter stress symptom frequency checklist: self-applied and group (KSSFC). Biological analysis was carried out on blood, urine and saliva samples which were collected on 11 occasions. Stress hormones, neurotrophins, cytokines and cardiovascular markers are planned to be measured (**Table 2.3**). In this pilot study, a molecular evaluation was carried out on blood samples of 3 time points (predeparture, month 1 and month 5) by means of gene expression analysis using qPCR. Saliva samples, which were collected according to RNeasy Protect Saliva mini kit protocol (QIAgen), were also processed.

2.6 Statistics

The analysis of the expression data of the parabolic flight study and BEACON study compared to control samples was carried out with Genex Enterprise Software 5.3.1. The software normalized the measured Cq-values of the target genes with those of the reference genes by considering the PCR efficiency. Raw Cq values were converted to $\log_2$ (relative quantities compared to the control group). A two-tailed paired T-test was carried out with a significance level set to * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Data are presented as mean percentage of $\log_2$ (fold change).

Microarray data from saliva samples were processed with Genespring 11.5.1. A two-tailed unpaired T-test was carried out to compare the high input treatment with the low input treatment with a significance level set to $p<0.05$. The time points between individuals were analyzed with a two-tailed paired T-test with $p<0.05$.

3. Results

3.1 Pilot study: BEACON

Total RNA was extracted and quality control was measured in 121 blood samples. The average concentration of total RNA was 119 ± 37 ng/µL. The A260/280 ratio was 2.0 ± 0.0, A260/230 ratio was 2.1 ± 0.2 and average RIN values were equal to 8.7 ± 0.8 (**Supplemental data I**). Further analysis was carried out on baseline -, first month (T1) - and fifth month (T5) samples to gain a first insight in the adjustment to the environment in Antarctica and the isolation in the Concordia station. Gene expression changes were measured in 12 genes. CRP was excluded from the analysis because data was only obtained from a few samples. The signal reached the detection limit of the qPCR. All target genes were normalized to 5 housekeeping genes with an M-value lower than 0.5 according to geNorm software (**Table 3.1**).

Table 3.1: M-values of 5 housekeeping genes tested in blood for all individuals (n=18)

Gene name	M-value
RN18S1	0,273
HPRT1	0,211
B2M	0,197
ACT-B	0,147
GAPDH	0,147

Results, represented as Log_2(FC) in figure 3.1, showed significant changes in 4 genes when T1 and T0 samples were compared. On average, a 161.87% downregulation ($p < 0.001$) was observed in BDNF and a 19.62% upregulation ($p < 0.01$) in GABARAP. TNF showed a mean upregulation of 50.57% ($p < 0.05$) and TPH1 a mean downregulation of 73.68% ($p < 0.001$). When analyzing Log_2(FC) of T5 with T1 (**Figure 3.2**), two genes were significantly changed. IL-1β was 21.49% downregulated ($p < 0.05$) and TNF was 15.74% upregulated ($p < 0.05$). Results also showed heterogeneity between the individuals. In figure 3.3, an opposite expression for GABARAP, TNF and MAOA was demonstrated for one individual. It was always another individual that showed a different expression. HTR2α, IFN-α1, IL-1α, IL-1β, IL-6, IFN-α1 and NOS2 had many variability between the expression patterns of the individuals. Some individuals showed an increased expression while other a downregulation of the genes. Same findings were found in the results of T5 compared to T1 (**Figure 3.4**). In the significant genes (TNF and IL-1β), there was one individual differently expressed compared to the other subjects. Many variation in expression was observed between all individuals for the non significant genes.

Total RNA from 4 randomly chosen saliva samples were extracted to observe the RNA yield and quality (**Table 3.2**). All saliva samples represented a very low RNA yield (Mean total RNA= 18.6 ± 10.0 ng/µL) and many contamination (A260/280 ratio = 2.8 ± 1.8; A260/230 ratio = 0.1 ± 0.2). Saliva samples were not adequate for further processing.

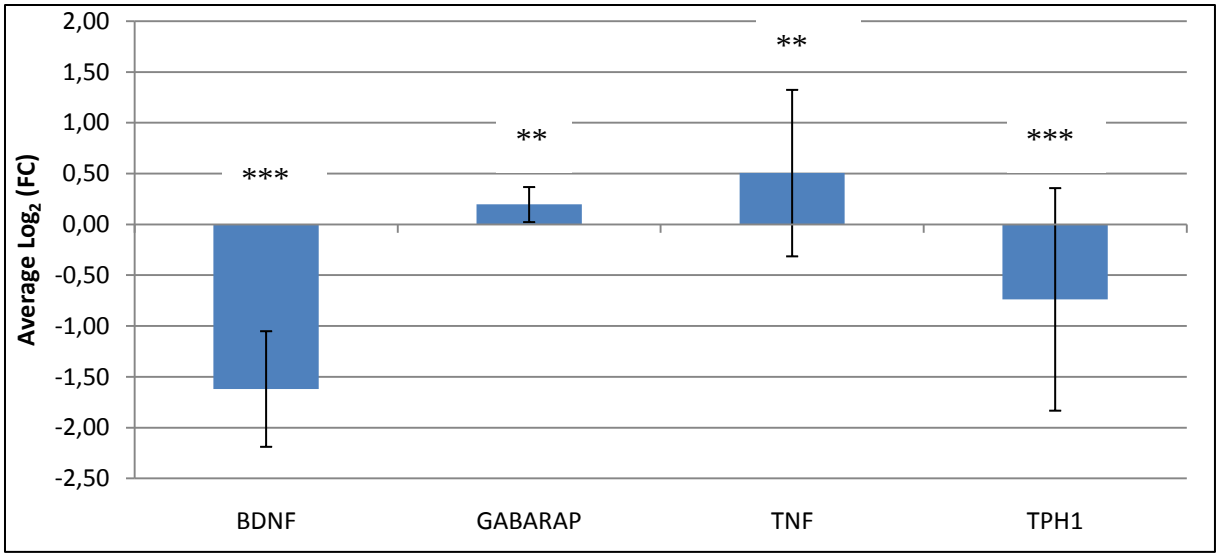


Figure 3.1: Averaged Log 2 gene expression changes (n=9) and standard deviation of 4 significant genes when comparing T1 and T0 samples. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$ of average Log_2 (FC)

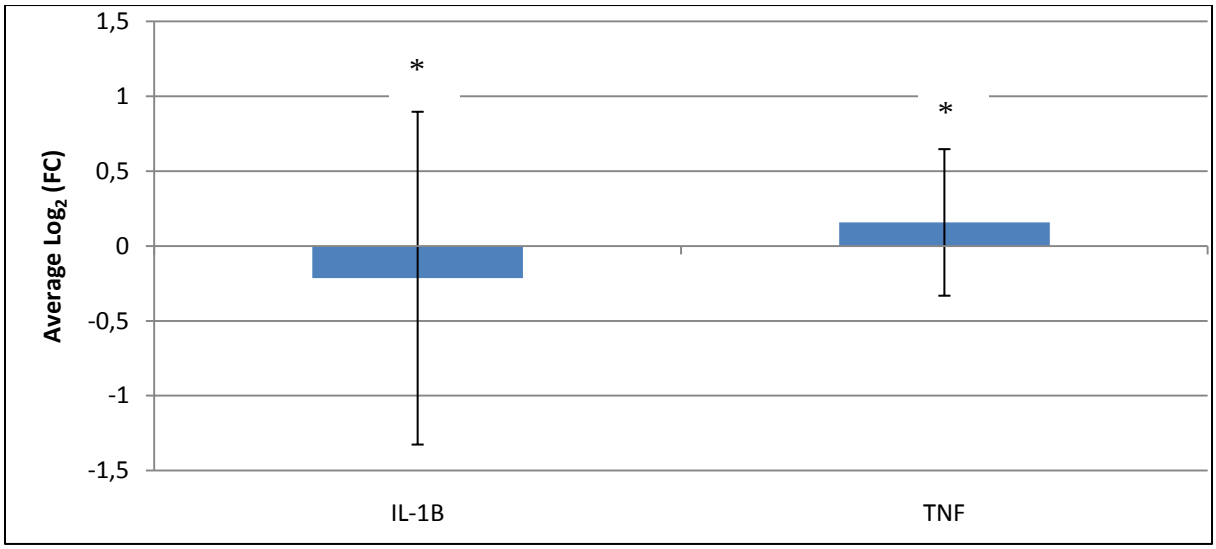


Figure 3.2: Averaged Log 2 gene expression changes (n=9) and standard deviation of 2 significant genes when comparing T5 and T1 samples. * = $p < 0.05$ of average Log_2 (FC)

Table 3.2: RNA yield and quality of 4 BEACON saliva samples

Sample ID	ng/ul	260/280	260/230
T8-P4	14.9	2.1	0,03
T8-K3	23.3	1.8	0,19
T8-Z10	6.7	5.6	0,01
T8-M6	29.6	1.9	0,34
Mean ± Stdev	18.6 ± 10.0	2.8 ± 1.8	0.1 ± 0.2

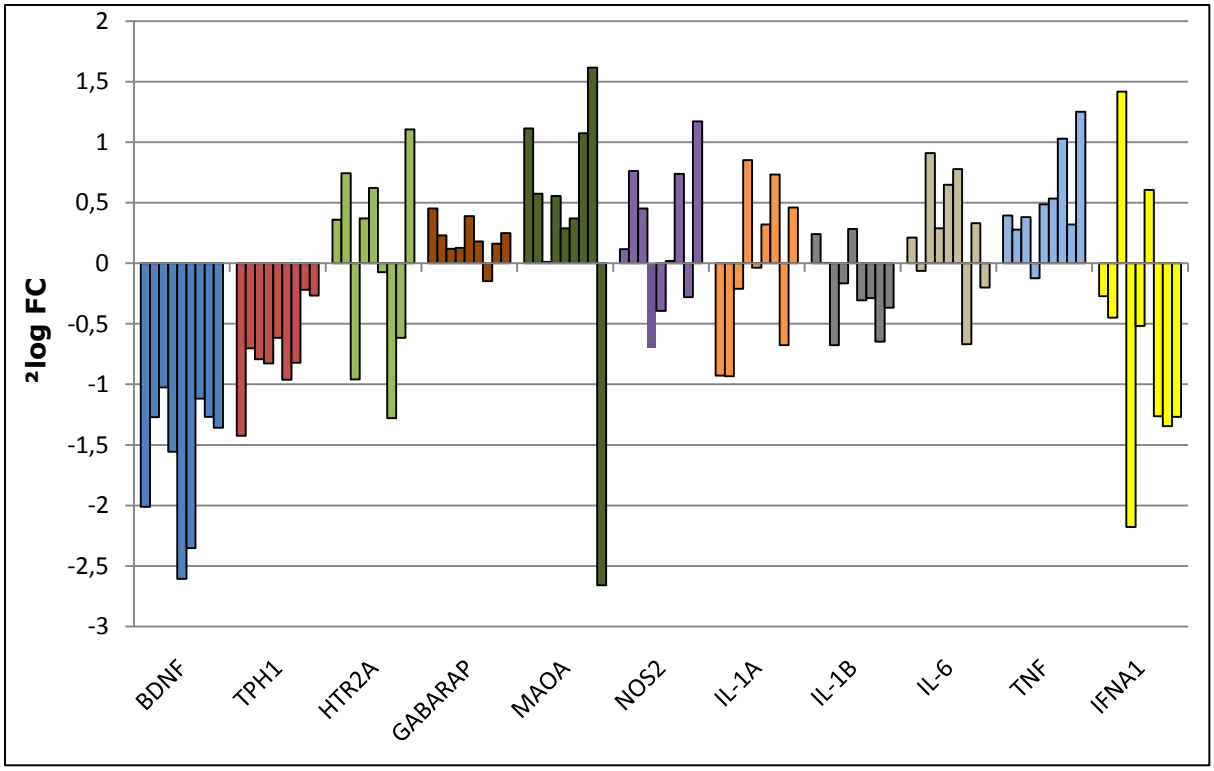


Figure 3.3: Blood gene expression changes of 11 selected genes of interest in 9 individuals. Samples collected in the first month of isolation (T1) were compared to the baseline samples (T0). Results are presented in Log_2 (Fold change).

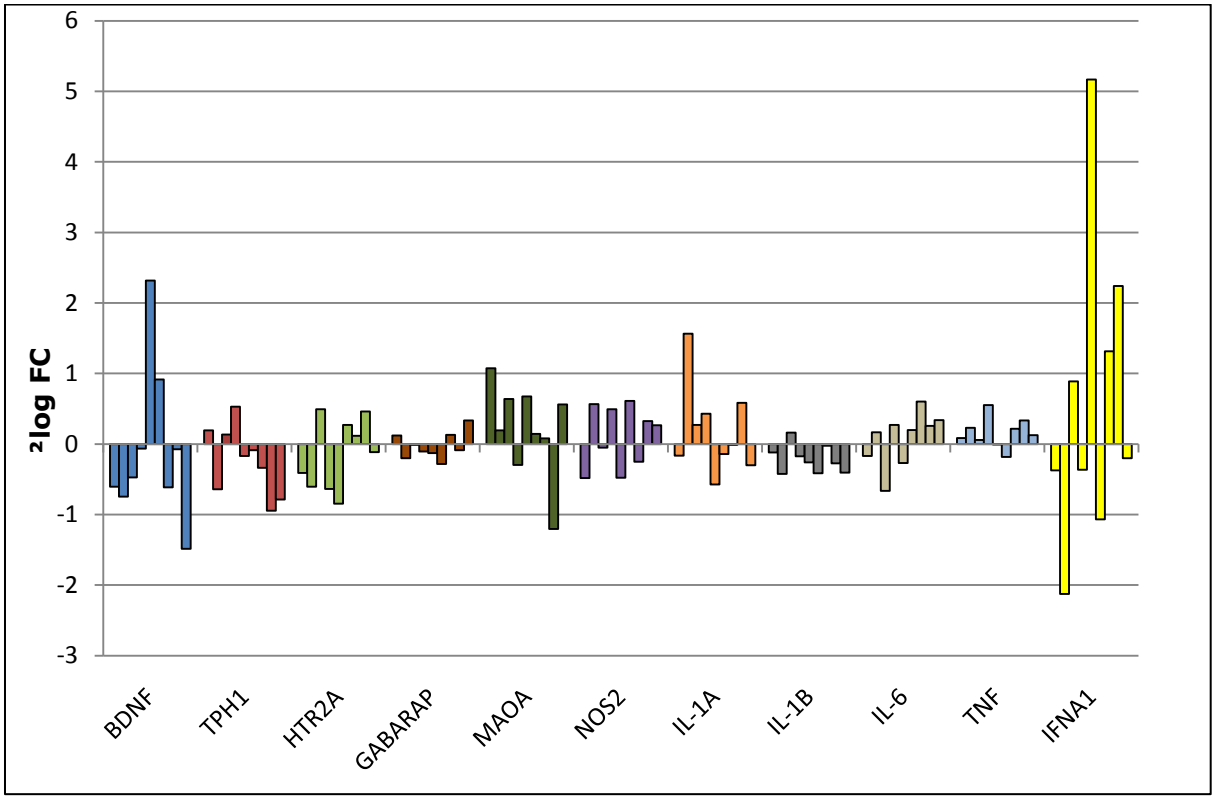


Figure 3.4: Blood gene expression changes of 11 selected genes of interest in 9 individuals. Samples collected in the fifth month of isolation (T5) were compared to the samples of the first month of isolation (T1). Results are presented in Log_2 (Fold change).

3.2 Feasibility of saliva gene expression analysis

It was clear out of the BEACON results (paragraph 3.1) that saliva collected and processed via RNeasy Saliva mini kit (QIAgen) was useless for real-time PCR analysis. Recently, a new kit called Oragene RNA for expression analysis self collection kit (DNA Genotek) was launched on the market. The feasibility of this kit was tested in this study to observe whether this sampling method can be used in future research.

3.2.1 RNA isolation and quality control

Eighteen healthy individuals were recruited to evaluate a sampling method for obtaining RNA from whole saliva (Oragene RNA from DNA Genotek). Total RNA from 1000 µL unstimulated whole saliva was extracted as described in materials and methods. The quality control was carried out with Nanodrop and Bioanalyzer 2100. Depending on the concentration of total RNA, a different bioanalyzer assay was used (Pico assay for ≤ 25 ng and Nano assay for ≥ 25 ng). On average, 628 ± 765 ng/µL of total RNA was obtained (n=18) and quality control results showed that most saliva samples (n=16) contained intact RNA with an average RIN value of 6.8 ± 1.3. Individual P1 and P12 had a very low RIN value of 3.8 and 4.0. This indicated that the RNA was degraded. No values were obtained for individual P7 and P18. No protein contamination (A260/280 = 2.0 ± 0.1) was observed but due to the wash steps with ethanol there was minor solvent contamination (A260/230 = 1.7 ± 0.4) (**Table 3.3**).

Table 3.3:Characteristics of individuals: Gender, Age, Total RNA amount of saliva (ng/µL), protein contamination (260/280 ratio), solvent contamination (260/230 ratioà and quality control (RIN)

ID	Gender	Age	ng/µL	260/280	260/230	RIN
P1	F	24	335	2.0	2.0	3.8
P2	F	20	48	1.8	0.9	8.1
P3	F	22	213	2.0	1.5	7.2
P4	F	21	129	2.0	1.5	7.8
P5	F	22	456	2.0	2.0	7.5
P6	F	26	416	2.0	2.0	7.2
P7	M	20	631	2.0	2.0	N/A
P8	M	20	137	2.0	1.5	7.4
P9	M	21	333	2.0	2.0	7.1
P10	M	29	2932	2.0	2.3	7.7
P11	M	24	2274	2.0	1.7	5.5
P12	M	23	442	2.1	2.1	4.0
P13	F	23	149	2.1	0.8	8.2
P14	M	23	736	2.1	2.2	6.7
P15	M	23	724	2.0	1.5	6.6
P16	F	24	487	2.0	2.1	7.1
P17	F	24	46	2.0	1.5	6.7
P18	F	24	816	2.1	2.0	N/A
Mean ± Stdev			628 ± 765	2.0 ± 0.1	1.7 ± 0.4	6.8 ± 1.3

3.2.2 Inter-intra variability of saliva sampling

The difference of stimulated and unstimulated saliva was compared within one individual and between individuals at different time points. Three stimulated and 3 unstimulated saliva samples were collected from 3 female subjects at two time points. The mean total RNA amounts of the stimulated and unstimulated samples were compared within each individual (**Figure 3.5**). Participant 1-3 showed a difference of respectively 358 ± 253 ng/µL, 1067 ± 755 ng/µL and 405 ± 286 ng/µL of total RNA between unstimulated and stimulated saliva samples. By studying the RNA amounts of the two time points in stimulated and unstimulated saliva, results revealed inter and intra variability between individuals and differences were observed between the time points (**Figure 3.6**).

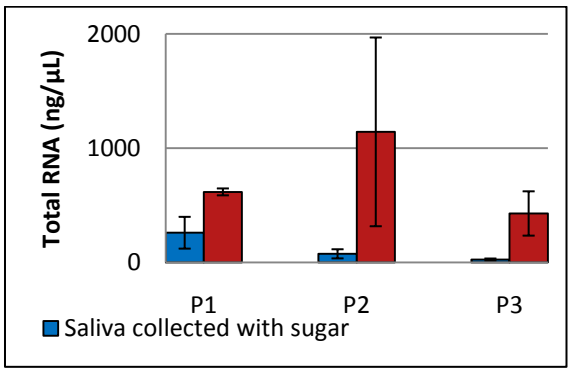


Figure 3.5: Mean total RNA amount of two time points and standard deviation in stimulated and unstimulated saliva of 3 female subjects.

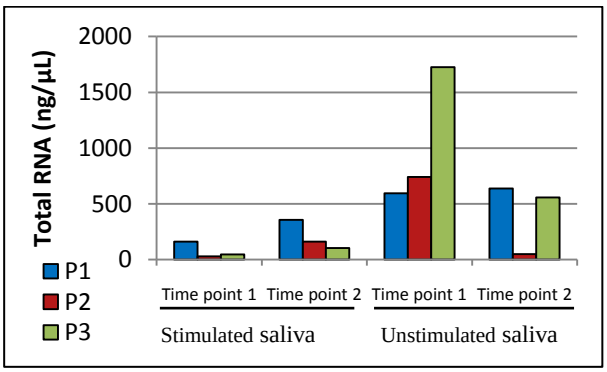


Figure 3.6: Total RNA amount of stimulated and unstimulated saliva at two time points in 3 female individuals

The degradation of RNA and the variation in total RNA amount between a reduced volume of saliva (250 µL) and normal volume (2 mL) was studied. Five individuals (4 females and 1 male) donated each 3 unstimulated saliva samples. All collection vessels were placed on ice. One sample was collected according to manufacturer's protocol, one sample had a reduced volume of saliva (250 µL) and one sample had a delay of 30 minutes before adding RNA stabilizer. The variability of total RNA amount was compared in all individuals between the sample collected as in the prescribed protocol and the samples with a reduced volume of saliva (**Figure 3.7**) and delay of adding RNA stabilizer (**Figure 3.8**). Reduced volume of saliva (250 µL) resulted in lower total RNA amounts in comparison to the RNA amounts of saliva collected according to manufacturer's protocol. Quality control results showed an A260/230 ratio = 0.84 ± 0.86 and a mean RIN value = 6.4 ± 0.7 of total RNA in 250 µL saliva from 4 individuals. The 260/230 ratio was much below the quality range of 2.0 due to the excess of RNA stabilizer added. When stabilisation buffer was added just 30 minutes after saliva sampling, total RNA amount was reduced for 3 out of the 5 participants. No solvent contamination was measured (A260/230 ratio = 1.9 ± 0.4) and RNA was of good quality (average RIN value = 6.6 ± 0.9).

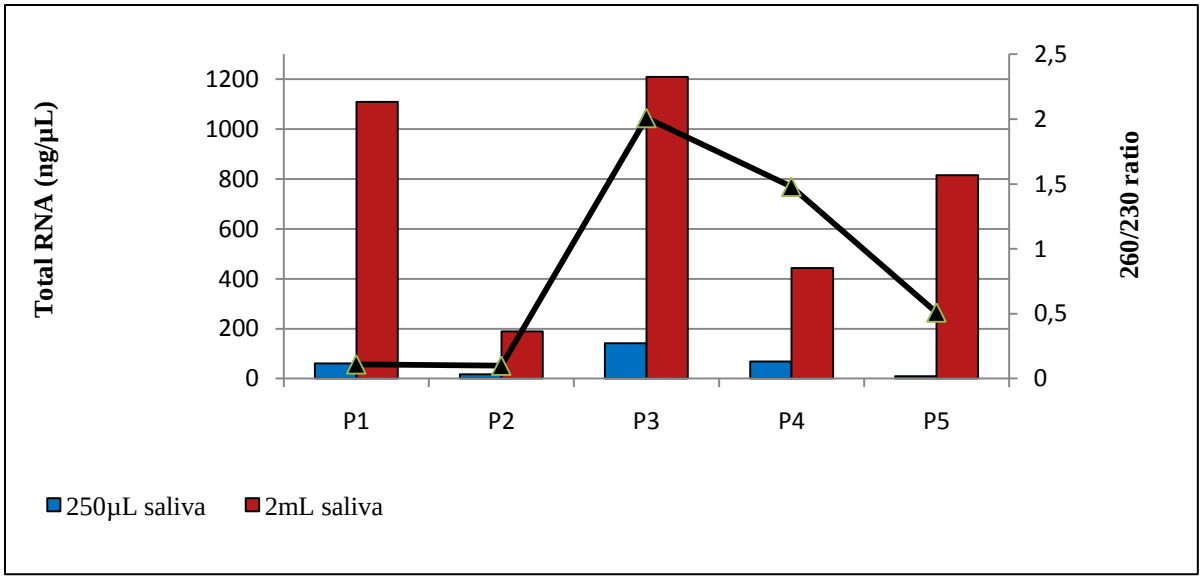


Figure 3.7: The change in total RNA amount was observed in reduced volume of saliva (250 µL) from 5 individuals. Left axis: total RNA amount (ng/µL). Right axis: 260/230 ratio

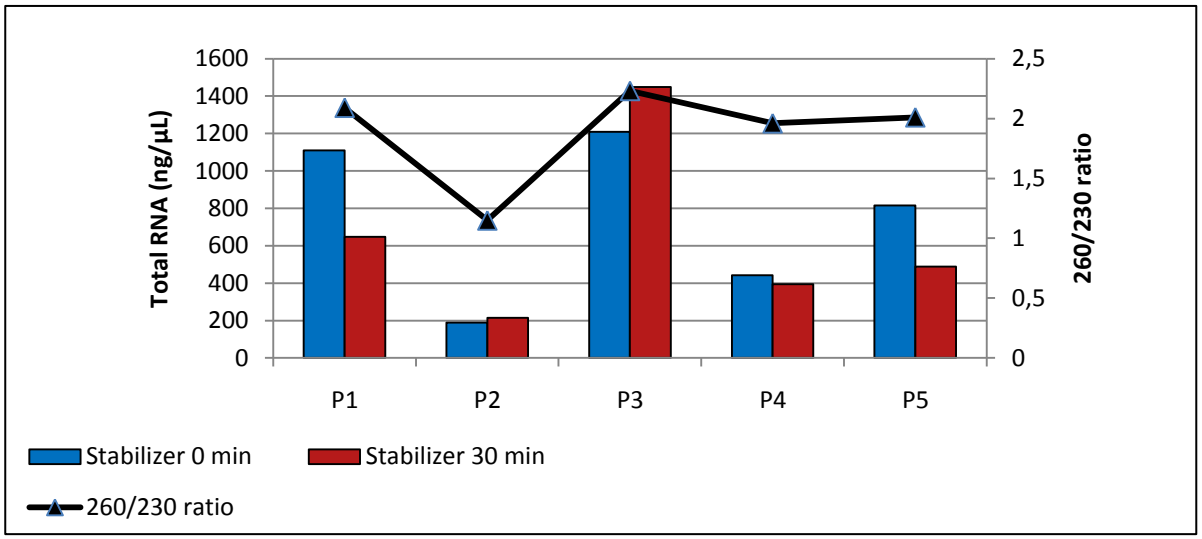


Figure 3.8: The change in RNA amount was observed in saliva samples with a delay of adding RNA stabilizer (30 minutes) in 5 individuals. Left axis: total RNA amount (ng/µL) Right axis: 260/230 ratio.

3.2.3 Integrity of salivary RNA

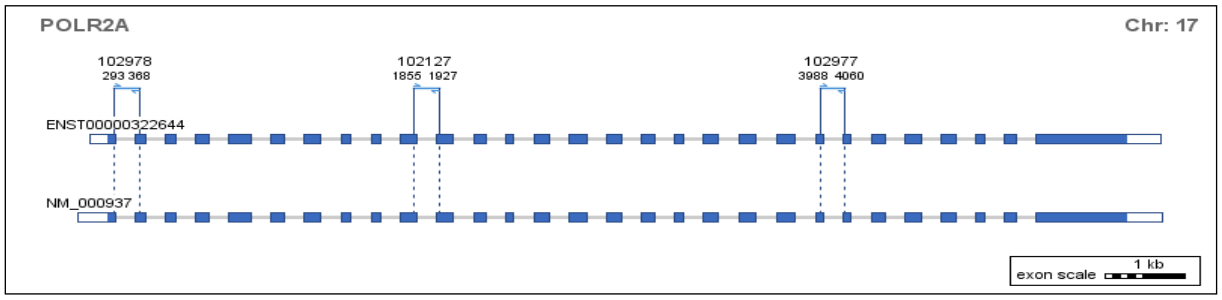
The integrity of salivary RNA was evaluated in unstimulated saliva from 3 female subjects collected at different days. Total RNA amount obtained from 1000 µL unstimulated whole saliva was on average 608 ± 520 (n=8). Quality control results showed that 6 from the 8 samples contained intact RNA with a mean RIN value = 6.9 ± 0.7. Two RIN values were not available because the software could not calculate the value based on the fluorescence signal measured. No protein contamination (A260/280 ratio = 2.1 ± 0.2) was observed but at time point 1 of P2, solvent contamination was measured (**Table 3.4**). To determine whether salivary RNA contained full length mRNA, a qPCR using 3 different primer pairs of polymerase RNA 2 polypeptide A (POLR2A) was carried out. The primer pairs were selected in a way the amplicons nearly spanned the full-length mRNA of POLR2A (**Figure 3.9-A**).

The qPCR was carried out in triplicate. Technical repeats of all Cp values ranged within 1 Cp (Standard deviation < 0.16). As shown in figure 3.9-B, Cp values of the primer pairs were detected in all individuals (n=3). Ratios of the averaged Cp values from each primer pair (POLR2A: 82/81; 82/83; 81/83) were taken for all samples (n=8). All ratios were equal to 1 which means that the RNA stayed intact from the 5'-3' region.

Table 3.4: Concentration and quality of total RNA of all saliva samples (n=8).

Participant	Time point	ng/µL	260/280	260/230	RIN
P1	1	596	2.1	2.1	7.3
	2	490	2.1	1.6	N/A
	3	639	2.0	2.2	7.1
P2	1	742	2.0	1.9	6.8
	2	65	2.4	2.4	N/A
	3	49	2.5	4.8	5,6
P3	1	1727	2.1	2.0	7.4
	3	559	2.0	2.4	6.9
Mean ± stdev		608 ± 520	2.1 ± 0.2	2.4 ± 1.0	6.9 ± 0.7

A



B

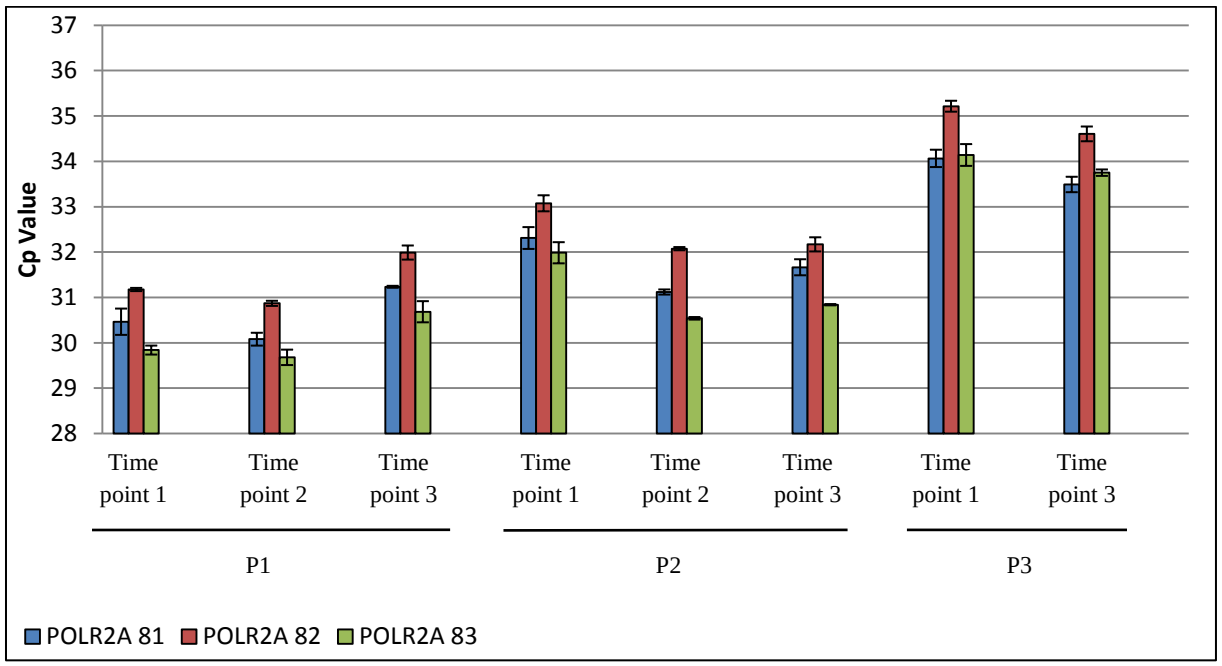


Figure 3.9: A: 5'-3' RNA strand of POLR2A and the different sections for the 3 primetime assays (ID 102978 = POLR2A 81; ID 102127 = POLR2A 82; ID 102977 = POLR2A 83). B: Ct values of 3 POLR2a probes in saliva from 3 female subjects collected on 3 different days.

3.2.4 Feasibility of qPCR and stability of housekeeping genes

The possibility to carry out gene expression analysis in saliva was tested for 17 genes of interest. Total RNA of a pooled saliva sample (n=2) was used for cDNA synthesis and detection of gene expression in qPCR. Eleven genes of interest and 5 housekeeping genes demonstrated Cp values below the detection limit (Cp = 40). Technical variation was always < 1 Cp value except for the TPH1 and NOS2. The TPH1 gene (2 values) and NOS2 gene (1 value) had some missing data because the Cp values were situated near the detection limit. HTR2A showed no Cp values and could not be detected in saliva (**Table 3.5**).

Table 3.5: Average Cp value of Genes of interest and Housekeeping genes

GOI	Average Cp	GOI	Average Cp	GOI	Average Cp	HK-gene	Average Cp
IL-1B	26.0	IL-1A	31.4	TNF	28.2	ACT-B	24.8
CRP	29.2	GABA	29.2	HTR2A	/	HPRT1	34.5
IL-6	35.4	BDNF	27.9			GAPDH	25.2
TPH1	37.9	IFNA1	26.8			RN18S1	17.0
NOS2	38.0	MAOA	33.6			B2M	24.7

The stability of 8 potential housekeeping genes was evaluated in saliva samples of 10 individuals. Expression stability for each housekeeping gene was analyzed using geNorm software. All investigated genes exhibited low average expression stability with M-values higher than 0.78 (**Figure 3.10**). This low stability was plotted in the range of Cp values of all individuals (n=10) for each gene (**Figure 3.11**). The RPLI3A gene showed the lowest Cp range equal to 3.0 and B2M the highest with a range of 7.5 Cp values (Right axis) between the 10 individuals. The variability of housekeeping genes between individuals resulted in no stable housekeeping gene for normalization using the geNorm procedure.

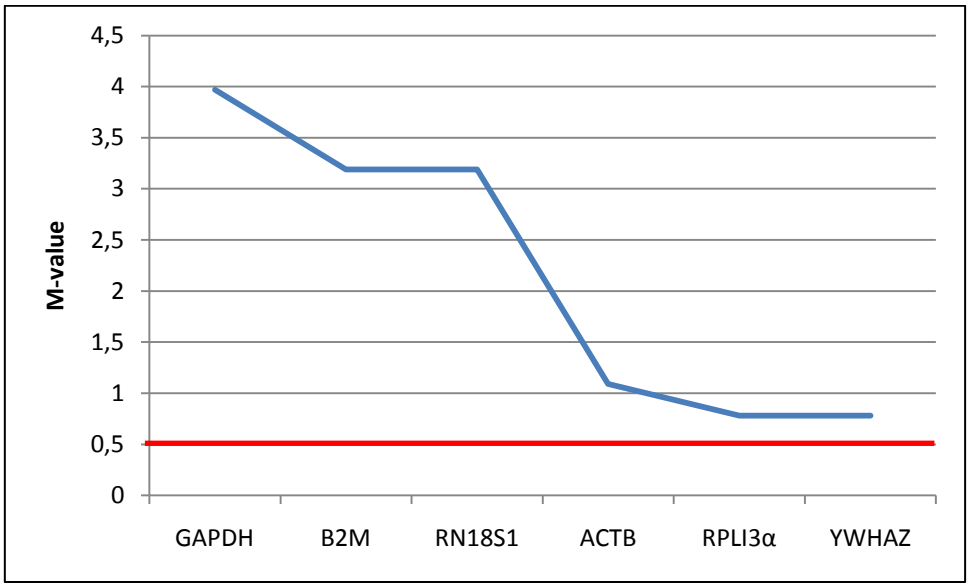


Figure 3.10: M-values of 6 housekeeping genes tested in saliva from 10 individuals. Treshold M-value < 0.5 (red line)

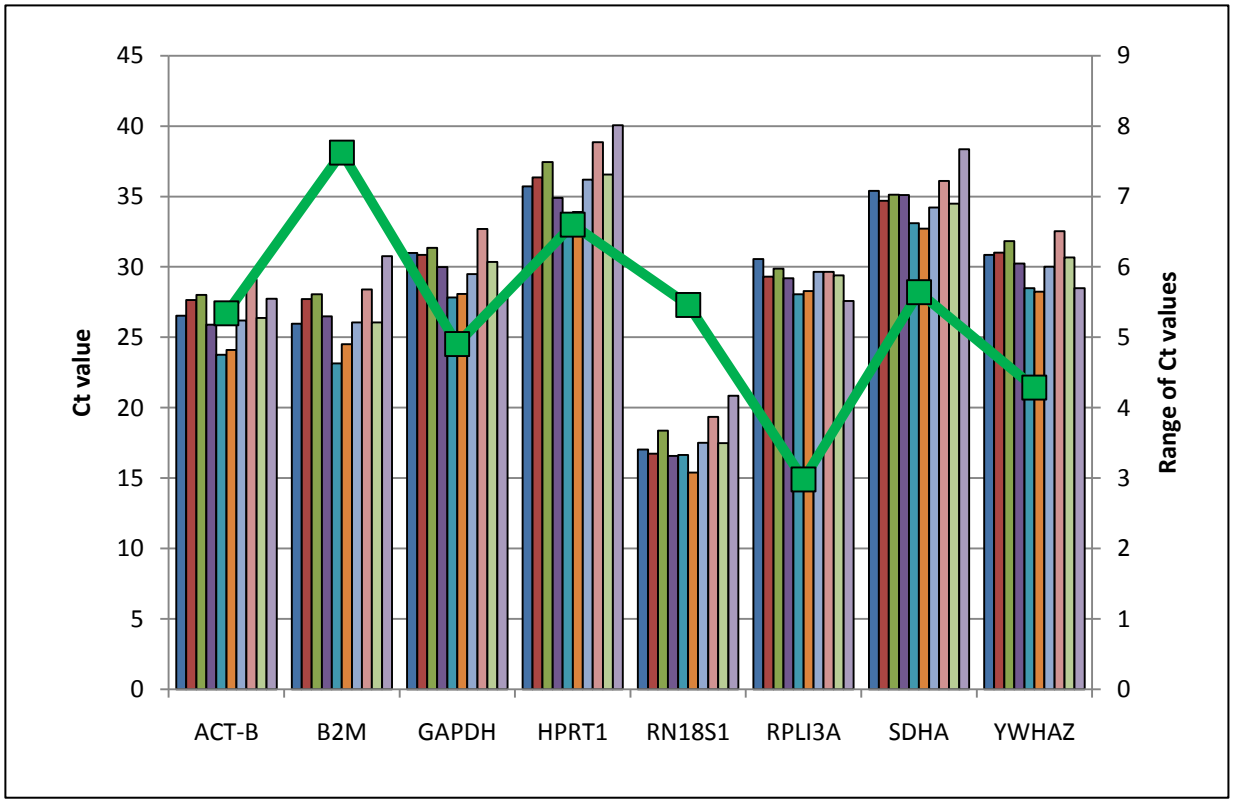


Figure 3.11: Left axis: Cp values of 8 housekeeping genes in saliva of 10 individuals. Right axis: Range of Cp values of 8 housekeeping genes between individuals (n=10).

3.2.5 Microarray profiling of salivary RNA

The feasibility of saliva for microarray profiling was examined in 4 individuals at two time points. Two hundred ng of total RNA was used for Cy3 labeling with Low input Quick Amp labeling kit (Agilent). After labeling, the average yield of Cy3 labeled cRNA was 4.68 ± 1.77 µg with A260/280 = 2.01 ± 0.03. The specific activity for all samples (n=8) was higher than 8.0 (averaged specific activity = 11.9 ± 3.1); which was the minimum activity needed for hybridization.

Salivary RNA profiles of all subjects (n=4) were obtained with 4x44K Whole human genome V2 arrays. An average of 40093 features were assigned as present on the array. These probe sets represented approximately 29954 different features that were mapped to an Entrez ID. For all individuals, genes lower than the 20% percentile were removed. This resulted in 13325 features that were still present in the 4 subjects (**Figure 3.12**). The 13325 features were ranked in % coefficient variation (CV) to observe the variability in expression present in saliva of all subjects (n=4) (**Figure 3.13**). A core gene set of 2276 stable genes were found in 10% CV of the 20-100% percentile intensity cut off. The amount of highly expressed genes (>90%) was equal to 1201 genes.

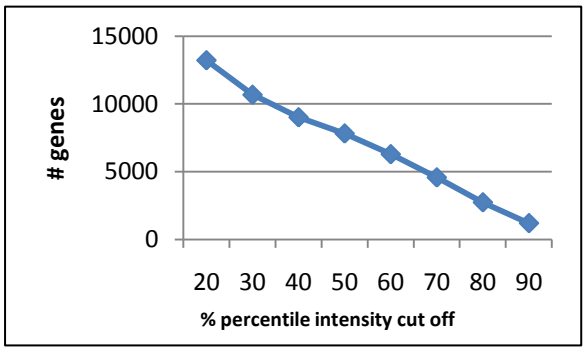


Figure 3.12: the number of genes present in 4 individuals at two time points in a 20-100% percentile intensity cut off from

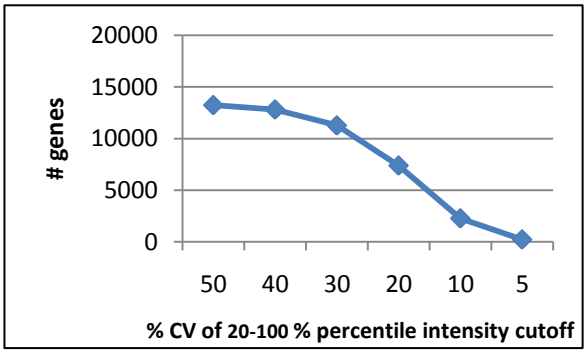


Figure 3.13: The number of genes present in 4 individuals at two timepoints. The % Coefficient variation of the 20%-100% percentile intensity cut off was observed.

The two time points were compared for 4 individuals. One individual was excluded from the analysis because an error occurred in grid positioning of the array. A paired T-test was carried out with a significance value ≤ 0.05 (**Figure 3.14**). All dots in the figure represented a gene. The Log2(FC) of all 13325 genes and the –Log10(p-value) were outlined in the figure. The dots of the genes with a p<0.05 were coloured red. Here, a significant effect was observed between the two time points but when the correction of Benjamini-Hochberg was included, all p-values were larger than 0.98.

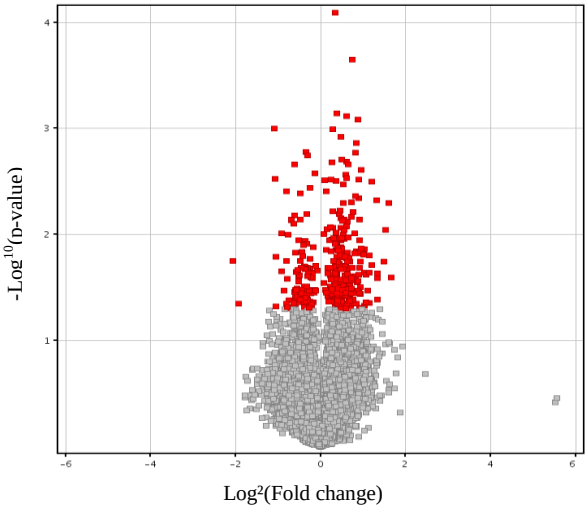


Figure 3.14: The comparison of two time points in 29954 features of 3 individuals.

In the second experiment, the input of total RNA for Cy3 labeling was 1 µg and the number of individuals was increased to n=10. The increased input of total RNA was used to examine whether a change in input of RNA alters the intensity of probes from the 4x44K Whole human genome V2 microarray. An average yield of 5.44 ± 2.0 µg of Cy3 labeled cRNA was obtained from ten individuals. The specific activity was on average 9.97. After hybridization and scanning, the data was extracted from feature extraction 10.7 software and processed with Genespring 11.5.1 (Agilent). Quality control results from feature extraction demonstrated that

10 out of the 12 arrays were of good quality. Two arrays were excluded because an error occurred in grid positioning. An average of 40093 features were present in the normalized data of the array. After removing positive/negative controls and probes without Entrez gene ID, 29954 features remained as present. A 20% percentile intensity cut off was taken to remove all low expressed genes (**Figure 3.15**). The 16827 features were used to obtain the most stable genes (% CV). A core gene set of 3854 genes was found in the 10% CV of most stable genes of the 20-100% percentile intensity cut off (**Figure 3.16**).

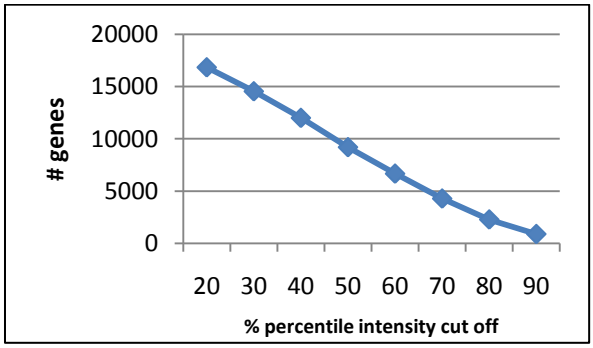


Figure 3.15: The number of genes present in 10 individuals in a 20-100% percentile intensity cut off of the normalized data.

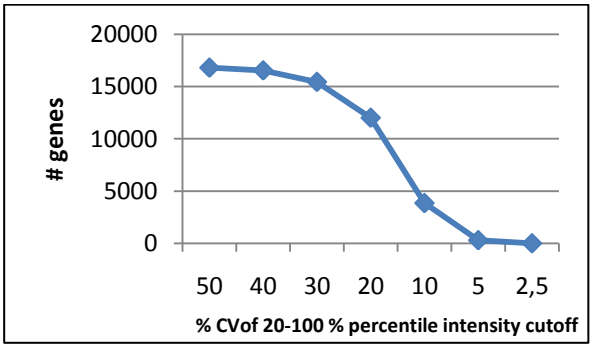


Figure 3.16: The number of genes present in 10 individuals in a % Coefficient variation (CV) of the 20-100% percentile intensity cut off data

The number of highly expressed genes (>90% percentile) was equal to 885 genes. IPA (Ingenuity® Systems, www.ingenuity.com) was used to identify biological functions, diseases and canonical pathways that were most significant in the data set (**Table 3.6**). Significance was represented by the range of p-values for all subcategories. The amount of molecules stands for the number of genes from the dataset present in a particular disease, function or disorder. The ratio characterizes the number of genes of the dataset present in a specific canonical pathway to the number of genes involved in the entire pathway. This is also presented by percentage of genes from the dataset in the canonical pathway .

In saliva, most genes were involved in basic processes such as cellular growth and proliferation (154 molecules), cell death (136 molecules) and protein synthesis (95 molecules). The molecules of these processes are often active in genetic disorders (202 molecules), immunological (169 molecules) and inflammatory diseases (180 molecules). Cancer was also one of the top diseases in which our dataset represented 222 molecules.

The 5 most significant canonical pathways were involved in cellular signalling. The Cdc42 signalling pathway, which is involved in cell cycle regulation was most significant (p=4.42E-06) followed by the hypoxia signalling in the cardiovascular system (p=9.74E-06) representing 12 genes. The other 3 pathways are mainly active in immune system regulation.

Different genes were also occupied in cardio-, hepato- and nephrotoxicity. Nineteen genes were related to renal necrosis or cell death and 11 genes had a function in hepatocellular carcinoma. Other functions in toxicity represented only 1-4 genes. The most significant pathway was involved in Liver necrosis/Cell death (p=9.12E-08).

Table 3.6: IPA functional and IPA canonical analysis of 885 genes. Significance is represented by the range of P-values for all subcategories. # Molecules is equal to the number of genes from the dataset present in the particular disease, function or disorder. The ratio characterizes the number of genes of the dataset present in the specific canonical pathway to the number of genes involved in the pathway. The ratio is also presented in percentage.

Top biological functions	P-value	# Molecules
Diseases and disorders		
Dermatological diseases and conditions	3.96E-22 - 3.79E-02	136
Genetic disorder	1.34E-15 - 4.26E-02	202
Immunological disease	1.89E-10 - 4.12E-02	169
Inflammatory disease	1.89E-10 - 3.34E-02	180
Cancer	2.48E-10 - 3.89E-02	222
Molecular and Cellular functions		
Protein synthesis	2.05E-68 - 3.11E-02	95
RNA Post-transcriptional modification	3.47E-10 - 3.47E-10	13
Cellular growth and proliferation	5.53E-10 - 3.39E-02	154
Cell death	2.83E-09 - 4.12E-02	136
DNA replication, recombination and repair	4.61E-06 - 2.38E-02	24
Physiological system development and function		
Hair and skin development and function	1.81E-03 - 1.34E-02	13
Organ morphology	1.81E-03 - 1.81E-03	2
Organismal functions	1.81E-03 - 1.03E-02	4
Respiratory system development and function	1.81E-03 - 3.34E-02	5
Tissue morphology	1.81E-03 - 1.81E-03	2
Top canonical pathways	**P-value**	**Ratio**
Cdc42 Signaling	4.42E-06	18/142(12.7%)
Hypoxia Signaling in the Cardiovascular System	9.74E-06	12/66 (18.2%)
OX40 Signaling Pathway	3.32E-05	10/61 (16.4%)
Graft-versus-Host Disease Signaling	3.62E-05	09/46 (19.6%)
Fcg Receptor-mediated Phagocytosis in Macrophages and Monocytes	9.49E-05	13/94 (13.8%)
Top tox lists	**P-value**	**Ratio**
Liver Necrosis/Cell Death	9.12E-08	24/162(14.8%)
NRF2-mediated Oxidative Stress Response	1.56E-04	21/208(10.1%)
Cardiac Hypertrophy	1.15E-03	22/256 (8.6%)
Hypoxia-Inducible Factor Signaling	2.51E-03	9/70 (12.9%)
Aryl Hydrocarbon Receptor Signaling	3.13E-03	14/146 (9.6%)
Top tox functions	**P-value**	**# Molecules**
Cardiotoxicity		
Cardiac Inflammation	9.14E-03 - 1.22E-01	4
Bradycardia	8.33E-02 - 8.33E-02	1
Cardiac Hypertrophy	1.60E-01 - 1.60E-01	1
Cardiac Stenosis	2.42E-01 - 2.63E-01	3
Cardiac Necrosis/Cell Death	4.56E-01 - 4.56E-01	1
Hepatotoxicity		
Liver Cirrhosis	1.81E-03 - 3.92E-01	8
Liver Hepatitis	3.32E-02 - 3.24E-01	9
Liver Necrosis/Cell Death	3.89E-02 - 1.32E-01	3
Liver Cholestasis	4.26E-02 - 4.95E-01	3
Hepatocellular Carcinoma	5.78E-02 - 5.78E-02	11
Nephrotoxicity		
Renal Necrosis/Cell Death	1.15E-02 - 1.60E-01	19
Renal Damage	4.26E-02 - 4.26E-02	1
Renal Proliferation	1.86E-01 - 5.02E-01	4
Renal Nephritis	2.76E-01 - 5.23E-01	3
Kidney Failure	3.71E-01 - 5.63E-01	4

GO enrichtment analysis, KEGG and REACTOME biocarta were carried out in ClueGO (Cytoscape, www.cytoscape.org) on the dataset of 885 genes that were most expressed in all ten individuals. A detailed network was created with very specific terms placed in GO levels 3-8, with only few associated genes but a high percentage of the uploaded genes. ClueGO visualized the selected terms in a functionally grouped annotation network that reflected the relationships between the terms based on the similarity of their associated genes. The size of the nodes reflected the statistical significance of the terms. The degree of connectivity between terms (edges) was calculated using kappa statistics [42] The leading terms, based on highest significance in GO terminology were involved in development of horny tissue (keratinisation), protein synthesis, immune system signalling, cellular processes and homeostasis. The most significant pathways found via KEGG pathway analysis were involved in bacterial infections (p=9.0 E-3). Other pathways were related to immune system dysregulation and Type I diabetis mellitus. REACTOME resulted in no significant signalling pathways (**Table 3.7**).

Table 3.7: Leading terms in gene ontology enrichment analysis and KEGG pathway of 885 genes in 10 individuals. Significance was calculated with a p≤ 0.05.

Leading term GO	P-value	Pathway KEGG	P-value
Keratinization	3.6 E-2	Pathogenic Escherichia coli infection	9.0 E-3
RNA biosynthetic process	2.8 E-2	Shigellosis	9.0 E-3
Translational elongation	8.6 E-7	Bacterial invasion of epithelial cells	9.0 E-3
Regulation of cytokine production	1.1 E-2	Viral myocarditis	5.0 E-2
Cellular response to cytokine stimulation	9.5 E-3	Cell adhesion molecules (CAM)	5.0 E-2
Response to lipopolysaccharide	2.8 E-2	Type I diatbetis mellitus	5.0 E-2
Positive regulation of cellular process	3.1 E-2	Autoimmune thyroid disease	5.0 E-2
Cellular protein metabolic process	1.7 E-2	Graft-versus-host disease	5.0 E-2
Homeostatic process	1.1 E-2	Antigen processing and presentation	5.0 E-2
		Phagosome	5.0 E-2

3.2.6 Differences between two Cy3 labeling treatments

The difference of probe intensity was observed between the samples labeled according Low input Quick Amp Labeling kit (Agilent) and High input Quick Amp Labeling kit (Agilent). The data of all individuals (n=4) were characterized according to amplification treatment and filtered based on Entrez Gene ID and positive/negative controls. The features with low intensity percentile cut off (< 20%) were removed from the normalized data. An unpaired T-test with corrected p-value = 0.05 was carried out to examine the variation between low input and high input (**Figure 3.17**). A significant change in probe intensity was observed between the two treatments in all 3 individuals (p<0.05). Most genes were higher expressed in the low input treatment but there was also an amount of genes which had a lower expression intensity in comparison to the high input treatment.

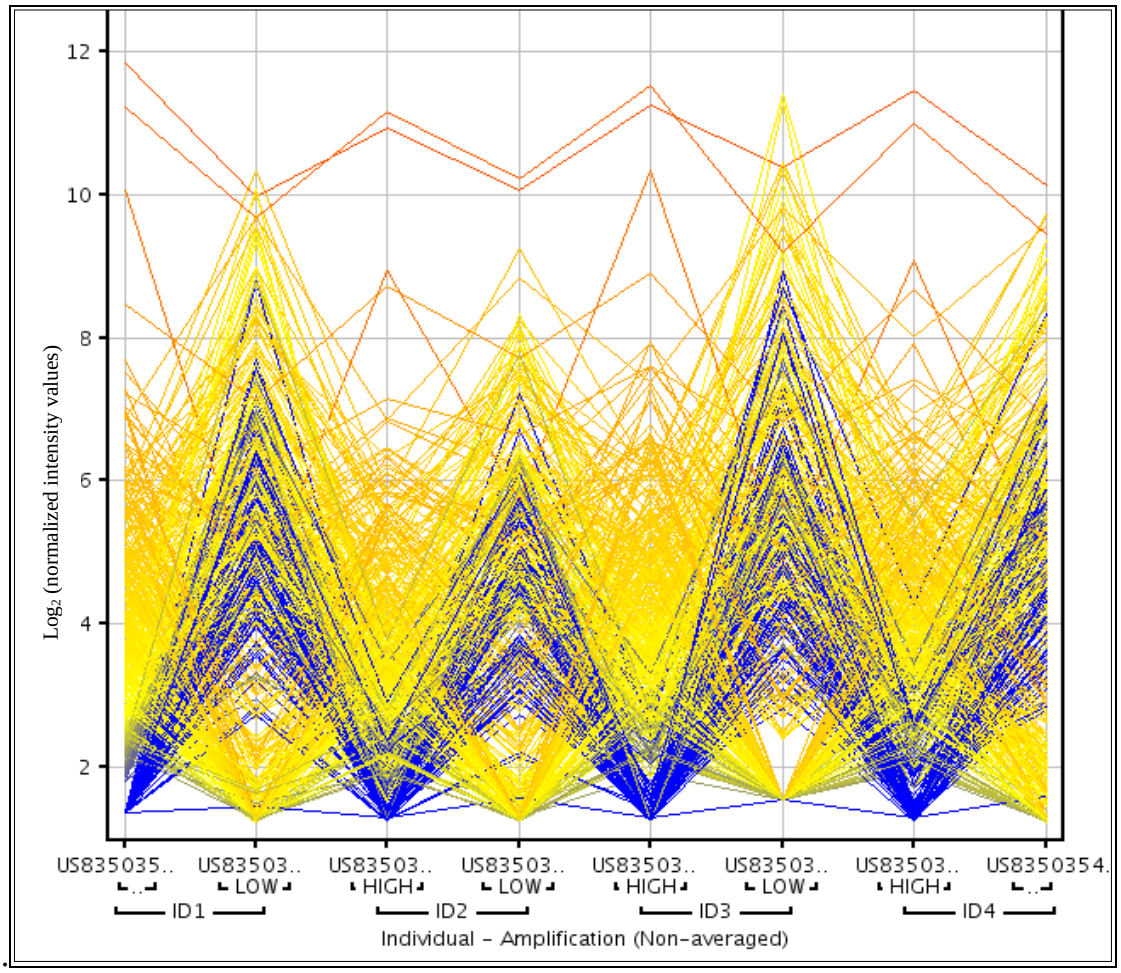


Figure 3.17: The difference in gene intensity of the 20-100% percentile intensity cut off data in each individual for low input and high input amplification.

3.3 Pilot study: Parabolic flight campaign

The effect of acute stress in a parabolic flight was investigated. Nine volunteers of good general health were signed up for the DLR parabolic flight campaign in November 2010. Blood samples were collected as described in materials & methods. Saliva samples were collected with Oragene RNA (DNA Genotek) to examine the appropriateness of this method in a first application. All individuals donated blood samples before – and after flight. Saliva samples were obtained from 8 individuals before flight and 6 individuals after flight. During the parabolic sequences, 4 volunteers were able to donate a saliva sample after 10 cycles and 3 volunteers after 20 cycles (**Supplemental data II**).

On average, 10.67 µg ± 4.38 of total RNA was extracted from 18 blood samples (9 before flight and 9 after flight). Total RNA was of good quality with an A260/280 ratio = 2.03 ± 0.04 and an average RIN value of 8.26 ± 0.73. After cDNA synthesis, gene expression analysis was carried out with qPCR. Gene expression changes of 12 selected genes of interest were compared between after flight and before flight data. Housekeeping genes were selected with geNorm software. An M-value≤0.05 was used as a parameter for stability of the housekeeping genes. All genes were normalized against 5 housekeeping genes (**Figure 3.18**).

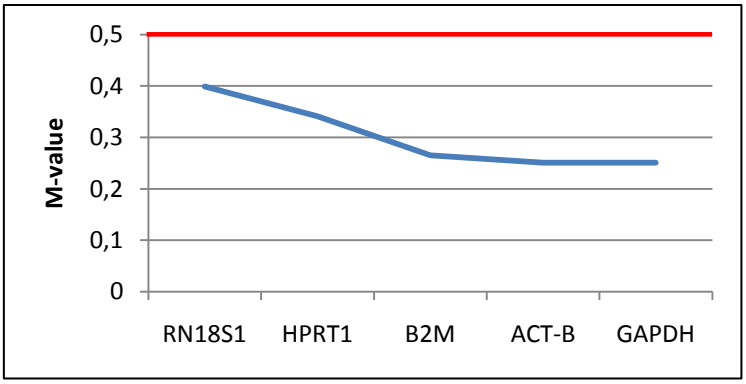


Figure 3.18: M-values of 5 housekeeping genes tested in blood for all individuals (n=18). Treshold below red line (M<0.5)

The CRP gene was eliminated from the study because data was only obtained for a few samples. Cp values of CRP ranged between 37 and 40 which is quite near the detection limit of the qPCR. Two individuals were excluded in IFN-α1 gene and one individual in IL-1α because no data was obtained from qPCR. The average Log_2(FC) of GABARAP and IL-1β showed a significant upregulation of respectively 43.96% ($p < 0.001$) and 60.03% ($p < 0.05$) after flight. IL-6 and TPH1 results demonstrated a significant downregulation with 44.96% ($p < 0.05$) and 73.67% ($p < 0.01$) (**Figure 3.19**). Results also showed individual variability in all genes. Each participant responded different to the acute stress experienced during the parabolic flight. For example, 2 individuals demonstrated no difference between before flight and after flight expression in IL-6 while 7 individuals showed a clear effect. For GABARAP, all individuals illustrated an upregulation but one participant showed a lower Log_2(FC) = 0.09 than the other subjects (Average = 0.44) (**Figure 3.20**).

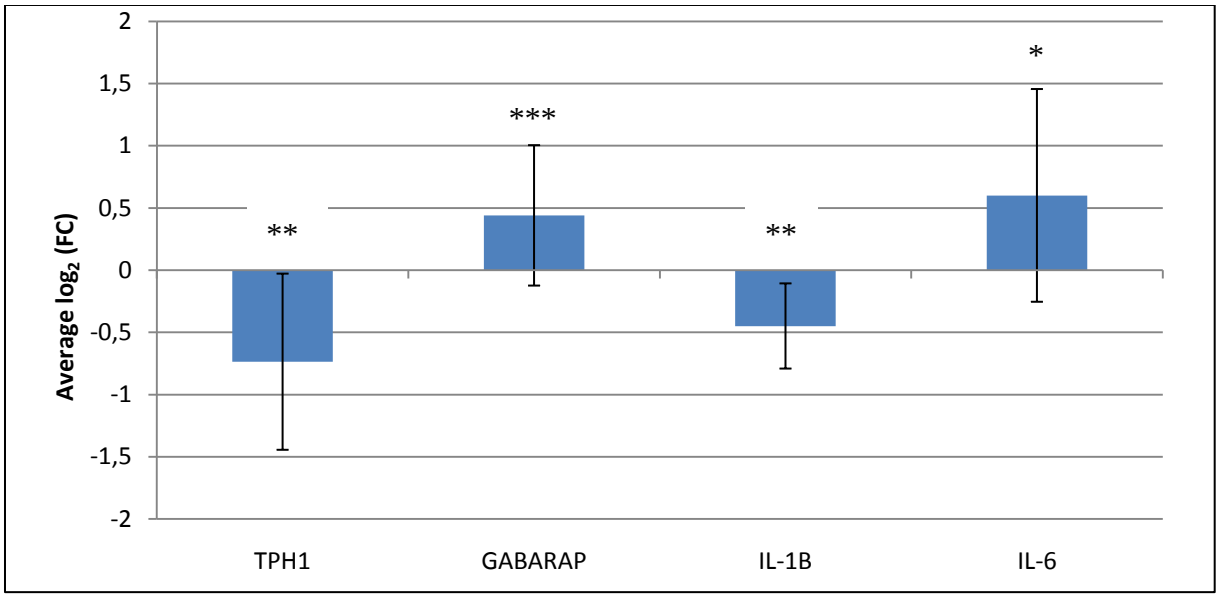


Figure 3.19: Signficant gene expression changes in blood of 9 individuals undergoing a parabolic flight. Results were presented as average Log_2(FC) with standard deviation. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$ of average ^{2}Log(Fold change)

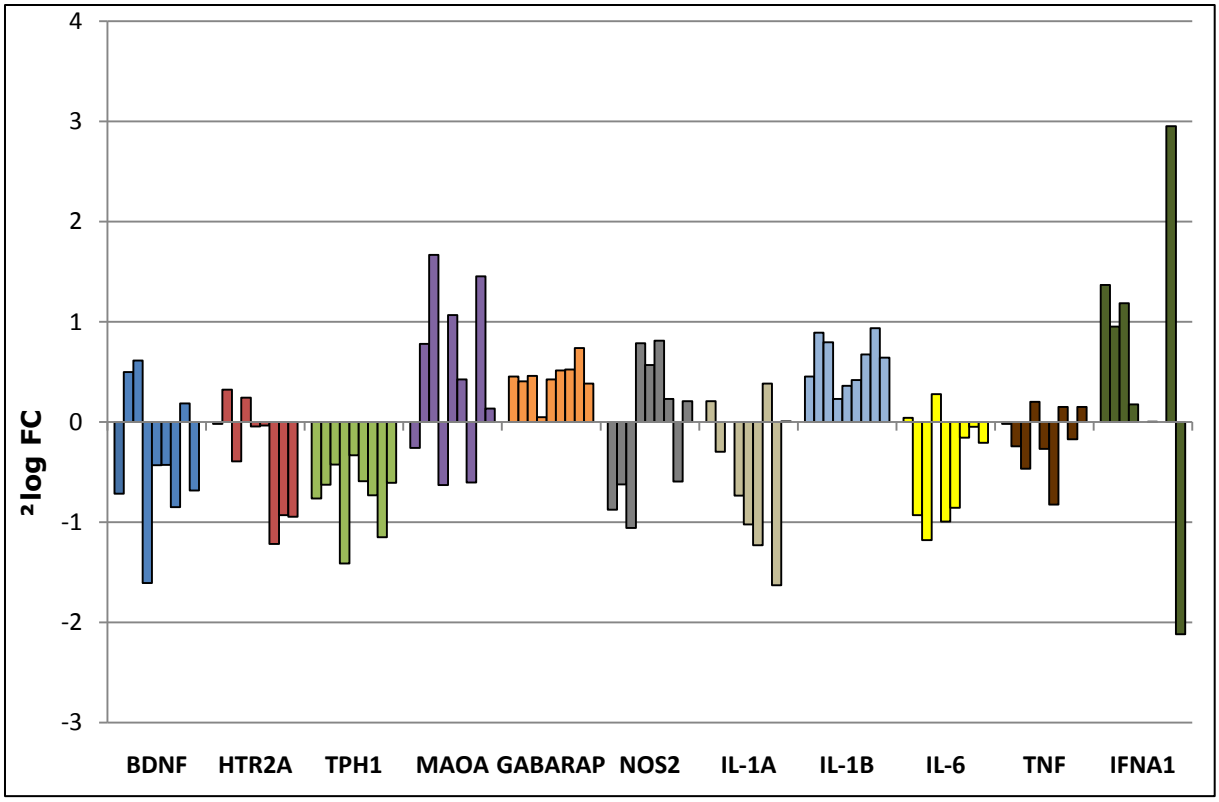


Figure 3.20: Blood gene expression changes of 11 selected genes of interest in 9 individuals undergoing a parabolic flight sequence. Results are presented in Log_2 (Fold change).

During the parabolic flight, saliva samples were collected for gene expression analysis via Oragene RNA technology. 7.53 ± 6.75 µg mean total RNA was obtained for the 21 saliva samples. Quality was measured with Nanodrop (A260/280 = 2.02 ± 0.07; n=21) and Agilent Bioanalyzer 2100 (RIN = 5.78 ± 1.67; n=17). On average, total RNA was of lower quality than in other experiments (± 1 RIN) (paragraph 3.2.1). Due to the heterogeneity found in all individuals and the many missing data (especially in flight), gene expression analysis was carried out on a set of 12 saliva samples (n=6 BF; n=6 AF). All housekeeping genes had an M-value larger than 0.5, which is the threshold value for indicating stability of a gene in all individuals. For normalization, no stable housekeeping gene was found via geNorm software. So, all genes were normalized to RN18S1 via the ΔΔCt method. qPCR data for gene expression analysis was not available for 5 of the 12 selected genes. MAOA and NOS2 signal reached the detection limit of Cp=40. No fluorescence signal was detected for HTR2α, TPH1 and IL-1β. Some individuals also had missing data for IL-1α (n=1), IL-6 (n=3), BDNF (n=4) and IFN-α1 (n=2). Comparing after flight samples to before flight samples, results revealed many variability between individuals for all analyzed genes (n=7). None of the 7 genes were significantly more or less expressed (**Figure 3.21**).

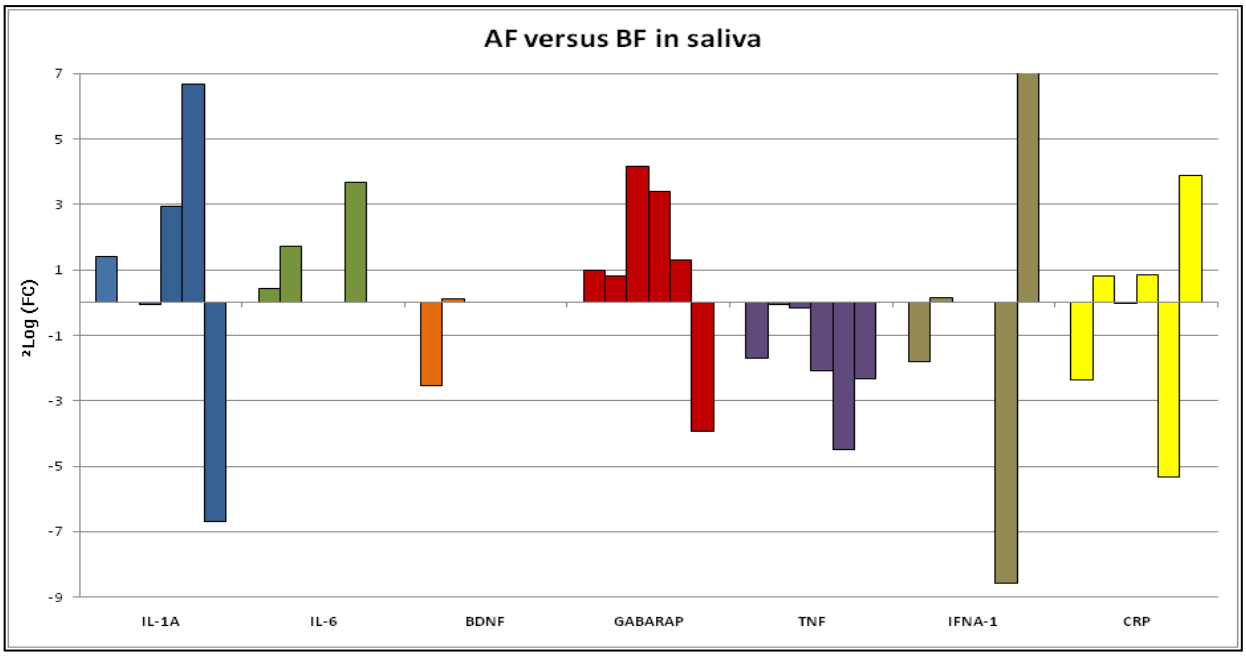


Figure 3.21: Saliva gene expression changes of 7 genes of interest in 6 individuals undergoing a parabolic flight sequence. Results are presented in Log_2 (Fold change).

4. Discussion

4.1 Adjustment difficulties to the Antarctic environment

High altitude exposure is characterized by hypobaric hypoxic environmental conditions that induce changes in both physiological and psychological responses. Hypoxia induced by a high altitude (4572 m) becomes a limiting factor for normal physical and mental performance [43]. In addition, impairments in a number of cognitive performances, in psychomotor performance, mental skills, reaction time, memory and logical reasoning have already been observed at altitudes above 3000 m [44]. From the present study, changes in gene expression levels were observed in the first month of isolation at the Antarctic station Concordia.

The stress experienced in the adjustment to the hypoxic environment has an influence on body processes. Reduced oxygen uptake decreases systemic blood circulation which results in a lower blood circulation and uptake of oxygen in the brain. BDNF is an important regulator of naturally occurring cell death in the peripheral nervous system [45-46] and also regulates neuronal development and maturation in the CNS [47]. Serotonin is a signalling system that also plays a role in many neuronal functions including survival, neurogenesis and synaptic plasticity. These two systems have the ability to regulate the development and plasticity of neural circuits involved in mood disorders. BDNF promotes the survival and differentiation of serotonin neurons. Conversely, serotonin reuptake inhibitors enhances BDNF gene expression [48]. It has been shown that changes in expression of a frequent nonsynonymous single nucleotide polymorphism in the BDNF gene is correlated with behavioural changes in humans and in mouse models. For example, this has been observed in abnormal feeding behaviour, alterations in episodic memory, and susceptibility to neuropsychiatric disorders of anxiety and depression [49]. BDNF is also involved in allergic diseases such as asthma [50] In our study, gene expression changes were measured in blood from healthy volunteers that were confined for ten months at the Antarctic station Concordia. Results demonstrated a significant downregulation of BDNF and TPH1 gene between the first month of isolation and baseline samples. TPH1 gene encodes for an enzyme involved in serotonin synthesis. As in literature has been shown that BDNF and serotonin interact and have a role in mood disorder. it is assumed that decreased expression of these genes result in an inefficient regulation of neuronal development and survival. This can probably be linked to altered mood and performance. The functional link cannot be proven yet at this stage because data from psychological tests are still in process.

Humans adapt to a rapidly changing environment due to their sophisticated cognitive abilities. The adaptation to the environment is enhanced by neuronal signalling pathways. The dynamics of neural networks is largely shaped by the activity pattern of interneurons. Most of these interneurons are GABAergic. Thus, a deeper understanding of the GABAergic control of neuronal network oscillations contributes to deeper insights into the neural representation of cognitive functions in normal brain. In addition, GABAA receptors has gained increased attention in analyzing GABAergic control of behaviour [51]. In our study, we have measured gene expression changes of GABARAP in peripheral blood of healthy volunteers. Increased GABARAP expression can probably lead to increased GABAergic control of behaviour.

4.2 The influence of confinement alters immune functioning

In the first month of isolation as well as in the fifth month of isolation we have demonstrated elevated expression of TNF-α. We also found decreased expression of IL-1β. Translational research already provided evidence that mood disorders are associated by disturbances in interacting immuno-inflammatory, metabolic, and neuroendocrine networks. Numerous studies document elevated pro-inflammatory circulating cytokines such as TNF-α in individuals with bipolar disorder as compared to healthy volunteers [52]. We presume that the elevated expression of TNF-α and decreased expression of IL-1β are caused by confinement. This will influence immune functioning of an individual which probably affect mood and performance.

4.3 Saliva as a biological matrix for transcriptomics, proteomics and metabolomics

In the past decade, saliva has already been used as a diagnostic fluid for the detection of different substances such as electrolytes, hormones, drugs and antibodies in human medicine. These components not only protect the integrity of the oral tissues, but also provide information of systemic and local diseases. For example, saliva is used in the diagnosis of oral and systemic viral diseases such as measles; mumps; rubella; hepatitis A, B and C; and HIV-1 and 2. Saliva also aids in the diagnosis of sarcoidosis, tuberculosis, lymphoma and Sjögren's syndrome. In addition, saliva is being used to monitor the level of selected chemicals introduced into the body such as alcohol, drugs and addictive substances [53]. The major advantages for using saliva in diagnosis are the relatively easy access for sampling in which sufficient amounts for analysis can be gathered. The costs of storage and shipping tend to be lower than those for blood or urine samples [54]. And saliva sampling is non-invasive, painless, possible any time, day or night because health care professionals are not needed to gather the saliva samples. So saliva collection can be accomplished under circumstances where blood collection is difficult or inadvisable. Most molecules present in blood and urine are found in saliva, although at concentrations one-tenth to one-thousandth of those in blood. Therefore disorders and diseases in which saliva has been used as diagnostic fluid were still. But in the past ten years, the development of technology has improved the experimental approaches (new amplification techniques and highly sensitive assays) which has eliminated the limitation to use saliva as a diagnostic tool for monitoring health or disease status. This progress has directed to the use of saliva in proteomics [55]. Many protein components have been studied in whole saliva including amylase, albumin, immunoglobulins, statherin for calcium homeostasis, mucins for lubrication and cystains (cysteine protease inhibitors) [56]. Previous studies also identified diagnostic biomarkers in saliva for pathological disorders such as cystic fibrosis, diabetes mellitus, periodontitis and Sjögren's syndrome [57-60]. In recent years, identification of diagnostic biomarkers in nucleic acids in most bodily fluids, including blood, urine and cerebrospinal fluid, have been explored and successfully adopted for use to monitor diseases. Via human saliva transcriptome analysis (Affymetrix microarray and RT-qPCR), Wong et al. revealed that whole saliva contains cell-free mRNA representing at least 3000 different mRNAs per person (and possibly more) and a salivary core transcriptome of 185 genes [61]. Certain concordance was found in saliva from each individual between the proteomes and transcriptomes [62]. This research group has mainly focused on the perspective of oral diseases and cancer [63-64]. In Li et al, 17 mRNAs were found to be

present in higher amounts in patients with oral cancer than in healthy persons. Out of this study, a prediction model based on salivary mRNA concentrations of 4 of these genes was developed. The model showed 91% sensitivity and specificity for oral cancer detection [63]. For the detection of pancreatic cancer, 4 genomic biomarkers have been discovered in the logistic regression model to differentiate pancreatic cancer patients from all non-cancer subjects with a 92.9% sensitivity and a 85.5% specificity [65]. Recently, 8 mRNA biomarkers and one protein biomarker have been found for breast cancer detection. The biomarkers, which were not affected by confounding factors, were pre-validated, yielding an accuracy of 83% sensitivity and 97% specificity on the preclinical validation sample set [66]. These studies have provided the first prove that 'cell-free' saliva can be used for the discovery of biomarkers. But little is known about the opportunity of 'whole' saliva for gene expression analysis using different techniques than Wong et al. [61]. In our study, the feasibility of whole saliva for gene expression analysis has been explored by using a new sampling approach (Oragene RNA from DNA Genotek) and technology (qPCR and Agilent Microarray technology).

4.3.1 Oragene RNA kit: a new sampling approach for collection of whole saliva

Saliva is produced by multiple salivary glands lying beneath the oral mucosa. Each day, the human salivary glands excrete almost 600 mL of serous and mucinous saliva containing minerals, electrolytes, buffers, enzymes and enzyme inhibitors, growth factors and cytokines immunoglobulins and other glycoproteins. Once saliva passes through the ducts and enters the oral cavity it mixes with blood cells, microorganisms and microbial products, oral epithelial cells and cell products, food debris and upper-airway secretions. Thus, saliva contains many substances which makes the composition of saliva more complex. Therefore it is accurate that at least one hour prior to sampling, subjects don't eat, drink or smoke and rinse their mouth with water to remove most debris. Our study used a new sampling approach, Oragene RNA kit (DNA Genotek), as a practical tool for collecting whole saliva. This new method has been examined because results from BEACON saliva samples demonstrated that the collection via RNeasy protect saliva mini kit (QIAgen) was inadequate for use.

Oragene RNA kit recommends the use of sugar for stimulating saliva production. In our study, human RNA was successfully isolated from as well stimulated as unstimulated whole saliva. But the RNA yield in unstimulated whole saliva was significantly higher than in stimulated whole saliva. The use of sugar stimulates saliva production which probably dilutes the saliva, making it a more watery fluid with a lower RNA yield. Therefore, we suggest to use unstimulated whole saliva in further research to obtain a higher yield of RNA.

In this study, saliva samples (2 mL) were obtained in the morning. The RNA yield showed many variation within one individual in time and between individuals. This probably can be explained by the diversity in saliva sampling. Every individual stimulates their saliva in another way. Some individuals produced a more watery saliva sample and other a more mucosal saliva sample. Some persons needed 5 spitting times while other only 2 spitting times. It was also clear that some adults had some trouble to collect 2 mL saliva in a short time period (5 minutes). Our experiments revealed no significant changes in time but reduced volume of whole saliva clearly lowered the yield and quality of RNA. An adequate volume of

saliva is necessary to obtain a sufficient amount of total RNA with a good quality. Therefore, we propose the use of saliva sponges in combination with Oragene RNA kit to facilitate saliva collection and to obtain a proper volume of saliva. We have already tested this method on 1 individual with success (**Supplemental data III**). As some adults already have difficulties to collect 2 mL saliva in a proper way, the application of saliva swaps would also be more useful when collecting saliva from children.

4.3.2 Integrity of salivary RNA

RNA integrity is a critical step in obtaining meaningful gene expression data. Using intact RNA is a key element for the application of modern molecular technology such as RT-PCR of microarray analysis. In our study, RNA quality was determined with NanoDrop and Bioanalyzer 2100. Some individuals had a very high RNA yield while other had a very low RNA yield. Depending on the quantity, different assays are needed to obtain reliable results. The Agilent RNA 6000 pico kit is recommended when total RNA yield ranged within 50-5000 pg/µL and Agilent RNA 6000 nano kit is used when the quantity of RNA ranged within 25 – 500 ng/µL. Depending on the derived results, a RIN value ≥ 6.0 was taken as a threshold value for qualitatively good RNA for use in qPCR and microarray profiling. The RNA integrity was also checked with the use of qPCR. Results showed intact RNA in all individuals, even in time, which confirmed the quality control of the Bioanalyzer 2100.

4.3.3 No stable housekeeping genes for data normalisation in RT-PCR

Data normalisation in RT-PCR is an important step in gene quantification analysis [67-68]. The reliability of a RT-PCR experiment can be improved by including an invariant endogenous control (reference gene) in the assay to correct for sample to sample variations in RT-PCR efficiency and errors in sample quantification. RT-PCR-specific errors in the quantification of mRNA transcripts are mainly caused by sample-to-sample variation, variation in RNA integrity, efficiency differences and cDNA sample loading variation [69]. This occurs especially when the samples have been obtained from different individuals, different tissues and different time courses, and will result in the misinterpretation of the derived expression profile of the target genes. Therefore, normalisation of target genes must be carried out to compensate intra- and inter-kinetic RT-PCR variations [70]. Previous studies which used saliva as biological matrix, carried out normalization via the ΔΔCT method with one gene (RN18S1 or GAPDH). In this method it does not matter if this gene varies in expression. However, the chance for getting significantly different results will be low due to the large expression variability. Despite this, the ΔΔCT method is feasible [61, 71-72]. To increase the specificity and reliability of normalization, we tested the stability of housekeeping genes with geNorm software. All housekeeping genes demonstrated many variation between individuals which resulted in no stable housekeeping gene via geNorm software. The variation in housekeeping genes can probably be explained due to the interference of bacterial RNA present in saliva. During RNA extraction of whole saliva, not only human RNA but also bacterial RNA are isolated. In this RNA pool, the content of bacterial RNA is not known and possibly differs between individuals. In qPCR, the standard input of cDNA is the same for all individuals. As the bacterial content differs between individuals, this will influence the actual input of human cDNA for qPCR. It is neseccary to

investigate this interference in future research. To characterize the bacterial content in whole saliva, DNA Genotek recommends to use the Bacterial DNA assay (PD_PR_065). No assay is currently available for the characterization for bacterial RNA. But there is some discussion if the bacterial DNA content can be used to identify the proportion of bacterial RNA in total RNA. So it is more likely to separate the bacterial RNA from human RNA during or after extraction. It is known that bacteria lack the relatively stable poly(A) tails found on eukaryotic messages. So it is possible to purify bacterial RNA from eukaryote RNA with magnetic beads via oligo (dT) selection. Another option is to use Bacterial RNA isolation from infected Eukaryotic hosts kit from Ambion. The kit separates bacterial RNA from eukaryotic RNA from any total RNA sample. After separation, the proportion of bacterial RNA in total RNA can be characterized. So in further experiments, an adjustment for the bacterial RNA content can be included.

4.3.4 A global profile of salivary RNA using Microarray technology (Agilent).

Wong et al. already showed that saliva can be used for biomarker discovery . This research group demonstrated the relevance of cell-free saliva for gene expression analysis with Affymetrix microarray technology. To our knowledge, this is the first report in which human RNA from unstimulated whole saliva is profiled with Agilent microarray technology. The probe sets on the 4x44K whole human genome array stand for approximately 41000 human genes. We discovered a core gene set of 3854 genes with a coefficient variation lower than 10% in unstimulated whole saliva of ten healthy subjects. The identified gene transcripts probably represent the universal transcriptome present in unstimulated whole saliva of normal individuals. The genes with a coefficient variation lower than 5% in all individuals are the most stable genes present in saliva and may therefore be a source for genes that can be used for normalisation purposes. In the 10 individuals, 885 genes have the highest expression intensity (>90%). These genes were categorized to their cellular and biological processes and molecular function via Ingenuity enrichtment analysis, GO enrichtment analysis and KEGG pathway analysis. As mentioned before, saliva has already been used in studies for genomic biomarker discovery for oral, breast and pancreatic cancer which showed a high sensitivity and specificity [63, 65-66]. In our dataset, many genes were found to be involved with cancer. This confirms the value of saliva for biomarker discovery in cancer research. It was also demonstrated that the genes in our dataset were involved in inflammatory and immunological diseases such as Dermatitis and Pancreatitis (**Supplemental data IV**). Out of the data it was clear that some auto-immune diseases were very related to genes present in saliva. For example, many genes were involved in rheumatoid arthritis and psoriasis. Another important disease, Diabetes mellitus, has also been observed in saliva. Studies have characterized glycation endproducts and measured immunoglobulins in saliva for predicting the incidence of Diabetes mellitus [58, 73-75]. Our results showed that genes present in our dataset were related to Type I diabetes mellitus. This suggests that in research saliva can be used for gene expression analysis on patients with Diabetes Mellitus Type I.

Communication within a cell and between cells are important for a good distribution of all processes. In saliva, genes are for the most part involved in cell cycle regulation, immune system signalling and hypoxia related cardiovascular signalling. Toxicity induced by an environmental exposure such as hypoxia can be observed in saliva. Salivary flow and

composition changes by acute high altitude hypoxia have already been examined [76].Cardiac risk has also been observed in saliva via an high sensitivity C-reactive protein assay [77]. An interesting gene, HSP90B1, can be detected in saliva. This gene encodes for the heat shock protein B1 enzyme which is involved in the regulation of hypoxia inducible factor 1α, the oxygen sensitive subunit [78]. This suggests that in future research it is feasible to use saliva as a diagnostic medium for predicting hypoxia related cardiac risk via gene expression analysis. Remarkably is that genes related to systemic toxicity also can be studied in saliva. Some genes were found in liver cirrhosis or renal necrosis. A recent study has used saliva for measuring inflammatory cytokines as an early signal for chronic kidney disease [79]. Also oxidative and compositional analysis has been carried out in saliva from kidney disease patients [80] A few genes in our dataset were also related to cardiac inflammation or hypertrophy. A saliva-based Nano-Biochip test is already being used for acute myocardial infarction [81]. Our study has revealed that saliva not only can be used for monitoring local diseases but also for examining systemic changes as for example renal necrosis or liver toxicity.

4.3.5 Differences in Microarray technology using two treatments

Agilent has two kits available for Cy3 microarray labeling. The Quick amp labeling kit uses an input of total RNA higher than 200 ng while the low input Quick amp labeling kit uses lower than 200 ng input of total RNA. In our study we have tested the difference in gene intensity between the two treatments. Expected was that when using a higher total RNA input was, the intensity of genes on the array would increase. Our results demonstrated altered intensity of genes treated with the low or the high input Quick amp labeling kit. The intensity differences demonstrated that some genes were undetectable with the high input treatment but with the low input treatment they were measurable. This was also observed in the opposite way. One of the possible causes to this difference, is that the arrays of the high input treatment showed a low quality but the arrays were still sufficient for data analysis according to feature extraction software. This needs to be investigated in more detail in future research.

4.3.6 Cellular characterization of saliva

Microarray technology has shown that genes related to systemic alterations could be measured in saliva. This can only be the case when the RNA present in saliva comes from white blood cells that entered the saliva or free RNA from death cells circulating in saliva. We have tried to visualize and count the cells present in unstimulated whole saliva using a light microscope and nucleocounter (**Supplemental data V**). Our results were too vague so one of the future perspectives is to characterize the cells in saliva using another colour staining or technology as for example FACS.

4.4 Stress during a parabolic flight affects immune system and neurotransmission

During a parabolic flight, humans experience and cope with conditions of weightlessness and hypergravity. Previous studies have already reported immune system dysfunction due to exposure to microgravity [82-83]. For example, it has been shown that one third of the participants experience motion sickness with symptoms of nausea and vomiting which is associated with impaired endocannabinoid system activations [84]. This system is a neurobiological mechanism present in a large extent of immune tissues. Endocannabinoids have various effects on immune cell function, some of which modulate cytokine release from macrophages or inhibit lymphocyte proliferation [85]. Parabolic flights are also associated with an alteration of innate immunity. Priming of polymononuclear leukocytes with respect to their tissue toxic capabilities are contributed to an increased susceptibility to inflammatory stimuli [86]. Research carried out on ground-based models have demonstrated effects on immune system cells such as reduced activation/proliferation [87], altered cytokine production, and altered signal transduction [88]. This has also been shown in spaceflight studies [89].

We have used a parabolic flight model to observe alterations in gene expression pattern from inflammatory genes and genes related to neuronal signalling. We normalized the qPCR data with the ΔΔCt method using RN18S1. Although this gene was not found to be stable according to geNorm software, it was recommended by the company DNA Genotek. Our results demonstrated that the gravitational stress experienced during a parabolic flight altered expression of inflammatory genes. IL-6 is a pro-inflammatory cytokine that induces IL-1β activity [90]. Downregulation of the IL-6 gene will result in the upregulation of IL-1β. In recent years, IL-1β and IL-6 were shown to play an active role in cellular events that induce structural changes at the synaptic level. Moreover, it has also been determined that these changes affect neurotransmission and ultimately modulate behaviour [91]. So the exposure to microgravity and hypergravity during a parabolic flight probably involves the activation of the cytokine network which can trigger, worsen or sustain altered higher brain functions in a susceptible individual.

GABARAP, a gene encoding for GABAa receptor associated protein, is also involved in neurotransmission. Control of neurotransmitter receptor expression and delivery to the postsynaptic membrane is of critical importance for neural signal transduction at synapses. GABARAP was reported to have an important role for movement and sorting of $GABA_A$ receptor molecules to the postsynaptic membrane [92]. During a parabolic flight, it was expected that the acute stress experienced by humans would decrease expression of GABARAP. This would influence neurotransmission negatively. In our study, expression of GABARAP was increased. We presume that the effect of stress on GABARAP is slowly and will be notified later in time.

Our study showed a down-regulated expression of TPH1. The TPH1 gene encodes for an enzyme involved in the synthesis of serotonin, a mono amine neurotransmitter, which regulates a wide repertoire of behaviours. In the adult, serotonergic neurotransmission

changes many brain functions including emotion, cognition motor function and pain sensitivity. Disturbances in serotonin signalling have been associated in an excess of psychiatric disorders such as depression [93] Here, we have subjected healthy volunteers to hypergravity and microgravity conditions. So we presume that the stress experienced during the parabolic flight sequence influence cognitive motor function and emotion negatively, resulting in altered behaviour and cognitive performance. Our study cannot confirm mood changes at this stage because cognitive data are still being processed by the German Sports University.

C-reactive protein is a marker for inflammation, meaning its presence indicates a heightened state of inflammation in the body. Inflammation (swelling) of the arteries is a risk factor for cardiovascular disease. It has been linked to an increased risk of heart disease, heart attack, sudden death, stroke, and peripheral arterial disease. It has also been linked to an increased risk of restenosis, or the re-closing of an artery that has been treated with balloon angioplasty. Studies have shown that CRP protein levels measured in serum seem to be correlated with levels of cardiac risk. In this study, we have found that CRP gene expression could be detected in saliva but not in blood samples. This suggests that in future research saliva probably can be used for the prediction of cardiac risk by measuring gene expression changes of the CRP gene.

In this parabolic flight campaign, we have demonstrated that whole saliva collected via Oragene RNA (DNA Genotek) can be used for gene expression analysis. No significant changes were observed in saliva but this can be explained by the fact that the data is too preliminary to draw any conclusion. Some individuals had difficulties to collect the right amount of saliva during and after flight. So a thorough investigation and optimization is needed to improve the use of saliva as biological matrix for gene expression analysis. As already suggested, saliva sponges for collection in combination with Oragene RNA (DNA Genotek) can be practical in difficult circumstances such as in a parabolic flight.

Conclusion

In this study, we investigated the changes in biological processes induced by stress in spaceflight analogues. Via two pilot studies we observed the impact of gravitational stress and the consequences of isolation on genes related to immune functioning and neurotransmission. Out of the results, it can be concluded that the stress induced by spaceflight conditions (such as microgravity and confinement) affect immune functioning and neurotransmission. This can probably influence mood and performance of an individual. These studies has provided a first insight of the influence of gravity and isolation on the gene expression pattern of inflammatory genes and genes related to neurotransmission. In the future, more genes related to hypoxia and neuronal signalling need to be tested to confirm the alterations seen in the adjustment to the environment. Comparing our results with the psychological data obtained during the confinement study will also give us more insight in the changes in mood and performance of the participants. This is an important basis for future research to explore the effect of the Antarctic environment and confinement on gene expression changes of the immune system and neuronal signalling. Our results will be used in the follow-up study in the winter-over campaign at Concordia (2010-2011).

In this project, the use of whole saliva collected via Oragene RNA (DNA Genotek) for gene expression analysis was also demonstrated. In particular, the aim was to observe the feasibility of whole saliva in real-time PCR and Agilent Microarray technology. It can be stated that the objectives were completed at all areas.

In a first phase, it was illustrated that unstimulated saliva collection provided more RNA than stimulated saliva. Decreased volume of saliva indicated a lower RNA yield and quality. So for further experiments, it was recommended to use an adequate quantity of unstimulated whole saliva to obtain a sufficient amount of qualitatively good RNA. Moreover, it was also proven that this collection method offered minor degration of RNA, which was confirmed through the assessment of RNA integrity via qPCR. Some individuals had difficulties to reach the level of saliva needed by spitting via the Oragene RNA kit. Therefore we propose the use of sponges for saliva sampling followed by the Oragene RNA kit to fasten and simplify collection. It can be concluded that saliva sampling via this method is satisfactory but optimization is still needed to improve the collection and processing.

In the second phase, the possibility of saliva for gene expression analysis was examined using qPCR. 5’ exonuclease assays for 12 target genes and 8 housekeeping genes were used to study their expression in saliva between individuals. From the results of the qPCR data, it was concluded that 11 target genes could be measured in saliva in exception of one gene which had reached the detection limit of the qPCR. The technical variability was within the range of 1 Cp value which proved a good reproducibility. From this, it can be diverted that most of the genes can be analyzed in saliva via qPCR technology depending on the mRNA content of that particular gene present. In previous studies, the $\Delta\Delta$ Ct method with one gene (GAPDH or RN18S1) was used for normalization. We investigated the possibility to use more genes for

normalization to improve specificity and reliability. The housekeeping genes were analyzed for normalization based on their stability between individuals. None of the tested housekeeping genes were adequate for normalization according to GeNorm software. This is probably caused by bacterial interference. In future research, the bacterial RNA content needs to be incalculated to adjust for this interference.

In a last phase, Agilent microarray technology was used to identify a global profile of the genes present in saliva. Here, it was concluded that 3854 genes present in saliva of ten individuals formed the core gene set. The genes with the highest expression (885 genes) were found to be involved in basic biological processes such as protein synthesis, cellular growth and proliferation and cell death but also in previously unnoticed pathways such as hypoxia signalling, renal necrosis, liver and cardiac toxicity. As systemic alterations can be measured in saliva, this suggests that the origin of human mRNA found in saliva needs to come from white blood cells or free circulating RNA. But still, this is not proven yet so it remains an important biological question that needs to be further elucidated.

Our results have provided the proof that whole saliva collected via Oragene RNA can be used for gene expression analysis via qPCR and Agilent microarray technology. The use of saliva as a biological matrix has gained increased attention as it can probably be used for biomarker discovery for systemic diseases. The new saliva sampling method, here described, is currently used in a second winter-over campaign at Concordia in 2010-2011 which studies the effect of confinement on sleep rhythm.

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Supplemental data

I. Results of all BEACON blood samples

Table I: RNA yield, 260/280 ratio, 260/230 ratio and RIN values for all BEACON blood samples

Sample ID	ng/ul	260/280	260/230	Rin
T0-01	152,31	2,08	2,2	8,50
T0-02	283,8	2,09	2,21	8,30
T0-03	175,79	2,1	2,19	7,60
T0-04	148,59	2,1	2,17	7,70
T0-05	247,8	2,04	2,34	7,80
T0-06	89,84	2,03	1,87	8,00
T0-07	145,83	2,03	1,95	8,80
T0-08	247,69	2,05	2,05	7,50
T0-10	84,42	2,14	3,32	8,20
T0-11	147,36	2,09	2,17	8,50
T0-12	122,73	2,02	2,18	7,50
T1-J2	96,23	2,04	2,18	9,40
T1-K3	96,4	2,03	2,2	8,50
T1-P4	113,05	2,02	2,14	9,30
T1-L5	118,44	2,03	2,18	8,70
T1-M6	94,7	2,03	2,12	9,60
T1-O7	66,54	2,05	2,2	9,30
T1-N8	143,7	2,02	2,17	9,00
T1-Z10	158,84	2,03	2,18	8,90
T1-Y11	93,09	2	2,03	9,00
T1-X12	137,8	2,02	2,21	9,10
T1-W13	79,85	2,03	2,21	9,10
T2-J2	119	2,05	2,08	9,10
T2-K3	134,98	2,03	2,07	8,70
T2-P4	91,09	2,03	2,11	9,10
T2-L5	37,95	2,02	1,81	4,70
T2-M6	93,54	2,02	2,05	9,40
T2-O7	97,09	2,02	2,03	9,40
T2-N8	132,23	2,04	2,22	9,40
T2-Z10	108,04	2,05	2,05	9,10
T2-Y11	78,45	2,02	2,23	9,50
T2-X12	92,48	2,04	2,09	9,40
T2-W13	46,59	1,99	1,89	9,20
T3-J2	119,53	1,99	2,13	8,60
T3-K3	104,77	1,98	2,13	7,90
T3-P4	95,34	2,04	2,1	8,40
T3-L5	124,98	2,06	2,1	8,20
T3-M6	136,2	2,07	2,14	8,70
T3-O7	82,24	1,98	2,05	8,80
T3-N8	99,11	1,99	2,11	8,90
T3-Z10	89,98	2,06	2,08	9,00
T3-Y11	99,85	2,06	2,08	8,80
T3-X12	115,02	2,05	2,13	8,50
T3-W13	71,28	2,03	2,01	9,00

Sample ID	ng/ul	260/280	260/230	Rin
T4-J2	161,39	2,08	2,19	9,30
T4-K3	152,36	2,09	2,18	8,70
T4-P4	72,71	2	1,5	9,60
T4-L5	104,8	2,01	1,65	9,10
T4-M6	91,84	2	1,59	9,60
T4-O7	134,68	2,1	2,14	9,50
T4-N8	164,24	2,08	2,13	9,30
T4-Z10	101,17	2,07	2,05	9,30
T4-Y11	82,73	2,05	2,17	9,50
T4-X12	130,9	2,06	2,13	9,30
T4-W13	86,14	1,99	1,58	9,40
T5-J2	165,49	2,03	2,18	7,80
T5-K3	124,14	2,02	2,17	8,70
T5-P4	118,84	2,04	2,17	9,20
T5-L5	115,85	2,04	2,24	9,10
T5-M6	170,66	2,05	1,81	9,50
T5-O7	112,03	2,03	2,15	9,60
T5-N8	165,43	2,05	1,81	9,30
T5-Z10	111,35	2,03	1,69	9,50
T5-Y11	95,72	2,04	2,16	8,00
T5-X12	107,14	2,02	1,68	9,20
T5-W13	95,83	2,04	2,26	8,20
T6-J2	170,12	2,03	2,13	9,30
T6-K3	191,79	2	2,05	8,50
T6-P4	132,42	2,07	2,19	9,00
T6-L5	128,48	2,04	2,34	8,80
T6-M6	139,73	1,9	2,09	9,30
T6-O7	155,94	2,08	2,18	9,30
T6-N8	131,43	1,99	2,1	9,50
T6-Z10	119,67	2,04	2,22	9,70
T6-Y11	71,87	2,08	2,21	9,10
T6-X12	125,29	2,1	2,29	9,10
T6-W13	72,53	1,96	2,05	9,20
T7-J2	132,38	2,02	1,65	9,20
T7-K3	123,1	2,01	1,66	8,30
T7-P4	96	2,02	1,65	8,70
T7-L5	153,28	2,03	2,16	8,30
T7-M6	96,6	2,05	2,17	9,00
T7-O7	117,37	2,09	2,34	9,10
T7-N8	134,08	2,02	1,75	9,30
T7-Z10	154,86	2,03	2,17	8,90
T7-Y11	89,73	2,02	2,17	6,70
T7-X12	147,5	2,06	1,79	9,00
T7-W13	84,81	2,03	2,21	9,10

Sample ID	ng/ul	260/280	260/230	Rin
T8-J2	130,67	2,03	2,18	8,80
T8-K3	125,64	1,98	2,22	8,30
T8-P4	106,93	2,03	1,88	8,40
T8-L5	109,91	2,05	2,19	7,90
T8-M6	112,01	2,05	2,16	9,00
T8-O7	121,17	2,04	2,19	9,40
T8-N8	124,85	2,02	2,23	9,40
T8-Z10	122,75	2,05	2,12	8,60
T8-X12	108,96	2,02	2,13	9,00
T8-W13	79,36	2	2,18	9,30
T9-J2	90,58	2,08	2,14	8,00
T9-K3	130,77	2,09	2,18	7,20
T9-P4	84,42	2,06	2,15	8,00
T9-L5	106,35	2,09	2,17	7,40
T9-M6	131,55	2,02	2,3	7,90
T9-O7	158,94	2,04	2,23	8,10
T9-N8	125,62	2,07	2,14	8,10
T9-Z10	157,21	2,06	2,09	7,90
T9-Y11	115,56	2,16	2,24	7,40
T9-X12	107,61	2,01	2,17	7,90
T9-W13	68,05	2,02	1,96	8,10
T10-J2	109,24	2,06	2,23	8,10
T10-K3	167,04	2,05	2,21	7,10
T10-P4	119,17	2,04	2,22	7,80
T10-L5	122,27	2,04	2,2	7,70
T10-M6	88,64	2,07	2,23	8,20
T10-O7	131,23	2,04	2,19	8,00
T10-N8	148,07	2,03	2,19	7,90
T10-Z10	94,99	2,04	2,23	8,10
T10-Y11	99,26	2,06	2,24	8,00
T10-X12	109,67	2,03	2,21	7,90
T10-W13	71,82	2,03	2,16	9,40

II. All samples obtained in the DLR parabolic flight campaign in November 2010

Table II: Overview of RNA yield, 260/280 ratio, 260/230 ratio and RIN value of blood and saliva samples obtained in the parabolic flight campaign of Cologne, November 2010

Saliva					
	ID	**(ng/µl)**	**260/280**	**260/230**	**(RIN)**
Before Flight	V1-BF	1672,03	2,02	2,13	7,80
	V2-BF	760,75	1,87	1,37	3,50
	V3-BF	1042,72	1,94	0,59	6,00
	V5-BF	669,14	2,05	2,02	N/A
	V6-BF	800,5	2,08	2,07	5,90
	V7-BF	724,85	2,08	2,11	N/A
	V8-BF	623,3	1,98	1,5	4,30
	V9-BF	107,64	2,1	1,63	N/A
10 Cycles	V1-P10	241,86	2	0,99	N/A
	V5-P10	199,17	1,99	1,72	4,80
	V6-P10	16,45	1,9	0,56	N/A
	V8-P10	171,92	2,07	1,43	4,80
20 Cycles	V2-P20	760,08	1,86	1,78	3,00
	V8-P20	64,01	2	1,13	5,90
	V9-P20	39,52	1,92	1,03	7,20
After Flight	V1-P30	1505,66	2,12	2,19	8,90
(30 Cycles)	V2-P30	981,7	1,92	1,97	4,40
	V5-P30	114,72	2,01	1,49	6,00
	V6-P30	93,21	2,05	0,4	5,80
	V8-P30	214,25	1,98	1,72	4,60
	V9-P30	494,47	2,04	1,8	6,60
Blood					
	ID	**ng/µl**	**260/280**	**260/230**	**Rin**
Before Flight	V1-BF	153,44	2,02	2,13	8,00
	V2-BF	118,29	2	2,34	8,30
	V3-BF	129,17	2,03	2,29	8,20
	V4-BF	227,86	2,03	2,2	5,90
	V5-BF	80,95	2,09	2,2	7,80
	V6-BF	175,26	2,01	2,3	8,00
	V7-BF	135,15	2,08	2,12	9,00
	V8-BF	59,35	2,01	2,14	8,90
	V9-BF	173,63	2,11	2,15	8,20
After Flight	V1-AF	82,17	2,05	2,19	8,30
	V2-AF	66,62	1,99	2,15	8,30
	V3-AF	77,23	2	2,1	7,90
	V4-AF	83,1	2,05	2,06	8,40
	V5-AF	76,2	2,04	2,1	8,20
	V6-AF	170,28	2,07	2,18	8,50
	V7-AF	94,66	2	2,13	9,20
	V8-AF	73,28	1,97	2,38	9,30
	V9-AF	157,69	2,01	2,14	8,30

III. Saliva sponges in combination with Oragene RNA kit

Materials & Methods

One individual was asked to refrain from eating, drinking and smoking for at least one hour prior to sample collection. The mouth was rinsed with water and 5 saliva sponges were used to collect whole saliva. The sample was then processed with minor modifications according to DNA Recovery rrom Saliva Sponges protocol from DNA Genotek (PD-PR-017).

Results

386.4 ng/µL total RNA was obtained from 1 mL saliva. No protein contamination was observed (260/280 ratio = 2) and minor solvent (ethanol) contamination was measured (260/230 ratio = 1.7). When using 1.75 mL of saliva, the RNA yield is equal to 1096.4 ng/µL. No protein or solvent contamination was found (260/280 ratio = 2.1; 260/230/ratio = 2.0) (Table IV).

Table III: RNA yield, 260/280 ratio and 260/230 ratio of saliva collected with 5 swaps in one individual.

Sample ID	ng/ul	260/280	260/230
saliva swap 1ml	**386,4**	2,0	1,7
saliva swap 1.75ml	**1096,4**	2,0	2,0

IV. Inflammatory and immunological diseases (IPA analysis)

Table IV: The amount of genes present in inflammatory and immunological diseases found by IPA analysis. Significance level was set to p<0.05

Functions Annotation	p-Value	# Molecules
lichen planus	1.89E-10	22
polyarticular juvenile rheumatoid arthritis	4.58E-10	20
juvenile rheumatoid arthritis	1.08E-09	23
hypersensitive reaction	2.31E-09	39
atopic dermatitis	1.88E-08	33
immediate hypersensitivity	7.00E-08	34
immunological disorder	1.22E-05	169
delayed hypersensitive reaction	1.08E-04	8
rheumatoid arthritis	7.69E-04	101
autoimmune disease	4.26E-03	131
immunological disorder of humans	2.65E-02	5
chronic granulomatous disease	3.30E-02	2
histiocytosis	3.34E-02	3
primary Sjogren's syndrome	4.12E-02	4

V. Cell characterization in saliva

Materials & methods

One participant was asked to refrain from eating, drinking and smoking for at least one hour prior to sample collection. The mouth was rinsed and ± 4 mL of unstimulated saliva was collected in a vessel on ice. 1 mL of unstimulated saliva was centrifuged at 1500 g for 10 minutes at 4°C. The supernatant was discarded and the insoluble pellet was washed twice with ice-cold PBS buffer (1 mL). The pellet was resuspended into 1 mL PBS and dilution series were made of the 1 mL pre-treated saliva and 1 mL of whole saliva (**Table 2.1**).

Table V: Dilution series of 1 mL unstimulated saliva in PBS

No dilution (1)	1:9 dilution (2)	1:99 dilution (3)
1 mL saliva	100 µL (1)	100 µL (2)
/	900 µL PBS	900 µL PBS
Total: 900 µL	Total: 900 µL	Total: 1000 µL

Cells of diluted samples of whole saliva and the resuspended pellet were counted with the nucleocounter and a cytospin for microscopic analysis was prepared and stained with standard May-Grunwald and Giemsa according to manufacturer's protocol. Trypan blue staining was used to indicate viability of the cells.

Results

Unstimulated whole saliva of one individual was used for measuring the amount of living cells via nucleocounter. The saliva sample was processed via two different methods as described in materials & methods. Results demonstrated that the detection limit of death cells ($> 2.0 \times 10^6$) was reached in whole, not diluted saliva. A dilution of whole saliva is therefore recommended. The 100x and 10x diluted, whole saliva samples had an amount of living cells = 1.9×10^6 - 2.9×10^6. The second procedure showed a lesser amount of living cells compared to whole saliva. The centrifugation steps in this procedure are probably the cause of the difference.

Table VI: The amount of living cells in unstimulated, whole saliva of one individual. The saliva sample was processed via two different methods and a dilution series was made.

	# living cells	
	Whole Saliva	Cell pellet dissolved in 1 mL PBS
Not diluted	<2,37.10^6	1,24.10^6
10 x diluted	2,9.10^6	1,39.10^6
100 x diluted	1,9.10^6	< 2,5.10^6

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Saenen, Nelly

Datum: **14/06/2011**