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Noise and diabetes: A morpho-functional and epidemiological study

Gonçalo Martins Pereira

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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

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NOISE AND DIABETES: A MORPHO-FUNCTIONAL AND EPIDEMIOLOGICAL STUDY

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To Beatriz and Tomás, and all my family

“One day mankind will have to fight the burden of noise as relentless as the pest and cholera”

Robert Koch, 1910

List of articles included in this thesis

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ABSTRACT

Introduction: Noise is a ubiquitous environmental and occupational risk factor known to cause hearing loss. However, exposure to this acoustic stimulus can also induce non-auditory systemic lesions. There is increasing evidence from longitudinal cohort studies in a limited number of countries that exposure to various types of noise is an independent risk factor for type 2 diabetes. In the sound spectrum, it is unknown whether high-intensity infrasound, which can affect internal systems and organs through body vibrations, induce changes in glucose metabolism, and, if they do, what are the pathophysiological mechanisms involved. On the other hand, and similarly to what has been done in other European cities, it is important to assess whether there is an association between type 2 diabetes and exposure to urban noise in the city of Lisbon, a city that has both a high prevalence of diabetes and high levels of noise levels higher than recommended.

Objectives: This thesis aimed to study the morphophysiological effects of high intensity infrasound exposure on glucose metabolism, hypothalamic-pituitary-adrenal endocrine axis, and hepatic lipid content in the Wistar rat animal model and study if an association exists between urban noise exposure and prevalence of type 2 diabetes, obesity, and hypertension in the city of Lisbon, Portugal.

Material and Methods: Both an experimental and an epidemiological study were designed. For the experimental morphophysiological study, 56 normal and glucose intolerant wild-type Wistar rats were randomly divided in two groups: one group not exposed and the other continuously exposed to high-intensity infrasound. Animals were sacrificed at three timepoints of exposure (1, 6 or 12 weeks). An intraperitoneal glucose tolerance test was performed, blood samples were collected and the pancreas, liver, and quadriceps femoris muscle were excised. Circulating insulin and corticosterone levels were determined, hepatic lipids extracted, and pancreatic and muscular tissue were routinely processed for histochemistry and immunohistochemistry with an anti-GLUT4 antibody, respectively. For the epidemiological study, data was retrieved from the 2008 Lisbon noise map regarding daytime, evening, and nighttime noise emission levels for each street in the city. After allocation of all roads to the respective parish of Lisbon, the noise emission for each parish was averaged for each day period. Prevalence of adults with type 2 diabetes, obesity, and hypertension in 2014, 2015 and 2016 in each parish of Lisbon was obtained from the Regional Health Administration of Lisbon and Tagus Valley. Prevalence as a percentage of population was determined using the number of residents in each parish determined in the 2011 and 2021 population census. ANOVA and Spearman's non-parametric correlation

coefficient were used in the experimental and epidemiological study, respectively, at the 5% significance level (α=0.05).

Results: Animals exposed to high-intensity infrasound had higher corticosterone levels than animals not exposed (p=0.043). Rats not exposed to high-intensity infrasound presented higher glucose area under the curve (AUC) than exposed animals (p=0.030). No differences were found on insulin concerning high-intensity infrasound exposure (p=0.531) or glucose intolerance (p=0.518). Glucose intolerant animals had higher pancreatic islet fibrosis ratio (p=0.007), after removing the effects of exposure duration. Regarding insulin-regulated glucose transporter GLUT4 in muscle, no differences were found in GLUT4 ratio concerning HII exposure (p=0.506). Statistical analysis did not show effects on hepatic lipid content due to high-intensity infrasound exposure (p=0.407) or glucose tolerance status (p=0.938). Concerning the epidemiological study, no correlations were found between daytime, afternoon or night-time noise exposure and the prevalence of type 2 diabetes mellitus, obesity, or hypertension, although correlations were found between the studied cardiometabolic diseases.

Conclusions: Continuous exposure to high-intensity infrasound increases corticosterone levels, thus being a risk factor for type 2 diabetes. Although continuous exposure to high-intensity infrasound did not induce short-term glucose intolerance, alteration of plasma insulin or peripheral insulin sensitivity through GLUT4 transporter, or changes in liver lipid content of normal and glucose-intolerant animals, further studies are needed with a longer period of noise exposure to assess these and other elements of type 2 diabetes pathophysiology. Although no association was found between exposure to diurnal, evening, and nocturnal noise exposure and the high prevalence of type 2 diabetes mellitus, obesity, and hypertension in the noisy city of Lisbon, further prospective cohort studies are needed due to the known impact of noise exposure on cardiometabolic disease risk.

RESUMO

Introdução: O ruído é um fator de risco ambiental e ocupacional ubíquo conhecido por causar hipoacusia. Contudo, a exposição a este estímulo acústico pode também induzir lesões sistémicas não-auditivas. Há evidência crescente, de estudos de coorte longitudinais num número limitado de países, que a exposição a vários tipos de ruído é um fator de risco independente para diabetes tipo 2. No espectro sonoro, desconhece-se se os infrassons de alta intensidade, que podem afetar sistemas e órgãos internos através de vibrações corporais, induzem alterações no metabolismo da glicose, e, caso o façam, quais os mecanismos fisiopatológicos envolvidos. Por outro lado, e à semelhança do que tem sido feito noutras cidades europeias, importa avaliar se existe uma associação entre a diabetes tipo 2 e a exposição ao ruído urbano na cidade de Lisboa, uma cidade que tem simultaneamente uma elevada prevalência de diabetes e níveis de ruído superiores ao recomendado.

Objetivos: Estudar os efeitos da exposição a infrassons de alta intensidade no metabolismo da glicose, no eixo hipotálamo-hipófise-adrenal e no conteúdo lipídico do fígado, num modelo animal com rato Wistar, e estudar a relação entre exposição ao ruído urbano e prevalência de diabetes tipo 2, obesidade e hipertensão arterial, na cidade de Lisboa, em Portugal.

Materiais e Métodos: Para o estudo animal morfo-fisiológico experimental, 56 ratos Wistar normais e intolerantes à glicose foram divididos aleatoriamente em dois grupos iguais: um grupo continuamente exposto a infrassom de alta intensidade e outro não exposto. Os animais foram sacrificados em três pontos temporais de exposição (1, 6 ou 12 semanas). Foram realizados testes de tolerância à glicose intraperitoneal e recolhidas amostras de sangue para determinação da concentração de insulina e corticosterona. O fígado, pâncreas, e músculo quadricípite femoral foram excisados. Os lípidos hepáticos foram quantificados, o tecido pancreático foi processado para avaliação histoquímica e o tecido muscular foi processado para avaliação imunohistoquímica, com anticorpo anti-GLUT4. Para o estudo epidemiológico, foram obtidos a partir do mapa de ruído de 2008 os níveis de emissão de ruído diurno, vespertino e noturno para cada rua da cidade de Lisboa. Após atribuição de todas as estradas à respetiva freguesia de Lisboa, procedeu-se ao cálculo da média das emissões sonoras de cada freguesia para cada período do dia. A prevalência de adultos com diabetes tipo 2, obesidade e hipertensão em 2014, 2015 e 2016 em cada freguesia de Lisboa foi obtida junto da Administração Regional de Saúde de Lisboa e Vale do Tejo. A prevalência calculada em percentagem da população foi determinada a partir do número de residentes de cada freguesia apurado nos censos populacionais de 2011 e

2021. A ANOVA, ao nível de significância de 5% (α=0,05), foi usada no estudo experimental e o coeficiente de correlação não paramétrico de Spearman no estudo epidemiológico.

Resultados: Os animais expostos ao infrassom de alta intensidade apresentaram níveis de corticosterona mais elevados do que os animais não expostos (p=0,043). Ratos não expostos ao infrassom de alta intensidade apresentaram maior área sob a curva de glicose do que os animais expostos (p=0,030). Não foram encontradas diferenças na concentração de insulina quanto à exposição a infrassom de alta intensidade (p=0,531) ou intolerância à glicose (p=0,518). Animais intolerantes à glicose apresentaram maior rácio de fibrose dos ilhéus pancreáticos (p=0,007), após a remoção dos efeitos do tempo de exposição. Em relação ao transportador de glicose regulado por insulina GLUT4 no músculo, não foram encontradas diferenças no rácio de GLUT4 em relação à exposição ao infrassom de alta intensidade (p=0,506). A análise estatística não mostrou efeitos sobre o conteúdo de lípidos hepáticos devido à exposição ao infrassom de alta intensidade (p=0,407) ou ao estado de tolerância à glicose (p=0,938). Em relação ao estudo epidemiológico, não foram encontradas correlações entre a exposição ao ruído diurno, vespertino ou noturno e a prevalência de diabetes mellitus tipo 2, obesidade ou hipertensão, embora tenham sido encontradas correlações entre as doenças cardiometabólicas estudadas.

Conclusões: A exposição contínua ao infrassom de alta intensidade aumenta os níveis de corticosterona, sendo assim um fator de risco para diabetes tipo 2. Embora a exposição contínua ao infrassom de alta intensidade não tenha induzido a curto prazo intolerância à glicose, alteração da insulina plasmática ou da sensibilidade periférica à insulina através do transportador GLUT4, ou alterações no conteúdo lipídico do fígado de animais normais e intolerantes à glicose, são necessários mais estudos com um período mais longo de exposição ao ruído para avaliar esses e outros elementos da fisiopatologia da diabetes tipo 2. Embora não tenha sido encontrada associação entre a exposição ao ruído diurno, vespertino e noturno e a alta prevalência de diabetes mellitus tipo 2, obesidade e hipertensão na barulhenta cidade de Lisboa, são necessários mais estudos de coorte prospetivos devido ao conhecido impacto da exposição a ruído no risco de doença cardiometabólica.

1. INTRODUCTION

INTRODUCTION

1.1. Basic acoustics

Acoustics is the branch of physics dedicated to the study of sound and related phenomena [1]. Sound is generated when a medium (whether gas, liquid or solid) is disturbed by particle motion, creating pressure variations in such medium [1,2]. From a physical perspective, sound and noise are the same entity, differing on the negative subjective-psychological reaction by the listener [3].

Sound energy radiates from a fixed source with a similar pressure pattern, but in a three-dimensional fashion. This pattern is essentially characterized by three mathematically interdependent parameters – frequency, wavelength, and wave speed [1,2].

Sound frequency is defined as the number of pressure variations per second, most denoted as Hertz (Hz). The frequency of a particular sound, together with the wave speed, makes it possible to calculate the distance between two contiguous pressure peaks, defined as wavelength, using the formula depicted below [1,2]:

$$\text{Wavelenght } (\lambda) = \frac{\text{Speed of sound (meters)}}{Frequency\ (Hz)}$$

The human ear generally hears the frequencies ranging between 20 and 20.000 Hz, although with varying sensitivity in this range. Sounds with frequency below 200 Hz are categorized as low-frequency sounds and infrasound, and frequencies above 20.000 Hz are labelled as ultrasound [4,5].

The incremental variation above and below atmospheric pressure is called sound pressure and is measured in units of Pascal (Pa). Since measuring sound in Pa is complex, a logarithmic scale was adopted, using the Decibel (dB) as a unit of sound pressure level. The hearing threshold of 20 μPa is used as the reference level and is defined as 0 dB [1,2].

Since the human ear is not equally sensitive to all sound frequencies, several weighting scales have adjusted sound pressure levels with frequency in accordance with sensitivity scales. These weighting scales are mathematical functions used to emphasize frequencies where animals (human and non-human) are more sensitive and de-emphasize frequencies where animals are less sensitive [6].

Although several weighting scales exist, the A-weighting scale (expressed as dBA) is the most widely used in environmental studies because it matches the human hearing frequency sensitivity [6]. Like the human ear, dBA values discount low-frequency sounds so these cannot be correctly evaluated using the conventional A-weighting scale and are, therefore, often misrepresented in these studies [4,5]. To better estimate the amount of low-frequency energy in a signal, the C-weighting scale (expressed as dBC) is more useful [1].

Traveling away from its source, a sound wave is affected by atmospheric, topographic, and ground conditions, both outdoors and indoors. These changes are expressed as reflection, refraction, diffraction, and diffusion [1,2].

Reflection occurs when a sound wave encounters a sharp discontinuity in medium density and reflects at the interface between the medium changes. Unlike reflection, refraction involves changing the course of a sound wave into the new medium condition rather than returning to the incident medium. Diffraction occurs when a sound wave encounters a barrier that causes to sound wave to bend and thus to lose sound energy. Finally, diffusion refers to the even distribution of sound energy after a sound wave reflects off an uneven or convex reflective surface [1,2].

Indoor environments present unique acoustic phenomena such as echoes, room modes (room resonance) and reverberation [1]. Echoes occur due to the reflection of a sound wave on a distant surface, causing the original sound wave and the reflected sound wave to be heard separately. Reverberation is the reflection of sound waves in non-parallel surfaces, creating a sound environment with relatively constant sound level due to the superposition of such echoes. Contrary to reverberation, room resonance occurs between parallel reflective surfaces producing different sound pressure levels at different locations of the room [1,2].

1.2. Low-frequency noise and infrasound

Low-frequency noise and infrasound are defined as sounds with frequencies below 200 Hz and 20 Hz, respectively [7,8].

There are several ubiquitous sources of low-frequency noise and infrasound in the environment. Some result of natural phenomenon such as wind, ocean waves, vulcanic eruptions or earthquakes. Other sources are anthropogenic such as industrial installations, low-speed machinery, motor vehicles, and wind turbines [9].

There is a common misconception about the inaudibility of infrasound since sounds with lower frequencies can still be heard with a sufficiently high sound pressure level [9], as shown in Figure 1.

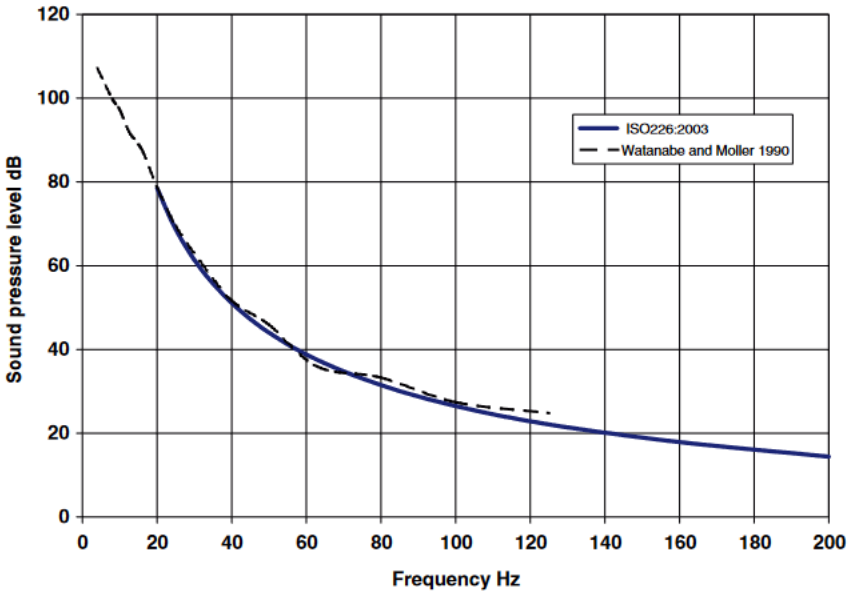

Figure 1. Hearing threshold levels in the infrasonic and low-frequency range [9]

Hearing range differs among animals. Studies comparing the auditory sensitivity of different animal species in the low-frequency and infrasonic range have shown broad differences between them. For example, rats have shown poorer infrasonic hearing than humans, considering different sound pressure levels [10,11].

Due to their long wavelength, infrasonic frequencies can propagate over large distances without reflection by obstacles. Also, they are hardly attenuated through dissipation, which refers to the transformation of kinetic energy into thermal energy and can elicit body vibrations and resonance in body cavities with higher sound intensities [1,7,9], thus affecting internal systems and organs. Like humans, animals such as rats can perceive also high-intensity (≥110 dB) infrasound as vibrations [12].

1.3. Low-frequency noise and infrasound effects on health

Noise pollution has been increasing all over the world, mainly in urban environments, mainly due to sources such as road, rail, and air traffic [13-15]. Several studies have shown that noise pollution causes adverse effects on human health beyond the auditory system [16].

In Europe, noise was estimated the third environmental risk factor with major impact on public health [17].

Among the sound spectrum, there has been a growing interest on the health effects of high-intensity infrasound (frequency <20 Hz and a sound pressure level >90 dB) exposure in the general population [18]. Sounds with a low frequency component are nowadays much more prevalent, especially due to the expansion of technology all over the world. The human ear's low sensitivity to low frequencies together with the lack of regulation for sounds in this frequency range can make us more vulnerable to its deleterious effects [1]. The World Health Organization (WHO) Regional Office for Europe has acknowledged that low-frequency noise (which includes high-intensity infrasound) may represent an environmental problem, and that research should focus on its outcomes due to the low quantity and heterogeneous nature of the existing studies [19].

In humans, exposure to acoustical environments rich in high-intensity infrasound (such as the ones present in industrial settings) has been shown to cause extra-auditory effects such as sleep disturbance, annoyance, psychological stress, and cardiovascular disease, including ischemic cardiomyopathy, heart failure, hypertension, arrythmia and stroke [20-22]. To our knowledge, no studies have found an association between exposure to high-intensity infrasound and type 2 diabetes. Nevertheless, longitudinal cohort studies have found that urban transportation noise exposure (which includes high-intensity infrasound, among other sound frequencies) increases the risk of type 2 diabetes [16].

Most human studies focus on environmental and wind turbine sources of high-intensity infrasound. These studies show that nighttime exposure to this acoustic element impacts sleep, decreasing deep and continuous sleep and leading to morning tiredness. Besides its metabolic burden, this impact on sleep increases the prescription of sedatives and antidepressants, especially in older adults [21-22].

Exposure to high-intensity infrasound is also associated with higher levels of annoyance, particularly in individuals with certain personality traits, which leads to higher blood pressure and a higher risk of myocardial infarction and stroke [21-22].

However, due to the limited number of existing studies with small samples and different outcome measurement, there is an overall moderate quality of evidence, reinforcing the need for further studies [18], including experimental studies using animal models [23,24]. This need also arises from the lack of understanding of the pathophysiological processes relating exposure and outcome.

Experimental studies in rats exposed to low-frequency noise, which includes high-intensity infrasound, showed endocrine alterations such as cytological changes in adrenal glands, suggestive of increased steroidogenic activity [25].

On the cardiovascular system, exposure to low-frequency noise induces myocardial dysfunction [26], ultrastructural alterations and decrease in cardiac connexins in both the atria and ventricles [27,28]. Long-term exposure also leads to focal thickening of the intima, disruption of the internal elastic lamina and a proliferation of smooth muscle cells in the intima of major arterial vessels, such as the aorta and femoral artery [29]. Concerning lymphatic vessels, long-term exposure to low-frequency noise induces thickening of the vessel wall, with severe lumen dilation and disruption of the valvular apparatus [30].

Other experimental findings include impaired hippocampus-dependent learning and memory [31] and increased rates of chromosomal aberrations in the bone marrow cells and elevated content of low-molecular-weight DNA in blood plasma [32].

On the respiratory system, loss of tracheal and bronchial ciliated cells [33,34] and a decrease in pleural microvilli [35] has been described because of low-frequency noise exposure.

Rats exposed to low-frequency noise also exhibit changes in the digestive system such as disruption of salivary glands acinar structure with quantitative and qualitative alterations in saliva [36], increased tooth wear [37], odontoblast lesion and formation of reparative tertiary dentin [38], morphologic alterations in the periodontium [39], superficial erosions in gastric mucosa [40] and destruction of microvilli and superficial erosions in duodenal mucosa [41].

A consistent report in experimental studies has been the non-inflammatory proliferation of collagen in blood vessel walls, myocardium, liver, digestive tract, trachea, lungs, and serous membranes [42].

1.3.1. Pathophysiological mechanisms

Although the mechanisms by which high-intensity infrasonic noise affects biological systems remain unclear, there are currently two main proposed models. One model focuses on the annoyance induced by exposure to this acoustic aggressor and the sustained reactive neuroendocrine stress response [43] while the other focuses on the body vibrations and resonance of internal organs induced by high-intensity infrasound, leading to physical disruption of tissues and organs [44]. Whether both models act in an independent, simultaneous, or synergic manner is still unknown.

The first model considers the role of high-intensity infrasound as a physical and psychological stressor, triggering the neuroendocrine sympathetic–adrenomedullary and hypothalamic–pituitary–adrenal axes. This results in an increase of glucocorticoids and catecholamines which have a hyperglycemic effect [45].

Chronic exposure to glucocorticoids negatively impacts pancreatic endocrine functions and peripheral insulin sensitivity, leading to weight gain, fat redistribution, increased free fatty acid circulation, decreased muscle mass, and increased gluconeogenesis and endogenous glucose production [46,47]. These changes lead to progressive insulin resistance in the main insulin-sensitive tissues, namely adipose tissue, skeletal muscle, and liver, and thus to chronic hyperglycemia [46,47], which are the foundations of type 2 diabetes pathophysiology [48].

These negative effects have an individual variability since noise sensitive individuals seem to be more susceptible than non-sensitive individuals to the adverse effects of noise exposure [49]. This finding, termed noise sensitivity, describes physiological and psychological internal states, which increase the degree of reactivity to noise [50].

Considering the second model of infrasonic noise-induced biological damage, several authors suggest that the mechanical stimulus provided by body vibrations produced by high-intensity infrasound disturbs mechanical homeostasis in normal tissue architecture and function [42].

These mechanical disturbances are transmitted to the plasma membrane or the interface between the cytoskeleton and cell-matrix adhesions of endothelial, epithelial, and interstitial mesenchymal cells such as organ-specific fibroblasts [51]. Following a biochemical pathway, this transmission activates several intracellular signaling pathways through mechanosensitive ion channels and G-protein coupled receptors [52]. The mechanical disturbances can also be directly transmitted to the nuclear membrane through the cytoskeleton (mechanical pathway), resulting in transcriptional changes [53]. The combination of the biochemical and mechanical pathways transiently or persistently alters cellular repair responses resulting in extracellular matrix remodeling and tissue fibrosis [51]. This mechanism, named mechanotransduction, is thought to function as a mechanical stabilizer of the organ [54].

Although previous studies have shown that fibrosis due to infrasonic noise mechanotransduction occurs without inflammatory signs, other authors found that fibrosis is decreased with corticosteroid administration, raising the possibility of an underlying inflammatory mechanism [55].

1.4. Diabetes Mellitus

Diabetes mellitus, usually abbreviated as diabetes, is a chronic heterogenous metabolic disorder with a complex pathogenesis, characterized by elevated blood glucose levels or hyperglycemia, resulting from abnormalities in either insulin secretion or insulin action, or both [56].

Diabetes can be classified into four general categories [57]: Type 1 diabetes, caused by autoimmune beta-cell destruction, leading to absolute insulin deficiency; Type 2 diabetes, due to an inadequate beta-cell insulin secretion (relative insulin deficiency) and insulin resistance; Gestational diabetes mellitus, diabetes diagnosed in the second or third trimester of pregnancy that was not present prior to gestation; and specific types of diabetes due to other causes, such as monogenic diabetes syndromes, diseases of the exocrine pancreas, and drug- or chemical-induced diabetes.

Type 2 diabetes accounts for 90–95% of all diabetes [57]. In recent years type 2 diabetes has become a major public health threat worldwide and continues to increase in numbers and significance across all regions of the world [58].

Since 1990, the global burden of diabetes has increased significantly reaching a global incidence, prevalence, and disability-adjusted life-years (DALYs) of 22.9 million, 476.0 million, and 67.9 million in 2017, with a projection to 26.6 million, 570.9 million, and 79.3 million in 2025, respectively [59]. Further estimates expect a continuous prevalence increase by 2045, with 693 million people living with diabetes worldwide [60]. More worrisome is perhaps the estimate that almost half of all people (49.7%) who live with diabetes will still be undiagnosed [60].

In Portugal, the first National Health Examination Survey (INSEF 2015) determined that the overall national prevalence of diabetes was 9.9%, a number higher than the global and European estimates [61]. Also, the estimate for undiagnosed diabetes in Portugal is as high as 43.6% [62].

These epidemiological trends are accompanied by a substantial global and national economic burden, with rising direct and indirect costs [63,64].

1.4.1. Pathophysiology and risk factors

Type 2 diabetes is a complex and heterogenous disease characterized by deficient insulin secretion by pancreatic islet beta-cells in the context of impaired insulin sensitivity, named insulin resistance [65,66].

The first element in the pathophysiology of type 2 diabetes is insulin resistance in the three main extra-pancreatic insulin-sensitive organs: liver, skeletal muscle, and adipose tissue [65,66]. Some authors believe that abnormal insulin sensitivity may precede the clinical diagnosis of type 2 diabetes by up to 15 years [67].

Skeletal muscle insulin resistance results from impaired downstream insulin signaling pathways [68], mainly in the so-called Akt isoform-AS160-GLUT4 axis [69]. Insulin resistance may stem from a deregulated insulin receptor substrate 1 (IRS1)-phosphoinositide-3-kinase (PI3K)-AKT isoform, a deregulated AS160 expression or phosphorylation leading to an improper dissociation from glucose transporter storage vesicles or an inability to bind to their 14-3-3 proteins, thus not allowing it to execute its function [69]. All these lead to an improper GLUT4 translocation and aberrant glucose uptake, as well as a defective glycogen synthesis [68-70].

As such, insulin-responsive glucose transporter type 4 (GLUT4) protein plays an important part in skeletal muscle insulin resistance and whole-body glucose metabolism since it is the most abundant glucose transporter in skeletal muscle and adipocytes [71,72].

Adipose insulin resistance results from a diminished insulin receptor tyrosine kinase activity that also leads to an impaired GLUT4 translocation to the cellular membrane and promotes the activation of lipolytic enzymes. These changes increase lipolysis, decrease glucose uptake, and enhances free fatty acid release into plasma [68].

Insulin resistance in liver is traceable to defects at the level of the insulin receptor and therefore affecting all subsequent hepatocellular insulin signaling pathways, leading to impaired glycogen synthesis and increased glucose production, lipogenesis (causing liver steatosis) and proinflammatory protein synthesis [68].

To counter the impaired insulin sensitivity, an increase in insulin production and secretion (hyperinsulinemia) occurs as an effective compensatory mechanism to preserve insulin action in mild and moderate insulin resistance (euglycemic hyperinsulinemia) [67].

However, a progressive decline of the beta-cell function and mass occurs over time. There are several proposed mechanisms for beta-cell loss and dysfunction (Figure 2): reduced beta-cell number, due to a defective proliferation and increased beta-cell apoptosis; beta-cell exhaustion, arising from endoplasmic reticulum and oxidative stress, due to an increased insulin production; and compromised beta-cell identity, by dedifferentiation (beta-cells reverting to a more immature state, similar to earlier stages in their normal development) and transdifferentiation (conversion of beta-cells into other islet cell types, such as delta-cells or glucagon-expressing alpha-cells) [73,74].

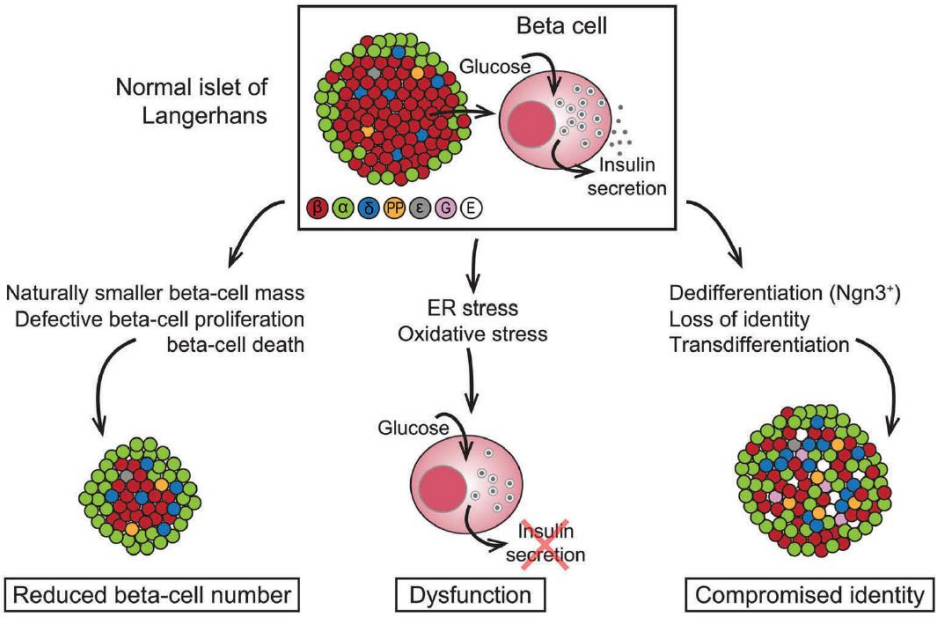

Figure 2: Models for beta-cell failure in type 2 diabetes [74]

Reductions in both beta-cell mass and beta-cell function progressively results in reduced insulin release, which becomes insufficient for maintaining normal glucose levels (hyperglycemic hyperinsulinemia), marking the clinical and laboratory signs of type 2 diabetes mellitus [57,67]. Following the natural history of type 2 diabetes, beta cells are eventually unable to secrete enough insulin, resulting in overt hyperglycemia (hyperglycemic hypoinsulinemia) [67].

Also contributing to type 2 diabetes pathophysiology, insulin resistance in kidneys leads to renal gluconeogenesis and renal glucose reabsorption, both of which promote hyperglycemia [75]. Recent studies have also shown a complex crosstalk between beta-cells and gastrointestinal hormones (both incretins and decretins), gut microbiota, and nervous system [76]. The gut microbiome has been investigated in type 2 diabetes and studies show that dysbiosis is strongly correlated with insulin resistance [77,78].

Several risk factors for insulin resistance, beta-cell dysfunction, and beta-cell loss have been established, such as genetic and epigenetic factors, obesity, hypercaloric diet, smoking, alcohol intake, physical inactivity, psychological stress, and aging. As such, type 2 diabetes results from a complex interaction of environmental, biological, and behavioral risk factors [79].

Due to the increased prevalence, incidence, morbidity, and mortality of type 2 diabetes worldwide [59], there has been particular attention on its environmental determinants to counteract the epidemiological trend [80]. Environmental characteristics are thought to increase exposure to risk factors of type 2 diabetes by enhancing or restricting behavioral, psychological, and physical stressors [81].

Several environmental characteristics that increase risk of type 2 diabetes have been studied over the years, with particular emphasis on noise pollution [81,82].

1.4.2. Noise as an environmental risk factor for diabetes

Systematic reviews and meta-analysis have identified an increased risk of type 2 diabetes mellitus associated with transportation noise exposure (including high-intensity infrasound, among other sound frequencies) in a time-dependent manner [81,83-85]. These systematic reviews and meta-analysis are based on large-scale cohort studies in a small number of cities in countries such as Denmark, Canada, and Switzerland which have specific social, urban, and demographic characteristics [81,83-85].

Sørensen et al. [86] found, in the population-based Danish cohort (n=57,053), that a 10-dB higher level of average road traffic noise at the time of diagnosis of diabetes type 2 and during the 5 years preceding diagnosis was associated with an increased risk of incident diabetes after adjusting for potential confounders such as age, body mass index, waist circumference, education, air pollution, and lifestyle characteristics.

In a prospective study using a German nationwide cohort, Heidemann et al. [87] found during a mean of 12.1 years of follow-up that people exposed to intense residential traffic noise had a twofold higher risk of type 2 diabetes after adjusting for potential confounders.

Clark et al. [88] found a positive association between residential transportation noise and incidence of diabetes, in a population-based prospective cohort study (n=380,738) of metropolitan Vancouver residents, remaining after adjustment for environmental co-exposures including traffic-related air pollutants.

In the Swiss Cohort Study on Air Pollution and Lung and Heart Diseases in Adults (SAPALDIA), Eze et al. [89] found in its 2631 participants that road and aircraft noise were independently associated with incident diabetes, with an estimated relative risk of 1.35 and 1.86, respectively.

Investigating the association between road traffic noise and diabetes incidence, Roswall et al. [90] found in 50,534 individuals enrolled into the Danish Diet, Cancer and Health cohort a hazard ratio of 1.08 (1.04–1.13) per 10 dB for 5-year exposure in fully adjusted models.

Ohlwein et al. [91] found in the German Heinz Nixdorf Recall study, including 3,396 participants of age 45-75 years, that a 10-dBA increase in outdoor or indoor road traffic noise was associated with a relative risk of 1.09 of developing type 2 diabetes, independent of air pollution exposure.

Studying the Ontario Population Health and Environment Cohort (n=914,607), Shin et al. [92] concluded that between 2001 and 2015, each 10 dBA increase for the 24-hour day was associated with an 8% increase in incident diabetes mellitus, with robust results to all sensitivity analyses and adjusting for traffic-related air pollutants, such as ultrafine particles and nitrogen dioxide.

To the best of our knowledge, there are no similar studies in Lisbon. In this city, the capital and largest city of Portugal, the population is exposed to high levels of noise throughout the day, originating from road traffic, due to the high volume of daily passengers entering the city, and from rail and air traffic, due to the high number of railway stations and the location of Lisbon airport on the outskirts of the municipality, just 5km from the center [19]. Due to the high prevalence and burden of type 2 diabetes in Portugal [61,62,64] there is the need to explore related environmental risk factors such as noise exposure so that mitigation strategies can be applied [93].

The epidemiological evidence linking noise exposure and type 2 diabetes is in line with findings in experimental studies. In these studies, glucose intolerance, insulin resistance, fasting hyperglycemia, dyslipidemia and alterations in insulin signaling in the skeletal muscle have been identified because of noise exposure with frequencies higher than 200 Hz [94-97]. All these findings are important components of type 2 diabetes pathophysiology [65,66].

Cui et al. [95] also referred increased levels of glycogen and triglycerides in the liver of noise-exposed rats that may lead to non-alcoholic fatty liver disease, a marker of metabolic dysfunction and risk factor for liver fibrosis, cirrhosis, and cancer [98]. This accumulation of lipids in visceral fat is a key player in metabolic derangement and an important risk factor for type 2 diabetes and metabolic syndrome [65,66].

All biological models for the pathophysiology of noise-induced metabolic disease have focused on the effects of noise as a physical and psychological stressor, triggering the neuroendocrine stress pathways that increase glucocorticoids and catecholamines and,

thus, promote hyperglycemia [99-101]. Chronic exposure to glucocorticoids and catecholamines can lead to chronic hyperglycemia and, as such, promote hyperinsulinism and insulin resistance [65,66].

In this regard, a particular focus has been given to nocturnal noise exposure. Besides the activation of the neuroendocrine stress pathways this exposure leads to a circadian desynchronization of the sleep-wake cycle and thus to consequent adverse cardiometabolic outcomes [102].

However, considering the different frequencies in the sound spectrum, whether high-intensity infrasound exposure induces these changes in glucose metabolism and liver lipid content is still unknown.

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2. OBJECTIVES

OBJECTIVES

Current experimental and observational studies point to a causal relationship between noise exposure and risk of type 2 diabetes. Experimental studies have focused on noise exposure with frequencies higher than 200 Hz, leaving the effects of high-intensity infrasound (frequency <20 Hz and sound pressure level >90 dB) exposure on glucose metabolism still unknown.

Thus, we will conduct:

1. An experimental morpho-functional study of high-intensity infrasound exposure in both glucose tolerant and intolerant Wistar rats, to further deepen our understanding of the pathophysiological mechanisms between high-intensity infrasound exposure and type 2 diabetes. In this study we will:
    1.1. Study the hypothalamic-pituitary-adrenal endocrine axis to ascertain whether stress hormones are increased with exposure to this acoustic element.
    1.2. Study the pancreatic tissue, due to its role in insulin production and response to peripheral insulin resistance, to verify whether exposure to high-intensity infrasound leads to morphological alterations in islets and changes in insulin production.
    1.3. Study the skeletal muscle and its insulin-responsive glucose transporter type 4 (GLUT4) to establish whether exposure to high-intensity infrasound leads to morphological alterations and insulin resistance, by decreasing GLUT4 quantity in muscle.
    1.4. Study if exposure to high-intensity infrasound alters the amount of lipids in the hepatic tissue since an increase (liver steatosis) is an established marker of insulin resistance.
2. A cross-sectional study in the city of Lisbon, which presents high levels of noise throughout the day, to ascertain whether urban noise in this city, rich in high-intensity infrasound, is associated with components of metabolic syndrome, such as type 2 diabetes.

3. RESULTS

3.1. HIGH-INTENSITY INFRASOUND EFFECTS ON GLUCOSE METABOLISM IN RATS

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HIGH-INTENSITY INFRASOUND EFFECTS ON GLUCOSE METABOLISM IN RATS

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Abstract

Recent focus has been given on the effects of high-intensity infrasound (HII) exposure, and whether it induces changes in pancreatic morphology and glucose metabolism is still unknown. As such, we have studied the impact of HII exposure on glucose tolerance, insulin sensitivity, pancreatic islet morphology, muscle GLUT4 and plasma insulin and corticosterone levels. Normal and glucose intolerant wild-type Wistar rats were randomly divided in two groups: one group not exposed to HII and the other continuously exposed to HII. Animals were sacrificed at three timepoints of exposure (1, 6 or 12 weeks). An intraperitoneal glucose tolerance test was performed, blood samples were collected and the pancreas and the quadriceps femoris muscle were excised. Circulating insulin and corticosterone levels were determined and pancreatic and muscular tissue were routinely processed for histochemistry and immunohistochemistry with an anti-GLUT4 antibody. Animals exposed to HII had higher corticosterone levels than animals not exposed. No differences were found on insulin concerning HII exposure or glucose intolerance. Glucose intolerant animals had pancreatic islet fibrosis and no differences were found in GLUT4 ratio concerning HII exposure. In conclusion, we found that continuous exposure to HII increases stress hormone levels without inducing glucose intolerance in rats.

Keywords: Infrasound; glucose intolerance; rat; insulin; GLUT4; corticosterone

Introduction

Noise pollution is a ubiquitous hazard, present in environmental and occupational settings, known to cause adverse effects on human health [1]. Among the sound spectrum, there has been a great interest on the health effects of high-intensity infrasound (frequency <20 Hz and a sound pressure level >90 dB) exposure in the general population [2]. Due to their long wavelength, infrasonic frequencies are hardly attenuated through dissipation and can induce body vibrations and resonance in body cavities, thus affecting internal systems and organs [3,4].

In humans, exposure to acoustical environments rich in high-intensity infrasound (such as the ones present in industrial settings) has been shown to cause extra-auditory effects such as annoyance, sleep disturbance, psychological stress, and cardiovascular disease, including ischemic cardiomyopathy, heart failure, hypertension, arrythmia and stroke [5]. Experimental studies in rats report an increase of collagen fibres without inflammatory signs in blood vessel walls, trachea, lungs, and serous membranes [6]. Other findings include impaired hippocampus-dependent learning and memory [7], loss of tracheal and bronchial ciliated cells [8,9], decrease in pleural microvilli [10], myocardial dysfunction and decrease in cardiac connexins [11,12], disruption of salivary glands acinar structure with quantitative and qualitative alterations in saliva [13] and cytological changes in adrenal glands suggestive of increased steroidogenic activity [14].

Systematic reviews and meta-analysis have identified an increased risk of type 2 diabetes mellitus associated with noise exposure in a time-dependent manner, with a stronger association for long-term exposure [15-18]. However, considering the different frequencies in the sound spectrum, whether high-intensity infrasound exposure induces changes in glucose metabolism is still unknown. For this reason, we have studied the morphophysiological impact of high-intensity infrasound exposure on glucose metabolism of normal and glucose intolerant rats. We measured glucose tolerance, insulin sensitivity, pancreatic islet morphology and fibrosis, skeletal muscle GLUT4 ratio and plasma insulin levels. We have also studied the impact of this exposure on the rat hypothalamic-pituitary-adrenal endocrine axis, a main element in stress response, by measuring plasma corticosterone levels.

Results

Effects of high-intensity infrasound on glucose tolerance, insulin sensitivity and insulin response during IPGTT in rats

Concerning glucose intolerance before high-intensity infrasound exposure, we found that G2, glucose intolerant, animals presented higher glycemia than G1, normal, animals ($p<0.001$). Baseline glycemia of the intraperitoneal glucose tolerance test, at the 2h timepoint, for G1 group had a mean value of 124 mg/dL ± 18.4 mg/dL and for G2 group had a mean value of 158 mg/dL ± 30.6 mg/dL (Table 1). Glucose AUC respective to the baseline was higher in G2, when compared to G1 ($p<0.001$), thus G2 animals were considered glucose intolerant (Figure 1B).

Table 1 – Distribution of animals per experimental groups. Mean values and standard deviation for glycemia at the intraperitoneal glucose tolerance test 2h timepoint, expressed as milligrams per deciliter (mg/dL), for each experimental group of normal (G1) and glucose intolerant (G2) animals, either kept in silence (s) or exposed to high-intensity infrasound (i).

G1 (n=28)			G2 (n=28)		
124 ± 18.4 mg/dL			158 ± 30.6 mg/dL		
G1s (n=14)	1 wk (n=4)	138 ± 13.8 mg/dL	G2s (n=14)	1 wk (n=4)	149 ± 45.5 mg/dL
	6 wks (n=5)	154 ± 20.5 mg/dL		6 wks (n=5)	155 ± 17.4 mg/dL
	12 wks (n=5)	144 ± 21.3 mg/dL		12 wks (n=5)	168 ± 25 mg/dL
G1i (n=14)	1 wk (n=4)	157 ± 27.8 mg/dL	G2i (n=14)	1 wk (n=4)	151 ± 17.6 mg/dL
	6 wks (n=5)	142 ± 20.8 mg/dL		6 wks (n=5)	152 ± 22.3 mg/dL
	12 wks (n=5)	142 ± 14.2 mg/dL		12 wks (n=5)	157 ± 11.5 mg/dL

No differences were found in final glucose AUC between G1, normal, and G2, glucose intolerant rats ($p=0.907$), after controlling for baseline glucose AUC. However, animals not exposed to high-intensity infrasound presented higher glucose AUC than exposed animals ($p=0.030$), regardless of their glucose tolerance status and after removing the effects of baseline insulinemia. Animals exposed to 1 week of high-intensity infrasound presented higher glucose AUC when compared to animals exposed to 12 weeks of high-intensity infrasound ($p=0.047$) (Figure 1).

Regarding plasma insulin levels, no differences were found on plasma insulin levels concerning infrasound exposure (p=0.531) or glucose intolerance (p=0.518). After controlling for infrasound exposure duration, no differences were found between fasting insulin levels and insulin levels 30 minutes after glucose administration (p=0.124) (Figure 2A).

Regarding glucose/insulin ratio, no differences were found concerning infrasound exposure (p=0.596) or glucose intolerance (p=0.883) (Figure 2B). Also, no differences were found on HOMA-IR regarding infrasound exposure (p=0.318) or glucose intolerance (p=0.402) (Figure 2B). Similarly, no differences were found on QUICKI regarding infrasound exposure (p=0.163) or glucose intolerance (p=0.464) (Figure 2C).

Analysis of plasma corticosterone levels in rats

Considering plasma corticosterone levels, no differences were found between glucose tolerant and glucose intolerant animals (p=0.674). However, animals exposed to high-intensity infrasound presented higher plasma corticosterone levels than animals not exposed (p=0.043) (Figure 2E).

Effects of high-intensity infrasound on insulin signaling in rat muscle

Regarding the quantity of GLUT4 transporter in skeletal muscle, G2 animals presented lower values than G1 animals (p=0.002). This finding supports the fact that G2 animals were indeed glucose intolerant, as confirmed by the baseline and subsequent intraperitoneal glucose tolerance tests. No differences were found in muscle GLUT4 ratio concerning high-intensity infrasound exposure (p=0.506) or concerning duration of high-intensity infrasound exposure (p=0.230) (Figure 2D).

Effects of high-intensity infrasound on rat pancreatic and muscle tissue morphology

The pancreatic tissue of normal animals, G1 group, both kept in silence and exposed to high-intensity infrasound, presented a regular micro-anatomy without cellular alterations throughout the established timepoints. Slight alterations and a small increase in the quantity of collagen fibers, both in the pancreatic islets and the exocrine parenchyma, was observed at the 6-week and 12-week timepoint in animals kept in silence (G1s) and animals exposed to high-intensity infrasound (G1i). This fibrosis on the endocrine and exocrine parenchyma

was considered medium degree and was more pronounced in the glucose intolerant group (Figure 3).

In glucose intolerant animals, G2 group, both kept in silence and exposed to high-intensity infrasound, the pancreas presented regular micro-anatomy, scarce collagen fibers and slight capillary congestion at the 1-week timepoint. A moderate increase in the quantity of collagen fibers in the pancreatic islets was observed in glucose intolerant animals not exposed to high-intensity infrasound (G2s) at the 6- and 12-week timepoint with a severe increase in the quantity of collagen fibers in the exocrine parenchyma at the same timepoints. Glucose intolerant animals exposed to high-intensity infrasound (G2i) had a more severe fibrosis, mainly in the periductal islets, than their counterparts kept in silence throughout the established timepoints. No cellular alterations were observed in glucose intolerant animals kept in silence and exposed to high-intensity infrasound at any timepoint (Figure 4).

Histochemical analysis of pancreatic islet fibrosis show that animals exposed to high-intensity infrasound did not present different levels of pancreatic islet fibrosis when compared to animals not exposed ($p=0.651$). However, higher pancreatic islet fibrosis was found in glucose intolerant animals, when compared to glucose tolerant animals ($p=0.007$), after removing the effects of exposure duration (Figure 2F).

Regarding skeletal muscle morphology, no differences were found between groups in different timepoints, and all animals had a regular micro-anatomy (Figure 5). Muscle fibers were organized in bundles and the nuclei were located peripherally. The sarcoplasm appeared uniform and no cellular alterations, inflammatory cell infiltration, fibrosis or necrosis were identified.

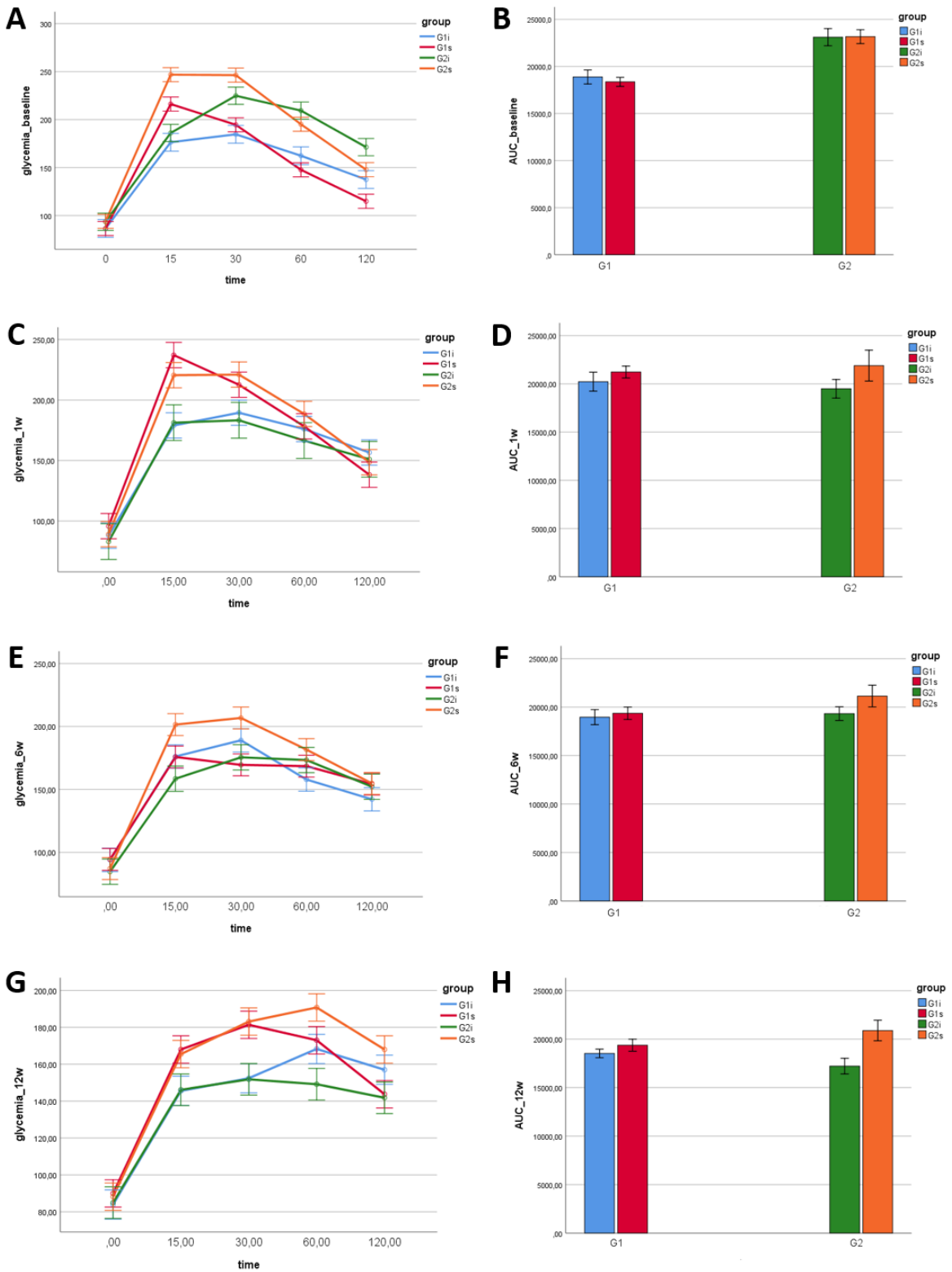

Figure 1 – Intraperitoneal glucose tolerance test curve and respective area under the curve (AUC). Means ± SE for each timepoint of the intraperitoneal glucose tolerance test curve (A, C, E, G) and the respective glucose AUC (B, D, F, H) of each experimental group of normal (G1) and glucose intolerant (G2) animals, either kept in silence (s) or exposed to high-intensity infrasound (i).

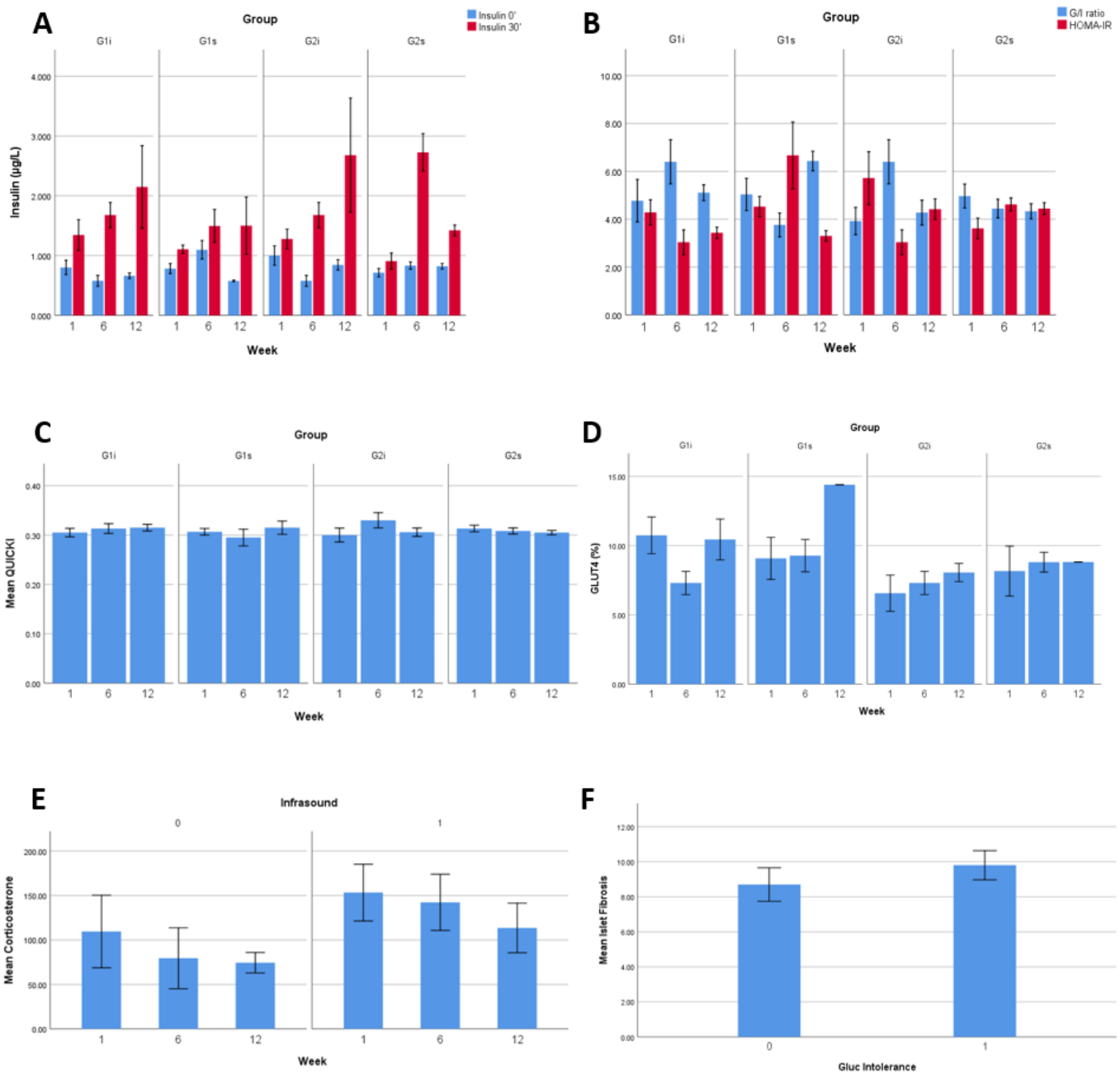

Figure 2 – Plasma insulin and corticosterone levels, glucose/insulin ratio, HOMA-IR, QUICKI, muscle GLUT4 ratio and islet fibrosis ratio. Means ± SE of plasma insulin levels (A), glucose/insulin (G/I) ratio (B), HOMA-IR (B), QUICKI (C), muscle GLUT4 ratio (D), plasma corticosterone levels (E) and pancreatic islet fibrosis ratio (F), in normal (G1) and glucose intolerant (G2) animals, either kept in silence (s) or exposed to high-intensity infrasound (i), throughout the established experimental timepoints.

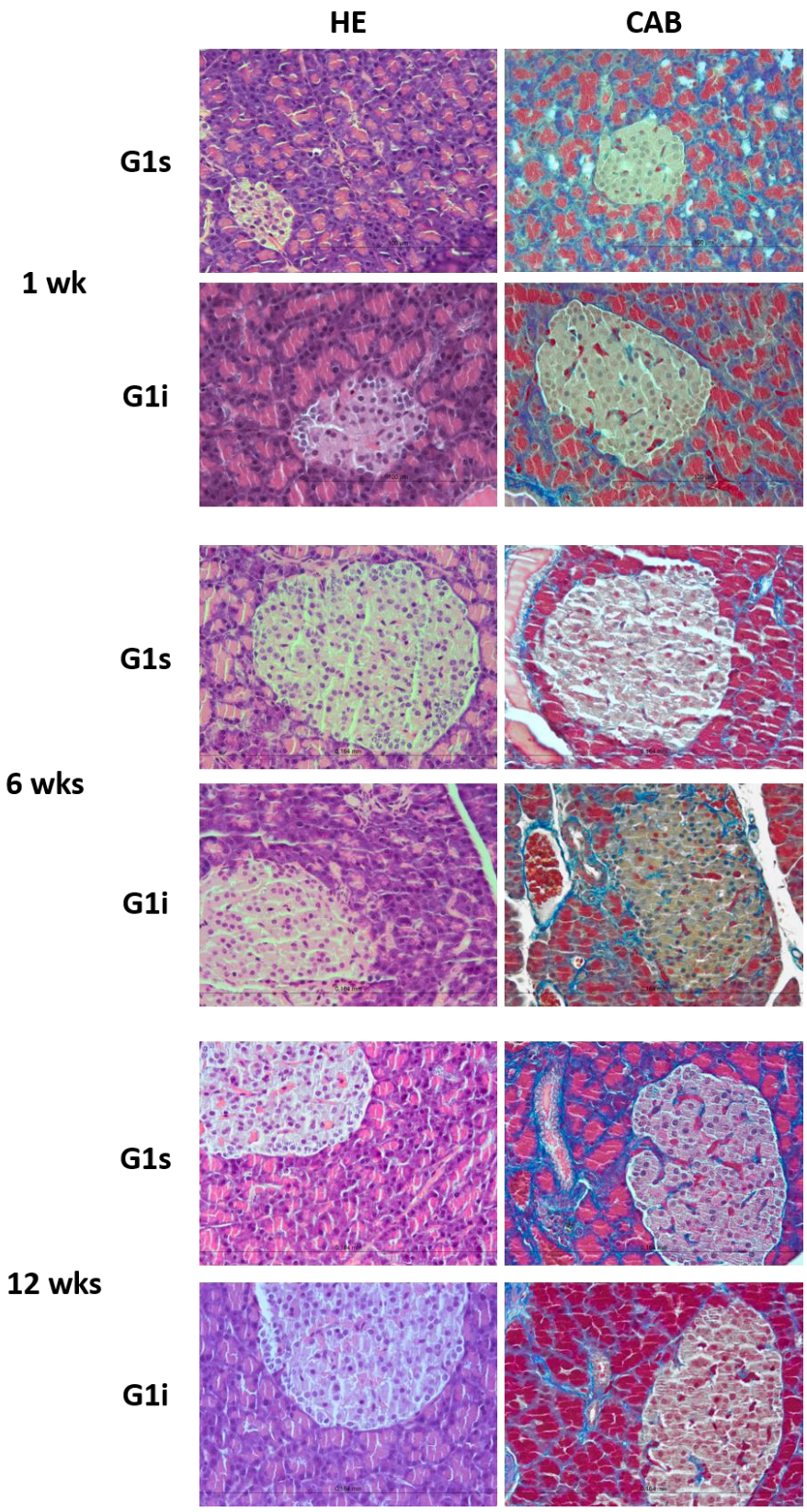

Figure 3 – Pancreatic morphology of normal rats. Representative images of pancreatic sections (40x objective), stained with hematoxylin-eosin (HE) and chromotrope aniline blue (CAB), of normal (G1) animals, either kept in silence (s) or exposed to high-intensity infrasound (i), throughout the established timepoints.

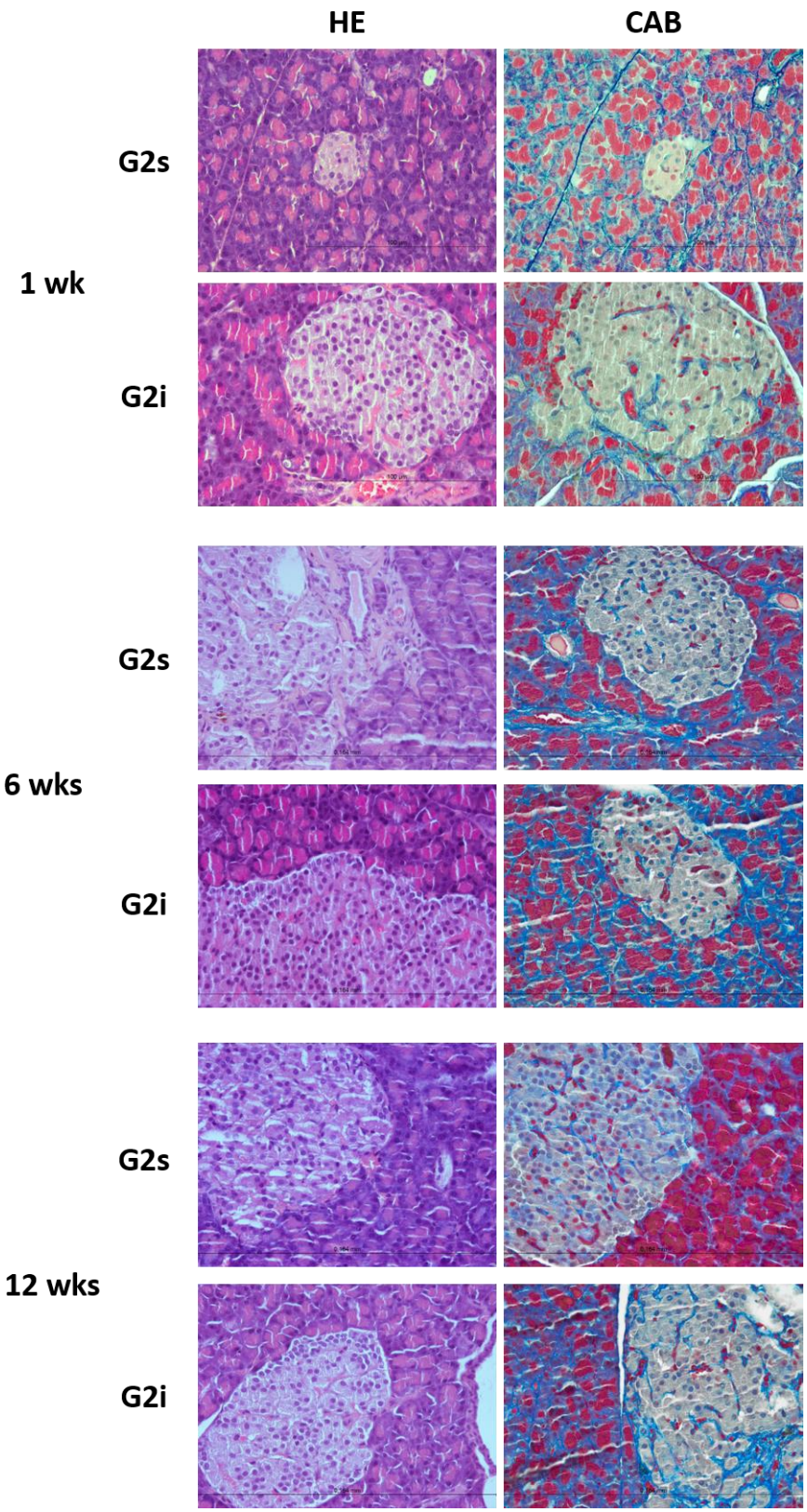

Figure 4 – Pancreatic morphology of glucose intolerant rats. Representative images of pancreatic sections (40x objective), stained with hematoxylin-eosin (HE) and chromotrope aniline blue (CAB), of glucose intolerant (G2) animals, either kept in silence (s) or exposed to high-intensity infrasound (i), throughout the established timepoints.

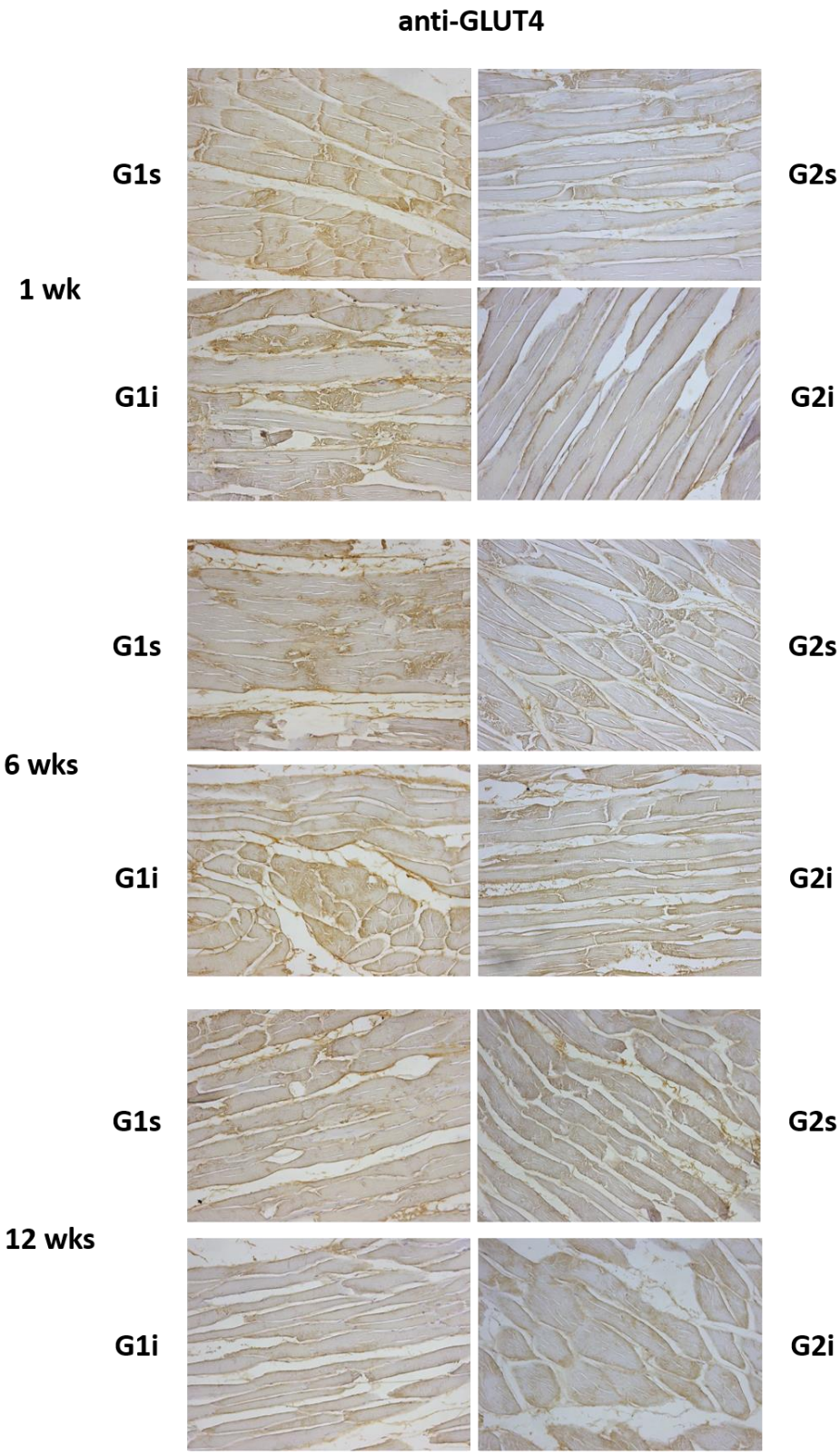

Figure 5 – Muscle morphology and anti-GLUT4 immunohistochemical analysis in rats. Representative images of quadriceps femoris muscle sections (40x objective), with immunohistochemical staining using anti-GLUT4 specific antibody, of normal (G1) and glucose intolerant (G2) animals, either kept in silence (s) or exposed to high-intensity infrasound (i), throughout the established timepoints. GLUT4 is stained brown.

Discussion

Our morphophysiological study aimed to investigate whether continuous exposure to infrasonic noise was able to induce alterations in glucose metabolism and pancreatic morphology in rats. Results showed that exposure to this environmental and occupational hazard increases plasma corticosterone levels, without inducing alterations in glucose tolerance, insulin levels or peripheral insulin sensitivity. Also, no morphological changes were observed in pancreatic and skeletal muscle tissue due to exposure to high-intensity infrasound.

The mechanisms by which infrasonic noise affects biological systems are still unclear. Currently there are two main proposed models for the systemic damage induced by high-intensity infrasound exposure. One model relates to the annoyance induced by exposure to this aggressor and the sustained reactive neuroendocrine stress response. The other relates to the mechanic effects of body vibrations and resonance of internal organs induced by high-intensity infrasound that may lead to physical disruption of tissues [6,19]. Whether both models act independently or simultaneously is still unknown.

Considering the first model, high-intensity infrasound acts as a physical and psychological stressor, triggering the neuroendocrine sympatho–adrenomedullary and hypothalamic–pituitary–adrenal axes that increase corticosterone and catecholamines and, thus, promote hyperglycemia [20]. Chronic exposure to corticosterone can lead to chronic hyperglycemia and, as such, promote hyperinsulinism and insulin resistance [21,22]. Previous studies in rats have shown that exposure to industrial noise, rich in high-intensity infrasound, decreases the volume of the adrenal zona fasciculata together with depletion of adrenal lipid droplets, both suggestive of increased stimulation of adrenal steroidogenesis of glucocorticoids [14]. In our study we have found that high-intensity infrasound increases plasma corticosterone levels, the main stress hormone in rats [23]. Although the intraperitoneal route used to assess glucose tolerance increases corticosterone levels in rats [24], since all rats were submitted to the same protocol, the stress induced by the IPGTT was similar in all experimental groups and, therefore, unlikely to influence our data. The fact that no hyperinsulinism and insulin resistance were observed as a result from exposure to high-intensity infrasound and from increased plasma corticosterone levels could point to a delayed response to this stressor and we argue that a longer period of exposure would be needed to observe changes in insulin production, insulin resistance and glucose tolerance [25], as seen in type 2 diabetes mellitus pathophysiology [26].

Animal exposure studies focusing on audible noise with higher frequencies found an increase in insulin resistance, fasting hyperglycemia, dyslipidemia, and alterations in insulin

signaling in the skeletal muscle [25,27-29]. Other studies show that exposure to high-frequency audible noise has no influence in glucose tolerance [25,28]. Our animals exposed to high-intensity infrasound had lower glucose AUC, and thus higher glucose tolerance, than their counterparts kept in silence. We interpret this finding as a reactive response, by the pancreas and/or the peripheral insulin receptors and insulin-regulated transporters, that occurs in the presence of this aggressor. In fact, compensatory increases in insulin production and pancreatic islets beta cell mass [30] have been reported as a response to several injury models, whether by enhanced function or increase in the number of beta cells by self-replication or conversion of other pancreatic islet cell types to beta cells [31]. Nevertheless, we have not found cellular alterations in the endocrine pancreas or differences on plasma insulin due to high-intensity infrasound exposure.

Concerning a possible reactive response in peripheral insulin-regulated glucose transporters, we have not found significant differences in muscle GLUT4 ratio between animals exposed to high-intensity infrasound and animals kept in silence. This finding suggests that alterations in other peripheral insulin receptors or insulin-regulated glucose transporters could be at play, namely on adipose and hepatic tissue [32]. The liver plays an important part in glucose homeostasis through insulin clearance, maintaining a homeostatic level of insulin [33]. However, the chronic hyperinsulinism that accompanies increased beta cell mass also leads to hepatic steatosis [33] and high-intensity infrasound does not appear to alter the hepatic lipid content of both normal and glucose intolerant animals [34].

Considering the direct effects of high-intensity infrasound in tissues, other exposure animal studies showed that continuous exposure results in proliferation of collagen fibers and tissue fibrosis [12,13]. Several authors suggest that the body vibrations and resonance produced by high-intensity infrasound represents a mechanical stimulus that activates intracellular signaling pathways resulting in extracellular matrix remodeling and fibrosis [6,35]. This mechanism, called mechanotransduction, may function as a mechanical stabilizer of the organ [6,35]. Oliveira et al. [13] documented perivasculo-ductal fibrosis in the rat parotid gland, as well as disruption of acinar structure, generalized vacuolization and signs of necrosis that were associated with quantitative and qualitative alterations in saliva production. Due to certain anatomical and functional similarities between the parotid gland and the pancreas [36], similar effects could also be expected due to high-intensity infrasound exposure. However, we have not observed an increase in collagen fiber quantity in pancreatic islets with exposure to this stressor. The rat pancreas presents several segments with different macroscopic appearance, from a relatively compact splenic segment to a duodenal segment dispersed within the mesentery [37]. We argue that the

deep location of the rat pancreas segments in the abdominal cavity could protect this organ from body vibration due to high-intensity infrasound and thus from tissue damage [14].

The fibrosis found in pancreatic islets of glucose intolerant animals could be either the result of glucose intolerance or the result of the glucose intolerance method used in this study. Glucose intolerance has an aggravating effect on pancreatic fibrosis caused by chronic inflammation from other pancreatic pathologies [38]. Regarding the used method, streptozotocin displays selective pancreatic beta cell toxicity and induces beta cell death [39]. This process induces tissue injury and local inflammatory response that activates pancreatic stellate cells with subsequent extracellular remodeling and deposition of collagen fibers [40,41].

Future studies should appraise the ultrastructural effects of high-intensity infrasound exposure in the pancreatic and skeletal muscle, through transmission electron microscopy [42]. Although immunohistochemistry allows the quantification of muscle GLUT4, other quantitative methods such as western blot could be of use, due to their greater reproducibility and sensibility [43]. Besides the intraperitoneal route used to determine glucose tolerance, the use of the oral route would provide information regarding the incretin effect, and thus allow a better understanding of the pancreatic insulin release [44]. A longer period of high-intensity infrasound exposure would allow us to better observe changes in glucose metabolism and refine the understanding of the pathophysiology of infrasound-induced metabolic dysfunction and type 2 diabetes mellitus [25]. Since high-intensity infrasound exposure effects on glucose metabolism are still unknown, a positive control was difficult to establish for this acoustic element. Our results will help establishing positive controls in future studies. Despite the small number of animals per group, in each of the 12 experimental groups, the number of animals in each group was sufficient to perform the adequate statistical analysis for the chosen design.

In conclusion, continuous exposure to high-intensity infrasound increases corticosterone levels, without inducing glucose intolerance and alteration of plasma insulin or peripheral insulin sensitivity through GLUT4 transporter. This environmental stimulus can act as a cofactor in metabolic dysfunction and type 2 diabetes mellitus in a time-dependent manner. These results highlight the importance of further research concerning the metabolic effects of high-intensity infrasound, due to its ubiquitous diurnal and nocturnal presence in a common daily life and its possible action in type 2 diabetes mellitus pathophysiology, whose importance is yet to be determined.

Methods

Animals

Study design and sample size estimation were carried out as described previously [34]. In short, fifty-six wild-type male Wistar rats were acquired from Charles River Laboratories (Saint-Germain-sur-l'Arbresle, France), aged 11 weeks, and with an average weight of 376 g ± 18.3 g. Only male rats were included to avoid uncertain sex-dependent differences on the outcomes. Upon arrival and throughout the entire study, the animals were kept in standard cages, exposed to a light/dark cycle of 12h and had free access to food and water. All animals passed the Preyer reflex test, a simple method to estimate auditory function [45].

After a one-week acclimation period the animals were randomly assigned using a free access online software [46] into two groups: G1 (no treatment, n=28) and G2 (glucose intolerance, n=28). Glucose intolerance was induced through a high-fat diet and the administration of low-dose streptozotocin (HFD/STZ rat model) because this model is considered to mimic the human disease [47]. G1 and G2 animals were then fed standard rat chow, to eliminate further differences between groups, and were randomly assigned into two subgroups each: G1s (no treatment, silence, n=14), G1i (no treatment, infrasound, n=14), G2s (glucose intolerance, silence, n=14) and G2i (glucose intolerance, infrasound, n=14). Animals from G1i and G2i were continuously exposed to high-intensity infrasound while G1s and G2s animals were kept in similar conditions but without high-intensity infrasound exposure. Intraperitoneal glucose tolerance tests were performed and animals euthanized in all experimental groups after 1, 6 or 12 weeks of high-intensity infrasound exposure. Animals were randomly distributed as shown in table 1.

Experimental design and planning were performed with full compliance to the PREPARE guidelines [48]. Animal procedures were approved by the Portuguese National Authority for Animal Health and the local Animal Welfare Body (project nº 204/2017). All handling and care of the animals was performed humanely and alleviating animal suffering by authorized researchers (accredited with FELASA Category C) and was done in accordance with the EU Commission on Animal Protection for Experimental and Scientific Purposes (2010/63/EU) and with the Portuguese legislation for the same purpose (DL 113/2013). The study was carried out in compliance with the ARRIVE guidelines [49].

Glucose Intolerance

Glucose intolerance was induced following the protocol by Furman [50]. Animals were fed a high-fat diet (D12492 diet, Research Diets Inc., USA) for 3 weeks. On a caloric basis, the high-fat diet consisted of 60% fats, 20% carbohydrates and 20% protein (5.21 Kcal/g energy density) whereas the standard rat chow (D10001 diet, Research Diets Inc., USA) consisted of 12% fats, 67% carbohydrates and 21% protein (3.86 Kcal/g energy density). After three weeks of high-fat diet, low-dose streptozotocin (STZ, Sigma-Aldrich, USA) 40 mg/kg was prepared in a sodium citrate buffer 50mM, pH4.4, and was administered intraperitoneally after a fasting period of 6-8 hours. Animals had unlimited access to water during fasting period.

Glucose intolerance was confirmed through an intraperitoneal glucose tolerance test following the protocol established by Ayala et al. [51]. Animals were considered glucose intolerant if they presented a plasma glucose > 140 mg/dL at the 2h timepoint of the test [51]. The results of the intraperitoneal glucose tolerance test were expressed as the baseline area under the curve (AUC) for each animal.

High-Intensity Infrasound Exposure

High-intensity infrasound exposure was performed as previously published [52]. In short, pseudo-random waveform in the 2 to 20 Hz decade band was designed with Matlab based on a bandpass-filtered 30-s maximum length sequence segment. The total sound pressure level and the spectral characteristics of the resulting acoustic pressure waveform were monitored throughout the experiment and showed an average sound pressure level of 120 dB ± 3 dB in the 30-s time window. The exposure was continuous (24h/day) to reflect the ubiquitous diurnal and nocturnal presence of high-intensity infrasound in a common daily life [53].

Intraperitoneal Glucose Tolerance Test and Plasma Insulin

At the respective timepoint and before euthanasia, an intraperitoneal glucose tolerance test was performed, following the protocol established by Ayala et al. [51]. Animals were fasted overnight for 12h and the fasting blood glucose level was determined through a sample collected from the caudal vein in a standard glucometer (Freestyle Precision, Abbott, USA). A 20% glucose solution was administered intraperitoneally (1ml/Kg) and the blood glucose was measured at 15-, 30-, 60- and 120-minutes post-injection. The glycemia recorded

during the test was used to evaluate glucose tolerance to the glucose challenge. The results of the intraperitoneal glucose tolerance test were expressed as both a time course of glycemia measurements and as the area under the curve (AUC) for each animal. The time course of glycemia measurements and the AUC was averaged for each experimental group (figure 1).

In addition to blood glucose measurements during the intraperitoneal glucose tolerance test, blood samples from the caudal vein were taken before and 30 minutes after glucose administration to assess plasma insulin levels. Following centrifugation, the resulting plasma was separated and kept frozen for later analysis. Plasma insulin levels were measured using commercially available ELISA kits (Rat Insulin ELISA kit 10-1250-10, Mercodia), according to the manufacturer's instructions and guidelines. Insulin levels are expressed as micrograms per liter (µg/L). Fasting levels of glucose and insulin were used to calculate the glucose/insulin ratio, the homeostasis model assessment insulin resistance (HOMA-IR) and the quantitative insulin check index of insulin sensitivity (QUICKI), which are the most common indirect methods used to measure insulin sensitivity [54].

Tissue Harvest

After the intraperitoneal glucose tolerance test, animals were euthanized by inhalation of carbon dioxide. This method is an acceptable method for rodent euthanasia and was performed considering the most recent recommendations [55]. Blood samples were collected and the pancreas and quadriceps femoris muscle were dissected, excised, and immersed in a 4% (vol./vol.) buffered paraformaldehyde solution.

Plasma Corticosterone

Blood collected at sacrifice was centrifugated and the resulting plasma was separated and kept frozen for later analysis. Plasma corticosterone levels were measured using commercially available ELISA kits (Corticosterone ELISA kit ab108821, Abcam), according to the manufacturer's instructions and guidelines. Corticosterone levels are expressed as nanograms per milliliter (ng/mL) of plasma.

Immunohistochemistry and Morphological Analysis

After routine processing for light microscopy, five micrometer paraffin-embedded slices of the pancreatic tissue sample were made and dyed according to the hematoxylin-eosin (HE)

and chromotrope aniline blue (CAB) techniques. Histological images were acquired with a Leica DM500 light microscope and a Leica DFC290 HD camera (Leica Microsystems CMS GmbH, Wetzlar, Germany) using a 40x objective. The morphological analysis was blinded to the groups and performed by an experienced endocrine pathologist, that examined the slides in random order and divided the observations as part of one of four groups: no alterations, slight, medium, and severe.

For the histochemical analysis of pancreatic islet fibrosis, three random images of equal area containing pancreatic islets were selected from each animal and analyzed using the ImageJ software [56], as described previously [12]. In short, for each image the pancreatic islets were isolated, and their blue pixel content was measured relative to the total islet area using a color deconvolution method [57]. The non-staining sections in interstitial spaces were excluded from quantification. The ratio of fibrosis area to islet area was calculated and the mean ratio of the three images was obtained for each animal.

For the immunohistochemistry analysis, a standard immunohistochemical technique was followed as described previously [12], using a rabbit polyclonal anti-GLUT4 antibody (abcam, ab654). Three random images of equal area containing GLUT4 immunostaining were selected from each slide, captured with a Leica DM500 light microscope and a Leica DFC290 HD camera (Leica Microsystems CMS GmbH, Wetzlar, Germany), and analyzed using ImageJ software. For each image, a threshold method was used to determine the number of brown pixels, corresponding to GLUT4 staining, relative to the total tissue area (interstitial spaces were excluded) and the ratio of GLUT4 area to muscle area was calculated and averaged for each animal. The researcher doing the pancreatic islet fibrosis ratio and GLUT4 quantification was blinded to the groups. Although a western blot would have quantitatively measured GLUT4 transporter in a more reproducible and sensitive manner that immunohistochemistry [43], since muscle GLUT4 measurement was hypothesized after the rats' sacrifice, no frozen muscle tissue was available to quantify GLUT4 using western blot.

Statistical Analysis

A univariate general linear model (ANCOVA) was used for data analysis. Final glucose AUC, GLUT4 ratio, pancreatic islet fibrosis ratio, plasma corticosterone and plasma insulin levels were included as dependent variables. The two nominal main factors established were infrasound exposure and glucose tolerance status. Duration of infrasound exposure and fasting insulin levels were included in this analysis as a covariate. The assumptions of normality variance homogeneity of the dependent variables were assessed using the

Shapiro-Wilk test and the Levene test, respectively. Data analysis was performed with the software IBM SPSS Statistics for Windows, version 26 (IBM Corp., Armonk, NY, USA), at the 5% significance level (α=0.05).

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3.2. EFFECTS OF HIGH-INTENSITY INFRASOUND ON LIVER LIPID CONTENT OF RATS

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EFECTS OF HIGH-INTENSITY INFRASOUND ON LIVER LIPID CONTENT OF RATS

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Abstract

Previous experimental studies show that exposure to noise with high and audible frequencies causes multiple metabolic alterations, such as increased liver glycogen and triglycerides. However, the effect of exposure to sound with lower frequencies, such as high-intensity infrasound (frequency <20 Hz and sound pressure level >90 dB), on the liver lipid content is still unclear. As such, we aimed to study the effect of exposure to high-intensity infrasound of both normal and glucose intolerant rats on the liver lipid content. For this study, 79 wild-type male Wistar rats were randomly divided into two groups: G1, no treatment, and G2, induced glucose intolerance. Each of these two groups was randomly divided in two subgroups: s (animals kept in silence) and i (animals continuously exposed to high-intensity infrasound noise). At three noise-exposure time-points (1, 6 and 12 weeks) the rats were sacrificed, the liver was excised and hepatic lipids extracted. Data analysis was performed using a two-way ANOVA ($p=0.05$). No significant effects due to interactions between the several factors exist on the liver lipid content ($p=0.077$). Moreover, no significant effects due to infrasound exposure ($p=0.407$) or glucose tolerance status ($p=0.938$) were observed. Our study shows that continuous exposure to high-intensity infrasound has no influence on the lipid content of the liver of both normal and glucose intolerant animals. This finding reinforces the need for further experimental studies on the physiological effects of infrasound due to its possible hazardous effects on human health.

Keywords: Infrasound; glucose intolerance; liver lipids; liver steatosis; rat

Introduction

Noise pollution is an important environmental and occupational risk factor known to cause several adverse effects on human health beyond the auditory system [1]. In Europe, noise was estimated the third environmental risk factor with major impact on public health [2]. The World Health Organization (WHO) Regional Office for Europe has acknowledged that low-frequency noise, below 200 Hz (including infrasound), represents an environmental problem and that research should focus on its outcomes [3].

In previous experimental studies, metabolic abnormalities such as glucose intolerance, insulin resistance, fasting hyperglycemia, dyslipidemia and alterations in insulin signaling in the skeletal muscle have been identified as a consequence of noise exposure with frequencies higher than 200 Hz [4-6]. Cui et al. [4] also referred increased levels of glycogen and triglycerides in the liver of noise-exposed rats that may lead to non-alcoholic fatty liver disease, a marker of metabolic dysfunction and risk factor for liver fibrosis, cirrhosis and cancer [7,8]. This accumulation of lipids in visceral fat is a key player in metabolic derangement and an important risk factor for type 2 diabetes and metabolic syndrome [9]. However, it is still unknown whether exposure to lower frequencies, namely high-intensity infrasound (frequency <20 Hz and sound pressure level >90 dB), induces the same changes in hepatic lipid content.

Therefore, we aimed to investigate if exposure to high-intensity infrasound induces changes in the liver lipids on both normal and glucose intolerant rats and to define the contribution of each of these factors to such outcome.

Material and Methods

Animals

Experimental design and planning were performed with full compliance to the PREPARE guidelines [10]. When applied, animal procedures were approved by the Portuguese National Authority for Animal Health (project nº 204/2017). All handling and care of the animals were performed by authorized researchers (accredited by FELASA Category C) and was done in accordance with the EU Comission on Animal Protection for Experimental and Scientific Purposes (2010/63/EU) and with the Portuguese legislation for the same purpose (DL 113/2013).

In compliance with the 3Rs principles [11], this study shares data and resources with a larger study of infrasound-induced pancreatic fibrosis, for which the sample size was

estimated based on a priori power analysis using G*Power 3 software [12] for a minimum statistical power of 80% (unpublished data). Thus, tissue samples were collected from randomly selected seventy-nine animals of the original sample of one hundred and fifty-six wild-type male Wistar rats acquired from Charles River Laboratories (Saint-Germain-sur-l'Arbresle, France), aged 11 weeks and weighing 375.95g ± 18.29g. Only male rats were included in order to avoid uncertain sex-dependent differences on the outcomes. They were housed in conventional cages, two animals per cage, with a 12h light/dark cycle (lights on at 8am) and had free access to food (standard rat chow) and water.

After a one-week acclimation period the original sample of one hundred and fifty-six animals were randomly assigned using a free access online software [13] into two groups: G1 (no treatment) and G2 (glucose intolerance). For this study, 39 animals were randomly selected from G1 and 40 animals randomly selected from G2 (table 1).

Table 1 – Number of animals per experimental group and mean values and standard deviation for glycemia at intraperitoneal glucose tolerance test 2h timepoint, expressed as milligrams per deciliter (mg/dL), for each experimental group of normal (G1) and glucose intolerant (G2) animals, either kept in silence (s) or exposed to high-intensity infrasound (i).

G1 (n=39)			G2 (n=40)		
123.59 ± 18.39			158.05 ± 30.58		
G1s (n=19)	1 wk (n=6)	138.25 ± 13.77	G2s (n=20)	1 wk (n=6)	148.63 ± 45.54
	6 wks (n=6)	154.25 ± 20.45		6 wks (n=7)	154.75 ± 17.43
	12 wks (n=7)	143.75 ± 21.31		12 wks (n=7)	168.00 ± 25.04
G1i (n=20)	1 wk (n=6)	156.63 ± 27.79	G2i (n=20)	1 wk (n=6)	151.00 ± 17.57
	6 wks (n=7)	142.14 ± 20.75		6 wks (n=7)	152.17 ± 22.34
	12 wks (n=7)	141.83 ± 14.23		12 wks (n=7)	157.00 ± 11.45

Glucose Intolerance

Glucose intolerance was induced through a high-fat diet (D12492 diet, Research Diets) and the administration of low-dose streptozotocin (HFD/STZ rat model) because this model is considered to mimic the human disease [14]. The protocol for glucose intolerance was performed as described by Furman [15]. In short, animals were fed a high-fat diet, with 60% of calories coming from fats, for 3 weeks. After this period STZ (STZ, Sigma) 40mg/kg was prepared in a sodium citrate buffer 50mM, pH4.4, and was administered intraperitoneally after a fasting period of 6-8 hours, with unlimited access to water.

Glucose intolerance was confirmed through an intraperitoneal glucose tolerance test (G2 animals with mean value for glycemia 158.05 mg/dL ± 30.58 mg/dL at 2h timepoint Vs. G1 animals with mean value for glycemia 123.59 mg/dL ± 18.39 mg/dL at 2h timepoint, table 1 and supplemental material) following the protocol established by Ayala et al. [16]. G1 and G2 animals were then fed standard rat chow and were randomly divided in two subgroups each (table 1): G1s (no treatment, silence, 19 animals), G1i (no treatment, infrasound, 20 animals), G2s (glucose intolerance, silence, 20 animals) and G2i (glucose intolerance, infrasound, 20 animals). Animals from each of the four groups were randomly divided into three infrasound exposure timepoints and euthanized after 1, 6 and 12 weeks of exposure (animals were randomly distributed as stated in table 1). Before euthanasia, glucose intolerance was again confirmed through an intraperitoneal glucose tolerance test (mean values for glycemia at 2h timepoint and standard deviation for each experimental group in table 1 and supplemental material) following the protocol established by Ayala et al. [16].

Infrasound Exposure

Infrasound exposure was performed as previously described by Oliveira et al. [17]. Animal cages were placed in a soundproofed room, measuring 217×211×195 cm, in front of a noise generator consisting of a subwoofer that reproduced a continuous (24h/day) sound signal, previously recorded in a cotton-mill room from a large textile factory of Northern Portugal. This sound signal was processed offline, applying LabVIEW and Matlab systems.

With the objective of creating a strong subsonic acoustic field in the room, a pseudo-random waveform in the 2-Hz to 20-Hz decade band was filtered from the recorded sound signal with Matlab based on a bandpass-filtered 30-s maximum length sequence segment. The waveform was used to excite an array of two infinite baffles mounted 18-in. 300-W-rated magnetodynamic subwoofers, by means of a 2×600-Wheavy-duty quasi-dc voltage output audio power amplifier. Subsequently, with the aim of exploiting as much as possible the available subwoofers dynamic range at this frequency range with an acceptable amplitude distortion, the waveform was iteratively nonlinearly treated with moderate compression expansion and further filtering (in order to reduce the crest factor to approximately 2.0 times). The total sound pressure level and the spectral characteristics of the resulting acoustic pressure waveform were monitored, and the results were an average sound pressure level of 120 dB in the 2-20 Hz with a tolerance of ±3 dB in a 30 second time window in the entire compartment. As to the spectral boundedness of the produced sound field the result was 80 dB total out-of-band average sound pressure level (-40 dB lower). Groups not exposed to infrasound were kept in a similar room but in silence.

Liver Lipid Content

At the respective timepoint, all rats were euthanized by inhalation of carbon dioxide. Liver was excised and hepatic lipids were extracted according to the protocol established by Folch et al. [18]. Samples of approximately 15mg were obtained and homogenized in a chloroform/methanol solution (v/v, 2:1), shaken for 20 min at room temperature and then centrifuged at 1200 rpm, at 4ºC, for 10 min. Small volumes of 0.9% NaCl were added and centrifuged to separate both phases. The lower phase was evaporated with nitrogen and dried at 100ºC with weighting every 10 minutes until weight stabilized. Results are expressed as milligrams of lipids per gram of liver.

Statistical Analysis

A univariate general linear model (two-way ANOVA), with dependent variable defined by lipid content and two nominal main factors defined by infrasound exposure and glucose tolerance status, was used for data analysis. The assumptions of normal distribution and variance homogeneity of the lipid content distribution were checked using the Shapiro-Wilk test and the Levene test, respectively. Data analysis was performed with the software IBM SPSS Statistics for Windows, version 26 (IBM Corp., Armonk, NY, USA), at the 5% significance level (p=0.05).

Results

The mean values and standard deviation of lipid content in the liver are illustrated on table 2 and in figure 1. The effect of age as a covariable on hepatic lipid content was discarded due to a non-significant Pearson correlation between both variables (r=0.137, p=0.228). Despite this non-significant correlation, the duration of noise exposure was included as covariable in a general linear model with two main factors (high-intensity infrasound exposure and glucose tolerance status), after validation of the assumption of homogeneity of variance (Levene test, p=0.460). It should be noted that the assumption of normal distribution of lipid content hold, except in one subgroup (Shapiro-Wilk test, p=0.026), in which however no severe symmetry was detected. Furthermore, no significant interaction between the covariate and the main factors included in the model was observed. The results show that no significant effects due to interactions between the several factors exist on the liver lipid content (p=0.077). Moreover, no significant effects due to high-intensity infrasound exposure (p=0.407) or glucose tolerance status (p=0.938) were observed.

However, and despite not being statistically significant (p=0.077), our results may suggest the existence of an interaction between factors, that is, that the response to noise exposure with regard to the hepatic fat content depends on the metabolic condition of the animals, which is also suggested by figure 2. Nevertheless, these conclusions must be considered with caution, in light of the above.

Table 2 – Mean values and standard deviation for liver lipid content, expressed as milligrams of lipids per gram of liver in normal (G1) and glucose intolerant (G2) animals, either kept in silence (s) or exposed to high-intensity infrasound (i) at different timepoints. No significant effects on the liver lipid content were observed, due to interactions between factors (p=0.077), infrasound exposure (p=0.407) or glucose tolerance status (p=0.938).

Group		Timepoint of sacrifice	Liver Lipid Content
No treatment (G1)	G1s	week 1	54.78 (± 7.91)
		week 6	49.00 (± 8.23)
		week 12	59.00 (± 8.58)
	G1i	week 1	54.13 (± 19.91)
		week 6	49.50 (± 5.65)
		week 12	51.71 (± 9.52)
Glucose intolerance (G2)	G2s	week 1	51.20 (± 3.49)
		week 6	48.33 (± 4.89)
		week 12	50.40 (± 11.06)
	G2i	week 1	48.00 (± 17.09)
		week 6	56.20 (± 10.64)
		week 12	61.00 (± 4.65)

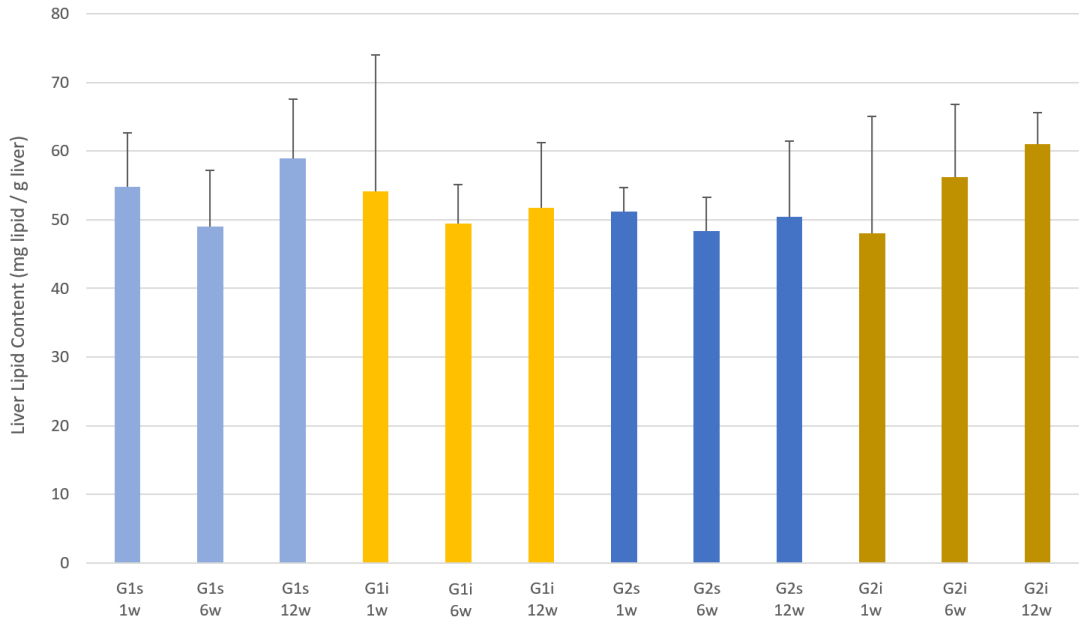

Figure 1 – Liver lipid content of normal (G1) and glucose intolerant rats (G2) kept in silence (s) and exposed to high-intensity infrasound (i) at different timepoints. A non-significant effect on the liver lipid content due to interactions between factors (p=0.077) was observed, as well as due to glucose tolerance status (p=0.938) or noise exposure (p=0.407).

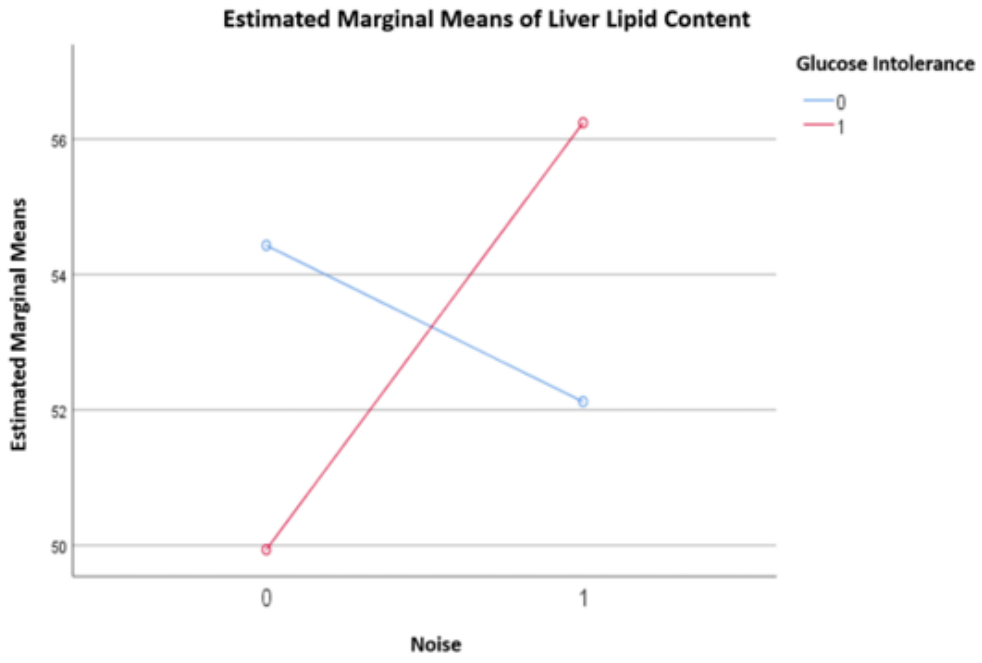

Figure 2 – Comparison of estimated marginal mean values of liver lipid content in relation with noise exposure and glucose tolerance status. No significant effects were observed on the liver lipid content due to interactions between factors (p=0.077).

Discussion

Our study aimed to investigate whether chronic exposure to high-intensity infrasound could trigger metabolic changes in the liver, namely on its lipid content, in both normal and glucose intolerant rats. Our results show that there is no influence from such exposure on this outcome, although in glucose intolerant rats the liver lipid content is slightly increased, which may be due to chance, but also reinforces the need of further evaluations to address if the presence of glucose intolerance may be an additional risk factor for the alterations induced by chronic exposure to high-intensity infrasound.

High-intensity infrasound exposure studies in laboratory animals addressing liver lipid content are scarce since most studies focus on audible noise with higher frequencies [4-6]. In these studies, an increase in hepatic concentration of glycogen and triglycerides has been described [4]. Several theoretical models for the association between audible noise exposure and metabolic changes have been developed, focusing on the role of noise as a stressor, and as a trigger of the neuroendocrine pathways that promote hyperglycemia, insulin resistance and fat accumulation [19,20]. Liver steatosis can also result from endoplasmic reticulum stress, through impaired fatty acid oxidation and disturbance of the unfolded protein response [21].

Previous experimental studies support the role of audible noise exposure as a liver stressor [22,23]. These studies have demonstrated that chronic stress associated with an enriched diet increases the levels of total cholesterol and triglycerides in the liver, as well as hepatic inflammation and oxidative stress [22], aggravating the induced nonalcoholic fatty liver disease from steatosis to steatohepatitis [23].

Infrasound is a mechanical vibration wave with a frequency range below 20 Hz, originated by natural phenomena and man-made sources, such as industrial installations, low-speed machinery and music [24,25]. Due to its wavelength, infrasound can propagate over very large distances without being reflected or absorbed by obstacles and is hardly attenuated through dissipation [24]. As such, infrasound can induce body vibrations and resonance in body cavities, thus affecting internal systems and organs [26].

There is evidence from high-intensity infrasound exposure studies in laboratory animals that chronic exposure results in proliferation of the connective tissue matrix and collagen fibers in animals; this fibrotic response has been documented in several organs, such as the heart, lung and glands of rats chronically exposed to industrial-type noise [27-30]. Oliveira et al. [31] documented the same alterations in the liver connective tissue, on centrolobular regions without disruption of the organ architecture, as a result of the exposure to high-

intensity infrasound. This is thought to be a response to the body vibrations induced by infrasound and may function as a mechanical stabilizer of the organ [30].

However, there is a common misconception about the inaudibility of infrasound since sounds with lower frequencies can still be heard with an increase of the sound pressure level [24,26]. Higher pressure levels, as the ones used in our study, can elicit both body vibration and hearing response from the animal model used [32,33]. As such, we cannot exclude animal stress due to audible noise with subsequent activation of the neuroendocrine pathways. To answer this question, future studies should assess clinical and behavioral signs along with corticosterone, the primary stress hormone in rodents, to examine the stress response of the experimental animal [34].

The major limitation of our study is the number of animals, as the study sample was drawn from an original sample estimated for a larger metabolic experimental study on infrasound-induced pancreatic fibrosis (according to the 3Rs principles), as stated in section 2.1. [11]. On the other hand, the experimental protocol allows the assessment of interactions between other important variables studied. We also considered the effect of aging on our experimental protocol, since lipogenesis and fat accumulation leading to liver steatosis are both part of the natural aging process of the liver [35,36]. Accordingly, we used age-matched animals, as control groups, and the effect of time as a covariable was discarded due to a non-significant correlation between both variables. We have found a discrete, non-significant increase, of liver lipid in the glucose intolerant rats that may be due to chance. Nevertheless, and although we had 79 animals, future studies should consider the possibility of this additional risk factor for the alterations induced by chronic exposure to high-intensity infrasound.

In summary, our study shows that continuous exposure to high-intensity infrasound has no influence on the liver lipid content of both normal and glucose intolerant animals. Within the limitations of our study, these results reinforce the importance of further research concerning the effects of high-intensity infrasound, a ubiquitous element, on the liver due to its possible hazardous effects on human health.

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3.3. URBAN NOISE EXPOSURE AND CARDIOMETABOLIC DISEASES: AN EXPLORATORY CROSS-SECTIONAL STUDY IN LISBON

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URBAN NOISE EXPOSURE AND CARDIOMETABOLIC DISEASES: AN EXPLORATORY CROSS-SECTIONAL STUDY IN LISBON

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Abstract

Introduction: Urban noise pollution has been associated to an increased risk of developing metabolic syndrome. Nevertheless, existing observational studies relating noise exposure and metabolic syndrome are based on non-generalizable cohorts. Lisbon remains a noisy city where this association has not been evaluated, and for this reason we studied the relation between exposure to urban noise and prevalence of type 2 diabetes mellitus, obesity, and hypertension. *Methods*: Diurnal, evening, and nocturnal noise emission levels were obtained for each street in the city from the Lisbon noise map. After allocation of all roads to the respective parish of Lisbon, the noise emission for each parish was averaged for each day period. The number of adult patients with type 2 diabetes mellitus, obesity, and hypertension in 2014, 2015 and 2016 in each parish of Lisbon was obtained from the Regional Health Administration of Lisbon and Tagus Valley. Prevalence as a percentage of population was determined using the number of residents in each parish determined in the 2011 population census. Spearman's non-parametric correlation coefficient was used due to non-normal distribution in the variables, at the 5% significance level ($\alpha=0.05$). *Results*: No correlations were found between daytime, afternoon or night-time noise exposure and the prevalence of type 2 diabetes mellitus, obesity, or hypertension, although correlations were found between the cardiometabolic variables. Nevertheless, noise levels in Lisbon were above the legally established limit and the World Health Organization guidelines for environmental noise exposure in the European region. *Conclusion*: Our results do not agree with previous studies and should be faced as preliminary due to a strong biological plausibility for an association between noise exposure and cardiometabolic diseases and to encourage further studies, with longitudinal cohorts.

Keywords: Urban Noise, Diabetes Mellitus, Hypertension, Obesity, Lisbon

Resumo

Introdução: A poluição sonora urbana tem sido associada a um risco aumentado de desenvolver síndrome metabólica. No entanto, os estudos observacionais existentes que relacionam a exposição a ruído e a síndrome metabólica são baseados em coortes não generalizáveis. Lisboa continua a ser uma cidade ruidosa onde esta associação não foi avaliada, pelo que estudámos a relação entre a exposição ao ruído urbano e a prevalência de diabetes mellitus tipo 2, obesidade e hipertensão. *Métodos*: Os níveis diurnos, vespertinos e noturnos de emissão de ruído foram obtidos para cada rua da cidade a partir do mapa de ruído de Lisboa. Após atribuição das ruas à respetiva freguesia, procedeu-se ao cálculo da média das emissões sonoras de cada freguesia para cada período do dia. O número de doentes adultos com diabetes mellitus tipo 2, obesidade e hipertensão em 2014, 2015 e 2016 em cada freguesia foi obtido junto da Administração Regional de Saúde de Lisboa e Vale do Tejo. O coeficiente de correlação não paramétrica de Spearman foi utilizado devido à distribuição não normal nas variáveis, ao nível de significância de 5% (α=0,05). *Resultados*: Não foram encontradas correlações entre a exposição ao ruído diurno, vespertino ou noturno e a prevalência de diabetes mellitus tipo 2, obesidade ou hipertensão, embora tenham sido encontradas correlações entre as variáveis cardiometabólicas. No entanto, os níveis de ruído em Lisboa situaram-se acima do limite legalmente estabelecido e das diretrizes da Organização Mundial de Saúde para a exposição ao ruído ambiente na região europeia. *Conclusão*: Os nossos resultados não concordam com estudos anteriores e devem ser encarados como preliminares devido a uma forte plausibilidade biológica para uma associação entre exposição ao ruído e doenças cardiometabólicas e para estimular novos estudos, com coortes longitudinais.

Palavras Chave: Ruído Urbano, Diabetes Mellitus, Hipertensão, Obesidade, Lisboa

Introduction

Noise pollution has been increasing all over the world, mainly in urban environments [1], due to sources such as road, rail, and air traffic [2]. Observational studies in these populations have associated noise exposure to negative effects that go beyond the auditory system [3], and longitudinal cohort studies have shown that noise pollution specifically increases the risk of developing metabolic syndrome [4-6].

Metabolic syndrome is a pathological condition that includes central obesity, insulin resistance (or type 2 diabetes mellitus), hypertension and dyslipidemia [7], in a complex interaction between individual genetic predisposition and environmental factors [8-10]. Observational studies in cities such as Stockholm and Toronto showed that noise exposure is associated with an increased risk of obesity, hypertension, diabetes and dyslipidemia [6].

Due to the increased prevalence, morbidity, and mortality of metabolic syndrome worldwide [11], there has been particular attention on environmental determinants, namely noise, to counteract the epidemiological trend [12]. However, observational studies have been carried out in a small number of cities in countries such as Denmark, Sweden, Norway, Canada, and the United Kingdom, having a limited external validity, as these have specific social, urban, and demographic characteristics [6]. Thus, studies are relevant to estimate the association between exposure to urban noise and its cardiometabolic effects in different populations [13, 14]

Lisbon, the capital and largest city (and municipality) in Portugal, has a territory of about 85km2 and a population of approximately 550,000 individuals, divided into 24 parishes [15]. The population of Lisbon is exposed to high levels of noise throughout the day, originating from road traffic, due to the high volume of daily passengers entering the city [16], in rail and air traffic, due to the high number of railway stations and the location of Lisbon airport on the outskirts of the municipality, just 5km from the center [16], and with the air corridor tearing the city in half.

This sound environment, characterized by excessive daytime and nighttime noise, has changed little since 2008 despite the monitoring and intervention programs on urban noise in the city of Lisbon, placing Portugal and in particular the city of Lisbon in a very unfavourable situation in terms of noise levels under European air traffic legislation, according to the European Environment Agency [16]. Lisbon is the second worst European capital regarding exposure to air traffic noise, in terms of the Lden indicator (average of 24 hours weighted by day, evening and night periods), with 15% of the population in Lisbon municipality exposed to levels above 55 dB, and to the Ln indicator (night noise between 11 pm and 7 am), with 10% of the population exposed to levels above 50 dB [16].

To the best of our knowledge, there are no studies that verify the relationship between noise exposure and metabolic syndrome in the city of Lisbon. Therefore, we carried out a exploratory cross-sectional study to evaluate the association between exposure to urban noise and the prevalence of type 2 diabetes mellitus, obesity and hypertension in Lisbon.

Materials and Methods

Study protocol and data provision were approved by the Ethics Committee of the Regional Health Administration of Lisbon and Tagus Valley, IP (ref. 1723/CES/2017). The association between the different variables was verified at the level of each of the 24 parishes ("freguesias") of the city of Lisbon: Ajuda, Alcântara, Alvalade, Areeiro, Arroios, Avenidas Novas, Beato, Belém, Benfica, Campo de Ourique, Campolide, Carnide, Estrela, Lumiar, Marvila, Misericórdia, Olivais, Parque das Nações, Penha de França, Santa Clara, Santa Maria Maior, Santo António, São Domingos de Benfica and São Vicente.

Urban noise exposure in Lisbon

European Directive 2002/49/CE and Portuguese legislation (Decree-Law 146/2006) instructed municipalities to draw up noise maps to assess noise emissions from road, air, and rail traffic in three reference periods – day, evening, and night. Since 2008, the municipality of Lisbon has publicly released its noise map, drawn up in accordance with legally established criteria (shown in Fig. 1). A detailed description of the development of these noise maps can be found elsewhere [17]. More recent noise maps have shown that noise levels in Lisbon have been similar throughout the years [15].

Fig. 1. A – Lisbon noise map in 2008 showing the estimated noise exposure for the combined diurnal, evening, and nocturnal emissions (adapted from [15]); B – Outline of the boundaries of each parish in Lisbon (retrieved from [54]); C – Lisbon noise map in 2008 with the outline of the boundaries of each parish in Lisbon (adapted from [15,54]).

For the distribution of noise levels across the 24 parishes of Lisbon, measurements of noise emissions from the 2008 Lisbon noise map were considered for the three reference periods, obtained in the various streets of the city. Each street was assigned to the respective parish, and if a street crossed two adjacent parishes, it was assigned to both. After all streets were allocated, the average noise emissions for each parish were calculated for each reference period. The mean values and standard deviations of noise emission from each parish and reference period are shown in Table 1.

Table 1. Mean values and standard deviations of noise emission (dBA) in each parish of Lisbon for each reference period (diurnal, evening, and nocturnal).

Civil Parish	Diurnal (dBA)	Evening (dBA)	Nocturnal (dBA)
Ajuda	81.29 (± 2.09)	79.77 (± 2.00)	72.96 (± 1.91)
Alcântara	86.16 (± 1.90)	85.25 (± 1.96)	80.23 (± 2.02)
Alvalade	84.00 (± 2.10)	82.41 (± 1.85)	75.52 (± 1.59)
Areeiro	80.99 (± 2.03)	79.15 (± 1.90)	72.69 (± 1.77)
Arroios	79.98 (± 2.21)	78.23 (± 2.13)	72.22 (± 2.05)
Avenidas Novas	80.29 (± 1.69)	78.77 (± 1.78)	72.49 (± 1.88)
Beato	80.65 (± 1.74)	79.14 (± 1.99)	72.64 (± 2.24)
Belém	83.80 (± 1.82)	81.70 (± 1.73)	75.14 (± 1.64)
Benfica	83.73 (± 1.89)	81.89 (± 1.84)	75.43 (± 1.79)
Campo de Ourique	81.25 (± 1.91)	79.48 (± 1.94)	72.91 (± 1.97)
Campolide	85.36 (± 1.67)	84.54 (± 1.86)	79.33 (± 2.04)
Carnide	84.45 (± 2.11)	83.07 (± 2.02)	76.73 (± 1.92)
Estrela	82.82 (± 1.93)	80.96 (± 2.03)	74.48 (± 2.12)
Lumiar	86.49 (± 2.05)	85.52 (± 1.85)	80.42 (± 1.65)
Marvila	82.37 (± 1.99)	80.60 (± 1.96)	74.14 (± 1.93)
Misericórdia	81.97 (± 1.78)	80.22 (± 1.82)	74.15 (± 1.86)
Olivais	82.48 (± 1.65)	80.58 (± 1.75)	74.10 (± 1.84)
Parque das Nações	82.21 (± 2.23)	80.10 (± 1.99)	73.62 (± 1.75)
Penha de França	79.17 (± 1.87)	77.64 (± 1.78)	71.17 (± 1.68)
Santa Clara	86.92 (± 2.04)	86.52 (± 2.13)	82.34 (± 2.22)
Santa Maria Maior	81.17 (± 1.79)	78.32 (± 1.92)	72.23 (± 2.04)
Santo António	80.60 (± 1.58)	78.53 (± 1.85)	72.93 (± 2.11)
São Domingos de Benfica	85.69 (± 2.01)	84.86 (± 1.97)	79.24 (± 1.92)
São Vicente	79.21 (± 1.92)	77.56 (± 1.99)	70.98 (± 2.07)

Prevalence of type 2 diabetes mellitus, obesity, and hypertension in Lisbon

Data were obtained from the Regional Health Administration of Lisbon and Tagus Valley on the number of adult patients with type 2 diabetes mellitus (identified by the International Classification of Primary Care – ICPC – code T90), obesity (ICPC code T82), uncomplicated hypertension (ICPC code K86), and complicated hypertension (ICPC code K87) in 2014, 2015 and 2016 for each parish in the municipality of Lisbon. To calculate prevalence as a percentage of population for each parish and each year, the population of each parish of Lisbon in the 2021 census [18] was subtracted to the corresponding data in the 2011 census [18], to obtain the estimated population variation for each parish and each year between 2011 and 2021 (supplementary material). As such, the estimated population of each parish in the city of Lisbon in 2014, 2015 and 2016 was obtained (supplementary material) and prevalence as a percentage of population was calculated. The prevalence of type 2 diabetes mellitus, obesity, uncomplicated and complicated hypertension in 2014, 2015 and 2016 for each parish is shown in Table 2.

Table 2. Prevalence of type 2 diabetes mellitus, obesity, uncomplicated and complicated hypertension in 2014, 2015 and 2016 for each parish in Lisbon. Values are presented as a percentage of population (%).

Parish	DM2 2014	DM2 2015	DM2 2016	Obesity 2014	Obesity 2015	Obesity 2016	HTA nC 2014	HTA nC 2015	HTA nC 2016	HTA Comp 2014	HTA Comp 2015	HTA Comp 2016
Ajuda	7.2	8.7	8.8	3.8	5.8	6.3	18.1	21.6	21.0	3.0	4.8	5.4
Alcântara	4.6	7.5	7.6	1.4	4.5	5.1	12.2	20.8	20.9	1.1	3.3	3.6
Alvalade	5.1	5.2	5.1	4.5	4.9	5.4	15.7	15.9	15.4	2.3	2.4	2.6
Areeiro	3.8	4.1	4.1	2.9	3.6	4.2	10.9	11.6	12.0	1.6	1.7	1.8
Arroios	5.1	5.3	5.2	3.9	4.8	5.3	14.8	15.4	15.0	1.7	1.8	1.8
Avenidas Novas	3.7	4.0	4.0	2.7	3.5	3.8	11.7	12.0	12.1	1.9	2.0	2.1
Beato	3.4	6.9	7.1	4.5	10.8	12.5	9.5	19.8	19.6	1.0	3.2	3.5
Belém	2.4	4.0	4.2	0.9	2.9	3.3	7.8	13.7	14.1	1.0	2.3	2.8
Benfica	8.2	7.8	8.0	9.0	9.6	10.7	24.4	23.7	24.0	4.9	4.7	5.2
Campo de Ourique	6.0	6.0	6.0	7.7	8.8	9.2	19.4	19.6	19.4	3.6	4.0	4.1
Campolide	6.3	6.6	6.7	5.0	5.7	6.1	17.4	18.0	18.0	2.9	3.2	3.2
Carnide	5.7	5.8	5.9	7.4	8.3	8.7	16.7	17.0	17.2	3.1	3.0	3.2
Estrela	4.4	4.5	4.5	2.1	2.5	3.0	16.1	16.3	16.3	1.6	1.8	1.8
Lumiar	4.4	4.6	4.7	4.3	4.8	5.1	12.8	13.4	13.5	1.9	2.0	2.1
Marvila	5.6	8.6	8.8	5.4	10.6	11.7	16.5	24.7	24.7	1.2	2.9	3.1
Misericórdia	5.4	5.3	5.7	3.5	4.9	6.8	14.5	15.2	15.7	1.4	1.8	2.4
Olivais	8.3	8.2	8.2	5.0	6.7	7.6	20.2	20.6	20.7	3.0	3.3	3.4
Parque das Nações	2.4	1.6	1.4	9.9	7.4	7.2	3.8	3.8	3.9	1.2	0.8	0.7
Penha de França	4.7	6.7	6.7	0.7	0.8	1.0	14.7	20.2	19.8	1.5	2.9	3.1
Santa Clara	4.5	4.8	4.9	3.5	4.0	4.5	10.4	11.2	11.7	1.6	1.8	1.9
Santa Maria Maior	6.7	6.9	6.9	3.4	4.7	7.2	15.6	16.6	17.1	1.1	1.2	1.3
Santo António	5.1	5.3	5.4	4.7	5.8	6.2	15.9	16.3	16.5	2.1	2.4	2.5
São Domingos de Benfica	5.0	5.3	5.4	5.1	5.7	6.2	16.2	16.5	16.8	3.0	3.1	3.2
São Vicente	5.0	6.5	6.5	3.1	5.8	6.3	12.2	17.5	17.4	1.9	3.0	3.2

Statistical analysis

The nonparametric Spearman's rank correlation coefficient was used for data analysis using type 2 diabetes mellitus, obesity, uncomplicated and complicated hypertension prevalence in 2014, 2015, and 2016 and diurnal, evening, and nocturnal noise emissions for each civil parish as variables. This test was used because deviation from a normal distribution was significant ($p=0.008$) for nocturnal noise and strongly suggested ($p=0.059$) for evening noise, as shown by the Shapiro-Wilk test of normality. As a surrogate analysis, the parametric Pearson correlation coefficient was also computed for the same associations. The analysis was performed using the IBM SPSS Statistics for Windows software, version 26 (IBM Corp., Armonk, NY, USA), at a significance level of 5% ($\alpha=0.05$).

Results

Noise levels in Lisbon decreased throughout the day, with the lowest values observed at night (as shown in Table 1). The noisiest parish in the city of Lisbon for all reference periods was “Santa Clara”.

No correlations were found between diurnal, evening, or nocturnal noise exposure and the prevalence of type 2 diabetes mellitus, obesity, or hypertension in any of the studied years (shown in Table 3). The magnitude and significance of these associations were essentially the same using the Pearson correlation coefficient. Consistent correlations were found between prevalence of type 2 diabetes mellitus, obesity, uncomplicated and complicated hypertension throughout the studied years, as shown in Table 4.

Table 3. Spearman’s correlation coefficient (rs) and its significance (p) between diurnal noise emissions, evening noise emissions, nocturnal noise emissions and prevalence of type 2 diabetes mellitus (DM2), obesity, uncomplicated hypertension (HTA nC) and complicated hypertension (HTA Comp) in 2014, 2015 and 2016 in the 24 civil parishes of Lisbon. Statistically significant correlations are highlighted in bold.

		DM2 2014	DM2 2015	DM2 2016	Obesity 2014	Obesity 2015	Obesity 2016	HTA nC 2014	HTA nC 2015	HTA nC 2016	HTA Comp 2014	HTA Comp 2015	HTA Comp 2016
Diurnal (dBA)	r_s	0.035	-0.079	-0.045	0.229	-0.004	-0.047	0.130	-0.060	-0.063	0.147	0.132	0.123
	p	0.872	0.714	0.834	0.281	0.984	0.829	0.543	0.781	0.768	0.492	0.539	0.565
Evening (dBA)	r_s	0.000	-0.092	-0.058	0.231	0.001	-0.068	0.128	-0.053	-0.055	0.173	0.166	0.160
	p	0.998	0.668	0.787	0.278	0.995	0.751	0.550	0.806	0.799	0.420	0.439	0.456
Nocturnal (dBA)	r_s	0.021	-0.106	-0.068	0.228	-0.006	-0.082	0.134	-0.076	-0.072	0.174	0.142	0.135
	p	0.923	0.622	0.751	0.285	0.979	0.704	0.534	0.725	0.738	0.415	0.509	0.529

Table 4. Spearman's correlation coefficient (rs) and its significance (p) between prevalence of type 2 diabetes mellitus (DM2) and obesity, uncomplicated hypertension (HTA nC) and complicated hypertension (HTA Comp) in 2014, 2015 and 2016 in the 24 civil parishes of Lisbon. Statistically significant correlations are highlighted in bold.

		Obesity 2014	Obesity 2015	Obesity 2016	HTA nC 2014	HTA nC 2015	HTA nC 2016	HTA Comp 2014	HTA Comp 2015	HTA Comp 2016
DM2 2014	r_s	0.433	0.446	0.529	0.876	0.625	0.632	0.597	0.501	0.463
	p	**0.035**	**0.029**	**0.008**	**0.000**	**0.001**	**0.001**	**0.002**	**0.013**	**0.023**
DM2 2015	r_s	0.210	0.496	0.564	0.593	0.934	0.938	0.181	0.709	0.703
	p	0.325	**0.014**	**0.004**	**0.002**	**0.000**	**0.000**	0.398	**0.000**	**0.000**
DM2 2016	r_s	0.215	0.511	0.581	0.584	0.936	0.944	0.158	0.719	0.722
	p	0.312	**0.011**	**0.003**	**0.003**	**0.000**	**0.000**	0.460	**0.000**	**0.000**

Discussion

Our study aimed to investigate a possible cross-sectional association between exposure to urban noise and prevalence of type 2 diabetes mellitus, obesity, and hypertension in the city of Lisbon. We found no association between the considered factors, unlike other studies where authors found an increased risk of cardiometabolic diseases in populations exposed to noise [19-26].

There is a strong biological plausibility for an association between noise exposure, type 2 diabetes mellitus, obesity, and hypertension. Noise exposure acts as a physiological and psychological stressor, activating the neuroendocrine pathways such as the hypothalamus-pituitary-adrenal axis and the sympathetic nervous system, with subsequent release of cortisol and catecholamines [27]. Long-term noise exposure leads to chronic hypercortisolism and hyperglycemia and, as such, promotes hyperinsulinism, insulin resistance, dyslipidemia, central adiposity, endothelial dysfunction, oxidative stress, inflammation, and thrombotic predisposition [28-30], which lead to the development of type 2 diabetes mellitus, obesity, and hypertension [31,32]. These effects are particularly pronounced with exposure to nocturnal noise. Nocturnal noise exposure can decrease sleep duration, reduce sleep quality, and disrupt circadian rhythm which are strongly and independently associated with adverse metabolic effects such as obesity and type 2 diabetes mellitus [33-35].

Our study found high levels of diurnal, evening, and nocturnal noise in Lisbon. These noise levels were above the legally established limit (Decree-Law 146/2006), which define an

exposure limit value of 65 dB for daytime noise and 55 dB for nocturnal noise, and the recent World Health Organization guidelines for environmental noise in the European region [36]. Although we did not find a correlation between the factors, these high noise levels observed throughout the day could lead to an increase in negative cardiovascular and metabolic health outcomes in the future [37].

Analyzing our results, it is important to consider that there is a significant number of daily commuters in Lisbon that are exposed to daily noise levels of this city but, if diagnosed with any of the studied diseases, are counted in their respective residential municipality and not in Lisbon. Also, there can be a considerable difference between noise exposure assessments based on the residence and the noise exposure measured by the method we used, allocating streets that cross several parishes and that may have various sound intensities due to their large extension [38,39].

The cross-sectional design does not allow the establishment of a causal inference between the studied cardiometabolic diseases and noise exposure [40], which may also constitute a limitation. The specific associations between residential transport noise and diabetes arise mainly from longitudinal studies, following up large population-based cohort of residents in urban regions [41]. Interestingly, this association holds even after adjusting for environmental co-exposures, including traffic-related air pollutants [42-45]. Thus, our study was an exploratory pilot study and aimed to establish preliminary evidence for future studies [40]. This is particularly relevant if we consider that strong earlier evidence exists.

Considering hypertension and obesity, the evidence of a relationship with road traffic noise is quite robust and is dose-dependent [23-25, 46-52]. Although we did not found an association concerning these factors, we should take into account that the prevalence of the studied cardiometabolic diseases in each civil parish of Lisbon was based on electronic health records from primary care. This creates a selection bias, excluding people with difficult access to healthcare and people covered by private insurance [53].

In summary, no association was found between exposure to diurnal, evening, and nocturnal noise exposure and the prevalence of type 2 diabetes mellitus, obesity, and hypertension in Lisbon. These results highlight the need for further research on the cardiometabolic effects of exposure to urban noise in Lisbon, due to its possible harmful effects on human health.

Conclusion

No correlations were found between exposure to urban noise in Lisbon and prevalence of cardiometabolic diseases in any of the studied years. Noise levels were above the legally established limit and the recommended environmental noise exposure by the World Health Organization. More studies are needed on the cardiometabolic effects of urban noise exposure in Lisbon, especially longitudinal cohort studies with better cardiometabolic disease data and characterization of noise exposure in each parish of the city of Lisbon.

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4. DISCUSSION AND CONCLUSIONS

DISCUSSION AND CONCLUSIONS

This thesis aimed to study the association between exposure to high-intensity infrasound and type 2 diabetes. For this purpose, both an experimental and an epidemiological study were designed.

For the experimental morphophysiological study, the Wistar rat animal model was used. This choice was based on the adequacy of rodents as an animal model for type 2 diabetes studies [1]. Although there are several animal models used in diabetes studies such as non-mammalians (such as Zebrafish, *Caenorhabditis elegans*, and Drosophila), rodents, large animals (such as dogs and pigs), and non-human primates, for the past two decades, small rodents, including rats, have become the most widely used preclinical animal model to study metabolic disorders [1].

Another reason for choosing this animal model was due to the large number of experimental studies focusing on the effects of high-intensity infrasound (as the one present in industrial settings) on several other Wistar rats' tissues and organs [2-16], making it a suitable model for a critical comparison of our results.

In the experimental study [17,18], Wistar rats were continuously exposed to high-intensity infrasound (24h/day). Most previous studies have exposed Wistar rats to high-intensity infrasound for only 8h/day (40h/week), to replicate an occupational exposure [2-16]. However, due to the ubiquitous diurnal and nocturnal presence of this acoustic stressor in a common daily urban human life [19], we opted for a continuous exposure.

Regarding the results of the experimental study, there are currently two main proposed models for the negative effects induced by exposure to high-intensity infrasound. According to one model, exposure to this acoustic aggressor induces annoyance with a consequent reactive neuroendocrine stress response [20]. The second model focuses on the physical proprieties of high-intensity infrasound that lead to body vibrations and resonance of internal organs and therefore to physical disruption of tissues and organs [21].

In the first article [17], results show that continuous exposure to high-intensity infrasound increases plasma corticosterone levels, which is the main stress hormone in rats [22]. This was a major finding because it highlights the role of high-intensity infrasound as a stressor, activating the neuroendocrine stress response [20,23], and as such provides support to the role of endogenous glucocorticoids on the negative effects of high-intensity infrasound exposure [24,25]. Although previous studies have hypothesized this possibility no such evidence has been produced so far [2-16].

Chronic exposure to glucocorticoids is known to negatively impact pancreatic endocrine function and peripheral insulin sensitivity, leading to progressive insulin resistance [24,25]. Glucocorticoids induce pancreatic beta cell death and dysfunction by decreasing glucose sensitivity [24]. The three main extra-pancreatic insulin-sensitive organs, such as liver, skeletal muscle, and adipose tissue are also affected by chronic exposure to glucocorticoids [24,25]. On skeletal muscle, glucocorticoids cause an improper insulin-responsive glucose transporter type 4 (GLUT4) protein translocation to the cellular membrane with a consequent decrease in glucose uptake [24,26]. Insulin resistance in liver due to chronic exposure to glucocorticoids leads to an increase in lipogenesis, causing liver steatosis, among other consequences [24,26].

The impaired pancreatic endocrine function and peripheral insulin resistance caused by chronic exposure to glucocorticoids lead to chronic hyperglycemia [24-26], the hallmark of type 2 diabetes [27]. As such, environmental exposures that increase stress hormones can be considered risk factors for type 2 diabetes [28-31].

Nevertheless, in the first article of this thesis, no hyperinsulinism or decrease of GLUT4 in skeletal muscle were observed as a result from exposure to high-intensity infrasound, despite the increase in plasma corticosterone levels [17]. In the second article of this thesis, long-term exposure to high-intensity infrasound did not increase the lipid content in hepatic tissue [18]. These findings are not consistent with type 2 diabetes and could point to a delayed response to this acoustic stressor and that a longer period of exposure, beyond 12 weeks, would be needed to observe changes in insulin production, insulin resistance and glucose tolerance [32]. In fact, type 2 diabetes pathophysiology develops over many years, with abnormal insulin sensitivity preceding the clinical diagnosis of type 2 diabetes by up to 15 years [33]. Considering this and considering that one rat month is comparable to three human years [34], we estimate that at least a high-intensity infrasound exposure of 20 weeks would be needed to observe insulin resistance and hyperglycemia in Wistar rats.

An interesting finding in the experimental study was that animals exposed to high-intensity infrasound had lower glucose AUC, thus higher glucose tolerance, than their counterparts kept in silence [17]. We argue that this higher glucose tolerance may be an acute reactive response, whether by the pancreas and/or by peripheral insulin receptors and insulin-regulated transporters, occurring in the presence of this aggressor. In fact, compensatory increases in insulin production and pancreatic islets beta cell mass [35] have been reported as a response to several injury models, whether by enhanced function or increase in the number of beta cells [36]. However, we did not find cellular alterations in the endocrine pancreas or differences on plasma insulin due to high-intensity infrasound exposure [17],

pointing to changes in peripheral insulin-sensitive tissues [24,25]. Future studies should clarify these pending questions on the peripheral insulin-sensitive tissues and explore the ultrastructural effects of high-intensity infrasound exposure in the pancreatic islets, through transmission electron microscopy [37].

Besides the negative effects of high-intensity infrasound mediated by the induced neuroendocrine stress response [20,23], this acoustic stressor is known to cause body vibrations and resonance of internal organs, leading to the physical disruption of tissues and organs [21]. In fact, the main consistent finding of previous experimental high-intensity infrasound exposure studies has been an increase of collagen fibers in the extracellular matrix without inflammatory signs [2-16].

Our experimental study did not show an increase in collagen fiber quantity in pancreatic islets with exposure to high-intensity infrasound [17]. Concerning pancreatic morphology, Wistar rats present four pancreatic segments with different macroscopic appearance, from a relatively compact splenic segment to a duodenal segment dispersed within the mesentery [38]. Since several of these segments are deeply located in the abdominal cavity [38], we argue that this deep location could protect the pancreas from body vibrations due to high-intensity infrasound exposure and thus from tissue damage. Previous experimental studies have shown that the adrenal gland of Wistar rats exposed to industrial noise rich in high-intensity infrasound, which is an organ anatomically close to the pancreas, also did not show fibrosis [16].

Pancreatic islet morphology is also slightly different across the species with humans presenting a heterogenous distribution of beta, alpha, and delta-cells, and rats presenting an islet with beta-cells concentrated at the islet core, surrounded by alpha and delta-cells [39,40]. This concentration of rat pancreatic beta cells in the islet core could also be an added protecting factor against the vibration induced by high-intensity infrasound exposure.

An increase in the collagen fiber quantity of pancreatic islets was, however, found in glucose intolerant animals [17]. This finding could be either be due to glucose intolerance or the result of the glucose intolerance method used in this study. Glucose intolerance has a known aggravating effect on pancreatic fibrosis caused by chronic inflammation from other pancreatic pathologies [41]. Concerning the used method, streptozotocin displays selective pancreatic beta cell toxicity and induces beta cell death [42]. This process induces tissue injury and local inflammatory response that activates pancreatic stellate cells with subsequent extracellular remodeling and deposition of collagen fibers [43,44]. These questions must be addressed in future studies with specific markers of pancreatic stellate cells [43,44].

Due to the complexity of type 2 diabetes pathophysiology [45,46], other elements should be focused on future studies such as gut-derived incretin hormones. These hormones play an important part in glucose metabolism and incretin effect is diminished or absent in type 2 diabetes [47]. Future studies should also use the oral glucose tolerance test along with the intraperitoneal route to determine glucose tolerance, as this would provide important information regarding the incretin effect in response to high-intensity infrasound exposure and allow a better understanding of the pancreatic insulin release [48].

The kidney also plays an important role on glucose metabolism, through gluconeogenesis, renal glucose consumption and glucose reabsorption in the proximal tubules [49]. This organ has gained attention in type 2 diabetes pathophysiology since impairment of these renal metabolic functions contribute to insulin resistance and hyperglycemia [45,46]. There is evidence that exposure to low-frequency noise, including infrasound, induces changes in Wistar rats' kidney glomeruli such as disruption of podocyte organization [50]. However, the biochemical and functional consequences of these morphological changes are yet to be determined. Future studies must address this point and assess both the kidney morphology [51] and function [52]

In recent years, changes in intestinal microbiota have been implied as a player in type 2 diabetes pathophysiology [53]. Although the exact mechanisms behind the impact of dysbiosis on host metabolism are yet to be deciphered at the molecular level [54], changes in bowel permeability, endotoxemia, interaction with bile acids and changes in the proportion of brown adipose tissue have been identified in several studies [55]. Previous studies have shown that chronic exposure to high-frequency noise alters gut microbiota composition from Proteobacteria to Actinobacteria, which may mediate progression to glucose dysregulation [56]. Since the effects of high-intensity infrasound exposure on intestinal microbiota are still unknown, future studies using molecular profiling methods, based on microbial 16S ribosomal RNA gene sequencing [57], are required.

The epidemiological study of this thesis aimed to investigate whether an association between exposure to urban noise and prevalence of type 2 diabetes mellitus existed in the city of Lisbon [58]. This association was also studied for other comorbidities related to type 2 diabetes, such as obesity and hypertension [59]. To the best of our knowledge, there are no previous studies that verified this association in Portugal.

In the third article of this thesis, no association was found between exposure to diurnal, evening, and nocturnal noise exposure and the prevalence of type 2 diabetes mellitus, obesity, and hypertension in Lisbon [58], contrary to other longitudinal studies, in other

cities, where authors found an increased risk of cardiometabolic diseases in populations exposed to noise [60-62].

This result may be due to the high prevalence of diabetes and uniformly high noise levels in all parishes of the city of Lisbon, preventing statistically significant associations, as well as some methodological limitations of our epidemiological study. First, due to the lack of similar studies in Lisbon, we opted for an exploratory cross-sectional pilot study to establish preliminary evidence for future longitudinal studies [63]. Second, a selection bias must be taken into consideration since there is a significant number of daily commuters in Lisbon [64] and because prevalence was based on electronic health records from primary care, excluding people with difficult access to healthcare and people covered by private insurance [65]. Third, the existence of different noise assessment methods can create a significant difference between estimated and real noise exposure [66].

An alarming finding in our epidemiological study was the high levels of diurnal, evening, and nocturnal noise in Lisbon, as high as 86.92 dB [58]. These noise levels exceeded the legally established limit in Portugal (Decree-Law Nº 9/2007, of January 17th), which define an exposure limit value of 55 dB for daytime noise and 45 dB for nocturnal noise in residential areas [67], and the recent World Health Organization guidelines for environmental noise in the European region [68]. According to these guidelines, at nighttime road traffic noise must be limited to 45 dB(A), railway noise limited to 44 dB(A) and aircraft noise limited to 40 dB(A) [68].

Particularly worrisome are the excessive nighttime noise levels found in Lisbon, ranging between 70.98 dB and 82.34 dB [58], since nocturnal noise exposure is strongly and independently associated with adverse metabolic effects such as obesity and type 2 diabetes mellitus [69].

Noise exposure during nighttime reduces sleep quality by shortening the times of phase 3 and 4 deep sleep and REM phases. Another consequence is a decrease in sleep duration by inducing frequent awakenings, such as vegetative, motorial and EEG arousals. All these changes in sleep disrupt the endogenous circadian rhythm by changing the secretion of activating hormones, or stress hormones, such as adrenal cortisol and catecholamine [70].

Several studies show that nighttime noise exposure directly and indirectly alters glucose metabolism by increasing hepatic glucose production and reducing glucose uptake in muscles, changing pancreatic α-cell and β-cell function, and changing the secretion of appetite-regulating hormone, such as ghrelin and leptin, from the gastrointestinal tract and adipose tissue, thus promoting food intake [71].

Although we did not find a correlation between noise exposure and prevalence of cardiometabolic diseases in Lisbon, these high noise levels observed throughout the day could lead to an increase in negative cardiovascular and metabolic health outcomes in the future [72], due to the increase in glucocorticoids associated with high-intensity infrasound exposure that we observed in our study [17]. As such, further studies are needed, particularly prospective cohort studies [73].

Some mitigation strategies have been proposed to reduce the burden of urban noise on type 2 diabetes risk [74,75]. Reducing road traffic noise can be achieved by combining measures such as replacing combustion engine cars with battery-driven electric cars, building of noise barriers along busy roads in densely populated areas, paving of road and highways with noise-reducing asphalt, developing and promoting low-noise tires and reducing speed limits [76].

Measures to reduce aircraft noise include planning air traffic routes overlying densely populated areas, banning nighttime flights, and implementing descent procedures that limit noise emissions. For railway noise mitigation several measures have been proposed such as replacing cast-iron block breaks with composite materials and implementing night bans [74].

Apart from these measures that limit noise sources, measures related to city and building planning have also been proposed such as setting homes and essential services away from heavily trafficked routes, designing houses to improve sound attenuation, installing sound-reducing windows, and increase green space areas [75,77]. Also, due to the risk of type 2 diabetes and other cardiometabolic diseases, regular health screenings should be implemented in noise-exposed populations to mitigate the burden of these diseases [78].

The recent and ongoing COVID-19 pandemic has led governments to impose national and local lockdowns to control the spread of SARS-CoV-2 [79]. Although some controversies and objections remain over the cost-benefit ratio of lockdowns [80], recent studies have observed a decrease in environmental noise [81-83] and individual sound exposure [84] associated to lockdowns. Whether this reduction in noise exposure is associated with potential future benefits for human health should be the focus of further studies.

Our morpho-functional and epidemiological study on the biological effects of high-intensity infrasound exposure allowed the following conclusions:

- Continuous exposure to high-intensity infrasound increases corticosterone levels. Due the known effects of glucocorticoids on pancreatic dysfunction and insulin resistance, this increase in corticosterone should lead to the consideration of long-term exposure to high-intensity infrasound as a risk factor for type 2 diabetes.
- The observed increase in corticosterone supports the role of high-intensity infrasound as an acoustic stressor and the neuro-endocrine stress response pathways as a route for the negative effects associated with high-intensity infrasound exposure. Since previous studies have provided extensive evidence of tissue fibrosis induced by body vibrations associated with high-intensity infrasound, we cannot exclude that this mechanism may be at play, even though pancreatic and muscle fibrosis have not been observed.
- Our study showed that a period longer than 12 weeks of high-intensity infrasound exposure may be needed to assess elements of type 2 diabetes pathophysiology in the Wistar rat animal model, since continuous exposure to high-intensity infrasound in this period did not induce alterations of plasma insulin, short-term glucose intolerance, decrease of skeletal muscle GLUT4 transporter, or increase in liver lipid content.
- Prospective cohort studies are required in the noisy city of Lisbon to establish whether a causal relationship exists between urban noise exposure and metabolic syndrome, since no cross-sectional association was found between exposure to diurnal, evening, and nocturnal noise and the prevalence of type 2 diabetes mellitus, obesity, and hypertension in this city.

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