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Changes of growth factors under the influence of hypobaric hypoxia and physical activity

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List of Abbreviations

a.m.	ante meridiem
AMS	acute mountain sickness
BMI	body mass index
C	celsius
CNS	central nervous system
COPD	chronic obstructive pulmonary disease
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGFR	endothelin growth factor receptor
EPC	endothelial progenitor cell
FGF-2	fibroblast growth factor 2
FGFR	fibroblast growth factor receptor
g	gram(s)
G-CSF	granulocyte colony stimulating factor
GH	growth hormone
GM-CSF	granulocyte macrophage colony stimulating factor
H	hour(s)
HACE	high altitude cerebral edema
HAPE	high altitude pulmonary edema
Hb	hemoglobin
Hct	Hematocrit
HIF	hypoxia inducible factor
HPH	HIF prolyl hydroxylase
IFN	interferon
IGF-1	insulin-like growth factor 1
IGF-1R	insulin-like growth factor 1 receptor
IGF-2R	insulin-like growth factor 2 receptor
IGFBP-2	insulin-like growth factor binding protein 2
IGFBP-3	insulin-like growth factor binding protein 3
IL	interleukin

IP	interferon gamma-induced protein
kg	kilogram(s)
l	liter(s)
km	kilometer(s)
LLS	lake louise score
m	meters
Max	maximum
M-CSF	macrophage colony stimulating factor
MCP	monocyte chemoattractant protein
MFI	median fluorescence intensity
mg	milligram
µg	microgram
µl	microliter
Min	minimum
min	minute(s)
ml	milliliter
MIP	macrophage inflammatory protein
mmHg	millimeters of mercury
NA	not available
ng	nanogram
NO	nitric oxide
NS	not significant
O2	oxygen
p	p-value
p53	protein 53
PAH	pulmonary arterial hypertension
PDGF	platelet-derived growth factor
pg	picogram
p.m.	post meridiem
pO2	barometric oxygen pressure
Rpm	revolutions per minute
RIA	radio immunoassay

SIRS	severe inflammatory response syndrome
STAT	signal transducer and activator of transcription
sVEGFR	soluble vascular endothelial growth factor receptor
sIL-2R	soluble interleukin 2 receptor
TGF-β1	transforming growth factor β1
TGF-βR	transforming growth factor β receptor
TNF	tumor necrosis factor
UK	United Kingdom
USA	United States of America
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VHL	von Hippel-Lindau tumor suppressor

1. Summary

Rationale

At high altitude, individuals are at risk of suffering from symptoms of compromised immunity. This is associated with acute mountain sickness (AMS), high altitude pulmonary edema (HAPE), and high altitude cerebral edema (HACE) [1]. Exercise can provoke symptoms related to AMS, as well [2]. Studies implicate that hypoxia as well as exercise influence the human immune response by triggering angiogenesis and pro- and anti-inflammatory processes[3]. There is little data on the immune response on a molecular level, however. Therefore, the present study had following objective:

Objective

To explore blood cytokine and growth factor changes under the influence of hypobaric hypoxia alone and of physical exercise under both normobaric normoxia and hypobaric hypoxia.

Methods

The study used a non-randomized, non-blinded design with two parallel groups and an additional group. Participants conducted a resting trial (group 1, n=25) and/or exercise trial (group 2, n=18) both at sea level under normoxia (Bangor, UK; 69 m) and at high altitude after a 14-day-trek to 5050 m under hypobaric hypoxia (Hidden Valley Base Camp, Dhaulagiri Circuit, Western Himalayan mountains, Nepal).

Each trial included peripheral blood sampling either at rest (group 1) or before and 180 minutes after a 12-minute incremental step test (group 2).

Additionally, blood was sampled from participants before and directly after a one-day climb from Hidden Valley Base Camp (5050 m) to Damphus Peak (6035 m) and back (group 3, n= 4).

Blood was analyzed via Luminex xMAP Multiplexing Technology and radio immunoassay. Growth factors VEGF, EGF, FGF-2, TGF-β1, G-CSF, GM-CSF, IGF-1, IGFBP-1, and IGFBP-3 were measured.

Results

In group 1, hypobaric hypoxia alone caused an increase in VEGF ($p = 0.039$), EGF ($p = 0.002$), TGF-β1 ($p < 0.001$), G-CSF ($p = 0.029$), and GM-CSF ($p = 0.035$). Furthermore, a decrease in IGF-1 ($p < 0.001$) and IGFBP-3 ($p < 0.001$) was found. Markers were measured before and after each exercise trial (group 2 and 3). In group 2, EGF ($p = 0.031$) decreased after exercise under

normoxia at 69 m. In group 3, no changes were seen. Other cytokines and growth factors of groups 1, 2, and 3 were unchanged. While hypobaric hypoxia is a potent stimulant of cytokines and growth factors, physical activity seems to have minor effects on these biomarkers.

Conclusions

The present study provides field data about changes in growth factors upon exposure to hypobaric hypoxia and physical exercise. It is known that cytokines and growth factors are influenced by hypoxia promoting angiogenesis, cell growth and repair, inflammation, and cancer [3-5]. Hypobaric hypoxia proved to be a potent trigger of significant changes in most cytokines and growth factors at rest. This may indicate the immunological response to counteract inflammation triggered by hypoxia and adapt to tissue hypoxia. Even though there is evidence that exercise amplifies the immunosuppression imposed by tissue hypoxia [6], this study shows that physical activity has only a minor impact on biomarkers both at sea level and high altitude.

2. Introduction

"Your attitude, not your aptitude, will determine your altitude"

Hilary Hinton Ziglar

Altitude is categorized into intermediate altitude [1500-2500 meters (m)], high altitude (2500-3500 m), very high altitude (3500-5800 m), and extreme altitude (> 5800 m) [1]. About 50 million people of the American continent and 80 million people of the Asian continent permanently live above 2500 m [7]. Apart from permanent high altitude residents, people also travel to high altitude for business reasons or in order to pursue recreational activities.

However, at altitude, individuals are at risk of experiencing potentially incapacitating symptoms which are associated with an increased incidence of acute mountain sickness (AMS) [8]. This apparent compromise of immunity limits work capacity and people are at risk to develop more severe clinical symptoms associated with diseases such as high altitude pulmonary edema (HAPE) and high altitude cerebral edema (HACE), ultimately putting them at risk of death. With increasing altitude, atmospheric pressure drops constantly, and inspired oxygen content is reduced. As a result, tissue hypoxia causes various changes in the autonomic nervous system and endocrine functions are evoked, which ultimately affects the human immune system.

Apart from hypoxia, exercise has also a certain impact on the immune system. [2, 9, 10] Heavy or prolonged exercise can provoke symptoms related to AMS including symptoms of the common cold or upper respiratory tract infection symptoms. This apparent compromise of immunity [11] can occur at sea level in immunocompetent people. In subjects who experience chronic hypoxic sates, such as patients suffering from chronic lung or heart disease or people dwelling at high altitude, exercise can precipitate more severe deterioration of health. Therefore, the combination of high altitude hypobaric hypoxia and physical exercise represents a stressor which has a greater impact on immunity than that of either hypoxia or exercise alone [12-14].

With increasing altitude, atmospheric pressure drops constantly, resulting in reduced inspired oxygen content. Consequently, tissue hypoxia poses stress to the homeostasis of the human body. Apart from hypoxia, exercise has also a certain impact on the immune system. [2, 9, 10] It is suggested that exercise under hypoxic conditions provokes a more pronounced immunological response than exercise at normoxia.

The human immune system incorporates both innate and adaptive immune responses in order to provide host defense. The complex immunological processes are controlled and regulated at various sites and stages by a multitude of factors of which growth factors play a vital role. Therefore, the present investigation highlights potential links between the exposure to hypobaric hypoxia and changes in the growth factor milieu. Furthermore, growth factor levels after physical exercise both at sea level (69 m) and high altitude (5050 m) were studied. Insights are applicable to clinical conditions involving chronic respiratory and/or cardiovascular diseases.

2.1. Hypoxia-Inducible Factor (HIF)

Hypobaric [total ambient gas pressure less than 760 millimeters of mercury (mmHg)] hypoxia (inadequate oxygen tension at the cellular level) leads to either cell death or triggers a variety of survival mechanisms. Hypoxia-inducible factor (HIF) represents the main mediator of adaptation to hypoxia. [15-18]

The HIF-pathway has been identified to regulate several hundred genes by direct or indirect modulation of gene expression [7]. Activation of HIF is shown in figure 1.

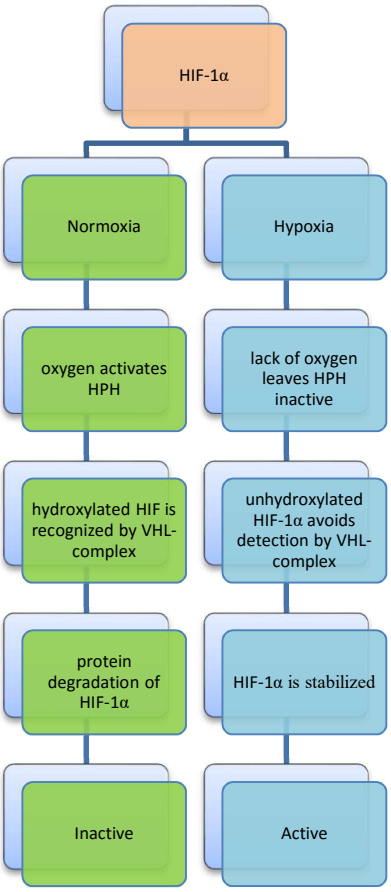

Figure 1 HIF-1α

Under normoxic conditions, HIF prolyl hydroxylase (HPH) is activated by oxygen, subsequently hydroxylating HIF-1α. The von Hippel-Lindau (VHL)-complex recognizes hydroxylated HIF-1α and causes degradation of HIF-1α. Under hypoxic conditions, the lack of oxygen leaves HPH inactive. The VHL-complex does not detect unhydroxylated HIF-1α, which is stabilized and remains in its activated state. [19-21]
HIF-1α Hypoxia Inducible Factor 1α, ***HPH*** HIF prolyl hydroxylase, ***VHL*** von Hippel-Lindau

Active HIF-1α translocates to the nucleus and binds to hypoxia response elements of several target genes. Resulting transcription products include factors involved in angiogenesis [e.g., vascular endothelial growth factor (VEGF), transforming growth factor (TGF)-β1], cell growth [e.g., TGF-α, platelet-derived growth factor (PDGF], inflammatory response (e.g., cyclooxygenase-1, IL-6), oxygen supply (e.g., erythropoietin), and metabolic processes such as adenosine triphosphate synthesis (e.g., glucose transporter-1). [19-21]

2.2. Growth Factors

Cytokines were first described in 1972 by Igal Gery and Byron Waksman as “lymphocyte activating factor[s]” [22]. Nowadays, cytokines are defined as soluble factors of variable size which are produced and released by many different cell types and exert manifold behavioral and

functional effects on target cells. Once released, cytokines may act on another cell in a paracrine or endocrine way or on the releasing cell itself in an autocrine way. Furthermore, cytokines function as integral membrane proteins. [3] Growth factors and cytokines are often used synonymously which makes clear linguistic distinction difficult. In this work, however, growth factors are not used synonymously for, but represent a subgroup of cytokines.

Growth factors comprise a complex family of biological factors that are produced by many different cell types in order to control cell growth, division, maturation, proliferation, migration, chemotactic attraction, and inhibition of mentioned actions, respectively [23]. After binding to their receptors, growth factors utilize specific multivariate signaling pathways which they share among each other. It has to be taken into account that once a growth factor has been secreted it has pleiotropic effects. That means that its actions are not limited to one type and effects are not limited to one specific cell type. [24, 25] Growth factors bind to receptors that are located either on only one specific type of cell or to receptors that are present on almost every cell. In the latter case, the cell type itself defines the property of the growth factor. Growth factors are not only pleiotropic but also fulfill their specific tasks in an overlapping manner. This highlights that the growth factor network as an immunological entity is based on a multiplication of function. For instance, each of the colony-stimulating factors [e.g., interleukin (IL)-3, granulocyte colony stimulating factor (G-CSF), granulocyte macrophage colony stimulating factor (GM-CSF), macrophage colony stimulating factor (M-CSF)] is derived from a distinct gene and binds to its specific receptor, their biological properties, however, overlap markedly. At last, a remarkable feature of growth factor biology is the ambiguity in effects which may be both health promoting and damaging. [23]

Growth factors are involved in the modulation of cell function, cell survival, and apoptosis [23], exert neuroprotective and neurotrophic effects on the central nervous system (CNS) [26], act as angiogenic factors or inhibit angiogenesis, and regulate the expression of matrix metalloproteinases, and both fibrinolytic and fibrinogenic factors [4]. Growth factors promote cancer progression [27-31] and are used therapeutically to stimulate leucocyte production following chemotherapy or bone marrow transplantation [32]. Figure 2 provides an overview of characteristics of the growth factors investigated.

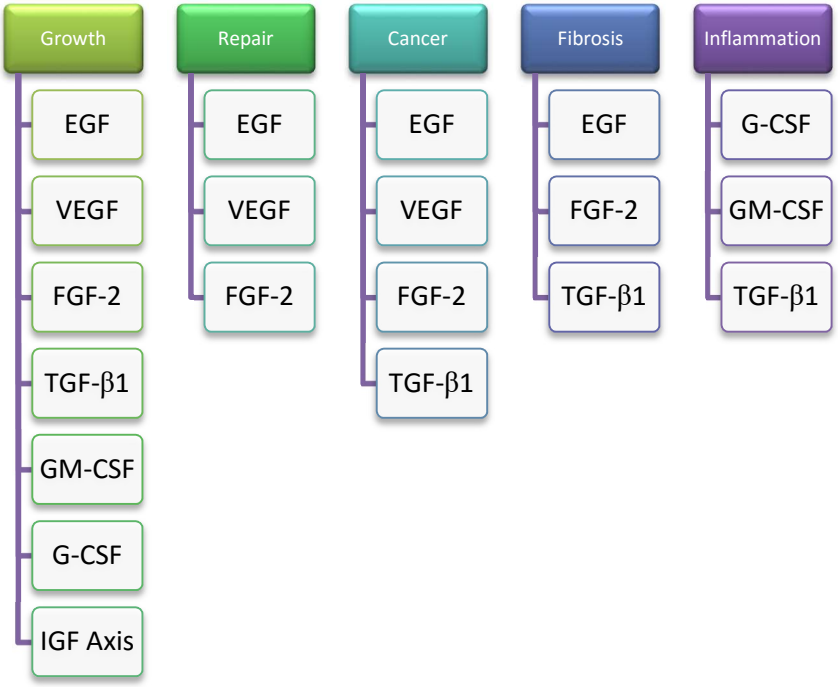

Figure 2 Growth factor functions

Functional groups illustrate the effects of growth factors including involvement in cellular growth and repair, cancer, fibrosis, and inflammation. It highlights both their pleiotropy and multiplication of function [3, 4].

2.3. Growth Factor Groups

The present study investigates growth factors of all major growth factor groups including vascular growth factors such as epidermal growth factor (EGF), VEGF, fibroblast growth factor 2 (FGF-2), and TGF-β1, hematological growth factors such as G-CSF and GM-CSF, and growth factors of the IGF-axis such as insulin-like growth factor 1 (IGF-1), insulin-like growth factor binding protein 2 (IGFBP-2), and insulin-like growth factor binding protein 3 (IGFBP-3).

2.3.1. Vascular Growth Factors

Vascular growth factors have been identified to be both inducers (EGF, VEGF, FGF, and TGF-β) and inhibitors [soluble vascular endothelial growth factor receptor (sVEGFR)] of angiogenesis [33].

2.3.1.1. Epidermal Growth Factor (EGF)

EGF is produced in both a membrane bound and soluble form by various cells including epithelial cells and fibroblasts [23, 34]. EGF is responsible for proliferation of epidermal, epithelial and endothelial cells. Its most important roles are promotion of wound healing processes which are brought about by its chemoattractant effects for fibroblasts and epithelial cells [35] and

induction of angiogenesis [23]. Uncontrolled signaling via the vascular endothelial growth factor receptor (EGFR) pathway such as overexpression of the EGF receptor has shown to promote tumor cell motility, adhesion, and metastasis [34, 36, 37]. Furthermore, EGF has been identified to play a role in tissue fibrosis [4].

Hypoxia potently stimulates the production of EGF which is implicated in pulmonary arterial hypertension by promoting proliferation, resistance to apoptosis, and migration of pulmonary vascular cells [20].

2.3.1.2. Vascular Endothelial Growth Factor (VEGF)

The VEGF family includes VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, placental growth factor, and various alternative splicing products of VEGF-A [4, 38]. VEGF is expressed ubiquitously but is predominantly released in the lungs, cerebral choroid plexus, and in platelets [39, 40].

VEGF expression is induced by various stimuli including growth factors (e.g., EGF, FGF-2 IGF-1, TGF-β1) and hypoxia [30, 38]. After binding to its receptors VEGF promotes chemotaxis and vascular permeability via VEGFR-1 [41] and cell proliferation and survival, mitogenic activities as well as vascular permeability via VEGFR-2 [42, 43]. VEGF is inactivated by soluble scavenger receptors (sVEGFR-1, sVEGFR-2) [30, 38]. Net effects of VEGF are cell survival and angiogenesis [41, 44]. However, VEGF has also been identified to be involved in pathophysiologic processes such as atherogenesis, restenosis [45], tissue edema [39], tumorgenesis [41], and metastatic spread of cancer [46, 47].

Hypoxia potently stimulates the release of VEGF (via HIF-1α) which results in neovascularization and angiogenesis through enhanced capillarization and subsequent increase in oxygen delivery to peripheral tissues [30, 48]. Physical exercise is another stimulus changing VEGF concentrations under hypobaric hypoxia [48].

2.3.1.3. Fibroblast Growth Factor 2 (FGF-2)

FGF-2 is one of 22 currently known members of the FGF-family [49]. It is widely expressed in many tissues and cell types [50] and reaches high concentrations in brain tissue [51] and the pituitary gland [52, 53]. FGF-2 regulates the expression of its own receptors (up-regulation of FGFR1 and FGFR4 and down-regulation of FGFR2 and FGFR3) which has a net inhibitory effect on cell differentiation [54]. FGF signaling pathways are intertwined with various other growth factor pathways including the ones of IGF and TGF-β [55]. FGF-2 promotes cell growth [41], neurogenesis both in embryonic and adult CNS cells [56-58], morphogenesis, wound healing,

angiogenesis, pro-inflammatory actions [50], atherogenesis [45], lung and liver fibrosis [3], and tumor growth and progression [58-60]. Under hypoxic conditions, FGF mediates the proliferation of vascular cells [20].

2.3.1.4. Transforming Growth Factor β 1 (TGF-β1)

The TGF-β family includes three isoforms (TGF-β1, -2, and -3). TGF-β1 is the most widely expressed isoform and is involved in the control of multiple biological processes including cell growth and differentiation, homeostasis, and modulation of immunological processes. Respective receptors include TGF-β receptor 1 to 3 (TGF-βR-1, -2, -3).[41, 61, 62] TGF-β1 is released from endothelial cells, epithelial cells, fibroblasts, regulatory T-cells, and hepatocytes [62, 63] and exerts both growth-promoting [4] and -inhibitory [41] effects on target cells. It regulates the cell cycle by freezing the cell cycle at the G1 stage in order to halt proliferation, induce differentiation, or promote apoptosis [64]. In analogy, TGF-β1 both stimulates and inhibits immunological processes [62]. It is a potent stimulator of chemotaxis demonstrated by migration of monocytes, lymphocytes, neutrophils, and fibroblasts [4, 65] but also inhibits the activation of lymphocytes and monocyte derived phagocytes [66, 67]. TGF-β1 is a crucial factor in angiogenesis and has both growth-promoting and -inhibitory effects on angiogenesis [4, 68]. Interaction with other growth factors is demonstrated by the up-regulation of VEGF by TGF-β1 along with a concomitant down-regulation of VEGFR-2 which impedes VEGF-mediated effects. The net effect of TGF-β1 is therefore anti-proliferative. [41, 69, 70]

Uncontrolled TGF-β1 signaling is implicated in pathologies such as inflammation, autoimmune disorders [71, 72], fibrosis [4], and cataracts [71, 72]. Extensive studies have been undertaken with respect to oncogenesis, where TGF-β both serves as a proto-oncogene and a tumor suppressor [62]. TGF-β is often co-expressed with VEGF in cancer [41]. TGF-β1 is not only involved in homeostasis in healthy lung tissue by regulating pulmonary cell proliferation and modulating the immune response, but also involved in asthma, COPD, lung fibrosis, and lung cancer [73-75].

Hypoxia induces TGF-β1-mediated angiogenesis causing proliferation and migration of vascular endothelial cells [62, 68, 76]. Furthermore, physical exercise induces TGF-β-mediated tissue repair [77, 78].

2.3.2. Hematological Growth Factors

2.3.2.1. Granulocyte-Colony Stimulating Factor (G-CSF)

While G-CSF is produced by all tissues in the body, the most relevant sites of production include macrophages, endothelial cells, fibroblasts, and related mesenchymal cells. The G-CSF receptor is expressed by all neutrophils and their precursors in the bone marrow, by neurons in the brain and spinal cord, endothelial cells, placental cells, activated T-lymphocytes, and many non-hematopoietic tumor cell lines [79]. G-CSF induces proliferation and promotes chemotaxis in all cells of the granulocyte lineage in bone marrow cells and activates their immunological functions such as superoxide production, phagocytosis, and bactericidal killing [80]. Apart from effects on the hematopoietic system, G-CSF acts also as a promoter of neurogenesis [81]. G-CSF both has pro-inflammatory (modulation of T-lymphocyte development and function) [79] and anti-inflammatory (triggering the release of IL-4 and IL-6) [82-84]effects. G-CSF plays a role in rheumatoid arthritis[79] and cancer [85, 86].

Under hypoxic conditions, G-CSF has been shown to be involved in cell-survival and protection from ischemic injury in brain [26]. Additionally, physical exercise, exemplified by an Ironman triathlon race, causes an up-regulation of G-CSF [87].

2.3.2.2. Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF)

GM-CSF is secreted by macrophages, T-lymphocytes, mast cells, NK cells, endothelial cells, fibroblasts [88], chondrocytes, and smooth muscle cells [89] and regulates various processes of both innate and adaptive immunity including proliferation, differentiation, activation, and survival of hematopoietic stem cells including myeloid and lymphoid cells [90]. Moreover, it has been shown that in response to a stimulatory signal (e.g., lipopolysaccharide, cytokines, and antigen) GM-CSF triggers the production of major inflammatory cytokines such as IL-1 and tumor necrosis factor (TNF)-α and growth factors including G-CSF and macrophage colony-stimulating factor [91].

On the one hand, anomalous expression of GM-CSF production or aberrant receptor function are implicated in pathologies including chronic myeloid leukemia, myeloproliferative diseases, tissue fibrosis (via induction of TGF-β1 release) [4], asthma, rheumatoid arthritis [89], juvenile myelomonocytic leukemia, chronic myelomonocytic leukemia, and alveolar proteinosis, on the other hand, GM-CSF represents a therapeutic approach in Crohn´s disease through its induction of immunological processes aimed against cancer cells and promotion of innate immunity [90].

2.3.3. The Insulin-Like Growth Factor Axis

Insulin like growth factors (IGFs) comprise a family which is structurally very closely related to insulin. The IGF axis consists of IGF-1 and IGF-2, the respective receptors IGF-1R and IGF-2R, IGF binding proteins (IGFBPs)-1 to 10, and IGFBP proteases. [92] The majority of the IGFs are bound to IGFBPs [93, 94] (75% are bound to IGFBP-3 [95]). IGFBPs have a higher affinity to IGF than IGF receptors to IGF [96]. IGFBPs regulate either positively or negatively IGF-mediated effects by releasing or binding them [93, 94]. IGFBPs transport IGFs, regulate their efflux from the circulation, prolong their half-lives, and modify their metabolic clearance [97]. On a local level, IGFBPs exert paracrine and autocrine control resulting in modulation of local IGF effects, altered local growth and metabolism, and activation of various signaling pathways [98]. IGFBP proteases lower the affinity with which IGFBPs bind IGFs [96]. Both the production of IGFs and IGFBPs is influenced by the growth hormone (GH) [98].

IGF-1 exists in two isoforms (IGF-1Ea and IGF-1Ec). IGF-1Ea is produced mainly in the liver upon GH stimulation and acts systemically in an endocrine fashion. IGF-1Ec is produced by various extrahepatic cell types and acts locally as an autocrine/paracrine growth factor under the control of different hormones. Systemic IGF-1Ea has a longer half-life than local IGF-1Ec due to glycosylation which protects it from proteolytic degradation. [99]

Unbound (bioactive) IGF-1 is positively correlated with total IGF-1 levels and IGFBP-3 levels, while it is negatively correlated with levels of IGFBP-1 and IGFBP-2. Insulin reduces levels of bioactive IGF-1 which presumably is due to simultaneous insulin-induced increases of IGFBP-2 which in turn binds and inactivates IGF-1. Reduced levels of unbound (bioactive) IGF-1 trigger the up-regulation of GH via a negative feedback loop. [100]

IGFBP-1 production is dependent on [96] and negatively correlated with insulin [101].

IGF-1 is a vital factor of pre- and post-natal body growth and is especially important in growth of bone and muscle [93, 94, 99]. IGF-1 is involved in pathologies such as growth deficiency [94], osteoporosis [99], oncogenesis [6], atherosclerosis [100], and muscle mass wasting [99] which is a feature of malnutrition, critical illness and sepsis [93].

IGFBPs are momentous adjuncts, because they extend IGF-1 half-life from estimated 10 minutes to 15 hours when bound to IGF-1 [97]. Of the family of high-affinity IGF binding proteins (IGFBP-1 to 10) [92] regulatory effects of IGFBP-1 and IGFBP-3 on IGF-1 are highlighted in this work.

While both IGFBP-1 and IGFBP-3 is mainly produced in the liver [94, 96], the majority of IGFs (75-80%) are bound to IGFBP-3 and only small quantities of them are bound by IGFBP-1 [95].

IGFBPs regulate the half-life of IGF-1 and its interaction with the IGF-1 receptor.

The following features of IGFBP-3 are exemplary for IGFBPs. Growth inhibitory effects are brought about by IGFBP-3 binding IGF-1 and thus scavenging it from the IGF-1 receptor. The IGFBP-3-IGF-1-complex is contained to the vascular compartment and even is able to guide IGF-1 to specific tissues and cell types. Growth stimulation, on the other hand, is brought about by liberating IGF-1 from IGFBP-3 through either proteolysis of IGFBP-3 or binding of IGFBP-3 to extracellular matrix proteins. [93, 98] Furthermore, actions not involving IGFs have been described for most IGFBPs and include binding to integrins or cell-membrane receptors [99, 102, 103]. While IGFBP-1 is involved in vasodilation by activation of nitric oxide (NO) production [103], IGFBP-3 induces apoptosis via protein 53 (p53) [6] and is involved in wound healing [98]. Additionally, IGFBP-3 is involved in cancer as a tumor-suppressor both in a direct and indirect (via TGF-β) way [98]. Furthermore, IGFBP-3 inhibits VEGF-mediated angiogenesis [104].
Physical exercise stimulates the GH-IGF-1-axis, which has anabolic effects [105] and is involved in tissue remodeling upon muscle damage [77, 78].
Various studies provide evidence for a hypoxia-induced up-regulation of IGFBP-3 [98, 106]. Hypoxia indirectly up-regulates IGFBP-1 levels which represents a mechanism in atherosclerosis [107]. Studies imply that hypoxia causes fetal growth restriction. In pregnant women, IGFBP-1 levels were significantly higher at high altitude than at sea level. It is assumed that placental hypoxia caused by hypobaric hypoxia at high altitude triggers a HIF-1-mediated up-regulation of IGFBP-1 in order to limit IGF-mediated fetal growth. This IGFBP-1-mediated growth restriction attenuates fetoplacental hypoxia and ultimately prevents fetal death. [108, 109] Intrauterine growth restriction may predispose to preterm birth which in turn is associated with low birth weight and low growth velocity in childhood. Eventually, perinatal growth restriction may be a predisposing factor for disorders such as cardiovascular disease or diabetes. [110] Another study reported that transcription levels of IGF-1 or IGFBP-3 in vertebrate embryonic cells were unchanged by hypoxia, however [101].

Having presented the most poignant facts about growth factor physiology, the present study aims to highlight less investigated issues. Therefore, following hypotheses arose.

3. Hypotheses

1. Does hypobaric hypoxia at high altitude (5050 m) induce changes in growth factor levels when compared to sea level (69 m)?
2. Does physical exercise induce changes in growth factor levels at sea level (69 m) and/or high altitude (5050 m)?

4. Materials and Methods

All samples and entailing data sets have been acquired during both data collection sessions in Bangor [United Kingdom (UK)] in June 2008 and an international medical expedition (Hidden Valley Expedition 2008) in the Western Himalayan Mountains (Dhaulagiri Circuit, Nepal) in September 2008. Both were organized by Medical Expeditions. Data presented in this study originates from a pool shared by the cross-over study of Jennifer Landerer presenting complementary findings with respect to cytokine interaction during a high altitude (5050 m) sojourn.

4.1. Materials

4.1.1. Equipment

Sterican® 20-Gauge needle (B. Braun Melsungen AG, Melsungen, Deutschland), EDTA Vacutainer™ (BD, Franklin Lakes, New Jersey, USA), Heparin Vacutainer™ (BD, Franklin Lakes, New Jersey, USA), Eppendorf tubes of 1.5 ml (Eppendorf AG, Hamburg, Germany), pipette (Genex B) (Genex Laboratories, Torquay, UK), centrifuge (Eppendorf MiniSpin) (Eppendorf AG, Hamburg, Germany), pulse oximeter (Nonin Onyx 9500) (Nonin Medical Inc., Plymouth, Minnesota, USA), barometric pressure wrist watch (Suunto X3HR) (Suunto Oy, Vantaa, Finland), ratings of perceived exertion (Borg, 1982) and mood (Feeling scale -5/+5: Hardy and Rejeski, 1989), SigmaStat and SigmaPlot (Systat Software, Inc., San José, California, USA), heart rate monitor (Polar S120) (Polar, Kempele, Finland), Eppendorf Thermomixer comfort (Eppendorf AG, Hamburg, Germany), Luminex 100 (Luminex Corporation, Austin, Texas, USA), hemoglobin-photometer (HemoCue Plasma/Low Hb Photometer, Sweden/UK), centrifuge (HaematoSpin 1400, Hawksley, UK), hematocrit reader (Tube reader, Hawksley, UK), new Neubauer chamber.

4.1.2. Chemicals

20-plex Premixed Human Cytokine Milliplex Map Panel Immunoassay (Cat. # SPRPMX21) including EGF, FGF-2, G-CSF, GM-CSF, VEGF and TGF-β1 Single Plex Kit (Cat. # TGFB-64K-01), Human Cytokine / Chemokine Standard, Human Cytokine Quality Controls 1 and 2, Serum Matrix, Assay Buffer, Wash

Buffer, Human Cytokine Detection Antibodies, Streptavidin-Phycoerythrin (Merck Millipore, Billerica, Massachusetts, USA), and Sheath Fluid (Luminex).

4.2. Methods

4.2.1. Study Population

The study formed one of a series completed on the "Hidden Valley Expedition 2008" to Nepal. The study was approved by both the Coventry Ethics Committee and the Nepal Health Research Council, and all participants provided written informed consent.

All 26 subjects of the study were healthy moderately active expedition members. The differing total numbers of participants in the 3 groups are due to the fact that individual subjects voluntarily took part in either all groups or two or only one and due to the fact that some subjects only took part at sea level (69 m) but not at high altitude (5050 m) for differing reasons (e.g., exhaustion, illness). That is why for instance in group 2 as many as 10 subjects took part at sea level (69 m), but only 8 subjects at high altitude (5050 m). For further demographic data, see table 1.

Table 1 Demographic data

The table shows demographic data of expedition members who participated in the present study. Values are expressed as mean (standard deviation), except for gender distribution (absolute numbers).
M male, ***F*** female, ***BMI*** body mass index [body weight in kilograms per square meters (kg x m2)]

	Whole Group	Group 1 Resting	Group 2 Exercise	Group 3 Damphus Peak
Sex, M : F	16 : 10	15 : 10	5 : 3	4
Age, years	33.1 (± 11.74)	32.8 (± 11.33)	32.8 (± 8.44)	26.0 (± 3.91)
BMI, kg x m2	23.33 (± 12.90)	22.48 (± 13.57)	27.12 (± 14.43)	14.25 (± 11.78)

4.2.2. Study Design

The study shows a non-randomized, non-blinded design with two parallel groups and an additional third group. For the explanation for the differing total numbers of participants in the 3 groups see "4.2.1. Study Population". Further detail is shown in table 2.

Table 2 Number of participants in the 3 groups studied

The table shows the distribution of participants and the strength of the different groups both at sea level (69 m) and high altitude (5050 m). Values are expressed as absolute numbers of participants.

	Whole Group	Group 1 Resting	Group 2 Exercise	Group 3 Damphus Peak
Total	26	25	10	4
Sea level (69 m)	25	25	10	-
High altitude (5050 m)	18	18	8	4

Group 1 members represent the reference group at rest. This group was comprised of 25 participants at sea level (69 m) and 18 at high altitude (5050 m).

Group 2 members underwent an incremental exercise test and totaled to 10 at sea level (69 m) and 8 at high altitude (5050 m).

In addition, a third group of 4 participants underwent a one-day climb from Hidden Valley Base Camp (5050 m) to Damphus Peak (6035 m) and descending back to Hidden Valley Base Camp.

The difference in altitude from ascent to Damphus Peak (6035 m) to descent to Base Camp (5050 m) totaled to 1970 meters.

In accordance with ethical approval from Coventry University Ethics Committee, participants gave their written informed consent to participate in the study in order to verify that they were fully informed of the study's requirement and had been notified of and understood the possible risks. In addition, participants completed a health screen questionnaire prior to conducting each of the two trials.

It has to be taken into account that subjects may or may not have participated in other double-blinded studies of which one applied carbohydrate supplementation and the other inhaled Iloprost.

4.2.3. Inclusion and Exclusion Criteria

Inclusion criteria were normal hemoglobin and hematocrit values (quantified prior to commencing the expedition). Subjects were also asked to refrain from consuming food for at least 2-3 hours prior to exercise. All subjects were non-smokers over the age of 18 years.

Exclusion criteria were a history of cardiovascular, respiratory, nervous, renal, liver, skeletal/muscular and metabolic disease. Furthermore, none of the participants suffered from immunosuppression

from HIV, was on any regular medication, or had suffered recently an allergic reaction. Other medical conditions were well controlled and individuals were free of symptoms at the time of the expedition's departure.

4.2.4. Data Collection Protocol

The study consisted of two trials of data collection. The first trial was conducted at Bangor University, UK in July/August 2008 [69 m, barometric oxygen pressure ($p0_2$) 760 mmHg] and the second trial took place at Hidden Valley Base Camp, Nepal in October 2008 (5050 m, $p0_2$ 550 mmHg).

4.2.5. Expedition Itinerary

The main route of ascent corresponded to the Dhaulagiri Circuit in the Western part of the Nepalese Himalayas. On the way to Hidden Valley Base Camp at 5050 m expedition members were exposed to various climatic conditions such as hot weather zones, humid subtropical climate, monsoon rain, and finally a permafrost zone above 3000 m. Expedition members were divided into four trekking groups, each of which departed on a different day. While figure 3 shows the complete expedition itinerary, figure 4 shows the corresponding altitude profile. For starting and end points of the expedition itinerary see table 7 in the appendix.

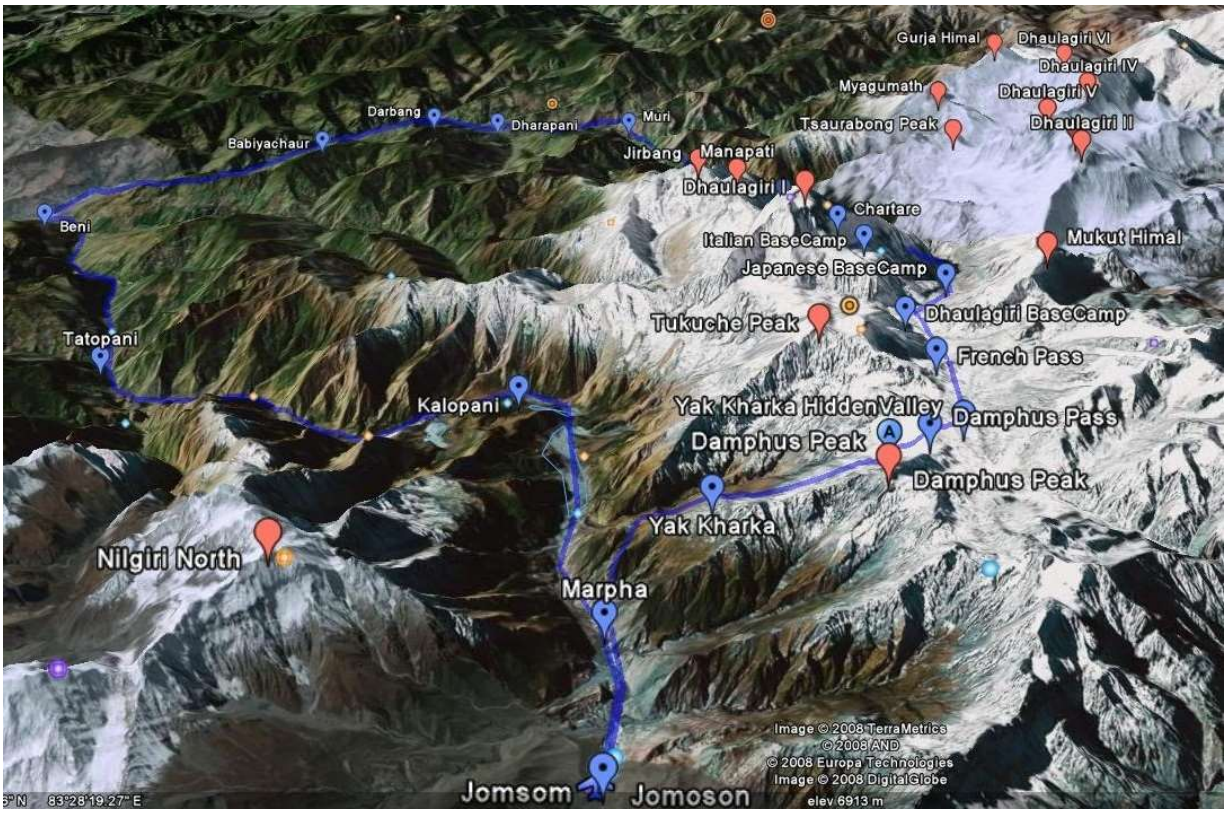

Figure 3 Expedition itinerary

The expedition itinerary is outlined in blue and surrounding peaks in red. Locations titled do not correspond to starting and end points of each expedition day. [111]

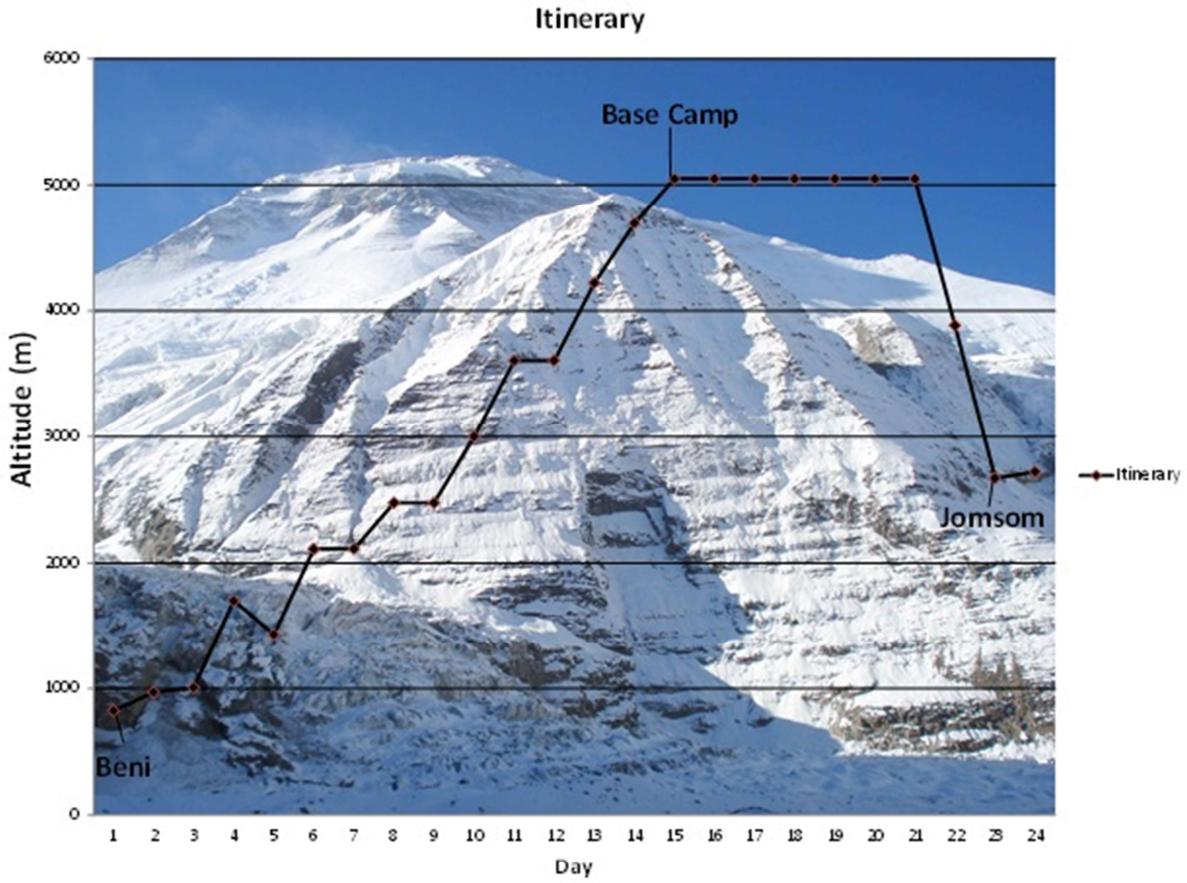

Figure 4 Altitude profile

The expedition in the Nepalese Himalayas started at Beni (826 m), went via the Dhaulagiri Circuit for 14 days in order to arrive at Hidden Valley Base Camp (5050 m). After 6 days of research the expedition went on and ended in Jomsom (2720 m).

4.2.6. Collection of Blood Samples

4.2.6.1. Set-up

Baseline resting blood samples were taken from a population of 25 participants (10 women, 15 men) in Bangor (UK, 69 m) and of 18 participants (7 women, 11 men) in Hidden Valley Base Camp (Nepal, 5050 m) who were all overnight fasted (minimum 8 hours fast). Participants remained in a seated position for 15 minutes prior to venipuncture from an antecubital vein by means of a 20-Gauge needle. A total amount of 6 ml blood was distributed into 1 EDTA and 1 Heparin Vacutainer^{TM} of 3 ml each.

Subjects who underwent an incremental step test were comprised of a population of 8 participants in Bangor (69 m) and 8 participants in Hidden Valley Base Camp (5050 m). Participants were fasted at least for 2-3 hours prior to the first and remained fasted until the last phlebotomy.
After the first phlebotomy an incremental step test was undertaken. Each participant stepped on and off a 25 centimeter metal step over a period of twelve minutes in total subdivided into three intervals of four minutes each. An acoustic beeping signal indicated the step-frequency which was for the first interval 13 steps per minute, for the second 26 steps per minute, and with initiation of the third interval the beeping signal was turned off and the subject was asked to apply a step-frequency as high as possible. One entire stepping cycle consisted of putting one foot on the step with the first beep, followed by the second foot with the second beep, so that the participant was standing with both feet on the step. With beep three and four, the feet were brought down in the same fashion one by one. Every minute the leading foot was changed in order to prevent exhaustion of one particular lower limb. Immediately prior to and during exercise heart rate (Polar S120, Polar, Kempele, Finland), ratings of perceived exertion and mood (Feeling scale -5/+5) were recorded at minute intervals. Four more blood samples were taken directly after, 15 min, 90 min, and 180 min after cessation of exercise. A total of 30 ml of blood was collected per participant.
A supplementary third group was formed by four subjects (all male) who undertook a one-day climb from Hidden Valley Base Camp (5050 m) to Damphus Peak (6035 m). After an overnight fast, blood samples were taken from participants at 03:30 a.m. (local time) prior to ascent and directly after return from the Damphus Peak summit. During their climb participants did not remain fasted. Meters covered on ascent and descent totaled to 1970. Duration of ascent and descent averaged to 12 hours (from 03:30 a.m. until 3:30 p.m.).

4.2.6.2. Processing of Blood Samples

Immediately after venipuncture the utilized EDTA Vacutainer™ was aliquoted into 2 Eppendorf tubes of 1.5 ml each by means of a pipette (Genex B). Same procedure was applied to the utilized Heparin Vacutainer™. Within 30 minutes after venipuncture Eppendorf tubes were spun in a centrifuge (Eppendorf MiniSpin) for 10 minutes at sea level (69 m) and for 5 minutes at high altitude (5050 m) at a relative centrifugal force of 12,000. Afterwards, plasma from the 2 EDTA and 2 Heparin aliquots was distributed to 6 and 2 Eppendorf tubes of 0.5ml each, respectively.

4.2.6.3. Storage and Transport of Blood Samples

After processing, blood samples were sealed in plastic bags and kept at -26° Celsius (C) in a freezer. At Hidden Valley Base Camp (Nepal, 5050 m), the portable freezer was insulated with a styrofoam case and cooled via a gas cartridge on the day of descent. Transportation of the freezer by foot to the nearest town with electrical supply for cooling took 8 hours. During transportation a remote sensor in the freezer allowed for real time temperature readings and re-cooling via a gas cartridge provided stable temperatures of at least -10° C or below. Subsequently, samples were transported by plane from Nepal to the UK, where samples from the first trial were added and finally sent via air mail to the laboratory in Homburg (Germany).

4.2.7. Immunoassay

In order to analyze specimen Luminex xMAP Multiplexing Technology was employed. Following growth factors were measured simultaneously: VEGF, EGF, FGF-2, TGF-β1, G-CSF, GM-CSF.
In cooperation with the University of Gießen, courtesy of the laboratory of Prof. Dr. Blum, IGF-1, IGFBP-1, IGFBP-3 were analyzed by means of a radio immunoassay (RIA) [112]. In RIA, the analyte (antigen) is measured by coupling antibodies specific for the antigen to a test tube. In a second step, the patient serum containing the antigen is added and antibodies bind the antigen. Non-bound antigen is subsequently removed by washing. In a further step, radiolabeled antibodies specific to the antigen are added. After washing, radioactivity is determined. [113, 114]

4.2.7.1. Luminex xMAP Multiplexing Technology

Luminex allows simultaneous analysis of exact quantities of a large number of small-volume samples. It is a highly specific, sensitive, and reliable assay. [115, 116]
Luminex is based on flow cytometry and employs as the key principle the fluorescent color coding of microspheres (polystyrene particles) allowing their identification and precise correlation with their respective population through optical analysis. Through combination of red and infrared fluorescent dyes 100 (10^2) different fluorescent color tones are created. One unique bead class is made up by one microsphere dyed with one of the 100 different fluorescent color tones and its specific capture antibody. Specific reaction partners (analytes) bind to the microsphere´s capture antibody. The resulting complex (bead + capture antibody + analyte) is recognized by a detection antibody and subsequently labeled with so-called reporter molecules. The median fluorescence intensity of the

reporter molecule is then used to quantify the amount of analyte bound to the bead. In this manner, a single sample of a volume as small as 25 µl can be incubated with up to 100 different bead classes at the same time on a 96-well-microtiter plate.

After the binding reaction is completed, microspheres are suctioned from the microtiter plate. While two lasers are directed at the particle stream, mirrors direct fluorescence discriminator signals emitted from microspheres and detection signals emitted from reporter molecules to detectors. Figure 5 shows the set-up of the bead (microsphere) technology. Photodiodes classify the microspheres and detect side scatter signals of the particles, which allows determination of the particle size and exclusion of fluorescent contaminants or microsphere aggregates (e.g., doublets) from the results, respectively. A special laser excites the fluorescent dye of the reporter molecules and the intensity of the emitted light allows for quantification of the analyte bound to a particular microsphere. Finally, optical signals are translated into digital signals. With the Luminex analysis system, up to 20,000 events per second can be amplified, processed and recorded. [117]

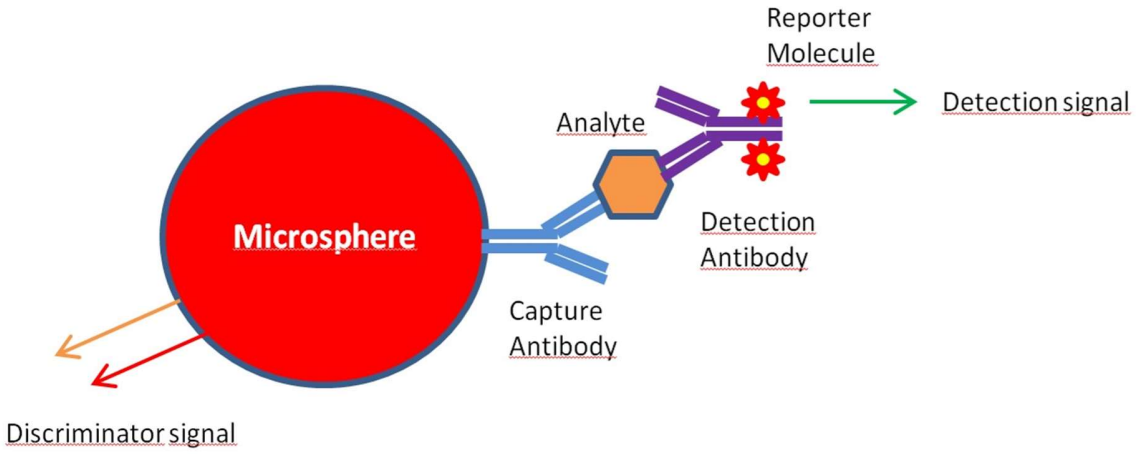

Figure 5 The bead (microsphere) technology of Luminex

Luminex employs as the key principle the fluorescent color coding of microspheres (polystyrene particles) allowing their identification and precise correlation with their respective population through optical analysis. One unique bead class is made up by one microsphere dyed with one of the 100 different fluorescent color tones and its specific capture antibody. Specific reaction partners (analytes) bind to the microsphere´s capture antibody. The resulting complex (bead + capture antibody + analyte) is recognized by a detection antibody and subsequently labeled with a so-called reporter molecule. While two lasers are directed at the particle stream, mirrors direct fluorescence discriminator signals emitted from microspheres and detection signals emitted from reporter molecules to detectors. Photodiodes classify the microspheres and detect side scatter signals of the particles. The median fluorescence intensity of the reporter molecule is then used to quantify the amount of analyte bound to the bead. Optical signals are finally translated into digital signals. [117]

4.2.7.2. Assay

Before analysis, samples were prepared as recommended by the manufacturer's instructions (see figure 6). Premixed antibody-immobilized beads were sonicated for 30 seconds and vortexed for 1 minute. Quality controls 1 and 2 were generated by adding 250 µl deionized water, mixing and vortexing the vials (rest before use: 5-10 min) and pipetting them into test tubes. At room temperature 30 ml of wash buffer were diluted with 270 ml deionized water. 1.0 ml of deionized water was mixed into the bottle containing lyophilized Serum Matrix (rest before use: 10 min). Adding 250 µl deionized water resulted in human cytokine standard (10,000 pg/ml). Working standards were generated by serial dilutions of the standard dilution (10,000 pg/ml). Five test tubes were filled with 200 µl of Assay Buffer (0 pg/ml). 50 µl of the 10,000 pg/ml standard was added to tube 1 (= 2000 pg/ml). 50 µl of tube 1 was added to tube 2 (= 400 pg/ml). 50 µl of tube 2 was added

to tube 3 (= 80 pg/ml). 50 µl of tube 3 was added to tube 4 (= 16 pg/ml). 50 µl of tube 4 was added to tube 5 (= 3.2 pg/ml).

Standard Concentration (pg/mL)	Volume of Deionized Water to Add	Volume of Standard to Add
10,000	250 µL	0

Standard Concentration (pg/mL)	Volume of Assay Buffer to Add	Volume of Standard to Add
2000	200 µL	50 µL of 10,000 pg/mL
400	200 µL	50 µL of 2000 pg/mL
80	200 µL	50 µL of 400 pg/mL
16	200 µL	50 µL of 80 pg/mL
3.2	200 µL	50 µL of 16 pg/mL

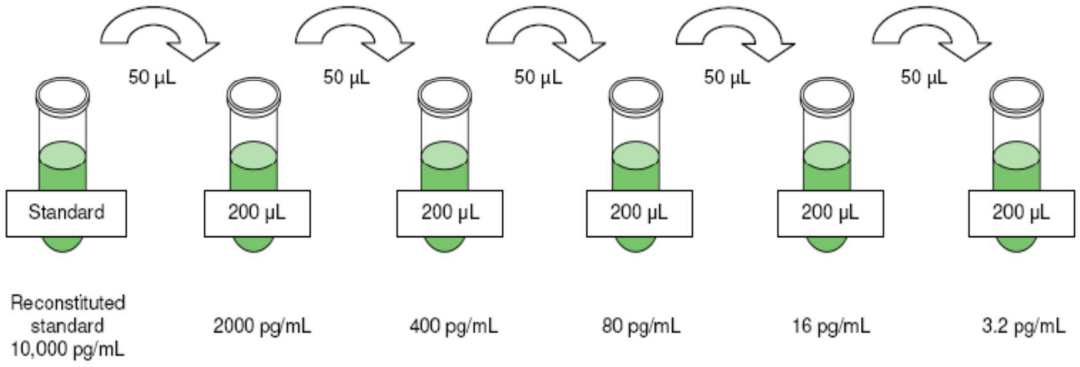

Figure 6 Assay preparation

Before analysis, samples were prepared as recommended by the manufacturer's instructions. 1.0 ml of deionized water was mixed into the bottle containing lyophilized Serum Matrix and 250 µl deionized water was added which resulted in the human cytokine standard (10,000 pg/ml). Working standards were generated by serial dilutions of the standard dilution (10,000 pg/ml). Five test tubes were filled with 200 µl of Assay Buffer (0 pg/ml). 50 µl of the 10,000 pg/ml standard was added to tube 1 (= 2000 pg/ml). 50 µl of tube 1 was added to tube 2 (= 400 pg/ml). 50 µl of tube 2 was added to tube 3 (= 80 pg/ml). 50 µl of tube 3 was added to tube 4 (= 16 pg/ml). 50 µl of tube 4 was added to tube 5 (= 3.2 pg/ml). [118]

pg pictogram, ***ml*** milliliter, ***µl*** microliter

After adding 200 µl of Assay Buffer into each of the 96 wells the microtiter plate was sealed and mixed on a plate shaker (Eppendorf Thermomixer comfort) at 500 revolutions per minute (rpm) for 10 minutes at room temperature (20-25° C). Assay Buffer was then removed and 25 µl of each

Standard and Quality Control solution were pipetted into the respective wells. Assay Buffer was used for the 0 pg/ml standard (background). Each sample well was filled with 25 µl of Assay Buffer and 25 µl of Serum Matrix solution was added to each of the Background, Standards, and Quality Control wells. Eventually, 25 µl of each sample was pipetted into the appropriate wells. The bottle containing the premixed beads was vortexed and 25 µl of the bead mix were added to each well.
After incubation (60 min at 20-25° C) on the plate shaker the fluid phase was removed from the microtiter plate and each well was washed 2 times with 200 µl of Wash Buffer.
Before a second incubation process (30 min at 20-25° C) began, 25 µl of Detection Antibodies were added to each well. After pipetting 25 µl of Streptavidin R-Phycoerythrin into each well, a third incubation period (30 min at 20-25° C) on the plate shaker began. All fluid was removed from the microtiter plate and each well was washed 2 times with 200 µl of Wash Buffer. Each well was then filled with 150 µl of Sheath Fluid, the plate was put on a plate shaker for 5 minutes, and ultimately inserted into the Luminex 100™ IS. The Median Fluorescent Intensity (MFI) data was saved and growth factor concentrations in samples were calculated.

TGF-β1

The assay for TGF-β1 equals to the assay mentioned above. In the following chapter only alterations from the protocol are portrayed:
Quality controls 1 and 2 were reconstituted with 250 µl Assay Buffer and mixed and vortexed (rest before use: 5-10 min).
Wash Buffer was brought to room temperature and mixed until salts were brought into solution. 30 ml of Wash Buffer was diluted with 270 ml deionized water.
1.0 ml of deionized water and 4.0 ml of Assay Buffer were added to the bottle containing lyophilized Serum Matrix (rest before use: 10 min) and mixed. Of this mixture 0.5 ml were added to 0.5 ml Assay Buffer in order to get the final dilution of 1:10 of the working Serum Matrix used for Standards and Controls.
Samples were centrifuged and 1 part of sample was diluted with 4 parts of Sample Diluent (e.g., 20 µl sample and 80 µl Cat. # LTGF-SD) (dilution 1:5). To each 25 µl of the 1:5 diluted samples 2.0 µl of 1.0 N HCl was added in order to have the pH drop below 3.0. After mixing, acidified samples were shaken at room temperature for 15 min and finally diluted with Assay Buffer (Cat. # L-MAB) in a 1:6

ratio in order to get a final sample dilution of 1:30. These diluted samples were then immediately added to the sample wells.

For the preparation of TGF-β1 standard 250 µl Assay Buffer was added (= 10,000 pg/ml), mixed, and vortexed for 10 seconds (rest before use: 5-10 min).

Working standards of 2500, 625, 156, 39, and 9.8 pg/ml were generated by adding 150 µl of Assay Buffer to five tubes and carrying out serial dilutions as described above.

After preparation of the wells as mentioned earlier, the plate was prepared for incubation on a plate shaker at 20-25° C. After two incubation periods (2 h and 1 h, respectively) all fluid contents were removed, each well was filled with 100 µl of Sheath Fluid, and the plate was put on the plate shaker for another 5 minutes. Final steps were equal to the ones of the protocol mentioned above.

For well maps of both assays see figures 10 and 11 in the appendix.

4.2.7.3. Software

Data was analyzed using Luminex IS 2.3 and both MFI and calculated concentration values were reported for each analyte.

4.2.8. Statistical Method

Statistical analysis was performed using SigmaStat 3.1 (Systat Software, Point Richmond, CA, USA). Differences between sea level (69 m) and high altitude (5050 m) values as well as between pre-exercise and post-exercise values were analyzed using the Wilcoxon test. Values of $p < 0.05$ were considered statistically significant. Results are presented as median (Min; Max). 3.2 pg/ml, the lowest dilution on the standard curve, was considered to be the lower limit of detection.

4.2.9. Additional Data

While leucocyte counts were obtained by means of microscopic visualization in a new Neubauer chamber, data about differential counts was provided kindly by the laboratory of Heartlands hospital (Birmingham, UK).

Hemoglobin content of blood samples was measured by means of a hemoglobin-photometer (HemoCue Plasma/Low Hb Photometer). By means of a centrifuge (HaematoSpin 1400) and a hematocrit reader (Tube reader), hematocrit measurements were obtained.

Data sets from the participants´ data collection booklets were provided by the University of Bangor (Wales, UK). Data include cold symptoms, Bristol stool scale, resting heart rate, arterial oxygen saturation, barometric pressure, and the Lake Louise score (LLS).

Cold symptoms in the last 24 hours were reported by the participant in person in the evenings throughout the entire sojourn. For each of the following items participants gave subjective score points with 0 = none, 1 = mild, 2 = moderate, and 3 = severe: sneezing, runny nose, blocked nose, sore throat, cough, chilliness, and malaise.

Gastrointestinal voiding was self-reported on a daily basis by employing the Bristol Stool Scale (number of motions per day and consistency of motions on a 1–7-point Likert scale) [119].

Resting heart rate and arterial oxygen saturation was recorded both in the mornings while fasted and seated for 10 minutes and in the evenings while seated for 10 minutes by means of a pulse oximeter (Nonin Onyx 9500). Barometric pressure was read from a wrist watch (Suunto X3HR).

The LLS was filled in by the participant in person in the mornings throughout the entire sojourn. For each of the following five symptoms participants gave subjective score points in increasing severity from 0 to 3: headache, gut symptoms, fatigue/weakness, dizzy/lightheaded, difficulty sleeping (see table 3).

Table 3 Lake Louise score

The Lake Louise score uses five symptoms, each scoring from 0 to 3 with increasing severity in order to detect AMS [120].

Score	Headache	Gastrointestinal symptoms	Fatigue/ Weakness	Dizziness/ Lightheadedness	Difficulty Sleeping
0	None	None	None	None	Slept as well as usual
1	Mild	Poor appetite or nausea	Mild	Mild	Did not sleep as well as usual
2	Moderate	Moderate nausea or vomiting	Moderate	Moderate	Woke many times, poor night's sleep
3	Severe	Severe nausea and vomiting, incapacitating	Severe	Severe	Could not sleep at all

The diagnosis of AMS is based on a rise in altitude within the last four days and the application of the scoring system according to the LLS. In order to diagnose AMS, headache is obligatory and at least one other symptom of the LLS has to be present (gastrointestinal symptoms, fatigue/weakness, dizziness/lightheadedness, difficulty sleeping). Finally, a total score of 3 or more from the questionnaire confirms the diagnosis of AMS. 3 to 5 points mean mild AMS and scores of 6 and above signify severe AMS. [120, 121]

5. Results

In the following sections results are presented according to the three sets of points of time of data collection compared. At first, data from resting subjects was analyzed and sea level (69 m) values were related to high altitude (5050 m) values. Furthermore, data from subjects who undertook a standardized incremental exercise test was utilized to check values before exercise against values 180 minutes after cessation of exercise. At last, data obtained from a one-day climb totaling to an altitude difference of 1970 meters was evaluated to compare values before ascent with values after descent.

As the major finding, this study found that growth factors showed significant changes predominantly when comparing sea level (69 m) values with high altitude (5050 m) values in resting subjects. There was no major impact on growth factor levels through exercise both at sea level (69 m) and at high altitude (5050 m).

5.1. Group 1 – Resting at Sea Level (69 m) versus High Altitude (5050 m)

Table 4 shows data from participants that were at rest [25 at sea level (69 m) and 18 at high altitude (5050 m)].

Table 4 Growth factors, differential counts, and additional data of resting subjects at sea level (69 m) versus high altitude (5050 m)

Median (Min; Max) rounded values of 25 subjects are shown [25 at sea level (69 m) and 18 at high altitude (5050 m)]

G-CSF granulocyte colony stimulating factor, ***GM-CSF*** granulocyte macrophage colony stimulating factor, ***EGF*** epidermal growth factor, ***VEGF*** vascular endothelial growth factor, ***TGF-β1*** transforming growth factor β 1, ***FGF-2*** fibroblast growth factor 2, ***IGF-1*** insulin-like growth factor 1, ***IGFBP-1*** insulin-like growth factor binding protein 1, ***IGFBP-3*** insulin-like growth factor binding protein 3, ***O_2*** oxygen, ***pO_2*** barometric oxygen pressure, ***Hb*** hemoglobin, ***Hct*** hematocrit, ***BMI*** body mass index, ***NS*** not significant, ***NA*** not available, ↑ increase, ↓ decrease, ***p*** p-value

Variable (units)	Sea Level (69 m)	High Altitude (5050 m)	p	↑/↓
G-CSF (pg/ml)	35.5 (14.8; 77.2)	50.3 (21.9; 161.3)	**0.029**	↑
GM-CSF (pg/ml)	3.2 (1.9; 50.0)	4.0 (1.9; 48.8)	**0.035**	↑
EGF (pg/ml)	5.8 (2.9; 138.5)	10.7 (3.2; 2047.2)	**0.002**	↑
VEGF (pg/ml)	160.5 (11.4; 616.1)	362.5 (11.4; 1520.2)	**0.039**	↑
TGF-β1 (pg/ml)	2566.8 (310.8; 9265.2)	17116.5 (294.0; 75043.2)	**< 0.001**	↑
FGF-2 (pg/ml)	98.9 (3.2; 1057.4)	98.9 (3.2; 1222.2)	**NS**	↑
IGF-1 (µg/l)	167 (96.3; 360)	130 (71.8; 212)	**< 0.001**	↓
IGFBP-1 (ng/ml)	8.5 (1.8; 72.7)	9.3 (1.9; 46.1)	**NS**	↑
IGFBP-3 (mg/l)	2.6 (1.6; 4.2)	2.1 (0.8; 3.6)	**< 0.001**	↓
Leucocytes/µl	3050.0 (1150.0; 7050.0)	3800.0 (1150.0; 10850.0)	**NS**	↑
Lymphocytes (%)	51.0 (20.0; 72.0)	39.0 (16.0; 64.0)	**0.048**	↓
Monocytes (%)	3.0 (0.0; 7.0)	4.0 (0.0; 8.0)	**NS**	↑
Neutrophils (%)	45.0 (23.0; 73.0)	56.0 (36.0; 81.0)	**0.022**	↑
Eosinophils (%)	3.0 (0.0; 12.0)	1.0 (1.0; 4.0)	**NS**	↓
Basophils (%)	3.0 (3.0; 3.0)	NA	**NA**	**NA**
O_2 Saturation (%)	96.0 (74.5; 98.5)	80.0 (61.0; 96.0)	**< 0.001**	↓
pO_2 (mmHg)	760.0 (760.0; 760.0)	554.0 (554.0; 555.0)	**< 0.001**	↓
Hb (g/l)	140.0 (95.0; 169.0)	159.5 (120.0; 196.0)	**< 0.001**	↑
Hct (%)	40.0 (29.0; 49.0)	44.3 (36.3; 53.3)	**NS**	↑
BMI	23.3 (19.0; 28.7)	22.9 (18.7; 34.5)	**NS**	↑

In subjects that were at rest (group 1), significant changes occurred when comparing sea level (69 m) data with values from 5050 m. Factors affected by these changes included G-CSF, GM-CSF, EGF, VEGF, TGF-β1, IGF-1, and IGFBP-3. However, no significant changes were observed in FGF-2 and IGFBP-1. Analysis of differential counts showed significant changes in neutrophils and lymphocytes. Not significantly altered were leucocytes, monocytes, and eosinophils. No information could be obtained

about basophil levels because they were below detection ranges. Additionally, O_2 saturation and partial oxygen pressure along with hemoglobin counts did show significant changes. However, neither BMI nor hematocrit changed significantly.

In resting subjects, a generalized rise of most of the growth factors was observed at high altitude (5050 m) when compared to values at sea level (69 m). Of the vascular growth factors investigated, EGF ($p = 0.002$), VEGF ($p = 0.039$), and TGF-β1 ($p < 0.001$) showed a significant rise at high altitude (5050 m). Although FGF-2 levels decreased at high altitude (5050 m) the changes were not of statistical significance. Levels of hematological growth factors G-CSF ($p = 0.029$) and GM-CSF ($p = 0.035$) were significantly higher than at sea level (69 m). Of the insulin axis, IGF-1 ($p < 0.001$) and IGFBP-3 ($p < 0.001$) showed a significant decrease at high altitude (5050 m), while IGFBP-1 levels showed a nonsignificant increase when compared to sea level (69 m) values. Analysis of differential blood counts produced significant changes shown by a decrease in lymphocytes ($p = 0.048$) and an increase in neutrophil counts ($p = 0.022$) at high altitude (5050 m). Finally, significant results of additional data include a decrease in partial oxygen pressure ($p < 0.001$) and oxygen saturation ($p < 0.001$), while hemoglobin levels were significantly elevated ($p < 0.001$) at high altitude (5050 m). Table 4 presents all statistically significant data in group 1 subjects. For corresponding box plots see figures 12 to 23 in the appendix.

5.2. Group 2 – Exercise at Sea Level (69 m) versus High Altitude (5050 m)

Table 5 shows all data of subjects who underwent an incremental exercise step test both at sea level (69 m) and at high altitude (5050 m).

Table 5 Growth factors, differential counts, and additional data of exercising subjects at sea level (69 m) and high altitude (5050 m)

Median (Min; Max) rounded values of 8 subjects are shown.

G-CSF granulocytes colony stimulating factor, ***GM-CSF*** granulocyte macrophage colony stimulating factor, ***VEGF*** vascular endothelial growth factor, ***FGF-2*** fibroblast growth factor 2, ***TGF-β1*** transforming growth factor β 1, ***EGF*** epidermal growth factor, ***IGF-1*** insulin-like growth factor 1, ***IGFBP-1*** insulin-like growth factor binding protein 1, ***IGFBP-3*** insulin-like growth factor binding protein 3, ***O_2*** oxygen, ***pO_2*** barometric oxygen pressure, ***Hb*** hemoglobin, ***Hct*** hematocrit, ***BMI*** body mass index, ***NS*** not significant, ***NA*** not available, ↑ increase, ↓ decrease, ***p*** p-value

Variable (units)	Sea level (69 m)				High altitude (5050 m)			
	Pre	180 min post	p	↑/↓	Pre	180 min post	P	↑/↓
G-CSF (pg/ml)	35.5 (14.8; 74.1)	30.5 (21.9; 71.0)	NS	↓	35.5 (25.4; 74.1)	41.3 (21.9; 154.0)	NS	↑
GM-CSF (pg/ml)	3.2 (3.2; 50.0)	3.2 (3.2; 80.1)	NS	↑	3.2 (3.2; 32.2)	3.2 (1.9; 40.2)	NS	↑
VEGF (pg/ml)	141.9 (16.0; 431.2)	122.8 (16.0; 520.4)	NS	↓	219.9 (16.0; 1520.2)	91.7 (16.0; 1101.3)	NS	↓
FGF-2 (pg/ml)	57.5 (3.2; 415.4)	114.5 (47.8; 372.5)	NS	↑	120.2 (63.7; 270.5)	94.7 (41.5; 181.7)	NS	↓
TGF-β1 (pg/ml)	2908.4 (762.0; 8755.2)	2050.8 (294.0; 7922.1)	NS	↓	9630.9 (2702.7; 17907.3)	2398.7 (678.3; 8299.2)	NS	↓
EGF (pg/ml)	3.9 (3.2; 18.0)	3.2 (3.2; 15.5)	0.031	↓	4.1 (3.2; 25.9)	3.2 (3.2; 21.9)	NS	↑
IGF-1 (µg/l)	148.5 (102.0; 182.0)	139.5 (107.0; 209.0)	NS	↓	143.0 (91.9; 174.0)	144.5 (91.3; 211.0)	NS	↑
IGFBP-1 (ng/ml)	8.7 (1.8; 28.0)	13.2 (3.0; 32.0)	NS	↑	8.5 (1.9; 37.3)	8.1 (4.1; 30.3)	NS	↓
IGFBP-3 (mg/l)	2.3 (1.6; 4.0)	2.9 (1.9; 3.7)	NS	↑	2.1 (1.5; 3.6)	2.5 (1.6; 4.8)	NS	↑
Leucocytes/µl	3800.0 (2400.0; 6600.0)	6450.0 (4350.0; 9750.0)	0.016	↑	4125.0 (3400.0; 5400.0)	7325.0 (5900.0; 16400.0)	0.008	↑
Lymphocytes (%)	44.0 (20.0; 72.0)	42.5 (14.0; 49.0)	NS	↓	45.5 (30.0; 60.0)	37.0 (32.0; 64.0)	NS	↓
Monocytes (%)	3.5 (0.0; 5.0)	4.0 (0.0; 12.0)	NS	↑	2.5 (1.0; 9.0)	2.0 (1.0; 4.0)	NS	↓

Table 5 continued

Variable (units)	Sea level (69 m)				High altitude (5050 m)			
	Pre	**180 min post**	**p**	**↑/↓**	**Pre**	**180 min post**	**P**	**↑/↓**
Neutrophils (%)	5.0 (23.0; 65.0)	54.0 (35.0; 81.0)	NS	↑	52.5 (35.0; 59.0)	60.0 (34.0; 68.0)	NS	↑
Eosinophils (%)	2.0 (0.0; 12.0)	1.5 (1.0; 5.0)	NS	↓	2.0 (1.0; 4.0)	1.0 (1.0; 1.0)	NS	↓
Basophils (%)	NA	NA	NA	NA	NA	NA	NA	NA
O_2 Saturation (%)	95.8 (94.5; 97.5)		NA	NA	86.3 (75.0; 96.0)		NA	NA
pO_2 (mmHg)	760.0 (760.0; 760.0)		NA	NA	553.0 (553.0; 555.0)		NA	NA
HB (g/l)	144.0 (97.0; 159.0)	151.0 (103.0; 157.0)	NS	↑	166.0 (152.0; 184.0)	179.5 (150.0; 183.0)	NS	↑
Hct (%)	42.8 (31:0; 45:0)	40.5 (38.0; 44.0)	NS	↓	48.5 (45.5; 51.5)	NA	NA	NA
BMI	23.6 (22.6; 24.8)		NA	NA	24.7 (21.4; 34.5)		NA	NA
AMS	0.0 (0.0; 0.0)		NA	NA	0.0 (0.0; 0.0)		NA	NA

Two factors measured in exercising subjects presented values of statistical significance. While this study could show a significant decrease after 180 minutes after exercise at sea level (69 m) for EGF, it could not reach a significant magnitude at high altitude (5050 m). Secondly, the leucocyte count showed a significant increase 180 minutes after exercise both at sea level (69 m) and high altitude (5050 m).

5.2.1. Sea Level (69 m) – Pre versus 180 Minutes Post Exercise

Significant changes occurred in EGF (p = 0.031) and leucocytes (p = 0.016). Median (Min; Max) rounded values for EGF were 3.9 (3.2; 18.0) pre exercise and 3.2 (3.2; 15.5) 180 minutes after exercise (see figure 7). Leucocytes showed a median increase from 3800.0 (2400.0; 6600.0) to 6450.0 (4350.0; 9750.0) 180 minutes after exercise (see figure 8).

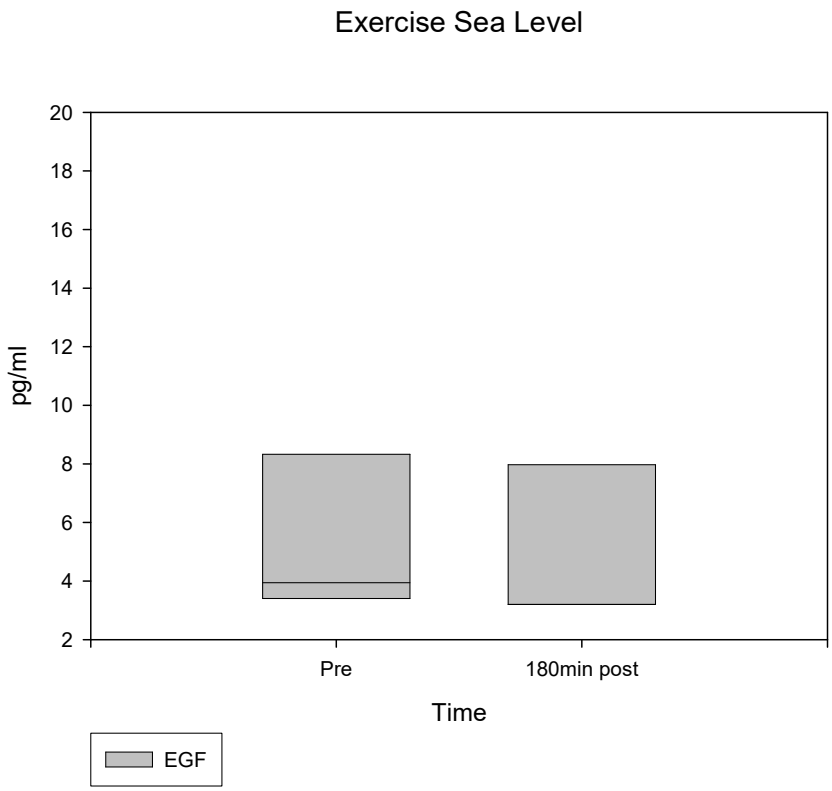

Figure 7 EGF – Exercise at sea level (69 m)

EGF levels changed significantly (p = 0.031) at sea level when levels before exercise 3.9 (3.2; 18.0) were compared to levels 180 minutes after exercise 3.2 (3.2; 15.5) [Median (Min; Max) rounded values].

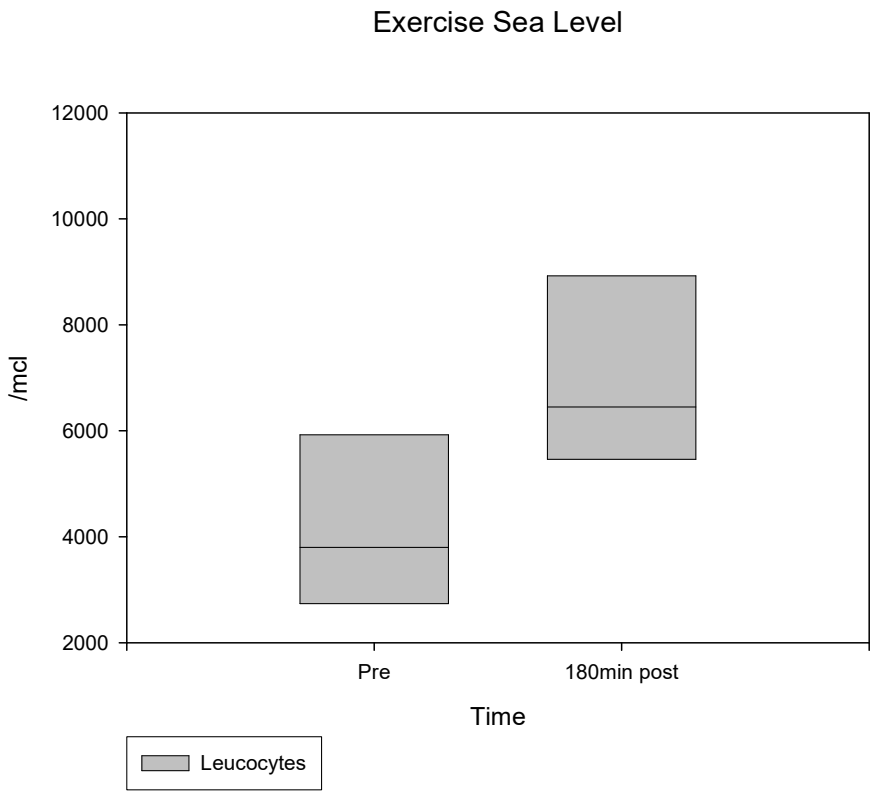

Figure 8 Leucocytes – Exercise at sea level (69 m)

Leucocyte levels changed significantly (p = 0.016) at sea level when levels before exercise 3800.0 (2400.0; 6600.0) were compared to levels 180 minutes after exercise 6450.0 (4350.0; 9750.0) [Median (Min; Max) rounded values].

Nonsignificant changes of growth factors at sea level (69 m) in exercising subjects are shown in the synoptical table 5.

5.2.2. High Altitude (5050 m) – Pre versus 180 Minutes Post Exercise

At high altitude (5050 m) no significant changes occurred in growth factors when levels before exercise were compared with levels 180 minutes after cessation of exercise (see table 5). However, leucocytes showed significant changes (p = 0.008) with median (Min; Max) values of 4125.0 (3400.0; 5400.0) pre and 7325.0 (5900.0; 16400.0) 180 minutes post exercise (see figure 9).

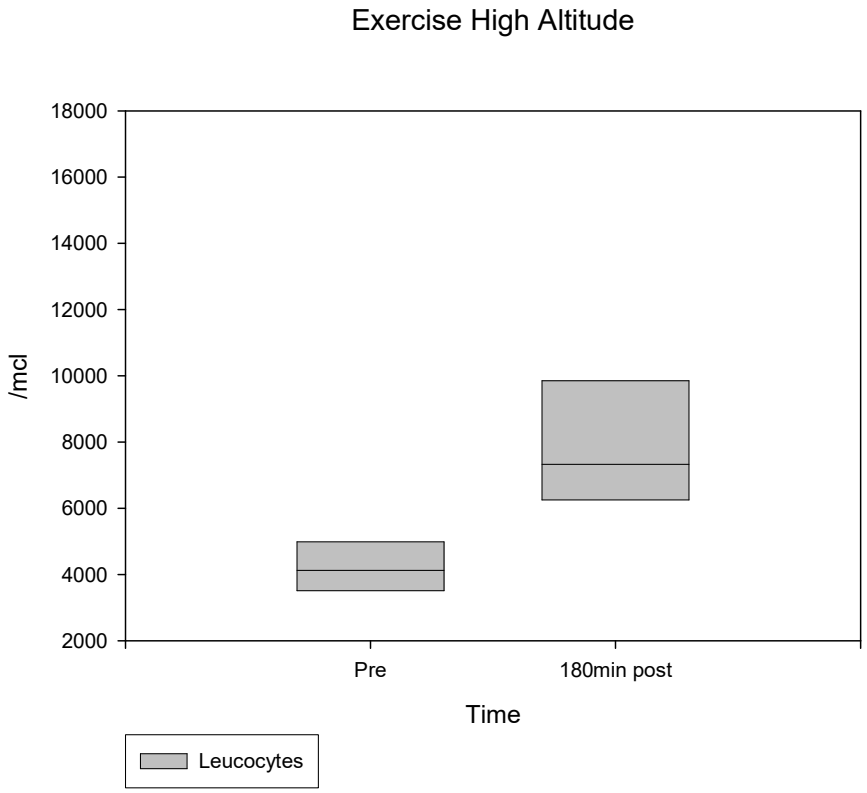

Figure 9 Leucocytes – Exercise at high altitude (5050 m)

Leucocyte levels changed significantly (p = 0.008) at sea level when levels before exercise 4125.0 (3400.0; 5400.0) were compared to levels 180 minutes after exercise 7325.0 (5900.0; 16400.0) [Median (Min; Max) rounded values].

5.3. Group 3 – Damphus Peak (6035 m)

No significant changes, neither in growth factors, nor in differential counts nor in additional data, could be shown after a bout of intense exercise while climbing Damphus Peak at 6035 m (see table 6). The difference in altitude from ascent to descent totaled to 1970 meters.

Table 6 Nonsignificant changes of growth factors, differential counts, and additional data after a one-day climb

Median (Min; Max) rounded values of 4 subjects are shown.

G-CSF granulocyte colony stimulating factor, ***GM-CSF*** granulocyte macrophage colony stimulating factor, ***EGF*** epidermal growth factor, ***VEGF*** vascular endothelial growth factor, ***TGF-β1*** transforming growth factor β 1, ***FGF-2*** fibroblast growth factor 2, ***IGF-1*** insulin-like growth factor 1, ***IGFBP-1*** insulin-like growth factor binding protein 1, ***IGFBP-3*** insulin-like growth factor binding protein 3, ***O_2*** oxygen, ***pO_2*** barometric oxygen pressure, ***Hb*** hemoglobin, ***Hct*** hematocrit, ***NS*** not significant, ***NA*** not available, ↑ increase, ↓ decrease

Variable (units)	Pre	Post	p	↑/↓
G-CSF (pg/ml)	33.0 (28.8; 45.4)	42.1 (38.8; 67.8)	NS	↑
GM-CSF (pg/ml)	3.2 (1.9; 5.0)	3.2 (3.2; 5.6)	NS	↑
EGF (pg/ml)	14.1 (11.2; 18.0)	10.8 (5.5; 13.4)	NS	↓
VEGF (pg/ml)	16.0 (11.4; 338.3)	16.0 (16.0; 386.8)	NS	↑
TGF-β1 (pg/ml)	23620.8 (4891.8; 39217.2)	11647.2 (9463.2; 14625.3)	NS	↓
FGF-2 (pg/ml)	22.3 (3.2; 80.9)	71.0 (34.2; 155.8)	NS	↑
IGF-1 (µg/l)	178.0 (162.0; 209.0)	173.0 (133.0; 213.0)	NS	↓
IGFBP-1 (ng/ml)	7.8 (4.7; 14.2)	15.2 (7.1; 24.1)	NS	↑
IGFBP-3 (mg/l)	3.0 (2.4; 3.6)	2.3 (2.0; 2.7)	NS	↓
Leucocytes/µl	5250.0 (3300.0; 6300.0)	9550.0 (6450.0; 14400.0)	NS	↑
Lymphocytes (%)	49.0 (39.0; 60.0)	24.0 (24.0; 24.0)	NA	NA
Monocytes (%)	7.0 (5.0; 9.0)	3.0 (3.0; 3.0)	NA	NA
Neutrophils (%)	39.5 (33.0; 46.0)	72.0 (72.0; 72.0)	NA	NA
Eosinophils (%)	4.5 (2.0; 6.0)	1.0 (1.0; 1.0)	NA	NA
Basophils (%)	NA	NA	NA	NA
O_2 Saturation (%)	NA	NA	NA	NA
pO_2 (mmHg)	553.0 (553.0; 553.0)	553.0 (553.0; 553.0)	NS	→
Hb (g/l)	170.0 (160.0; 182.0)	175.0 (175.0; 175.0)	NA	NA

6. Discussion

In recent years, substantial progress has been made toward the identification and understanding of the molecular environment generated by growth factors. The data this study produced provides insight into hypoxia- and exercise-induced modifications of growth factor levels.

The main aim of this study was to test the novel idea that members of all major growth factor groups, i.e. vascular growth factors, hematological growth factors and growth factors appertaining to the IGF-network are affected in concert by hypobaric hypoxia. To our knowledge, this study is the first to investigate the regulation of circulating growth factors in humans when exposed to hypobaric hypoxia compared to normobaric normoxia and effects of physical exercise under these circumstances. For latter, the time course of changes in the concentrations of serum growth factors before and after a standardized incremental step test both at sea level (69 m) and at high altitude (5050 m) was therefore examined.

As the major finding, growth factors showed significant changes predominantly when concentrations at sea level (69 m) were compared to high altitude (5050 m) values. While hypoxia generated pronounced effects on the growth factor milieu in resting subjects (group 1), exercise did not elicit significant changes in most growth factors at sea level (69 m) (group 2). At high altitude (5050 m), exercise in hypobaric hypoxia (group 2) did not produce any significant changes in growth factor levels. Growth factors measured at 5050 m both before and after return from a summit (6035 m) approach (group 3) did not show any significant changes, either.

This study adds valuable data to the understanding of mechanisms of growth factor regulation in humans. New insights are applicable to the understanding of the pathophysiology of clinical conditions such as chronic lung and/or heart disease or tumor pathologies as well as potential new treatment strategies. Furthermore, insights may be used to improve altitude training employed by athletes, pilots, or military personnel.

In the subsequent sections, methodological aspects and findings of the present study are contrasted with those of other studies and discussed below.

6.1. Methodological Aspects

The employment of Luminex xMAP multiplex assay system deserves recognition as a methodological disparity when contrasting findings of this study to those that have utilized other assays with different limits of detection. As a consequence, statistical reports with respect to means, standard deviation, medians, minimum and maximum values, interquartile ranges, and percent changes may not be directly comparable. Owing to the fact that manufacturers constantly produce improved products and thus provide researchers with more sensitive and/or more specific assays, assay specifications have to be taken into account when comparing results across studies. For instance, the assay used in this study is now available with higher sensitivity and specificity, which was not at disposal at the time the study was carried out. Furthermore, instead of running samples repeatedly, each sample was analyzed only once. This method holds the danger of missing inter- and intra-assay variations.

In comparison to other studies such as the one of Laufs et al. who employed only young and healthy men [122], the present study conducted exercise tests also in healthy, moderately active subjects but they represented a more heterogeneous group with relation to both age (median 37.5 years) and sex (3 women and 5 men). This adds to the representativeness of this study.

It is important to note that the time course of changes in tissue oxygenation does not necessarily correspond with changes in serum levels of growth factors. The lack of significant changes in growth factor levels may be due to removal from the circulation by rapid receptor binding [123]. Moreover, up- or down-regulation of growth factors may be on a local level via autocrine/paracrine signaling rather than on a systemic level via endocrine signaling [38, 52, 124].

Furthermore, growth factors produced locally might not only translocate to the systemic circulation but also to the lymphatic system. Although lymphatic vessels ultimately join blood vessels, growth factor levels are likely to be modulated at sites of high concentrations of immunological cells such as lymph nodes. [125]

A further benefit of this study is that this study was conducted under hypobaric hypoxic conditions at high altitude (5050 m) instead of under controlled environmental conditions in so-called

(normobaric) hypoxia chambers. Since participants of the study were obligated to multiple varying stressors including psychological stress, environmental extremes, disturbed sleep architecture, malnutrition, and weight loss, this study is more representative for clinical conditions including AMS, HAPE, HACE, chronic obstructive pulmonary disease (COPD), and congestive heart failure, just to name a few.

6.2. Discussion of Results

The following section will illuminate the role of growth factors in a hypoxic environment and in response to physical activity.

6.2.1. Growth Factors and Hypoxia

The present study investigated whether there is a change in concentrations of growth factors under hypobaric hypoxia at high altitude (5050 m) when compared to normobaric normoxia at sea level (69 m) values. The expedition´s altitude profile allowed for adequate acclimatization since maximum daily increase of altitude where participants slept amounted to 300 meters. Nonetheless, about 25 % of participants suffered from acute mountain sickness on the day after arrival at base camp (5050 m). This phenomenon and other signs of reduced immune function such as impaired wound healing are common traits in mountaineers at high altitude. Other than that, Walsh et al. allege that high altitude appears to have no further marked effects on immune function. [126] The present study, however, very well proved that hypoxia affects neuroendocrinological and immunological responses. This is represented, in part, by the increase of vascular growth factors including EGF ($p = 0.002$), VEGF ($p = 0.039$), and TGF-β1 ($p < 0.001$).

The present study is in line with previous studies proving that exposure to high altitude (5050 m) hypobaric hypoxia potently stimulates the production of EGF which rose in the present study significantly ($p = 0.002$). EGF is involved in cellular events such as resistance to apoptosis, proliferation, and migration of pulmonary vascular cells. Investigations of clinical conditions such as pulmonary arterial hypertension (PAH) could also prove a strong association with elevated EGF levels [20]. By linking hypoxic EGF-induction in our field study with the role of EGF in PAH this study substantiates clinically present effects of hypoxia on a molecular level.

Discussion

The present study showed that there is a significant rise in VEGF concentrations at high altitude (5050 m) when compared to sea level (69 m) in resting subjects ($p = 0.039$). This is in line with previous studies [127, 128]. Moreover, validity of this study´s data is confirmed by studies of Heits et al. who investigated VEGF concentrations in healthy humans under resting conditions[129] and Gunga et al. [48] who gave similar ranges of VEGF alterations.
The reason for these findings is given by the fact that hypoxia is a potent stimulator of growth factor release. VEGF is one of the key factors of angiogenesis and vascular development in the setting of tissue hypoxia which is mediated by HIF-1α. VEGF-mediated capillarization enhances the delivery of oxygen in peripheral tissues and thus aids adaptation to hypoxia [32, 43] Furthermore, hypoxia is known to up-regulate pro-inflammatory cytokines such as IL-6 which in turn is known to increase VEGF levels [130]. Therefore, it can be argued that increases in VEGF levels are triggered not only directly by hypoxia but also indirectly via the hypoxia-mediated initiation of inflammatory processes [130].

Hypoxia induces epithelial to mesenchymal transition in a TGF-β1-dependent manner and raises the ratio of bioactive to total levels of TGF-β1 [76]. The present study is in line with previous studies [76, 131] and confirms a significant rise of TGF-β1 plasma levels in resting subjects at high altitude (5050 m) when compared to sea level (69 m) data ($p < 0.001$).

Various reports confirm that hypoxia induces FGF production causing proliferation of vascular cells [20, 124, 132] and facilitates responsiveness of endothelial cells to FGF-2 [133]. In the present study, however, changes in FGF-2 levels were not significant. One reason could be that opposing factors interfere with FGF-2 production. Indeed, IGF and TGF-β1 intertwine with FGF-2 at certain points of their signaling pathways [55]. TGF-β1 opposes hypoxia-induced FGF-2-mediated growth promoting effects [41] and therefore it may be that increased levels of TGF-β1, as seen in the present study, interfere with FGF-2 at an earlier stage of signal transduction.

In the present study, hematological growth factors, both G-CSF ($p = 0.029$) and GM-CSF ($p = 0.035$), showed a significant rise at high altitude (5050 m) when compared to sea level (69 m). This is substantiated by clinical studies investigating hematological growth factors in hypoxic conditions. In cerebral ischemia, it has been shown that both G-CSF and G-CSF receptor are up-regulated in

neuronal cells. Neuronal injury due to hypoxia elicits G-CSF release which represents a neuroprotective mechanism because G-CSF promotes neurogenesis and angiogenesis [79, 134]. Since high altitude induces multiple processes involving the immune system and endocrinological system, it is difficult to identify whether changes are triggered by either hypoxia or the initiation of inflammatory processes, or both.

To our knowledge, this study is the first to describe a direct correlation between increasing GM-CSF levels upon exposure to low levels of atmospheric oxygen. GM-CSF was significantly elevated at high altitude (5050 m) when compared to sea level (69 m). A possible explanation for the increase in both G-CSF and GM-CSF under hypoxic conditions is the fact that both factors are key regulators of the production of erythroid and megakaryocytic progenitor cells [91, 135-137]. Both types of progenitor cells have been identified to be up-regulated under tissue hypoxia[1, 138]. The purpose of up-regulation of GM-CSF remains to be investigated. It can be assumed, however, that increased levels of GM-CSF bolster the generation of both immunologically active cells and cells involved in enhanced tissue oxygenation (as seen in erythropoiesis).

The present study found significant decreases in IGF-1 ($p < 0.001$) and IGFBP-3 ($p < 0.001$) in resting subjects upon exposure to high altitude (5050 m). Hansen et al. found no changes in IGF-1 or IGFBP-3 in vertebrate embryonic cells under hypoxia [139]. Yamada et al. showed increases in IGFBP-3 mRNA upon exposure to both increasing levels of hypoxia and hypoxic exposure time [98]. The present study describes changes in the IGF axis in serum blood, while the other two studies targeted embryonic cells and mRNA products, respectively. While the studies of Hansen et al. and Yamada et al. present in vitro data of the IGF axis, the present study illustrates in vivo changes of the IGF axis. Since all studies looked at the IGF axis on a different level, findings might rather complement than contradict each other.

On the one hand, the present study found no significant changes in IGFBP-3 levels after physical exercise both under normoxia and hypoxia, on the other hand, resting subjects showed a significant decrease of IGFBP-3 concentrations upon exposure to hypoxia ($p < 0.001$). Considering this study´s findings and the study of Hug et al. [140], hypoxia appears to be the crucial factor to produce significant changes in IGFBP-3, while exercise seems to play a minor role.

Since both IGF-1 ($p < 0.001$) and IGFBP-3 ($p < 0.001$) decreased significantly under hypoxia, data of the present study is in line with previous findings that stated that IGF-1 and IGFBP-3 are positively correlated [100].

Previous reports stated that IGFBP-1 is up-regulated via HIF-1 upon exposure to hypoxia [108, 109]. Furthermore, Arafat et al. stated that IGFBP-1 and IGF-1 are negatively correlated. [100] Therefore, decreasing IGF-1 levels, as seen in the present study ($p < 0.001$), are associated with increasing IGFBP-1 levels. Even though IGFBP-1 was not significantly changed under hypoxia, there was a tendency towards elevated IGFBP-1 levels. Woods et al. confirmed that resting morning cortisol levels are elevated at high altitude (> 5000m) [141]. Taking into account that Cianfarani et al. reported that cortisol is positively correlated with IGFBP-1 and negatively correlated with IGF-1 [142], potentially elevated cortisol levels at the time of blood sampling would explain this study´s findings of not significantly increased levels of IGFBP-1 and significantly decreased levels of IGF-1 ($p < 0.001$) in resting subjects at high altitude (5050 m). One possible explanation of IGF-1 reduction at high altitude (5050 m) is that increased cortisol concentrations reduce proteolysis of IGFBP-3 and thus increase IGFBP-3 levels. Since IGFBP-3 binds IGF-1, the net effect is reduced IGF-1 bioavailability [104].

6.2.2. Growth Factors and Exercise

While there are many studies that investigated either hypoxia-induced or exercise-induced changes in growth factors, there are few studies that have investigated changes in growth factor concentrations after physical exercise in hypobaric hypoxia. Summarized insights of various studies lead to the assumption that exercise amplifies the immunosuppression imposed by tissue hypoxia and that growth factors play a role in this setting. [6, 127] In the present study, hypoxia at rest caused significant changes in most growth factors, additional stress through exercise, however, failed to cause significant changes in most growth factors both at sea level (69 m) and high altitude (5050 m) (see tables 4 and 5, respectively).

The present study investigated whether there is a change in growth factor levels after physical exercise when compared to resting values. Neither the 12-minute exercise protocol applied both at sea level (69 m) and at high altitude (5050 m), nor a 12-hour climb from Hidden Valley Base Camp (5050 m) to Damphus Peak (6035 m) and back produced significant changes in growth factor levels.

Discussion

Prolonged physical activity, as seen in endurance exercise, produces large changes in plasma levels of cytokines (e.g., increases in IL-6, IL-8, and IL-10 [143] and increases in IL-1rα, TNF-α, and IL-1β [144]) and it is therefore likely that it affects all growth factors investigated. However, in the present study a prolonged physical activity represented by a 12-hour journey (ascent from 5050 m to 6035 m and subsequent descent to 5050 m) failed to produce significant changes in growth factors. This is likely to be caused by too small a sample size (4 subjects) to produce significant conclusions.

In the present study short term exercise (12 minutes) did not produce significant changes in growth factor levels, neither at sea level (69 m), nor at high altitude (5050 m). One reason could be that during an exercise bout of 12 minutes there is not enough time to generate a considerable magnitude of oxidative stress and energy demand, i.e. represents a too weak stimulus to elicit significant changes.

In addition, the rate of clearance of growth factors from the circulation into the urine after exercise seems to affect their plasma concentrations. In that sense, it is reasonable to presume that, when compared to short-term exercise, prolonged physical activity causes a stronger redistribution of systemic blood flow to exercising muscles. This exerts more pronounced restrictions on renal blood flow which in turn causes a prolonged inhibition of the clearance of growth factors from circulation. [11, 145, 146] Accordingly, it is assumed that the longer the period of exercise, the slower the clearance of growth factors from the circulation and thus the higher growth factor concentrations after exercise. Taking into account that the present study employed an exercise bout of 12 minutes it can be assumed conversely that absent significant changes in growth factor levels owe to too short a duration of physical activity. In other words, plasma growth factors are presumably cleared too fast from the blood stream during and after short term exercise to be detected in significant amounts. Accordingly, it can be assumed that during the 12-minute incremental step test growth factors travel from blood to peripheral tissues, therefore measurements reveal at this point reduced plasma levels of growth factors. In the post-exercise phase pre-exercise plasma levels are restored by migration of growth factors from bone marrow to the circulation. [147] Further studies are needed in order to investigate clearance of growth factors from circulation.
Another issue is exercise-induced ischemia of the gastrointestinal tract which could result in disintegration of gut mucosa and bacterial invasion into the systemic circulation. Bacteremia might

result in elevated levels of endotoxin lipopolysaccharide [145, 148] which is a potent stimulant of immunological responses including changes in growth factors. [149, 150] In consideration of this mechanism, endurance exercise is more likely to induce bacterial translocation than short-term exercise, as employed in the present study, which may be another reason for nonsignificant changes of growth factors in the present study.

Other putative mechanisms for undetected growth factor changes are on the one hand a restricted systemic release of growth factors in response to exercise-induced tissue hypoxia, on the other hand a very short half-life of growth factors in the systemic circulation.
It has been shown that exercise negatively influences cellular immunity by reducing circulating numbers of T- and B-lymphocytes. Amongst others, these changes are related to alterations in the balance of the growth factor milieu. To date, the clinical significance of these immunological changes in response to exercise lack explanation. [151]

In agreement with the notion that suppression of the immune system is induced by physical exercise which in turn increases susceptibility to symptoms of infection such as upper respiratory tract infection, cellular changes are observed during and immediately after exercise.

With respect to differential blood counts, this study´s findings confirm previous reports of a marked lymphocytosis after exercise [151]. Leucocytes were both at sea level (69 m) ($p = 0.016$) and at high altitude (5050 m) ($p = 0.008$) significantly elevated 180 minutes after cessation of exercise. Walsh et al. pointed out that leucocytes drop below pre-exercise levels during the early stages of recovery and levels return to resting values within 24 hours [126]. This leads to the conclusion that immunological changes are in progress even 180 minutes after cessation of exercise.
Various reasons for nonsignificant changes in other factors of the differential count after exercise exist. Since, for instance, the magnitude of the increase in neutrophils is subject to intensity and duration of exercise [151], it can be assumed that an incremental physical exercise of a total duration of 12 minutes is insufficient to produce pronounced changes even 180 minutes after cessation of exercise. Other reports argue that down-regulation of the neutrophil response after an ascent to high altitude (in this case from 1780 m to 3198 m) serves to prevent exercise-induced inflammatory destruction of tissues that might otherwise be aggravated by cytotoxic effects of neutrophils [152].

Discussion

Acute exercise triggers a monocytosis which persists only for about 120 minutes [151]. In the present study, post-exercise samples were taken 180 minutes after cessation of exercise. It is possible that if levels of monocytes were initially increased, they had already returned to baseline levels at 180 minutes after cessation of exercise and therefore no significant changes could be measured.

VEGF has been shown to have inflammatory [41] and angiogenic [30, 48] effects upon exercise. This study adds novel data to the scarce knowledge about changes in the production and release of VEGF upon exercise under different environmental conditions. This study´s data is in line with previous reports with respect to basal VEGF concentrations [48, 129] ranging at rest from 2.93 to 138.49 pg/ml at sea level (69 m).

Furthermore, this study could verify previous findings of other reports of unchanged plasma concentrations of VEGF after exercise [48, 122, 147, 153].

Even though the scope of the incremental step test employed was not identical with a marathon race or a 1500 m run as employed by Bonsignore et al. [147] or 30 minutes of intensive running as employed by Laufs et al. [122], this study shares the same findings. Möbius-Winkler et al. employed an exercise protocol similar to ours (incremental graded exercise on a cycle ergometer) and were able to show an initial significant rise in VEGF levels 10 minutes after exercise, which lost statistical significance already 15 minutes post exercise, however [154]. This again complies with the present study which found no significant changes of VEGF levels 180 minutes after cessation of exercise. Contrasting this study´s findings, two other studies reported a significant decrease of VEGF levels immediately after a marathon race of which one took place at sea level [143] and the other at high altitude [48]. The decrease in VEGF levels after exercise compared to pre-exercise data may be explained by a concomitant increase in levels of VEGF binding proteins (sVEGFR-1) and an increased binding affinity of VEGF to its receptors at the endothelium [155, 156]. A possible explanation for unchanged concentrations of VEGF in the present study is a concomitant rise in endogenous corticosteroid levels which inhibits VEGF release [48].

It is well documented that VEGF is a mediator of ischemia-induced regulation of endothelial progenitor cells (EPC) [122]. Interestingly, analogous to the present study´s results that exercise did not change VEGF levels, Laufs et al. found that exercise was not correlated with VEGF-induced EPC regulation. Since this study proved, however, that hypoxia alone produces a significant increase in

VEGF levels (p = 0.039), this study disproves Laufs et al. who state that it is not likely that ischemia plays also a role in the exercise-induced EPC up-regulation [122].

A systematic review by da Silva and coworkers features a list of studies dealing with exercise- and hypoxia-induced changes of VEGF. Studies treated the issue of exercise-induced mobilization of EPC as a marker of vascular repair and angiogenesis which is, at least partly, VEGF-dependent. EPC mobilization is more pronounced in long and ultra-long duration exercises (e.g., half-marathon, marathon, ultramarathon training) than in maximum exercise regimens (e.g., conventional cardiopulmonary exercise test) or submaximum exercise regimens (utilizing a certain percentage of the anaerobic threshold and/or maximum VO_2max as reference with a maximum duration of one hour). Furthermore, while VEGF plasma levels changed significantly in studies using long/ultra-long duration exercise protocols, most studies using maximum intensity exercise did not show significant changes in VEGF plasma levels. [157] This is in line with this study, since no significant changes in VEGF in the groups undergoing maximum (Damphus peak group 3) or submaximum exercise (12-minute incremental step test) were found. Additionally, long duration exercise was represented by the ascent to base camp (5050 m) which lasted 14 days and produced significant changes in most growth factors including VEGF (p = 0.039). This leads to the assumption that in the present study maximum and submaximum exercise protocols were too low in intensity and/or too short in duration to produce significant changes in the growth factor milieu [157].

This study complies furthermore with Gunga et al. who also did not find changes in VEGF concentrations after physical exercise in normobaric normoxia. In contrast to the present study, they showed a decrease of VEGF immediately after a marathon run at high altitude (12 participants running 42.2 kilometers (km) each, each one starting at a different altitude ranging from sea level up to 4722 m). [48] Arguably, this may be only an immediate response which changes direction in the hours following physical exercise. In fact, the study of Schobersberger et al. reports a rise in VEGF levels which persists even five days after an ultramarathon (distance 67 km, peak altitude 2739 m, altitude difference 2300 m) [130]. Asano et al. showed a biphasic trend of VEGF levels with an initial decrease after altitude training at 1886 m at day 10, followed by an increase which reached peak values at day 19 of the regimen. VEGF levels returned to normal by 1 month after return to sea level. [158] In the present study, VEGF levels significantly increased upon exposure to hypoxia (p = 0.039) but did not change after exercise at sea level (69 m) or high altitude (5050 m). Therefore, this study

provides valuable data for the identification of triggers of VEGF changes. Thus, it can be assumed that hypoxia has a more potent effect on VEGF levels than exercise.
Multiple studies have been conducted investigating exercise and subsequent changes in immune response and growth factors, respectively. Unlike most research projects, this study targeted not only VEGF but also other growth factors, thus widening the view upon alterations in growth factors involved in exercise. Morici et al. found that VEGF and TGF-β1 levels did increase after exercise [159]. However, this study found that VEGF and TGF-β1 do not change significantly after exercise.

Hematological growth factors including G-CSF and GM-CSF did not change significantly upon physical exercise. This study´s findings are supported by other studies which did not show significant changes in G-CSF after exercise [159, 160] and by Suzuki et al., who did not find statistically relevant changes in GM-CSF levels directly after exercise, either [11]. However, Suzuki et al. presented data about elevated levels of G-CSF in two separate studies. The first study confirmed increased levels of G-CSF 10 minutes after a marathon race [11]. The second study confirmed G-CSF up-regulation both 30 minutes and 14-20 hours after an Ironman triathlon race (maximum race time 10 hours and 30 minutes) [87]. In a third study Suzuki et al. demonstrated that both endurance and short-duration exercise promotes the secretion of G-CSF, M-CSF, IL-8, and MCP-1. Furthermore, they highlighted that changes of mentioned growth factors and cytokines are more prominent in urine than changes seen in blood plasma. [145] One explanation of the discrepancy towards this study´s findings may be the fact that the exercise protocol employed (exercise time of 12 minutes) did not reach intensity levels of a marathon or Ironman triathlon race.

Although Hirose et al. showed that 12 minutes of eccentric exercise of elbow flexors induced significant muscle damage (rises in creatine kinase and myoglobin), changes in cytokines in general were relatively small. However, G-CSF levels were significantly increased 1 hour, 3 hours, and 3 days post exercise. [125]

Multiple studies corroborate exercise-induced subclinical states of severe inflammatory response syndrome (SIRS) characterized by an amplified cytokine response. Physical exercise induces muscular damage as characterized by increasing levels of creatine kinase and myoglobin and promotes the release of various factors such as cytokines and growth factors (e.g., G-CSF) [11]. As stated before, G-

CSF plays a dual role (both pro- and anti-inflammatory effects) in immunomodulation of physical exercise.

With regard to the IGF axis the present study found no significant changes in levels of IGF-1, IGFBP-1, and IGFBP-3 upon physical exercise, neither at normoxia, nor at hypoxia. This is in line with the studies of Hug et al., who examined elite cyclists and did not find significant changes in IGFBP-3 levels after exercise under normoxic conditions [140] and Spruit et al., who examined COPD patients on a cycle-ergometer under normoxic conditions and did not find significant changes in IGF-1 levels, either [161].

On the one hand, it has been shown that exercise suppresses GH causing a decrease in IGF-1 levels [99], on the other hand, there is evidence that exercise induces GH release engendering an increase in IGF-1 concentrations [98]. In addition, contradicting reports describe either a decrease or a nonsignificant increase in IGF-1 during long-term exercise [162-164].

Physical exercise reduces the IGF-1/cortisol ratio in athletes, which suggests an increase of catabolic processes due to physical exercise [165]. Moreover, exercise under hypoxic conditions induces IGFBP-3 down-regulation [140], which causes a concomitant decrease of IGF-1 levels [100].

Various studies support the hypothesis that an acute bout of physical activity increases energy expenditure and thus increases pro-inflammatory cytokine levels, which in turn decrease IGF-1 levels. After successful adaptation, pro-inflammatory cytokine levels decrease and entail a rise in IGF-1 levels even higher than before exercise which represents an “anabolic rebound”. [166]

Additionally, about 8 to 30 hours elapse between the release of growth-hormone-releasing factor from the hypothalamus and ultimate IGF-1 release. Thus it can be deduced that local changes in IGF-1 do not reflect systemic changes. [161] This shows that after physical activity changes in IGF-1 levels are time-sensitive and that in this study significant systemic changes of IGF-1 might not have been detected by data analysis 180 minutes post exercise. In order to gain more insight into changes of IGF-1 in both systemic and local levels, future studies are needed which incorporate muscle biopsies into their methodology. Furthermore, it can be assumed that initial decreases of systemic IGF-1 levels do not mirror local levels of IGF-1 which might be higher upon acute physical exercise [166]. With regard to that, in the present study, absent changes in IGF-1 may be also explained by the fact that local production of IGF-1 in skeletal muscle is increased without having any effects on systemic

levels of IGF-1. This underscores the differences in autocrine/paracrine signaling and endocrine signaling of growth factors. [93]

Another explanation for the present study not to show significant changes in IGF-1 levels might be a relative GH insensitivity, i.e. IGF-1 levels do not increase albeit the presence of increased GH levels [167, 168].

As stated above, IGF-1 levels are altered by nutritional states [94, 99]. Proportional to GH levels, IGF-1 levels are highest at puberty and gradually decline during adulthood [99]. Despite a relatively low mean age of participants of 28.5 years, it has to be taken into account that age-related differences in the GH set-up have not been accounted for by this study.

In the present study, the mean BMI of subjects was 23.33. Whether or not BMI influences IGF-1 levels is controversially discussed. Both decreased and increased levels of IGF-1 have been found in obese subjects when compared to non-obese individuals [169]. Additionally, the study of Viesti A. Collares et al. pointed out that IGF-1 levels differ in different tissues. For instance, while IGF-1 levels were higher in subcutaneous fat of obese subjects (BMI 47.46) when compared to the control group (BMI 24.04), this relation was reversed in visceral fat. [169]

6.3. Limitations of the Study

The power of the present study and thus its representativeness was weakened by the fact that total numbers of participants differed in the 3 groups. This is paradigmatic for field studies especially when performed in remote areas and under severe psychological and physical stress. Individual subjects voluntarily took part in either all groups or two or only one and some subjects only took part at sea level (69 m) but not at high altitude (5050 m). This was due to differing reasons including exhaustion, illness, or intake of drugs which then met exclusion criteria.

At high altitude (5050 m), due to dependency on solar power and limited access to electricity, samples were spun for 5 instead of 10 minutes at a relative centrifugal force of 12,000 x g. Furthermore, due to malfunctions of the centrifuge on day 15 (see figure 4), samples taken that day could not be centrifuged but were handled in the following way instead: After phlebotomy blood sample tubes were left in a vertical position for sedimentation. After 1 hour the supernatant was pipetted into test tubes of 1.5 ml in volume and put in the freezer at -26˚C. These circumstances are paradigmatic for aggravating conditions for scientific research in remote areas such as high altitude.

In group 3 subjects, blood was sampled at 3:30 a.m. which contrasts sampling times of the day in groups 1 and 2 (between 8 a.m. and 3 p.m.). While diurnal variation in cortisol release potentially influences growth factor levels [170], Laufs et al. did not find changes in cortisol or VEGF levels after a 30 minute exercise trial [122]. However, neither member of group 2 nor of group 3 did present major changes in growth factor levels after physical exercise. This is in line with Peake et al. who did not find significant changes in growth factor levels after exercise, even though the potential influence of diurnal variation of cortisol was minimized by conducting all trials between 11:00 a.m. and 12:00 p.m. [160].

This study was part of a broader range of investigations. Some subjects took part in both the present study and other research projects. For instance, one project asked subjects to drink carbohydrate supplements [171] in order to assess energy balance which plays a major part in hormonal and immunological processes [140, 172, 173]. Another group asked subjects to inhale iloprost while performing a cycle ergometer test. Both studies were double-blinded and have potential effects on exercise performance and thus also bear the potential to alter hormonal and immunological processes with respect to growth factors.

The present study is potentially confounded by subjects who received alimentary carbohydrate supplementation. Thissen et al. found that malnutrition leads to decreased IGF-1 levels [174] and Freedland et al. confirmed these findings in a mouse model via deprivation of alimentary carbohydrate [175]. Freedland et al. also found the absence of alimentary carbohydrate produced higher IGFBP-3 levels and a lower IGF-1:IGFBP-3 ratio when compared to mice with normal (Western) or reduced carbohydrate intake [175]. However, firstly little is known about normal ranges of variation of IGF-1, IGFBP-2, and IGFBP-3 levels [176] and secondly total energy intake and expenditure is strongly correlated with body size, the extent of physical activity, and metabolic efficiency. Therefore, it is not clear to what extent carbohydrate supplementation interacts with the IGF axis. Underfeeding is much more potent in decreasing IGF-1 levels than overfeeding is in increasing IGF-1 levels [177]. Since subjects experienced severe physical stress during the entire sojourn which was associated with weight loss, carbohydrate supplementation rather attenuated reduced alimentation rather than producing hyperalimentary states. Therefore, potential interactions of increased carbohydrate intake with growth factor levels of the IGF axis are if at all of minor magnitudes.

This study is potentially confounded by subjects who inhaled Iloprost which possesses anti-inflammatory and immunomodulating actions. Considering that subjects participated in the present study at least 24 hours after inhalation of Iloprost and that inhaled Iloprost has a maximum duration of action of 60 minutes [178], potential interactions of Iloprost with growth factors at time of blood sampling are if at all of minor magnitudes. Another fact adds to the small (if at all present) effects of Iloprost on growth factor levels found by previous studies [179-182]. In the present study, subjects inhaled Iloprost over several minutes instead of receiving it intravenously or orally over several days or implementing animal or cell culture models as applied in cited studies.

Potential confounders which might influence growth factor levels include, but are not limited to, genetic determinants [48], gender [4, 93], age, alcohol use, and hormone replacement therapy [183].

6.4. Conclusion and Recommendations

The human organism is subjected to multiple stressors when going to high altitude. Physical exercise amplifies the stress imposed on the human body. Clinical symptoms of disease may be reflected on a cellular level by altered levels of growth factors. Whether underlying pathological processes involve infectious agents or not is not clearly identifiable. Muscle damage as well as adaptive mechanisms may share the same growth factor signaling pathways as the immunological response towards infection. Hormonal, endocrinological, neurological and immunological responses might signal via idiosyncratic as well as collectively utilized pathways at the same time. Current knowledge is not sufficient in order to provide a holistic view of physiological and pathophysiological implications of growth factors and their network. However, this study contributes to the understanding of intricate growth factor-mediated processes.

Since host survival depends on efficient immune functions, the immune system has evolved redundant processes in order to provide for maximum safety. Redundancy complicates clear identification of mechanisms involved in immune function. It remains to be investigated how great the loss or gain of immune function has to be in order to produce changes in host defense and disease susceptibility or severity. [151]

Given the fact that growth factors are pleiotropic and form part of a highly complex network, not only absolute changes in growth factor concentrations but the contextual dependence has to be

taken into account when interpreting these processes. Stressors such as hypoxia or exercise engender dynamic effects disturbing the homeostasis of the growth factor network. Paradigmatic is the effect of TGF-β on CD4+ T-lymphocyte differentiation. TGF-β alone causes differentiation of T_{H0}-lympocytes (non-polarized) towards regulatory T-lymphocytes. TGF-β in concert with IL-6, however, drives the differentiation of T_{H0}-lympocytes towards T_{H17}-lympocytes [65].

High altitude represents an environment where multiple stressors affect the human immune response. The altitude-induced change of many growth factors measured in the present study provides evidence that subacute exposure to hypoxia tends to stimulate the neuroendocrine and immune system. Controversial reports give an account of equivocal immunological processes initiated upon exposure to hypobaric hypoxia [152]. Furthermore, changes in the growth factor milieu cannot clearly be assigned to immunological or metabolic processes. While, for instance, chronic changes in blood concentrations of growth factors appear to give an account of activated or inhibited cell growth, migration, or chemotactic attraction of cells, it remains to be shown whether (sub)acute changes in growth factors levels reflect the same processes or are representative for a new concept of growth factor interactions with respect to hormonal, endocrinological, and immunological processes. Further investigation is needed to elucidate whether the function of growth factors at altitude extends beyond their currently accepted roles in adaptation.

Likewise, changes in the growth factor milieu may be an expression of metabolic rather than immunological responses to stressors such as hypoxia as well as physical exercise.

Most exercise studies are conducted in humans and analyze blood samples. In order to grasp the extent of both systemic and local changes in growth factors, other types of specimen need to be incorporated, as well. Parallel measurements of, for instance, urine, saliva, or cell tissue biopsies might contribute greatly to the current knowledge of growth factor environment and network.

It has to be acknowledged that measurements at given times always can only reflect a growth factor concentration at that point in time. Furthermore, the lack of methods of constant sensing and measuring of growth factor concentrations aggravates the understanding of interactions of growth factors among one another and with other tissues.

Besides *in vitro* studies, *in vivo* studies are required in humans because they possess the advantage of the applicability to human pathologies.

Discussion

The dynamic response of growth factors to acute physical stress by exercise appears to be complex and does not follow linear courses of time. This has to be borne in mind when evaluating investigations of exercise training-induced responses in growth factors. There are studies like the one of Schobersberger et al., who measured constantly elevated levels of VEGF at time intervals of 5 minutes, 2 hours, 24 hours, 2 days, and 5 days after exercise [130]. In contrast, the present study showed no significant changes 3 hours after exercise which indicates that any potential changes in growth factor concentrations are not sustained at 3 hours after cessation of exercise.
Whether or not growth factor concentrations were similarly reduced or elevated earlier during or after exercise remains to be investigated by future studies.

7. Appendix

Table 7 shows the expedition itinerary with starting and end points and corresponding altitudes of each expedition day.

Table 7 Expedition itinerary

The expedition itinerary is outlined by showing starting and end points of each expedition day with corresponding altitudes.

BC Base Camp, ***m*** meters

Day	From	To	Altitude (m)			Comments
			Start	End	Difference	
1.	Beni	Babichar	826	970	*144*	
2.	Babichar	Phedi	970	1007	*37*	
3.	Phedi	Muna	1007	1701	*694*	
4.	Muna	Jugapani	1701	1433	*268*	
5.	Jugapani	Bogara	1433	2110	*677*	
6.	Bogara	Bogara	2110	2110	*0*	Rest day
7.	Bogara	Dobang	2110	2475	*365*	
8.	Dobang	Dobang	2475	2475	*0*	Rest day
9.	Dobang	Salagari	2475	3000	*525*	
10.	Salagari	Italian BC	3000	3606	*606*	
11.	Italian BC	Italian BC	3606	3606	*0*	Rest day
12.	Italian BC	Japanese BC	3606	4220	*614*	
13.	Japanese BC	Dhaulagiri BC	4220	4691	*471*	
14.	Dhaulagiri BC	Hidden Valley BC	4691	5050	*359*	
15.	Hidden Valley BC	Hidden Valley BC	5050	5050	*0*	Research
16.	Hidden Valley BC	Hidden Valley BC	5050	5050	*0*	Research
17.	Hidden Valley BC	Hidden Valley BC	5050	5050	*0*	Research
18.	Hidden Valley BC	Hidden Valley BC	5050	5050	*0*	Research
19.	Hidden Valley BC	Hidden Valley BC	5050	5050	*0*	Research
20.	Hidden Valley BC	Hidden Valley BC	5050	5050	*0*	Research
21.	Hidden Valley BC	Yak Kharka	5050	3885	*1165*	
22.	Yak Kharka	Marpha	3885	2670	*1215*	
23.	Marpha	Jomson	2670	2720	*50*	

Appendix

Figures 10 and 11 show the well map according to the manufacturer's user guide.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0 pg/ml Standard (Back-ground)	internal Standard										
B	0 pg/ml Standard (Back-ground)	internal Standard										
C	3.2 pg/ml Standard	QC 1 Control										
D	16 pg/ml Standard	QC 2 Control										
E	80 pg/ml Standard	Sample 1										
F	400 pg/ml Standard	Sample 2										
G	2000 pg/ml Standard	Sample 3										
H	10000 pg/ml Standard	Etc.										

Figure 10 Well map

The well map for growth factor analysis via Luminex according to the manufacturer's user guide. 96 wells are filled with standard, serial dilutions of standard, quality controls, and samples. [118]

QC quality control, ***pg*** pictogram, ***ml*** milliliter

	1	2	3	4	5	6	7	8	9	10	11	12
A	0 pg/ml Standard (Back-ground)	625 pg/ml Standard	QC 2 Control									
B	0 pg/ml Standard (Back-ground)	625 pg/ml Standard	QC 2 Control									
C	9.8 pg/ml Standard	2500 pg/ml Standard	Sample 1									
D	9.8 pg/ml Standard	2500 pg/ml Standard	Sample 1									
E	39 pg/ml Standard	10000 pg/ml Standard	Sample 2									
F	39 pg/ml Standard	10000 pg/ml Standard	Sample 2									
G	156 pg/ml Standard	QC 1 Control	Etc.									
H	156 pg/ml Standard	QC 1 Control										

Figure 11 Well map TGF-β1

The well map for TGF-β1 analysis via Luminex according to the manufacturer's user guide. 96 wells are filled with standard, serial dilutions of standard, quality controls, and samples. [184]

QC quality control, ***pg*** pictogram, ***ml*** milliliter

Following graphs show significant growth factor changes in resting subjects at sea level (69 m) and high altitude (5050 m).

Hematological growth factors G-CSF ($p = 0.029$) and GM-CSF ($p = 0.035$) showed a significant rise at high altitude (5050 m) (see figures 12 and 13).

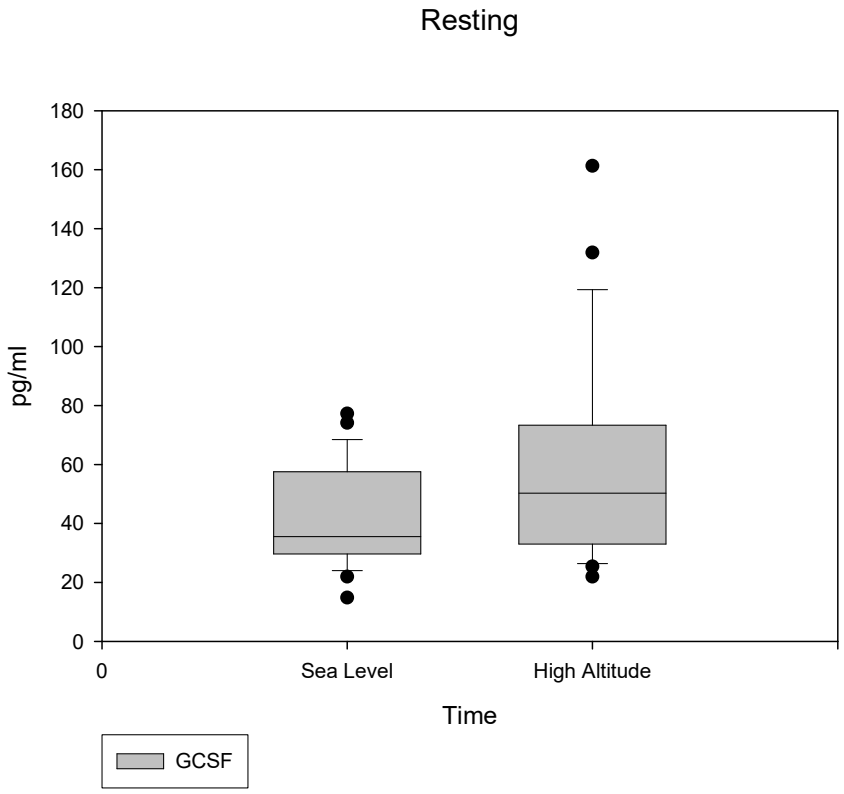

Figure 12 G-CSF at rest

G-CSF levels increased significantly (p = 0.029) in resting subjects when levels at sea level (69 m) 35.5 (14.8; 77.2) were compared to levels at high altitude (5050 m) 50.3 (21.9; 161.3) [pg/ml, Median (Min; Max) rounded values].

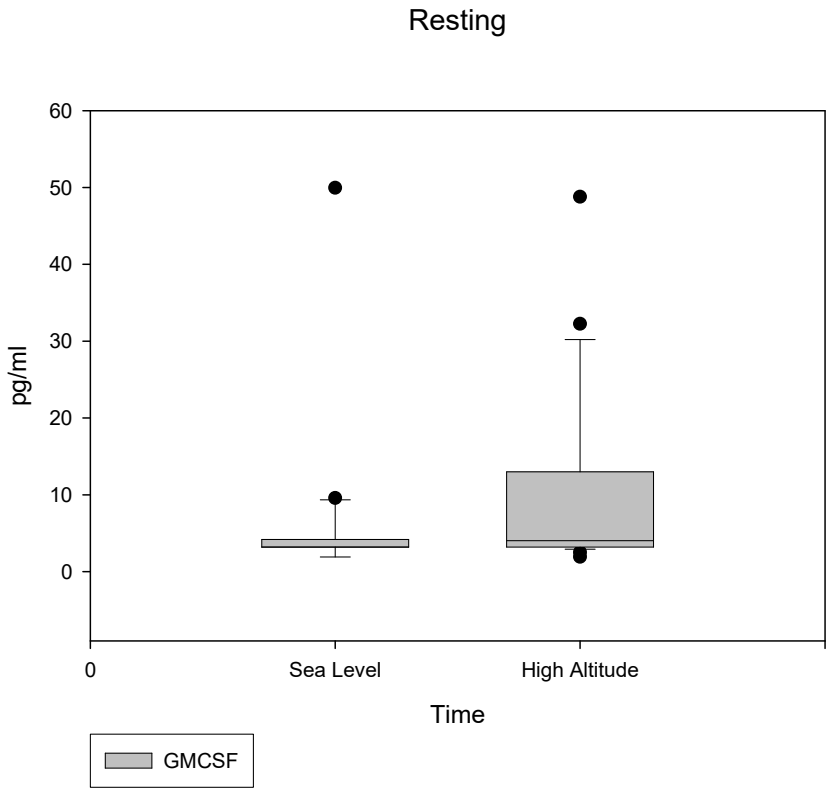

Figure 13 GM-CSF at rest

GM-CSF levels increased significantly (p = 0.035) in resting subjects when levels at sea level (69 m) 3.2 (1.9; 50.0) were compared to levels at high altitude (5050 m) 4.0 (1.9; 48.8) [pg/ml, Median (Min; Max) rounded values].

Significant increases of the vascular growth factors were shown in EGF (p = 0.002), VEGF (p = 0.039), and TGF-β1 (p < 0.001) at high altitude (5050 m) (see figures 14 through 16).

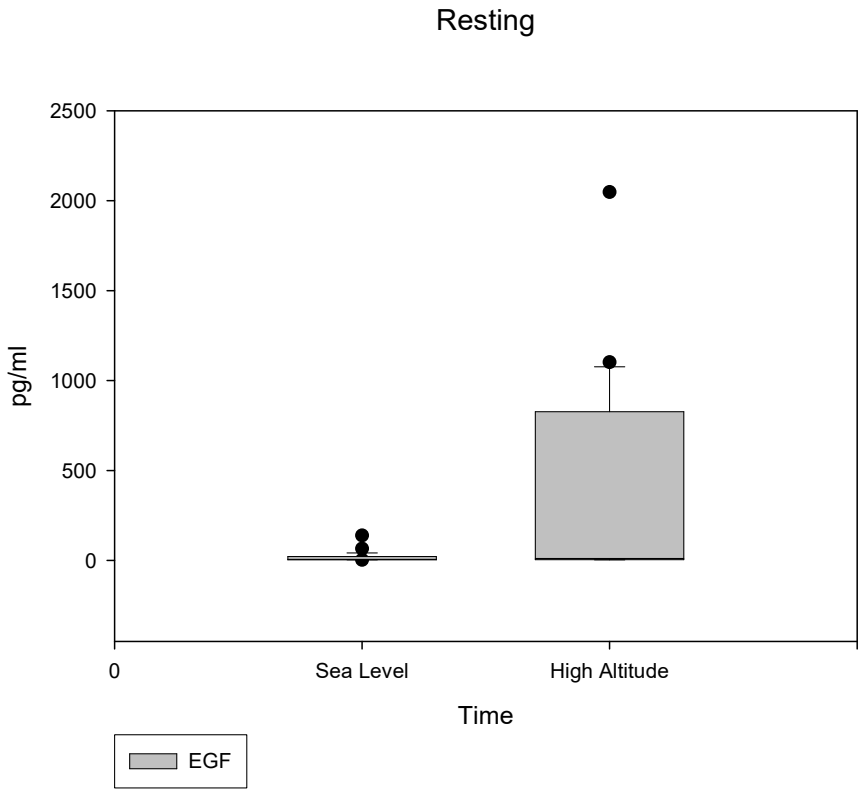

Figure 14 EGF at rest

EGF levels increased significantly (p = 0.002) in resting subjects when levels at sea level (69 m) 5.8 (2.9; 138.5) were compared to levels at high altitude (5050 m) 10.7 (3.2; 2047.2) [pg/ml, Median (Min; Max) rounded values].

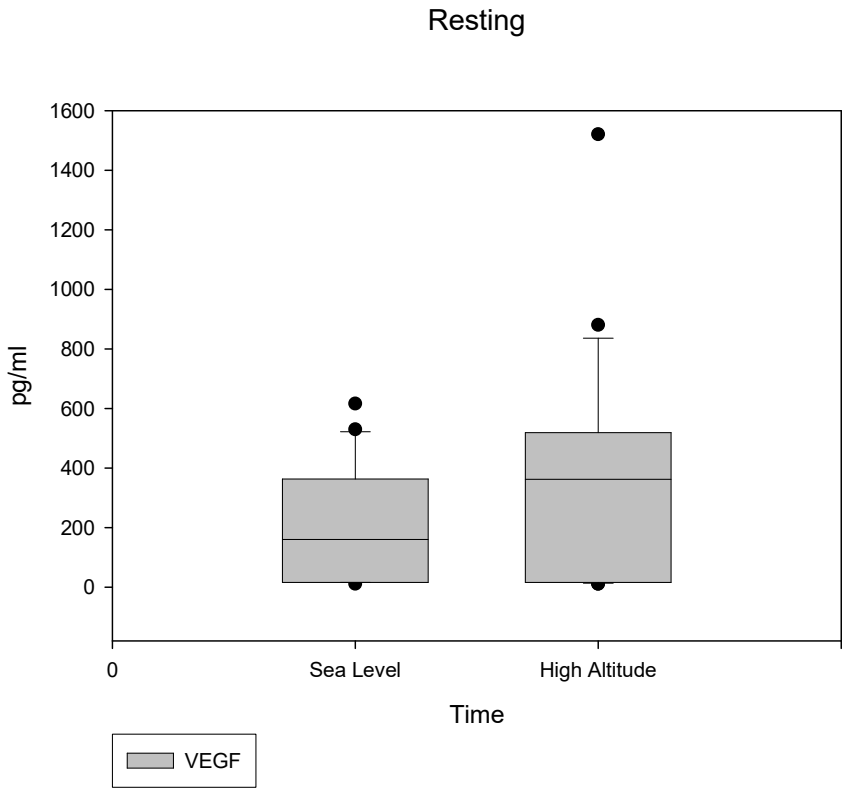

Figure 15 VEGF at rest

VEGF levels increased significantly (p = 0.039) in resting subjects when levels at sea level (69 m) 160.5 (11.4; 616.1) were compared to levels at high altitude (5050 m) 362.5 (11.4; 1520.2) [pg/ml, Median (Min; Max) rounded values].

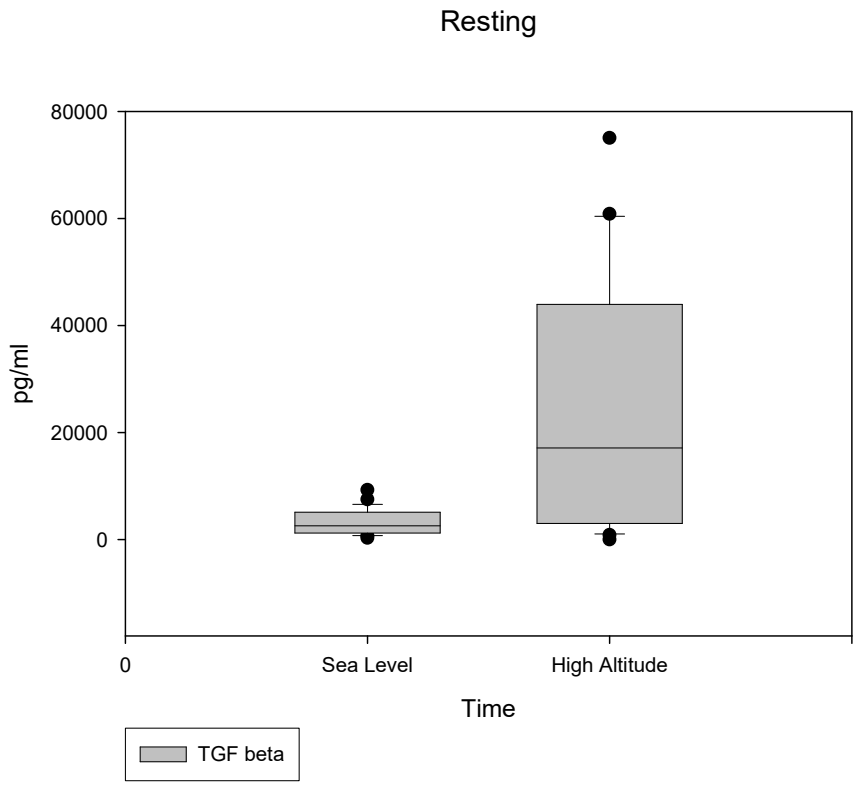

Figure 16 TGF-β1 at rest

TGF-β1 levels increased significantly ($p < 0.001$) in resting subjects when levels at sea level (69 m) 2566.8 (310.8; 9265.2) were compared to levels at high altitude (5050 m) 17116.5 (294.0; 75043.2) [pg/ml, Median (Min; Max) rounded values].

Of the insulin axis, IGF-1 ($p < 0.001$) and IGFBP-3 ($p < 0.001$) showed a significant decrease at high altitude (5050 m) when compared to sea level values (see figures 17 and 18).

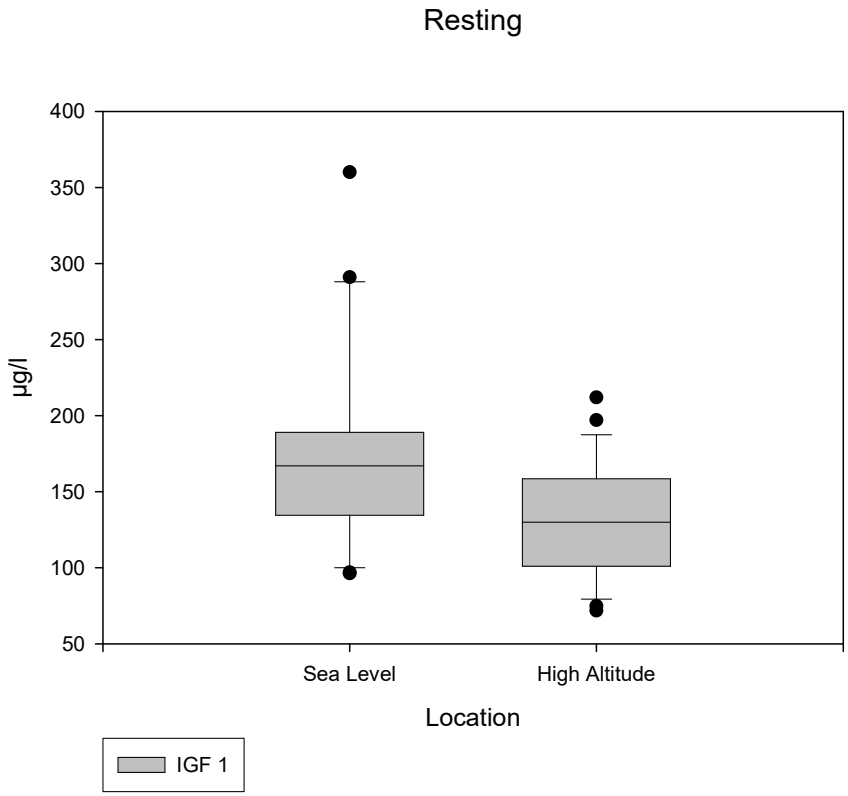

Figure 17 IGF-1 at rest

IGF-1 levels decreased significantly ($p < 0.001$) in resting subjects when levels at sea level (69 m) 167 (96.3; 360) were compared to levels at high altitude (5050 m) 130 (71.8; 212) [µg/l, Median (Min; Max) rounded values].

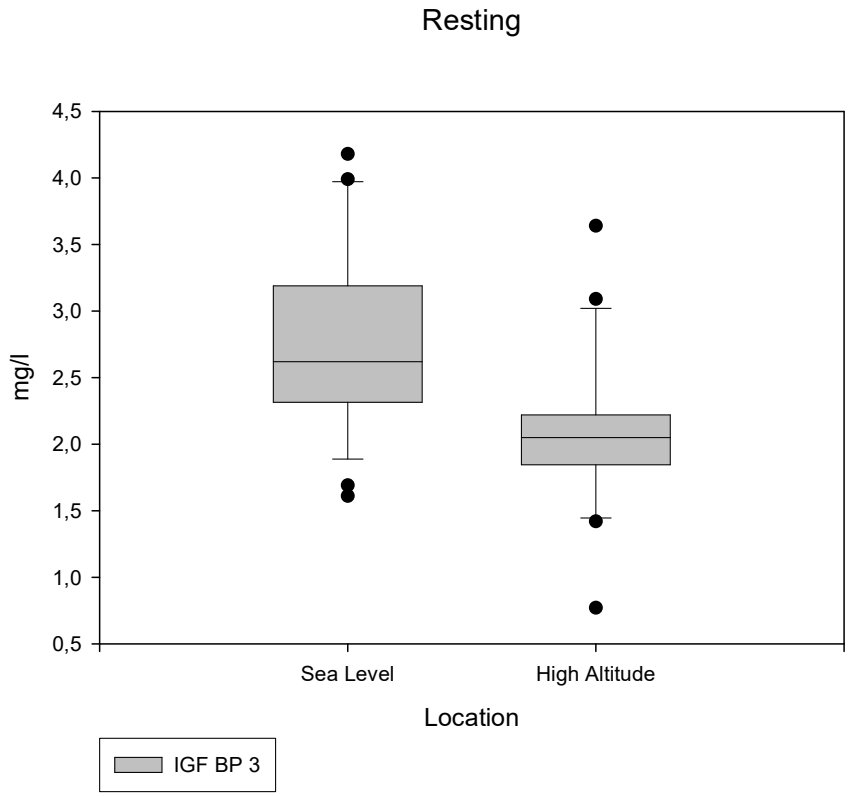

Figure 18 IGFBP-3 at rest

IGFBP-3 levels decreased significantly ($p < 0.001$) in resting subjects when levels at sea level (69 m) 2.6 (1.6; 4.2) were compared to levels at high altitude (5050 m) 2.1 (0.8; 3.6) [mg/l, Median (Min; Max) rounded values].

Apart from growth factors, also differential blood counts showed some significant changes. Lymphocytes ($p = 0.048$) were significantly decreased and neutrophil counts ($p = 0.022$) were significantly increased at high altitude (5050 m) (see figures 19 and 20).

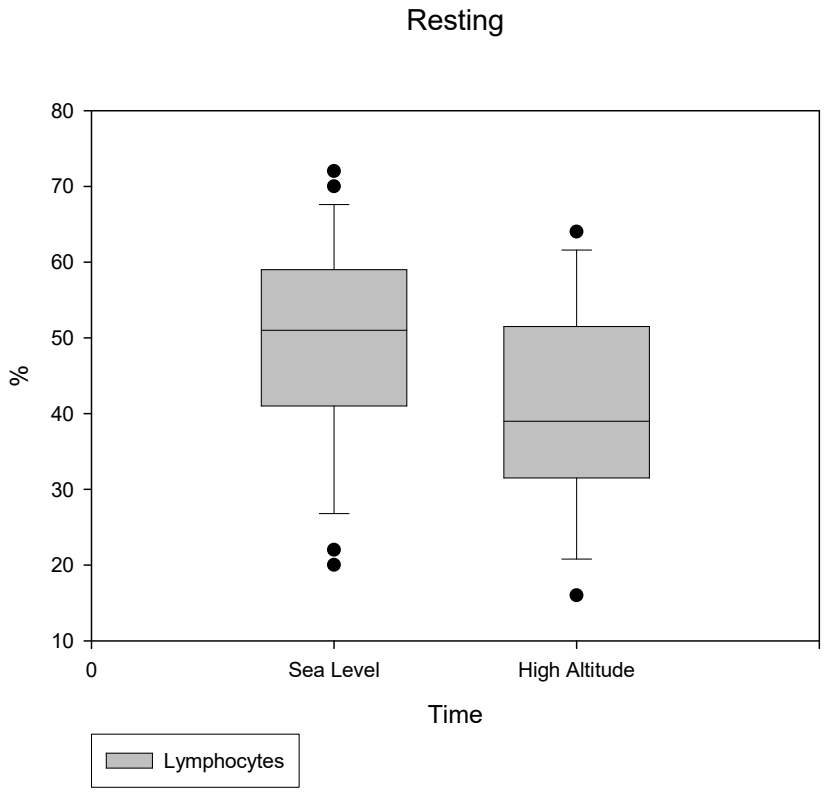

Figure 19 Lymphocytes at rest

Lymphocyte levels decreased significantly (p = 0.048) in resting subjects when levels at sea level (69 m) 51.0 (20.0; 72.0) were compared to levels at high altitude (5050 m) 39.0 (16.0; 64.0) [%, Median (Min; Max) rounded values].

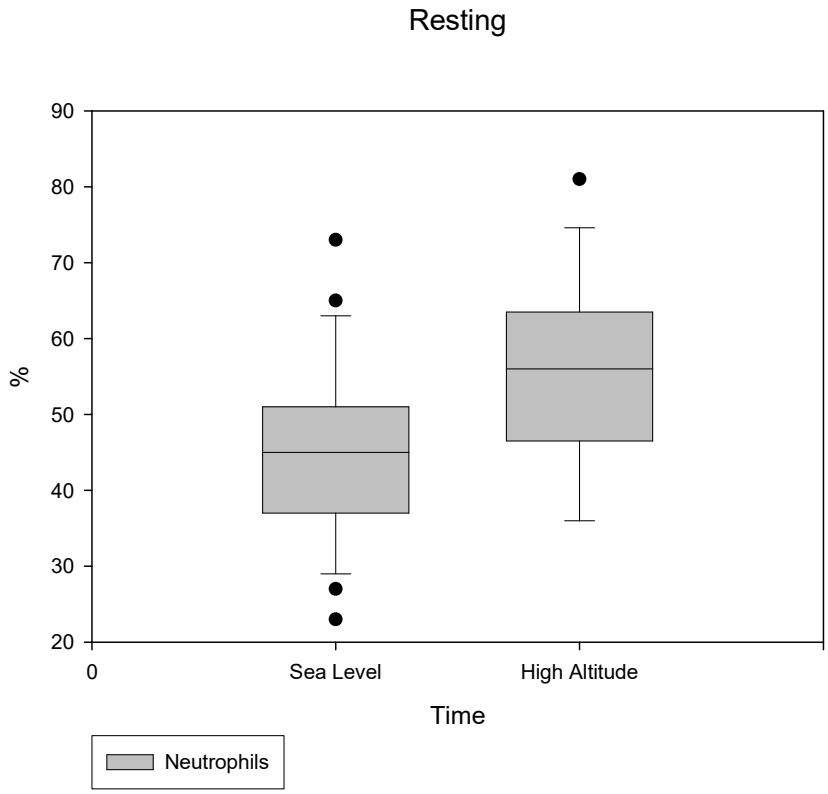

Figure 20 Neutrophils at rest

Neutrophil levels increased significantly ($p = 0.022$) in resting subjects when levels at sea level (69 m) 45.0 (23.0; 73.0) were compared to levels at high altitude (5050 m) 56.0 (36.0; 81.0) [%, Median (Min; Max) rounded values].

While partial oxygen pressure ($p < 0.001$) and oxygen saturation ($p < 0.001$) were significantly decreased at high altitude (5050 m), hemoglobin levels were significantly increased ($p < 0.001$) (see figures 21 through 23).

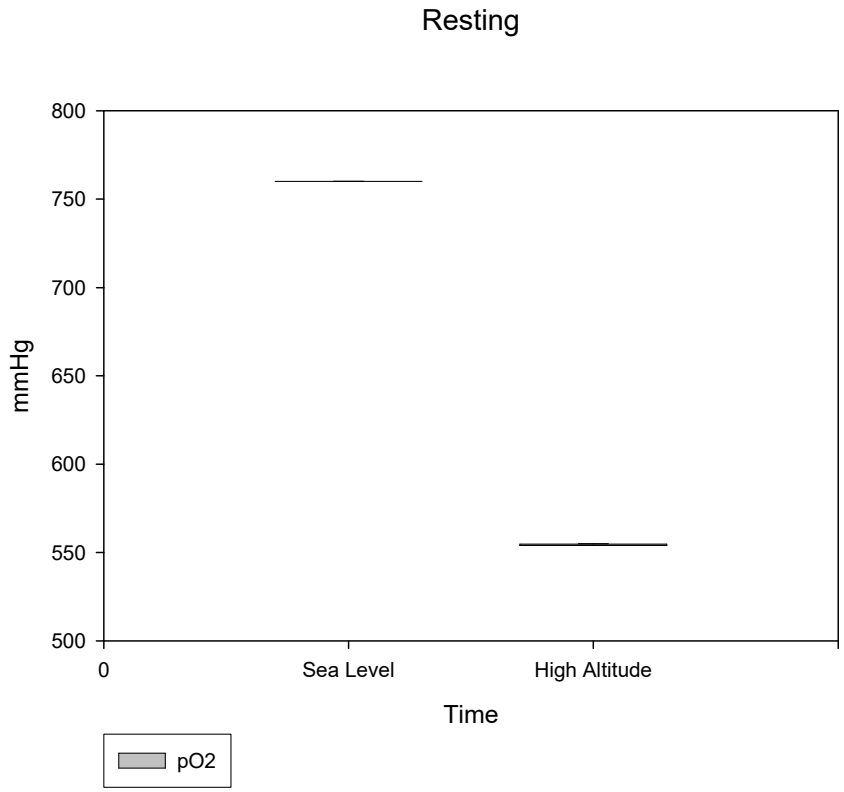

Figure 21 pO_2 at rest

pO_2 decreased significantly ($p < 0.001$) in resting subjects when levels at sea level (69 m) 760.0 (760.0; 760.0) were compared to levels at high altitude (5050 m) 554.0 (554.0; 555.0) [mmHg, Median (Min; Max) rounded values].

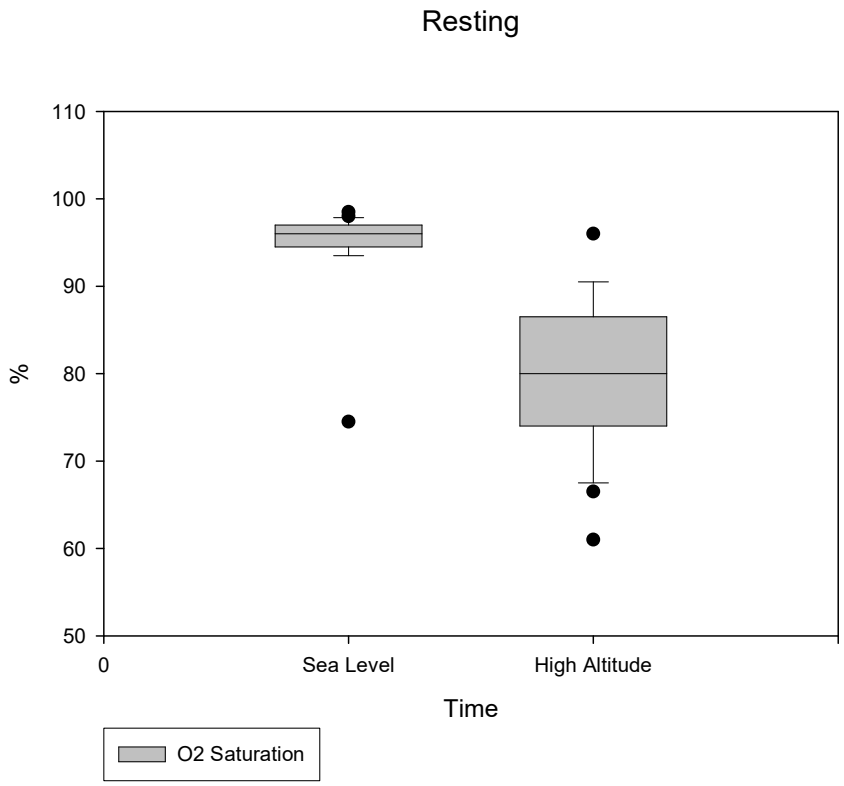

Figure 22 O_2 saturation at rest

O_2 saturation decreased significantly ($p < 0.001$) in resting subjects when levels at sea level (69 m) 96.0 (74.5; 98.5) were compared to levels at high altitude (5050 m) 80.0 (61.0; 96.0) [%, Median (Min; Max) rounded values].

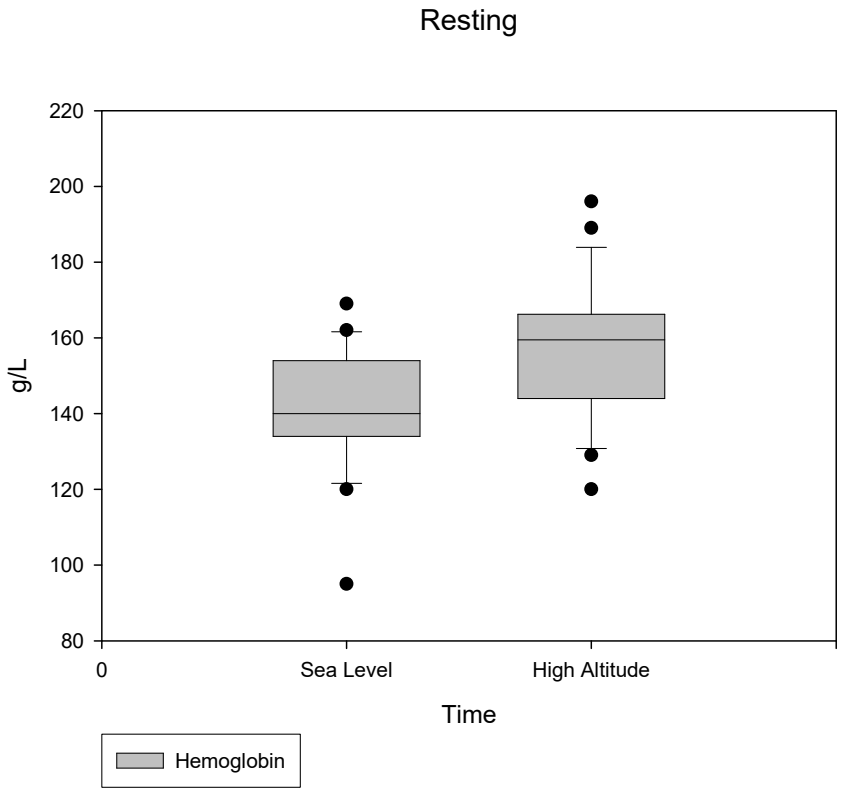

Figure 23 Hemoglobin at rest

Hemoglobin increased significantly ($p < 0.001$) in resting subjects when levels at sea level (69 m) 140.0 (95.0; 169.0) were compared to levels at high altitude (5050 m) 159.5 (120.0; 196.0) [g/l, Median (Min; Max) rounded values].

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