Universitat Autònoma de Barcelona

Mediterranean forests and health: analysing the interactions between forest chemistry and humans

Albert Bach Pagès

MEDITERRANEAN FORESTS AND HEALTH

Analysing the interactions between forest chemistry and humans

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PhD thesis // September 2020

Supervisors: Roser Maneja // Josep Peñuelas // Joan Llusià
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In collaboration with

Note: British spelling and conventions are used in the Introduction and conclusions. US English is used in chapters 1 to 4.

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And into the forest I go, to lose my mind and find my soul"

John Muir

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And now, with your permission, I switch to Catalan to address the following acknowledgements

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Preface

This thesis is the result of a four-year PhD project developed in the Environmental Science and Technology Institute of the Autonomous University of Barcelona (ICTA-UAB), in Barcelona, Spain. The supervisors of this work are Roser Maneja, Josep Peñuelas and Joan Llusià.

This research included fieldwork, laboratory analysis and clinical trials. All the process embraced the contributions of several specialists from different research and health centres: *Centre de Recerca Ecològica i Palicacions Forestals* (CREAF), *Centre Tecnoloògic i Forestal de Catalunya* (CTFC), *Hosptital de Sant Celoni*, *Servei d'Anàlisi Química* (SAQ) and IRSI Caixa.

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Overall, chapter 3 serves as an introduction to the field of research. It identifies the gaps and questions arisen from the current body of literature. Focussing more on the potential chemical links between forests and human health, chapter 4 constitutes a first-step analysis of the availability of monoterpenes in Mediterranean forests. Chapter 5 makes a step forward in this line by analysing the absorption of these chemical compounds by humans during a forest walk. Lastly, in the Chapter 6, the thesis sounds out the role of forests as stress mitigators.

Abstract

Interest in understanding if and how the exposure to forests leads to human health benefits is growing among the studies analysing the effects of nature on human health. This topic is not only being addressed by the scientific community but also by the civil society and other sectors resulting in the incorporation of "health provision" objectives in the international and national forest agendas, strategies, management plans and policies. Although the current body of literature has underlined the benefits of these ecosystems on different human body systems and functions, studies on the mechanisms and pathways leading to the health outcomes are still scarce and inconclusive. Furthermore, Mediterranean forests appear to be barely studied in comparison with others (mainly Asian forests), where abiotic variables and forest ecosystems may differ.

The main goal of this thesis is to assess to what extend Mediterranean forests can be linked to human health. More particularly, the specific aims of the thesis are: A) analysing which forest variables have been linked with effects on human health; B) characterising the Mediterranean forest air monoterpene concentrations below the canopy; C) studying the human absorption of monoterpenes in the forest; and D) investigating the potential of forests to regulate human stress markers.

Chapter 3 is articulated from a systematic review of the current literature to assess the extent to which forests have been studied and described in detail and the extent to which relationships between forest variables and health effects have been reported. The analysis underlines the lack of forest descriptions as well as the high heterogeneity of forest variables' description. Patterns among the articles could not be identified correlating the broader forest variable (forest type) and the most studied health variables identified (blood pressure, pulse rate or/and cortisol levels).

The growing body of research on this topic, has argued that biogenic volatile organic compounds (BVOCs), particularly monoterpenes, may be potential determinants of the human health effects induced by forest exposure. Chapter 4 characterizes the total monoterpene air concentrations at

nose height in a Mediterranean holm oak forest. Results show a strong variability of the total monoterpene air concentrations in season and daytime with its peak during July and August. These two months displayed two average maxima in their diel cycles; during early morning and at early afternoon. Monoterpene air concentrations were strongly related with temperature and solar radiation. The concentrations registered are similar or higher than in previous *ex situ* studies showcasing the effects of forests on human health.

In chapter 5, we step further in the chemical bonds between forest and people by analysing the monoterpene absorption by humans during forest exposure. Results showed no significant changes in monoterpene blood concentrations for the forest and control group. However, a negative significant relationship between absorption and baseline blood concentration of the most abundant forest air monoterpenes, alpha-pinene and beta-pinene, was found in individuals visiting the forest, i.e. higher absorption was found the lower the baseline blood concentration was. We also found significant correlations between the absorption of the different monoterpene compounds.

Finally, chapter 6 studies the evolution of human stress markers during an 8-hours exposure. Our results show: A) a significant decrease in cortisol saliva concentrations since the second hour until the end compared to basal time; B) a significant increase of alpha amylase activity after the first hour of exposure compared to basal time that remained stable during the rest of the study; C) a significant decrease of IgA from the fourth hour of exposure compared to the basal time. The findings of this chapter support the physiological and psychological relaxing effects of forest exposure.

Overall, the findings of this thesis underline the need to incorporate forest variables descriptions in the ongoing studies and call for the development of more interdisciplinary research teams. Considering our results regarding forest chemistry and stress mitigation, we conclude that Mediterranean forests can constitute suitable habitats for preventive health initiatives although further research is essential to advance the comprehension of the mechanisms an pathways involved in the equation forests-human health to provide valid data for the healthcare community, civil society and policy makers.

Resum

El creixent interès per comprendre els efectes de l'exposició al bosc en la salut humana ha captat l'atenció no només de la comunitat científica, sinó també de la societat en general i d'altres sectors. La incorporació d'objectius de "provisió de salut" en agendes, estratègies, plans de gestió i polítiques forestals són en alguns exemples a nivell internacional i nacional. Tot i que les investigacions recents destaquen els beneficis d'aquests ecosistemes en diferents sistemes i funcions del cos humà, els mecanismes i les vies per les quals es donen aquests efectes positius estan poc estudiats o els resultats són encara inconclusius.

L'objectiu principal d'aquesta tesis és analitzar les interaccions entre els boscos mediterranis i la salut humana. En particular, els objectius específics de la tesi són: A) analitzar quines variables forestals han estat vinculades amb efectes en la salut humana; B) caracteritzar les concentracions de monoterpens en l'aire per sota la copa dels arbres en un bosc mediterrani; C) estudiar l'absorció humana de monoterpens en el bosc; i D) investigar el potencial dels boscos com a reguladors de l'estrès.

El capítol 3 parteix d'una revisió sistemàtica de la literatura existent per tal d'analitzar el grau de detall amb què es descriuen els boscos i si s'han observat relacions entre variables forestals i efectes en la salut de les persones. Els resultats subratllen la manca de descripcions dels boscos i una gran heterogeneïtat en la descripció de les variables en els estudis de l'àmbit de recerca. A més, no es van identificar patrons en els articles que correlacionessin la variable forestal "tipus de bosc" amb les variables mèdiques més estudiades (pressió sanguínia, ritme cardíac i nivells de cortisol).

La recerca actual sobre aquest tòpic ha identificat els compostos orgànics volàtils d'origen biogènic, en particular els monoterpens, com a determinants potencials dels efectes en la salut induïts pels boscos. El capítol 4 caracteritza les concentracions totals de monoterpenes en l'aire d'un alzinar mediterrani per sota la copa dels arbres. Els resultats reporten una gran variabilitat

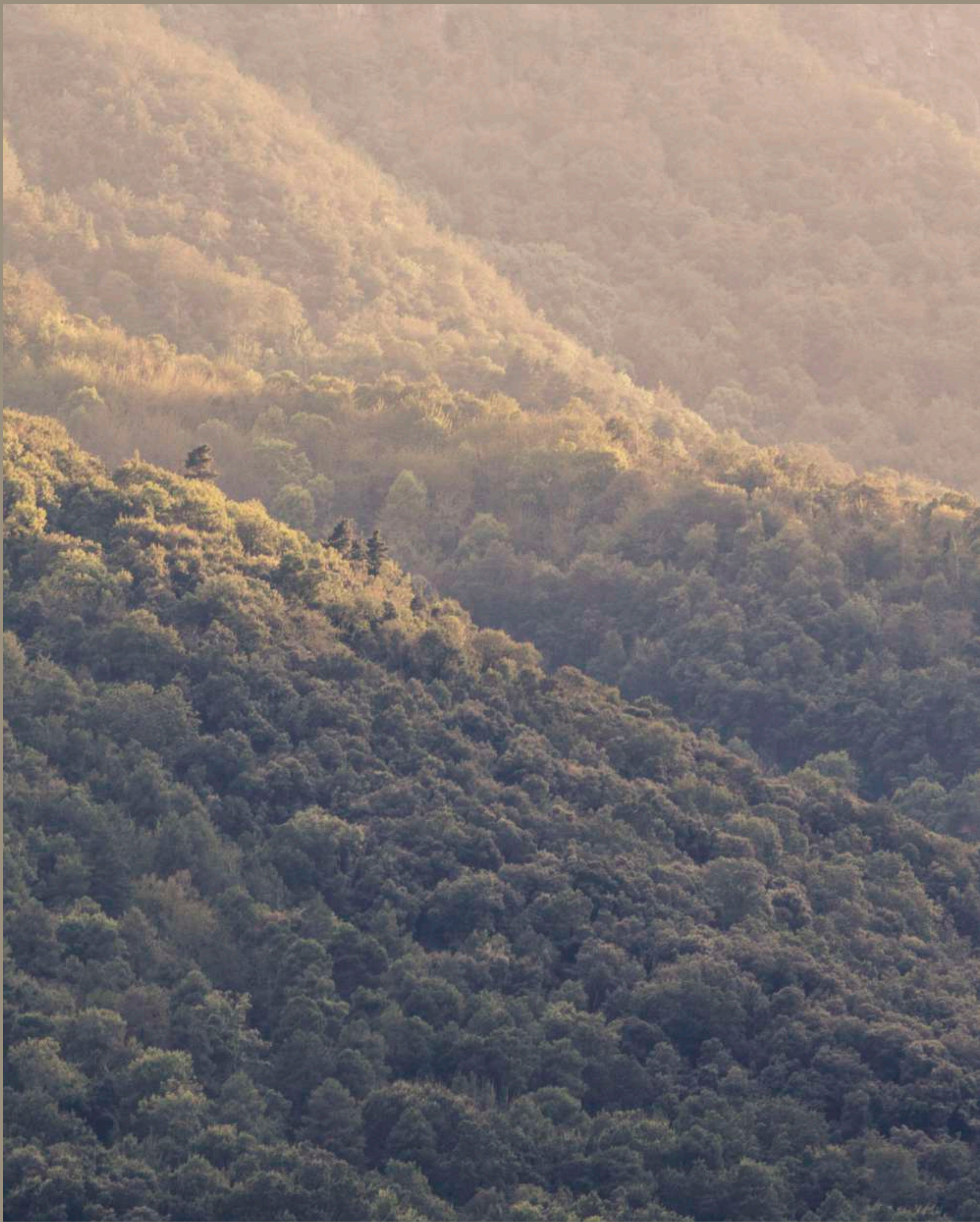
estacional i diària d'aquestes concentracions amb els pics més alts durant els mesos de juliol i agost. Durant aquests dos mesos, el cicles diaris mostren dos màximes: durant les primeres hores del matí i a primera hora de la tarda. A més a més, les concentracions de terpens en l'aire es mostren fortament correlacionades amb la temperatura de l'aire i la radiació solar. Les concentracions registrades són similar i, fins i tot, superiors a les investigacions prèvies que destaquen els efectes dels boscos en la salut.

En el capítol 5, es fa un pas endavant en l'estudi dels lligams químics entre boscos i persones analitzant l'absorció de monoterpens pels humans durant una estada al bosc. Els resultats no mostren diferències significatives en les concertacions de monoterpens en sang pels grups exposat al bosc o a la ciutat. No obstant això, es va identificar un relació negativa significativa entre l'absorció i les concentracions basals dels compostos més abundants en el bosc: alfa- i beta-pinè. Es van trobar també correlacions significatives entre l'absorció dels diferents compostos.

Finalment, el capítol 6 estudia l'evolució de diferents marcadors d'estrès durant una estada de 8 hores al bosc. Els resultats mostren: A) una baixada significativa dels nivells de cortisol a partir de les 2 hores d'exposició fins al final en comparació amb els valors basals; B) un augment significatiu de l'activitat de l'alfa-amilasa després de la primera hora d'exposició forestal que es manté estable fins al final de l'estudi; C) una disminució significativa de l'IgA des de la quarta hora d'exposició en comparació amb els nivells basals. Aquests descobriments donen suport al paper dels boscos com a mitigadors de l'estrès tant a nivell fisiològic com psicològic.

En conclusió, els resultats d'aquesta tesi subratllen la necessitat d'incorporar la descripció de variables forestals en els estudis d'aquest camp i aboquen al desenvolupament de més recerca en aquest camp interdisciplinari. En referència a la química forestal i a la mitigació de l'estrès, la tesis conclou que els boscos mediterranis poden constituir hàbitats adequats per desenvolupar iniciatives orientades a la medicina preventiva malgrat cal més investigació per avançar en la comprensió de les vies i dels mecanismes involucrats en l'equació boscos i salut humana i així aportar més dades contrastades al sector sanitari, la societat civil i els responsables de la presa de decisions.

CONTENTS



Chapter 1

Introduction

1.Introduction

More or less frequently, during childhood or adulthood, together with family or individually, most of us experience contact with forests. These wooded ecosystems represent somehow an important part of our life, even unconsciously. Almost everyone has had comforting or restorative experiences when in contact with nature and particularly with forests. Illustrative examples about the present and the historical interaction between forests and people's health and well-being can be found in ancient recovery medical centres located in forests, woodland areas protected in regard to their health benefits, or traditional medicine practices that were developed taking into account some of the processes taking place in these habitats.

1.1. Forests on Earth

There are several definitions of forests [1]. Among them, a widely accepted one is the definition used by the Food and Agriculture Organization of the United Nations (FAO): "Land spanning more than 0.5 hectares with trees higher than 5 meters and a canopy cover of more than 10 percent, or trees able to reach these thresholds in situ. It does not include land that is predominantly under agricultural or urban land use". These ecosystems cover approximately 4.06 billion hectares, 31% of the world's land surface. Forests are not evenly distributed around the globe: five countries host more than half of the world's forest cover (Russia, Brazil, Canada, the United States of America and China) and 66% of the world's forests are located within the borders of only ten countries. According to the FAO recent reports, nowadays deforestation and forest degradation still play out at alarming rates. Although deforestation rates have decreased during the last 3 decades, approximately 420 million forest hectares have been lost through conversion to other land uses since 1990. Since then, primary forest worldwide area has decreased by over 80 million hectares. The main driver of deforestation and forest degradation is agricultural expansion (primarily cattle ranching, cultivation of soya bean and oil palm and local subsistence agriculture)

[2]. Current research also highlights the threats that forest are facing associated with climate and global change [3–6].

Before digging deeper on the interactions between forests and human health when being in contact to these ecosystems, we should briefly consider the role of forests in sustaining life in the planet Earth.

Forests are fundamental concerning many of the essential conditions for life. Forests are crucial in absorbing a substantial amount of the CO_2 we, humans, are increasingly emitting to the atmosphere, and therefore they mitigate climate change. In addition, forests play a crucial role in another critical element: water. These ecosystems support the regulation of the atmospheric and terrestrial water cycle all over the world, influencing rainfall patterns and fixing water in soil through their root system [7]. Thus, forest become indispensable to guarantee our clean water demand [8]. They also become important concerning soil stability. Tree structures help on minimising the effects of extreme meteorological events and on fixing the soil, thus reducing the effects of floods, wind and storms, among others [9,10]. Furthermore, forests are reservoir for a vast number of terrestrial biodiversity, particularly in tropical areas where this type of habitat is the most species-rich [11,12]. This biodiversity and other values of forest are intimately linked to food production and security. In agroforestry and silvopastoralism, forests provide water and microclimate regulation, shade, protection from wind, soil protection, nutrient cycling, biological pest control and pollination which support agricultural, livestock, forestry and fishery production [13]. Forests not only influence cloud formation and rain patterns all over the world, but they are also key in many air processes that shape climate locally and globally. On one hand, forests are crucial stores of atmospheric carbon [14]; and take a relevant part in climate-change impacts mitigation [12]. Therefore, in some areas of the world it is not strange that re-forestation has been spotted as a valid strategy to mitigate climate change and its impacts [15,16]. On the other hand, forests help filtering and cleaning the air from a vast number of pollutants which also act as green-house gases and contribute to global warming [17–19]. This has also a direct impact on air quality and therefore on human health, since air pollution remains one of the main challenges for humanity nowadays. According to the World Health Organization, there are 4.2 million deaths every year as a result of exposure to outdoor air pollution, mostly due to cardiorespiratory diseases [20].

1.2. Effects of forests on human health

Once the relevance of forests within the Earth life context is emphasised, it is time to dig into the interactions between these ecosystems and human health. During the last decades, the interest in the connection between forests and people's health has increased leading to an emerging number of studies that provide evidence of the benefits of exposure to these forested ecosystems [21–25]. Overall, the results of these studies report effects of forests on the following body systems and functions: cardiovascular, endocrine, immune, respiratory, nervous, as well as the impact on mental disorders and psychological well-being [25–32]. Forest exposure has also generally been strongly correlated with stress regulation [30,33].

At a physiological level, and related to the *cardiovascular* system, forests have been shown to decrease blood pressure (systolic and diastolic) [34–38], pulse rate [37,39–41], heart rate [42,43] and other parameters and variables like the Heart Rate Variability (HRV), the high frequency of HRV or the concentrations of plasma Endothelin-1 among others [42–45]. Ideno et al., conducted a systematic review and meta-analysis including 20 trials and concluded that forest environments could correlate to lower levels of blood pressure [26].

Concerning the *neuro-endocrine* system, several studies have addressed the effects of forest exposure on stress biomarkers like cortisol, adrenaline, alpha-amylase, immunoglobulin-A, chromogranin-A, dopamine or norepinephrine [22,24,39,46–53] and showed that exposure to forests reduce the levels of these markers compared to the exposure to urban settings [24,39,40,46,50–53]. From the current existing literature, a recent review and meta-analysis concludes that exposure to forests can significantly affect cortisol levels inducing stress reduction [29]. These findings are specially relevant since chronic and acute stress, low capacity to recover from it, as well as states derived from suffering it, have been linked to different long-term pathologies and psychological disorders [54–58].

The effects of forest exposure to the *immune* system are one of the most remarkable findings. Among other authors, Dr. Qing Li is one of the forefathers of this field and has contributed with a great number of publications [31,59–66]. Generally, his research reports a significant increase in human natural killer (NK) cells activity and percentages; and in the expression of intracellular cytolytic molecules, perforin, granzyme A (GrA) and granulysin (GRN); as well as a lower percentage of T cells after forest exposure, particularly to forest chemicals compounds. These findings are in line with other investigations that suggest that forest exposure may induce an enhancement of the immune NK cells response and an activation of NK cells [67,68]. Previous research have also studied the beneficial effects on other parameters of the immunological and inflammatory systems as the Interleukin (IL)-6 , IL-8, IL-1β, tumour necrosis factor α (TNF-α) and C-reactive protein (CRP) [44,52,67,69,70].

Regarding the *respiratory* system, the following health variables or parameters have been shown to modulate after forest exposure: Forced vital capacity (FVC); Forced expiratory volume in the first second (FEV1); Forced expiratory volume in six seconds (FEV6); Cardio-ankle vascular index (CAVI); Fractional exhaled nitric oxide (FeNO); Pulmonary and activation-regulated chemokine/CC-chemokine ligand-18 (PARC/CCL-18); TIMP metallopeptidase inhibitor 1 (TIMP-1); and Surfactant protein D (SP-D) [52,71,72]. Jia et al., conducted a randomized controlled trial with COPD patients and observed significant lower levels of the parameters PARC/CCL-18 and TIMP-1 in the forest group after treatment, which may indicate that forest exposure could partly enhance COPD patients' health [52].

The *nervous* system variations in forest ecosystems have been addressed in different ways: from sleep quality assessment to dopamine levels, brain bioelectrical activity via Electroencephalography (EEG), cerebral oxygenated haemoglobin (HbO_2) and deoxygenated haemoglobin (HHb) [38,40,73]. Overall, these studies show significant positive effects on brain activity (by decreasing the activity of the sympathetic nerves and increasing the parasympathetic nerves) and underline the mental relaxation induced by forests. At a metabolic level, research has pointed out the regulatory effects on adiponectin and Dehydroepiandrosterone sulphate (DHEA-S) levels derived from forest exposure [40,74].

Although research on the benefits of forest exposure is increasing, evidence is not yet robust to draw certain conclusions linking forest to the recovery of certain pathologies. This is mainly because the great majority of the studies performed so far address physiological variables but not clinical parameters that can be directly linked to a curative effect or a symptom-improvement of particular pathologies. In addition, repeated and long-term exposure to forests are scarcely addressed among literature. Furthermore, most of the studies to the date, are conducted with small numbers of participants [75], which makes it difficult to develop powerful statistical analysis and to deal with the variability associated with experimental studies within an environment that presents many variables that can be hardly controlled. Although randomized controlled trials are lately increasing in this field of research, non-randomized controlled trials are still a significant part of the studies [25] making it difficult to control the effects observed in the forest environments. As assessed by Wen et al., the methodological quality of randomized controlled trial studies is significantly higher than the studies using other methodological approaches [25]. Furthermore, exposure time and type (includes a great number of activities from forest walking to forest bathing) varies substantially among the researches [25,75].

Forest bathing or *Shinrin-yoku* idea was created by the Ministry of Agriculture, Forestry and Fisheries of Japan in 1982 and it is commonly understood as staying in the forest environment and taking in the atmosphere of the forest in expectation of potential curative or therapeutic effects [25,51]. This exposure type and other pro-active exposures (as described in Bach et al., 2020) may present confounding variables in the effects of forest exposure itself since participants develop activities that may induce health effects *per se* (yoga, meditation and mindfulness, among others). All these limitations call for further research to identify benefits of forest exposure and to unveil whether or not the reported benefits are induced exclusively by forest exposure [76]. Overall, current research is highlighting the positive physiological effects of forest exposure, but more research is still needed to build a robust body of literature that provides relevant data to the public health and healthcare community in regard to clinical practice guidelines and preventive medicine.

While *psychological* effects of forest exposure evade from the scope of this thesis, it has to be mentioned that research is also supporting the benefits of these ecosystems on mental health and psychological wellbeing [77,78]. One of the most studied impacts is on the emotional states [34,35,37–44,52,79–81] where the widely used Profile of Mood States (POMS) and other techniques have proved the effects of forests decreasing the emotional states of "anger-hostility", "tension-anxiety", fatigue, "depression" and increasing "vigour" and other positive emotions. Regarding *mental disorders*, research has underlined also the decreases in anxiety and depression levels linked to forest exposure [37,38,41,82–86].

1.3. Forest-health equation: Mechanisms and Pathways

Now that the potential benefits have been exposed, two main questions arise: *What is it on forest that produce this physiological and psychological changes?* And *How do we interact with these forest elements and features?* First of all, it has to be stated that little information is currently available to respond to these questions [75]. This is the fundamental reason why the first chapter of this thesis "How Should Forests Be Characterized in regard to Human Health? Evidence from Existing Literature" is developed. Rather than summarising all the elements and features from forest that have been related to human health outcomes (see chapter 1), here we would like to elucidate the need for further research in the field of ecology, chemistry, microbiology and applied management to build up more integrative and comprehensive analysis. Nowadays, many questions are set on the table: *Are different tree species presenting different beneficial effects? Are biodiverse forests more beneficial than monocultures? Are mature forests more suitable than young-stand forests?* Addressing these and other questions that link health to forest composition, structure and management is crucial since health promotion is now starting to be integrated into international and national forest agendas (forests have already an essential role in the United Nations Agenda in the related sustainable development goal (*SDG) number 15, Life on Land*), leading to changes in strategies, plans and management [21,28,87]. Some authors have highlighted the shift in forest priorities from production and conservation to recreation and promotion of health [87,88]. If health provision is to be integrated into management plans, laws and projects, managers, policymakers and the healthcare community will need relevant data to better understand the specific mechanisms and pathways by which forests' elements and features can affect human health.

Several authors have hypothesized and described the potential pathways by which forests interact with people [89–92]. The way to sort and categorise the key elements that conform the suggested pathways varies notoriously from each approach. While some reviews connect the pathways to the five-sense forest experience [21], human-nature evolutionary perspectives [93,94] or other social visions [95], others break the pathways into exposure, elements and features, physiological and psychological states, behaviours and conditions and health outcomes [91]. From this last approach, in this thesis we have as well considered the individuals expectations when visiting the forest, the exposure types, the effects of forest management in the equation and split the forests determinants in elements and characteristics (Figure 1).

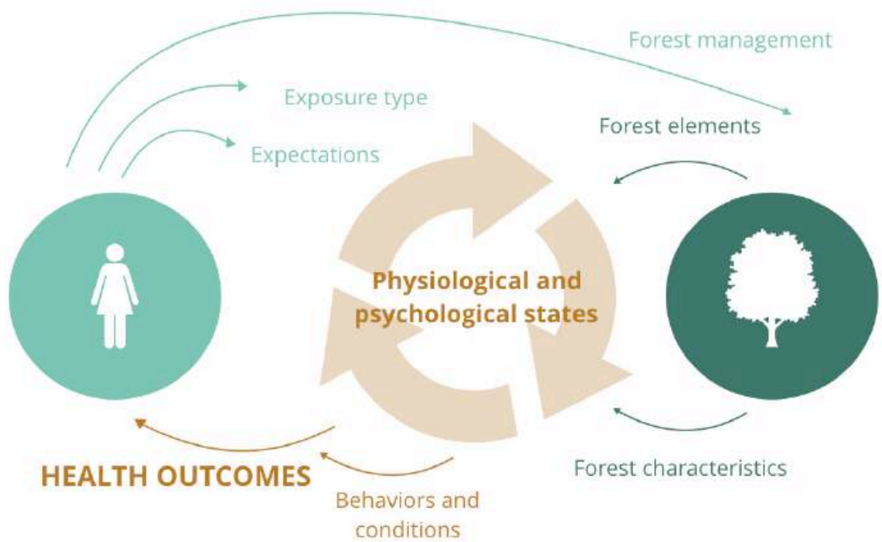


Figure 1. Forest and human health interaction pathway.

Within this diversity of approaches, there is a key element that stands out among others: forest chemistry. This element is sounded out by many of the pathways described by literature linked to forest features or related to the olfactory stimuli of forests. At this point, we want to take the opportunity to further describe this element from the forest that constitutes the central axis of this thesis.

Forest environments present a great number of biogenic volatile organic compounds (BVOCs). These chemical compounds are naturally emitted by plants and play diverse roles at multiple scales at plant ecology level; from cellular protection and defence at foliar level, through chemical signalling at ecosystem level, up to influencing rainfall at local and regional scale (Figure 2) [6,96]. Likewise, BVOCs profoundly affect biosphere-atmosphere interactions by atmospheric reactivity, aerosol growth processes, cloud formation and, therefore, radiative balance [97,98]. Monoterpenes are part of the isoprenoid class, the largest class of BVOC, and are major components of forest atmospheres [99,100]. These compounds are produced by plants as a defensive mechanism against environmental stress and herbivory [99,101,102] and allow intra- and inter-plant communication acting as signals [103,104]. Monoterpenes can have different properties which vary from insect-proof to bactericidal, depending on their concentrations and proportions [105–107].

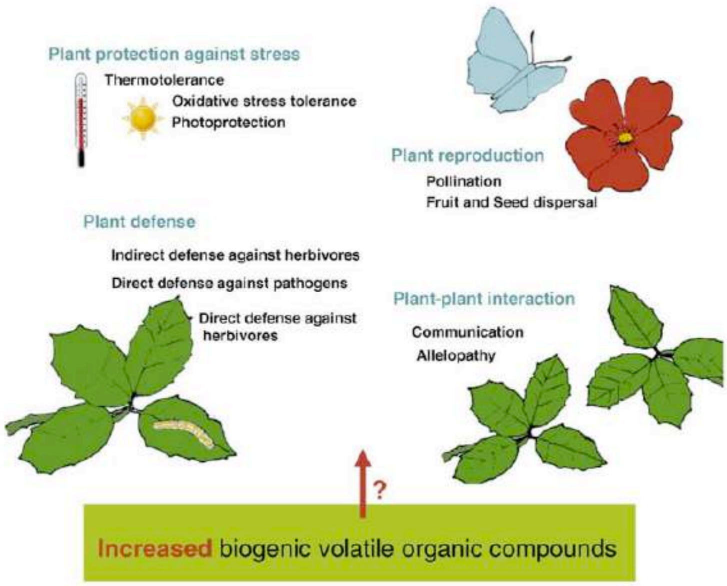


Figure 2. BVOCs role in plant physiology. From Peñuelas and Staud, 2010.

Monoterpenes have been shown to interact with human health in different ways, from stress relief to an effect on the immune function. Inhaling monoterpenes has been shown to decrease blood pressure and cortisol levels [108], improve antibiotics efficiency [109] or boost the immune system [110–112] especially by increasing the percentage and activity of NK cells [60,61,65,66]. In general, monoterpenes that relevantly affect cellular and animal systems have shown anti-inflammatory, anti-tumorigenic or neuroprotective activities [113]. So far, only one study has analysed the absorption of monoterpenes in blood after forest exposure, identifying the monoterpene species present in coniferous-forest atmosphere in serum samples of individuals after walking in the forest [114]. They also identified an increase in the amount of ◎-pinene in the serum after the individuals walked in the forest, as well as differences in monoterpene composition and abundance between coniferous and broad-leaved forests air [114]. Lee *et al*. (2018) reported that the mean atmospheric concentration of monoterpenes at 1.5 meters height was higher in a natural than a tended forest [110] suggesting that vegetation characteristics derived from management may affect terpenes' concentrations under the canopy. Although some studies have identified certain elements like BVOCs to be potential determinants of the health effects induced by forest exposure [65,66], few have unveiled the mechanisms and pathways by which forests interact with human health [113].

In addition to these chemical compounds related to forest, new advances in technologies and scientific techniques have identified a new forest element: the microbiome of forest air. Craig et al. suggested a potential interaction between this airborne biocomponent and human microbial communities and the health effects that could be derived from it. Furthermore, the forest airborne microbiome may present a co-dependence with monoterpenes since these chemical compounds present bactericidal and antifungal properties [105–107] which might interact with forest airborne within the forest ecosystem and as well in the human body such as skin, intestinal and lung). These authors have thus opened a debate on the possibility that the microbiota of natural environments could influence human physical and mental health and well-being [115].

1.4. Thesis motivation: Mediterranean forests and Health

The present thesis pursues to provide scientific data to contribute to filling the gaps and answering the questions underlined among this introductory chapter. Furthermore, we focus the attention on the forest of the Mediterranean area, particularly in the northern part of the Mediterranean Sea. Nowadays, most of the research concerning forests and human health has been developed in Asian countries (mainly Japan, South Korea and China) [75,116]. Although some of the studies are laboratory experiments that could apply to any forest condition, a vast number of the *ex-situ* researches have been carried out in Asian countries where biotic and abiotic conditions differ from other areas of the planet.

We placed the research area in Mediterranean forests in Catalonia (North-East of the Iberian Peninsula). These are forests that have been highly altered by human activity during several centuries [117]. Currently, 64.1% of the Catalan territory is covered by wooded ecosystems from which 1.350.980 ha are forests [118]. A wide range of forest types can be found within the area, but *Pinus halepensis* and *Quercus ilex* are the most abundant species [119,120]. 75.1% of the forested surface of Catalonia has a private ownership and nearly 60% of these private lands are not managed [121]. This might be due to the low valorisation of Mediterranean products, the slow tree growth and limited timber production typical from the area, which results in owners' discouragement and lack of interest to manage their wooded properties [117]. The reasons why we place our attention in this Mediterranean region are diverse. On one hand, these forests are particularly under-valued. Although efforts have been made in this region to highlight the role of forests on biodiversity conservation [122–124], mitigation of climate change [125], water regulation [126] and pollution removal [127,128], society still has not grown a global awareness about the relevance of these forests. On the other hand, the current lack of forest management and the afforestation derived mainly from land abandonment is causing an uncontrolled growth of forests, with the subsequent increase of forest biomass which is ultimately translated in a higher risk of (great) forest fires [129,130], a loss of biodiversity and an increase of water sequestration. For the aforementioned reasons, we conduct this thesis to study human health links to Mediterranean forests in an attempt to bring up a new forest value and a novel vision to the Mediterranean society towards their own forests.

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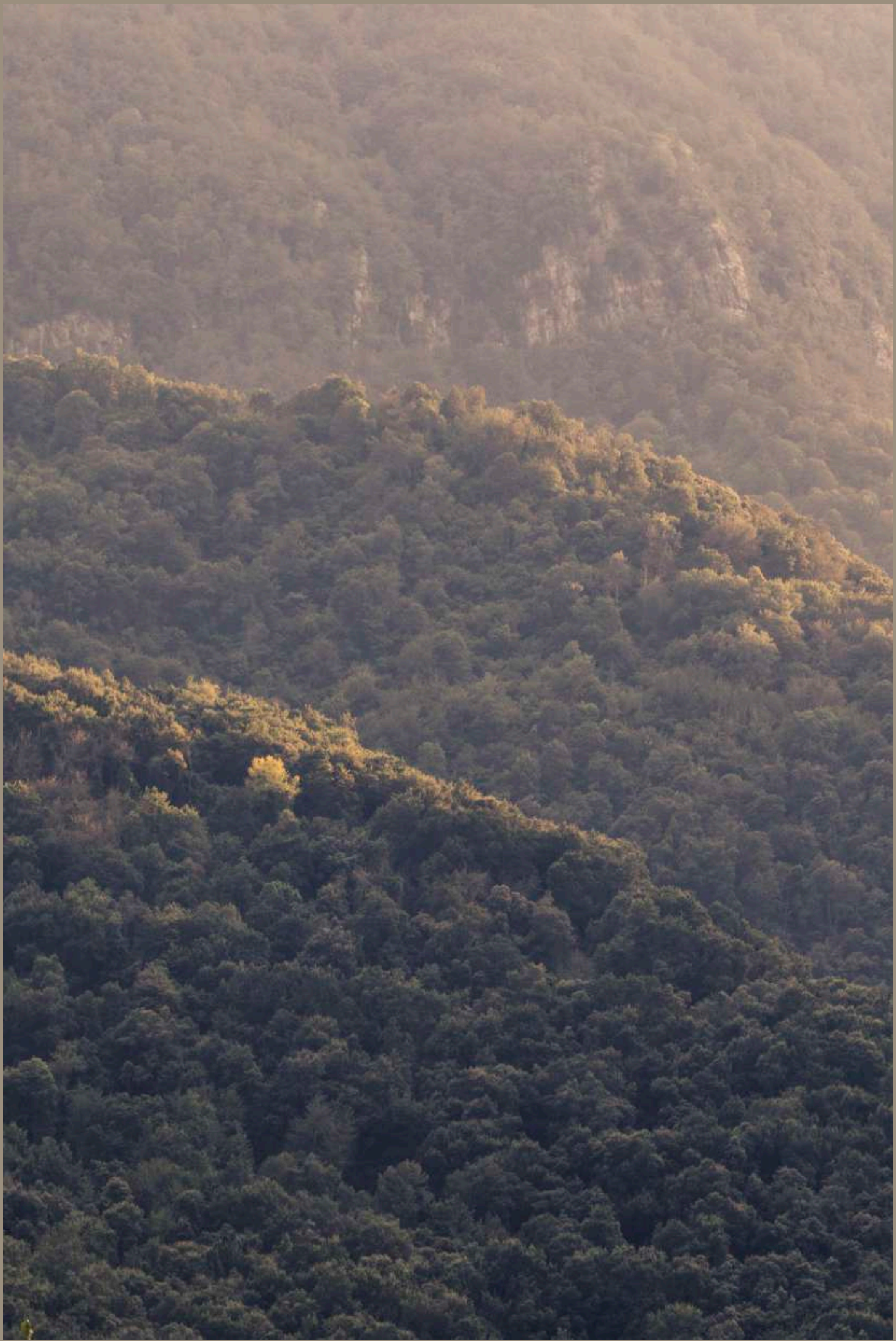


Chapter 2

Goals and research questions

2. Goals and research questions

The main goal of the present thesis is to assess to what extend Mediterranean forests can be linked to human health. More particularly, the specific aims of the thesis are: A) analysing which forest variables have been linked with effects on human health; B) characterising the Mediterranean forest air monoterpene concentrations below the canopy; C) studying the human absorption of monoterpenes in the forest; and D) investigating the potential of forests to regulate human stress markers.

To address the aforementioned general and specific goals, we have split them into different *Research Questions (RQ)* which have been the backbone of the thesis. Thus, we present below the description of the thesis structure together with the research questions.

RQ1. *Which forest elements and features can be consistently related with human health from the research conducted in experimental studies with humans?*

In order to answer the research question 1, the first chapter is based on a systematic review that aims at analysing which elements and features from forest (forest variables) have been linked with effects on human health in the scientific literature. The results of this chapter were compelled in a manuscript which was published in the *International Journal of Environmental Research and Public Health* in February 2020.

RQ2. *Are monoterpenes air concentrations in Mediterranean holm oak forests comparable to other studies where effects on human's health were underlined?*

The availability of these chemical compounds in Mediterranean forests air constitutes the first and essential step to study the monoterpenes absorption. In this sense, chapter 2 characterised the total monoterpene concentrations at nose height in a Mediterranean holm oak forest during the annual emission peak. This research was published in the *International Journal of Environmental Research and Public Health* in June 2020.

RQ3: *Can humans absorb monoterpenes emitted by trees during a 2 hour forest exposure?*

Chapter 3 aims to describe the basic interactions between monoterpenes and humans. To do so, we have focussed on the absorption of these chemicals compounds by humans when walking through the forest. This research has been submitted to the *Journal of Pharmaceutical and Biomedical Analysis* in July 2020 and it is currently under revision.

RQ4: *To what extent humans' physiological stress can be mitigated when visiting a Mediterranean forest?*
Last research chapter's goal is to describe the role of Mediterranean forests as potential stress mitigators through a field experimental study. Thus, chapter 4 is based on a field experimental study that aimed at analysing the variation and evolution of a combination of human salivary stress biomarkers during forest exposure. This research has been submitted in the *Environment and Behaviour Journal* in May 2020 and it is currently under revision.

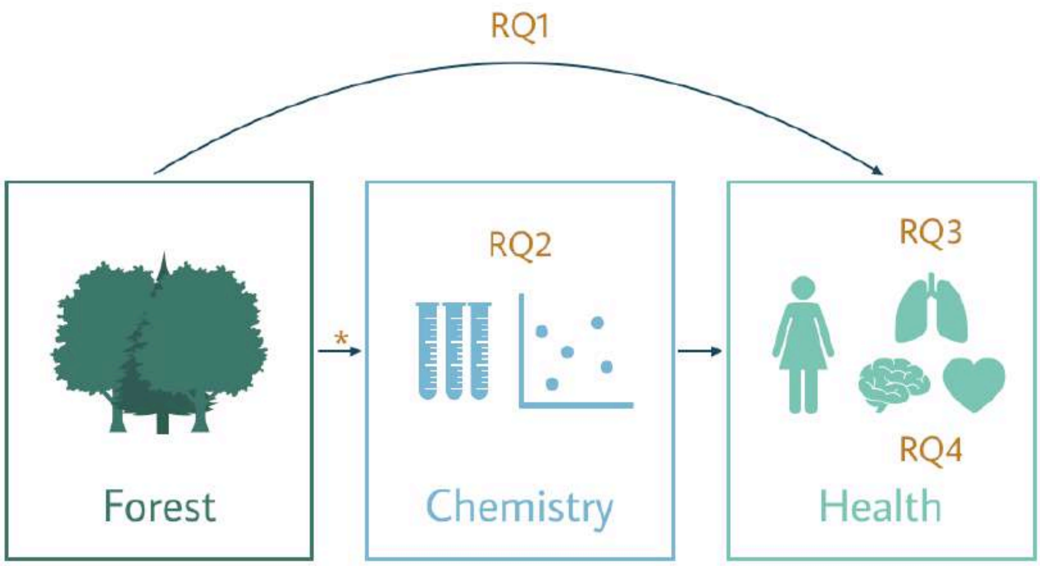
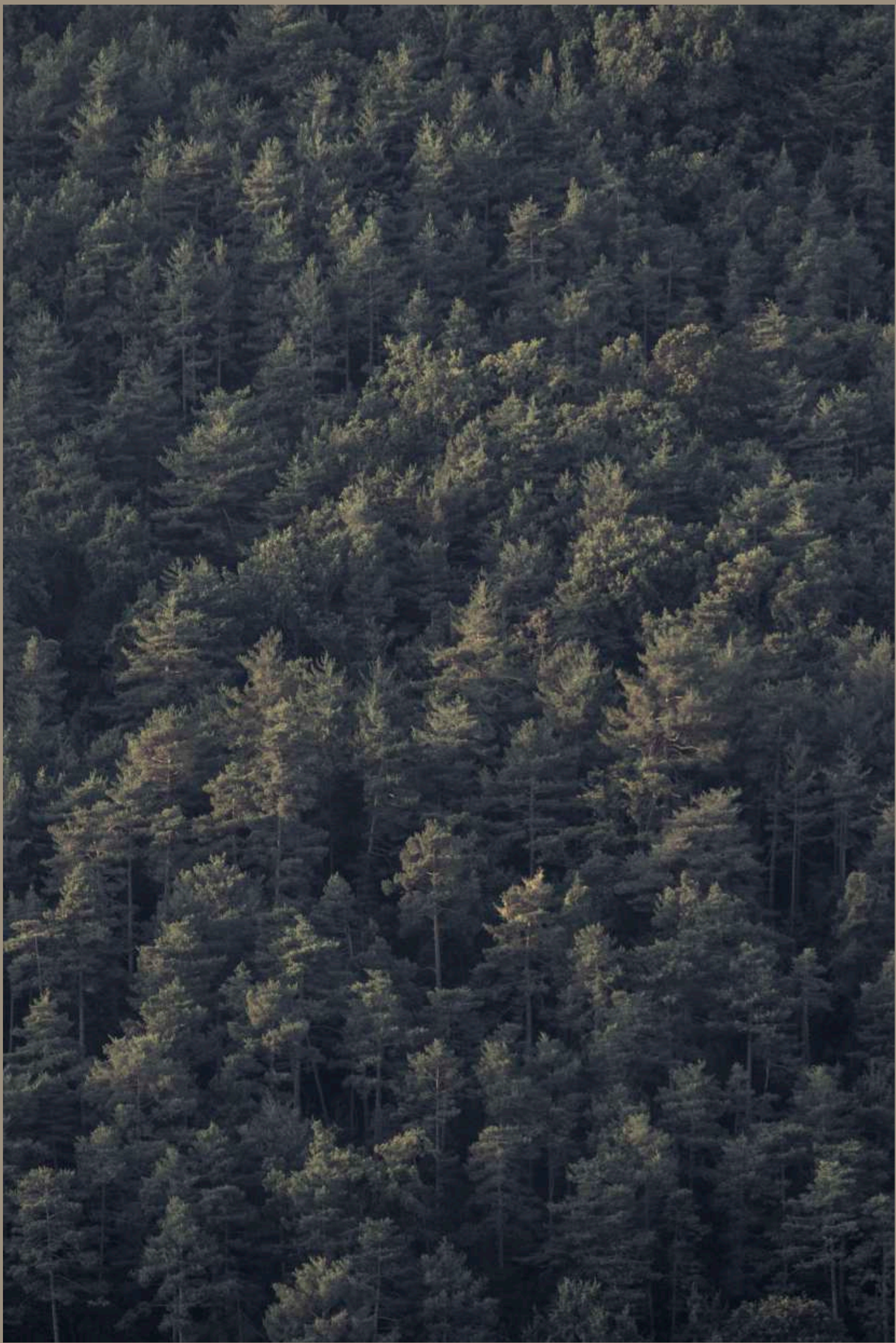


Figure 3. Research topics diagram and research questions (RQ).

Initially, the thesis integrated a chapter between chapters 1 and 2. This was a chapter that prospected the effects of different forest variables (such as forest type, composition or structure) as modulators of forest chemistry (monoterpenes mainly) (marked as an asterisk in figure 3). Although we are convinced about the relevance of this chapter, due to the several restrictions derived from the Covid-19 situation faced during the last part of the thesis, it has been technically impossible to include it. Nevertheless, this research will be finalised and published as a research article as soon as the situation allows it.



Chapter 3

How Should Forests be Characterized in Regard to Human Health? Evidence from Existing Literature

Abstract: The potential of forests as a source of health has been addressed by the scientific community and is now being considered in national forest strategies, management plans and policies. Studies identifying the mechanisms by which forest characteristics may induce these effects on human health are nevertheless scarce. This systematic review of literature on forests and human health with real-life human exposure was conducted to assess the extent to which forests have been studied and described in detail and the extent to which relationships between forest variables and health effects have been reported. The analysis underlines the lack of forest descriptions in 19.35% of the 62 studies selected for review as well as the high heterogeneity of forest variables' description. Patterns among the articles could not be identified correlating the broader forest variable (forest type) and the most studied health variables identified (blood pressure, pulse rate or/and cortisol levels). These findings, together with previous ex situ researches, suggest the need to ameliorate and incorporate more accurate descriptions of forest variables within human health studies to provide data for forest management and the potential use of these habitats for preventive medicine and clinical practice guidelines.

Keywords: forest exposure; *shinrin-yoku*; forest characterization; human health; forest management; preventive medicine

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1. Introduction

Interest in the connection between forests and human health is increasing among studies analyzing the effects of nature on human health [1–3]. The potential of forests as a source of health has led to numerous studies that provide evidence of the benefits of exposure to these forested ecosystems [1,3,4]. Previous reviews that have compiled the results from these studies report the effects of forests on the following body systems and functions: cardiovascular, respiratory, endocrine, immune, nervous, as well as the impact on mental disorders and psychological well-being [5–9]. Forest exposure has also generally been strongly correlated with stress regulation [9,10]. Although some studies have identified certain elements like biogenic volatile organic compounds (BVOCs) to be potential determinants of the health effects induced by forest exposure

[11,12], few have unveiled the mechanisms and pathways by which forests interact with human health [13].

Health promotion is now starting to be integrated into national forest agendas leading changes in strategies, plans and management [1,7,14]. Some studies have highlighted the shift in forest priorities from production and conservation to recreation and promotion of health [14,15]. If health provision is to be integrated into management plans, law and projects, managers, policy-makers and the healthcare community will need data to better understand the specific mechanisms and pathways by which forests' variables can affect human health.

The aim of this review was thus to analyze the available literature on forests and human health to assess:

- the extent to which forests are studied and described in studies of human health;
- the extent to which patterns can be identified between forest variables and physiological health effects.

2. Materials and Methods

Studies published before May 2019 were systematically reviewed following the *Guidelines for Systematic Review and Evidence Synthesis in Environmental Management* [16].

2.1. Review Scoping

Previous reviews were analyzed to identify relevant terms for configuring a Boolean search: Cho et al. (2017), Hansen et al. (2017), Meyer and Bürger-Arndt (2014), Oh et al. (2017) and Song et al. (2016) [3,6,9,13,17]. The keywords identified were divided into three main blocks: (a) habitat (synonyms of forested environments and terms referring to these areas), (b) activity (action developed in forests that have been tested seeking health outcomes) and (c) health effects (effects of forest exposure on body and health systems or functions). A scoping study was finally conducted using the terms obtained in the review analysis to assess the relevance of each keyword.

2.2. Searches

The keywords identified were combined with Boolean operators considering the three blocks mentioned above to generate the following search: ((forest OR forests OR forested OR woodland*

OR jungle OR rainforest) AND (exposure* OR visit* OR bath* OR walk* OR recreation* OR "forest-walking" OR "spending time" OR trip OR "forest-air bathing" OR healing) AND ("human health" OR wellbeing OR well-being OR stress OR health) NOT (biomass OR "forest health" OR "random forest" OR fire OR virus OR "climate change" OR soil OR carbon OR parasite OR radiation OR pathogen)) OR (shinrin-yoku OR shinrinyoku OR "shinrin yoku").

Shinrin-yoku is commonly understood as being in the forest environment and taking in the atmosphere of the forest in expectation of potential curative or therapeutic effects [18]. This term was thus incorporated in the search but was not combined with other keywords as it implies a wide range of activities or actions in a forested environment for health purposes.

The Web of Science (WOS) and PubMed were selected for the systematic search. Terms were searched in WOS for topic without any other filter, but terms were searched in PubMed for all fields containing the terms and sorted by clinical studies and trials, comparative and observational studies and randomized control trials. The results from PubMed were also filtered by experiments only with humans and articles only written in English.

2.3. Screening

The combined searches identified 3445 articles (Figure 1). Titles, abstracts and keywords were screened, and duplicates and off-topic articles were excluded. Only articles published between 1900 and 2019 and correlating any forest (including urban or virtually imaged) with any variable of human health or well-being were eligible.

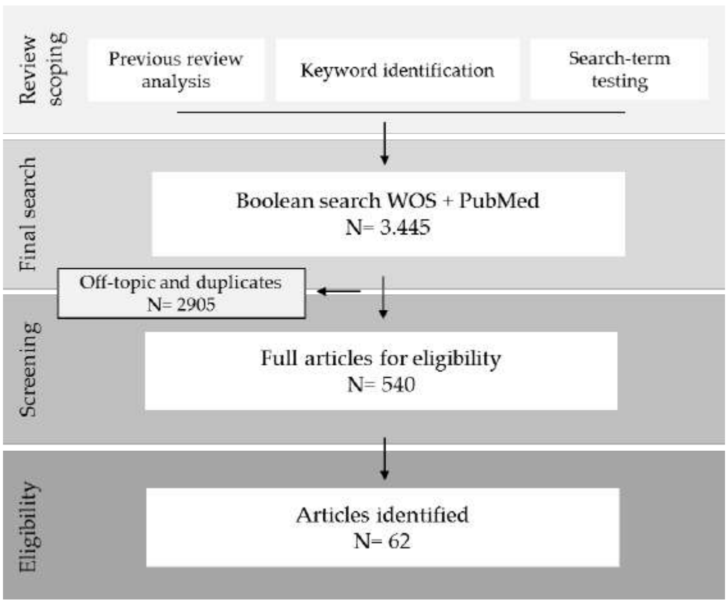

Figure 1. Flow diagram of article selection for the systematic review.

2.4. Eligibility

Articles were examined for title, abstract, keywords and methods, if needed. Inclusion considered the following criteria:

- experiments with a real-life exposure to forests;
- experimental studies with humans;
- articles containing quantitative objective measurements of physiological health variables.

Laboratory experiments and studies with only virtual images or videos; studies with qualitative, self-rated or self-perceived health issues; reviews and articles using broad-scale spatial data were all excluded. In this study forests are considered as areas covered by trees which are not predominantly under agricultural or urban land use. Therefore, we did not include studies conducted in plantations or urban forests. A total of 62 articles were ultimately included in the analysis.

2.5. Data Collection

Three authors gathered the information from each article using a codebook, with consensual definitions and consultation to resolve differences. Inter-code consistency was tested by each author by reviewing three random articles.

Details of variables were collected from each study based on article basics, exposure characteristics, forest descriptions and health outcomes. Items from each group are presented below.

- Article basics: *authors*, *year* of publication, *title*, *country* where the study was conducted, *number of participants* and *study design*. This last item, concerning the methodology used in the study, was further classified into three categories: before-after (B-A) studies, control trials (CTs) (randomized or not) and comparative studies (when different forests or types of groups were compared). This distinction was made to better register the records of health variables for a more comprehensive analysis. Effects before and after forest exposure, between urban and forest environments or even between forest types could thus be compared.

- Exposure characteristics: we suggested the following *exposure type* classification considering three levels: (a) passive exposure (when participants just stayed in the forest, sitting or viewing the landscape, or passively walked around the forest); (b) active exposure (when participants did any kind of moderate to vigorous-intense physical activity such as running, cycling or any other sport) and (c) pro-active exposure (when the activities and actions conducted could induce mental or physical well-being effects by itself, without necessarily being in the forest, e.g., yoga, meditation). *Exposure time* was also recorded and ranked in minutes (≤60 min), hours (≤24 h), days (≤365 days) and years (>1 year).
- Forest descriptions: forest information from the articles were gathered and sorted into eight categories: *forest type* (taken from the species composition or dominance if not explicitly mentioned in the text), *abiotic variables measured* (e.g., temperature, humidity, light intensity, wind speed), *general forest description* (referring to any broad information about the forest, such as the surface of the forested area, the conservation status or any geographical information like the altitude), *forest species described* (tree species composition), *forest age*, *management strategy (*any information about the management regime develop in the studied forest*)*, *forest variables described* (e.g., tree density, biodiversity, BVOCs, vegetation structure, diameter at breast height, basal area) and *forest variables measured* (if numerical values of the measurements were recorded). All data were gathered into these representative categories of the detail degree of description for a forest ecosystem. We assumed forest type as the broadest description of a forest, and the measurement of a particular forest variable as the most accurate and precise approach for describing a stand.
- Health outcomes: measured health variables were registered for each article. The effects of forests in B-A studies were assessed and classified at four levels: *increases* (if the value of a variable increased significantly after a visit to the forest), *decreases* (if the value of a variable decreased significantly after a visit to the forest), *non-significant* (NS) results after the trial and *mixed effects* (when changes were significant but increased for some participants while decreased for others). Similarly, the effect of a forest was assessed for CTs and classified as: *Higher* (if the value of a variable was significantly higher in the forest than the urban group), *Lower* (if the value of a variable was significantly lower in the forest than the urban group), *Non-significant* (NS) after the trial and *Mixed effects* (when changes were significant but were lower for some participants and higher for others). Significant levels were set at $p < 0.05$.

Health variables were not registered if an article did not explicitly present statistical analysis of values before and after exposure or a comparison between the tested environments.

2.6. Data Analysis

Descriptive and exploratory analyses were used to identify patterns among the data collected. We analyzed the frequency in which different forest variables were described in the existing literature sorting them out in categories according to the degree of detail description and produced summary statistics. We developed pivot tables to examine the relationships between forest and human health variables. These relationships were obtained only for the most commonly studied health variables registered in this review: blood pressure (diastolic and systolic), pulse rate and cortisol levels (blood and saliva). These variables were also surveyed in two recent reviews, which found strong correlations with exposure to forest ecosystems [5,8].

3. Results

3.1. General Overview

An overview of the selected articles' basics is presented in Table S1. Ninety percent of the articles were conducted in Asian countries (53.23% of the total in Japan, 19.35% in South Korea, 12.90% in China and 4.84% in Taiwan). Only six of the 62 studies were conducted in Europe (three in Sweden and one each in Poland, Denmark and Spain). The number of participants involved in the studies varied from seven [19] to 625 [20]. Exposure time also varied greatly among the studies, from 10 min to repeated exposures for six years. Most of the articles (59.68% of the total) considered short exposure times (minutes or hours), whereas 40.32% investigated the health impact of one day or longer exposures. For exposure type, 79.03% of the articles analyzed the effects of forests during a passive exposure to a forest environment (motionless, viewing or passively walking only), 20.97% studied the effect of a pro-active exposure (e.g., yoga, mindfulness) and none of the articles assessed the effects during an active exposure (doing any intense physical activity). Of the total, 64.52% were CTs, 24.19% were B-A tests and the remainder were comparative studies.

3.2. Forest Variables

We found no consistent and uniform consensus between the descriptions of the forest ecosystems and human health (Table 1). The forest where the analyzed studies were conducted was not described in 12 out of the 62 selected articles. Considering forest type, 33.87% of the studies did not provide this information, even though our requirements for this item description were broad. The forest types studied in the articles were: coniferous, broad-leaved (evergreen and deciduous), bamboo and mixed forests. Forest age, which varied from 10 to 120 years, was reported in 10 articles. Seventeen articles provided information for described or measured forest variables. From two articles providing the management regime of the studied forest, only one described the technique applied in the stand. The specific forest variables identified were: tree density (five articles), diameter at breast height (two articles), biodiversity level (one article), species dominance (one article), tree height (one article), BVOCs concentrations (five articles), air quality (four articles) and pollutant concentrations (one article). The main BVOCs identified by the five studies that reported concentrations were alpha- and beta-pinene, tricyclene, camphene, limonene, camphor, alpha-phellandrene, carene and isoprene [21–25].

Table 1. Details of forest descriptions. Symbol "*" indicates that the article provides the forest description information. Manag. Strategy, management strategy; Forest var. described, forest variables described; and Forest var. measured, forest variables measured.

Reference	(-) Detail of Description (+)							
	Forest Type	Abiotic Variables	Forest Description	Forest Species	Forest Age	Manag. Strategy	Forest Var. Described	Forest Var. Measured
[26]	*	*	*	*	*	*	*	*
[25]	*	*	*	*	*		*	*
[27]	*	*	*	*	*		*	*
[28]	*	*	*	*	*		*	*
[29]	*	*	*	*			*	*
[30]	*	*	*	*			*	*
[31]	*	*	*	*			*	*
[32]	*	*	*	*			*	*
[33]	*	*	*	*			*	*
[34]	*	*		*			*	*
[22]	*	*		*			*	*
[35]	*	*	*				*	*
[24]	*			*			*	*
[36]			*				*	*
[23]	*	*					*	*
[21]		*					*	*
[37]	*	*	*	*	*		*	
[38]	*	*		*		*		
[39]	*	*	*	*	*			
[18]	*	*		*	*			
[40]	*	*		*	*			
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3.3. Health Variables

Among the 62 studies, 103 health variables were recorded (Table S2), including measurements of the same variable in different samples (e.g., blood, urine, saliva). The measured variables mainly belonged to the endocrine/reproductive, cardiovascular, metabolic, nervous, respiratory and immune systems. The most commonly studied health variables were blood pressure (23 B-A and 20 CT records on diastolic blood pressure (DBP) and 24 B-A and 18 CT records on systolic blood pressure (SBP) from all selected articles), pulse rate (PR) (12 B-A and 9 CT records among all selected articles) and cortisol levels (11 B-A and CT records in studies measuring blood and saliva levels from all selected articles).

Nearly 55% of the studies provided information about the potential effect of forest type on blood pressure (Tables 2 and 3). Mixed forests were studied the most for both DBP and SBP in B-A studies, where only one study reported an increase in SBP after forest exposure. The DBP records showed no consistent patterns, specifically referring to the effects of mixed forests (six non-significant records vs three records of decreases). For SBP, more consistency can be observed with six decrease records vs two non-significant. Only single decrease records were identified in coniferous and bamboo forests for both blood pressures.

Table 2. Effects of forest type on blood pressure in the before-after studies. (A) diastolic blood pressure (DBP) and (B) systolic blood pressure (SBP). NS, not significant.

A	**Forest type**	***Effect on Diastolic Blood Pressure (DBP)***				
		Decrease	**NS**	**Increase**	**Mixed Effects**	**Total**
	Broad-leaved	-	1	-	1	2
	Coniferous	1	-	-	-	1
	Bamboo	1	-	-	-	1
	Mixed	3	6	-	-	9
	Total	5	7	-	1	13
B		*Effect on Systolic Blood Pressure (SBP)*				
		Decrease	NS	Increase	Mixed effects	Total
	Broad-leaved	-	2	-	-	2
	Coniferous	1	-	-	-	1
	Bamboo	1	-	-	-	1
	Mixed	6	2	1	-	9
	Total	8	4	1	-	13

Table 3. Effects of forest type on blood pressure in the control trials. (A) Diastolic blood pressure (DBP) and (B) systolic blood pressure (SBP). NS, not significant.

A	**Forest type**	***Effect on Diastolic Blood Pressure (DBP)***				
		Lower	**NS**	**Higher**	**Mixed Effects**	**Total**
	Broad-leaved	1	1	-	1	3
	Coniferous	3	-	-	-	3
	Bamboo	1	-	-	-	1
	Mixed	1	5	-	-	6
	Total	6	6	-	1	13
B		*Effect on Systolic Blood Pressure (SBP)*				
		Lower	NS	Higher	Mixed effects	Total
	Broad-leaved	1	1	-	1	3
	Coniferous	1	1	-	-	2
	Bamboo	-	1	-	-	1
	Mixed	-	6	-	-	6
	Total	2	9	-	1	12

Contrasting these records with CTs measurements in the cities (Table 3) indicated that the blood-pressure levels were not higher in forests than the cities in any of these studies, although one study reported mixed effects among participants in a broad-leaved forest. Three records complemented the contribution of coniferous forests to blood pressure, with lower levels after forest exposure compared to city exposure for DBP. In contrast, the previous B-A decrease records for mixed forests were not supported by the CTs records, where only one DBP record was lower for forest than city, whereas the rest indicated non-significant differences between the two environments.

The effects on PR are presented in Table 4. The B-A records did not follow a specific pattern. CTs records of PR, however, were lower in three studies comparing mixed forests to cities.

Table 4. Effects of forest type on pulse rate (PR) in the (A) before-after studies and the (B) control trials. NS, not significant.

A	Forest type	*Effect on Pulse Rate*				
		Decrease	**NS**	**Increase**	**Mixed Effects**	**Total**
	Broad-leaved	-	-	-	1	1
	Mixed	2	3	1	-	6
	Total	2	3	1	1	7
B		Lower	NS	Higher	Mixed effects	Total
	Broad-leaved	1	1	-	-	2
	Coniferous	1	-	-	-	1
	Mixed	3	-	-	-	3
	Total	5	1	-	-	6

Few of the B-A studies reported cortisol levels. Cortisol measurements were reported for only two forest types: broad-leaved and mixed forests (Table 5). The B-A studies did not identify a decrease in cortisol levels for broad-leaved forests, but levels were lower for broad-leaved forests than cities. Likewise, cortisol levels were consistently lower in the B-A and CT records for mixed forest, even when compared to cities.

Table 5. Effects of forest type on cortisol levels in the (A) before-after studies and the (B) control trials. NS, not significant.

A	Forest type	*Effect on Cortisol Levels*				
		Decrease	**NS**	**Increase**	**Mixed effects**	**Total**
	Broad-leaved	-	2	-	-	2
	Mixed	2	-	-	-	2
	Total	2	2	-	-	4
B		Lower	NS	Higher	Mixed effects	Total
	Broad-leaved	4	1	-	-	5
	Mixed	3	-	-	-	3
	Total	7	1	-	-	8

4. Discussion

This systematic review was conducted to analyze the details of forest descriptions among studies of these ecosystems’ potential effects on human health and to identify patterns between forest and health variables, when possible. The number of participants, exposure time and type were highly variable among the 62 selected articles. The impacts on health were generally positive, while a considerable number of studies showed non-significant results. Most studies addressed physiological health variables during short periods of time in small samples of healthy populations mainly focusing on parameters with low specificity for clinical decisions.

This review found that: a) 19.35% of the articles lacked any forest description; b) the descriptions in the articles that did provide this information were highly heterogeneous and c) no pattern was identified in the data between health variables from the three most studied variables (blood pressure, PR and cortisol level) and the basic level of forest description considered: forest type. From the total, 66.13% of the articles described the forest type or procured information to estimate it. Other reviews analyzing the link between nature, forests and human health have also reported this scarcity of confounding factors description of forest environments [17,78], perhaps due to the belief that these data are not yet relevant. Descriptions of forest ecosystems for determining effects on human health may nevertheless become crucial with the increasing interest from many spheres of the society. This topic has both attracted the attention of the scientific community in recent decades [1,2] and has become an emerging priority for policy makers, managers and planners. Examples can be found at international level, where the use of forests to foster human health has been included in the agendas of forest policy (e.g., IUFRO Task Force on Forest and human Health, 2007), in Asian countries such as Japan and South Korea [1,14] and in some European countries [7]. Some studies predict that forest priorities can change from production and conservation to recreation and health promotion [14,15], so detailed information about the characteristics of these habitats should be provided for its management and its potential use for preventive medicine.

Some studies have begun to address the relevance of forest characteristics to human health. Saito et al. (2019) assessed the differences in various health variables between exposure to an unmanaged forest and a managed forest [26]. Blood pressure and saliva cortisol levels decreased significantly for both forests after a stress stimulus but records were significantly lower in the managed than the unmanaged forest [26]. Another study reported that health responses differed between an unmanaged and a managed forest, with significantly more favorable acute insulin reactions and levels of oxidative stress in the unmanaged forest, underlying more profound beneficial effects in the unmanaged than the managed forest [25]. These two studies, however, did not use a control group in an urban setting. Sonntag-Öström et al. (2014) reported that heart rate was significantly lower for three forest environments (a forest by a lake, an open forest with exposed bedrock and a closed spruce forest) when compared to a city and in particular, it was significantly lower for the forest by the lake than the other forest environments [39]. DBP in the same study did not differ significantly between the city and the forest with exposed bedrock but was significantly lower for the forest by the lake and the spruce forest than the city [39]. An et al. (2004) analyzed through digital images the different effects of stand density for two forest types, showing higher frontal brain activity related to greater stand density and brain relaxation when viewing lower stand density in coniferous forests while for the broad-leaved forest images, 50% stand density was related to stability of brain activity and PR, whereas 100% density was associated with more active electroencephalogram [79]. Blood pressure, heart rate, body temperature and oxygen saturation did not differ significantly before and after forest exposure in a comparative study contrasting the health effects of a mature and a young forest [37]. The results of these studies together with our findings indicate the need for further research and the need to integrate descriptions and measurements of forest characteristics in studies of human health.

In regard to forest variables, some studies have identified BVOCs as a key element in these ecosystems [12,25,80]. Terpenes, sometimes called phytoncides, are the largest class of naturally occurring organic compounds and the major components of forest atmospheres [81]. These compounds are produced by plants as a defensive mechanism against environmental stress and herbivory [81,82,83]. Terpenes that relevantly affect cellular and animal systems have shown anti-inflammatory, anti-tumorigenic or neuroprotective activities [13]. Only five of the studies in our review measured terpene levels in forest air, identifying mainly alpha- and beta-pinene, tricyclene, camphene, limonene, camphor, alpha-phellandrene, carene and isoprene [21–25]. Lee et al., (2018) significantly associated alpha-phellandrene with an acute insulin reaction, consistent with another in vivo study where alpha-phellandrene increased immune responses [84]. Although the included studies of Dr. Li did not directly associate the health outcomes observed with terpenes, a previous in vitro study did, reporting a significant increase in human natural killer (NK) cells activity and in the expression of intracellular cytolytic molecules, perforin, granzyme A (GrA) and granulysin (GRN) by phytoncides [11]. Komori et al., (1995) similarly reported the effects of citrus fragrance in forests on the immune and endocrine systems, analyzing NK cells activity and urinary cortisol and dopamine levels [85]. Li et al. (2009) reported significantly higher NK activity and percentages of NK, perforin, granulysin and granzyme A/B-expressing cells, as well as significantly lower percentage of T cells and concentrations of adrenaline and noradrenaline in urine when phytoncides were vaporized in hotel rooms at night [12]. These findings together indicate that increased NK activity in subjects visiting a forest may be partially due to forest terpenes [21,22]. Another study found that inhaling oils containing terpenes significantly decreased SBP, DBP and cortisol levels [86]. Surprisingly, as far as we know, only one study has analyzed the absorption of terpenes in blood after forest exposure [87]. The authors of this study identified the monoterpenes species present in coniferous-forest atmosphere in serum samples of the subjects who were walking in the forest [87]. They also identified an increase in the amount of alpha-pinene in the serum after the subjects walked in the forest as well as differences in monoterpene composition and abundance between coniferous and broad-leaved forests air [87]. Lee et al. (2018) reported that the mean atmospheric concentration of phytoncides was higher in a natural than a tended forest (25.58 vs 18.44 ng/m^3, respectively) [25]. Studies are increasingly providing evidence of the role of these compounds in human health, but more research is needed to describe and predict the composition and abundance of terpenes under forest canopies, to analyze the absorption and metabolism of these chemicals by humans and for identifying the mechanisms leading to health effects.

This review could not find any pattern in the data between the three most studied health variables (blood pressure, pulse rate and cortisol level) that could account for the basic level of forest description considered: forest type. These findings are similar to those of other reviews that considered the effects of exposure to nature on mental health [78] or the benefits of forests to health and well-being [17]. Although not including any forest variable in the analysis, blood pressure and cortisol levels have been surveyed by two recent systematic reviews which conducted meta-analyses and significantly linked forest exposure with the decreases of these health variables [5,8]. Thus, the scarce descriptions of forest variables identified in our review that could affect objectively measurable physiological health variables may therefore apply generally. We identified a weak relationship in SBP records among B-A trials showing a decrease in mixed forests. This was not supported by the analyzed control trials and might be due to the broaden categorization of these forest type. Encouraging future research to develop a more integrative approach is thus essential, both analyzing the effects of forests and characterizing ecosystems to describe the pathways that may induce health effects to provide data and tools for forest managers, policy makers and planners in coordination with healthcare professionals.

5. Limitations

The limitations of language may have been important when developing the systematic search because many of the publications were in Asian languages. The high variability of participant number, exposure time, forest type and study design may have induced analytical biases among the data collected. The assumption of forest type when tree composition or dominance were provided may also have added biases to the analysis due to the scarcity of basic forest descriptions. We were also conscious of the challenge to identify patterns and relationships by analyzing studies of real-life exposure to natural forested areas where subject reactions and health effects may be influenced by different sensory stimuli, seasonal variabilities, meteorological conditions, exposures and performances of the activities conducted or other inputs not previously considered. Only three health variables among the studies could be screened. Nevertheless, other health variables not considered in this review may also be relevant to human health in future studies. These in situ studies nevertheless provided relevant data for understanding the connection between human health and forest ecosystems, even though the effects of forests on humans may presumably be derived from different inputs, components and characteristics of forest environments.

6. Conclusions

This systematic review underlines the lack of forest variables descriptions among studies of forests and human health and highlights that it is still premature to make any sort of conclusions with respect to data patterns due to the high heterogeneity within the studies performed so far. Furthermore, no consistent relationships between forest type and health variables (blood pressure, pulse rate and cortisol levels) could be identified from the existing literature.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Table S1:Overview of the analyzed articles basic information., Table S2:Health variables registered in the articles analyses classified by systems or functions .

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Suplementary Material

Table S1. Overview of the analyzed articles basic information.

Authors and reference	Year	Country	Study type	Exposure type	Exposure time	N Participants
Bang et al. [1]	2017	South Korea	Control trial	Passive	Weeks	118
Bielinis et al. [2]	2019	Poland	Before-after	Pro-active	Days	21
Chen et al. [3]	2018	Taiwan	Before-after	Pro-active	Days	16
Chun et al. [4]	2017	South Korea	Control trial	Pro-active	Days	59
Dolling et al. [5]	2017	Sweden	Comparative	Passive	Months	27
Han et al. [6]	2016	South Korea	Control trial	Pro-active	Days	61
Hassan et al. [7]	2018	China	Control trial	Passive	Minutes	60
Horiuchi et al. [8]	2013	Japan	Comparative	Passive	Hours	48
Horiuchi et al., [9]	2014	Japan	Comparative	Passive	Minutes	15
Horiuchi et al. [10]	2015	Japan	Before-after	Passive	Hours	54
Im et al. [11]	2016	South Korea	Control trial	Passive	Hours	41
Jia et al. [12]	2016	China	Control trial	Passive	Days	20
Joung et al. [13]	2015	South Korea	Control trial	Passive	Minutes	7
Kim, et al. [14]	2015	South Korea	Before-after	Passive	Days	11
Kobayashi et al. [15]	2015	Japan	Control trial	Passive	Minutes	625
Kobayashi et al. [16]	2017	Japan	Control trial	Passive	Minutes	348
Lee and Lee [17]	2014	South Korea	Control trial	Passive	Hours	43
Lee et al. [18]	2009	Japan	Control trial	Passive	Minutes	12
Lee et al. [19]	2011	Japan	Control trial	Passive	Minutes	12
Lee et al. [20]	2014	Japan	Control trial	Passive	Minutes	48
Lee et al. [21]	2018	South Korea	Comparative	Pro-active	Hours	79
Li et al. [22]	2007	Japan	Before-after	Passive	Days	12
Li et al. [23]	2008	Japan	Before-after	Passive	Days	13
Li et al. [24]	2008	Japan	Control trial	Passive	Days	12
Li et al. [25]	2011	Japan	Control trial	Passive	Days	17
Li et al. [26]	2016	Japan	Control trial	Passive	Hours	19
López-Pouza et al. [27]	2015	Spain	Comparative	Passive	Days	30
Mao et al. [28]	2012	China	Control trial	Passive	Days	20
Mao et al. [29]	2012	China	Control trial	Passive	Days	24
Mao et al. [30]	2017	China	Control trial	Passive	Days	33

Mao et al. [31]	2018	China	Control trial	Passive	Days	20
Morita et al. [32]	2011	Japan	Before-after	Passive	Hours	71
Ochiai et al. [33]	2015	Japan	Before-after	Pro-active	Hours	17
Ochiai et al. [34]	2015	Japan	Before-after	Passive	Hours	9
Ohe et al. [35]	2017	Japan	Before-after	Pro-active	Days	43
Ohtsuka et al. [36]	1998	Japan	Before-after	Passive	Years	237
Park et al. [37]	2007	Japan	Control trial	Passive	Minutes	12
Park et al. [38]	2008	Japan	Control trial	Passive	Minutes	12
Park et al. [39]	2009	Japan	Control trial	Passive	Minutes	12
Park et al. [40]	2010	Japan	Control trial	Passive	Minutes	280
Saito et al. [41]	2019	Japan	Comparative	Passive	Minutes	17
Seo et al. [42]	2015	South Korea	Before-after	Pro-active	Days	48
Shin and Choi [43]	2019	South Korea	Control trial	Passive	Minutes	10
Song et al. [44]	2013	Japan	Control trial	Passive	Minutes	485
Song et al. [45]	2015	Japan	Control trial	Passive	Minutes	20
Song et al. [46]	2015	Japan	Control trial	Passive	Minutes	92
Song et al. [47]	2017	Japan	Before-after	Pro-active	Days	26
Song et al. [48]	2017	Japan	Control trial	Passive	Minutes	20
Song et al. [49]	2019	China	Control trial	Passive	Minutes	60
Sonntag-Öström et al. [50]	2014	Sweden	Control trial	Passive	Minutes	20
Sonntag-Öström et al. [51]	2015	Sweden	Control trial	Pro-active	Days	86
Stigsdotter et al. [52]	2017	Denmark	Control trial	Passive	Minutes	51
Sung et al. [53]	2012	South Korea	Control trial	Pro-active	Days	56
Toda and Takeshita [54]	2015	Japan	Control trial	Passive	Minutes	20
Toda et al. [55]	2013	Japan	Control trial	Passive	Minutes	20
Tsao et al. [56]	2018	Taiwan	Control trial	Passive	Days	11
Tsunetsugu et al. [57]	2007	Japan	Control trial	Passive	Minutes	12
Wang et al. [58]	2018	Japan	Control trial	Passive	Hours	28
Wu et al. [59]	2017	China	Control trial	Passive	Days	33
Yamaguchi et al. [60]	2006	Japan	Control trial	Passive	Days	10
Yu et al. [61]	2016	South Korea	Before-after	Pro-active	Days	24
Yu et al. [62]	2017	Taiwan	Before-after	Pro-active	Hours	128

Table S2. Health variables registered in the articles analysis classified by systems or functions.

Endocrine/reproductive
Glucose (serum concentration)
Glycated hemoglobin (HbA1C)
Insulin
Adiponectin
Estradiol
Progesterone
Dehydroepiandrosterone sulfate (DHEA-S)
Cardiovascular
Diastolic blood pressure (DBP)
Systolic blood pressure (SBP)
Pulse pressure (SBP-DBP)
Cardio-ankle vascular index (CAVI)
Heart rate variability (HRV)
Mean heart rate (HR)
Standard deviation of normal to normal beat interval (SDNN)
High-frequency (HF) band
Low-frequency (LF) band
LF/HF ratio.
Homocysteine (HCY)
Renin-angiotensin system
Angiotensin II receptor type 1 (AT1)
Angiotensin II receptor type 1 (AT2)
Angiotensinogen (AGT)
Renin
Angiotensin (ANG)
Endothelin-1 (ET-1)
Brain natriuretic peptide (BNP)
Cerebral oxygenated hemoglobin (HbO2) and deoxygenated hemoglobin (HHb)
N-terminal pro-B-type natriuretic peptide (NT-proBNP) (serum concentration)
Metabolic
Body fat
Low-density lipoprotein (LDL) cholesterol
High-density lipoprotein (HDL) cholesterol
Remnant-like particle (RLP) cholesterol
Stress
Cortisol (serum, saliva)
Chromogranin-A (CgA) (saliva)
Adrenaline (urinary, blood)
Noradrenaline
Salivary amylase (sAMY) activity (and its natural logarithm, $\log_e$ sAMY).
Oxidative stress/carcinogenesis
Hydroperoxides (reactive oxygen metabolites of the d-ROM test)
Hydrogen peroxide (H_2O_2)
8-hydroxy-2'-deoxyguanosine (8-OHdG)
Malondialdehyde (MDA)
Superoxide dismutases (SODs) (total in serum)
Tissue inhibitor of metalloproteinase (TIMP-1) (serum concentration)
Nervous
Noradrenaline
Dopamine

Sleep quality assessment
Total time in bed
Sleep latency
Total sleep duration
Actual sleep
Immobile minutes
Sleep efficiency
Brain bioelectrical activity
Beta waves
Cerebral oxygenated hemoglobin (HbO_2) and deoxygenated hemoglobin (HHb)
Necker cube pattern control task (spontaneous reversals and focused reversals)
Respiratory
Forced vital capacity (FVC)
Forced expiratory volume in the first second (FEV1)
Forced expiratory volume in six seconds (FEV6)
Fractional exhaled nitric oxide (FeNO)
Pulmonary and activation-regulated chemokine/CC-chemokine ligand-18 (PARC/CCL18) (serum concentration)
Surfactant protein D (SP-D) (serum concentration)
Hematological/immunological/inflammatory
Red blood cell (RBC) count
Hemoglobin (Hb) (total)
Platelet count
White blood cell (WBC) count
CD8+ cells (proportion)
Natural killer (NK) cell count (total) and activity (proportion of activating NK cells)
NK T-like cells (proportion)
T cells (proportion)
Perforin production (total and proportion in CD8+ cells and NK cells)
Granulysin (GRN) (total and proportion)
Granzyme A and B (GrA/B) production in peripheral blood lymphocytes (PBL) and specifically in CD8+, NK and NKT-like cells (total and proportion)
Granulocytes (proportion)
Monocytes (proportion)
Macrophages (proportion)
Lymphocytes (proportion)
Interleukin-6 (IL-6)
Interleukin-8 (IL8)
Interferon-y (IFN-y)
Interleukin-1β (IL-1β)
C-reactive protein (CRP)
Tumor necrosis factor α (TNF-α)
Secretory immunoglobulin A (s-IgA) (saliva concentration)
Thymus and activation-regulated chemokine (TARC/CCL17)
Macrophage-derived chemokine (MDC/CCL22)
Musculoskeletal
Bone density

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Chapter 4

Human Breathable Air in a Mediterranean Forest: Characterization of Monoterpene Concentrations Under the Canopy

Abstract: Monoterpenes have been identified as potential determinants of the human health effects induced by forest exposure. The present study characterizes the total monoterpene concentrations at nose height in a Mediterranean Holm oak forest located in North-East Iberian Peninsula during the annual emission peak (summer and autumn: June to November) using a Proton Transfer Reaction – Mass Spectrometry (PTR-MS). Results show a strong variability of the total monoterpene concentrations in season and daytime. The concentration peak appears during July and August. These two months displayed two average maxima in their diel cycles: One during early morning (from 6:00 to 8:00, 0.30 ppbv for July and 0.41 ppbv for August) and another one at early afternoon (from 13:00 to 15:00, 0.27 ppbv during July and 0.32 ppbv during August). Monoterpene concentrations were strongly related with the temperature (exponentially) and solar radiation (rectangular hyperbolic relationship). The concentrations registered here are similar or higher than in previous ex situ studies showcasing the effects of forests on human health. These findings provide relevant data for the scientific and healthcare community by improving the understanding of monoterpene dynamics at nose height and suggesting further research on the effects of forests on human health, particularly in the Mediterranean region.

Keywords: monoterpenes; forest exposure; PTR-MS; forest chemistry; BVOCs; forest bathing

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1. Introduction

Interest in understanding if and how the exposure to forests leads to human health benefits is growing among the studies analyzing the effects of nature on human health [1–4]. However, evidence connecting particular forest variables and health effects is still inconclusive, due to the lack of forest descriptions and the high heterogeneity of approaches and results within the studies performed so far [5]. Furthermore, Mediterranean forests appear to be scarcely studied in comparison with Asian ones [5], where abiotic variables and forest ecosystems may differ. This growing body of research has argued that biogenic volatile organic compounds (BVOCs) may partly explain the health effects induced by forest exposure [6–9]. BVOCs emitted by forests have diverse roles at multiple scales; from cellular protection and defense at the foliar level, through to chemical signaling at the regional level, up to influencing rainfall at the ecosystems scale [10]. These compounds profoundly affect biosphere–atmosphere interactions by atmospheric reactivity, aerosol growth processes, cloud formation, and therefore radiative balance [11,12].

Monoterpenes are part of the isoprenoid class, the largest class of BVOCs and major components of the forest atmospheres [13]. These compounds are produced by plants as a defensive mechanism against environmental stress and herbivory [13–15]. Additionally, monoterpenes are highly reactive compounds that have a high yield in secondary organic aerosol formation [16]. Monoterpene emission vary across plant species and functional type [17,18], which further changes with an altered physiological plant state [19], leading to distinct spatial [20] and temporal [21,22] emissions worldwide. Monoterpenes have been shown to interact with human health in different ways, from stress relief to an effect on the immune function. Inhaling monoterpenes has been shown to decrease blood pressure and cortisol levels [23], improve antibiotics efficiency [24], or boost the immune system [8,25,26], especially by increasing the percentage and activity of natural killer cells [7,9,27,28]. Furthermore, monoterpenes that relevantly affect cellular and animal systems have shown anti-inflammatory, antitumorigenic, or neuroprotective activities [29].

To our knowledge, only one study has analyzed the absorption of monoterpenes in blood after forest exposure, by identifying the monoterpene species present in a coniferous forest atmosphere in serum samples of individuals after walking in the forest [30]. The study demonstrated an increase in the amount of α-pinene in the serum after the individuals walked in the forest as well as differences in monoterpene composition and abundance between coniferous and broad-leaved forest air [30]. In addition, Lee et al. (2018) reported that the mean atmospheric concentration of monoterpenes at a 1.5-m height was higher in a natural than a tended forest [8], suggesting that vegetation characteristics derived from management may affect terpenes' concentrations under the canopy.

While studies are increasingly providing evidence on the role of monoterpenes in human health, research is needed to describe and predict the composition and abundance of monoterpenes at nose height under forest canopies, analyze the absorption and metabolism of these chemicals by humans, and identify the mechanisms leading to health effects. To address this gap, this article characterized the total monoterpene concentrations at nose height in a Mediterranean holm oak forest during the annual emission peak and posterior months and sheds light on the potential effects of such concentrations on human health.

2. Materials and Methods

2.1. Measurement Site

The study site was located in Montseny Natural Park—Biosphere Reserve (NE Iberian Peninsula, Spain) in a Holm oak forest dominated by *Quercus ilex* (1100 per ha), which is highly representative of the montane holm oak (*Quercus ilex* L.) forests in the northern Mediterranean regions [31], Figure 1. At Montseny, due to coppicing until the 1950s and the posterior selective thinning, holm oaks have nowadays become dense forest of resprout origin [32]. The average diameter at breast height (DBH) for *Quercus ilex* was 16.36 ± 0.917 cm. Other species like *Pinus halepensis* or *Erica arborea* were identified in the study area but not abundantly. Concerning the forest structure, vegetation cover at the 8-m height was 100% while the layers below did not exceed coverages of 10% except for heights of 0.25 and 0.5 m, where the understory was dense and presented coverages of 80%. This forest area has an altitude range from 415 to 550 m.

Figure 1. Sampling site.

The habitats within a 500-m radius around our sampling site are presented in Table 1 and illustrated in Figure 2.

Table 1. Habitat type, surface, and percentages of the total surface per habitat within a 500-m radius from the sampling point.

Habitat	Surface (ha)	Percentage (%)

Holm Oak Forest	33.63	43.50
Cropland	16.83	21.77
Mixed forest (holm oak + deciduous oaks)	9.97	12.90
Urban and industrial areas	5.41	6.99
Bushland	4.94	6.39
Riparian vegetation	4.19	5.42
Cork oak forest	2.34	3.03
TOTAL	77.31	

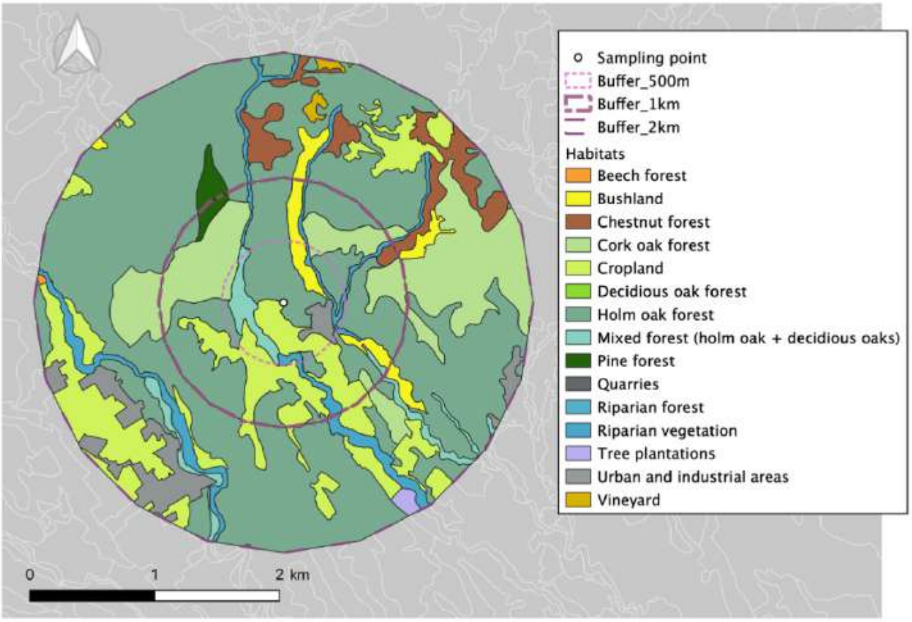

Figure 2. Study area: Sampling point, buffers (500 m, 1 and 2 km), and habitats within the zone.

2.2. Meteorological Conditions

At the sampling site (41°43′43.29″ N,2°26′24.35″ E, 422 m a.s.l.), there was a manual meteorological station with daily values of temperature at 8:00 am, and daily maximum and minimum temperature and precipitation. In order to complement the data with higher frequency datasets, we obtained meteorological data from two other stations of the Servei Meteorològic de Catalunya (i.e., Catalonia's Meteorological Service): Radiation and humidity from Puig Sesolles station (41°46′25.00″ N, 2°26′15.90″ E, 1668 m a.s.l.) 5 km away from our sampling site, and temperature from Tagamanent station (41°44′51.43″ N, 2°18′10.64″ E, 1030 m a.s.l.) 12 km away from the sampling site. The vapor pressure deficit was calculated with the solar radiation data from Puig Sesolles and the temperature data from Tagamanent.

2.3. PTR-MS Sampling Methodology

Measurements of the total monoterpene concentrations were performed with a PTR-MS (Ionicon Analytic GmbH, Innsbruck, Austria) from 26 June to 15 November 2019. One isolated 10-m Teflon tube (OD ¼) was installed at 1.5 m attached with a rope to a holm oak tree. Air was sucked to inside the Park Office where the PTR-MS was located by a pump (JUN-AIR, Benton Harbor, MI, USA). The PTR-MS was operated at standard conditions (2.2 mbar drift pressure, 600 V drift voltage, 127 Td) [33]. A catalytic converter (Supelco, Bellefonte, USA Inc. with platinum pellets heated to 380 °C) was used to monitor the background impurities of the PTR-MS. The background signal for each compound was calculated once every hour. Background values were interpolated over the time of the measurements. Humidity-dependent calibrations (using bubbled zero air to dilute the standard, regulated as close as possible to ambient humidity conditions) were performed using a gravimetrically prepared multicomponent standard, including methanol (*m*/*z* 33), acetonitrile (*m*/*z* 42), acetaldehyde (*m*/*z* 45), acetone (*m*/*z* 59), isoprene (*m*/*z* 69), MACR (*m*/*z* 71), MEK (*m*/*z* 73), and α-pinene (*m*/*z* 137), with several dilution steps (Riemer Environmental Inc., Miami, USA).

The PTR-MS technique separates per mass and not per compound, so other compounds than monoterpenes with the same mass might be measured together. However, sporadic GC-MS screening at the site confirmed the presence of monoterpenes (i.e., *m*/*z* 137) in ambient air, more specifically α-pinene and β-pinene.

2.4. Data Analysis

Igor Pro (Wavemetrics Inc., Portand, OR, USA) was used for the calculations of BVOCs concentrations, data time series treatment, graphing, and descriptive statistics. We conducted a generalized linear model (GLM) to determine the effect of the available atmospheric data (temperature, precipitation, relative humidity, solar radiation, ozone, and vapor pressure deficit) on monoterpene concentrations using R (Version 1.2.5033). Monoterpene concentrations were log-transformed to ensure normality of the residuals. A $p < 0.05$ was considered significant.

3. Results

Figure 3 shows the complete time series of the total monoterpene concentrations measured in this study from June to November 2019, which ranged from 0.02 to 0.13 ppbv (25 percentile and 75 percentile), with maximum values reaching 1.24 ppbv. Total monoterpenes had a clear seasonality with higher concentrations during summer as well as a strong daily pattern with highest concentrations during daylight.

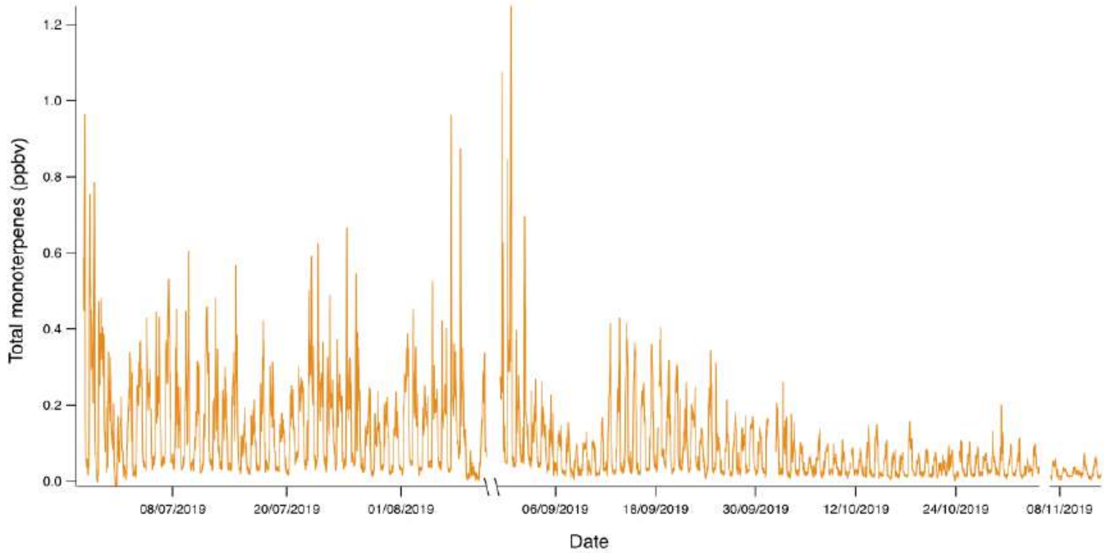

Figure 3. Total monoterpene air concentrations during the sampling period (from June to November 2019). The gaps are due to instrument lack of power in the sampling site.

The average monthly diel cycles (Figure 4) showed that August and July had the highest concentrations. These two months displayed two average maxima in their diel cycles, one during early morning (from 6:00 to 8:00, 0.30 ppbv for July and 0.41 ppbv for August) and another one at early afternoon (from 13:00 to 15:00, 0.27 ppbv during July and 0.32 ppbv during August). These peaks were not identified in the rest of the sampled months (September, October, and November), where total monoterpene concentrations increased with the solar radiation and a peak around 14:00, coinciding with the temperature diel cycle peak. Additionally, concentrations values stayed relatively constant during night, with 0.05 ppbv for July and August decreasing to 0.02 ppbv in November.

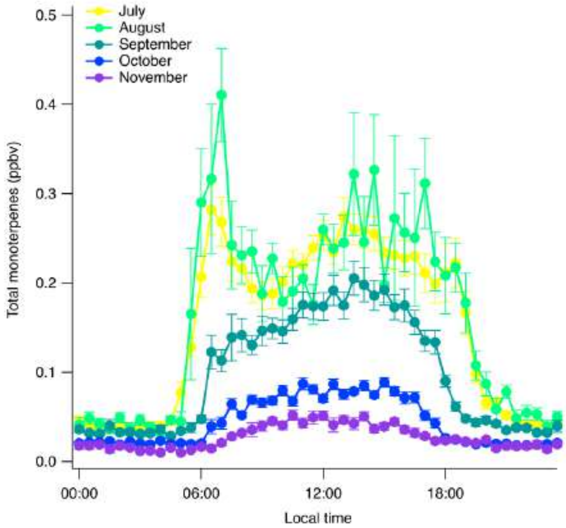

Figure 4. Half-hour average diel cycle of total monoterpene concentrations per month. Error bar represents the standard error.

Additionally, Figure 5 shows the monoterpene concentrations were strongly related with temperature (exponentially) and solar radiation (rectangular hyperbolic relationship). The evolution of the meteorological variables correlated with the monoterpenes is presented in Figure S1. The GLM analysis demonstrated a significant effect of solar radiation (t-value = 22.770; p-value = 2×10^{-16}), temperature (t-value = 21.645; p-value = 2×10^{-16}), humidity (t-value = 9.506; p-value = 2×10^{-16}), and vapor pressure deficit (vpd) (t-value = 6.236; p-value = 4.82×10^{-16}) (Figures S2 and S3 and Table S1).

4. Discussion

The present study analyzed the under-canopy variations of monoterpene concentrations in a Mediterranean holm oak forest in Montseny Natural Park—Biosphere Reserve (NE Iberian Peninsula) continuously from June to November 2019 using a PTR-MS. This is the longest time series of total monoterpene concentrations ever measured in a Mediterranean holm oak forest at the nose level. During the sampled period, we identified seasonal and daily patterns. The highest concentrations occurred during summer, and diel cycles changed among months, with peaks in early morning in summer and peaks at midday in the entire period. The total monoterpene variations were significantly linked to abiotic/atmospheric variables, such as temperature and solar radiation, as well as to humidity and vapor pressure deficit (vpd).

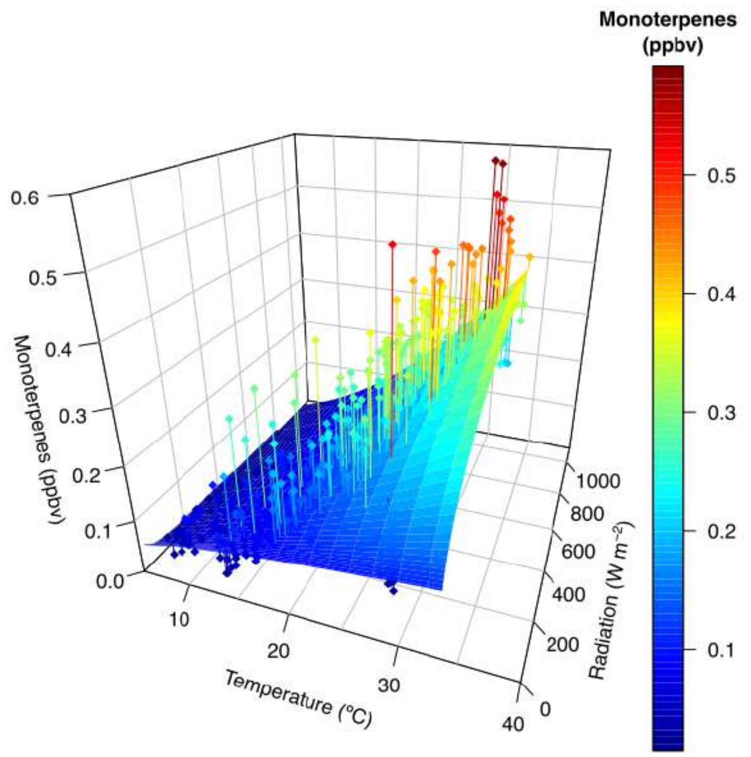

Figure 5. 3-D relationship of monoterpene concentrations with temperature and radiation.

The highest monoterpene concentrations occurred during July and August. These results are consistent with other studies that found higher monoterpene concentrations above the canopy level in summer in an holm oak forest (*Quercus ilex* L.) from the same Mediterranean region [34,35]. Previous studies in other regions have as well observed higher monoterpene emissions during summer period [36,37]. Furthermore, monoterpene emission rates measured at the leaf or branch level have proved to be higher from July to August in *Quercus ilex* [34,35,38,39]. This seasonality of monoterpene concentrations is associated to the seasonality of plant monoterpene emission [19,40] mostly linked to the seasonal changes in temperature [41,42] and other meteorological variables, such as solar radiation [17,43,44], which explains as well the decrease during the autumn months (October and November).

We found higher monoterpene concentrations during daytime, with maxima at early morning (from 6:00 to 8:00) and at early afternoon (from 13:00 to 15:00). This early morning peak has been previously reported at a nearby site above forest canopies [38,45,46]. This peak has been attributed to the biogenic monoterpene emissions at the site, which have not yet been influenced by mountain breeze (which starts a few hours later in the day) [46]. The peak at around 14:00 is also related to the biogenic origin of monoterpenes, which is enhanced at maximum temperature and solar radiation. In fact, the maximum at 14:00 coinciding with the temperature peak has been reported previously for areas with light- and temperature-dependent monoterpene-emitting trees, such as tropical trees [47,48]. This differs from other forest sites, including Mediterranean, that documented the diel cycle of the monoterpene concentration to peak at nighttime [37,49,50]. This inconsistency is due to the fact that holm oaks emit monoterpenes in a light- and temperature-dependent manner (so called de *novo* biosynthesis) and not from storage pools at night, like boreal coniferous species [17,43], and thus maximum emissions occur during the middle of the day [51].

Monoterpene emissions are well-known to be affected by atmospheric variables, such as air temperature, solar radiation, and air humidity [17,43,44]. Our results from the GLM analysis support this fact by showing a strong effect of temperature, solar radiation, humidity, and vpd on monoterpene concentrations. Ambient concentrations of a volatile organic compound can be affected by the strength of emissions (either biogenic or anthropogenic), the effectiveness of air mixing, and the strength of sink processes, including deposition and chemical degradation. However, the strong effect of temperature and solar radiation on the observed concentrations suggests the dominance of a biogenic source for total monoterpenes. Further on, as the site is dominated by *Quercus ilex* forests, a dominant monoterpene emitter tree species, we assume that the main origin for the observed concentrations comes from the monoterpene emission of this tree species. This further supports the biogenic origin of the measured concentrations, similar to a previous study at a nearby site [46]. Furthermore, the daily patterns of the concentrations could as well be partly associated to atmospheric vertical stability and wind speed and direction as shown in previous studies [52]. Reasonable interpolation from existing weather stations in this regard could not be provided in the present study, but further research is warranted at this regard.

Our results report similar monoterpene concentrations to another study that has measured these compounds under coniferous forest canopies in Japan [53]. The most abundant monoterpenes in Mediterranean holm oak forests are ◎-pinene, sabinene, limonene, camphene, and ◎-phellandrene [38]. Some of these monoterpene species have been previously found to be absorbed by humans after a 60-min walk through a coniferous forest with a six-fold increase of the ◎-pinene peak in blood after forest exposure [30]. These monoterpenes have been related with physiological changes in humans, in particular ◎-phellandrene has been significantly associated with an acute insulin reaction [8]. Some ex situ and in vitro studies have shown enhancing effects of monoterpenes on the human immune system but at higher monoterpene concentrations than those we registered in our study (approximately from 50 to 1900 ppbv) [7,9]. Nevertheless, the concentrations we report here (from 114 to 725 ngm^{-3} during the whole sampling period and up to 2230 ngm^{-3} in August) happen to be comparable [53] or much greater [27,28] than in previous in situ studies that identified similar health outcomes to the ones observed in the in vitro and ex situ experiments. The concentrations of monoterpenes of our studied Mediterranean forest are also higher than other in situ studies characterizing the forest atmosphere under the canopy [54] or testing the effects of forests on other human systems and functions [8]. Taken together, these findings suggest that the Mediterranean holm oak forests constitute a suitable forest environment to develop further research on the effects of monoterpenes on human health.

5. Conclusions

This study analyzed the monoterpene concentrations at nose height in a Mediterranean Holm oak forest from June to November. We identified a strong variability of the total monoterpene concentrations in season and daytime with its peak during summer. Additionally, the monthly average diel cycles showed two main peaks at early morning (from 6:00 to 8:00) and early afternoon (from 13:00 to 18:00) during July and August. Monoterpene concentrations significantly correlated with air temperature, solar radiation, air humidity, and vapor pressure deficit (vpd).

If BVOCS play a key role in the interaction between forest and human health as highlighted in the literature, our analysis provided relevant information concerning the availability of these compounds at the nose level, thus suggesting that humans walking in the studied forest may be subject to a potentially high absorption of monoterpenes into their bloodstream, especially at early morning and from midday. Our findings contribute to improving the understanding of monoterpene dynamics under the canopy and enhancing the development of more applied research on the effects of forests on human health, particularly in the Mediterranean region.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: Evolution of the meteorological variables during the sampling period; Figure S2: Correlation between air humidity and monoterpenes concentrations; Figure S3: Correlation between air vapor pressure deficit (vpd) and monoterpenes concentrations; and Table S1: Outcomes from the GLM conducted to assess the effect of air temperature, solar radiation, air humidity and vapor pressure deficit (vpd) on monoterpenes concentrations.

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Supplementary material

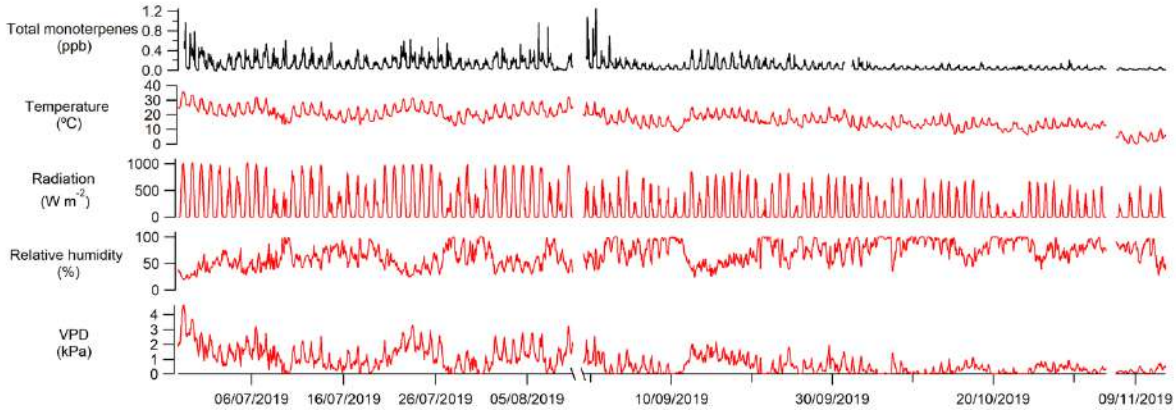


Figure S1. Evolution of the meteorological variables during the sampling period.

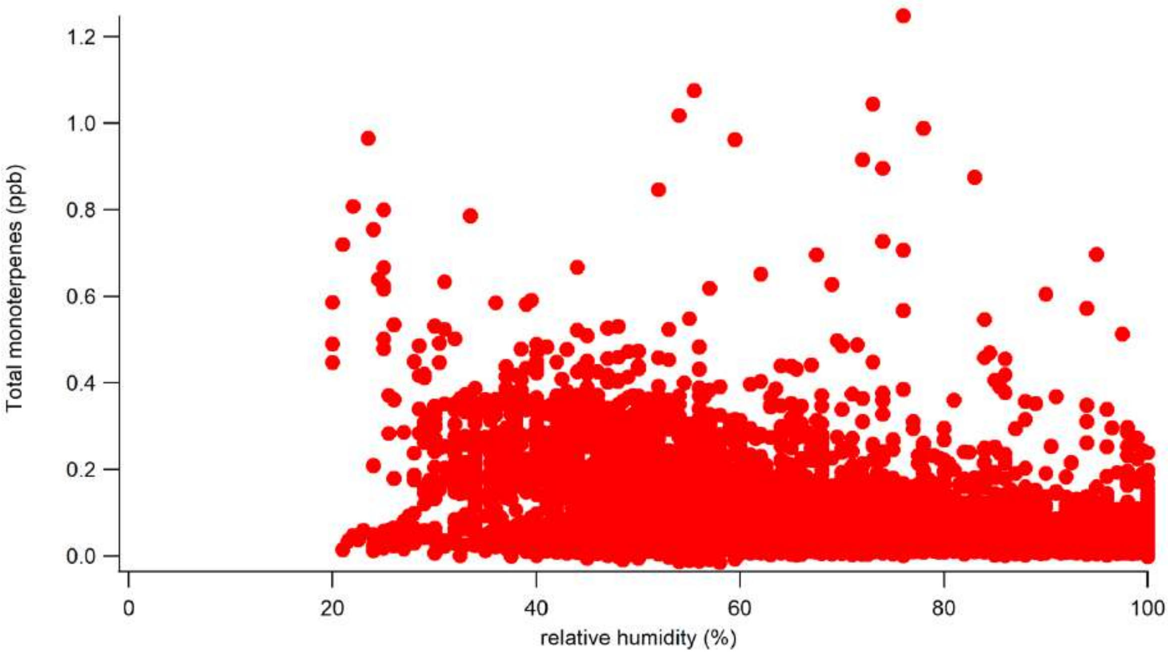
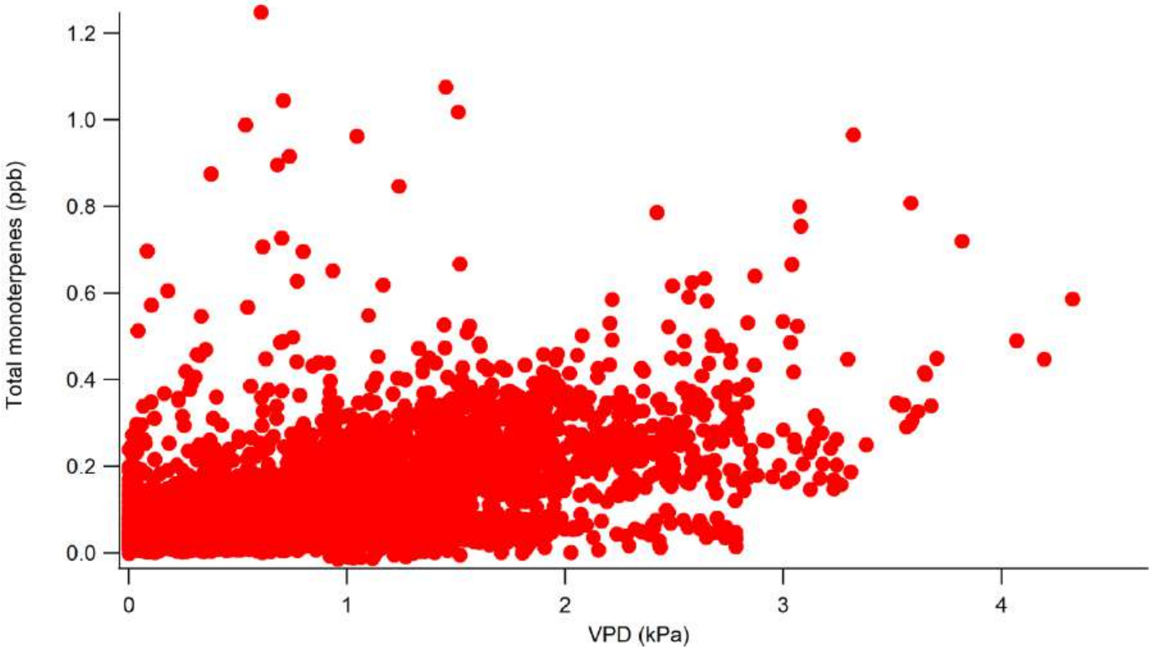


Figure S2. Correlation between air humidity and monoterpenes concentrations.

66. 

67. Figure S3. Correlation between air vapor pressure deficit (vpd) and monoterpenes concentrations.

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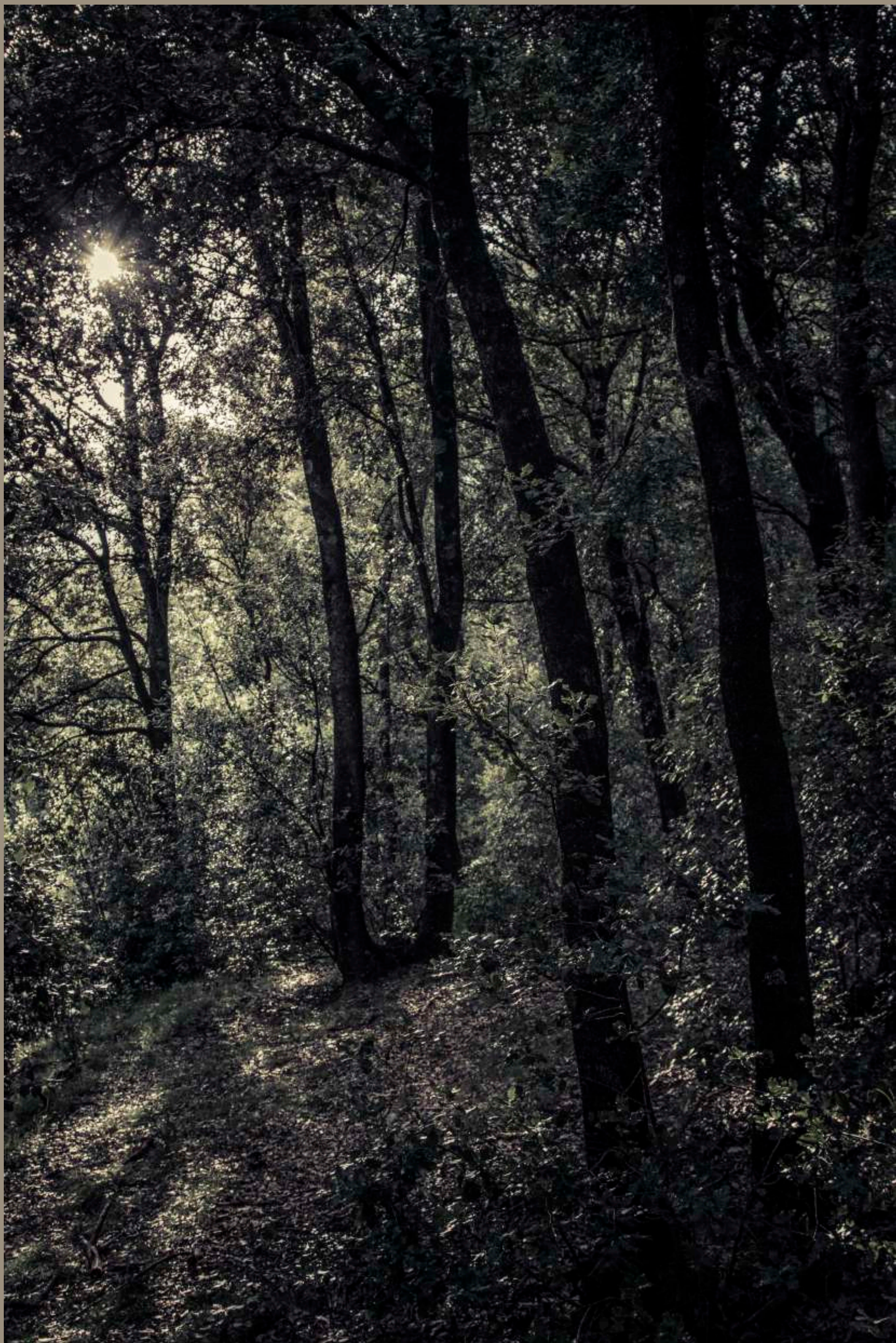
73. **Table S1**. Outcomes from the GLM conducted to assess the effect of air temperature, solar radiation, air humidity and vapor pressure deficit (vpd) on monoterpenes concentrations. Please note that a log conversion was applied to the monoterpenes concentrations. Thus, the estimates can not be interpreted straight forward.

	Estimate	Std. Error	t value	p value
Temperature	0.0317485	0.0014668	21.645	2,00E-16
Solar radiation	0.0141676	0.0006222	22.770	2,00E-16
air humidity	0.0073928	0.0007777	9.506	2,00E-16
vpd	0.0055614	0.0008918	6.236	4.82e-10

74.



Chapter 5

Human absorption of monoterpenes after a 2-hours forest exposure: a field experiment in a Mediterranean holm oak forest

Abstract: The current body of literature points monoterpenes as one of the determinant factors of the interaction between forests and human health. The present study aims at analyzing the monoterpene absorption by humans during a 2-hours forest exposure in a Mediterranean holm oak forest focusing on the four most abundant monoterpene compounds: alpha-pinene, beta-pinene, alpha-phellandrene and limonene. Participants' blood samples were collected before and after exposure to forest or urban environment (control). We conducted air and blood sampling using cartridges and head space method and determined the monoterpene compounds through CG-MS. We identified the four compounds in forest air during the experimental study being alpha-pinene the monoterpene with the greatest concentration. Results show no significant changes in monoterpene blood concentrations for the forest and control group. However, a negative significant relationship between absorption and baseline blood concentration of the most abundant forest air monoterpenes, alpha-pinene and beta-pinene, was found in individuals visiting the forest, i.e. higher absorption was found the lower the baseline blood concentration was. Although no significant lineal correlation could be spotted between the vital variables and the monoterpene absorption, we found significant correlations between the absorption of the monoterpene compounds. This attempt, first in a Mediterranean holm oak forest, can serve as a starting point and constitute a valuable contribution for further research in regard to experimental design and laboratory analysis.

Keywords: monoterpenes; forest exposure; CG-MS; forest chemistry; BVOCs; forest bathing, blood samples

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1. Introduction

Biogenic Volatile Organic Compounds (BVOCs) have been identified as potential determinants of the human health effects induced by forest exposure [1–4]. These compounds make part of the isoprenoid class, the largest class of BVOC and major components of forest atmospheres [5]. Monoterpenes emitted by forests have multiple roles at different scales; from cellular protection and defense at foliar level, through chemical signaling at regional level, up to influencing rainfall at an ecosystem scale [6]. These compounds are produced by plants as a defensive mechanism against environmental stress and herbivory [5,7,8] and their emissions vary across plant species and functional type [9,10], which further changes with altered physiological plant state [11] leading to distinct biogenic emissions worldwide [12] that can change over time [13,14].

Numerous ways of interaction between monoterpenes and human health have been reported; from stress relief to an effect on the immune function. Inhaling monoterpenes have been shown to decrease blood pressure and cortisol levels [15], improve antibiotics efficiency [16] or boost the immune system [3,17,18] especially by increasing the percentage and activity of natural killer (NK) cells [2,4,19,20]. In general, those monoterpenes that relevantly affect cellular and animal systems have revealed anti-inflammatory, anti-tumorigenic or neuroprotective activities [21].

So far, only one study has analyzed the absorption of monoterpenes in blood after forest exposure identifying the monoterpene compounds present in coniferous-forest atmosphere in serum samples of individuals in Japan [22]. The main results show: differences in monoterpene composition and abundance between coniferous and broad-leaved forests air; and an increase in alpha-pinene levels in serum after participants walked in the forest [22].

While studies are increasingly providing evidence of the role of monoterpenes in human health, Mediterranean forests are rarely studied in this regard [23] even though previous research have identified remarkable monoterpene emissions and concentrations above [24–27] and below the canopy [28]. In this sense, advanced research is crucial to analyze the absorption and metabolism of these chemicals by humans and identify the mechanisms leading to human health effects.

The aim of the current study was to analyze the monoterpene absorption by humans in a Mediterranean holm oak forest. For this purpose, we selected the four most abundant monoterpenes compounds in a Mediterranean holm oak forest (alpha-pinene, beta-pinene, alpha-phellandrene and limonene) [24] and also the four ones that have been identified as the most relevant in the forest-health pairing [21] according to the scientific literature. We analyzed the monoterpene absorption during a 2-hours walk at the end of July when monoterpene atmospheric concentrations are highest in these Mediterranean forests [28].

2. Methods

2.*1. Participants*

A total of ten subjects (6 females and 4 males) were recruited from the Autonomous University of Barcelona. Inclusion criteria were: A) aged between 20 and 40 years old; B) index of body mass between 19 and 25; C) non-smokers (at least 6 months before the study); D) no respiratory pathology (asthma, chronic bronchitis or respiratory allergies); and E) no pathologies affecting the immune system during the study. Two days before the study participants were asked not to consume alcohol to control for the effects of it. Furthermore, we prohibited the use of any perfumed soap, shampoo, air freshener or products potentially containing the odours caused by the studied monoterpenes. After providing the study description and fully inform participants about the study objectives and design, written informed consent was received from each participant in advance. This study was approved by the Research ethics committee of Granollers Hospital, Spain, on the 26th of June 2018 (CEIC Code 20182020).

2.2. Study site

The forest site was located in Montseny Natural Park - Biosphere Reserve (NE Iberian Peninsula) (Figure 1) in a Holm Oak forest dominated by *Quercus ilex* (10000 trees per hectare) and *Pinus pinea* (500 trees per hectare). The average Diameter at Breast Height (DBH) for *Q. ilex* was 5.175±0.461cm and 31.787±2.341 cm for *P. pinea*. Concerning forest structure, vegetation cover at 8 meters height was 95% while the layers below did not exceed coverages of 35% except for ground layer at 25 cm that covered 80% of the area at that height. This forest area has an altitude range from 860 to 972 m. During the study records taken under the canopy level registered an average temperature of 23,87±0,18°C and an average relative humidity of 53,70±0,50% during the sampling period.

The city center of Sant Celoni, approximately 10 km away from the forest site, was chosen as urban environment (control). This is a city of approximately 17.904 inhabitants with a surface area of 65.2km^2. During the control exposure participants walked around 4 km through a route that evaded the presence of street trees in order to avoid non desirable bias.

Figure 1. Location and site scenarios: urban environment (left picture) and forest site (right picture).

2.3. Experimental design

We conducted a randomized controlled trial experimental approach to assess the absorption of monoterpenes by humans during a 2-hours forest walk. Participants' blood samples were collected before and after the experiment. The study was carried out the 26th of July 2019 and started at 9:00 in the Hospital of Sant Celoni (Barcelona Metropolitan Area), where the vital variables (weight, height, blood pressure (BP), heart rate (HR) and peak expiratory flow (PEF)) and the first blood samples were collected avoiding any previous contact with the forested area. Participants were then randomly assigned to either the city group (control) or the forest group (treatment). The city group remained in the hospital for 30 minutes and then started the 2-hours walk from the hospital while the forest group was taken to the forest site by car (25 minutes trip) and then proceeded to the walk in the forest. Participants were asked to walk through the forest or the city at their pace, rest and sit if they were tired. Intense physical activity such as running or conducting any sport was forbidden. After the 2-hours walks, the city group returned to the hospital were blood samples were collected whereas forest group samples were collected in the forest area (Figure 2).

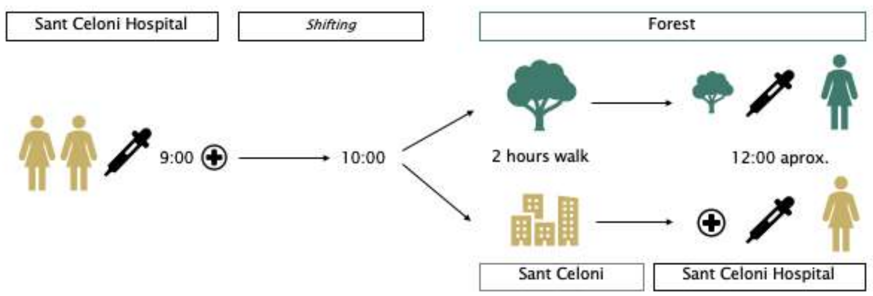

Figure 2. Sampling design diagram.

2.4. Air sampling and analysis

Forest air was collected at a height of 1.6 meters (nose height) using a stainless steel tube (89 mm in length and 6.4 mm external diameter) filled with adsorbents, 115 mg of Tenax® **TA** and 230 mg of SulfiCarb®, separated by sorbent-retaining springs fixed using gauze-retaining springs and closed with air-tight caps (Markes International Inc. Wilmington, USA). Samples were collected using a Q_{max} air-sampling pump (Supelco, Bellefonte, Pennsylvania). The flow was measured with a flowmeter Bios Defender 510 fluxometer (Bios International Corporation, Butler, USA) and regulated with a metallic valve. The sampling time was 120 min, and the flow varied between 309 and 230 mL/min depending on the metallic tube adsorbent. We took a couple of sampling tubes to the field site the same day without opening the air-tight caps to be used as blank samples. The hydrophobic properties of activated adsorbents minimized any sample displacement by water. The terpenes were not chemically transformed in these tubes, as determined by reference to trapped standards (alpha-pinene, D^3-carene, limonene). Prior to air sampling, the tubes were conditioned twice during 30 min at 350 °C with a stream of 100 mL min^{-1} of purified helium. The trapping and desorption efficiency of aforementioned standards was 99%.

The BVOCs trapped in the metallic tubes were thermally desorbed with an automatic sample processor (TD Autosampler, Series 2 Ultra, Markes International Inc. Wilmington, USA) using a coupled injector with a cryotrap (Unity, Series 2, Markes International Inc. Wilmington, USA) and connected to a Gas Chromatograph (7890A, Agilent Technologies, Santa Clara, USA) with a mass spectrometer detector (5975C inert MSD with Triple-Axis Detector, Agilent Technologies). A full-scan (between 35 to 350 m/z) method was used in the chromatographic analyses.

The working conditions in the Markes system of desorption of the samples were as follows: Flow path temperature of 200 ºC, the minimum carrier pressure of 5 KPa and GC cycle time of 30 min. The standby split of 50 ml min^{-1}. The pre-desorption conditions were as follows: prepurge time 0.1 min with a Split of 20 ml min^{-1}, and a dry purge of the tube during 2 min. The tube/sample desorption time was 30 min with a temperature of 320 ºC. The cryotrap was maintained in line with a flow of 50 ml min^{-1} and a split of 2 ml min^{-1}. The cryotrap settings were as follows: cryotrap low temperature was -25 ºC. Before trap heating, trap remained in pre-cryotrap fire purge during 2 min and the cryotrap flow was 50 ml min^{-1} with a splitless. The heating rate was 40°C s^{-1} to cryotrap high of 320 °C. This temperature was maintained during 7 min with splitless.

The cryofucused desorbed sample was injected into the capillary column (HP 5MS, 30m x 0.25 µm x 0.25 mm) with a transfer line at 250ºC. Following sample injection at 35ºC (initial time 3 min), the oven temperature was increased stepwise at 15ºC min^{-1} to 150ºC and maintained for 5 min, at 50ºC min^{-1} to 250ºC and maintained for 5 min and finally at 30ºC min^{-1} to 280ºC and maintained for 5 min. Total run time was 30 min, and the helium column flow was 1 ml min^{-1}.

The identification of terpenes was performed by a comparison of the mass spectra with published spectra (Wiley275 and Nist05a libraries) and known standards, while quantification of the peaks was conducted using the fragmentation product with mass 93 [24,29]. Calibration curves for quantification were prepared with commercial standards of the most abundant monoterpenes in the samples: alpha-pinene, alpha-phellandrene, betha-pinene and limonene (Fluka Chemie AG, Buchs, Switzerland). Terpene calibration curves were always highly correlated ($r^2 \geq 0.95$) in the relationship between signal and terpene concentration. The four monoterpenes had very similar sensitivities (differences were less than 5%).

2.5. Vital variables collection

Prior to intervention (forest or city exposure), blood pressure (BP), heart rate (HR) and peak expiratory flow (PEF) were measured for all the participants. All measurements were carried out by medical staff at 9:00 in an indoor room of the Sant Celoni Hospital to avoid previous contact with monoterpenes from forest. BP and HR were measured using a portable digital sphygmomanometer (Visomat comfort 20/40, ROCHE DIAGNOSTICS, St. Cugat del Vallès, Spain) at the right upper extremity. The PEF was measured using a peak flow meter (Sibelmed Datospir Peak 10, SIBEL. Barcelona, Spain). Participants were provided with the recommended protocol for the respiratory test: in a standing position, the person conducts a deep inhalation and then a maximum forced exhalation for 3 seconds. The process was repeated twice per participants and the maximum value was selected. In order to assess the influence of regular exposure to natural areas, participants were asked for the regularity of their visits to natural areas. We categorized the answers as follows: A) 3-4 times a week; B) 1-2 times a week; C) once a month and D) once every 3 months.

2.6. Blood samples analysis

In total we collected 54 mL of blood per participant (27 mL before the intervention and 27 mL after) using 9- and 4,5-mL blood sampling tubes K2E(EDTA) (ref:367525, BD Vaccutainer). The medical staff conducting the blood collection was asked not to use perfumes or any other perfumed products that could interfere the sampling. The tubes were agitated just after collection and kept cold using dry ice until stored at the laboratory at -20ºC. We had previously conducted a stability test comparing fresh and frozen blood samples and reported no significant differences between analysis (data not shown).

As monoterpenes are ubiquitous at higher or lower concentration in the environment, the laboratory maximized the following preventive measures to avoid samples contamination:

- Limitation of the use of cleaning products in the laboratory during the study to reduce terpene levels in the blank method.
- Preparation orders was followed as: blanks, samples and standard solutions.
- Argon (Ar) was used as a cleanning gas to remove the potential monoterpenes levels in the air of the SPME.

We used a stock solution of 3-Carene at a concentration of 0.2 ppm using ethanol solvent as internal standard. Mix standard stock solutions of alpha-pinene, beta-pinene, alpha-phellandrene at concertation of 0.2 ppm were prepared using ethanol solvent. All standard and internal standard solutions were stored at 5°C.

Firstly, blank vials were prepared adding 8mL of water to a 20mL vial of SPME previously filled with argon gas. Quality Control (QC) vials were set adding 8mL of blood to a 20mL vial of SPME previously filled with argon gas and then spiked with 20µL of 3-Carene (0.2ppm) as internal standard. The Mix standard-500ppt vials were prepared adding 8mL of water to a 20mL vial of SPME previously filled with argon gas and then spiked with 20µL of Mix standard (0.2ppm).

Finally, sample vials were prepared adding 8mL of homogenized and thawed blood sample to a 20mL vial of SPME previously filled with argon gas and then it is spiked with 20µL of 3-Carene (0.2ppm) as an internal standard. Immediately after the preparation, the sample vials were capped, shaken and stored at 5ºC before analysis.

Samples analysis was carried out as follows:

SPME fibers condition

Blanks method;

QC1 / QC2

Pair of Sample-1 (Before/After)

Pair of Sample-2 (Before/After)

QC3 / QC4

Mix standard-500ppt

Blanks Method

All vials in a sequence were prepared daily.

To ensure the proper analysis, the following parameters QA/QC are applied:

1. Retention time: We considered only monoterpenes peaks with values about ± 0.02 min on retention time of standard MIX-500ppt.
2. Blank method: We ensured that the monoterpenes laboratory levels were negligible compared to samples.
3. Sensibility method: It was verified that the signal for low concentration values at 500ppt of all analytes had sufficient instrumental sensitivity for purposed identification.
4. Reproducibility method: the ratio of monoterpenes and the Internal Standard between two consecutive QC Controls must have a variability of less than 25%.

The instrumental parameters used during the analysis process are described in Table S1.

2.7. Data analysis

Air and blood samples chromatograms were analyzed with Agilent ChemStation G1074A HP. We estimated blood concentrations of monoterpenes compounds as follows:

1) We calculated the Response Factor (RF) of each compound from the standards and the internal standard (IS).

$$RFcompound\ a = \frac{Area\ compound\ a\ \times\ Concentration\ IS}{Area\ IS\ \times\ Conentration\ compound\ a}$$

2) Concentrations were then estimated as follow:

$$Concentration\ compound\ a = \frac{Area\ compound\ a\ \times\ Concentration\ IS}{Area\ IS\ \times\ RF\ compound\ a}$$

The data of each participant was plotted showing the increments and decrements in the absorption levels for each monoterpene (see Figure 4). Outliers were assessed through boxplots and values above Q3 + 3 x Interquartile range (IQR) or below Q1- 3 x IQR were considered as outliers. Boxplots showed that at least one outlier was present in each monoterpene distribution. Due to the little data available, we did not exclude the outlier observations from the analyses. Significant differences of the baseline values between the forest and the city group were tested with independent t-test. Data normality was assessed with a Shaphiro-Wilk test. Although most of the data was normally distributed according to the outcomes of the test, we expressed the data in histograms and qqplots (See Annex S1) to further assess its distribution. The treatment effects were tested using an ANOVA for parametric data sets. The homogeneity of variance assumption was checked using the Levene's and the Box's M-test. For all five outcome variables (Alpha-pinene, Beta-pinene, Alpha-phellandrene, Limonene and all four monoterpenes compounds together) there was homogeneity of variance (*p-value*>0.001). Finally, potential linear correlations between monoterpenes absorption and vital variables were sounded considering absorption as Absorption = (after concentration - before concentration) / before concentration. Absorption was also assessed in regard to baseline values via SMA regression analyses. Data analyses were performed using the rstatix package from R software (Version 1.2.5033). A *p-value* < 0.05 was considered significant.

3. Results

The two groups (forest and city) were well balanced at baseline and showed no significant differences for age, height, weight, blood pressure (systolic blood pressure; SBP, diastolic blood pressure; DBP), heart rate and lung capacity (Table S2).

The total air concentration of the 4 studied compounds was 7.10 ppbv. Alpha-pinene was the monoterpene compounds that showed the greatest air concentration in the forest site (3.65 ppbv) whereas alpha-phellandrene presented the lowest air concentration (0.27 ppbv) (Fig. 3).

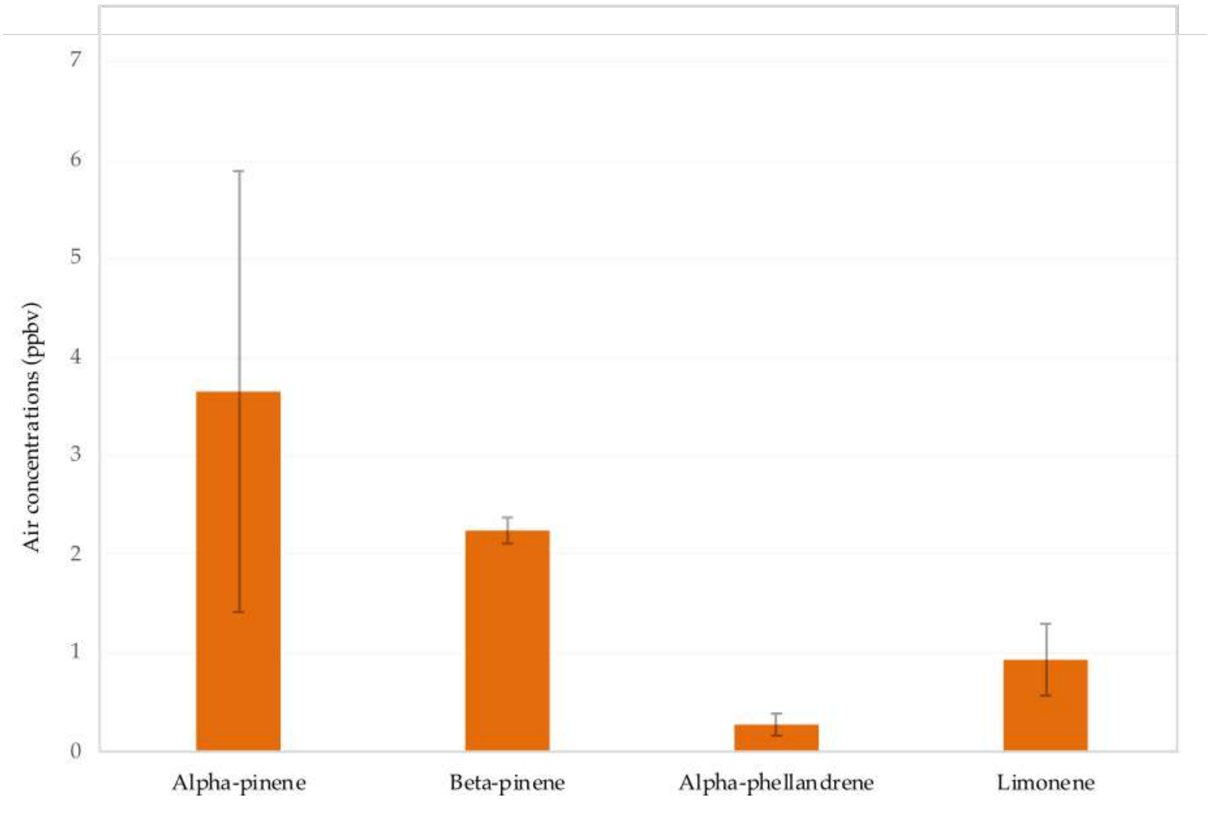

Figure 3. Monoterpenes compounds air concentration during the experimental study.

The absorption of monoterpenes was not different between individuals visiting the forest and individuals visiting the city (Figure 4 for individual responses, Figure 5 for group responses, and Table 2 for statistics for each one of the monoterpenes, alpha-pinene, beta-pinene, alpha-phellandrene, limonene, and for all four monoterpenes together).

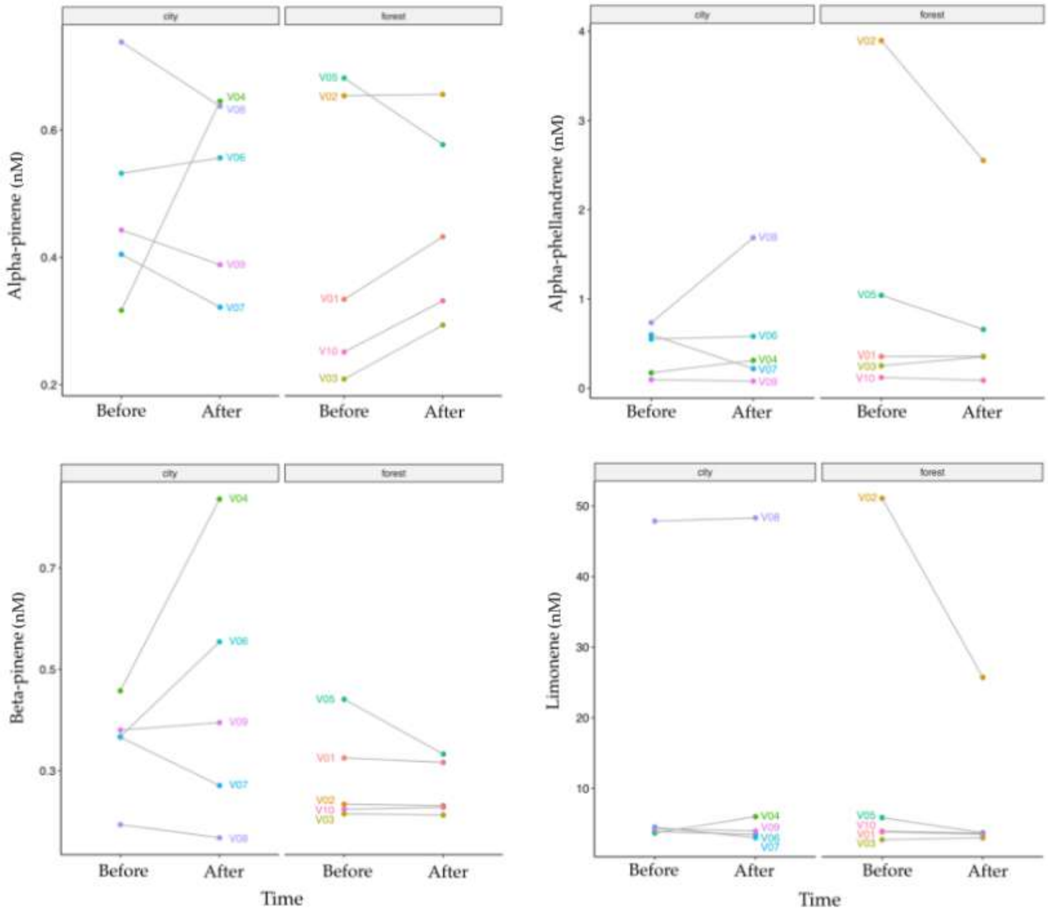

Figure 4. Dotplots of monoterpene blood concentrations before and after forest or city exposure.

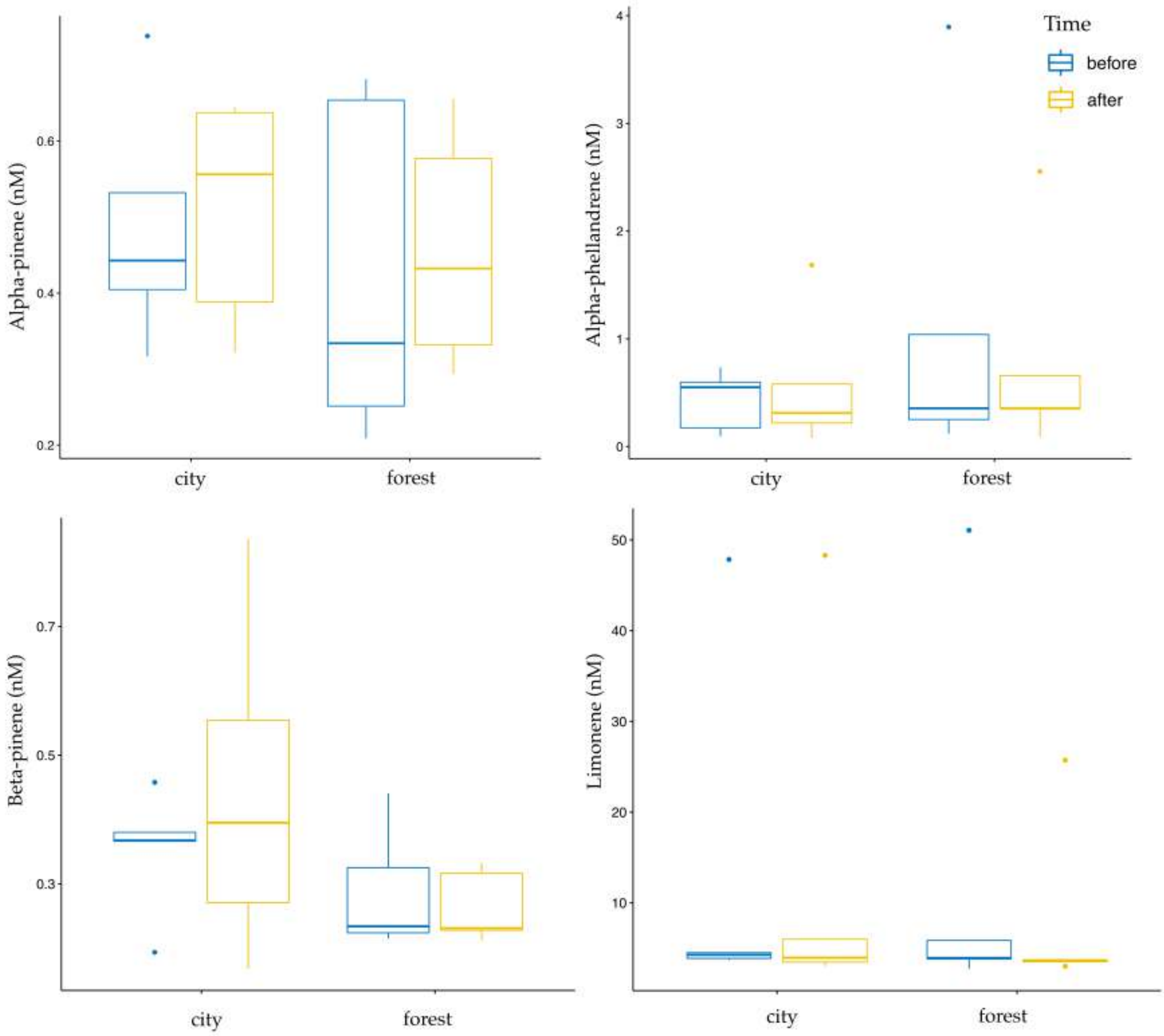

Figure 5. Boxplot of monoterpene blood concentrations before and after forest or city exposure.

However, it was observed that the participants of the forest group presenting lower baseline alpha and beta-pinene concentrations experienced greater absorptions during the exposure while participants with higher baselines decreased them. As it can be seen in Figure 6, there were negative relationships between the absorption and the baseline values of alpha-pinene (estimate = -0.977; *p-value* = 0.004) and beta-pinene (estimate = -0.924; *p-value* = 0,02).

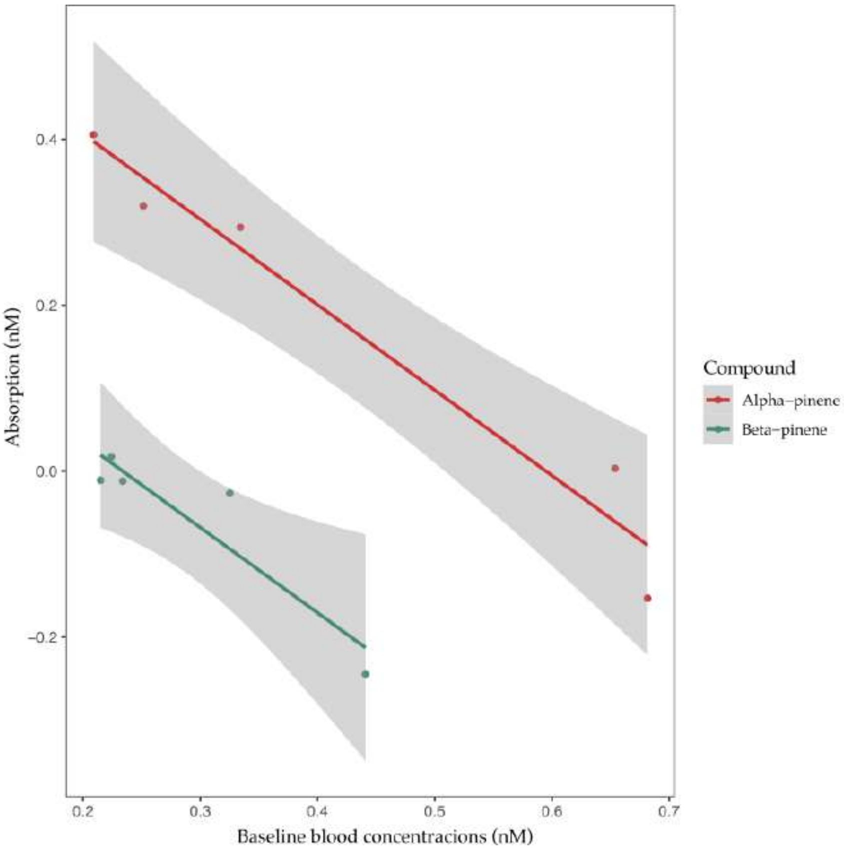

Figure 6. Relationship of the absorption of alpha and beta-pinene with baseline blood concentration for the individuals visiting the forest.

Table 2. Pre-exposure values, Post-exposure values and *p-values* assessed by Two-factor mixed-design ANOVA for Alpha-pinene, Beta-pinene, Alpha-phellandrene, Limonene and all monoterpenes absorption. Note: Level of significance *p-value* ≤ 0.05.

Outcome measure	**Groups**	**Pre-exposure mean with standard deviation**	**Post-exposure mean with standard deviation**	**p= Main effects for groups**
Alpha-pinene	City Forest	0.487 ± 0.16 0.426 ± 0.23	0.510 ± 0.15 0.458 ± 0.16	0.594
Beta-pinene	City Forest	0.353 ± 0.09 0.288 ± 0.10	0.445 ± 0.26 0.264 ± 0.06	0.183
Alpha-phellandrene	City Forest	0.430 ± 0.28 1.132 ± 1.58	0.575 ± 0.64 0.801 ± 0.99	0.468
Limonene	City Forest	12.843 ± 19.57 13.490 ± 21.03	12.954 ± 19.79 7.92 ± 9.96	0.850
All monoterpenes	City Forest	14.113 ± 19.79 15.336 ± 22.71	14.483 ± 20.34 9.447 ± 11.03	0.328

The linear correlations between monoterpenes absorption (expressed as delta) and vital variables (height, weight, SBP, DBP, HR and lung capacity) revealed no lineal associations between these variables (Figure S1). When looking at the increase or decrease of blood monoterpenes concentrations in regard to lung capacity, results show diverse patterns. The participant with a higher lung capacity (610 L/min) experienced an increase of alpha-pinene after forest exposure but a decrease in beta-pinene, alpha phellandrene and limonene. The participants from the forest group showing an increase of alpha-pinene present different lung capacities (from

510 to 410 L/min). Concerning usual exposure to natural areas, although one participant (V05) regularly visiting natural areas (3-4 times a week) showed great concentrations of the studied monoterpenes, the same link could not be identified for the rest of participants, neither among the control group. Nevertheless, we found significant positive correlations between the absorption of monoterpenes; particularly, between alpha-pinene and limonene (R=0.835) and beta-pinene (R=0.748), and between limonene and alpha-phellandrene (R=0.702) and beta-pinene (R=0.736) (Figure S1).

4. Discussion

This randomized controlled trial was carried out to analyze the forest monoterpenes absorption by humans during a 2-hours forest walk in a Mediterranean holm oak forest. This is the first attempt developed in this forest type at this region. The methodology developed allowed to successfully identify and determine the four most abundant monoterpenes compounds (alpha-pinene, beta-pinene, alpha-phellandrene and limonene) in the forest air and in participant's blood. Monoterpene absorption did not present a clear pattern among participants, neither the two studied groups (city or forest). Significant variations of monoterpenes concentration in blood could not be spotted. Nevertheless, we identified a negative correlation between absorption and participants' baseline values of alpha- and beta-pinene in the forest group.

The monoterpene air concentrations registered during the study are consistent with a recent study that found a similar range of monoterpene concentration in an holm oak forest from the same natural protected area [28] and others conducted at the same forest type and region [24,25,27]. Our results identify as well alpha-pinene as the most abundant monoterpene compounds among the other studied compounds, what has been also observed by studies in holm oak forests [24] and other forest types [22]. The air concentrations of monoterpenes of the studied Mediterranean forest are higher than other *in situ* studies characterizing the forest atmosphere under the canopy [30] or testing the effects of forests on other human systems and functions [3]. Considering these facts, the air concentrations of monoterpenes reported here suggest that the Mediterranean holm oak forests constitutes a suitable environment where monoterpene absorption by human could take place according to previous experiments [22].

The methodology developed in our study enabled the identification an estimation of the selected monoterpenes compounds in blood samples. We could not find significant effects of treatment (forest/city) on monoterpenes absorption. These were unexpected results since alpha-pinene and limonene can be found in exhaled air and urine and are known to be rapidly distributed in tissues [31–33]. Our results are also inconsistent with a previous study that reported an increase of six fold of alpha-pinene levels after 60-minutes' walk through a coniferous forest in participants serum samples [22]. Regarding the forest group, we identified significant correlations between alpha- and beta-pinene absorption and the participants baseline values of these two compounds which are in addiction the monoterpenes compounds with greater air concentration during the study. This observed trend could be supported by the aforementioned study that found an increase in alpha-pinene blood concentrations up to levels similar to forest air concentrations [22]. The concentrations we reported are similar to the ones identified in the previous study at some point of the range [22], nevertheless, blood alpha-pinene concentration after forest exposure differ notoriously. In parallel, baseline concentrations of all the selected monoterpene compounds in our study were different among participants. As pointed out by some authors, these could be explained by the uptake of foods, perfumed/fragranced products and monoterpenes emitted by the street trees from the participants' living areas [22]. Therefore, we strongly encourage future researches to keep participants under the same conditions (space, food and activities) some days before collecting the samples and developing the experiments, and besides consider baseline concentrations of each individual as covariable as we have performed here.

The fact that we could not identify significant increases or decreases of monoterpenes concentrations in participants blood samples after forest exposure could be due to different reasons. First of all, it should be mentioned that the sample size taken in this study (5 participants in the forest and 5 in the city) is limited and therefore it is not a powerful statistical test in order to spot patterns among variability. The potential variability associated with SPME and GC-MS analytical methods was mitigated by using an internal standard. Since results still present a high variability, we strongly recommend conducting further research with higher samples size if logistically and economically possible.

We found no significant correlations between monoterpene absorption and the vital variables (Height, Weight, SBP, DBP, HR or Lung Capacity) even though monoterpene absorption via inhalation has been already proved [31,34]. Nevertheless, we found significant correlations between the absorption of different monoterpene compounds. This is consistent with a previous study that found higher alpha-pinene levels in mice brain and liver when inhaled with other components in comparison to single-component inhalation [35].

5. Conclusions

This randomized controlled test analyzed the monoterpene absorption by humans during a 2-hours forest exposure in a Mediterranean holm oak forest focusing on the four most abundant monoterpene compounds: alpha-pinene, beta-pinene, alpha-phellandrene and limonene. We identified the four compounds in forest air during the experimental study being alpha-pinene the monoterpene with the greatest concentration. No significant changes in monoterpene absorption could be found for the city (control) and forest group (treatment). Nevertheless, higher absorptions of alpha-pinene were found in the forest group participants with lower baseline monoterpene blood concentrations. Although no significant correlation could be spotted between the vital variables and the monoterpene absorption, we found significant correlations between the absorption of the different monoterpene compounds.

The current body of literature highlights monoterpenes as one of the forest factors determinants of the interaction between these ecosystems and human health. Although several studies have conducted *in vivo* an *in vitro* analyzes of this interaction reporting beneficial effects, we could not describe the basic absorption of these compound *ex situ*. Nonetheless, we are convinced that our attempt will serve as a starting point and constitute a valuable contribution for further investigations in this emerging research field.

Supplementary Materials: The following are available at the end of this chapter , Figure S1: Correlation plots. Table S1: Instrumental parameters of SPME-GC-MS. Table S2: Vital variables at baseline

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Can Surell forest to conduct the present study. Last but not least, we would like to thank all the stuff from SAQ (Servei d'Anàlisi Química-UAB) for all their tireless efforts to conduct the laboratory experiments under the best conditions.

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Supplementary material

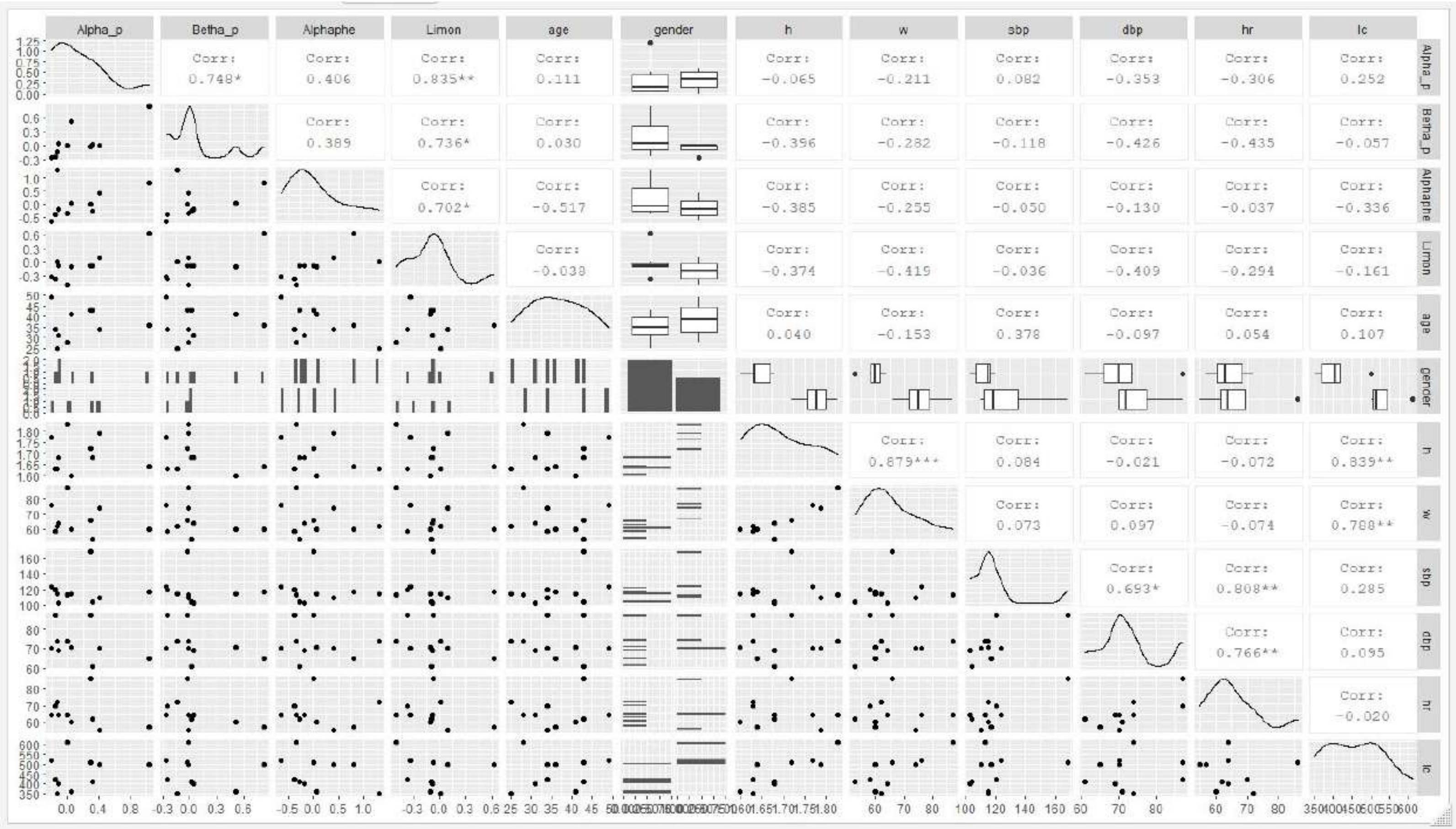


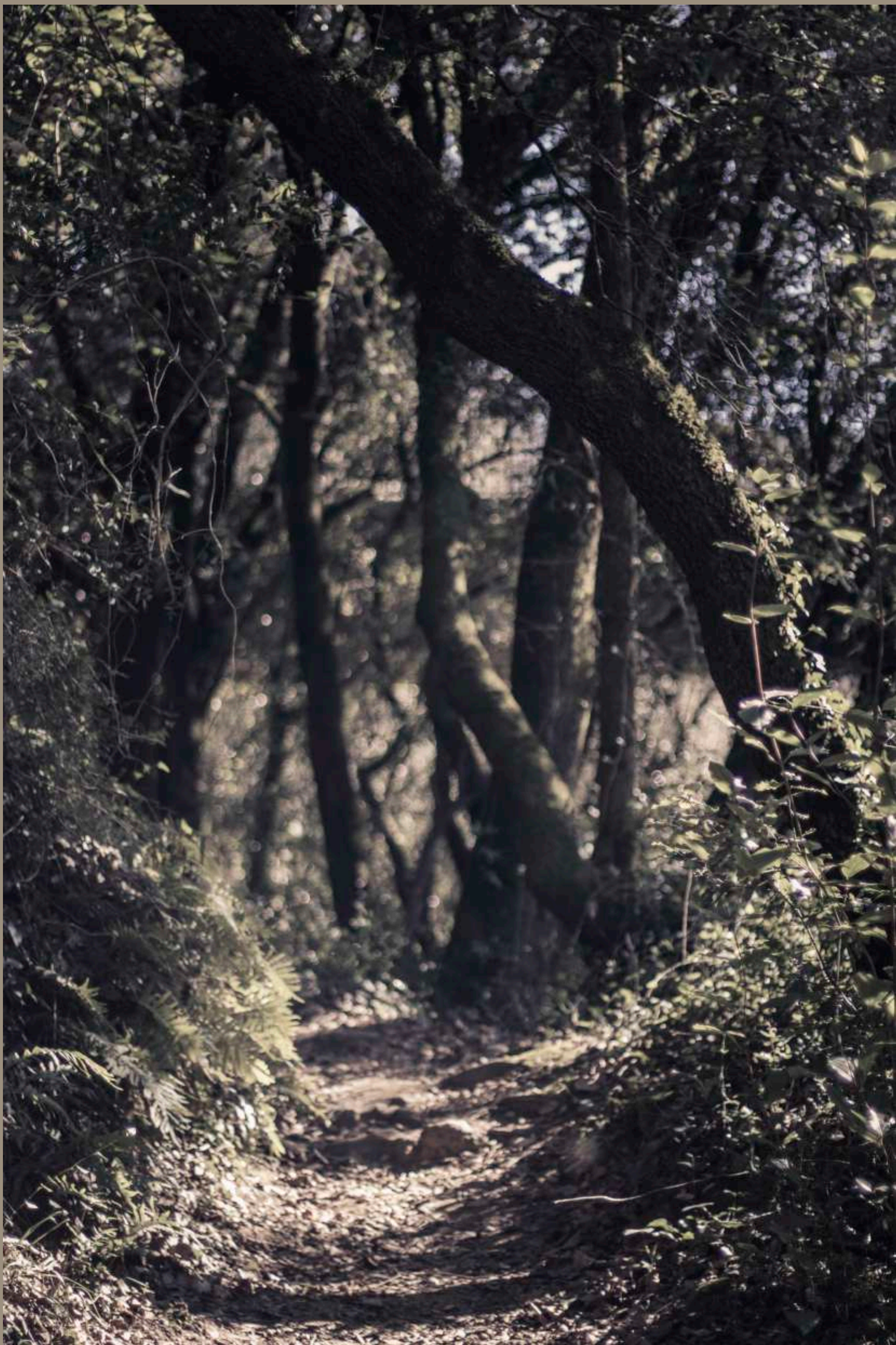
Figure S1. Correlation plots. Lineal correlations between the vital variables and the monoterpenes absorption. Delta here is expressed as: Delta = (after concentration - before concentration) / before concentration. Variables description: Alpha_p (delta alpha-pinene); Beta_p (delta beta-pinene); Alphaphe (delta alpha-phellandrene); Limon (delta limonene); h (height); w (weight); sbp (systolic blood pressure); dbp (diastolic blood pressure); hr (heart rate); lc (lungs capacity (l/min)).

Table S1: Instrumental parameters of SPME-GC-MS

Instrumental parameters of SPME-GC-MS
SPME: Combipal, CTC Analytics
SPME fiber: 75µm CAR/PDMS (ref:57343-U), Supelco.
Pre-Incubation time: 10 min
Incubation temperature: 45°C
Extract time: 60 min.
Desorption time: 4 min.
Fiber Bakeout time: 15 min.
Fiber Bakeout temperature: 300°C
GC: HP 6890 Series II GC System, Agilent Technologies
Inlet mode: Splitless (4min)
Inlet Temperature: 290°C
Column oven temperature: 35°C (5min), 5°C / min to 100°C (0min), 20°C /min to 300°C (5min)
Carrier gas: Helium
Mode: Constant Flow
Flow:0.9mL/min
Septum Merlin amb diafragma SPME 23 ga.(ref:5182-3444), Agilent Technologies.
Liner SPME Injection Sleeve 0.75 mm ID (ref:2-6375.05), Supelco.
Column: ZB-5 (30m x 0.25mm x 0.25µm), Phenomenex
Run time: 33 min
MS: HP5973, Agilent Technologies
Interface temperature :280°C
Source temperature: 230°C
Quad temperature: 150ºC
Solvent delay: 0min
Adquisition mode: SIM
SIM mass (m/z): 93
Electron Ionization: 70 eV

Table S2: Vital variables at baseline (average and standard error) and Independent *t*-test p-value. SBP (systolic blood pressure), DBP (diastolic blood pressure)

Groups	**Age**	**Gender M**	**F**	**Height**	**Weight**	**SBP**	**DBP**	**Heart rate**	**Lung capacity**
City	36.4 ±9.20	1	4	1.65±0.06	64.4±6.69	114.8±7.56	69.8±3.27	63.4±5.63	426±79.24
Forest	36.4±6.50	3	2	1.73±0.08	67.6±13.46	123±25.80	75.80±11.25	67.40±11.69	490±80.93
p-value	1	-		0,196348	0,651381	0,527605	0,307629	0,517675	0,242023



Chapter 6

Evolution of human salivary stress markers during an 8-hours exposure to a Mediterranean Holm Oak forest

Abstract: The current study analyses the evolution of different human stress markers during an 8-hours exposure to a Mediterranean Holm Oak forest. We conducted a pre-post study with thirty-one subjects in which saliva samples were collected before the exposure (baseline) and after 1, 2, 4 and 8 hours. Our results show: A) a significant decrease in cortisol saliva concentrations since the second hour until the end compared to basal time; B) a significant increase of alpha amylase activity after the first hour of exposure compared to basal time that remained elevated during the rest of the study; C) a significant decrease of IgA from the fourth hour of exposure compared to the basal time. These findings indicate an effect of forest exposure in salivary biomarkers of stress and provide relevant data for the scientific and healthcare community encouraging further research in the field.

Keywords

forest exposure, *shinrin-yoku*, cortisol, stress markers, forest environments

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1. Introduction

Chronic and acute stress, low capacity to recover from it, as well as states derived from suffering it, have been linked to different long-term pathologies and psychological disorders [1–5]. The potential of forests as a source of health has led to numerous studies that provide evidence of its benefits [6–8] and strongly correlate exposure to these ecosystems with stress regulation [9,10]. While several studies have proved that exposure to forests lowers stress markers compared to urban settings [7,11–16], other studies found no significant differences [17–20].

From the existing literature, a recent review and meta-analysis from Antonelli et al. (2019) concludes that exposure to forests significantly affects cortisol levels. Most of the studies included in the review considered short term exposures to forested ecosystems (from 15 minutes to 4 hours). Up to date, limited data is available to understand the effects of forests on stress hormones [21] and no studies assess the effect of long term exposures to forest environments.

Saliva sampling is widely used as a non-invasive method in stress studies preventing confounding stress-derived factors. In addition, saliva allows a repeated sampling over short time intervals facilitating an ongoing monitoring. Salivary cortisol is the most used stress marker within the studies analysing forest effects on stress regulation [21]. The levels of salivary cortisol can be useful to study psycho-physical benefits of a specific intervention on the so-called stress system because they are indicative of the hypothalamic pituitary–adrenal (HPA) axis activity and the integrated effect of an anti-stress practice upon a person's neuro-endocrinological system, [22,23]. Salivary alpha-amylase (sAA) is secreted by the salivary glands (mainly in the parotid) in response to adrenergic activity. Previous studies suggest sAA as a surrogate marker of the autonomic nervous system (ANS) [24,25] and have shown that sAA levels rise in response to both physical and psychological stress [26–28]. It has also been used to evaluate the possible positive effect of natural environment in humans [17,29]. Salivary immunoglobulin A (IgA) can be increased by both parasympathetic and sympathetic nerve system [30,31]. The IgA expression has been related to mental stress in humans [32]. Furthermore, saliva has also been used as a marker to evaluate oxidative stress in humans [33]. Although one study has analysed oxidative stress through malondialdehyde MDA [15], to authors knowledge, the total antioxidant capacity (TAC) has not been used in forests and human health studies and could constitute a relevant biomarker of oxidative stress.

The aim of this study was to analyse the variation and evolution of a combination of human salivary stress biomarkers (cortisol, alpha amylase and IgA) which reflect different systems involved in stress mechanism, during an 8-hours exposure to a Mediterranean forest ecosystem.

2. Materials and Methods

2.1. Participants

A total of thirty-one subjects (21 females and 10 males) were recruited from the Autonomous University of Barcelona and the Gym Chain "Duet Fit". Inclusion criteria were: A) aged between 20 and 40 years old; B) index of body mass between 19 and 25; C) none-smokers (at least 6 months before the study); D) No respiratory pathology (asthma, chronic bronchitis or respiratory allergies);

and E) no pathologies affecting the immune system during the study. Two days before the study participants were asked not to consume alcohol to control for the effects of it. After providing the study description and fully inform participants about the study objectives and design, written informed consent was received from each participant in advance. This study was approved by the Ethics Committee of Granollers Hospital, Spain, on the 26th of June 2018 (CEIC Code 20182020).

2.2. Study site

The study site was located in Montseny Natural Park - Biosphere Reserve (NE Iberian Peninsula) (Figure 1) in a Holm Oak forest dominated by *Quercus ilex* (2400 trees per hectare). The average Diameter at Breast Height (DBH) for *Q. ilex* was 16,41±1.037 cm. Other species like *Castanea sativa*, *Juniperus communis* or *Erica arborea* were identified in the study area but not abundantly. Concerning forest structure, vegetation cover at 8 meters height was 100% while the layers below did not exceed coverages of 10%. This forest area has an altitude range from 860 to 972 m. During the study records taken under the canopy level registered an average temperature of 23,87±0,18°C and an average relative humidity of 53,70±0,50% during the sampling period.

Figure 1. Site location and experimental scenario.

2.3. Study design

We conducted a pretest-posttest experimental approach to assess the effects of forest exposure on stress markers considering the pretest measurement (before forest exposure) as "baseline". The study was carried out the 20th of July 2018 and started at 9:30 in the Hospital of Sant Celoni (NE

Catalonia, Spain), where the first saliva samples were collected avoiding any previous contact with the forested area. Participants were then taken to the forest where a light breakfast was provided. At 11:00 participants began the forest exposure until 19:00 having lunch in the forest at 14:00. They were asked to walk through the forest, rest and sit if they were tired. Intense physical activity such as running or conducting any sport was forbidden. Participants were asked to inform if they conducted yoga, meditation or any other pro-active activity (as described in [34]). Once the experimental study started, saliva samples were collected after 1 hour, 2 hours, 4 hours and 8 hours of forest exposure leading to a total of 5 samples per participant including the baseline sample (Figure 2).

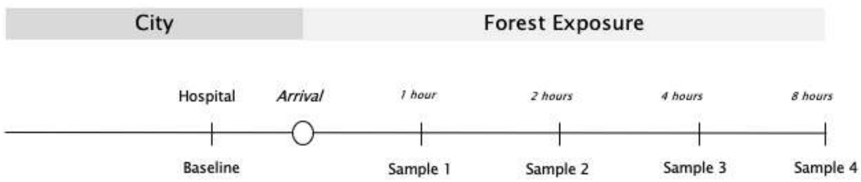

Figure 2. Sampling design diagram

2.4. Saliva sampling procedure

Saliva was collected during 1 min by passive flow under supervision, using 5 mL standard microcentrifuge polystyrene tubes with round bottoms (12 X 75 mm). To minimise any potential physiological effects about responses to salivary biomarkers, before the beginning of the saliva collection, participants were not allowed to eat, have coffee or caffeinated soft drinks [26]. Each sample was refrigerated or stored on ice until arrival at the laboratory. Afterwards, samples were weighed and then they were centrifuged at 4.500 x g for 10 min at 4°C to remove cells. The supernatant was transferred in 1.5 mL Eppendorf tubes and stored at -80 °C until analysis [27,35].

2.5. Laboratory salivary methods

Cortisol was evaluated by a solid-phase, competitive chemiluminescent enzyme immunoassay (Immulite; Siemens, Erlangen, Germany), displaying within-run and between-run imprecision lower than 10%, recovery rates between 92% and 120%, and a limit of detection of 0.2 nmol/l [36].

sAA activity was measured using a colorimetric commercial kit (Alpha-Amylase, Beckman Coulter Inc., Fullerton, CA, USA) following the International Medicine (IFCC) method [37,38], as previously reported and validated [36].

IgA was evaluated with a commercial ELISA kit (Bethyl, Montgomery, TX, USA), with a within-run and between run imprecision lower than 10%, recovery rates between 91% and 112%, and a limit of detection of 0.05 mg/l [39].

Two assays commonly used to evaluate the total antioxidant capacity (TAC) were used: Cupric reducing antioxidant capacity (CUPRAC) is based on the generation of a complex containing Cu^{2+} and one chelating agent, in this case the bathocuproinedisulfonic acid disodium salt, and its reduction to Cu^{1+} by the non-enzymatic antioxidants present in a sample [40]. Results were compared with a standard curve achieved with Trolox and were also expressed in millimoles of Trolox equivalents per litre. The ferric reducing ability of plasma (FRAP) measurement is based on the assay described by Benzie and Strain [41]. A reaction mixture containing ferric-tripyridyltriazine (Fe^{3+}) is reduced to the ferrous (Fe^{2+}) form by the non-enzymatic antioxidants provided by the sample. Ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2$) solution was used to produce a standard curve and compare with sample results that were expressed in millimoles of Fe^{2+} equivalents per litre.

2.6. Data analysis

The different salivary biomarkers concentrations were evaluated for normality of distribution, using the Kolmogorov–Smirnov test. As the results did not meet the normal distribution criteria, a non-parametric test of repeated measures one-way ANOVA with a Friedman's test and a Dunn's multiple comparisons post-test were used to compare the different levels of salivary biomarkers between sampling times. To study the possible effect of gender on the salivary levels, a two-way repeated measures ANOVA test and a Bonferroni post hoc test were performed for the 'sampling time-gender' influence. All statistical analyses were performed using a commercial statistics package (GraphPad Prism 6, GraphPad Software Inc., La Jolla, CA). A $P < 0.05$ value was considered significant.

3. Results

Salivary cortisol samples show a decreasing tendency during the 8-hours exposure to forest environment (Figure 3A). After one-hour exposure (0.305 μg/dL; range 0.24–0.44 μg/dL; 25-75th percentiles) data show a decrease although this is not significant in relation to baseline (0.522 μg/dL; range 0.38–0.60 μg/dL; 25-75th percentiles). For the samples collected after 2 (0.232 μg/dL; range 0.17–0.32 μg/dL; 25-75th percentiles), 4 (0.273 μg/dL; range 0.21–0.33 μg/dL; 25-75th percentiles) and 8 hours (0.148 μg/dL; range 0.11–0.22 μg/dL; 25-75th percentiles) in the forest, there is a significant decrease of salivary cortisol levels in comparison with the baseline measurements (p<0,0001). Comparing sampling times, significant decreases are found between the 1-hour and 2-h (p<0,05) and 8-h measures (p<0,05) as well as for 4-h and 8-h (p<0,0001).

Alpha amylase samples showed a significant increase at 1-hour (116820 Ul/L; range 57720–187140 Ul/L; 25-75th percentiles) and in the measures obtained after this exposure time compared to the baseline values (Figure. 3B). Salivary IgA levels (Figure. 3C) show a significant decrease at 4-h (24.41 mg/dL; range 17.48–39.99 mg/dL; 25-75th percentiles) and 8-hours (23.79mg/dL; range 11.56–37.67 mg/dL; 25-75th percentiles) measurements compared to the baseline values (p<0.001 and p<0.01 respectively). This decrease is also significant (p<0.05) when comparing both sampling times with sampling time 2-h (33.47 mg/dL; range 16.89–47.89 mg/dL; 25-75th percentiles).

The stress oxidative markers analysed did not present significant variations during the forest exposition. Salivary CUPRAC levels were 0.195 mmol/L (range 0.12–0.27 mmol/L; 25-75th percentiles) at baseline and were 0.197 mmol/L (range 0.12–0.27 mmol/L; 25-75th percentiles), 0.178 mmol/L (range 0.12–0.28 mmol/L; 25-75th percentiles), 0.191 mmol/L (range 0.14–0.28 mmol/L; 25-75th percentiles) and 0,198 mmol/L (range 0.14–0.28 mmol/L; 25-75th percentiles) at 1h, 2h, 4h and 8h after forest exposition, respectively. Salivary FRAP levels were 0.366 mmol/L (range 0.17–0.58 mmol/L; 25-75th percentiles) at baseline and were 0.414 mmol/L (range 0.16–0.58

mmol/L; 25-75th percentiles), 0.338 mmol/L (range 0.22–0.56 mmol/L; 25-75th percentiles), 0.375 mmol/L (range 0.24–0.54 mmol/L; 25-75th percentiles) and 0,392 mmol/L (range 0.23–0.61 mmol/L; 25-75th percentiles) at 1h, 2h, 4h and 8h after forest exposition, respectively.

There were no significant effects of gender on any of the evolutions of the analytes studied.

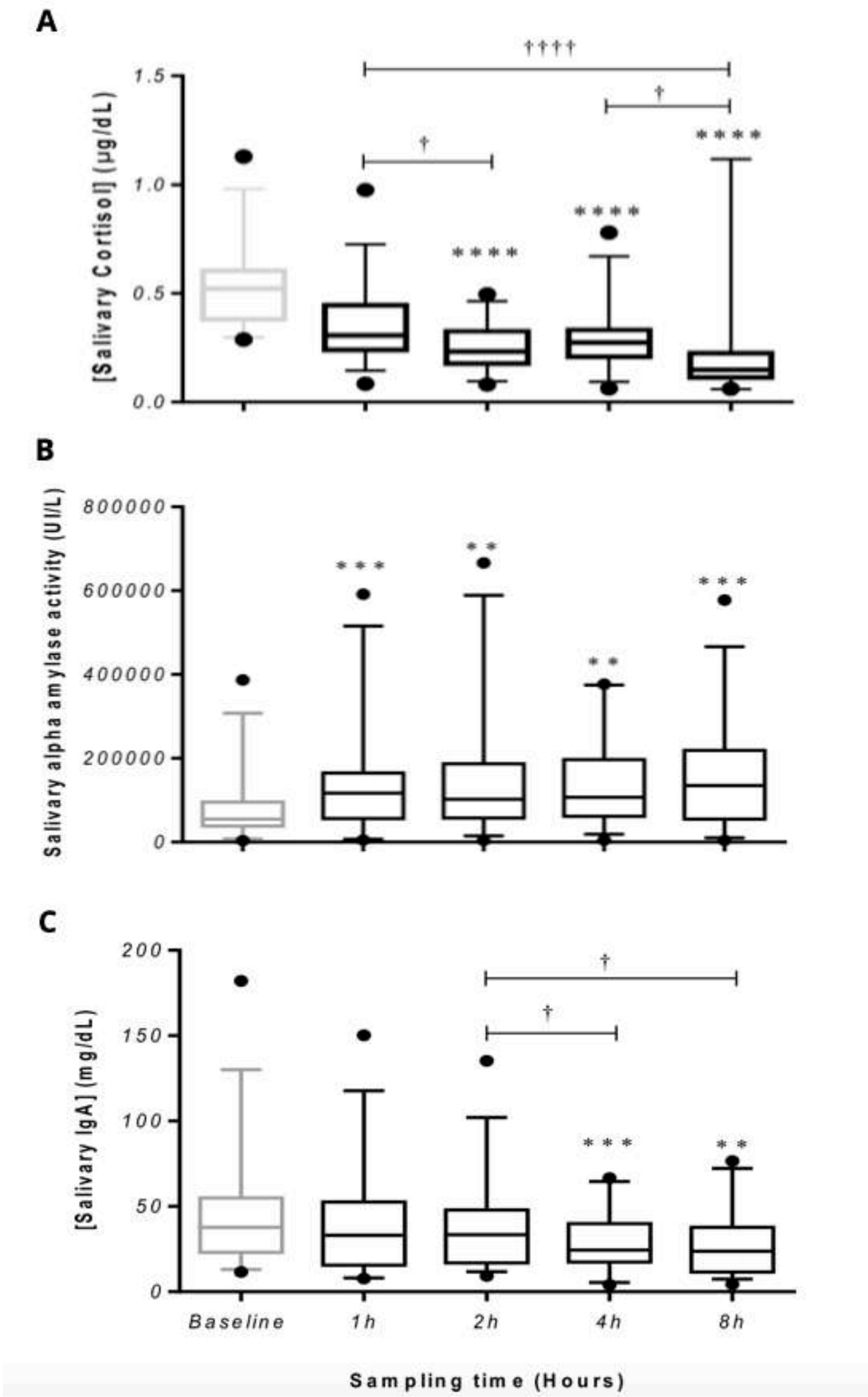

Figure 3. Cortisol (A), salivary alpha amylase activity (B) and IgA (C) changes box plots. The boxes (25th-75th percentiles) show the median concentrations of salivary marker (line within the box) and whisker plots represent the minimum (5th percentiles) and maximum values (95th percentiles). * $p<0.05$, **$p < 0.01$, ***$p< 0.001$ and ****$p< 0.001$ compared to baseline values. †$p < 0.05$, †† $p< 0.01$, †††$p< 0.001$ and †††$p< 0.001$ comparing between different sampling times.

4. Discussion

The present study analysed the evolution of a combination of different human salivary stress markers during an 8-hours exposure to a forest ecosystem. We found different trends depending on the stress marker: while cortisol and IgA showed a significant decrease during the forest exposure, alpha amylase increased significantly after it. In addition, the studied biomarkers showed different dynamics with sAA varying earlier while cortisol and IgA changing at later stages.

We observed a decreasing trend in cortisol levels during forest exposure, being significant after the first 2 hours. A significant cortisol decrease in serum after contact with forests has been previously reported (Ochiai et al., 2015). Our results are also in line with other studies that have showed lower salivary cortisol levels in the forest compared to urban settings [7,11–14,19,43]. The significant decrease of cortisol levels after 2 hours-exposure in our study could explain why some authors previously found no significant changes in salivary cortisol levels just after forest exposure, neither 20 or 40 minutes later [20]. This cortisol response found after 2 hours could be due to the fact that cortisol changes represent the HPA system that usually responds later to stress than other pathways also involved in the stress response such as the adrenergic systems. Overall the decrease in cortisol would indicate a decrease in the stress levels and therefore a benefit of the activity. On one hand, our results showing a significant decrease only after 2-hour exposure might be due to different factors: the trip from the hospital to the forest area as well as the acclimation and adaptation process of the volunteers could explain the delayed decrease of this hormone levels. Furthermore, physical exercise has been observed to induce cortisol levels increase [44]. On the other hand, the significant differences we found between sampling times show that the decreasing effect of forest exposure lasted for the 8 hours exposure. This is important to note since cortisol concentrations can rebound after a stimuli [45]. Thus, our results follow the findings of this field of study where no concentration rebounds have been reported so far.

We identified a significant increase in sAA concentrations during the first hour-exposure but no significant variations from that sampling time and on. Thus, we assume that sAA levels were increased and then remained stable during the forest exposure. Usually a decrease in cortisol is associated to a decrease in sAA; however, the increase of sAA found in our study could be due to the fact that participants conducted low intensity physical activity which it is known to increase sAA activity [46]. Furthermore, that initial and significant increase might be due to a physiological rise of activity, caused by the normal diurnal pattern of liberation produced after the pronounced decrease after awakening [47]. Regarding the stability and non-significant variation of this hormone after the first hour, a previous study where participants viewed a forest also obtained non-significant variations of salivary amylase [48]. However, other form of stress reduction interventions, such as yoga, has been reported to decrease sAA levels compared to baseline values [49] and one previous study found that salivary amylase was reduced in the forest in comparison to an urban setting after a 20-minute walk [29][29][29][29][29][29]. Although forests might constitute a habitat with less environment-derived stressors, other factors may play a role in salivary alpha amylase activity and further studies are needed to clarify these observed trends.

IgA concentrations showed a decreasing trend but only significant from the sampling time 4-hours when compared to baseline values. These delayed changes could be in line with those observed by Tsunetsugu et al., (2007) who found no changes in IgA concentrations when walking on or viewing a forest for 15 minutes. The response to stress of salivary IgA is complex [50] and the non-significant tendency toward an increase or decrease of this marker's concentrations in the present study at shorter periods is unclear. A plausible explanation could be that the higher initial levels were due to an immediate stress-effect related to transport, acclimation and adaptation to forest whereas return to a normal pattern was induced by a potential relaxing effect of forest exposure. Decreases in IgA after forest exposure have been previously described [50] and further studies should be performed to elucidate the cause and the possible implications of this decrease.

Overall, our results indicate that forest can produce changes in salivary human stress markers patterns with decreases in cortisol and IgA and increases in sAA. However, future research is needed to provide a better compression of the confounding factors as well as to improve our understanding of stress markers variation in longer exposures to forests. This information could provide essential information specially for the healthcare community in clinical practice guidelines and preventive medicine particularly in the field of stress management and its derived pathologies.

4.*1. Limitations*

We are aware about the different limitations that the experimental design faces. First, we did not include a control group in the study. This implies that we cannot control the potential effects of confusing factors associated with the forest neither isolate the regular variations of the circadian rhythmicity. Nevertheless, we prioritized a bigger sample size exposed to the forest (31 individuals instead of 15) and the analysis of the evolution of the forest-exposure itself not compared to an urban setting. Considering that several studies have found lower cortisol concentration in forest ecosystems compared to urban environments [7,11–14,19,43] we focussed in unveiling the variation of salivary stress markers in the forest during an 8-hours exposure rather than comparing again these environments against the urban ones. Considering the current literature, we also aimed at analysing whether these previously reported differences between forested and urban settings were due to a decrease of stress markers in the forest or simply to an increase in urban environments testing the forest environment itself.

In addition, it is well-known that the stress markers analyzed in our study follow a circadian rhythmicity [47,51]. However, Yamaguchi et al. (2006) showed that sAA activity circadian rhythm fluctuations were much smaller than the stressor-induced variations and that forest constitutes a good environment in which people experienced much less environment-derived stress, which enabled observations of exercise-induced physiological effects to be made. Another limitation is that pace and walking distance were not registered during the study. Given the variability we observed, especially at the beginning of the exposure, and the influence of physical exercise in stress markers concentrations in saliva, we strongly encourage future researches to include these parameters in their measurements. Despite the methodological limitations mentioned above, we are convinced that the design of the study provides relevant findings of long-exposure variations of human salivary stress markers in this Mediterranean forest ecosystem.

5. Conclusions

This experimental pre-post study reported: A) a decrease in cortisol saliva concentrations during an 8-hours exposure to forest significant from the second hour until the end; B) a significant increase of sAA activity during the first hour exposure and the stability of it during the rest of the study; C) a significant decrease of IgA from the fourth hour of exposure. These findings support the physiological (cortisol) and psychological (IgA) relaxing effect of forest exposure and provide relevant data for the scientific and the healthcare community encouraging further research in the field.

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Chapter 7

General discussion

7. Discussion

The contents of this dissertation have addressed different components and relationships of the forest and human health binomial. In this last chapter, we will discuss about the implications and contributions of our findings. The general discussion of this thesis dissertation is articulated in three sections: principal contributions, main drawbacks and future research.

7.1. Principal contributions

Thoroughly, the investigations developed in this thesis aimed at assessing to what extent Mediterranean forests can be linked to human health. More particularly, we worked towards analysing which forest variables have been associated to effects on human health; characterising monoterpenes air concentrations under the canopy in a Mediterranean holm oak forest; studying the human absorption of monoterpenes during forest exposure; and researching on the potential of forests to regulate human stress markers. In the following paragraphs, we will present the main contributions from this dissertation in regard to the research questions outlined at the beginning of this thesis.

RQ1. *Which elements and features can be consistently related with human health from the researches that have conducted experimental studies with humans?*

The systematic review highlighted the lack of forest descriptions and the heterogeneity of forest variable descriptions in studies about forests and human health, and pointed out that it is still premature to draw any sort of conclusions with respect to data patterns, due to the high heterogeneity among the studies performed so far. In addition, no consistent relationships between forest type and the most widely studied health variables (blood pressure, pulse rate and cortisol levels) could be identified from the existing literature. These findings supplement the outcomes from previous systematic reviews and meta-analysis that clearly stated the effects of forests on blood pressure, pulse rate and cortisol levels [1,2]. Concerning other health variables, the existing body of literature seems to generally contend that contact with forests can induce health benefits and particularly additive health effects when compared to the development of similar activities in urban environments [1–10], but no assessment of the forest variables has been conducted so far. In parallel, previous reviews [9,11], and a recent one published during the writing of this thesis [10], report results that are in line with our findings and underline the scarcity of confounding variables and forest ecosystem descriptions. All together, these findings are important, since the topic "forests and human health" has caught the attention of the scientific community [3,12] and is starting to become an emerging priority for managers, landscape planners and policy makers over the last decades. Examples can be found at international level, where the use of forests to foster human health has been included in the agendas of forest policy (e.g. IUFRO Task Force on Forest and human Health, 2007) in Asian countries such as Japan and South Korea [3,13] and in some European countries [14]. Additionally, some studies predict that forest priorities can change from production and conservation to recreation and health promotion [13,15], so detailed information about the characteristics of these habitats should be provided for its management and its potential use for preventive medicine.

In our systematic review we have identified previous research contrasting the effects of different forest features [16–20] and elements [19,21–27]. Nevertheless, these are incipient works that in some occasions lack an environment control or present a high variability of participant numbers, exposure time or type [28]. Forest composition and structure is strongly shaped by forest management, which also ultimately determines forest features and elements [29,30]. The effects of forest management regimes on human health outcomes has started to be addressed by emerging studies [17–19] that point out different health effects linked to the different regimes. Still, evidence arisen from these researches does not allow to visualise clear conclusions since some identify managed forests as the most beneficial, while others do so for the unmanaged ones. Moreover, stand age appears to be a key topic on the table nowadays. It is widely argued, especially among the civil society, that "mature" or ancient forests may have a greater contribution to health effects. Our review has identified only few studies reporting the stand age and only one that compares the effects of a young and a "mature" forest. In that single study, significant differences were spotted in favour of the mature in regard to fibromyalgia impacts, but no significant differences could be shown for the physiological parameters or the anxiety levels of participants [16]. Once more, no robust conclusions can be drawn from the current body of literature between particular forest variables and health benefits.

To sum up, we conclude that the design and development of a more comprehensive research approach to analyse the interactions between forests and humans surfaces as crucial. Thus, analysing the effects of forests and characterizing ecosystems to describe the pathways that may induce health effects becomes essential to provide data and tools for forest managers, policy makers and landscape planners in coordination with healthcare professionals.

RQ2. *Are monoterpenes concentrations below Mediterranean holm oak forest comparable, or higher than in previous in vivo, in vitro and ex situ studies where effects on human's health were underlined?*

The systematic review also allowed us to spot a particular element from forests that, according to the current body of research, may partly explain the health effects induced by forests: the biogenic volatile organic compounds (BVOCs), especially monoterpenes [19,21,22,31]. Several *in vivo*, *in vitro* and *ex situ* studies have underlined the properties and the effects on different body functions and systems [19,27,32–36]. These compounds have been widely studied above the canopy due to their role in climate and atmospheric interactions [37,38], whereas the concentrations below the canopy (where these could be available and breathable by humans) remain weakly studied. In this sense, most of the *ex situ* research conducted to characterise monoterpenes concentrations below the canopy and to analyse the interactions and effects of these chemicals with human health have been conducted in Asian forests [28], where abiotic and biotic conditions may differ from other areas of the planet.

Chapter 2 of this dissertation aims at filling this gap by analysing the under-canopy variations of monoterpene concentrations in a Mediterranean holm oak forest during the emission peak. We identified seasonal and daily patterns. The highest concentrations occurred during summer, and diel cycles changed between months, with peaks in early morning in summer and peaks at midday in the entire period. The total monoterpene variations were significantly linked to abiotic/atmospheric variables, such as temperature and solar radiation, as well as humidity and vapor pressure deficit (vpd). These results are in consonance with the emission and the above-canopy concentrations reported by previous studies conducted in the same forest type, the same region and the same particular forests [39–42]. Furthermore, the observed seasonality of monoterpene concentrations is associated to the seasonality of plant monoterpene emission [43,44], mostly linked to the seasonal changes in temperature [45,46] and other meteorological variables, such as solar radiation [37,47,48], as it could be also reported from our results. All these findings taken together suggest that monoterpenes emission and above-canopy measurements characterised in previous works may serve as an estimation to understand below-canopy monoterpene concentrations and the variations among seasons and days.

Among monoterpene compounds, alpha-pinene, sabinene, limonene, camphene and alpha-phellandrene are the most abundant in Mediterranean holm oak forests [39]. Some of these monoterpene species have been previously found to be absorbed by humans when forest walking [49]. These monoterpenes have been related with physiological changes in humans, in particular alpha-phellandrene, that has been significantly associated with an acute insulin reaction [19]. Some *ex situ* and *in vitro* studies have shown enhancing effects of monoterpenes on the human immune system, but at higher monoterpene concentrations than those we registered in our study [22,31]. Nevertheless, the concentrations we report here (from 114 to 725 ngm^{-3} during the whole sampling period and up to 2230 ngm^{-3} in August) happen to be comparable [25] or much greater [23,24] than in previous *in situ* studies that identified similar health outcomes to the ones observed in the *in vitro* and *ex situ* experiments. The air monoterpene concentrations in our studied Mediterranean forest are also higher than other *in situ* studies that characterize the forest atmosphere under the canopy [50] or test the effects of forests on other human systems and functions [19]. Taken together, these findings suggest that the Mediterranean holm oak forests constitute a suitable forest environment to develop further research on the effects of monoterpenes on human health.

RQ3: *Can humans absorb monoterpenes emitted by trees during a 2 hours forest exposure?*

In an attempt to study the basic interactions between humans and this Mediterranean forest chemistry we conducted a randomised controlled trial to analyse the monoterpene absorption by humans during a 2-hours forest exposure in a Mediterranean holm oak forest. We focused on the four most abundant monoterpene compounds: alpha-pinene, beta-pinene, alpha-phellandrene and limonene [39]. According to literature, these are some of the most relevant compounds in the forest-health pairing [35].

On one hand, we designed a combined methodology (Tenax and Sulficarb tube sampling, headspace methodology and GC-MS analysis) that presents novel insights in comparison to previous experimental setting from the same field of research [49]. As a methodological contribution of this thesis, we used an internal standard for the blood samples analysis, which allows to mitigate the variability of the headspace and GC-MS methods. Chapter 3 deeply describes the design developed with the aim that this may serve to improve further research in this field.

Some authors have discussed about the positive-results bias that the forest and human health field of research may face, since significant positive results are more attractive to be published in an emerging topic [10]. In this sense, this thesis reports as well the non-significant results from the analysis of monoterpene concentrations, willing that this may help as a starting point for other works or it will create more attention in the research agenda. We found no significant changes in monoterpene absorption for the city (control) and forest (treatment) groups. These were unexpected results since the studied compounds are assumed to be highly absorbed through the respiratory system [51–53]. These are also inconsistent with the only one study developed so far, that found an increase of alpha-pinene levels in participant's blood after forest walking [49]. Many differences are spotted between this previous study and ours. Firstly, their experiment was conducted in Japan and in a different forest type: a coniferous forest. As reported in the same study, forest types can present highly different monoterpene air concentrations [49]. In addition, few information is available in the study description in regard to forest structure and management: relevant information that has been observed to shape these chemicals air concentrations [19]. Furthermore, both researches (including ours) present a constrained sample size. Sample analysis was similar in the two works, although differently from the previous study, in our study we used the internal standard to improve methods accuracy as mentioned before. Taking all the findings from both works and considering the aforementioned differences, it is hard to draw conclusions in regard to *ex situ* human absorption of monoterpenes from the available research.

Nevertheless, we identified significant correlations between alpha- and beta-pinene absorption and the participants baseline values of these two compounds, which are, in addition, the monoterpenes compounds with greater air concentration during the study. Higher absorptions of alpha-pinene were found in the forest group participants with lower baseline monoterpene blood concentrations. This observed trend is aligned with the aforementioned study that only reports a significant increase for the most abundant compound in forest air [49]. It could be argued that participants with daily exposure to products with high monoterpene-perfumes show greater baseline concentrations [49] and when exposed to forest atmosphere, with lower air concentrations of monoterpenes, they experience no blood absorption of these compounds. In parallel, no significant correlation could be proved between the vital variables (weight, height, blood pressure, pulse rate and lung capacity) and the monoterpene absorption. Nonetheless, we found significant correlations between the absorption of the different monoterpene compounds. Although these correlations are not fundamental to the scope of this dissertation, they are consistent with a previous study that found higher alpha-pinene levels in mice brain and liver when inhaled with other components in comparison to single-component inhalation [54]. As stated, the findings of chapter 3 constitute a novel contribution to assess this absorption in Mediterranean forest and a substantial methodological contribution.

RQ4: *To what extent humans' physiological stress can be mitigated when visiting a Mediterranean forest?*

Among the effects on human health induced by forest, the stress regulation has been broadly studied [1,8,55]. These effects have been seen to happen at psychological [56–63] and physiological level [12,64,73,65–72]. Monoterpenes have also been related to this particular effect [22,32,34]. The findings of chapter 4 support the physiological and psychological relaxing effects of forest exposure. Moreover, the results contribute to increase the knowledge about the different aspects in the connection between forests and its role as stress mitigators. Most of the previous research proved that exposure to forests lowers stress markers compared to urban settings [67–73]. Nevertheless, the question arises: *Is it forest exposure that decreases stress biomarkers levels or is it exposure to urban environments that stresses us?* To aswer that, this dissertation first contribution is at showing the decrease of the selected stress markers (cortisol, IgA, sAA) in a forest environment. In addition, and to our knowledge, no such stress-related studies have been developed so far in this forest type: Mediterranean holm oak forest.

Another contribution of the experimental setting developed is the measurement of the evolution of the stress markers during 8-hours forest exposure while most of the previous research assesses the short-time exposures effects (from 15 minutes to 4 hours) [1]. In this sense, we reported: A) a decrease in cortisol saliva concentrations during the 8-hours exposure significant from the second hour until the end; B) a significant increase of sAA activity during the first hour of exposure and the stability of it during the rest of the study; C) a significant decrease of IgA from the fourth hour of exposure. Largely, the significant differences we found between sampling times show that the effect of forest exposure lasted for the 8 hours exposure. This is important to note since, for example, cortisol concentrations can rebound after a stimuli [74]. Thus, our results follow the findings of this field of study where no concentration rebounds have been reported so far. Complementarily, our results may explain why previous research analysing short-time forest exposure showed no significant results [12,64–66].

Overall, our results indicate that forest exposure can produce changes in salivary human stress markers patterns with decreases in cortisol and IgA and increases in sAA. However, the confounding factors that may influence these stress-mitigating effects need to be taken into serious consideration, even though forests might constitute a habitat with less environment-derived stressors. The information reported in Chapter 4 could provide essential knowledge especially for the healthcare community in clinical practice guidelines and preventive medicine, particularly in the field of stress management and its derived pathologies.

7.2. Main constraints

Science, and particularly research design, implies making choices to achieve some advances while at the same time facing constraints. This section presents the different boundaries of the thesis. Rather than listing again all the limitations from each section, here we present a synthesis of the most conspicuous drawbacks from a global standpoint.

First of all, one of the main limitations that this dissertation and the topic face is the fact that most of the research has been developed in Asian-speaking countries, which may constrain the transferability of the results [28]. In addition, and perhaps due to this language-related point, there is also a lack of well-defined and integrated terminology in the forest and human health field as also identified by this thesis and other authors [28,75]. This clearly drowns the availability of the ongoing studies and the reported findings and limits the knowledge at many steps of the research; mainly in the research design and the results discussion.

The lack of forest descriptions and high heterogeneity of forest variables' descriptions in the literature is also a major constraint of this dissertations, since it challenges the comparison of the findings with previous works (especially in chapter 3 and 4).

Additionally, this thesis confronts as well diverse limitations at a methodological level. Generally, one of the main challenges in science is achieving a representative sample to gain statistical power and extrapolate the results [76]. In our experimental studies (chapter 3 and 4), sample size is clearly a limiting factor as discussed within the chapters. It has been defiant to recruit people that were willing to participate in a study involving biological samples collection and to orchestrate and develop experimental studies with specialists in different fields to conduct simultaneous data and sample collections.

Finally, and linked to sample size constraints, there is one last issue that always remains on the table. Although it may sound typical and redundant, it must be stated that budget represents a crucial limitation in this kind of studies and that strongly determines sample size.

7.3. Future Research

Globally, the constraints identified underline the need of further exploration in this emerging field of research that comprehends the forest and human health links. In this section we expose the main questions that arose during the thesis and we present the diagram from the "Goals and research questions" chapter complemented with the identified gaps for future research (Figure 1).

Firstly, we appeal the urgent need to integrate descriptions and measurements of forest characteristics in studies of human health [28][75], in order to identify and define the forest elements and features involved in the forest-health equation and to step further in the characterisation of the pathways taking place when humans are in contact with forests.

As highlighted within the thesis, the forest and human health binomial can be approached from several perspectives. Here, we call for a more integrative and comprehensive research that analyses the links between these ecosystems and people from a holistic approach. Thus, we endorse the creation of inter- and multidisciplinary research groups that can build up bridges between the different disciplines involved in this field: ecology, chemistry, microbiology, clinical and human biology, epidemiology and medicine, among others. Furthermore, the integration of other stakeholders from the civil society would increase the potentiality and the transferability of the research projects.

In parallel, many questions remain still unanswered regarding the dynamics and the health effects of BVOCs and particularly monoterpenes. On one hand, how forest structure may shape forest airborne chemistry remains poorly studied. Assuming that forest management can strongly modify forest structure, and thus forest under canopy chemistry [19], further research is required to provide data for forest managers, landscape planners and policy-makers, especially when health provision objectives are emerging in forest policy agendas [3,13,14]. In this sense, future investigation should sound out the effects of forest management regimes on the resulting forest elements and features characteristics and on the particular effects on human health and well-being.

One of the main constrains in the dialogue between forest specialists and the medical community is the fact that medical research methodologies demand many resources and the healthcare community requires rigorous data to integrate new initiatives [75]. This may be hardly achieved by the environmental and forest sectors. Thus, we appeal the medical sector to invest more resources in this field of research since forest interventions may conform a valid tool for preventive medicine and its implications may have further positive impacts at a broader scale; even at economic level [77]. By doing so, samples size, long term and repeated exposure and the effects on different pathologies could be addressed.

Lastly, even though not attended in this dissertation, the interaction between forest air microbiome and humans' microbiome has been spotted [78]. Human microbiome appears to be relevant in regard to different pathologies; for example, lungs microbiome dynamics play an important role in chronic obstructive pulmonary disease (COPD). Therefore, we encourage future research to unveil the potential interactions between these two microbiome communities.

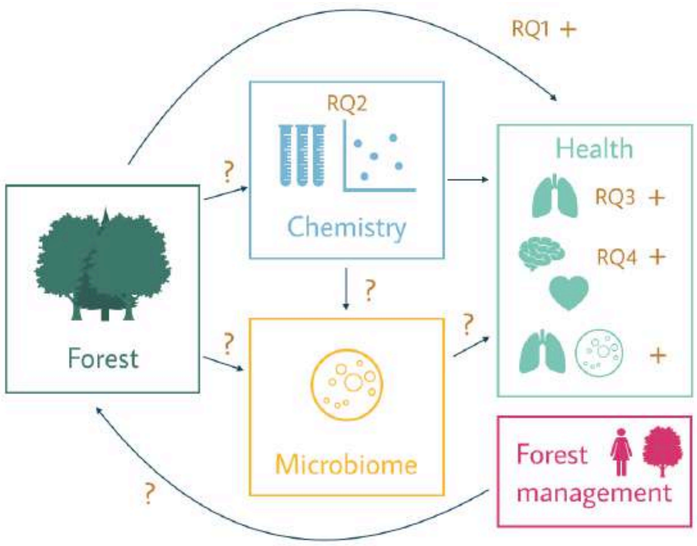

Figure 1: Gaps for future research integrated in the "Goals and research questions" chapter diagram. "?" refers to unexplored links while "+" calls for further research to complement the current data available.

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Chapter 8

Conclusions in brief

8. Conclusions in brief

Overall, this thesis has analysed the interactions between two highly complex systems: forests and human health. We have approached these connections from different perspectives although there are more elements and pathways to be considered and that may explain, all together, the global effects that we generally experience when in contact with forests. Below we present the general conclusions of this PhD dissertation:

- There is a lack of forest descriptions and a high heterogeneity of forest variables' descriptions among the current body of literature.

- No consistent relationships between forest type and health variables (blood pressure, pulse rate and cortisol levels) could be identified from the existing literature.

- We identified a strong variability of the total air monoterpene concentrations in season and daytime, with their peak during summer in a Mediterranean holm oak forest.

- Within the identified summer monoterpene peak, two main peaks at early morning (from 6:00 to 8:00) and early afternoon (from 13:00 to 18:00) were observed.

- Monoterpene air concentrations correlated with air temperature, solar radiation, air humidity and vapor pressure deficit (vpd).

- The monoterpene air concentrations we report in the Mediterranean holm oak forest are comparable or greater than in previous *in situ* studies that identified similar health outcomes to the ones observed in the *in vitro* and *ex situ* experiments.

- Our findings suggest that the Mediterranean holm oak forests constitute a suitable forest environment to develop further research on the effects of monoterpenes on human health.

- No significant changes in monoterpene blood concentrations after forest exposure could be found for the city (control) and the forest (treatment) group.

- Higher absorptions of alpha-pinene were found in the forest group participants with lower baseline monoterpene blood concentrations.

- Although significant correlations between the vital variables and the monoterpene absorption could not be spotted, we found significant correlations between the absorption of the different monoterpene compounds.

- The human stress markers showed different evolutions among the 8-hour forest exposure: A) decrease in cortisol saliva concentrations during the 8-hours exposure significant from the second hour until the end; B) significant increase of sAA activity during the first hour exposure that remained during the rest of the study; C) significant decrease of IgA from the fourth hour of exposure.

- Forests can produce changes in salivary human stress markers patterns supporting the physiological (cortisol) and psychological (IgA) relaxing effect of forest exposure.