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RESEARCH ARTICLE

Evidence of Limonene in Breath Samples in Men from the World's Highest City

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ABSTRACT

A study to measure the composition of exhaled air was carried out in subjects living in the highest city in the world: La Rinconada in Peru.
People living at high altitude have a strongly disturbed metabolism and have to deal with major physiological disturbances. It is very likely that these subjects develop a disturbance of their redox system. It was therefore undertaken to study their ability to resist oxidative stress by measuring markers in the exhaled air. The traditional biomarkers encountered in this case are the alkanes resulting from the oxidative degradation of unsaturated fatty acids.
The breath was collected on carbotrap tubes thanks to the Exp'Air sampling system. The analyses were carried out by GC-MS. In addition to some alkanes, witnesses of the particular conditions of their exposure to the low quantities of oxygen present in the air, it was discovered surprisingly the presence of a compound rarely detected in the exhaled air, limonene. Hypotheses are presented but no certainty exists as to the origin of this compound in the breath. Further studies are underway to determine the conditions and circumstances of occurrence of limonene in the exhaled air of these subjects chronically exposed to hypoxia.

Keywords: Volatile organic compounds; alveolar breath; limonene; alkanes; gas chromatography-mass spectrometry; Rinconada; chronic mountain sickness

Introduction

Over the world, more than 80 million of people live in a high-altitude environment (i.e., above 2,500 m) and are chronically exposed to hypoxia [1], the magnitude of which exponentially increases with the altitude[2]. The highest known city in the world, La Rinconada, is located in the southeastern of Peru, at an altitude of 5,100-5,300 m[3]. In this gold mining city, more than 50,000 inhabitants permanently live in a highly hypoxic environment, but also in crowded and precarious sanitary conditions[3 4]. To deal with this permanent hypoxic stress, it is well known that native highlanders have developed over time several adaptive responses, including, especially in Andean population, high levels of hemoglobin concentration ([Hb]) compared to lowlanders, in order to preserve the amount of oxygen delivered to the tissues[5]. Thus, the highest levels of hemoglobin mass ever measured in healthy humans have been reported in La Rinconada[6], where more than 40% of inhabitants exhibit an excessive erythrocytosis (EE), according to the current consensus statement (i.e., [Hb] ≥ 21 g/dL for males or ≥ 19 g/dL for females)[7 8]. This increase in [Hb], contributing to an increase in blood viscosity [9], may trigger the occurrence of clinical symptoms and lead to the development of chronic mountain sickness (CMS), the most prevalent form of maladaptation to chronic hypoxia in highlander populations[10]. Thereby, a prevalence of CMS near 14% has been previously reported in La Rinconada[7]. Highlanders suffering from CMS may develop chronic pulmonary hypertension, which may secondarily evolve toward right heart failure[11]. Furthermore, CMS has been shown associated with oxidative-nitrosative stress and systemic vascular dysfunction[12].

Formation of free radicals and lipoperoxidation occur at the onset of cellular damage[13]. Reactive oxygen species (ROS) are constantly formed in the human body and are removed by antioxidants. Tissue destruction and degeneration can result in increased oxidation damage, by such processes as metal-ion release, phagocyte activation, lipoxygenase activation and disruption of mitochondrial electron transport chains. Non-invasive methods indicating the load of ROS at metabolic equilibrium as well as under conditions of oxidative stress are important to estimate the extent of potential damage by ROS in humans.

In this mind research focused on the measurement on n-pentane which is a hydrocarbon gas produced by peroxidation of Omega-6 fatty acids (linoleic family) and which can pass through the lungs into the expired air[14 15 16 17 18]. However the effect of oxygen on markers of oxidative stress has not been totally elucidated because previous studies have yielded conflicting results [19].

Spectrum of alkanes was investigated to replace ethane and pentane determination to reinforce the specificity of the method[20]. This first step constrained Phillips et al. to measure new breath markers of oxidative stress in healthy subjects. The criterion standard of oxidative stress was the breath methylated alkane contour (BMAC), a three-dimensional plot of the alveolar gradient of C4-C20 alkanes and monomethylated alkanes produced by lipid peroxidation[21]. This method has already been carried-out to investigate the oxidative stress in younger as well as in older humans. Breath markers of oxidative stress as BMAC were significantly increased both in younger and in older subjects which may be a normal physiological response in youth[22].

From another point of view, Mathews et al. described volatile organic compounds (VOCs) that were routinely detected and measured in system blank, including pentane and low concentrations of other aliphatic, aromatic, and some oxygenated compounds. Pentane, benzaldehyde, and acetophenone were the major background contaminants and arose from the Tenax-GC itself. Moreover a number of chemicals presumed to be exogenous origin also showed marked increased[23].

Recently, a study investigated multiple chemical sensitivity (MCS) by analysing volatile organic compounds (VOCs). The authors conclude that breath testing is a valuable tool to investigate the hypoxia-related VOC profile, facilitating MCS diagnosis[24].

In addition, it has been described that limonene in exhaled breath is elevated in hepatic encephalopathy[25 26].

Limonene, with the gross formula $C_{10}H_{16}$, is a terpene hydrocarbon present in many essential oils from which it can be obtained by distillation. At room temperature, it is a colourless liquid with a bright, fresh, clean orange odour, characteristic of citrus fruits.

Limonene is used as a solvent in cleaning products, food manufacturing, perfumery and personal care products, and as an insecticide.

Limonene gets its name from the lemon, which, like other citrus fruits, contains considerable amounts of this chemical compound, which is largely responsible for their fragrance.

Limonene is a chiral molecule, and, as with many chiral molecules, biological sources produce a specific enantiomer. The main industrial source, orange, contains d-limonene ((+)-limonene), which is the dextrorotatory R-enantiomer. Eucalyptus and peppermint, on the other hand, contain l-limonene ((-)-limonene), which is the levorotatory S-enantiomer. The racemic limonene is known as "dipentene ".

Limonene can promote wound healing and anabolism, while improving stress, depression, inflammation, oxidative stress, spasms and viral infections. In addition, it has a variety of anti-cancer and anti-tumor mechanisms.

Materials and methods

Participants

Highlanders. 41 male highlanders, inhabitants of La Rinconada (5,100-5,300 m), all native from the Altiplano high-altitude area (above 3,500 m) and permanently living in La Rinconada from more than 3 years were included in the present study.

Lowlanders. Alveolar gas measurements were also performed in 7 healthy male lowlanders, partially acclimatized to high altitude, travelling from low-altitude residency (Grenoble, France, altitude: 200 m) to La Rinconada and staying at high altitude for 7 days. All lowlanders were scientists involved in the Expedition 5300 research program. They were all no smokers and did not take any medications, except acetazolamide (for 5 of them) in order to prevent acute mountain sickness[27].

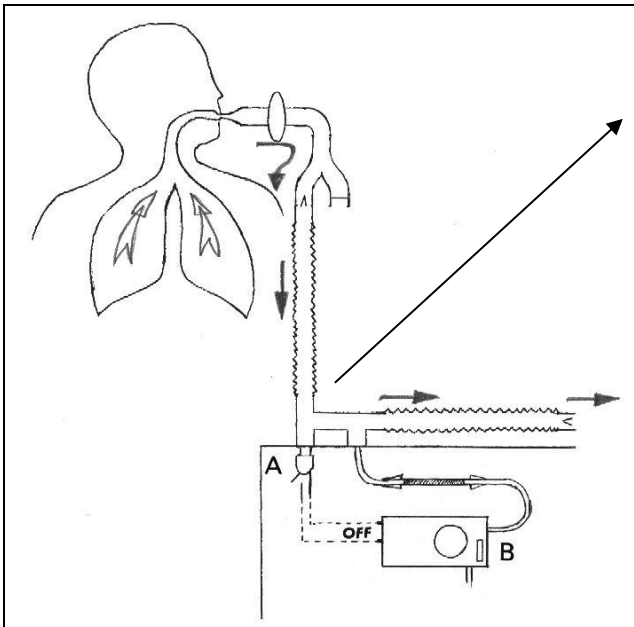
Demographic and physiological measurements

For all highlanders included in the study, demographic and lifestyle data were collected. Hemoglobin concentration was measured from a capillary blood sample (Hemocue AB, Angelholm, Sweden) and oxygen saturation (SpO_2) was measured by a finger sensor (NELLCOR OxiMaxN-65, TycoHealthcare, CA) after a 5-minutes rest period in a sitting position. The presence and the severity of the seven clinical symptoms of CMS (i.e., breathlessness and/or palpitations, sleep disturbance, cyanosis, dilatation of veins, paresthesia, headache, tinnitus) were scored from 0 (no symptom) to 3 (severe symptom) according to the Quinghai score. Based on the current international consensus, EE was defined as an [Hb] $\geq$ 21 g/dL. The total Quinghai CMS score was computed as the sum of the points given by each symptom, more 3 points in presence of EE. According to the current international consensus, a CMS diagnosis was made in case of a total Qinghai CMS score >5, including the presence of EE; Mild CMS corresponding to a 6-10 points score, moderate CMS to 11-14 points and severe CMS to a score > 15[8].

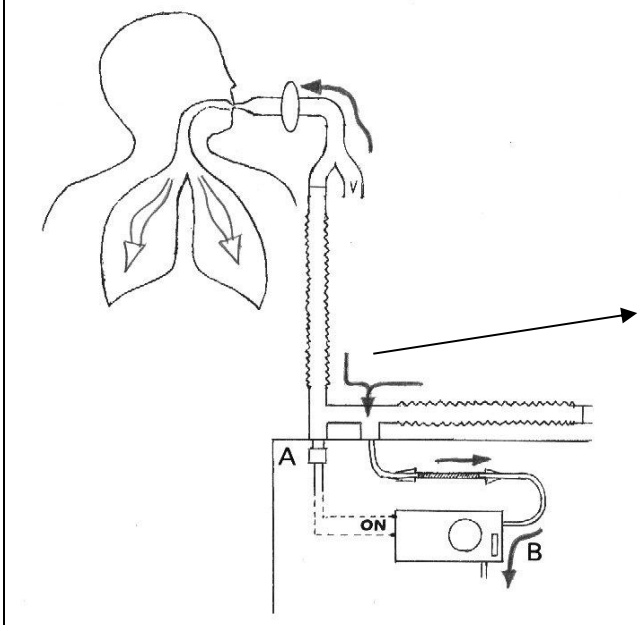
Breath sampling

In the past, archaic methods for breath collection were used, for example a spirometer coupled with a CO_2-adsorbant- chamber[28], or a gas-tight bag for entire exhalation ("Total breath")[28]. A recent paper presents the "single breath canister" as technique for breath sampling before halogenated VOCs measurements[29].

The technique carried-out in this study is a concentration method using a pump and active carbon containing cartridges. Subjects are breathing room air. "The breath end" is sampled by collecting only the final exhalation, to have a concentrated alveolar breath sample, as shown in fig.1 and fig.2, through a Carbotrap 200® glass tube (fig.3 and fig.4) during 2min of pump work[30] [31] [32] [33]. After collection by using the ambulatory system (fig.2), the adsorptive trap is removed and sealed in a screwtop glass storage container (fig.3)[34].



(a)
The expired breath is channelled through the pipe then evacuated. Only the final fraction remains in the pipe at the end of the expiration.
A breathing out pressure higher than 2 mbar imposes on electromagnetic sluice gate A a stop of the pump B.



(b)
During the breathing in, the pressure in the pipe decreases to a value lower or equal to 1mBar and causes the priming of pump B.
The alveolar breath is trapped through the cartridge Carbotrap 200®

Fig.1: Sampling system of final exhalation during breathing out (a) / in (b) for VOCs analysis

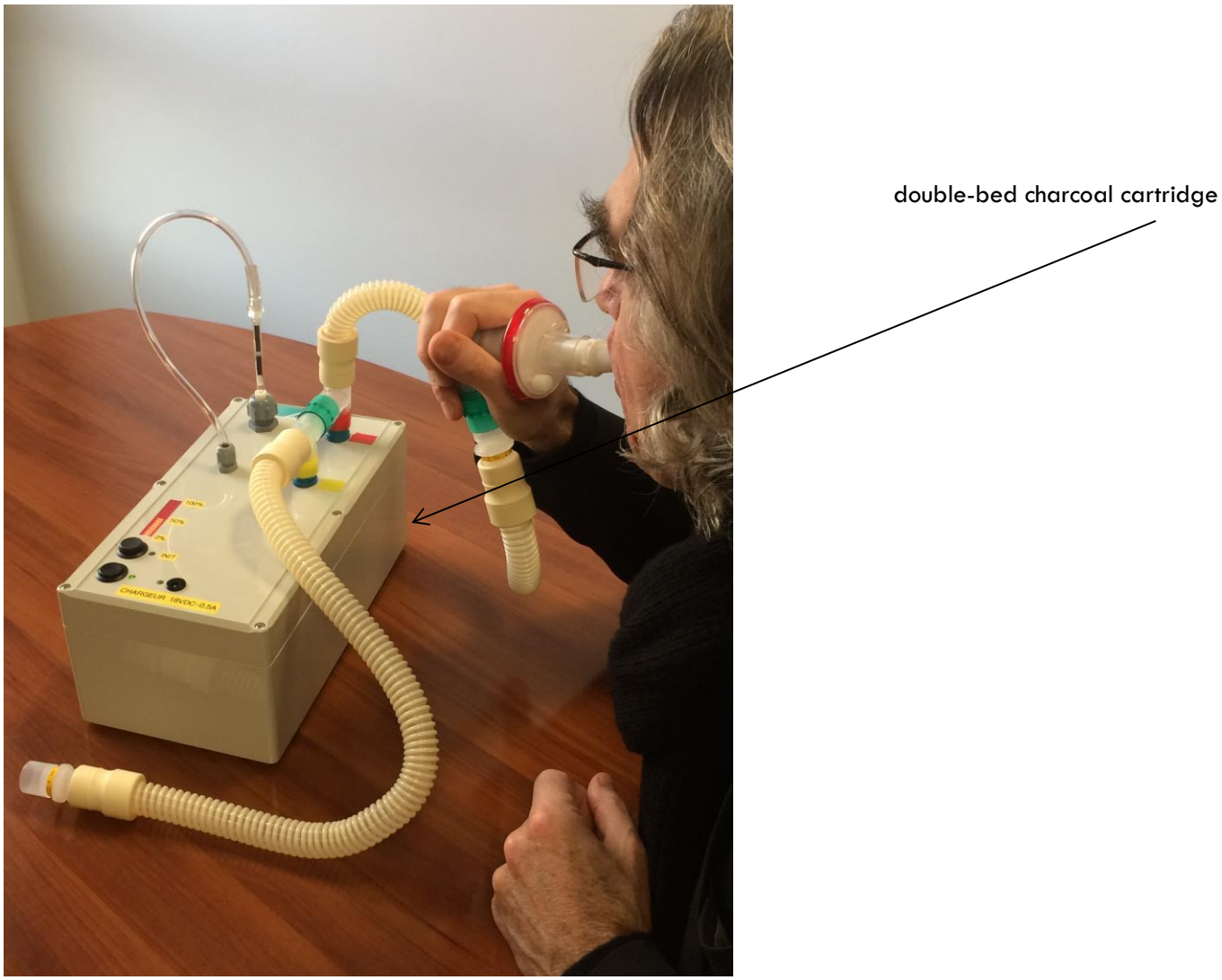


Fig 2: Picture of the ambulatory sampling system

Fig 3: The cartridge is packaged into an air tight screwed container

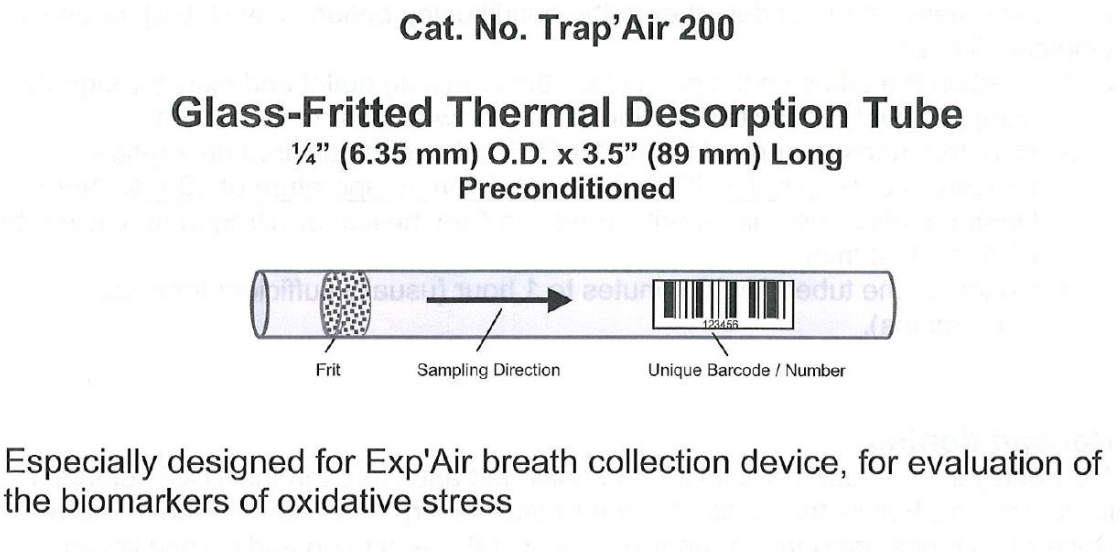


Fig 4: Glass-Fritted Thermal Desorption Tube

Sample analysis apparatus

Volatile hydrocarbons are concentrated on a specific double-bed charcoal cartridge (fig.4). The cartridge is then sent to the laboratory where it will be treated by a thermal desorber coupled to gas chromatography coupled with mass spectrometry detector (fig.5)

Fig 5: Shimadzu TD20 Thermal Desorption device coupled to GC-2010Plus and a mass detector QP 2010 SE

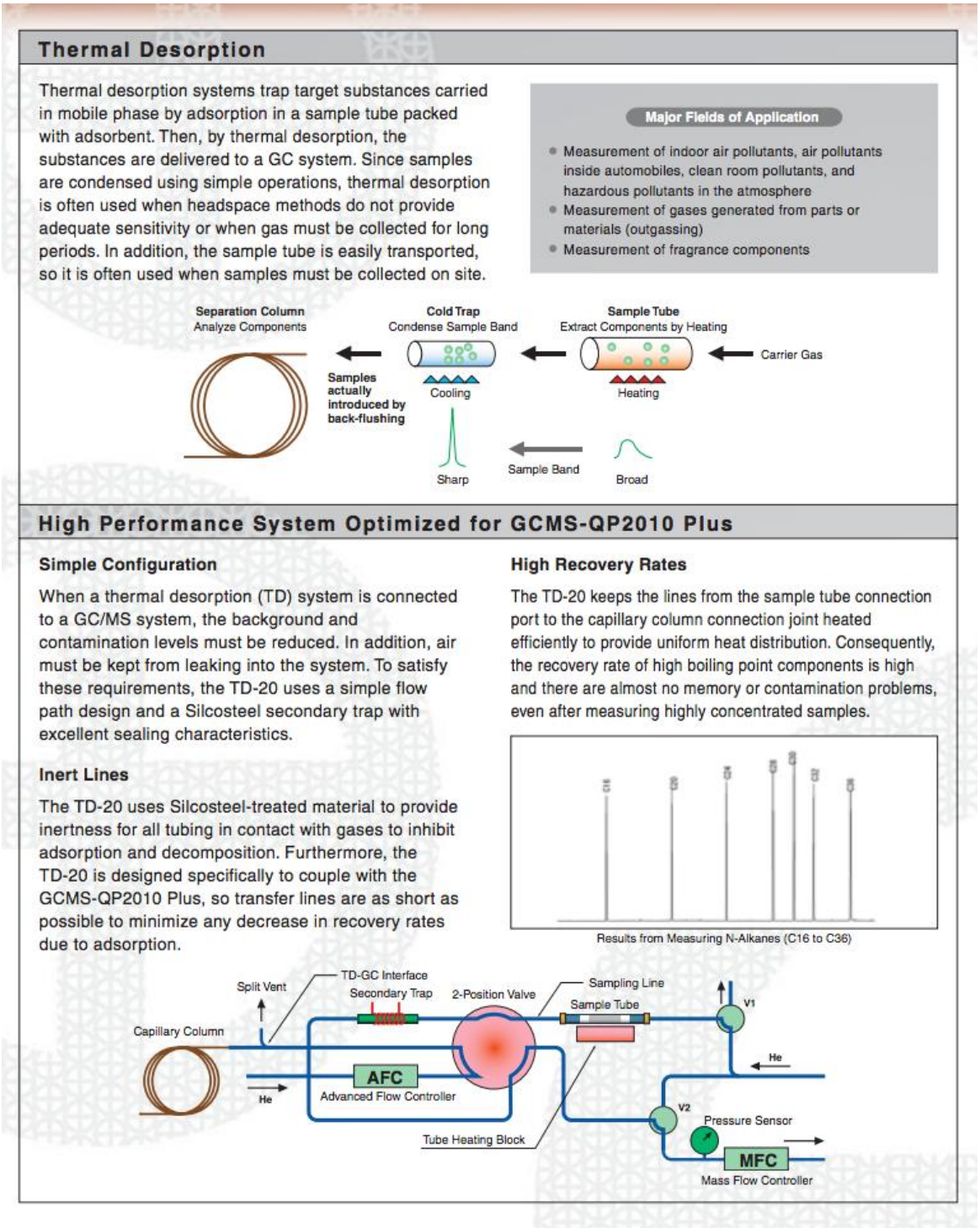


Fig 6: Shimadzu TD20 Thermal Desorption device: schema of principle

A thermal desorption adsorbent tube, Carbotrap 200® glass tubes filled with Carbotrap B and Carbosieve S-III (Supelco, Sigma-Aldrich, Saint-Quentin Fallavier, France), has the capacity for trapping and then releasing many airborne organic compounds (fig.6). The desorption efficiency is approximately 100% for trapped organic compounds. Carbotrap 200 tubes contain silanized glass beads, Carbotrap® (graphitized carbon black) and Carbosieved® SIII (a carbon molecular sieve). These tubes effectively retain small size

halogenated alkanes (C_1 and C_2) as well as larger molecules (up to C_{14}).

In the thermal Desorption Unit (Shimadzu TD20 Thermal Desorption device) the sample is delivered onto the analysis column undiluted (carrier gas: ultra pure helium; gas flow: 60 ml/min); by heating the adsorbent tube from 35°C to 380°C in 16 sec then steady-state 5 min at 300°C, the sensitivity is greatly enhanced.

VOC are analysed using a gas chromatograph Shimadzu 2010 Plus coupled with mass-spectrometric detector QP 2010 SE (Shimadzu) lead with GCMS Real time analysis software (Shimadzu).. Chromatograms are analysed by Total Ion Current (TIC). The gas chromatographic conditions are the following:

- Column SPB-5ms; 0.25 mm x 60 m, Thickness of film 1µm (N° 01)
- Carrier gas: ultra pure helium.
- Temperature of column: 35°C
- Program of the oven temperature: 35 °C during 11 minutes then 5°C until 170°C. Hold 32 minutes.
- Temperature of interface: 260°C
- Temperature of ionization source: 260°C
- Time of analysis: 60 min
- Acquisition of ratio m/z from 30 to 250.

Results

The study involved 48 subjects including 5 administrative staff, (expedition members) and 36 mine employees (Table 1).

Period measurement	subject	Groups
February 2020	CHMA	Rinconada
February 2020	MOCL	Rinconada
October 2020	ARFE	Rinconada
February and October 2020	QUEU	Rinconada
October 2020	MAEL	Rinconada
February and October 2020	RAWI	Rinconada
February 2020	MAAL	Rinconada
February and October 2020	MASI	Rinconada
February 2020	LOJO	Rinconada
February 2020	NAJU	Rinconada
February 2020	MAEF	Rinconada
February 2020	MAGE	Rinconada
October 2020	VAEM	Rinconada
October 2020	SUHE	Rinconada
October 2020	VIDA	Rinconada
October 2020	QURE	Rinconada
October 2020	QURU	Rinconada
February 2020	LEOS	Rinconada
February 2020	TUVA	Rinconada
February 2020	LIAR	Rinconada
October 2020	ANBE	Rinconada
February and October 2020	SATE	Rinconada
February and October 2020	CAER	Rinconada
October 2020	BEWA	Rinconada
February 2020	COJU	Rinconada
February 2020	QURO	Rinconada
February 2020	CHFR	Rinconada
February and October 2020	COED	Rinconada

February 2020	SUEL	Rinconada
October 2020	MIAL	Rinconada
October 2020	QUCE	Rinconada
February 2020	CUAB	Rinconada
February 2020	VIED	Rinconada
February 2020	HIRO	Rinconada
February 2020	CAIG	Rinconada
October 2020	PIAX	Expe5300 team
October 2020	VESA	Expe5300 team
October 2020	BRJU	Expe5300 team
October 2020	PIAU	Expe5300 team
October 2020	DOST	Expe5300 team
October 2020	STEM	Expe5300 team
October 2020	CHBE	Expe5300 team
October 2020	CHLU	Rinconada
October 2020	MACE	Rinconada
October 2020	QHFA	Rinconada
October 2020	RACR	Rinconada
October 2020	RACR2	Rinconada
October 2020	TRGO	Rinconada

Table 1: Subjects (and code name) involved in the study

The results of this study clearly show a significant concentration of limonene in the exhaled air of all subjects, including some controls taken at La Rinconada. Alkanes were also found (table 2).

February 2020

	Tr	COJU	CUAB	VIED	HIRO	CAIG	SUEL	CHLU	RACR	SATE	LIAR
Nonane	30,0	-	-	-	-	-	-	-	-	-	-
Decane	33,9	177490	162886	236545	246152	159927	215523	164849	152345	181871	160817
D-Limonene	35,5	982275	561840	637980	601111	556397	960472	709075	96949	650054	629769
Undecane	37,5	144374	108283	158557	168950	133080	136125	95414	-	99944	92276
Dodecane	41,1	152609	74222	123413	105556	108104	-	91878	-	77415	80193

	Tr	CAER	CHFR	LEOS	QURO	COED	QHFA	MAAL	QUEU	RAWI	MASI
Nonane	30,0	-	-	-	-	-	-	-	-	-	-
Decane	33,9	159946	189623	213059	150903	158426	106480	344546	379431	419072	325332
D-Limonene	35,5	680416	1106961	957981	783790	828610	351252	317724	234744	322806	317715
Undecane	37,5	91304	134438	133591	123054	136720	50577	65254	-	151297	67724
Dodecane	41,1	83075	151484	121786	128775	146240	-	66042	-	161727	68917

	Tr	TUVA	MACE	MOCL	RACR (2)	MAGE	MAEF	NAJU	TRGO	CHMA
Nonane	30,0	-	-	-	-	-	-	-	-	-

Decane	33,9	244644	349583	447718	498511	198767	393662	356592	135870	98133
D-Limonene	35,5	300406	130471	180331	285851	227867	364595	301834	474879	422570
Undecane	37,5	-	-	-	-	-	99960	-	53937	50166
Dodecane	41,1	-	-	-	103630	64618	70586	-	-	-

October 2020

	Tr	MIAL	PIAX	VIDA	ANBE	VESA	QUCE	BRJU	PIAU	MAEL	MASI
Nonane	30,0	-	-	315176	-	-	386903	-	-	-	-
Decane	33,9	401496	262717	476792	143691	262459	380545	256091	232709	244672	211948
D-Limonene	35,5	289625	-	1063351	114963	94266	1074914	137235	113849	439548	189955
Undecane	37,5	324554	289877	249424	89304	260841	218933	278904	333009	138843	139570
Dodecane	41,1	203944	252359	170400	-	170193	-	223869	228413	111131	108017

	Tr	DOST	COED	BEWA	SUHE	QUEU	RAWI	ARFE	STEM	CHBE	CAER
Nonane	30,0	-	-	-	-	-	-	-	-	-	-
Decane	33,9	127747	224645	163165	213097	205103	208604	160493	168775	148986	189560
D-Limonene	35,5	54595	160062	155915	329768	209751	307082	144491	-	-	127955
Undecane	37,5	162126	171865	105601	96873	124626	110893	122759	166798	160007	151547
Dodecane	41,1	151053	124364	70778	-	85165	79383	86352	175931	112718	86111

	Tr	QURE	VAEM	QURU	LOJO	SATE
Nonane	30,0	98308	-	-	-	-
Decane	33,9	171744	183804	-	138410	183942
D-Limonene	35,5	505915	184768	-	75171	321834
Undecane	37,5	80749	103670	-	119982	142819
Dodecane	41,1	-	57728	-	107349	143451

Table 2: Results (area under curve) of main products detected in exhaled breath.

Statistical analysis

54 samples were analyzed, which corresponds to the entire sample of analyzed data (including duplicate measurements in 6 highlanders after a 8-month interval. The number of subjects needed for this study was not calculated, considering that the discovery of limonene in exhaled air was incidental and that the entire population of workers at the mine was included.

The set of data (n=24) brings together different kinds of information as: the origin of the groups (ethnicity; altitude of life; consumption of alcohol or cigarettes…); their clinical or biological characteristics (CMS Score; SpO2; [HB]) and chemical assays of volatile species taken from the exhaled air of the subjects. All data, quantitative or qualitative, have been digitized, centered and reduced do be statistically analyzed. The comparison between data was made using a correlation analysis to identify the absence or presence of a relationship between two variables. The significance of the correlation coefficient is evaluated with a p-value calculated using a t-distribution with n-2 degrees of freedom. The formula for the statistic test is $t = \frac{r\sqrt{n-2}}{\sqrt{1-r^2}}$

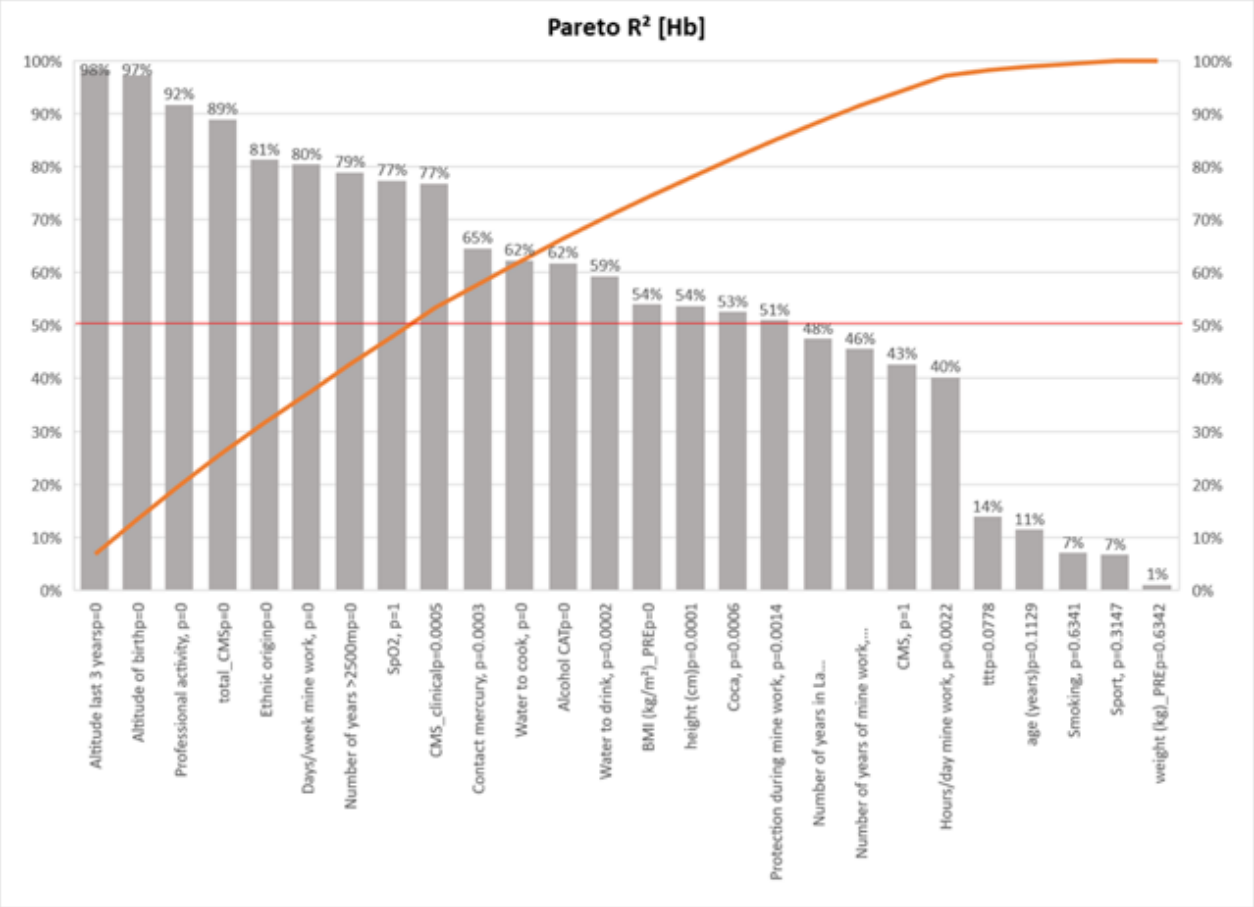
The value of the statistic test, t, is shown with the p-value giving the probability of no significance of R^2 for n-2 degree of freedom. The R^2 with p-values for each data are plotted as a Pareto chart (a histogram) by sorting from the most to the less significant relationships between two parameters.

Four correlations analysis were investigated:

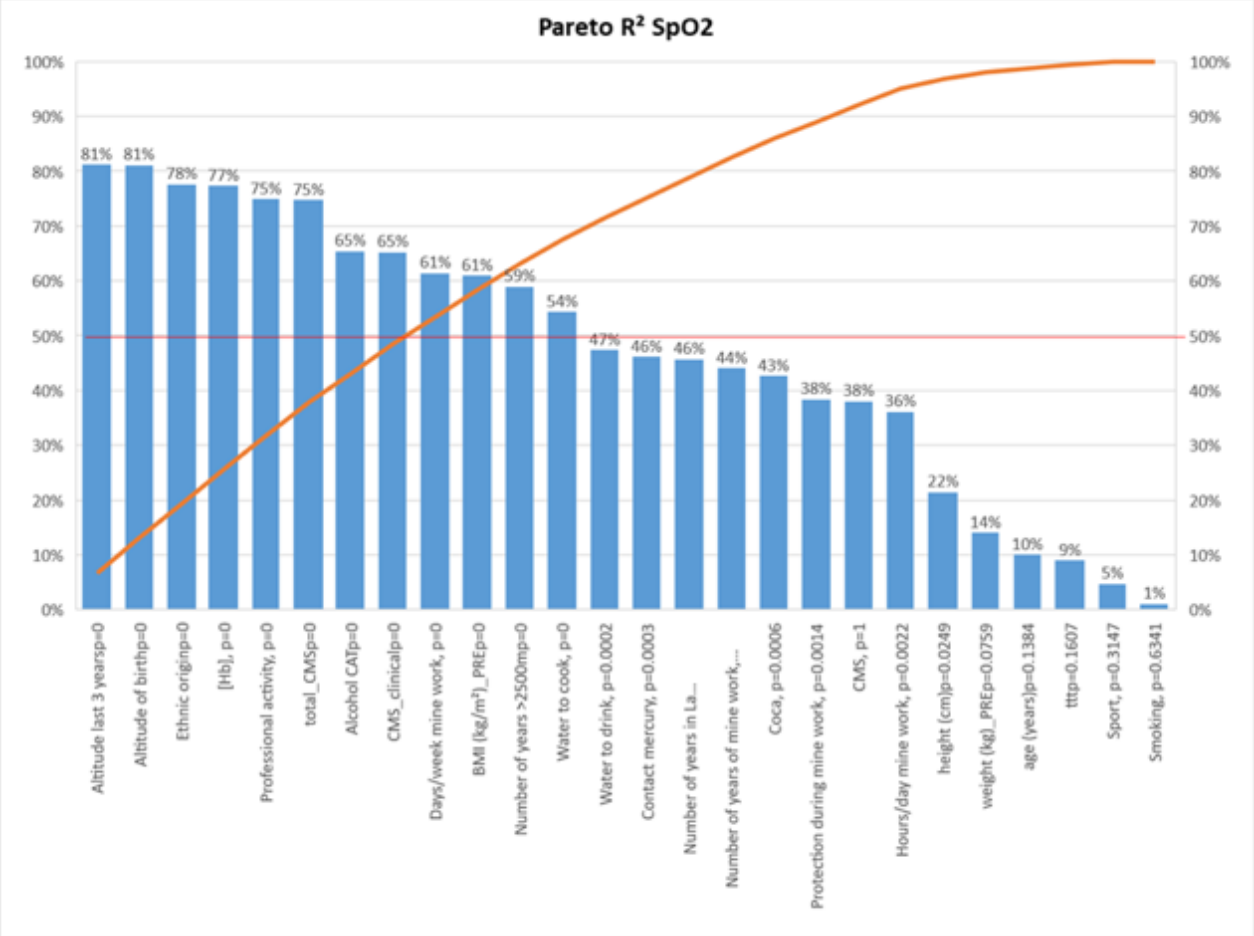
- First, the correlations between the categorical input data, in order to check if input data are independently associated. If input data have

relationship with $R^2 > 50\%$ ($p=0.0001$), a confusion will appear between them if a correlation is significant with output data, i.e clinical characteristics or chemical species. It appears that 9 input data out 21 have R^2 below 50%, can be reasonably considered without confusion, namely: age; weight; height; BMI; Smoking status; ttt; work time in the mine; Protection during mine work; physical activity.

- Second, the correlations between the categorical input data with respect to the clinical or biological characteristics, in order to associate the possible relationship between CMS clinical parameters and categorical input data. Regarding CMS clinical parameters, most of them are significantly correlated together ($R^2 > 40\%$; $p=0.001$), showing the expected implication of the clinical parameters to the CMS. Then focusing on [HB] and SpO2 as the main clinical CMS parameters, it appears that all categorical input data relative to highlanders are very significantly linked to [HB] and SpO2 ($R^2 > 75\%$, $p<1e\text{-}7$). Fig 7 shows the R^2 Pareto histogram showing the relative importance of input parameters in decreasing order of occurrences. Red line gives the threshold for $R^2>50\%$, $p=0.00013$.



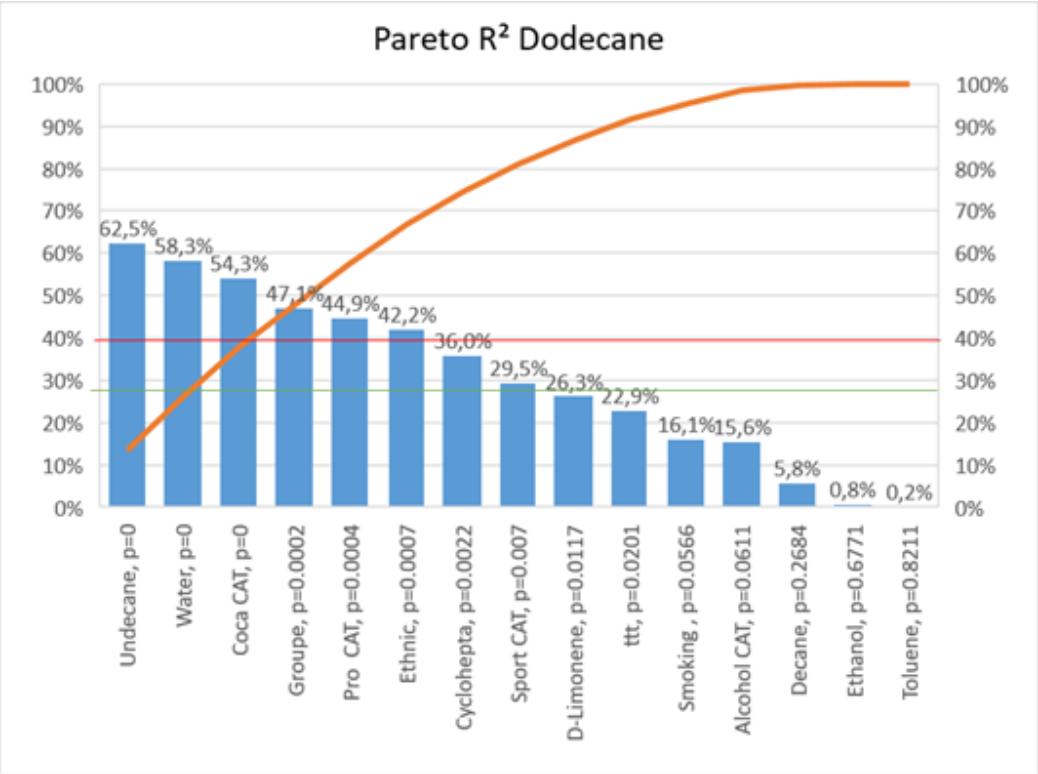
A



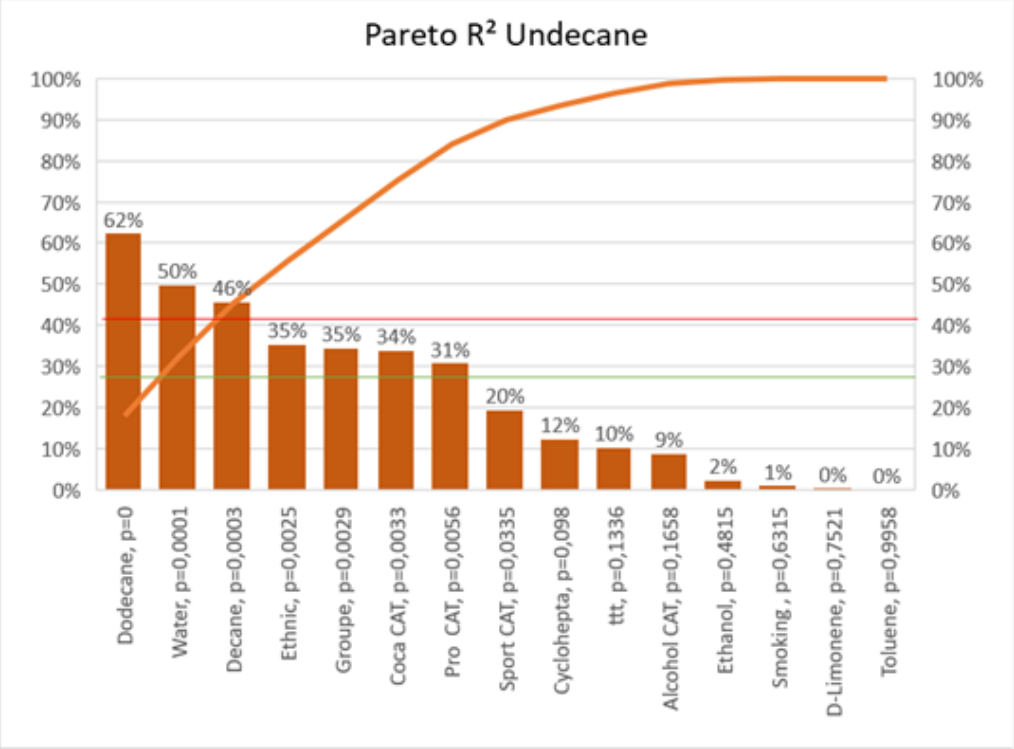
B

Fig 7: Pareto charts of R^2 for A) [HB] with respect to input data, B) SpO2 with respect to input data. The red line gives the threshold for $R^2>50\%$, p=0.00013. The orange curve shows with secondary vertical axis the cumulative impact.

- Third, the correlations between input data and chemical species were investigated. Four chemical species, dodecane, undecane, D-Limonene and 1,3,5-Cycloheptatriene give significant correlation ($R\geq27\%$, $p<0.01$) with categorical input data. Dodecane is the most significant with six strong relationships ($R^2>$ 40% $p<0.001$) with categorical parameters representatives of highlander subjects, Coca consuption; Number of years in La Rincondada; origin of drinking water (rainwater, glacier, mineral water); Professional activity; altitude of residence last 3 years; number of years working in the mine. Undecane gives also the same trend of relationship quite significant ($R^2>$ 32%, $p<0.005$), (Fig. 8).



A

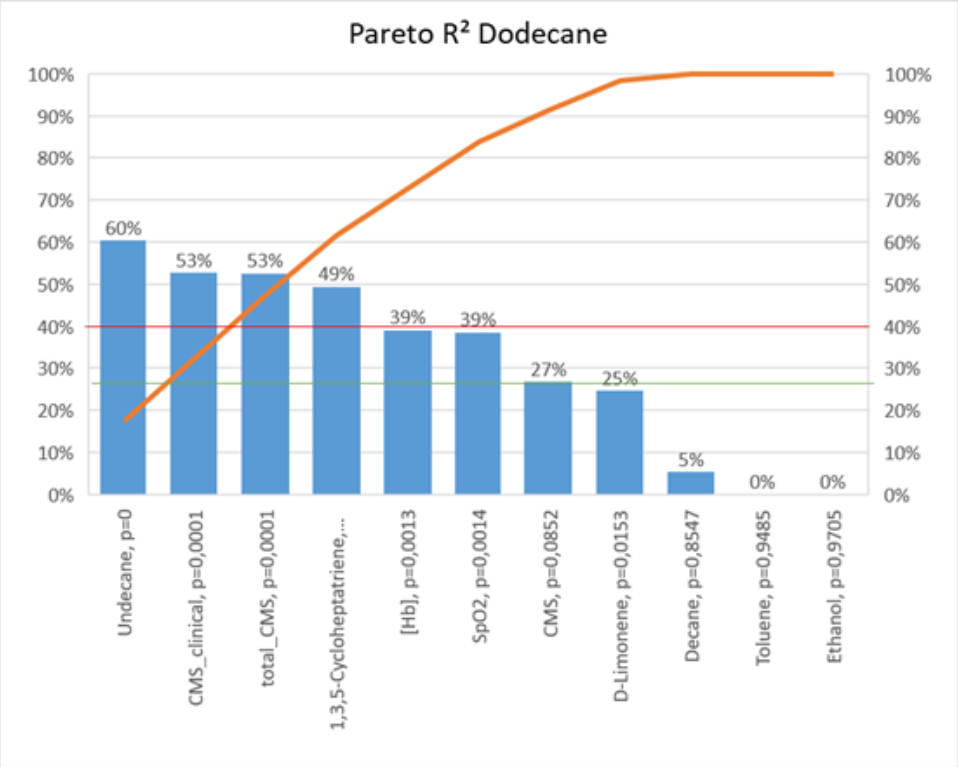


B

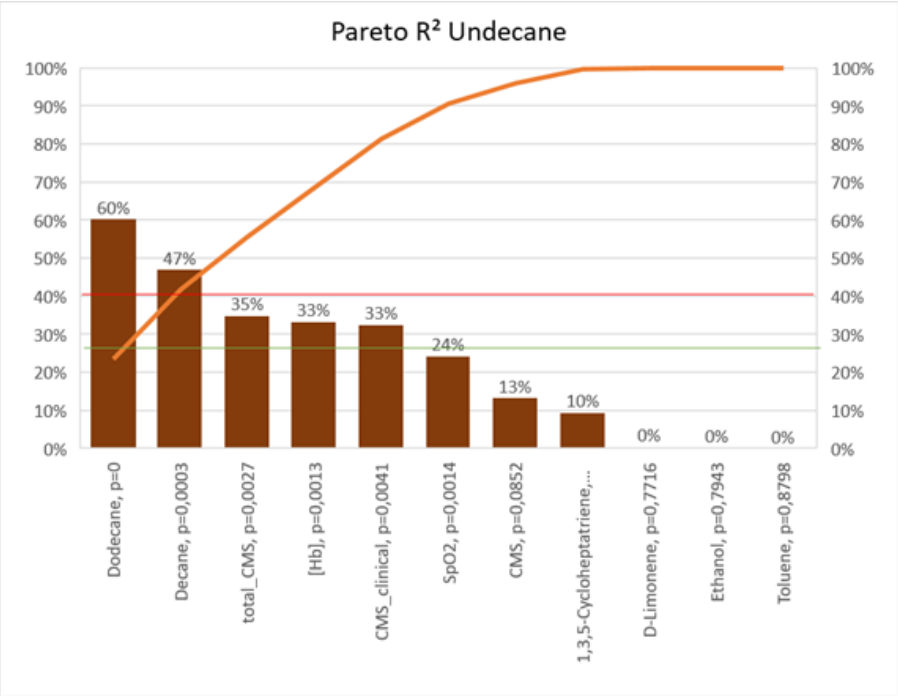
Fig 8: Pareto charts of R^2 for A) Dodecane with respect to input data, B) Undecane with respect to input data. The red and green lines, give respectively the threshold for R^2>40%, p=0.001 and R^2>27%, p=0.01The orange curve shows with secondary vertical axis the cumulative impact.

D-limonène gives also significant relationships (R^2> 27%, p<0.01) with two categorical parameters, Number of year in La Rinconada and Smoking status.

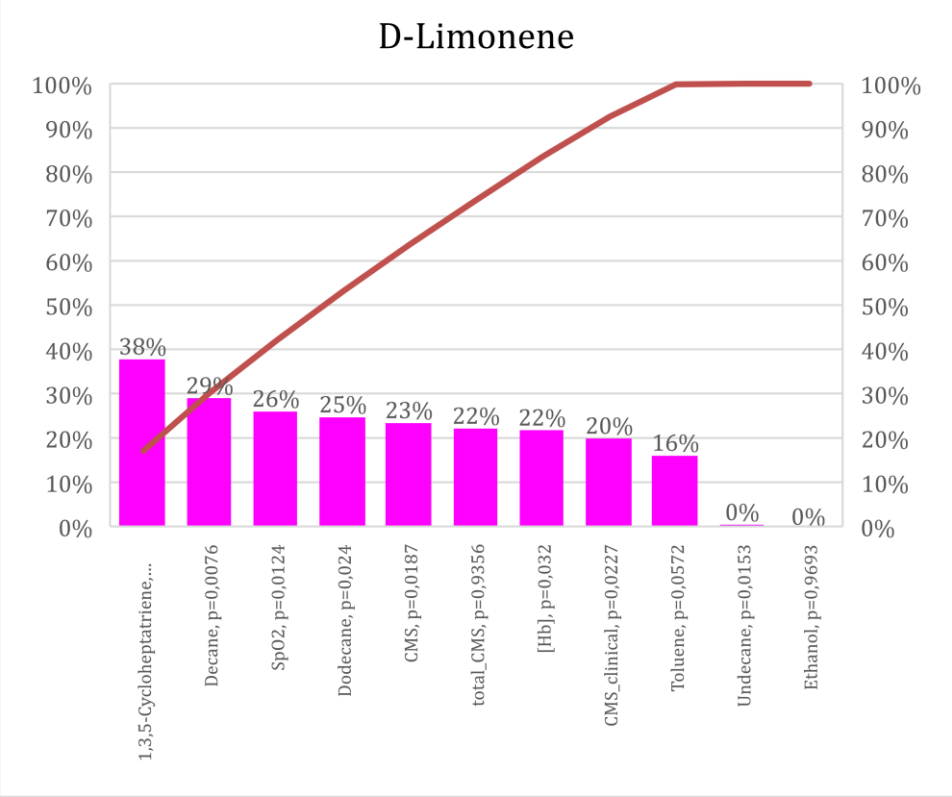
- Finally, correlation analysis was performed between chemical exhaled species together and with clinical parameters to show relationship possibly significant between the origins of exhaled chemicals. Dodecane, Undecane, 1,3,5-cycloheptatriene, Decane and D-limonene have significant relationship (R^2> 27%, p< 0.01) between them. Dodecane is significantly correlated with CMS_clinical (R^2>53%, p<0.001). Undecane is significantly correlated with total_CMS score, clinical CMP score and [HB] (R^2>33%, p<0.005); D-limonene is significantly correlated with SpO2 (R^2=26%; p=0.012); C.f R& Pareto histogram below.



A



B



C

Fig 9: Pareto charts of R^2 for A) Dodecane B) Undecane and C) Limonene with respect to clinical parameters and GCMS chemical species. The red and green lines, give respectively the threshold for $R^2>40\%$, $p=0.001$ and $R^2>27\%$, $p=0.01$. The orange curve shows with secondary vertical axis the cumulative impact.

Discussion

Limonene is not a substance classically found in exhaled air. It is not possible at this date to find a mechanism of endogenous production of this compound. However, the correlation studies detailed in the results of this study show coherence between its presence in the body and the physiological conditions of the tested subjects. We have several hypotheses to propose for discussion. Either by an excretion in the expired air related to a pathology such as cirrhotic encephalopathy. Or by absorption of this compound related to the food.

Exhaled limonene

Exhaled limonene levels are primarily affected by the presence of cirrhosis through reduced liver functional capacity, as indicated by limonene correlation with blood metrics of impaired hepatic clearance and protein synthesis capacity, without further alterations observed in subjects with hepatocellular carcinoma. This suggests that exhaled limonene is a potential non-invasive marker of liver metabolic capacity[35].

Breath terpinene, dimethyl sulfide, and D-limonene are potentially useful volatomic markers for stratifying non-alcoholic fatty liver disease[36].
Limonene has also been detected in Exhaled Breath Analysis in Diagnosis of Malignant Pleural Mesothelioma[37].

Limonene, methanol and 2-pentanone are breath markers for a cirrhotic liver. This study raises the potential to investigate these volatiles as markers for early-stage liver disease. By monitoring the wash-out of limonene following transplant, graft liver function can be non-invasively assessed[38].

Limonene in food

Various plant families contain Terpenes as their secondary metabolites. Monoterpenes constitute an important part of these secondary metabolites. D-limonene is a well-identified monoterpene that is commonly applied as a fragrance ingredient in essential oils. D-limonene is known to possess remarkable biological activities. It can be effectively used for treating various ailments and diseases. Due to its diverse functions, it can be efficiently utilized for human health[39].
A paper reviewed the microbial communities identified from spontaneous cocoa fermentations and focused on the yeast starter strains used in cocoa beans and their sensorial and flavor profile. The potential compounds that could have health-promoting benefits like limonene, benzaldehyde, 2-phenylethanol, 2-methylbutanal, phenylacetaldehyde, and 2-phenylethyl acetate were also evaluated as their presence remained constant after roasting[40] .

Limonene is also present in beverages fermented by *Saccharomyces cerevisiae* and notably beer[41].

In the present situation it is very difficult to draw conclusions about the origin of limonene in these subjects. The above hypotheses are all relevant and none can be ruled out. The most interesting is the cerebral origin of limonene synthesis *in vivo*. As it has been observed in the case of hepatic encephalopathies it is problematic that the cerebral hypoxia present in all these subjects is at the origin of limonene biosynthesis. The questions that remain are: i) how is limonene biosynthesized by the cells and ii) what is its role in the maintenance of homeostasis.

Conclusion

This study revealed the presence of oxidative stress-causing VOCs in exhaled air, such as alkanes, but also of a new biomarker, limonene. There is certainly a correlation between the presence of this biomarker and the physiological conditions of the subjects studied living at a very high altitude. Even if this study was carried out during two expeditions and shows reproducible results, confirmations will still have to be carried out to find the origin of limonene in the breath. The biological conditions of limonene production will also have to be clarified.

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