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Urineprint of high-altitude: Insights from analyses of urinary biomarkers and bio-physical-chemical features of extracellular vesicles

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ABSTRACT

Humans exposed to altitude hypoxia experience dysfunctions of the urinary system. As a non-invasive, easily manageable and informative biological sample, urine represents a relevant matrix for detecting clinical impairments of urinary system, as well as alterations of other systems and extracellular vesicles (EVs) biology during high-altitude expeditions. Nevertheless, gaps exist in the comprehensive assessment of dysfunction, molecular burden and EVs biology due to high-altitude acute exposure. This study aimed to find a biophysical and biochemical signature of urinary EVs for hypoxia-induced changes in urinary function, putatively accompanied by an oxinflammatory burden. Urine samples of 15 participants were sampled at low and high-altitude during an Alpine project (7 women and 8 men, aged 24-to-63 years and with BMI 17.93-to-30.76 kg/m^2) and analysed for: creatinin and albumin, lipid peroxidation, IL6, NO derivatives; atomic force microscopy and Raman spectroscopy were carried out after urinary EVs were isolated through sucrose-gradient ultracentrifugation. Albumin-to-creatinin ratio increased at high altitude, as did IL6 and 8-isoprostane. AFM showed a globular and flattened shape of EVs, although several samples were characterized by a lot of contaminants and EVs lost their prototypal spherical shape; EVs comprehensively maintained their morphology at high altitude. Raman spectroscopy revealed some typical phospholipidic-like pattern, often masked by contaminants of spectra that most often refer to high-altitude samples. Collectively, short-term exposure to altitude hypoxia increased renal concentrating ability, produced non-pathological impairment or renal function, and triggered an oxy-inflammatory burden with heterogeneous response of NO system. The combination of AFM and Raman spectroscopy revealed that EVs collected at high altitude more likely are fused together and incorporated into a sediment matrix, and contain contaminants peaks that make the purification process less efficient. The combination of analytical procedures as in the present study offers novel possibilities to detect the biological and clinical effects of high altitude on renal system.

1. Introduction

The impairment in urinary function due to high altitude is known since decades ago, although rather than hypoxia per se the effects were attributed to vibration, noise and temperature [1]. However, the effect on micturition due to hypoxia can be also exerted via neurological and humoral systems. As many expeditioners have experienced on themselves, humans exposed to acute hypoxia undergo diuresis and

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natriuresis [2]. Uroflowmetry and urodynamic test can reveal transient dysfunctions, although the pathological significance remains debated [3–5].

Urine is a non-invasive biological matrix that can serve for monitoring some human responses to hypoxia, in particular: 1) dehydration, as inferred from urine osmolality [6], and 2) renal acid-base compensation, which occurs to compensate respiratory alcalosis through HCO^{3-} excretion [7]. For what concerns biomarkers of urinary functions, glomerular filtration rate (GFR) is largely used, as it allows to achieve a simple, rapid and reproducible measurement from blood samples [8]. Blood creatinine and cystatin-C, despite being widely used, have limitations in determining the real GFR. Radiolabeled markers are cheap but unsafe. Inulin represents the most precise method but is difficult to be implemented in a clinical environment due to the costs and complexity of the procedure. Iothalamate is less precise than inulin but more convenient and easier to use. Iohexol represents a precise and safe method but yet to be implemented in humans. The ideal method should avoid 24-h urine collection, reduce the amount of blood, avoid radiolabeled substances, and be rapid. In addition, 24-h urine collection for assessing creatinine excretion can be difficult in field study, and sampling spot blood, although minimally, is an invasive method. Sampling spot urine can represent a good alternative by accounting for the feasibility and non-invasiveness of sampling during high altitude expeditions. Spot values can be used as a surrogate of continuous assessment for quantifying urinalysis parameters. Indeed, in most cases the measurement of urinary albumin and creatinine is performed on extemporaneous urine samples and the albumin/creatinine ratio (ACR) is calculated. Creatinine is a product of muscle metabolism normally excreted in the urine at a constant rate. Therefore, its quantity usually serves as an indicator of the concentration of the urine and it is used as a corrective factor, as for computing the albumin/creatinine ratios (ACR), that is used in the diagnosis of microalbuminuria, which indicates an alteration of the filter function of the renal glomerulus. To this extent, first morning void better represents 24-h collection than spot urine [9]. Indeed, recent guidelines advocate the use of ACR from spot urine, and a large sample study reported high agreement between early morning void and 24-h collection for quantifying ACR, with 88 % of participants in the three categories of <30, 30 to 300, and >300 mg/g [10]; the three categories refer to low, moderately increased and severely increased risk for kidney disease, respectively.

The usefulness of urine as a biological matrix is not limited to biomarkers that are clinically relevant in urinary medicine. Among a plethora of possible investigations, urine represents a valuable biological source for studying inflammatory markers in a non-invasive way that can be used in daily comparisons, thus limiting the burden for participants while providing an integrative measure of inflammation due to the presence of molecules which can cross the glomerular filtration barrier [11]. The combined study of inflammation and redox system disruption, named “OxInflammation”, has been demonstrated to be affected by the cumulative stressor of high altitude exposure and trekking by our group [12]. Our group also detected an altered oxi-inflammatory status as a response of running at hypoxia in samples of urinary extracellular vesicles (uEVs) [13].

The presence of EVs in urine has been known since decades ago. From the initial discovery of proteome cargoed by uEVs, the topical research has exponentially grown for the possibility of obtaining unexplored pathological biomarkers. However, given the heterogeneity of biochemical composition and the complex biophysical features of EVs, the identification of putative biomarkers is challenging. Beyond kidneys, bladder and urogenital tract, uEVs can refer to distant sites thereby providing a systemic readout of states, whose collection methods are non-invasive and allow to sample large quantities [14]. EVs thus reflect internal pathways; moreover, among their various functions, EVs serve as a mechanism for eliminating extracellularly toxic molecules [15]. Environmental factors of different nature influence physiological systems in ways that can be captured in part by the biophysical and molecular alterations of EVs. Among environmental stressors, a growing body of research investigated hypoxia-induced EVs, mostly focusing on cancer models, using incubation of cells with variable oxygen levels, and isolating EVs by ultracentrifugation protocols [16]. However, the growing interest in altitude travelling and the non-negligible presence of people living at high altitude rises the need to produce robust efforts in identifying the effect of hypobaric hypoxia on human EVs.

1.1. Aims of the study

Hypoxia affects urinary system and uEVs are increasingly considered as biomarkers of the renal system. Nevertheless, there is no specific evidence of the effect of hypoxia on uEVs in humans. Given this background, this study aimed: 1) to find a biophysical, biochemical or morphological signature of uEVs isolated from spot urine samples as an accessible and original monitoring for hypoxia-induced changes in urinary function, and 2) to find whether or not putative changes in uEVs are accompanied by a parallel dysfunction of urinary system with oxinflammatory burden.

2. Methods

2.1. Study design

As extensively reported elsewhere [17], the project “Monte rosa exploration & physiology” was carried out by trekking 4 days from Alagna Valsesia (1191 m a.s.l.) up to Capanna Regina Margherita (4556 m a.s.l.), thus encountering a range of temperature from −3.5 to 31.3 °c and of relative humidity from to 36 to 95 %. Urine samples were collected twice, at the lowest and highest altitude, on the same days for all participants.

2.2. Participants

As a whole, the expedition involved 15 participants, of which 7 women and 8 men, whose age ranged from 24 to 63 years and BMI from 17.93 to 30.76 kg/m^2. All of them took acetazolamide once daily 125 mg at 2370 m and 250 mg at both 3647 and 4556 m; 6 participants developed AMS as resulted from 2018 Lake Louise Acute Mountain Sickness Score; along with the massive reduction in peripheral saturation, on average heart rate and blood pressure both increased at high altitude [17].

2.3. Urine sampling and management

The pre-processing flow is reported following the recommendations of Urine Task Force of the Rigor and Standardization Subcommittee of the International Society for Extracellular Vesicles (ISEV) [18]:

1) Urine samples were collected in sterile containers as first void specimens in the early morning at lowest (Alagna Valsesia, 1191 m) and highest altitude (Capanna Regina Margherita, 4556 m), as a surrogate of 24-h collection achievable during field studies
2) At lowest altitude samples were stored in a freezer, at highest altitude samples were transported in boxes with ice packs down to lowest altitude in a few hours
3) All the samples were transported in boxes with ice packs to Chieti (duration of about half a day) for isothermal transport and before the night all the samples were inspected for integrity and then stored at −80 °C
4) Once thawed at 37 °C in water bath, a medium aliquote (9 ml) was allocated for EVs isolation and two small aliquots (1.5 ml each) were allocated for urinalysis and OxInflammation analysis

When applicable, the analytical procedures of uEVs are reported following the last report on “Minimal information for studies of

extracellular vesicles" (MISEV) [19].

2.4. Urynalisis

Albumin and creatinin dosage was carried out with automated clinical chemistry assays on Architect Plus (Abbott Laboratories, USA) by using in particular the immunoturbidimetric process for albumin and the kinetic alcaline picrate process for creatinin. Then, the values obtained in mg/l for albumin and mg/dl for creatinin were adjusted to obtain the albumin-to-creatinin ratio (ACR) in mg/g.

2.5. OxInflammation

Lipid peroxidation was detected by competitive immunoassay of 8-isoprostane concentration (8-isoPGF2α; Item No. 516360, Cayman Chemical, Ann Arbor, MI, USA). 8-isoPGF2α concentrations were determined using a standard curve. Samples and standards were read at a wavelength of 512 nm. This method has been previously described [12,20].

According to the manufacturer's instructions, IL-6 urinary levels were determined by an ELISA kit (Cayman Chemical, Ann Arbor, MI, USA, Item No. 501030), based on the double-antibody "sandwich" technique in accordance with the manufacturer's instruction [21,22]. NO derivatives, nitrate, and nitrite ($NO_2 + NO_3 = NOx$) were measured in urine samples by a colorimetric method based on the Griess reaction (Cayman Chemical, Ann Arbor, MI, USA, Item No.780001). Samples were read at 545 nm, and a standard curve assessed the concentration [21,22].

All the above samples and standards were read by a microplate reader spectrophotometer (Infinite M200, Tecan Group Ltd., Männedorf, Switzerland). The determinations were assessed in duplicate, and the inter-assay coefficient of variation was in the range indicated by the manufacturer.

2.6. Isolation of extracellular vesicles

The procedures was carried out as follows: 1) centrifugation at RCF 2000 x*g*, 20 min 4 °C; 2) surnatant centrifugation at RCF 10,000 x*g*, 30 min 4 °C, 3) addition of 1 ml of sucrose cushion 30 % (m/v PBS 1×) on fresh tube; 4) carefully addition of the collected surnatant; 5) centrifugation at RCF 100,000 x*g*, 90 min 4 °C; 6) surnatant remotion maintaining cushion volume; 7) addition of PBS 1× to reach the final volume and mixing of sample by turning it upside down; 8) centrifugation at RCF 100,000 x*g*, 70 min 4 °C; 9) Resuspension in ≈ 20–25 μl of PBS 1× (Fig. 1). The use of sucrose density gradient allows to obtain highly pure EVs samples, although this method is time consuming, requires special equipment and results in low yield. For the aim of this work, the use of a sucrose cushion represented the trade-off for limiting the disadvantages while maintaining those advantages that optimise further analyses. After resuspension, 1 μl was immediately used for nucleic acid-to-protein ratio (NPr), estimated by UV absorbance at 260 nm and 280 nm, respectively, by UV–vis spectrophotometry in a Nanophotometer NP80 (Implen, Germany), calibrating the instrument using sample elution buffer Blank Control™ (Implen, Germany) before the test [23]. The samples were stored at −80 °C until the further analyses.

2.7. Atomic force microscopy (AFM) investigation

Atomic force microscopy was used to study the size distribution and the morphology of EVs isolated from urine collected at low and high altitudes by using MultiMode 8 AFM microscope with Nanoscope V controller (Bruker, Billerica, Massachusetts, US). To analyze the urinary EVs, the samples were properly diluted with MilliQ water and deposited onto a SiO_2 substrate followed by drying at 37 °C for 2 h and then at room temperature overnight. The obtained samples were scanned in air environment by the silicon ScanAsyst-Air probe (triangular geometry, cantilever resonance frequency 70 kHz and nominal spring constant 0.4 N/m) and information on dimensions and morphology of the vesicles were obtained from images of 512 × 512 pixels collected with different scan sizes and elaborated using the Nanoscope Analysis 1.8 software.

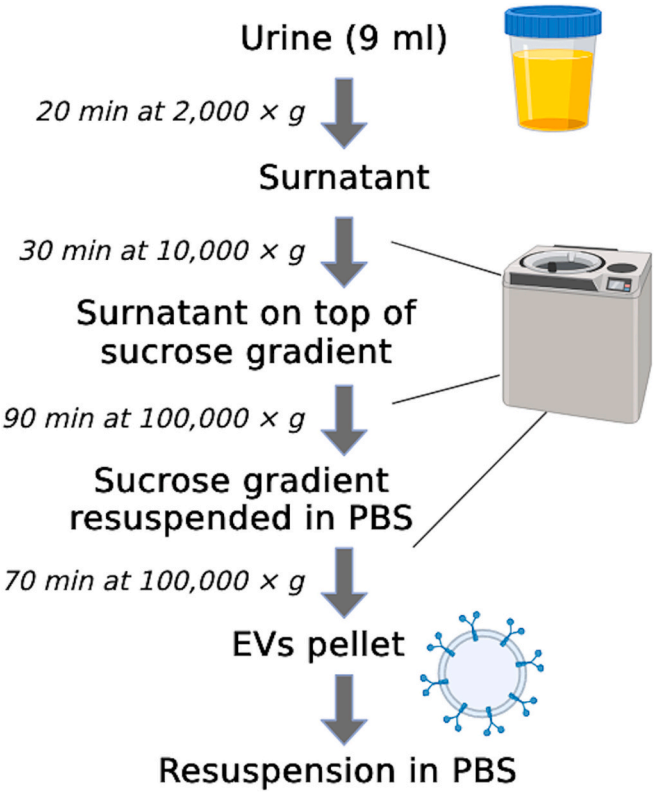

Fig. 1. Protocol of EVs isolation from urine samples. Image created with BioRender (www.biorender.com)

2.8. Raman spectroscopy analysis

Raman spectra of exosomes on SiO_2 substrate were collected using a confocal and high-performance Raman microscope (XploRA PLUS, HORIBA, Japan) with deep-cooled CCD detector technology. LabSpec (Horiba, Japan) was employed to control the Raman spectroscopic system and optimise and process the acquired data. Samples were analysed through a 532 nm laser, and 10 s and 10 accumulations were chosen to optimise spectra acquisition. Raman measurements were collected in a 550–3200 cm^{-1} range with 1800 line/mm grating. Furthermore, 10 mW of power was chosen, focusing on a 50× objective to avoid excessive surface heating. Eventually, data were manipulated using LabSpec (Horiba, Japan) to fit spectra through Lorentzian peak fitting. Thus, the data obtained are Lorentzian deconvolution of the obtained spectra.

2.9. Statistical comparisons

After assumption checks of normality of residuals and eventual remotion of outliers, a series of paired-sample *t*-test were carried out to compare low vs high altitude results. If assumption was violated, values were log-transformed before computing *p* values. Statistical analyses and graphs were made with GraphPad Prism (v. 10.1.1). Effect size was computed as Cohen's d for repeated mesures (dRM) that is effect size for within-subjects designs referred as Cohen's dz. controlled for the correlation between the two sets of measurements [24], as.

$d_{rm} = M_{diff}/\sqrt{(SD1^2 + SD2^2 - 2 \times r \times SD1 \times SD2)}$; Hedges' g for repeated measures (gRM) was also calculated through an online tool

(Uanhoro, J. O. (2017). Effect size calculators. *Available online at: https://effect-size-calculator.herokuapp.com/*.), as it is corrected for both correlation and sample size.

3. Results

3.1. Urynalisis

We already reported that urine osmolality did not significantly change at high altitude (612 ± 320 to 595 ± 201; $p = 0.879$, d = 0.041), while urinary pH increased at altitude (from 6.15 ± 0.69 to 7.13 ± 0.32; $p < 0.001$, d = 1.502) [25]. Herein we report urinalysis results based on albumin and creatinin, as shown in Fig. 2. 12 out of 15 participants had increased values of albumin at high altitude (mean difference: 2.14 mg/l, 95 % CI –2.51 to 6.79 mg/l; $p = 0.011$, dRM = 0.255; gRM = 0.244). Creatinin changes were more heterogeneous across participants, although most of them experienced a reduction or a negligible increase and only one experienced a substantial increase (mean difference: −17.3 mg/dl, 95 % CI –39.8 to 5.18 mg/dl; $p = 0.121$, dRM = 0.466; gRM = 0.595). As a result of creatinin and albumin changes, ACR substantially increased at high altitude (mean difference: 9.27 mg/g, 95 % CI 2.73 to 1.82; $p = 0.009$, dRM = 0.785; gRM = 0.700); at low altitude, only one participants had values over the clinical threshold of 30 mg/g and that participant remained over threshold at high altitude; instead, two participants that were under threshold at low altitude raised over the first threshold of microalbuminuria at high altitude.

Spot osmolality/creatinin ratio (Uosm/Ucr) increased at altitude (mean difference: 37.3 mOsm/mmol, 95 % CI 17.6 to 57.1 mOsm/mmol; $p = 0.001$, dRM = 1.090; gRM = 1.026); obviously, the inverse index (Ucr/Uosm) decreased at high altitude (mean difference: −0.034 mmol/mOsm, 95 % CI –0.056 to −0.013 mmol/mOsm; $p = 0.004$, dRM = 0.920; gRM = 1.013).

3.2. OxInflammation

As shown in Fig. 3, IL-6 consistently increased at high altitude (mean difference: 2.64 pg/ml, 95 % CI 1.56 to 3.72 pg/ml; $p < 0.001$, dRM = 1.554; gRM = 1.837), as did 8-isoprostane to a lower extent (mean difference: 155 pg/mg of creatinin, 95 % CI 22.6 to 287 pg/mg of creatinin; $p = 0.025$, dRM = 0.678; gRM = 0.788). Instead, NOx did not change homogeneously across participants (mean difference: 101 μM, 95 % CI –93.3 to 295 μM; $p = 0.282$, dRM = 0.300; gRM = 0.229).

3.3. Atomic force microscopy

AFM represents one of the main high-resolution imaging techniques to assess sample heterogeneity and morphological properties, as well as a useful method to confirm the efficiency of the isolation and purification protocols of EVs. The urinary EVs collected at low altitude (LA) and high altitude (HA) were subjected to the same dilution treatment with MilliQ water and deposited onto SiO_2 substrates, characterized by high surface flatness. As shown in AFM micrographs reported in Fig. 4 and Figs. S1 and S2 in the Supplementary Material, the isolation protocol resulted very effective for the EVs extraction from all the samples, except for MR1-LA where no vesicular structures were detected. Indeed, the AFM probe tracked the morphology of only salt crystals and the absence of EVs was further confirmed by the disappearance of C—H stretching deformation of lipid molecules in the region 2800–3200 cm^{-1} registered by Raman spectroscopy (see section *Raman spectroscopy*). At high scan sizes (10 μm × 10 μm), most samples were characterized by the presence of lots of sediments with micrometric dimensions in addition to smaller vesicular structures in isolated form. Zooming into the clean areas, the vesicles showed a globular and flattened shape, due to alteration induced by the drying step. In some cases, such as for MR5 LA, MR7 HA, MR9 HA, MR12 HA, MR13 HA and MR15 HA (Figs. S1 and S2 in the Supplementary Material), the samples were characterized by a lot of contaminants and EVs lost their spherical shape as they resulted fused and incorporated into a sediment matrix. Furthermore, the samples MR4, MR8 and MR10 showed the presence of flattened material spread over the surface which is consistent with the complete fusion of the vesicles with the consequent formation of bilayer films adhering onto the silicon substrate. The Particle Analysis tool of the software was used to detect and measure the size of the vesicles and was applied to several AFM images to collect data from the analysis of tens of particles. As reported in Table 1, the EVs populations showed a mean diameter in the range 70–250 nm, consistent with the presence of exosomes and small ectosomes. However, no correlation in mean diameters was detected for urinary EVs isolated at low and high altitude.

3.4. Raman spectroscopy

As a preliminary check before Raman spectroscopy, the 260/280 ratio from UV–vis spectrophotometry was registered immediately after the EV isolation, indicative of the relative composition of nucleic acid and protein, since phospholipids that mainly constitute vesicles' membranes are low-UV-absorbing molecules, thereby serving to evaluate the purity and relative protein or nucleic acid contamination. We used it as quality control, resulting in values with an average of 1.055 (95 % confidence interval: 0.865 to 1.245), in line with expectations and compatible with the presence of proteins and nucleic acids [23]. The Raman spectra of EVs isolated from urine collected at low and high altitudes were compared to highlight differences in uEVs composition. Table S1 in the Supplementary Material indicates well recognized Raman bands that have been previously observed in samples of exosomes.

In Fig. 5, the most representative spectra obtained through Raman spectroscopy of EVs are reported. The regions between 1000 and 1500 cm^{-1} and between 2600 and 3200 cm^{-1} are mostly related, as described in Table S1 of the Supplementary Material, to alkyl chains (L) mainly of lipidic origin [26]. Meanwhile, the region between 1500 and 1800 cm^{-1} is typically assigned to protein and aminoacidic stretching (P) [27]. Furthermore, the peak at around 1650 cm^{-1} is the fingerprint of primary amide [27–29], while other peaks in the P region are related to Trp, Phe and Tyr [28]. Each spectrum is reported in the Fig. S3-S14 of the Supplementary Material.

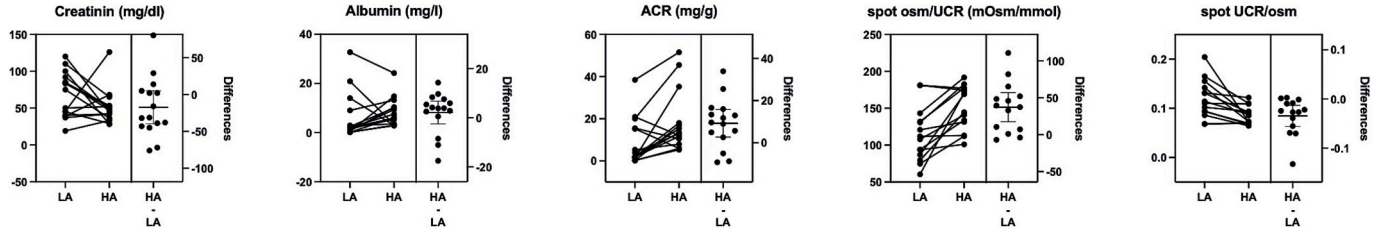

Fig. 2. Results of urinalysis; on each box, the left part represents the low vs high altitude individual comparison, while the right part represent individual high – low altitude differences.

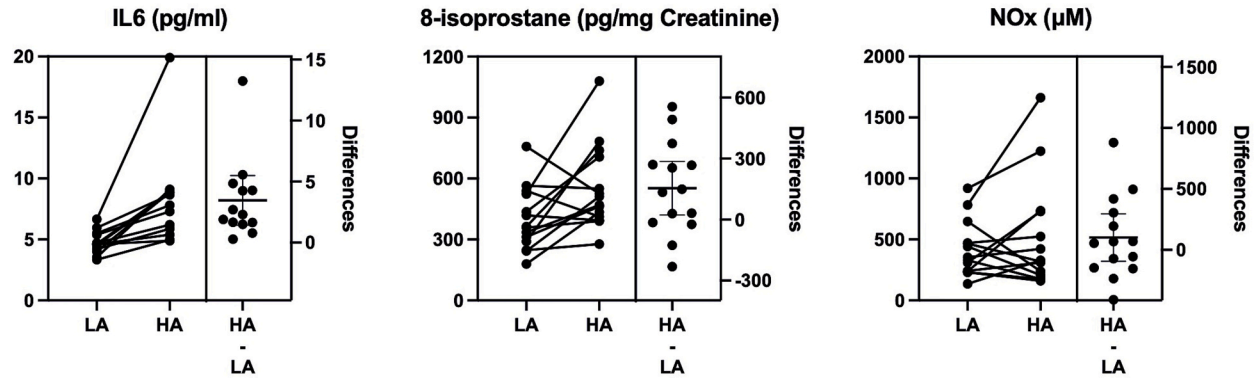

Fig. 3. Results of oxinflammatory biomarkers; on the left side of each box, the low vs high altitude individual comparison, while on the right side the individual high – low altitude differences.

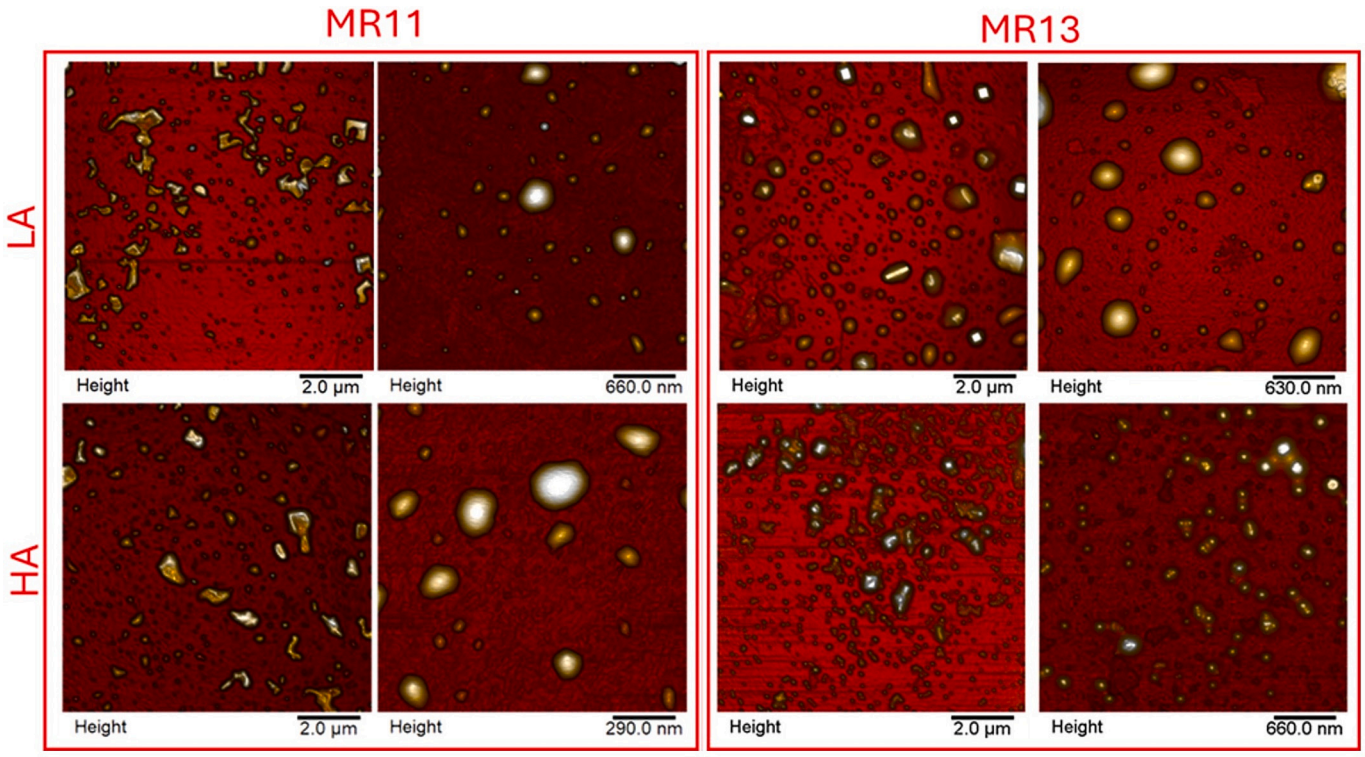

Fig. 4. AFM micrographs of topography of EVs isolated at low (LA) ad high altitude (HA) from the MR11 and MR13 urine samples collected during the Monte Rosa Expedition. For each condition two different micrographs were reported: an initial 10 μm × 10 μm scan size picture and a zoom into a smaller and particular area of the same region.

Table 1
Mean diameter (± standard deviation) values of urinary EVs isolated at low (LA) and high altitude (HA) measured by the particle analysis tool of the AFM Nanoscope Analysis software. Values are expressed in nm.

Sample	LA	HA	Sample	LA	HA	Sample	LA	HA
MR1	–	161.0 ± 70.0	**MR6**	122.3 ± 72.7	166.2 ± 103.4	**MR11**	107.2 ± 62.8	97.5 ± 59.1
MR2	103.2 ± 44.4	137.4 ± 61.7	**MR7**	221.5 ± 102.4	164.0 ± 64.9	**MR12**	96.5 ± 54.7	127.3 ± 45.9
MR3	–	71.9 ± 26.7	**MR8**	118.4 ± 59.3	252.6 ± 169.6	**MR13**	123.5 ± 96.1	111.6 ± 65.7
MR4	143.9 ± 40.5	134.2 ± 56.0	**MR9**	116.3 ± 67.4	91.0 ± 38.0	**MR14**	68.3 ± 55.2	123.9 ± 45.3
MR5	95.6 ± 43.8	100.4 ± 37.1	**MR10**	99.1 ± 42.3	143.3 ± 78.8	**MR15**	163.3 ± 91.5	82.7 ± 53.1

3.5. Summary report

A comprehensive account of the diverse issues pertaining to each sample, along with those encountered during the isolation of uEVs and subsequent analytical procedures, is provided in Table 2. Details of Raman spectroscopy are provided in the Supplementary Material.

4. Discussion

The topic of uEVs have attracted a massive interest in scholars and

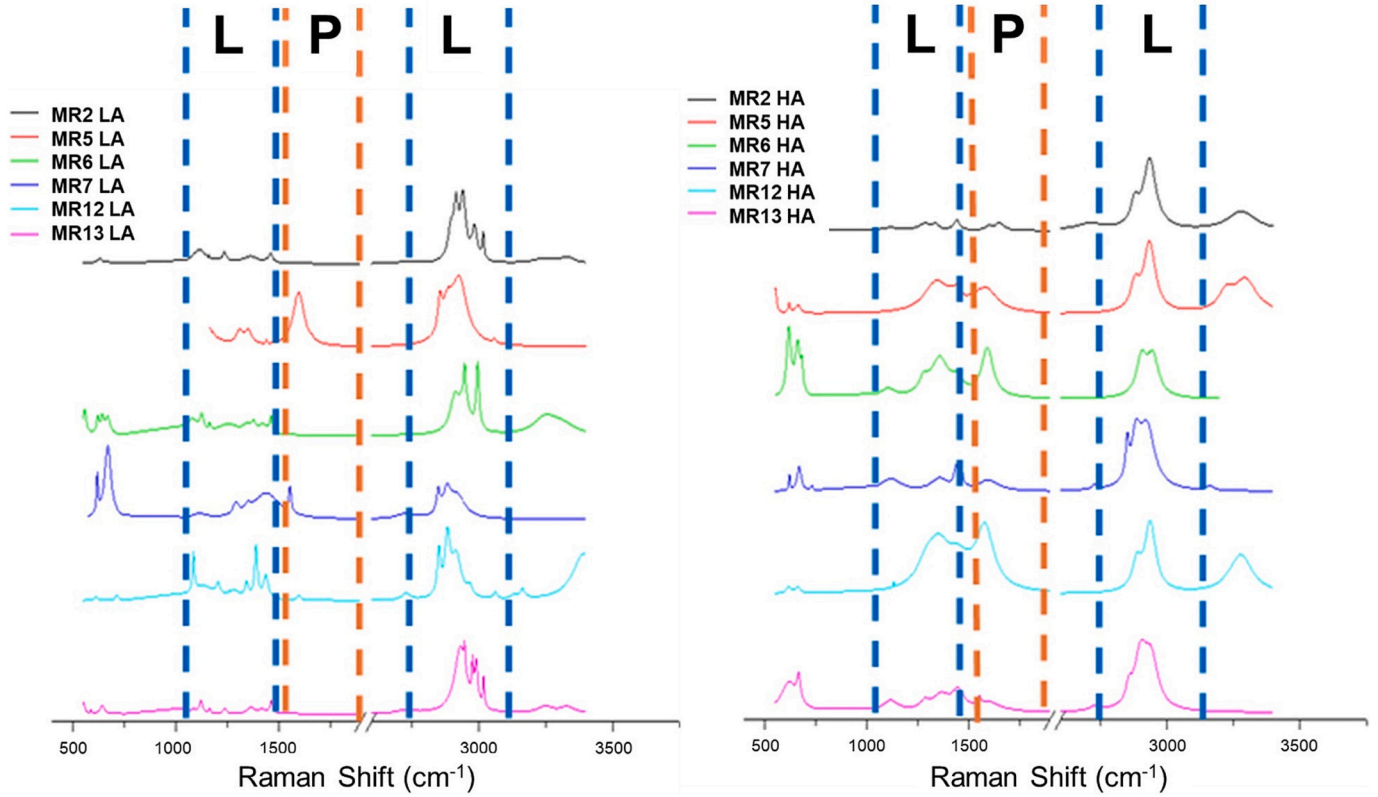

Fig. 5. Comparison between Raman spectra of some EVs isolated at low (LA, left panel) and high altitude (HA, right panel) from urine samples collected during the Monte Rosa Expedition. Orange lines highlight regions where aminoacidic and protein signals (P) are present, while the phospholipidic regions (L) are highlighted between blue lines, as reported in Table 2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

clinicians interested in kidney diseases. However, the possibility of using urine as a non-invasive sample has opened intriguing perspective beyond kidney pathophysiology only. For example, our group investigated uEVs to detect the effect of environmental exposure on metabolic pathways [30] and to identify the long-term effect of physical activity [31], and generally this ever-expanding scientific field has added to technical and medical focuses an ancillary physiological focus [32].

Firstly, we explored urinary function through several biomarkers. Albumin concentration increased at high altitude, while creatinin concentration tended to decrease. As a result, albumin-to-creatinin ratio substantially increased at high altitude. The increase of this parameter usually indicates a damaged kidney, with higher risk for kidney and heart failure; however, alterations usually anticipate subsequent development of nephropathy. Rather than considering ACR values as a proxy of existing endothelial dysfunction that reflects underlying macrovascular and microvascular disease as in clinic routine [33], we considered our results as transient sub-clinical kidney dysfunction. Uosm/Ucr serves as an estimation of renal concentrating function by normalizing urinary excretion of solutes to excretion of creatinin, assumed to be constant in non-pathological states. Greater values can refer to acute kidney injury or dehydration status, although water loading can acutely rise values [34]. Since we did not observe substantial changes in bodily hydration status and the changes in creatinin were not homogeneous, we can interpret our results of greater spot Uosm/Ucr as an increased renal concentrating ability at high altitude.

Usually, renal excretion function is estimated by serum creatinin, under the assumption that the urinary excretion rate of creatinin is steady. However, in situations where creatinin excretion changes, assuming daily renal load and urinary excretion of osmoles is stable, the Ucr/Uosm can serve as a plausible surrogate of urinary creatinin excretion rate. Indeed, neither spot urine creatinin nor osmolality per se represent adequately urinary excretion function, while spot Ucr/Uosm, as the relative value of excreted creatinin to osmoles is independent of actual urination intervals and volumes [35]. Diminished values should be considered negatively, and values lower than 0.08 account for highest risk of acute accumulation of creatinin in the body, as resulting from the cross-sectional study on 3316 health adults [35]. Therefore, our results of lower Ucr/Uosm can be interpreted as negative changes at high altitude that remained non-pathological.

4.1. OxInflammation

As is well known, the inflammatory state and ROS homeostasis are involved in the human hypoxic response, potentially also relevant to the mediation of diseases that induce a hypoxic state [20,36]. In particular, kidneys have an abundant blood supply: 20 %–25 % of cardiac output) and high blood flow; thus, they are susceptible to the effects of hypoxia [37]. Our data once again confirm the synergy of hypoxic stress with inflammation and, in this case, with lipid damage. Despite IL-6 and 8-isoprostane increased at high altitude, nitric oxides did not. These metabolites in spot urine have been demonstrated to be a surrogate of 24-h urine and representative of NO production [38]. Considering that NOx increased after a longer exposure to hypoxic trek [12], we may interpret our results as heterogeneous response of NO system to short-term altitude hypoxia. Nitrites and nitrates become poorly indicative of NO production when renal function is altered [39]; however, in the present study, we found only sub-clinical alterations of renal function. Therefore, further studies should clarify the time-dependent response of the NO system at hypoxia while specifying whether urinary NOx are altered when renal function is slightly affected.

Table 2
Summary report of urine quality and troubleshooting during the sequential processes.

ID	Sex	Age (y)	Quote	Urine inspection	Osmolality (mOsm/kg)	Isolation issues	AFM issues
MR1	m	31	LA	Dark yellow	829	No issue	No vesicles detected
			HA	No issue	568	No issue	Clean area full of spherical EVs
MR2	f	24	LA	Lots of sediment	795	No issue	Presence of contaminants with spherical EVs
			HA	No issue	538	No issue	Presence of contaminants with spherical EVs
MR3	m	36	LA	/	/	/	/
			HA	No issue	681	Possible contamination	Lots of spherical EVs with few crystals
MR4	m	34	LA	No issue	347	No issue	Presence of flattened material with few spherical and wrinkled EVs
			HA	Lots of sediment	1033	No issue	Presence of flattened material and crystals with few spherical EVs
MR5	f	25	LA	No issue	324	No issue	EVs embedded into a matrix of sediments and crystals
			HA	No issue	480	No issue	Lots of spherical and globular EVs
MR6	f	54	LA	No issue	523	No issue	EVs both in isolated and aggregated form with presence of debris
			HA	No issue	451	Not perfect sucrose level	Lots of spherical and well defined EVs
MR7	m	63	LA	Lots of sediment	1468	No issue	Lots of sediment with few large and spherical EVs
			HA	No issue	402	No issue	Presence of EVs fused and embedded into a matrix of sediments and crystals
MR8	f	29	LA		417	No issue	Lots of not spherical EVs in isolated form
			HA	Dark yellow	980	No issue	Presence of flattened material and debris with large EVs
MR9	m	29	LA	Lots of sediment, dark yellow	727	No issue	Lots of sediment with few spherical EVs
			HA	No issue	400	No issue	Lots of sediment with fused EVs
MR10	f	32	LA	Clear	93	No issue	Lots of sediments and crystals with few spherical EVs in isolated form
			HA	No issue	458	No issue	Presence of flattened material and debris with EVs in isolated form
MR11	m	24	LA	Lots of sediment	708	No issue	Lot of spherical EVs with debris
			HA	No issue	762	No issue	Presence of debris and spherical EVs
MR12	f	34	LA	Lots of sediment	649	No issue	Lots of sediments and crystals with spherical EVs in isolated form
			HA	No issue	510	No issue	Presence of EVs fused and embedded into a matrix of sediments and crystals
MR13	f	27	LA	No issue	588	No issue	Lots of spherical EVs in isolated form with some crystals
			HA	No issue	594	No issue	Presence of EVs fused and embedded into a matrix of sediments and crystals
MR14	m	51	LA	Lots of sediment	648	6 ml rather than 9 ml of volume	Lots of not spherical EVs in presence of crystals
			HA	No issue	671	No issue	Lots of globular EVs
MR15	m	26	LA	Lots of sediment	454	No issue	Lots of sediment with large EVs
			HA	No issue	477	No issue	Presence of EVs fused and embedded into a matrix of sediments and crystals

LA: low altitude; HA: high altitude; atomic force microscopy.

4.2. Insights and technical considerations from AFM and Raman spectroscopy

Despite the growing interest, the use of EVs in clinical applications is limited due to the lack of standardization in EVs isolation and analysis, especially when dealing with complex and highly heterogeneous biological samples. Different strategies for EVs characterization are currently available, including electron microscopy, atomic force microscopy (AFM), flow cytometry and various chemical/biological techniques which provide important information on vesicle morphology and composition. Furthermore, the technological advances in metabolomics provide a powerful approach to expand EVs research and the two main analytical techniques that have recently been applied to EVs metabolomics are nuclear magnetic resonance (NMR) and high-resolution mass spectrometry (MS) [40,41]. NMR is a highly reproducible spectroscopic method based on the energy absorption and re-emission of the atom nuclei in an external magnetic field. ^{1}H NMR is the most targeted nucleus for its natural abundance in biological samples [42,43]. One-dimensional NMR (1D-NMR) spectra using a single frequency axis is the most often applied method in metabolomics studies, even though to have a better peak resolution, the 2D-NMR method is sometimes applied to separate overlapping spectral peaks that cannot be identified with 1D-NMR, especially in case of complex biological matrices [44,45].

Mass spectrometry (MS) acquires spectral data in the form of a mass-to-charge ratio and a relative abundance of the measured compounds, which has been extensively studied for metabolomics. Compared to NMR, the MS method is considered with higher specificity and sensitivity given a wide range of instrumental and technical variants that are currently available for metabolite characterization.

On the other hand, recent studies have shown applications of Raman spectroscopy as a fast and alternative tool for characterization, compositional studies, and purity assessment of EVs derived from different tissues and biological fluids. Raman spectroscopy, based on anelastic scattering of light, is beneficial in being a label-free, chemical specific, and relatively non-destructive technique detecting molecular vibrations that can identify essential components present in EVs, such as membrane lipids, proteins and nucleic acids, without the need to disrupt the EVs before the measurements.

Considering this background, the effects of high-altitude exposure on the parameters of extracellular vesicle (EVs) isolated from urine were studied with a combination of complementary techniques. The EVs are important for physiological adaptations, also triggered by altitude hypoxia. The urinary EVs were obtained by a well standardized protocol of isolation by ultracentrifugation and characterized by AFM. As shown in AFM micrographs reported in Fig. 4 and S1-S2 of Supplementary Materials, the isolation protocol resulted very effective for the EVs extraction. Indeed, most of the samples revealed the presence of a high number of globular shaped extracellular vesicles present on the

substrates. In the majority of the samples, the EVs collected at high altitude showed a higher concentration of contaminants and salts with respect to EVs collected at low altitude, resulting in EVs fused together and incorporated into a sediment matrix.

Raman spectroscopy was also used to identify the presence of EVs molecular signatures or biomarkers in response to the exposure to high altitude. In line with AFM results, the isolation procedure resulted very effective for the EVs extraction, since most of the Raman spectra showed the typical phospholipidic pattern related to C—H stretching deformation of lipid molecules in the region 2800–3200 cm^{-1}. As reported in Fig. 5, the most significant part of those spectra are clear, confirming that Raman spectroscopy is a suitable technique to analyze purity in EVs obtained through the sucrose gradient technique, which contrasts with the literature [46]. However, as shown in Fig. 5, the Raman spectra of several samples collected at high altitudes seem to be more disturbed than their low altitude counterparts. Indeed, as explained in more detail in Supplementary Materials, EVs obtained by urine collected after the body has been stressed by altitude seem to contain contaminants peaks (i.e. peaks connected with aminoacids or nucleic acids) [46,47], making the purification process less efficient. The presence of more salts and contaminants outside the vesicles could be responsible of the distortion of the phospholipid CH_2 and CH_3 stretching bands recorded in some samples. Furthermore, shifts of these bands and changes in the ratio of peaks are usually associated with different interactions of phospholipids and guest inserted in the vesicle bilayer, as already widely described in the literature. Both changes refer to various packing and degrees of freedom of hydrocarbon chains of phospholipidic structures [48–52].

Fig. 6 reports the Raman spectra of the unique samples out of 15 pairs whose high-altitude sample spectrum appears less contaminated than the low altitude collected sample one. Indeed, as shown in Fig. 6, both the spectra, the one referring to HA sample and that referring to the LA sample, seem contaminated, but the shape of the band indicative of the phospholipid alkyl chain of the LA sample is clearly more disturbed and almost unidentifiable. This could be related, as in samples collected at high altitudes described broadly above, to a crown protein that shields EVs, reducing the contribution to the spectrum of the lipidic chain in favor of protein domain signals [53].

4.3. Limitations

We cannot exclude that changes in urinary pH affected uEV physiology and isolation, as reported [14]. The rise in urinary pH was expected, since metabolic acidosis is a normal compensatory of humans at high altitude in response to respiratory alkalosis. Acetazolamide exacerbates this mechanism by inhibiting carbonic anhydrase in the kidneys, resulting in bicarbonaturia and generating metabolic acidosis thereby attenuating the effects of respiratory alkalosis due to the hypoxic-induced rise in ventilation. This drug also induces diuresis and natriuresis [2]. However, during this expedition dehydration did not occur, as evidenced by urine osmolality and by other analyses [25], likely because trained staff instructed all participants how to avoid dehydration. This drug could have potentially influenced the results, by altering uEVs' release, cargo and subtype; however, all participants took the same amount of drug with the same time schedule, thus reducing the possible confounding effects. We cannot rule out the possibility that the non-use of protease inhibitors altered the protein composition of the sample; however, all the samples were processed identically, and not all EV proteins are prone to proteolysis in urine [14]. The necessity to transport urine from high to low altitude may have possibly altered the samples; however, the difference on storage between the samples obtained at low vs high altitude consisted in a few hours, i.e., the transport from Capanna Margherita to Alagna Valsesia; all the rest of sample storage and transportation was identical.

5. Conclusions

Under exposure to altitude hypoxia, the lower partial pressure of oxygen leads the body to implement a series of compensatory mechanisms, collectively affecting human physiology. After 4 days across from low to high altitude, a sub-clinical dysfunction occurred due to short-term stress on the kidneys; additionally, a synergy of hypoxic stress with inflammation and lipid damage was detected in urine samples. The combination of AFM and Raman spectroscopy allowed to verify the effectiveness and purity of uEVs isolation. In the majority of the samples, the EVs collected at high altitude contained more contaminants and

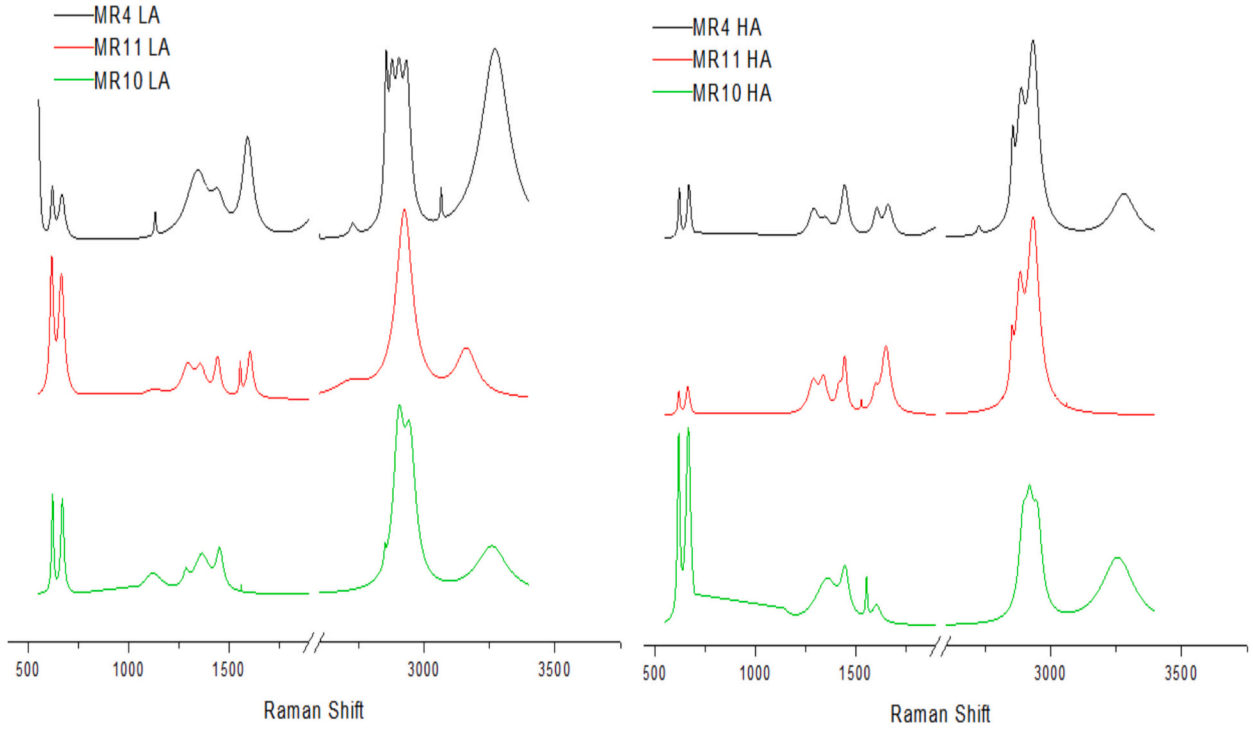

Fig. 6. Comparison between some samples collected at low altitude (LA) and high altitude (HA), on the left and on the right, respectively.

salts, resulting in EVs fused together and incorporated into a sediment matrix, with Raman peaks connected with aminoacids or nucleic acids that altered the typical phospholipidic pattern. The sub-clinical impairment of renal function at high altitude likely altered both the cargo of uEVs and the fluid in which uEVs were contained, thereby putatively altered crown protein that shields EVs. The high-altitude *urineprint* that includes clinical and oxinflammatory variable linked with EVs biology represents a promising avenue of research in the field of environmental physiology, with the potential for significant implications in the domain of translational medicine for hypoxia.

CRediT authorship contribution statement

Serena Pilato: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Simona Mrakic-Sposta:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Vittore Verratti:** Writing – review & editing, Supervision, Resources, Project administration, Conceptualization. **Carmen Santangelo:** Writing – review & editing, Investigation, Conceptualization. **Stefano di Giacomo:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis. **Samanta Moffa:** Writing – review & editing, Investigation. **Antonella Fontana:** Writing – review & editing, Supervision, Methodology. **Tiziana Pietrangelo:** Writing – review & editing, Supervision. **Federica Ciampini:** Writing – review & editing, Investigation. **Sofia Bonan:** Writing – review & editing, Investigation. **Pamela Pignatelli:** Writing – review & editing, Investigation, Conceptualization. **Carmine Noce:** Writing – review & editing, Investigation. **Pietro di Profio:** Writing – review & editing, Investigation. **Michele Ciulla:** Writing – review & editing, Investigation. **Danilo Bondi:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Fabrizio Cristiano:** Writing – original draft, Investigation, Formal analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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