VNIVERSITAT DE VALÈNCIA

FACULTAD DE MEDICINA
Departamento de pediatría,
obstetricia y ginecología
Programa de doctorado en medicina

Study of the relationship between life habits, circadian rhythm variations and metabolic disorders in childhood obesity

Estudio de la relación entre los hábitos de vida, las variaciones del ritmo circadiano y los trastornos metabólicos en la obesidad infantil

PhD thesis – Tesis doctoral

Marie Gombert

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Valencia, July 2022

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Tesis presentada para optar al Grado de Doctor por Doña Marie Gombert

Thesis submitted for the Degree of Doctor by Marie Gombert

Valencia, 3 de Julio del 2022

Directed by/dirigida por:

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CERTIFICA/N:

Que la presente memoria, titulada "Estudio de la relación entre los hábitos de vida, las variaciones del ritmo circadiano y los trastornos metabólicos en la obesidad infantil", corresponde al trabajo realizado bajo su dirección por Dña Marie Gombert, para su presentación como Tesis Doctoral en el Programa de Doctorado en Fisiología de la Universitat de València.

Y para que conste firman el presente certificado en Valencia, a 5 de Julio de 2022.

Fdo. Pilar Codoñer Franch

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Fdo. Stéphanie Bordenave Juchereau, en La Rochelle

Stéphanie BORDENAVE-JUCHEREAU

Abstract

Introduction: Numerous studies on nightshift workers evidence a link between circadian rhythms disruption and metabolic disorders. In parallel, other investigations mainly carried out on animal models or in human adults, show a link between the timing of life habits, insufficient duration and poor quality of sleep, and the development of obesity and its comorbidities. Nevertheless, little is known about the role played by these mechanisms in the current epidemic of childhood obesity. Therefore, we developed a study of the relationship between life habits, circadian rhythms of melatonin, and metabolism, in the context of childhood obesity. In complement, we also studied *in vitro* the impact of melatonin on the circadian rhythm of the genetic expression of adipocytes.

M&M: A transversal and analytical study was performed on 203 children between 7 and 16 years old, assigned to the control group or the overweight and obesity group. Anthropometric and clinical characteristics were collected, metabolic and inflammatory markers were measured in the plasma. Melatonin was assessed by immunoassay in saliva that was collected by the participants at home at three time points: 4h before sleep time, 2h before sleep time and after 1h of sleep. Questionnaires were used to collect information about life habits, chronotype, and life environment. The *in vitro* study was performed on human subcutaneous adipocytes, after 24h in culture with or without melatonin supplementation, RNA was extracted at four time points and quantified by rtqPCR for clock genes and metabolic genes.

Results: A lower increase rate of melatonin around sleep time was observed in children with overweight and obesity. In parallel, the *in vitro* study showed that adipocytes stimulated with melatonin present a greater amplitude in the circadian expression of clock genes and metabolic genes. In children, correlations and multivariate analysis showed interrelationships between variables from all the different categories: anthropometry, clinic, metabolism, inflammation, circadian rhythms, chronotype, life habits, and environment. A subsequent clustering analysis showed that among the individuals from the obesity group, a subgroup of individuals presented a better metabolic health in parallel of earlier life habits and a longer sleep duration. An algorithm showed that, among the parameters studied, poor sleep quality and duration and late meal timing were the strongest predictors of obesity. Another algorithm showed that melatonin nocturnal increase rate was as much a biomarker of obesity as classic markers such as adiponectin, omentin, ghrelin, or glucose.

Discussion: The present findings support that there is, in childhood obesity, a relationship between life habits, circadian rhythms of melatonin and metabolism. We observed that late chronotypes and life habits, short sleep duration, poor sleep quality, and eating more in the later part of the day, are associated to poorer metabolic health outcomes, in parallel of an altered nocturnal melatonin rise. Plus, the presence of screen devices in the sleep environment, as well as low education levels of the parents and precarious work situation of the father, appear as risk factors for the children in term of social jetlag, short sleep, late life habit, but also obesity and its metabolic alterations. These new findings emphasize the importance to address sleep, life habits timing, and life environment, in the development of measures of prevention and treatment of obesity.

Remerciements, agradecimientos

Ante todo, agradezco a los **miembros del jurado** el haber aceptado formar parte de ello, es un honor para mí.
Les deseo buena lectura.

Avant toute chose, merci aux **membres du jury** d'avoir accepté d'en faire partie, c'est un honneur pour moi.
Je vous souhaite une bonne lecture.

***

Quel est le point commun entre la neuroscientifique spécialiste du bonheur Manuela Martínez Ortiz, le moine bouddhiste Matthieu Ricard, frère Émile de Taizé, et l'auteure Delphine de Vigand ? La réponse, c'est que chacun d'eux m'a montré, à sa manière, que dire « **merci** », dans la vie, ça rend plus heureux. Du coup, chaque soir, j'écris mes trois « merci » de la journée. En une thèse, ça en fait, des mercis (5 carnets, plus de 3000 merci). Comme ça faisait trop long à inclure, en voici un résumé…

En premier, merci pour la chance de faire une thèse. Merci pour ces années passées à essayer de comprendre les humains, le dedans comme le dehors. Merci à la nature d'être si bien faite et passionnante à étudier, merci pour l'émerveillement ressenti à la découverte des mécanismes de « comment et pourquoi on stocke du gras ». Merci pour le moment où j'ai compris que je n'aurai pas assez d'une thèse pour comprendre, ni assez d'une vie, mais que c'est pas un problème.

Merci pour les opportunités qui sont venues avec, et pendant la thèse. Celle de retourner aux racines, vivre en Espagne, m'y sentir à la maison. Merci d'avoir été María en France, et Marie en Espagne. Merci pour Valencia, dont je suis tombée amoureuse en Erasmus et où j'ai été comblée de pouvoir revenir après le Master. L'odeur des fleurs d'orangers, la chaleur, Ruzafa, les palmiers, el Carmen, Blasco, Benimaclet, el Ayuntamiento, les « Signor Pirotecnic, pot començar la mascleta », le Turia, las monas de Pascua, le mocador des amoureux avec des fruits en mazapan dedans, le Marqués de dos Aguas enfin visité (après 47 échecs ça valait le coup), la heladeria la Romana (la millor del Món), la trop belle facultat de Medicina, les robes de falleras, le palau de la Musica, Aragon, Mestalla, le jeu de la fallera calavera, la calçotada, les aprems télétravaillées dans tous les jolis cafés de la ville, le Valencia Basket, les almuerzos con tortilla de patatas, la ciudad de Artes y Ciencias, et bien sûr, la Méditerranée au lever du soleil avant d'aller au labo, la Méditerranée quand il fait chaud après avoir ramené et rangé les échantillons de l'hôpital, la Méditerranée le soir pour passer un moment avec des copains… Merci aussi pour les cours : de trapèze et tissu aérien, de Valenciano, de guitare, de paddle/surf/plancha à voile, de salsa-bachata pour guiris-qui-apprennent-à-remuer-le-bassin, de couture, de natation pour se préparer au concours européen d'astronaute… Merci pour le café philo, les tandems de langue, les bénévolats à la croix rouge à l'arrivée de l'Aquarius, au festival du film des droits Humains, à créer un site web d'infos pratiques pour les nouveaux Erasmus et le carnet du Cuerpo Funcional, pour l'organisation de la rencontre de Taizé, et puis tout le Intimvestigation Project… Merci pour tout ça ! Je suis aussi très reconnaissante d'avoir pu faire un bout de ma thèse chez moi, à La Rochelle. Faire de la recherche avec celles qui m'ont appris ce que sont des acides aminés et des enzymes de restriction, et donner mes premiers cours sur les bancs où j'ai moi-même appris tout ça, quelle chouette expérience ! La pandémie et la fin de thèse à rallonge m'auront fait passer plus de temps que prévu à la maison. Quitte à être bloqué quelque part, autant que ça soit dans un bel endroit, avec l'océan et pleins de gens qu'on aime, non ?

En effet, c'est avant tout vous, les personnes avec qui j'ai fait ce chemin, vous l'avez rendu spécial. Alors à ceux qui ont été là le temps d'une étape, tout du long, ou qui l'ont rejoint et le continuent avec moi… Merci !
En premier lieu, bien sûr, merci à mes directeurs de thèse.

Merci **Pilar** Codoñer-Franch, modèle d'humain, de femme, de cheffe, de travailleuse, de rigueur, d'autorité naturelle, et, je veux le dire aussi, de tortilla (toujours la meilleure que j'aie mangée à ce jour, et mon n de tortilla consommées est plus grand que le n d'enfants recrutés dans cette étude). Merci pour l'exemple d'équipe que vous formez avec Elías, dans l'entraide et la bonne humeur. Merci de montrer qu'on peut être

cheffe de tout à la fac et à l'hôpital, et quand même donner priorité au plus important. Merci parce qu'avant la pandémie, la réunion d'équipe du mardi matin dans ton bureau, c'était chaque fois un des « merci » de ma journée. Merci pour toutes les opportunités, les conférences où tu m'as invitée, les personnes intéressantes que tu m'as présentées, les reviews écrites ensemble l'été. Merci de m'avoir permise de choisir mon sujet de thèse, conseillée tout le long, et m'avoir appris que « mes sucre, mes dolç !». Dommage qu'on n'ait pas toujours toujours réussi à se comprendre. L'essentiel est ailleurs, je te remercie et te souhaite une bonne continuation.

Merci Joaquin Carrasco Luna, alias **Ximo**, mi profesor de Erasmus, luego mi tutor de prácticas, y finalmente mi director de tesis. Merci por tu presencia diaria, tu buen humor, tu comprensión, tu entusiasmo por formar y transmitir, que hacen que sea tan agradable aprender a tu lado. Merci por la energía invertida en multiplicar los intercambios entre universidades europeas, y acoger siempre tan bien los internacionales como yo. Merci por aconsejarme sitios donde ir en Valencia ciudad y Comunidad. Merci por la Maroon 5 playlist de Jango mientras pipeteamos en el lab. Merci por la alegría que tienes cuando ves estudiantes tuyos querer seguir en investigación. Merci por enseñarme que hay que tener amigos por todas partes, sobre todo cuando en el laboratorio se te crecen hasta los enanos. Merci a ti también por el ejemplo de equipo con Ana, merci a los dos por compartir generosamente vuestras pasiones científicas, por hacerlas accesibles a todos, y siempre con una sonrisa. Gracies per tot això i mes, bona continuació!

Merci **Stéphanie** Bordenave-Juchereau, quand je t'ai interviewée en début de première année de fac pour mon PPP sur le métier d'enseignant-chercheur, je n'imaginais pas que tu deviendrais ma co-directrice de thèse. Merci d'avoir accepté, de m'avoir aidée à inventer une expérience compatible avec le travail de l'équipe BCBS, de m'avoir confié mes premiers cours de « Sciences du Médicament », merci pour le soutien en temps de galère et les conseils (je postulerai aux qualifs !). Bonne continuation.

Merci a todos los que habéis estado en el día a día en los años pasados en Valencia…

Primero a **Rosa**, merci por hacerme sentir, desde el día que entré a la facultad de medicina por primera vez, que siempre podría venirme a tu despacho a por una oreja atenta, un consejo, un abrazo, una solución a cualquier problema, o una información sobre "quien es tal persona" (porque para ello, en el mundo: hay Facebook. Pero en la Facultad, hay mucho más potente: hay Rosa). Merci por ser mi Mami de España, por cuidarme tanto. Merci por todo lo que haces para los demás discretamente y que les acaba cambiando la vida. ¡Me alegro por las próximas personas que entrarán por primera vez por este pasillo, o por cualquier pasillo donde estés tú, qué suerte tienen! Cuídate muchísimo :)

Vicky… Un merci a la vida por habernos puesto en el mismo lugar en el mismo momento, creo que la hemos sabido aprovechar. Mil merci por compartir, escuchar, cuestionar, enriquecer mis reflexiones sobre la ciencia a veces, sobre la vida muchas veces. Gente que entiende tan bien a las personas como tú, he conocido pocas, y por eso también fue un verdadero regalo charlar tantas veces contigo. Merci también por el día a día, por tu sentido de lo que es justo, por tu fiabilidad, por el dúo con Rosa en el lab y ahora como dragonas. Finalmente, un gran merci por compartirnos Chelva, ¡un recuerdo que se quedara grabado!

Un merci especial para **Clara**, una Golden-compi de piso y de día a día. Merci por las pizzas caseras y sus derivados serendipiteños, por cantar canciones de Måneskin conmigo, por las tardes de manualidades/decoración de la casa, por coserme una mascarilla de fallera, por el chun-chis en la playa, por las comidas-netflix, por las conversaciones super guays, por decir "feliz como una perdriz", "vámonos atomos", y "la fregación es un problema del futuro" … fue un placer vivir contigo! También te dedico especialmente la numeración de las presentes páginas, espero que te guste. Un merci a ti y a **Sara** también por enseñarme que Eurovision mola, por la mejor visita del Petronila's kindgom que incluyó la mejor narración de la campana de Huesca, merci a las dos por los momentos relajados en la Vitti o delante de un Brunch. Este último año Valenciano, a pesar de la muy presente palabra "toque de queda", fue mucho más guay gracias a vosotras. Merci!

Merci **Vanessa**, que gusto tener a una persona generosa, trabajadora, y complementaria, haciendo la tesis justo en paralelo a la mía! Merci por ayudar sin contar, por tu motivación, por los congresos juntas (¡los reales como el de trampa!), por siempre animarme con la tesis y mis proyectos locos. ¡¡Que acabes muy pronto y muy bien, mucho ánimo compi!!

Merci **Alvaro**, por todo el trabajo juntos en el lab, por tu serio, tu ayuda, y eficiencia. Merci por las mil etapas de desarrollo del protocolo DPPH desde el nanodrop hasta la caja de poliestireno, por compartir despacho en el "Bulevar nou" en la pandemia conmigo, por hablar a tu móvil mientras pipeteas cosas "oye Siri, ponme una alarma de aquí diez minutos", por comer chino mientras el anticuerpo se liga/el luminex lee, por compartir tus ideas y trucos de lab, por pipetear horas cantando la Maroon 5 playlist en Jango, por tu simpatía y también por tu sinceridad. ¡Que te vaya muy muy bien en Rotterdam, y en todas las etapas siguientes!

Un merci a las alumnas que se han venido de prácticas mientras estaba en el lab. Gracias por vuestras ganas de aprender, por ayudar con entusiasmo, por vuestro buen humor. **Maxime, Juliette, Laurine, Emanuela**, ha sido y será un placer cruzar vuestros caminos. ¡Siempre estaré encantada de ayudaros en lo que pueda!
Un merci también a los residentes, en particular **Sara, Izan, Mariola, y Pier**, por vuestras sonrisas, vuestras historias entretenidas de guardia, las buenas conversaciones, y más… Me alegro por vuestras pacientes, sé que están en buenas manos :)

Merci **Gemma** por tu ayuda con el nanodrop y por los almuerzos prepandemia, gracias a ti y a Vicky aprendí qué son las oposiciones en España. ¡Todo un programa! Te deseo lo mejor.
Merci **Loreto** et **Benja**, vous, vous n'avez pas besoin de traduction ! Merci de m'avoir aidée chaque fois que vous en avez eu l'opportunité. Bonne route et prenez bien soin de vous.

Jesús, María, Sonia, Layla, mis predecesores, merci por los consejos, por vuestro trabajo gracias al cual aprendí mucho, por el buen ambiente al cual contribuís en las comidas/almuerzos con el equipo, y merci porque viéndoos, he tenido aún más claro que quería hacer la tesis en POG. ¡Y qué felicidad que me toque ya! Os deseo un buen camino.
Merci a **Marina** y sus compis del IATA, a **Sandra** del Peset y de Glasgow, por vuestra ayuda en las peripecias del Luminex.

Merci **Gonzalo**, por tu profesionalismo y grandeza ante cualquier situación. Merci por estar, por tu escucha, tu comprensión, tus consejos, tus palabras. Merci, merci, merci. Fue un placer aprender de sueño y ritmos biológicos contigo, y qué guay que hayamos coincidido en el congreso de Roma. Merci por tu apoyo! Merci finalmente por ponernos en contacto con **Matías**, gran profesional que se ha hecho disponible para ayudarme a hacer una estadística de mejor calidad, merci.

Merci a las trabajadoras de pediatría del hospital, en particular **Belen**, **Ester**, **Carmen** y **Yolanda**, por vuestro trabajo y vuestra ayuda continua a lo largo del estudio. Merci también a las secretarias de la planta baja del Peset, que discretamente y siempre con una sonrisa, hacéis también que todo eso sea posible. Un merci a los **participantes del estudio y a sus familias**, merci por haber aceptado dormir peor para que pueda estudiar la hormona del sueño. Conoceros me motiva mucho a buscar cómo mejorar la prevención y el acompañamiento.

Merci también por todas las personas del mundo paralelo a la tesis.

Merci **Davide, Bob, Javivi, Jermin, Campo, Paco, Leti, Bogi, Sarah, Felix, Ivan, Irving, Fabri, Joel** y sus **cariñets,** los **italianos deportistas** del Turia, y a **Cécile** de las Arenas. Merci porque vuestro contacto también fue oportunidad para aprender y pasar buenos momentos.

Merci **Valli** por la convivencia en tu bonito piso, por enseñarme lo más Valenciano de Valencia, por la oportunidad del día de la mujer y la niña en la ciencia en Lliria, por el apoyo, por hacer que los platos queden bonitos, por tus amigas y el proyecto de investigación espárragos, por hacer cosas inspirantes lo mejor que puedes, por un día a día agradable… ¡Te deseo un buen viento!

Davinia, merci por los lunes por la mañana acogiendo a la semana juntas, y las despedidas del viernes, Taizé-style, me hizo mucho bien. Merci por ser otra persona que dedica su vida a buscar y transmitir, a tu manera. Merci por los guiños que hace la vida para señalarnos que estamos cerca. Merci por estar. Buen camino y hasta la próxima :)

Merci **Ana Peiro,** por hacerme sitio a tu lado en el escenario de la fábrica de Hielo, de las Naves (que, sepámoslo, no es lo mismo que La Nau…), y de tus videos. Me alegro de que vayamos juntas buscando formas de combinar nuestros amores de la ciencia y las mujeres. Muchos merci, y ahora… ¡A seguir en versión internacional!

Merci aux gens de l'étape au labo de La Rochelle.

Tout d'abord merci **Rémi** pour m'avoir partagé de ton temps, tes compétences et ton matos en culture cell, et puis aussi ton bureau, avec **Anne-Solène** et **Claire**. Merci et bonne continuation.

Merci à **Hugo** et **Béa**, mes grands copains de l'aventure BCBS. Merci pour votre accueil, vos conseils, et pour avoir assidument suivi les chapitres de mon épopée conclue par un adipocide. Merci pour les chouettes moments et avoir rendu si riche cette période. Bon vent à tous les deux !

Tous mes anciens profs, **Ingrid, Jean-René, Sophie, Isabelle L, Isabelle G, Jean-Marie, Stéphanie, Michel, Laurent**… Merci pour vos enseignements et de votre accueil au labo.

Merci à **Rodolphe**, **Maëlle**, **Arthur**, **Guig**… Parce qu'on se voit quand on se voit, mais j'aime mieux LR village en sachant que vous y êtes. Et merci de travailler à en faire un endroit encore plus chouette !

Merci aux **filles de la danse**, pour ce cocon bienveillant du jeudi soir.

A special merci for special people met during congresses in Austria, Italy, or Scotland…

Clare, merci not only for the great times we've had, but for the support, understanding and kindness. Merci again for introducing me to the amazing **Isabel**, **Tori** and **Roni**, you guys are walking sources of inspiration, and the weekend with you was fresh air in the middle of the last chapter of my thesis. A big merci, and to the pleasure to meet again.

Teja, talking about walking sources of inspiration… Merci so much for your generosity and support, it has been so refreshing meeting someone else who thinks "just jump into it, you'll see!". Merci Teja, I wish you the best.

Janice, I am so thankful I met you! Merci for your investing your energy in making people healthier, for your different background which gives you a different perspective and enriches the collaborations. I am looking forward to seeing what comes out of all of this, and to contribute when I can! Best of luck.

Ken and **Chris**, although it was impossible to come in person, I am very thankful for the opportunity of working with you, it has been very rich in learning. Merci! I look forward working in the US and hopefully seeing you around!

A merci to **EASO** for the opportunity of integrating the Public Health Task Force and attending such interesting events. Merci to **Harry Rutter** for transmitting your vision of public health management. Merci as well for sharing with me your personal ground rules regarding ecology and ethics when I was struggling to find my own.

Merci à **Taizé**. Merci à frère Roger pour avoir exprimé des intuitions qui m'accompagnent chaque jour. Merci à chacun de vous, les frères, qui faites vivre cette source. Je suis si chanceuse de pouvoir y venir de temps en temps, me rafraichir et me rapprocher de l'essentiel. Merci pour toutes les fois où vous m'avez accueillie, permise de vous aider, d'apprendre auprès de vous, rencontrer de si belles personnes avec qui je suis maintenant amie. Merci pour le soutien. Merci parce qu'avoir autant de tontons, c'est si riche que ça ne peut qu'être un cadeau du ciel. Merci aussi de vous laisser accueillir. Merci parce que vous aussi vous êtes des chercheurs, par votre vie. Merci pour l'inspiration et l'apaisement que ça m'apporte. Chercher ensemble, même loin, quelle joie !

Aux copains qui sont loin mais restent proches, vous êtes des pépites. Merci **Juju, Popo, Gagar, Julo, Teo, Camille, Kinga, Camille** et **François**, **Geoffrey, Lucile, Jeanne-Mallet**, pour votre bienveillance, votre présence par petite goutte, parfois par surprise, qui fait du bien.

Merci à mes piliers, les indéboulonnables qui sont là qu'il pleuve, qu'il vente, qu'il neige.

Ou qu'il fasse 100000°C en Andalousie. **Arianou** merci beaucoup. Fidèle au poste depuis tant d'année, j'suis si reconnaissante qu'on continue de se creuser les méninges ensemble pour se comprendre et comprendre les autres, c'est un cadeau.

Merci **Jérémie** pour cette riche amitié. Merci pour ta recherche à toi aussi, merci de me donner une petite fenêtre pour la suivre, et de m'aiguiller dans la mienne !

Parce qu'il y a des choses qui ne s'expliquent pas. Certaines personnes, qu'on les voit beaucoup ou pas beaucoup, elles sont importantes, c'est tout. Comme **Martin-Mallet** par exemple. Merci toi :)

Jeannette, ma voisine de partout et copine de toujours. Merci d'avoir toujours la même rigolade dans la voix, merci que ça soit toujours aussi simple que si on s'était quittées la veille, d'être présente même à distance quand c'est fun comme dans les coups durs. Merci !

Merci ma **Sasha**, pour tout ce que tu vois en moi, pour avoir souvent su m'aider à me remettre en question avec une grande douceur, pour ces heures à faire des listas, pour les merci commencés ensemble, pour les moments à faire les reines du dancefloor sur du reggaeton, pour ce truc magique de penser à ce qui est bon pour l'autre complètement indépendamment de ce que ça implique pour soi, pour avoir quelqu'un qui apprécie autant que moi la tortilla, le soleil et la mer… Simplement merci qu'il y ait une toi dans ma vie :)

Merci ma **Nina**, pour toutes les fois où tu apparais dans mes Merci. Merci d'être tout simplement là à chaque moment, les plus petits comme les plus grands. Merci pour cette année supplémentaire à être « Marie et Nina » à LR. Merci de chercher, toi aussi, et de le dire avec ta vie. Ça va mieux en sachant qu'où que je sois, tu es là. Merci d'être mon amie.

Merci, pour finir, à ma famille.

Merci **Païs**, pour les heures passées avec moi à observer la nature et faire des expériences à Lacanau, à planter des graines curiosité et de méthode scientifique. Merci **Mamina** pour tout ce que tu m'as transmis, petite et encore maintenant, merci de toujours essayer de comprendre les autres, et de semer une ambiance joyeuse et motivante là où tu passes.

Avec mes oncles, tantes, cousins et cousines, merci pour tout ce que vous m'avez apporté. Merci **Victoria** pour les chouettes discussions. Merci **Marick** et **Hervé** pour votre soutien en période de coup dur, et pour les bons moments l'été. **Gaëlle** merci d'avoir si souvent pris soin de moi à Toulouse, et de te construire un nid douillet où il fait bon aller se réfugier et/ou faire la fiesta.

Merci ma tata **Monique**, je suis heureuse qu'on ait pu passer du temps ensemble pendant ma période toulousaine, merci de m'avoir partagé un peu de ta belle vie. **Bertrand** et **Fred**, merci pour votre soutien et votre gentillesse. **Jean-Marc** merci beaucoup, ça m'a fait beaucoup de bien que tu sois là pour moi quand j'en ai eu besoin. Je suis heureuse d'être ta filleule.

Merci finalement à **Bon-Papa**, premier Gombert à s'être intéressé à la mélatonine, je te fais un petit clin d'œil scientifique. Je vous remercie, **Bonne-Maman** et toi, pour ce que vous m'avez transmis.

Enfin, merci **Oliv**, mon premier coéquipier, avec qui j'ai tant en commun. Merci pour tout ce que tu m'apportes, merci de prendre soin de notre lien, merci pour ton estime et ta confiance qui me donnent la force de faire des trucs chouettes.

Merci **Clem**, être ta grande sœur, quel cadeau. Merci de me donner envie d'être une meilleure personne, de m'avoir montré que si les diplômes étaient vraiment au mérite de quantité de travail, toi t'aurais 12 thèses. Merci de ta gentillesse et ton intégrité, et merci pour toutes ces trop belles illustrations qui me donneront toujours envie de rouvrir ce livre.

Mes mécènes inconditionnels, quelle chance j'ai de vous avoir, pour cet aspect-là aussi, merci.

Papou, merci de vivre sur ta planète du soin et de l'aide aux autres. Merci de ta tranquillité et ta confiance, de suivre ce que je fais scientifiquement et m'envoyer des publis, de faire de ton mieux. J'ai trop d'chance, et c'est pas un problème.

Maman, merci d'être un pilier, merci d'être là tout le temps, sur tant de plans. Sans tes boutades, tes reniflages, tes « moi je ferais ça à ta place mais tu fais ce que tu veux », tes câlins, tes WhatsApp, tes encouragements, tes petits mots dans la valise, tes appels, tes bisous, jamais j'aurais pu arriver jusque-là. Ça y est, la fin approche, enfin… Merci mille fois, pour tout, Mamounette.

Merci **Sylvain**, pour ta douceur, ton sourire, tes encouragements. Merci pour l'ouverture que tu m'apportes, merci de m'écouter parler de gras et de comment on pourrait prévenir l'obésité, merci de me partager tes passions, merci d'avoir pleins de questionnements existentiels et d'être aussi animé que moi, merci de me faire danser tout le temps alors qu'on sait pas danser, merci pour nos discussions enflammées sur tout et n'importe quoi, merci de me faire rire, d'être mon amoureux mais aussi mon meilleur ami, et merci d'avoir envie de partir à l'aventure à l'autre bout du monde avec moi.

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Abbreviations

BMAL1: and brain and muscle aryl hydrocarbon receptor nuclear translocator-like 1
BMI: Body mass index
C/EBPα: CCAAT/enhancer-binding protein α
CLOCK: circadian locomotor output cycles kaput
CRY1 and **CRY2**: Cryptochrome
CT: Cycle Threshold
ΔCT: Difference in cycle threshold compared to β-actin
DPPH: 2,2-diphenyl-1-picrylhydrazyl
E-box: enhancer box
ELISA: enzyme-linked immunosorbent assay
Γ-GT: γ-glutamyl transferase
HDL: high density lipoproteins
HOMA index: Homeostasis Model Assessment of insulin resistance
IL-6: Interleukin-6
LDL: low-density lipoproteins
MCP-1: monocyte chemotactic protein-1
PAI-1: plasminogen activator inhibitor-1
PCA: Principal Component Analysis
PER1, **PER2** and **PER3**: Period genes
PPARγ: peroxisome proliferator-activated receptor γ
RBP: Retinol binding protein
ROC: receiver operating characteristic
ROR/RZR: retinoid orphan receptors/retinoid Z receptors
SD: Standard deviation
vLDL: Very low-density lipoproteins

I. Introduction

I. 1. Childhood obesity, a public health issue of the modern era

Overweight and obesity define an abnormal or excessive accumulation of fat in the organism, which present risk for health.

Several methods exist to characterize this excessive accumulation of body fat mass, taking into consideration that the body composition evolves with age and puberty stage and is different according to the sex. On one hand, the lean and fat masses can be accurately measured by bioelectrical impedance analysis, nevertheless, this method requires expensive materials, limiting its availability worldwide. On the other hand, the body weight is easy to measure everywhere, and 70–85% of the body weight variations is in fact linked to the fat mass variations.[1] Therefore, an index based on the body weight for a given body height informs quite reliably on the body fat mass. The formula of the **body mass index** (BMI) is BMI = mass in kg / (height in m)^2. In adulthood, from a BMI of 25kg/m^2, an individual is considered to present overweight, and passed 30kg/m^2, to present obesity. During growth, the body mass and height are not proportionate so these criterions cannot apply. Therefore, the WHO indicates that the reference to use is the number of standard deviations (SD) to the mean BMI in a population of a same age and sex. Above +1SD, a child is considered to present overweight, and above +2SD, to present obesity.[2,3] In complement, other anthropometric characteristics as the circumferences of the arms, waist, and hips, can allow a more accurate prediction of the comorbidities associated with high BMI.[4]

The prevalence of obesity is increasing worldwide, including in childhood, with a rise from 4% in 1975 to 18% in 2016 in the children between 5 and 19 years old.[5,6] This global trend is represented in

Figure 1 with the increasing proportion of the overweight and obesity (in red and yellow) in the world population. This figure illustrates the trends in females, which look very similar as the equivalent figure of trends in males.[7] The overweight and obesity increase is frequently qualified as a global pandemic[8,9], and Southwestern Europe is touched in an even higher proportion than the global.[7]

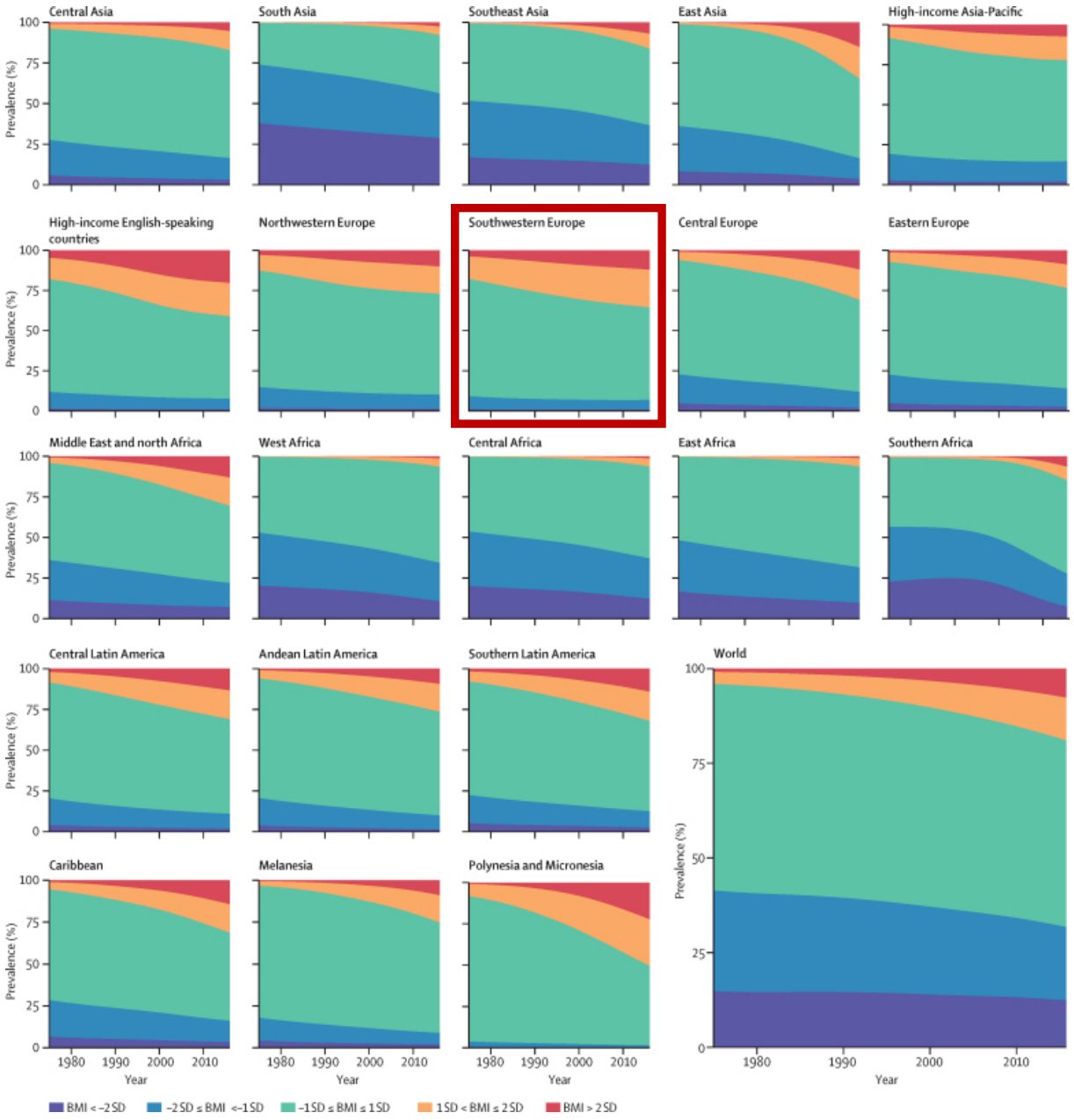


Figure 1. *Worldwide trends in obesity, overweight, normal weight and underweight in females.*

Adapted from NCD-RisC, 2017 with permission from the authors[7]*, Open license: Attribution 4.0 International (CC BY 4.0).*

According to the developed by the World Obesity Federation, this trend is very likely to persist in the future. In their Global Atlas on Childhood obesity, they predict that the number of children age 5-19 years old living with obesity will rise of 60% by 2030.[10] This same report also estimates the chances of countries to be meeting the WHO target of "no increase in childhood obesity prevalence by 2025". As an example, for Spain, the chances are estimated at only at 18%. This situation is preoccupying considering overweight and obesity result each year in millions of deaths[11], and the concerned individuals are also more vulnerable to die or develop more complications in other pathologies as clearly observed in the Covid-19 pandemics.[12–14] The

children with overweight and obesity have an increased probability to present obesity in adulthood,[15,16] but also other diseases such as type-II diabetes, steatohepatitis, cardiovascular diseases and cancers.[17–21] On the short term, this burden of ponderal overload is also associated with weakened cognitive functions, and these children are more prone to depression, impaired body-image, low self-esteem, stress, weight stigma, eating disorders and poor quality of life.[22–24]

To efficiently prevent obesity development, it appears essential to reach an accurate understanding of the elements favouring obesity development as well as the biological mechanisms involved. In the recent years, the importance of timing and rhythms in obesity physiopathology has been investigated, although substantially more in adulthood than in childhood. This work constitutes the first approach in children integrating together life habits, circadian rhythms, and metabolic homeostasis, and showing their relationship in childhood obesity.

I. 2. Metabolic health and life habits

The metabolism is defined as the sum of the reactions that take place within a living organism and that provide the energy necessary to live. First, the parameters conditioning the energy intake and energy expenditure are presented (**Figure 2**), followed by energy storage and a particular introduction to the adipose tissue physiology. Finally, the concept of homeostasis in energy, fat, and weight is discussed.

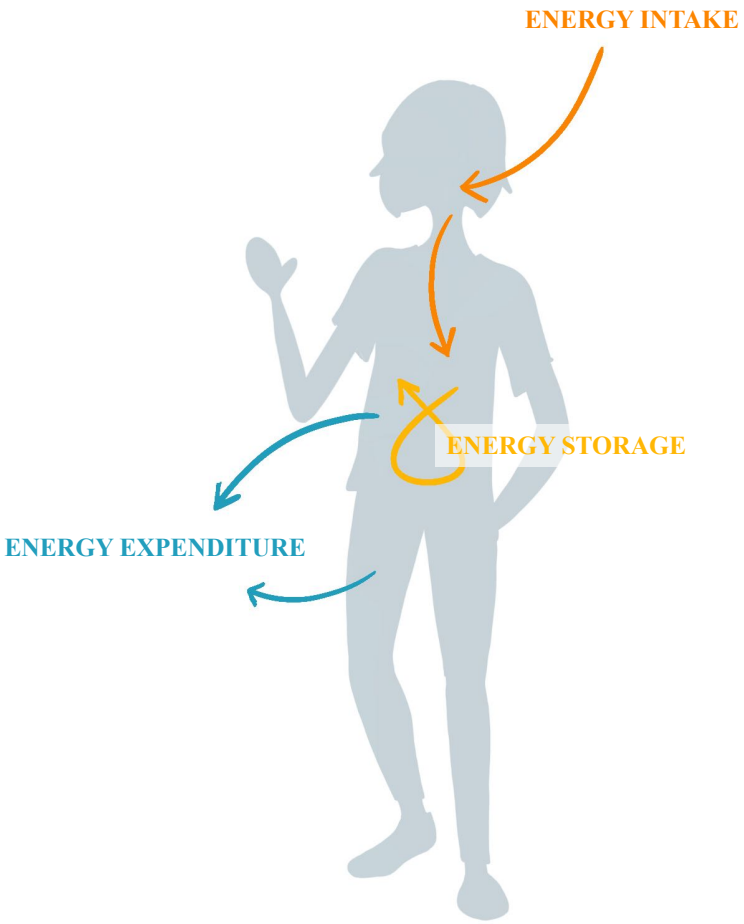


***Figure 2**. Schematic representation of the energy metabolism*

Scheme created by Marie Gombert and drawn by Clémence Gombert

I. 2.1. Energy intake

Energetic molecules as carbohydrates, lipids, and proteins, are absorbed by the organism through the digestive tract. The total energy intake of an individual depends on the regulation of the ingestion and the absorption processes.

I. 2.1.1. Nutritional ingestion

The occurrence of the nutritional ingestion, as well as the quantity and quality of nutrients ingested, is a behaviour which results from the integration by the central nervous system of biological, psychological, and external drivers.

a) Biological signals

The action of nutritional ingestion integrates biological signals from the organism indicating the energy available and the resources needed, in terms of quantity and quality.

The digestive system, storage organs, and the central nervous system, dispose of a variety of sensors which continuously detect the levels and type of nutrients that are ingested, stored, or available in the blood circulation. This information is communicated through nervous and endocrine signals to the central nervous system and gets integrated in the brain stem and in the hypothalamus. The arcuate nucleus of the hypothalamus is a key location of the regulation of the ingestion. This nucleus contains two groups of neurons with opposite actions: stimulating or inhibiting the hunger feeling. These neurons are themselves stimulated or inhibited by peripheral signals, and act by communicating with other nuclei of the hypothalamus such as the dorsomedial hypothalamus, lateral hypothalamus, paraventricular nucleus, ventromedial nucleus.[25] The peripheral signals involved in the arcuate nucleus activity are of endocrine and nervous nature:

- **Endocrine signals**

When the digestive tract is empty, ghrelin is produced by the cells of the stomach and proximal duodenum. It reaches high levels before meals which fall during the postprandial period.[26] This peptidic hormone acts directly as orexic factor on the arcuate nucleus. Leptin, in the contrary, has an anorexigenic action. It is produced by the adipose tissue in higher levels during the post prandial periods, and its synthesis is proportional of the volume of fat in the body.[27,28] Its action on the arcuate nucleus triggers the satiety feeling, which consequently reduces nutritional ingestion.[28] In addition, insulin, produced by the pancreas consecutively to a rise in glycemia, acts in a similar manner as leptin on the hunger regulation through hypothalamic signalling.[29]

The control of appetite and satiety occurs at the level of the nervous system and is integrated in the arcuate nucleus of the hypothalamus, in which there are two groups of neurons with opposing effects. On one hand, the neurons expressing the neuropeptide Y and the agouti peptide, promoting the consumption of foods that increase appetite and decrease energy expenditure. On the other hand, the proopiomelanocortin, and the cocaine- and amphetamine-regulated transcript neurons, with the opposite action.

- **Nervous signals**

Direct signal from the digestive tract is communicated to the central nervous system through the vagus nerve, for instance from the baroreceptors of the stomach which sense distensions, and the detection of glucose and lipide levels in the liver.[28] The distention of the stomach caused by food ingestion is sensed by baroreceptors, which in turn inhibits the ghrelin synthesis and therefore decreases the hunger feeling. Circulating nutrients are also detected directly in the brain to regulate nutritional intake. Glucose-sensitive neurons have been identified in the arcuate nucleus and the paraventricular nucleus.[30] Plus, lipids presence in the central nervous system may also diminish the nutritional ingestion through decreasing the expression of orexigenic neuropeptides in the hypothalamus.[31] Finally, the repeated lack of an essential amino acid in the diet is known to induce a repulsive behaviour towards foods which lack this amino acid.[32] This mechanism show that not only the quantity but also the content of the meals are driven by biological signals.

In summary, the human organism senses the levels of energy available and produces signals aiming to regulate energy ingestion through a modulation of the hunger and satiety feelings.

b) Psychological signals

The human feeding behaviour in does not only depend on the biological needs in energy. An important part lies in the integration of these signals, together with psychological signals.[25] These psychological signals include the reward system, which mediates the behaviours associated to pleasure-seeking associated with food intake.[33,28] Numerous other psychological parameters influence the nutritional intake such as emotions[25], memory[34,35], aversion or attractivity toward foods[36] the attention toward foods[37], the cognitive control of decision making[25,38] which is shaped by social trust.[39] Finally, in emergency conditions such as trauma, illness or injury, the hunger signal can be switched-off.[40]

c) External signals

The biological and psychological signals regulating feeding are modulated by elements from the environment. The availability of foods, the influence of peers, social norms, culture, or education, also condition the feeding behaviours.[41–43] In childhood and particularly at younger ages, the nutritional context is set by the caregivers. Consistently, the nutritional ingestion of the children is related to their own consumption and awareness.[44] The policies applied in the country regarding marketing and signalling of

unhealthy foods, together with sensibilization campaigns, also influence the awareness of the parent and the child.[45] Consecutively, it changes the content of the supermarket cart, the fridge, and the plate.[46,47]

In summary, the feeding behaviour is the result of the integration of biological signals, psychological signals, and environmental signals. All their different components form a network in which they regulate each other.[25]

I. 2.1.2. Nutritional absorption

The energy intake of the organism depends on the quantity and quality of nutrients that are not only ingested, but also absorbed. From the arrival in the mouth, foods undergo a succession of mechanical, chemical and enzymatic steps act in chain to be transformed into nutrients that are absorbable by the intestines.[48] In average 90% of the nutrients ingested are absorbed, but through the modulation of the motility of the digestive tract, and the expression of transporters at the surface of the enterocytes, the absorption can be regulated. This regulation has been shown to be influenced by hormones[49], digestive peptides[50,51], mental stress[52], the integrity of the intestinal barrier[53], and by the gut microbiota.[54,55]

It is to note that the mechanisms regulating ingestion and digestion are interconnected. For example, the sight, smell, or taste of a food trigger a cognitive and psychological response which stimulates the hunger feeling. In parallel, it signals the digestive system to prepare for food consumption, starting with the well-known saliva secretion increase in the mouth, as well as the production of digestive enzymes, hormones and motility regulation.[25]

As summarized in **Figure 3**, the nutritional intake depends in the energy ingested and absorbed. These processes depend on biological, psychological, and external signals.

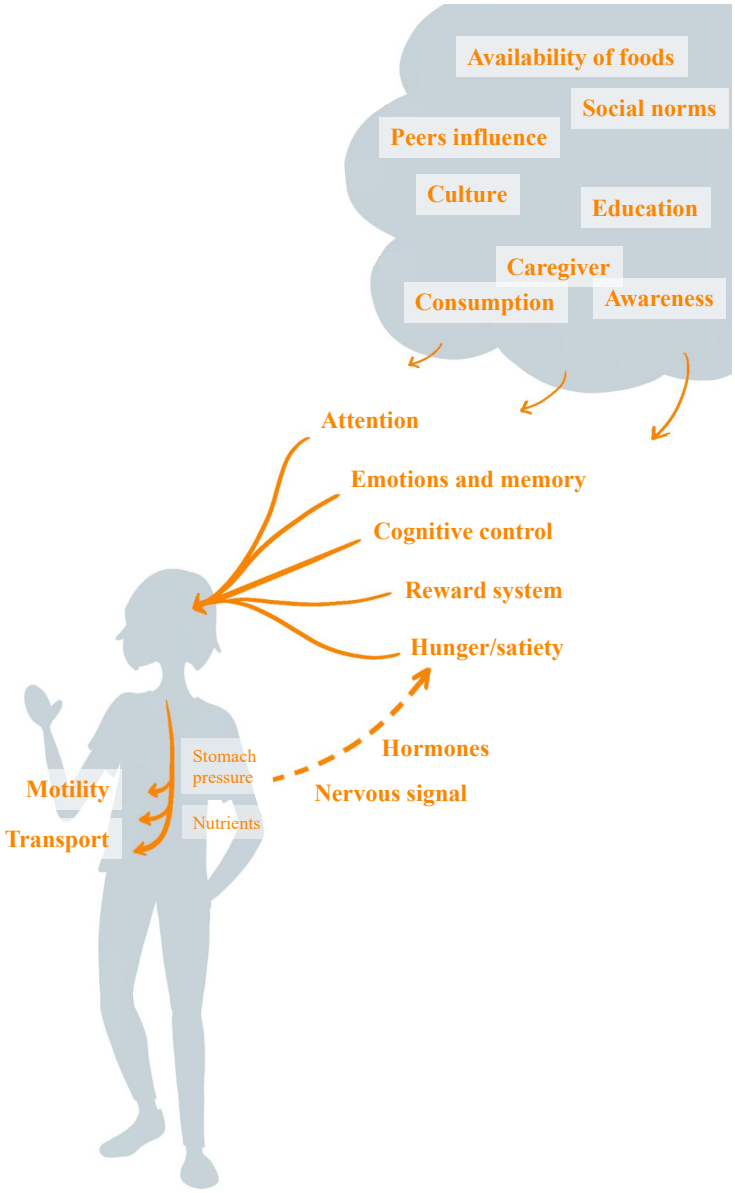


***Figure 3**. Summary of the elements influencing energy intake*

Scheme created by Marie Gombert and drawn by Clémence Gombert

I. 2.2. Energy expenditure

The nutrients absorbed are the substrates of biochemical reactions which generate an energy molecule that can directly be used by the cells: the adenosine triphosphate (ATP). The glycolysis, the Krebs cycle and the cellular respiration are reactions during which ATP is produced. The organism needs to provide each of its cells with the necessary energy for its basal maintenance, for the digestion, the temperature homeostasis, the specific needs of physical, mental activities, sleep, and in the case of children, growth.

I. 2.2.1. Basal metabolism

The basal metabolism corresponds to the energy expenditure necessary for the individual to survive, without doing any activity, but not asleep. It is quantified through the measurement of the basal metabolic rate, the metabolic expenditure at rest per unit of time. At rest implies being fully awake but lying down, fasted for 10 to 12 hours, in a thermo-neutral environment and free of emotional stress.[56] The average estimated percentage of basal metabolic rate per organ is of 27% for the liver, 19% for the brain, 18% for the skeletal muscle, 10% for the kidney, 8% for the heart, and 19% for the other organs. basal metabolic rate is lower in girls compared to boys, and decreases with age.[57] Lean mass is closely related to energy expenditure even at rest[58], and through its increase, regular physical activity practice increases the basal metabolic rate in the long term.[59]

I. 2.2.2. Digestion

The digestion process by itself consumes energy for the motility, secretions of fluids, enzymes, hormones, immune factors, and compounds of the mucosa. In addition, expenses are related to the postprandial metabolism of the nutrients. These processes elevate the energy expenditure of the organism, and the variation depends on the quantity and quality of the meal.[60]

I. 2.2.3. Temperature homeostasis

As human are homeotherm animals, the body temperature is actively maintained constant, around 37°C. The hypothalamus receives information from cutaneous and inner temperature sensors, and triggers adaptative responses. The body temperature decreases through radiation, evaporation of the transpiration, convection, and conduction of temperature. The vasodilatation of the peripheral vessels potentiates these mechanisms by increasing the quantity of blood present close to the body surface and participating to the temperature exchanges. Children present a higher body surface area relative to their mass compared to adults, therefore this mechanism is even more efficient for them.[61] Plus, other mechanisms are activated to regulate

body temperature in addition to this vasodilatation/vasoconstriction system, such as the horripilator muscles, muscular shivering, and the thermogenesis in the brown adipose tissue.

I. 2.2.4. Physical activity

a) Effect on metabolism

Physical activity increases the energy expenditure via muscular contraction, increase in heart rate and breathing rate. Body weight impacts the energy expenditure of physical activity as a heavier body cost more energy to move. The quantity of movement, frequency and duration spent in activity matter. In addition, the type of physical activity practiced conditions the level of intensity of the effort. School-aged children are frequently enrolled in extracurricular physical activities which intensity levels vary from an activity to another. A way to estimate physical activities intensity is through classification based on the type of mechanical action done by the muscles, from which can be deduced the level of cardiovascular response demanded by each sport.[62] In childhood, the energy expenditure related to activity is lower than in adulthood. An explanation may be that their body being smaller and lighter, they need fewer energy to move it.[63,64] In addition, studies with actigraphs show that compared to adults, children spend more time in activity per day, and their activity patterns differ too, characterized by intermittent, short and intense moments of activity.[64]

b) Intrinsic and environmental drivers of physical activity

As for the nutritional intake, the quantity of exercise varies according to different factors from biology, psychology, and environment. Genetics contributes to predispose for physical activity in daily life, and for the quantity of energy expenditure induced by an activity.[65] These predispositions may correspond to visible interindividual differences in personality or tempers, with certain children appearing to naturally be very dynamic and mobile while others are more calm. The body weight also modulates the energy expenditure as the same movement requires more energy if the body is heavier. In addition, the **social environment** influences the level of physical activity of the child through the direct contact with other persons[66], indirect influence through medias[67], the sociodemographic characteristics[68,66], and the urban or rural life environment[69,70].

I. 2.2.5. Mental activity and emotional status

Complex tasks requiring intense mental focus (such as reading a PhD thesis), may seem very energy consuming, but it appears that this process induces a very little increase in energy expenditure.[71] As the brain function already constitutes 19% of the basal metabolic rate, a possibility is that the increase in activity induced by the mental task represents a very small increase at the scale of the brain function, and is hardly detectable on the global metabolic rate. Nevertheless, emotions and mental stress do increase the energy expenditure significantly. Mental stress was shown to increase about 12% the metabolic rate.[72]

It is to note that the time spent doing mental activities are generally sedentary. From listening to the teacher at school to playing videogames, these activities have an impact on energy expenditure by the limited amount of movement performed during their duration. Studies tend to show that the effect on the energy expenditure may differ according to the types of screen devices. For instance, the use of television is thought to induce the most sedentary behaviour and is associated to an increase in snacking habits, while active videogames are estimated to induce the same level of energy expenditure as walking.[73] Gender is also associated with different patterns and types of screen use.[68]

I. 2.2.6. Sleep

Energy expenditure decreases during sleep. Therefore, the sleep duration changes the time spent in "low energy mode", which impacts the total energy expenditure over a day.[74] The energy expenditure varies according to the sleep stage. The lowest energy expenditure occurs during stage 2 and slow waves sleep, whereas the phases of rapid-eye-movement (REM) sleep and stage 1 are higher but remain significantly lower than during the wake period. Sleep also indirectly impacts energy expenditure during the day.

As feeding and physical activity, sleep behaviour results from biological, psychological, and external signals. Physiological factors play a role in sleep arousal, for instance the accumulation of adenosine in the basal forebrain[75], and the melatonin onset which will be further described and prepare the organs for the sleep period. But sleep is also conditioned by psychological elements of decision making, mental and emotional state, social schedules (in particular school and work time)[76], and the direct sleep environment including light, noise, presence of other individuals or animals…

In addition to its impact on energy metabolism, sleep constitutes a temporal niche for numerous key health functions that need to occur every day when the organs are at rest. For instance, cellular turnover in the organs takes place essentially while they are at rest during several hours, and was shown to be controlled by the circadian clock.[77,78] It is also the time of repair mechanisms to be performed, and sleep quality is necessary for inflammation levels to remain at healthy levels.[79]

I. 2.2.7. Growth

During childhood, growth constitutes an additional path of energy expenditure. Passed the first months of life, the daily energy expenditure mobilized for growth is negligeable compared to the basal metabolic rate, the thermic effects of meals and the physical activity.[80] Nevertheless, puberty is a period of important changes in metabolism regarding hormonal activity, body composition and psychological development, which influence activities and, indirectly, the energy expenditure.

As summarized in **Figure 4**, the energy expenditure depends on the basal metabolism, thermic regulation, digestion, growth, mental activity, and physical activity. These last elements are influenced by biological, psychological, and environmental elements.

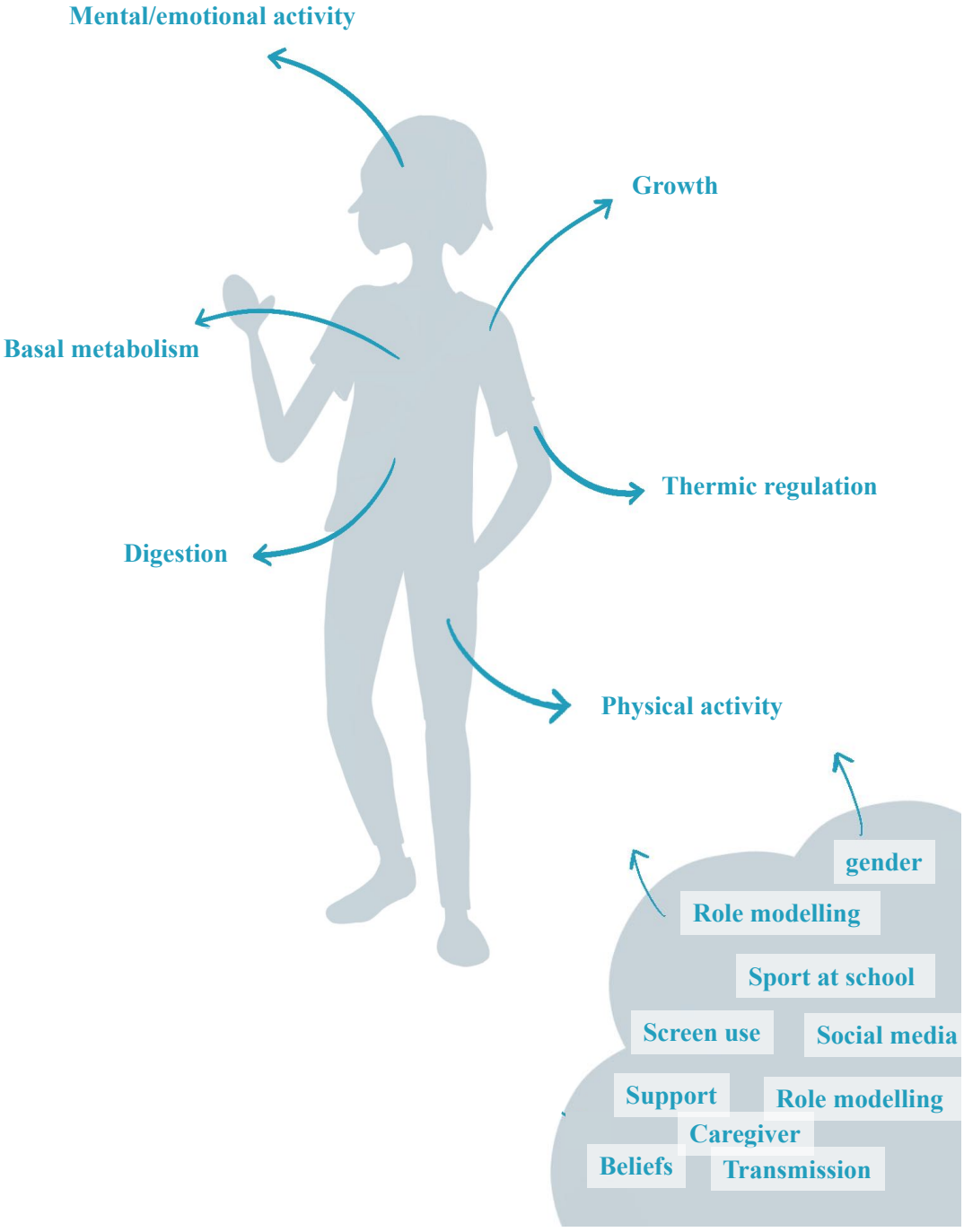


***Figure 4**. Summary figure of the elements influencing nutritional expenditure*

Scheme created by Marie Gombert and drawn by Clémence Gombert

I. 2.3. Energy storage

Energy is continuously expended, but energy cannot be continuously absorbed. Therefore, forms of energy storage are needed for survival.

I. 2.3.1. Cells, liver, muscles

A form of energy storage in the cells is by the presence of ion gradients also known as chemiosmosis. Nevertheless, the essential part of the energy is stored in form of covalent chemical bonds in diverse energetic molecules which characteristics and location are strategically distributed so that energy can be mobilized and expended in all situations. Energy is instantaneously available in the cells in form of ATP, which can be quickly restored by transfer from creatine phosphate during an intense effort. The glycogen, a branched glucose polymer is another form of energy storage which is mainly present in the liver and the muscles to be quickly remobilized. Finally, the triglycerides constitute the biggest energy storage. Formed a glycerol molecule is esterified by three fatty acids, triglycerides are present in the muscles and the liver in small quantity, and mainly stored in the adipose tissue.

I. 2.3.2. Adipose tissue

The adipose tissue plays an important role in metabolism through **energy storage** and **endocrine activity**. The normal proportion of body fat mass changes with age and puberty. At 6 years old, the body fat percentage is already higher in girls compared to boys. In boys, the adiposity increases during childhood until reaching a maximum before teenage growth spurt onset around 10-11 years of age and then decreases. In girls, the fat mass increases continuously during the entire childhood and adolescence.[81,82]

Body fat is composed of three main types of adipose tissue: the white adipose tissue, the brown adipose tissue, and the adipose tissue from the bone marrow.

The **white adipose tissue** is mainly composed of **adipocytes** which store the triglycerides in a single big lipid droplet which pushes the nucleus to the periphery of the cell, it appears mainly white in histology, hence its name. The white adipose tissue is located in part under the skin and in part inside the abdomen. The **subcutaneous white adipose tissue** plays a thermic and mechanic protective role and is also a source of energy. The **visceral white adipose tissue** allows triglycerides storage directly in the abdominal cavity and is also more metabolically active. The oestradiol produced mainly by women from puberty until menopause favours the accumulation of subcutaneous rather than visceral adipose tissue. This explains an important part of the **sex-difference in the repartition of fat accumulation** in men versus women in reproductive age, with

men presenting an increased belly volume and women an increased hips volume. Subcutaneous deposit is less associated with metabolic and cardiovascular disorders than visceral fat.

The second category is the **brown adipose tissue**. Its adipocytes contain several small lipid droplets and an important number of mitochondria which matrix contains iron and confers the brown colour. The mitochondria of the brown adipose tissue contain a high level of the uncoupling protein-1 (UCP-1) which uncouples the respiratory chain to produce heat. This thermogenic mechanism activated by cold exposure is particularly important in new-borns who are not able to shiver yet but is less useful in later life. Nevertheless, it remains present in most individuals in the neck and upper chest, and its activation improves insulin sensitivity and glucose homeostasis.[83,84] The brown adipose tissue is more metabolically active, has a higher density of mitochondria and blood vessels, generate more heat when activated, and a higher water-to-fat ratio compared to white adipose tissue. These characteristics allow to distinguish them using techniques as PET/CT scan, MRI, functional MRI, or thermal imaging.[85,86] Some researchers investigate pathways to trigger a "browning" or "beiging" of the white adipose tissue to improve metabolic health.

The third main type of adipose tissue is the one from the bone marrow. At birth, the bone marrow is mainly composed of red blood cells. With time, **marrow adipose tissue** accumulates and by 25 years of age, it composes 50 to 70% of the bone marrow and represents approximately 10% of the adipose tissue in lean individuals. The close location of fat and bone tissue is not their only common point. Indeed, adipocytes and osteoblasts are both derived from mesenchymal stem cells, and they are responsive to each other's signalling. There is a balance between bone and fat development, and the excess in one is frequently associated with insufficiency in the other.[87] Vitamin D is involved in bone health, but also impacts the adipose tissue function by regulating adipokines expression and inflammatory response.[88] A healthy exposure to day light is therefore necessary to contribute to bone but also adipose tissue physiology.[89]

Finally, some cellular factors play a key role in the adipose tissue development and function. The activation of the peroxisome proliferator-activated receptor γ (PPARγ) induces the genetic expression of compounds necessary for the adipogenesis and the storage of triglycerides. The transcription factor CCAAT/enhancer-binding protein α (C/EBPα) binds directly the leptin gene promotor. Together, C/EBPα and PPARγ play a key role on adipogenesis.[90] For instance, they are necessary to the expression of the enzyme lipoprotein lipase (LPL) which hydrolyses the triglycerides, and the adipocyte protein2 (aP2) which is a carrier for fatty acids.[91]

Although the adipose tissue was long considered only as a storage organ, it is now known that it is an endocrine organ. It is sensitive to metabolic signals from other organs and produces signalling molecules named adipokines. The first to be discovered was leptin in 1994, and since then hundreds of potentially secretory proteins have been identified.[92,93] Here are some of the key adipokines identified and their roles. As described, leptin acts through its receptors, present on different regions of the central nervous system and

in particular the hypothalamus, in order to decrease energy intake. In addition, it decreases triglycerides storage by inhibiting its synthesis, stimulating lipolysis and β-oxidation, and regulating gluconeogenesis. Adiponectin is another adipokine. It potentiates the insulin sensitivity by stimulating β-oxidation and regulating gluconeogenesis, and prevents atherogenesis through inhibiting the expression of adhesion molecules in blood vessels.[94] In addition to these hormones, some adipokines are immune factors such as the lectin omentin, the C-reactive protein (CRP), the interleukin-6 (IL-6), the monocyte chemotactic protein-1 (MCP-1) or the plasminogen activator inhibitor-1 (PAI-1).

In summary, the energy intake, expenditure and therefore storage, are impacted by nutrition, physical activity, sleep, mental and emotional activity, and exposure to temperature and light. Nevertheless, the energy exchanges in the human organism are far from being the passive consequence of nutritional intake and physical activity. Numerous external and internal signals are integrated, and dynamic regulations are activated to maintain a healthy balance. The homeostasis is the phenomenon by which a biological parameter is actively regulated at a beneficial level for the organism. As presented earlier, internal signals inform the central nervous system and the other organs on the energy status of the organism, and consequent metabolic adaptations are triggered. It is hypothesized that these signals are integrated in a form of homeostatic system regulating body fat mass or body weight.[1] A crucial aspect of life habits is their repetitiveness. They are defined “a settled or regular tendency or practice, especially one that is hard to give up” or “an automatic reaction to a specific situation”, habits correspond to stimulus–response or context–response mechanisms developed through repetition and learning.[95] In habits, the daily rhythms are key. Every day, the digestive functions are mobilized at similar hours, followed by the storage reactions. The wake/rest cycles orchestrate the patterns of energy expenditure. And once again, the metabolism is far from passively reacting to the external signals: the metabolism has a **clock**.

I. 3. Circadian rhythms: structure, synchronization, and metabolic control

A key characteristic of life on Earth surface is the 24h variations of the environment. This cyclic niche, or life context, induces a temporal repartition of the behaviours and exposures over the course of the day. Humans being essentially a diurnal animal, the main sleep phase takes place during the night and the active and feeding behaviours occur during the day. This pattern gets repeated every day. Logically, the metabolic adaptations triggered by these behaviours and exposures display 24h patterns as well. Nevertheless, these metabolic reactions are not only passively rhythmic as an exclusive consequence of cyclic inputs. On the contrary, inner clocks are present in the organism which self-synchronize with external day time. In turn, they regulate metabolic mechanisms, conferring to the organism the faculty to anticipate the adaptations to regular variations. The biological rhythms following a period of twenty-four hours are referred to as "circadian rhythms", a term coming from the Latin words *circa*, meaning "approximately", and *diem*, "day". In every tissue where in which they have been investigated, circadian oscillations have been found. They are therefore considered ubiquitous.[96] In humans, two main mechanisms ensure the structure and synchronization of the inner circadian rhythms: the clock genes, and melatonin's production and activity. These clocks rhythms are synchronized by external factors, the zeitgebers. The entrainment of the rhythms will be discussed, together with the interindividual differences known as the different chronotypes. Finally, the circadian regulation of metabolism will be presented.

I. 3.1. Clock genes

A group of genes named the **clock genes** form a double regulation loop which period lasts approximately twenty-four hours illustrated in **Figure 5**.

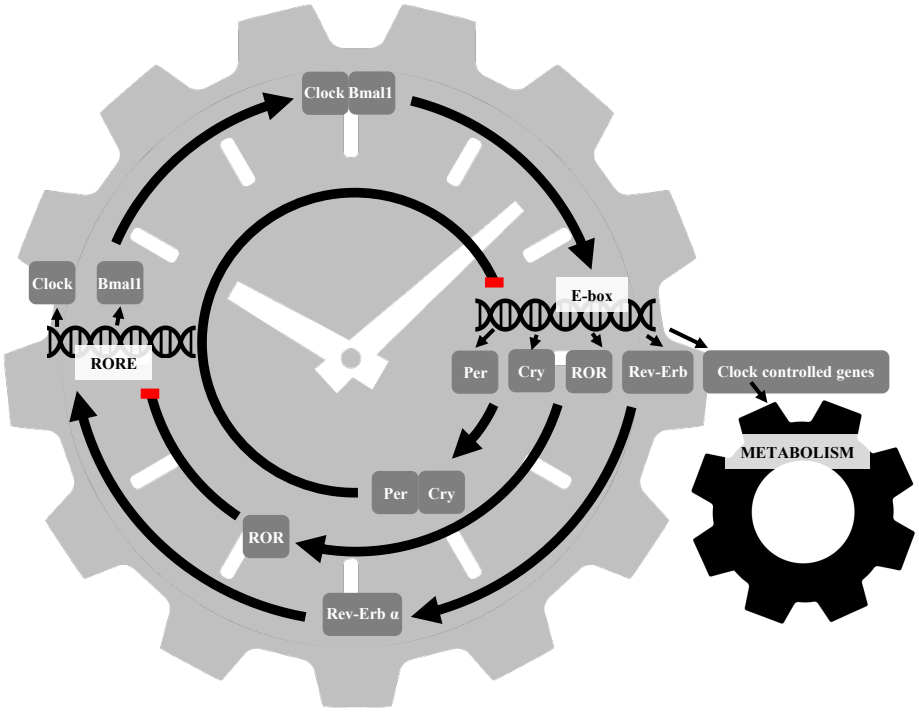


***Figure 5**. Clock genes circadian loops*

Scheme created by Marie Gombert

At the beginning of the subjective day, the genes circadian locomotor output cycles kaput (*CLOCK*) and brain and muscle aryl hydrocarbon receptor nuclear translocator-like 1 (*BMAL1*) are expressed and heterodimerized. The heterodimer binds on the enhancer box (E-box) located in the promoter region of the following genes to activate their expression: Period genes (*PER1*, *PER2* and *PER3*), Cryptochrome (*CRY1* and *CRY2*), and the orphan receptors REV-ERB and ROR genes. There is a competition between the protein REV-ERB which inhibits *BMAL1* expression, and the protein ROR which inhibits *BMAL1* expression, both by binding on specific response elements (RORE).[97] The inhibition of *BMAL1* expression prevents its dimerization with CLOCK. In parallel, the proteins PER and CRY accumulate, forming a complex that inhibits the activating limb and consequently the expression of *PER, CRY,* and *REV-ERBα*. The levels of REV-ERBα drop, which allow the expression of *BMAL1*. New CLOCK-BMAL1 dimers are formed, and another subjective day begins.[98,99] This mechanism is also known as the **transcription-translation feedback loops of clock genes**.[100]

In addition, several **post-translational reactions** are involved in the fine tuning of the clock. Indeed, reactions of phosphorylation, ubiquitylation, SUMOylation, O-GlcNAcylation, and acetylation can modify the durations and level of expression of the components of the loops.[96]

Under the promoters targeted by the components of the clock figure different genes, consistently called the clock-controlled genes (**Figure 5**). Among them figure transcription factors, receptors, hormones, carriers of the metabolism.[101] This mechanism confers a circadian rhythmic structure to the genetic expression which is maintained even in the absence of external variations. For example, in the adipose tissue, the leptin gene (LEP) presents circadian rhythms of expression. Clock genes and clock-controlled genes continue to oscillate even in constant conditions, and their rhythm is temperature compensated, meaning that the rhythm does not accelerates when the temperature increases or slows when temperature falls. Plus, *ex vivo* studies of cellular explants can be performed since these rhythms are maintained during several days out of the organism.

It is to note that other oscillating mechanisms exist independently from the genetic expression. In humans, they have been encountered in the red blood cells, which metabolism presents circadian rhythms while these cells do not comport a nucleus. These cycles are **redox-based** and involve the antioxidant proteins peroxiredoxins. These mechanisms are suspected to participate to circadian rhythms in other human cells too, by interacting with the transcription-translation feedback loop of clock genes.[100]

Together, these rhythmical and self-sustained mechanisms are therefore considered to form **cellular pacemakers**, they constitute the **peripheral clocks**. Another mechanism exists which is crucial in metabolic adaptation to daily variations: **the central clock**.

I. 3.2. The central clock and melatonin

Every day, the external light from the environment varies. In the morning, it contains a high proportion of blue wavelengths around 480 nm. These wavelengths stimulate the cells of the retina. In addition to the cones and rods, a third type of retinal cells is activated by the light signal: the intrinsically photosensitive retinal ganglion cells, containing the photopigment melanopsin. When activated, the photic information is transmitted by the retino-hypothalamic fibers to the suprachiasmatic nucleus of the hypothalamus, which is the location of the **central clock**. In turn, the neural stimulus is forwarded to the superior cervical ganglion, triggering norepinephrine release. It activates the adrenergic receptor, which increases the cAMP levels, resulting in the activation of the associated protein kinase A in the pineal gland. Inside the pinealocytes, the amino acid tryptophan undergoes three enzymatic reactions, including by the arylalkyl-amine N-acetyltransferase, to synthesize N-acetyl-5-methoxytryptamine, commonly known as **melatonin**. The light signalling pathway inhibits the expression the enzyme arylalkyl-amine N-acetyltransferase, which confers a rhythm to melatonin expression. When produced, this circadian hormone is released in the blood and flows all around the organism.

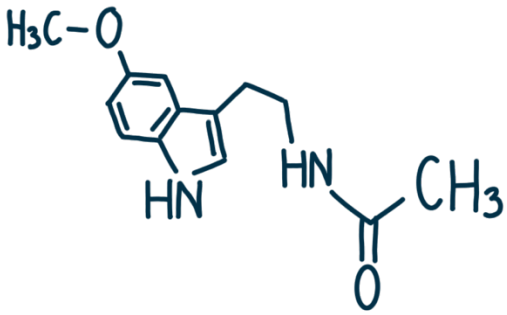


***Figure 6**. Melatonin molecule*

Melatonin is an indoleamine of amphiphilic nature (**Figure 6**), and a powerful antioxidant molecule.[102] The amphiphilic characteristic allows melatonin to flow directly in the blood, to reach the surface of the target cells, but also to passively diffuse through the cell membrane or enter through transporters. Produced in the last hours of the day, melatonin signals to the organs to prepare for the resting phase through different pathways[103]:

- On the cell surface, melatonin binds its specific receptors, MT1 and MT2, two G protein-coupled receptors respectively associated with proteins Gi and Gq.[104] They are present at the surface of numerous cell types, such as of the cardiovascular system on which melatonin plays an antihypertensive role, or on the endocrine system as in the adipose tissue or pancreas, in which melatonin participates to regulate the lipid and carbohydrate metabolisms.[104]
- Melatonin also acts directly inside the cells on other types of receptors such as the nuclear receptors of the group ROR/RZR (retinoid orphan receptors/retinoid Z receptors). In the immune system, it is

through their activation that melatonin contributes to the regulation of the metabolism of lymphocytes B, and of cytokines expression in mononuclear cells. [104]

- Melatonin interacts with intracellular proteins in the cytosol and organelles like the mitochondria. These interactions modulate the activity of enzymes, transporters, calcium-binding proteins, cytoskeletal and scaffold proteins. Plus, melatonin is proposed to regulate the peripheral clocks formed by the clock genes by regulating the activity of the proteasome.[105]
- Finally, melatonin can directly act biochemically as a scavenger of free radicals to decrease oxidative stress in the cells.[102]

Through these actions, melatonin communicates the external photoperiod to the organs, which synchronize their rhythms of clock genes and clock-controlled genes to be in phase with the cyclic needs and activities of the organism. For instance, the body temperature and the blood pressure drop at night consecutively to the melatonin signal.[106,107] These changes put the organism in the ideal dispositions for sleep onset.

At birth and during the first months of life, rhythmic melatonin is not yet produced by the infant, but is present in the breastmilk.[103] It reaches the highest nocturnal levels around 3 years of age, and then decreases during the entire life. An important part of melatonin drop occurs during childhood and is suggested to be due to an unchanged production of melatonin in a growing organism, inducing a dilution of the hormone.[108] In addition, crystalline absorption increases with age and pupil diameter decreases with age, which decreases retinal photoreception, from which depends melatonin expression.[109] Sex differences have been reported in some studies with a higher and earlier melatonin peak in women compared to men, but other studies did not observe these differences.[110,111]

Melatonin levels variation is frequently used as an indicator for circadian rhythms studies. Melatonin's production starts in the evening, peaks at night and is down in the morning. Therefore, it needs to be measured at several time points and during the dark photoperiod to provide reliable information about the individual's rhythms.[112] As melatonin flows in the blood and the other body fluids when produced, it can be easily measured in saliva. This makes possible the assessment of melatonin at several time points in a non-invasive manner. It can be performed directly by the subject, which makes possible a sample collection at home, in the usual life conditions.[113] The collection of samples in the life environment provides the most reliable information as the daily life habits and exposure are preserved, unlike when the experiment is performed in a sleep laboratory.

I. 3.3. Circadian rhythms

I. 3.3.1. Zeitgeber

The signals indicating the external time to the organs and their inner clocks are called *zeitgeber*, the German word for *time-giver* (**Figure 7**). They include the external light and temperature, feeding, sleeping, mental and physical activity.

***Figure** 7. Zeitgebers = external time cues to which the organism is sensitive*

It is said that the clocks can entrain to those signals, meaning using them as time indicators to actively enter in synchrony with the external day. The entrainment capacity depends on the relative strength of the clock and the zeitgeber. The zeitgeber strength corresponds to the difference between its minimum and its maximum, for example the lightning difference during the day vs night. When the central clock and the peripheral clocks are in phase, together and with their environment, it is said that they are aligned. These key terms and concepts definitions of circadian rhythms biology were clarified by Till Ronnenberg and Martha Merrow in the glossary of their review entitled *The Circadian Clock and Human Health*.[114]

I. 3.3.2. Chronotypes

There are interindividual differences in the tendency to engage in earlier or later behaviours. It is said that individuals are of early chronotype or late chronotype, or in a colloquial language, “morning-” or “evening-persons”. The chronotype can be assessed with equivalent efficacy through different tests such as the Munich ChronoType Questionnaire, which is based on times of sleep during work and free days, or via Horne and Ösberg’s morningness eveningness score, which is based on time preferences for several activities.[115] The individuals chronotypes depend on the duration of their inner day and on their entrainment capacity to the light/dark cycle.

Melatonin rhythmic expression is also regulated by the clock genes expressed in the suprachiasmatic nucleus, which ensures its rhythmic expression even in constant conditions. The period of the inner rhythms of humans is usually longer, and sometimes shorter, than 24h. An individual with a short inner day tends to eat early, feel awake and full of energy in the earlier part of the day, and feels the need to go to bed early. In

the contrary, individuals with longer inner days naturally tend to engage in later behaviours. Genetics, age and sex are linked to chronotype and inner day duration.[76,116] Chronotype evolves with age, as in early childhood the chronotype is early, and gets delayed during the entire period of growth, reaching its maximum around 19.5 years old for women and 21 for men. Then, it progressively becomes earlier with age until the end of the life. Approximately from the beginning of puberty until menopause age, women display in average earlier chronotypes than men.[117] These elements influence clocks characteristics such as amplitude or response characteristics, which changes the relative strength of the clock compared to the zeitgebers, and consequently, the entrainment capacity.[118]

On the other hand, the zeitgebers strength changes according to the environment, which modulates clock entrainment, and finally the chronotype. This is clearly illustrated by studies on children going camping. After a few days of contact with natural temporal cues, and in particular a strong light zeitgeber, the range of diversity in chronotype narrows, the inner days of the participants all become closer to the external day.[119] As illustrated in **Figure 8**, with a strong contrast exist between day and night light exposure, the individual's rhythms and behaviours (here, sleep) entrain earlier (A) than when this zeitgeber is weaker (B).

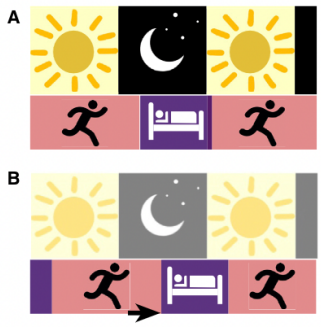


***Figure 8**. Early or late entrainment of wake/sleep cycle to light/dark cycle*

A) Strong contrast in light zeitgeber and early entrainment of the wake/sleep cycle. B) Weak contrast in light zeitgeber and later entrainment of the wake/sleep cycle. Adapted from Ronnenberg and Merrow with authorized license from the publisher (Licence n°5310720528873).[111]

Light exposure is the zeitgeber that has been the most studied and seems to be the most important driver of chronotypes[76] and in particular of the central clock entrainment. Nevertheless, other zeitgebers influence clocks entrainment. In our current society, sleep timing results in an important part from the social organization.[76] Therefore, through the moment we put our organism at rest and close our eyes, sleep mediates a time information from the social clock.[120] An important element in the entrainment of peripheral clocks of the digestive system, and more particularly the liver, is the timing of the nutritional intake. The liver clock may primarily entrain to the nutrition rhythm rather than the central clock signalling.[121] The sport and mental activity, through the release of glucocorticoids, are also able to influence the inner clocks rhythms. Indeed, **physical activity** in the two hours **before sleep** has been shown to delay circadian rhythm of wrist temperature[122], nevertheless it is not clear whether late physical activity practice constitute a risk factor for

circadian misalignment and obesity in childhood. Finally, temperature variations are proposed to modulate clocks rhythms through regulation of the phosphorylation of the clock genes.[123]

According to the time of the zeitgeber peak, the clocks rhythms will be shifted in different directions. It is considered that high light exposure, intense physical activity, or heavy meal, when happening in the earlier part of the day, entrain the inner clock towards an early chronotype. Inversely, engaging in these behaviours in the later part of the day entrains the clock towards a late chronotype.

I. 3.3.3. External factors influencing zeitgebers

It is to note that because our activities are scheduled in an important part in consequence to social organization, chronotypes are influenced by the social clock.

First, in a same time-zone, the social time is the same for everyone, whereas the dawn, dusk, or the sun's zenith do not fall at the same time in the eastern and western part of the time-zone. It was observed that in the countryside and small cities, the chronotype is strongly correlated with the east-west location of a time-zone, showing the high importance of the light zeitgeber on chronotype. In the other hand, in bigger cities, this correlation was still significant but weaker, showing a reduced importance of the light zeitgebers in these life places.[76] The strength of the light zeitgeber is decreased in urban areas, as during the days most of the activities happen indoors, and even once outside, the architecture can limit the direct contact with sunlight. In parallel, at night, the enlightenment is higher and so is the level of noise and activities opportunities. Finally, the temperature rhythms are different, as the buildings accumulate heat and release it once the sun is down. These factors influence sleep, in its timing, duration and quality, to the extent that New York City, in which these characteristics are pushed to the extreme, is even called "*the city that never sleeps*". In addition to the home location, the life context inside of the home influence the life habits timing. The presence of electronic devices with screens in the bedroom, or the habit to watch TV/play videogames before bed influences sleep onset. Indeed, even a light pulse before sleep can delay the rhythm of the suprachiasmatic nucleus and consequently melatonin expression.[124]

In childhood, meal and sleep timings are directly the consequence of the caregiver's schedule and own habits. This last depends on the culture and country, influenced by work schedules, school schedules, paediatrician/public health recommendations, awareness, ... And the timing of physical activity and mental/emotional signals are also related to the societal organization and social environment with school schedules, engagement in extracurricular activities, familiar and community support, ...

In summary, human circadian rhythms are structured in peripheral clocks, mainly composed of the clock genes, and of the central clock, which signals the photoperiodic time to the rest of the organs mainly through melatonin's action. These rhythms entrain to zeitgebers from the environment and life activities. Consequently, they confer rhythms to numerous mechanisms playing important roles in metabolic homeostasis.

I. 3.4. Circadian rhythms of the metabolism

In *The circadian regulation of food intake*, Etienne Challet explained clearly the two aspects through which metabolic regulation by the circadian clocks is beneficial: "*First, [the clocks] provide a temporal organization from the cellular level to the organism level. Therefore, circadian clocks facilitate the temporal occurrence of related functions, such as food intake and glycogenesis, and separate conflicting functions and behaviours, such as eating and sleep. Second, circadian clocks allow organisms and organs to anticipate or be in phase with foreseeable events from one day to another, such as sunrise and sunset or food availability*."[125]

The daily window of the nutritional ingestion, physical and mental activities correspond to the wake period, which in humans corresponds essentially to the light photoperiod, in contrary to sleep. In accordance with this organization, the circadian clocks orchestrate the interconnected mechanisms involved in metabolic homeostasis. Here are presented the circadian rhythms in the biology of energy intake, expenditure, and storage.

I. 3.4.1. Circadian rhythms in energy intake

Everyone has experienced being hungry, then being very very hungry, and a little while later, noticing that the hunger feeling went away as if "*the train had passed*". In addition, it is known that, for a same nutritional intake, according to the time of the day, the metabolic response is different.[126] These are clues that from a biological point of view, the organism has a variable capacity to welcome the meal over the day. Indeed, mechanisms regulating food ingestion as well as digestion are under circadian control.

A rise in glucocorticoids, controlled by the suprachiasmatic nucleus, has been shown to occur in the pre-prandial periods, in accordance with the meal schedules of the previous days. This endocrine signal is involved in food anticipatory activities and preparation of the digestive system.[125] Similarly, the gastrointestinal hormone ghrelin is present in high levels in the plasma before meals and more particularly in the early part of the day. Its levels decrease during the meals, increase again during the fasting periods, and its daily variations depend on circadian rhythms health. In parallel, Leptin, which peak of secretion occurs at the beginning of the night, is a mediator of the circadian regulation of rhythmic glucose levels in the blood. While glucagon and insulin appear not to play a role on the food anticipatory action from one day to another, they have a regulatory role on the hepatic clock.[125] It is to note that not only the secretion of these hormones is clock-regulated, but also the sensitivity of the tissues to their action. At each moment of the day, a different cocktail with various concentrations of the mentioned hormones influences the hunger feeling and the nutrients metabolization. As a result of their actions, the eating behaviour is influenced into driving the individuals to eat when the organism is prepared.

Once food is ingested, it undergoes successive mechanical, chemical, biochemical, and microbiological transformations making possible the absorption of nutrients, and the time dimension is critical in this process. Indeed, the digestive organs ensuring these modifications need to be timely activated one after the other to complete the process, and to be ready for use at the time of the day at which food is ingested. Consistently, circadian variations have been observed in the levels of the following digestive mechanisms: saliva production, mouth microbiota diversity, gastric emptying rate, digestive enzymes expression, digestive tube peristalsis, intestinal epithelial permeability, mucus and antimicrobial peptides, and gut microbiota metabolome.[127,128] These rhythms result from the joint action of melatonin and the peripheral clocks.

Once we understand the extent of this time regulation in the ingestion and digestion processes, it becomes clear that for the energy intake metabolism to function optimally, the central clock, peripheral clocks and eating behaviour need to be in phase. Consistently, certain moments of the day are better adapted than others for the body to process food. The dark photoperiod is temporally allocated to sleep, logically the organism is better adapted for the nutritional intake during the light photoperiod. In addition, in the light photoperiod, moments are more adapted than others. Scientific observations confirm the ancient adage "*Eat breakfast like a king, lunch like a prince, and dinner like a pauper*". Indeed, the postprandial metabolic response is found better: for equivalent meal contents, when the meal is consumed earlier than later[129], and for equivalent meal schedules, when most of the caloric content is consumed in the first meals of the days.[130]

I. 3.4.2. Circadian rhythms in energy expenditure

Through rhythmic expression of hormones and clock-controlled cellular activity, the functions of the organs and the type of metabolic reactions vary over the course of the day. Consequently, the **metabolic rate** varies as well on a circadian manner, with a nadir of O_2 consumption around 6AM and a maximum around 6PM.[131]

The **temperature homeostasis** also presents circadian rhythms. Each night, the core body temperature decreases of approximately 1°C, from ~37°C to ~36.2°C, whereas the peripheral temperature of the skin presents the opposite pattern. Indeed, around dusk and melatonin onset, the vasodilatation of the peripheral vessels allows the blood to come closer to the body surface, increasing the skin temperature. Temperature exchanges with the environment are potentiated, and the core temperature consequently decreases for the night. In the contrary, around dawn and cortisol onset, the vasoconstriction of the peripheral vessels keeps the blood away to the body surface, decreasing the skin temperature and limiting the temperature exchanges with the environment. Consequently, the core temperature increases for the day.[132] This temperature rhythm is maintained even in constant conditions.[133]

Important interindividual differences exist on the capacity to engage efficiently in **mental** and **physical activities** according to the type of the day. Indeed, Horne and Östberg's morningness and eveningness questionnaire is mainly based on the time preference for these activities.[134] For a same individual, circadian rhythms have been observed in physical components such as muscular strength and flexibility[135], and mental components such as alertness, performance and mood.[136] Let's illustrate this: at what time are you reading these lines? Is this the moment of the day at which you feel more mentally alert, attentive and efficient? The answers give you clues on your chronotype.

The efficacy feeling is not only linked to the time of the day, but also to the time already spent awake on this day (fatigue accumulation), and to the time since the last meal. Indeed, as introduced, the **digestive system** circadian rhythms at each step of the mechanic, enzymatic, chemicals and microbiological processes.[127,128] This activity being energy consuming, it participates to the daily variations in the energy expenditure.

I. 3.4.3. Circadian rhythms in energy storage

Given that energy intake and expenditure are clock controlled, it is no surprise that the subsequently stimulated metabolic pathways involved in energy storage and mobilization also present circadian rhythms. The light photoperiod is associated with increased glycogenesis and lipogenesis, while the sleep period is the time of lipolysis, glycogenolysis and gluconeogenesis.[125,137]

Focussing specifically the circadian rhythms in the adipose tissue, adipocytes express clock genes[138] and are sensitive to melatonin, as suggested by the presence of its receptor MT2 at their surface.[104] Adipocytes genes coding for key factors in adipogenesis and lipid metabolism are clock-controlled. This was observed in human adipose tissue for the adipogenesis factor PPARγ, glucocorticoid-related genes[138], and also lipid metabolism genes as LPL.[139] It is also the case of various adipokines including leptin, adiponectin, IL-6 and PAI-1.[140] The adipocytes insulin sensitivity also presents circadian rhythms, as it was observed that insulin stimulation does not trigger the same level of phosphorylation of Akt (protein in insulin signalling), according to the time of the day.[141] Melatonin contributes to this circadian rhythm of adipocytes physiology, as it was shown to modulate adipocytes insulin sensitivity[141] together with leptin[142] and adiponectin expression[143]. On rodents, it was shown that when melatonin is not produced (secondary to pinealectomy), the circadian expression of clock genes and metabolic genes is altered, with a modulation in the amplitude of the transcript rhythm.[144] These changes in rhythmic expression may be mediated by the regulatory role of melatonin on the proteasome's activity on the peripheral clocks.[105] In humans, little is known about the effect of melatonin on the circadian rhythms of clock genes and clock-controlled genes in the adipose tissue. Further studies on these mechanisms may nevertheless be strategic, as they may constitute a pathway through which circadian rhythms disruption may favour obesity development. Indeed, at the scale of the organism, melatonin mediates the seasonal changes of body weight in rodents. The weight decrease in winter is caused by an increased thermogenesis of the brown adipose tissue, consecutively to the increased melatonin levels during this season.[145] It is proposed that melatonin may promote thermogenesis and browning in humans as well.[146] In human, melatonin may also act on metabolic and weight health through acting on the toll-like receptors activating MyD88 and through regulating the pathways of leptin and insulin.[147,143]

I. 3.5. Chronodisruption

In 1960, Colin S. Pittendrigh published its generalizations on circadian rhythms, 16 principles according to which he proposed the chronobiology may be organized. In this work, he stated "circadian rhythms are inherent in and pervade the living system to an extent that they are fundamental features of its organization; and to an extent that if deranged they impair it".[148] In the 2000's, epidemiological studies started to support this assumption, showing a higher prevalence of cancer in shift-workers and flight personnel. This is when Thomas C. Erren and Russel J. Reiter proposed to employ the term **chronodisruption** to refer to the "relevant disturbance of the circadian organization of physiology, endocrinology, metabolism and behaviour, which links light, biological rhythms and the development of cancers with melatonin being a key biological intermediary".[149] This state of loss of synchrony between the different internal clocks, together and/or with the external clock can be caused by incoherent time signals from external factors or life habits. The same authors named these elements **chronodisruptors, and they** add that the strongest one appears to be exposure to light at night. Indeed, it inhibits melatonin expression, which consequently cannot play its synchronizing role on peripheral oscillators.[149] Plus, the metabolic responses to uncoordinated external signals are likely to be incoherent, which may be physiologically compensated, but in the long term, favour the development of cancers and other metabolic disorders as obesity.[114,149,150] Thomas C. Erren and Russel J. Reiter finally underline the need for relevant indicators of chronodisruption, and for researches on the links between this circadian rhythms alteration and its adverse health effects. To date, the knowledge is still scarce concerning the links between circadian rhythms and childhood obesity.

In summary, the circadian rhythms are strongly present among all the mechanisms composing the energy intake, expenditure, and storage. Consistently, the situation of chronodisruption is also tightly linked with metabolic unbalance which can lead to disorders, such as obesity.

I. 4. Link between obesity and chronodisruption

Here, obesity will be described from a metabolic and inflammatory point of view, and its causes will be presented integrating the current knowledge of the role played by life habits timing and circadian misalignment in the development of obesity (**Figure 9**).

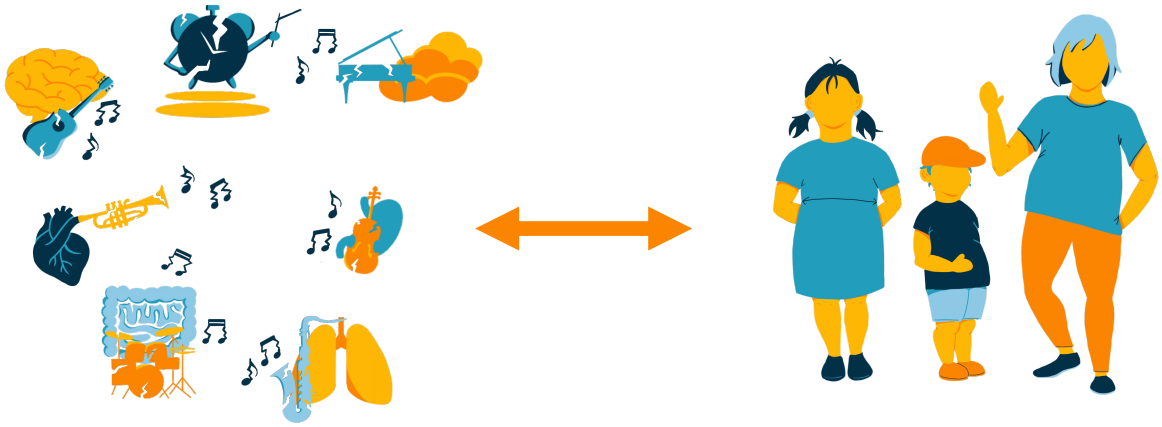


***Figure 9**. Chronodisruption and obesity*

I. 4.1. Life habits timing

If the increasing prevalence of overweight and obesity constitutes a proportionally growing preoccupation, it is because of their impact on health. In obesity, the adipose tissue which increases is the white adipose tissue, the main responsible for energy storage. In the contrary, the brown adipose tissue, which expends energy for thermogenesis is inversely proportional to the BMI.[151] Carrying extra kilograms, sometimes tens of kilograms, of an endocrine tissue conveys multiple consequences in link with the modified hormonal and inflammatory signal, the mechanical pressure on the other organs, and the overburden.

Metabolic changes occurring in obesity are reflected by the levels of key metabolic hormones in the blood. Leptin is found highly overexpressed, and a leptin resistance appears. It is associated with impairments in the processes regulated by leptin, such as energy intake, metabolic pathways and insulin sensitivity.[152] In parallel, adiponectin is found decreased in obesity, together with its roles in insulin sensitivity, anti-inflammatory, and anti-atherogenic actions. Omentin, produced in the visceral adipose tissue and playing an anti-inflammatory role is also decreased.[153] In the contrary, resistin, associated with insulin resistance, is increased. Obesity is frequently the context of high levels of insulin and insulin resistance.

These hormones are regulators of various metabolic pathways, therefore as a consequence of their levels change, the levels of characteristic blood metabolites change as well. Glucose levels tend to be higher despite the increase of insulin levels. Obesity is also associated with changes in the lipoproteins in charge of the lipid transport (cholesterol, fatty acids, triglycerides). Very low-density lipoproteins (vLDL) and low-density lipoproteins (LDL), transport lipids to the organs are present in high concentrations and are transported with apolipoprotein B. On the other hand, high density lipoproteins (HDL), which transport lipids to the liver for elimination, are decreased and are transported with the apolipoprotein A1.[154]

In parallel, inflammatory markers including CRP, IL-6, TNF-α, PAI-1 are higher than in lean individuals.[153,155] In a physiological situation, the basal levels of these immune factors are very low, and they punctually rise to trigger an acute and efficient reaction. In obesity, this basal level is upregulated. This basal low grade and chronic inflammation is typical of obesity, and figures among the main causes of the development of comorbidities. The production of reactive oxygen species also increases in obesity, while the expression of antioxidant enzymes such as catalase or superoxide dismutase decrease. These changes result in an overall increased **oxidative stress**, which damages the organs and also promote comorbidities development.[156,157] These changes have been found to be already present in childhood obesity.[158,159] Finally, the gut microbiota is modified in obesity. Impacted by the hormonal and inflammatory factors, but also by the behaviours of the host, the **gut microbiota** evolves in composition but also in its secretory metabolome, which in turn influences the host's health.[160]

It is to note that in **obesity**, the **circadian rhythm** of expression of numerous hormones, inflammation markers, blood pressure and gut microbiota is **altered**.[161]

The combination in the blood vessels of elevated circulating LDL, inflammation, and oxidative stress, constitutes the perfect ground for atherosclerosis. Indeed, LDL containing apolipoprotein B get oxidized and cross the endothelial layer of the arteries. The endothelial cells consequently produce cytokines such as **MCP-1**, which mobilize monocytes. Monocytes also cross the endothelium and differentiate into macrophages. They absorb the LDL and consequently become foam cells which accumulate between the endothelium and the smooth muscle of the arteria. In parallel, the endothelium mobilizes platelets. These mechanisms constitute atheromatous plaques which on the long term thicken the arteria vessels and increase thrombosis risk.[162] Through this process, obesity promotes the development of **cardiovascular disorders**.

In addition, various key elements of glucose homeostasis are commonly modified in obesity. As a result of the hormonal changes already mentioned, the muscles, liver and adipose tissue cells develop **insulin resistance**. For a same quantity of insulin, the glycemia decrease is less important than in normal conditions. Initially, the β-cells of the pancreas expend and increase their insulin production. After a time, there is a failure of β-cells, which may be triggered by hyperinsulinemia and fatty acids induced apoptosis. This stage characterizes **type-II diabetes**.[163]

Regarding these comorbidities, the disease stage is extremely rarely reached during childhood, nevertheless in numerous children with obesity they are already developing, as shown by the elevated risk factors detectable in the blood. And even at short term, **childhood obesity** has an **impact** on **several health compartments** such as puberty onset[164], bone health[165,166,167], respiratory function[168,169], physical capacities[170,171], cognitive performances[172,173], and psychological health[174, 175].

I. 4.2. The role of circadian rhythms

Certain **personal characteristics**, combined with an **obesogenic environment**, favour the development of obesity.

I. 4.2.1. Personal characteristics

Several personal characteristics, which include the **genome**, the **epigenome,** and the **microbiota** can be decisive in obesity development. Of all personal characteristics, the ones directly or indirectly related to the energy intake, expenditure and storage are susceptible to promote or prevent obesity development.

Genetic disorders such as the Prader-Willi syndrome, in which the individuals present hypotonia and hyperphagia, can be a monogenic cause of obesity.[176] But the obesity associated to a syndrome constitutes a small proportion of the people living with obesity. The inherited genetic predispositions to obesity mainly consist in **polymorphisms** of genes involved in the energetic homeostat. These different versions of key metabolic genes influence the efficiency of the related mechanisms, explaining why to a same signal, there are variations in individual responses. Among the polymorphisms which have been identified to favour obesity development figure the MC4R gene involved in hunger regulation, but also the genes of leptin, leptin receptor, adiponectin, ghrelin, adiponectin, and IL-6.[177,178] Interestingly, clock genes polymorphisms have also been linked to obesity.[179]

In addition to the genetic background of an individual, the **epigenetic modifications** of said key metabolic genes contribute to promote or prevent obesity. These modifications in the methylation of the DNA and acetylation of the histones condition the availability of the genes, and therefore their expression. Epigenetics is one of the paths through which early life conditions leave a mark which will influence health in later life. Even before birth, the mother's nutrition and metabolic health induce epigenetic modifications on the child's metabolic genes.[180] In addition, the composition of the early nutrition, meaning breastmilk or formula milk, influence the epigenome as well.[181] These modifications were found significantly associated with obesity in later life.[180] Numerous genes of the signalling pathways are concerned, and so are clock genes such as PER2, BMAL1 or CLOCK.[182]

Another individual health compartment which is influenced by the personal history and in particular early life, is the **microbiota**. At birth, we are colonized by our mother's microbiota. When the delivery is natural, the infant mainly receives the mother's vaginal microbiota, whereas in the case of a c-section, the infant receives the microbiota from the skin and the room. The early nutrition and the early life context in general, shape the microbiota development until it reaches a relatively stable state at around 3 years of age. This early life context therefore determines the microbiota composition in terms of diversity, proportion of certain strains, but also the overall metabolome or secretome of the microbiota, as well as its resilience.[183–

[185] This last characteristic is the capacity of the microbiota to recover a healthy stable state after experimenting a dysbiosis or loss of diversity due to a stress (period of unhealthy eating, use of antibiotics, mental stress impacting digestive health, chronodisruption…). This is one of the processes through which chronodisruption may lead to obesity development.[160,186]

Collectively, these elements constitute the characteristic which influence the tendency of an individual to gain or lose weight in a given context. Therefore, they explain the **interindividual differences** in body weight and composition that can be observed between persons living with comparable accesses to food and physical activity structures (**Figure 10**). Nevertheless, the substantial increase in obesity prevalence in the last decades cannot reasonably be attributed to genetic factors.

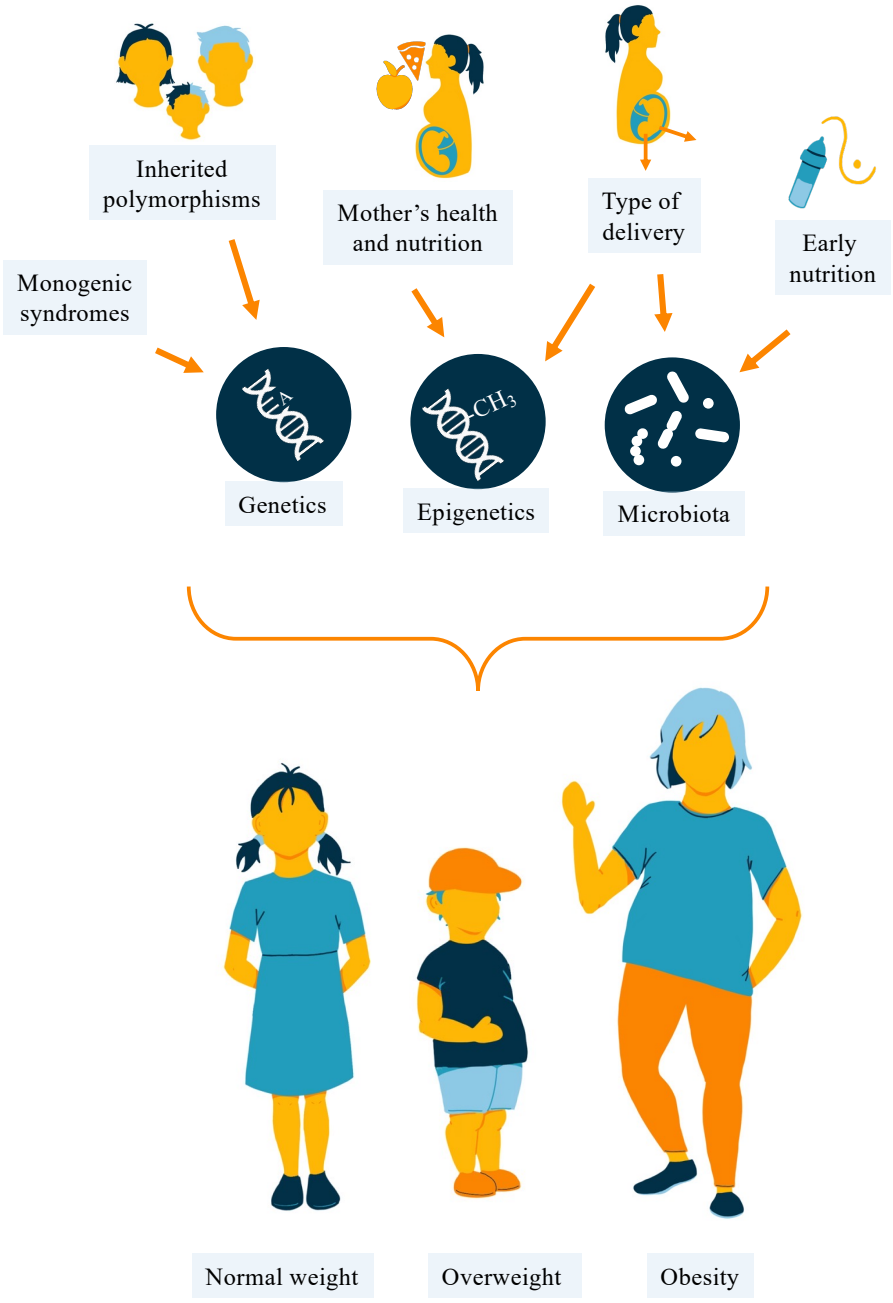


Figure 10. *Interindividual differences in the tendency to gain weight*

I. 4.2.2. Obesogenic habits

a) Life habits: key importance of timing

The balance between energy intake and expenditure is influenced not only by the quantity and quality of life habits, but also by its timing.

In the last decades, the understanding of the importance of sleep health in obesity development has risen. First, epidemiological studies, including in paediatric age, show that **short sleep duration** constitute a risk factor for increased central adiposity and obesity.[187,188] In addition, **late bedtime**, even independently from wake-up time, has been encountered to be associated with increased obesity and overweight.[189] It appears that the insufficient sleep duration and in particular the extra awake time in the evening is associated with increased hunger and appetite feelings, lower quality of the nutritional intake with lower fruits and vegetables consumption reported, and higher reported food intake rich in energy and poor in nutrients.[189,161] In parallel, sleep deprivation, through fatigue accumulation, perturbates the capacity and motivation to perform physical activity, increases the probability to be injured during an effort, and increases sedentary behaviours such as screen use.[189] This global effect of short sleep duration combining increased food intake and more sedentary habits has been observed on adolescents as well.[145] Nevertheless, further studies are needed on the childhood population to measure the effect of insufficient and late sleep on the hormones involved in the energy metabolism, and understand more accurately the pathways involved in these modifications of the energy balance.

Sleep timing also impacts the energy metabolism indirectly, through its influence on the timing of other activities, such as **meal timings**.[190] For an equivalent nutritional intake, late meal timings are associated with increased indicators of metabolic risk. Similarly, for equivalent mealtimes and total quantity ingested, eating a higher proportion of the nutritional intake during the latest meals of the day triggers poorer metabolic, immune and microbiota indicators. Irregular mealtimes, and a high time amplitude in nutritional intakes can trigger negative metabolic outcomes.[129,191–194] Few studies have investigated the relationship between meal timing and metabolic response during childhood, and the association studies available show mitigated results. Indeed, in a study from the United Kingdom, no association was found between late dinner and obesity in children, whereas another study from the USA found association only in certain groups of age.[195,196] Therefore, further studies are required to strengthen the understanding on the link between meal timing and metabolism in the paediatric population.

Globally, the individuals with a tendency to engage in late behaviours, or **late chronotypes**, present more frequently metabolic diseases such as obesity, cardiovascular disorders, or type-II diabetes.[187] Although studies on chronotypes and metabolism have mainly been conducted in adults, there is emerging evidence of the relationship between late chronotype and high BMI and metabolic alterations in childhood as well.[197] During adolescence, the late chronotype combined with the early school start is an important vector of **social**

jetlag, meaning a high difference of behaviour timing between school vs free days. In parallel, activities scheduled during the latter part of the day are more often associated with unhealthy behaviours such as alcohol and other drugs consumption, junk food intake or important screen exposure. Together, these elements may explain why individuals presenting later chronotypes tend to have a poorer sleep quality and health.[198] Social jetlag is itself a predictor for high BMI in adulthood.[199] This difference between inner and social clocks times is measured as a >1h delay in sleep behaviours between work/school days and free days, and is encountered in more than 70% of the population.[199] Nevertheless, the pathways through which social jetlag influences body weight and health remain to characterize.

b) The role of circadian rhythms

The late behaviours, as well as the use of screen devices or intense/stressful activity at night[200], can induce **misalignments** between the **central clock**, the **peripheral clocks**, and the **social clock**. This phenomenon appears to play an important part in the metabolic impairments associated to life habits timings. Circadian misalignment's impact on energy metabolism has been primarily observed on nightshift workers. From an epidemiological point of view, these individuals are more at risk of developing obesity, diabetes, cardiovascular diseases, and cancers.[201] A recent meta-analysis on shift work effect on obesity and overweight show that these workers tend to gain weight, and more particularly visceral fat.[202] Additionally, studies measuring the metabolic characteristics of individuals in nightshift work conditions have shown that compared to the control, they displayed a significant increase in the tendency to choose high-fat meals, although no significant increase in the total caloric intake was measured.[203] In addition, another study observed that the participants presented a decrease in the total energy expenditure, as well as a decreased diet-induced thermogenesis consecutively to nightshift.[204] They observed that the sleep rhythms was misaligned with the melatonin rhythm, and that the nutritional intakes occurred during the biological night. Interestingly, another study in a sleep laboratory suggests that the level of diet-induced thermogenesis depends primarily on the alignment between nutrition intake and circadian rhythms, rather than with sleep rhythm.[205] This is supported by studies on rodents with feeding restricted to biological night, which induces an increased body weight and decreased glucose tolerance even in absence of changes in the caloric intake and activity performed.[206] In addition, among rodents fed with high-fat diet, the ones kept in conditions inducing misalignment (constant light exposure) presented heavier weight and altered glucose metabolism.[207] In humans, night eating syndrome, characterised by an important part of the daily nutritional intake consumed during the night, is associated with obesity and binge eating tendency.[208] In individuals whose nutritional ingestion was usually spread over >14 consecutive hours over a day, restricting feeding to only 10-11 hours resulted in weight loss.[209] Further studies are necessary to precisely characterise the metabolic mechanisms modulated according to the extent of the daily eating time frame, and to its synchronization with internal rhythms.

Finally, the habits promoting obesity through circadian rhythms disruption are also influenced by the environment. During childhood, life habits times greatly depend on schooltime and familiar habits.

School time influences wake up time but not bedtime, and early school time promote sleep deficit.[210,211] School start times have even been directly associated to melatonin levels.[212] The other parameters which influence sleep duration are the transport duration to go to school, which if long induces an earlier wake time, while bedtime is influenced by the time of extracurricular activities, by dinner time, by the presence of stress or excitement sources before sleep, and by the presence of screens in the bedroom.[210] These different elements are influenced by social determinants including household education and income, parental context (two parents, single mother, …), minority ethnic background, family conflict, neighbourhood safety.[213,214] As an example, according to the French national institute of sleep and alertness, when parents are educated around sleep questions, the children sleep in average 22 more minutes per night.[215] Nevertheless, scarce information is available regarding the influence of these parameters on the alignment of the circadian rhythms of the children.

In summary, the existence of multidirectional relationships between life habits, circadian rhythms, and metabolism are progressively being identified.

II. Justification

Chronodisruption, generally measured through an altered melatonin expression, is associated with metabolic health disorders, including obesity.[161,204] The late life habits, high screen exposure, poor sleep and frequent social jetlag may play an important role in the current childhood obesity pandemic through an alterations of the circadian rhythms, yet, little is known about these relationships in the paediatric population.

It was observed that disrupted **melatonin expression**, as encountered in pituitary lesioned craniopharyngioma patients, constitutes a risk factor for obesity including in childhood. The concerned individuals present increased daytime sleepiness and low physical activity levels.[216] In children with a functional pineal gland, melatonin expression is delayed by exposure to screen devices at night.[109,124,217] It is to note that childhood is the moment when the melatonin expression is the most sensitive to the inhibition by light exposure.[218] In addition, videogames can constitute a source of excitement and stress which favour cortisol expression and may consequently delay melatonin expression through this path, and delay bedtimes.[219,220] Studies in adults suggest that the timing of nutritional intakes (time of the day, extent of the daily time range of food intake, relative caloric content in early vs late meals) and physical activity may influence the internal circadian rhythms, but no information is available on that regards in childhood.

Few studies have investigated the link between obesity and melatonin expression in childhood. In a sleep laboratory study in which melatonin was measured in 22 adolescents with obesity, no correlation was found between melatonin onset and BMI.[221] In another work on 149 adolescents with obesity, melatonin was measured in the first morning urine, and was found to be significantly lower in individuals with insulin resistance. In these individuals were also found later chronotypes and more social jetlag.[222] An investigation of the evolution of melatonin expression over evening, in at-home conditions, on a wider age panel, would therefore constitute a strategic step to deepen the understanding on the link between childhood obesity and circadian rhythms.

Indeed, circadian rhythms appear to play an important role in the physiology of the adipose tissue as key hormones, enzymes and transcription factors are clock-regulated. Melatonin is considered as the synchronizer of these peripheral rhythms. However, its precise impact on the circadian rhythm of the adipocyte's transcript has not been described yet. Further studies are therefore needed to understand the influence of melatonin on the acrophase, amplitude, and mesor of the genetic expression in the adipocytes.

This link between melatonin and metabolic health may participate to mediate adverse effects of sleep timing, duration, quality, as well as social jetlag, which have been found associated with childhood obesity.[198,199] Melatonin could also constitute a biomarker of obesity. Finally, external factors such as sociocultural and economic characteristics are known influence life habits. However, an innovative approach would be to consider their relationship with the timing of life habits, together with the circadian rhythms and the metabolic health of children. New tools of multivariate analysis are making this approach accessible.

In summary, investigating the multidirectional relationships between life habits and environment, circadian rhythms, and metabolic health, constitutes a key step to improve the understanding of childhood obesity and its development (**Figure 11**).

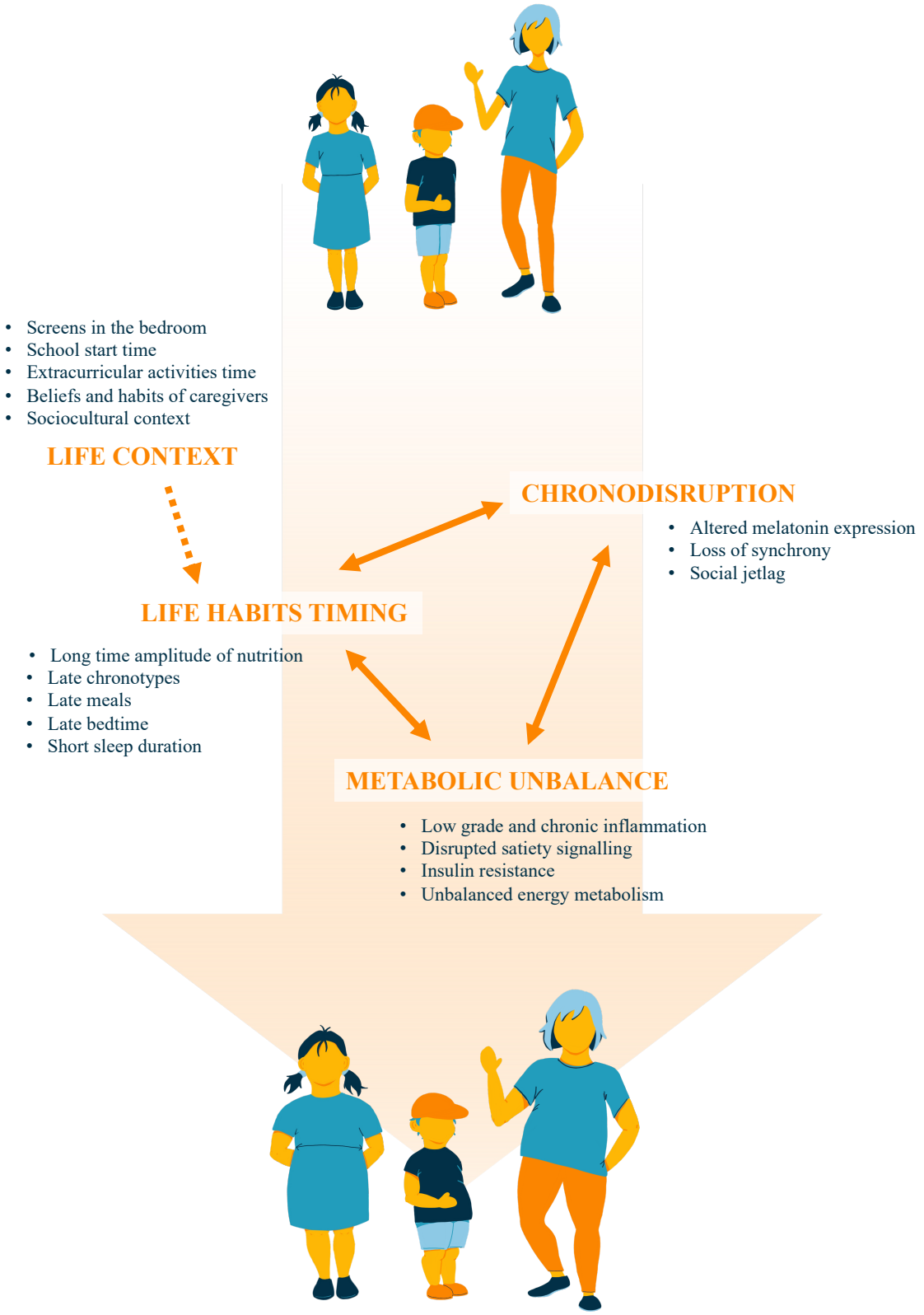


***Figure 11**. Model of childhood obesity development*

III. Hypothesis and objectives

III. 1. Hypothesis

The relationship observed in adulthood between chronodisruption, and obesity also exists in childhood. In children with obesity, melatonin variations are altered. This modified melatonin expression is associated with metabolic and inflammatory markers characteristic of obesity. In the adipose tissue, melatonin modulates the rhythm of expression of clock and metabolic genes.

There is a relationship between melatonin expression and the children's lifestyle habits, with respect to mealtimes, physical activity, sleep duration and hours, but also, screen use, and the familiar sociodemographic environment. Subgroups of children exist that present different melatonin levels of expression, in association with life habits and metabolic health markers. The timing of life habits participates to predict obesity and overweight. Melatonin expression level is a biomarker of obesity in childhood. Interrelationships exist between life habits, circadian rhythms, and metabolic health in childhood obesity.

III. 2. Objectives

The main objective of the present project is to assess the **relationships between life habits, circadian rhythms, and metabolic health during childhood**.

In the aim of reaching this main objective, secondary objectives are set as follows:

- Quantify the difference in evening melatonin expression between children with obesity and normal weight by testing a new approach of circadian rhythms measurement using an at-home saliva collection method for melatonin detection at three times over evening.
- Measure the effect of melatonin on the circadian expression of clock and metabolic genes in human adipocytes.
- Assess the relationship between melatonin expression and clinical, metabolic, inflammatory, circadian, and environmental factors related with obesity in children.
- Assess the relationship between chronotype and clinical, metabolic, inflammatory, circadian, and environmental factors related with obesity and overweight in children.
- Measure the relationship between dinnertime during school week and clinical, metabolic, inflammatory, circadian, and environmental factors related with obesity and overweight in children.

  Measure the relationship between sleep duration and clinical, metabolic, inflammatory, circadian, and environmental factors related with obesity and overweight in children.
- Measure the relationship between nocturnal screen exposure and clinical, metabolic, inflammatory, circadian, and environmental factors related with obesity and overweight in children.
- Identify subgroups of children sharing similar metabolic, circadian, life habits and environmental characteristics.
- Build a prediction model of obesity based on life habits and environment.
- Build a biomarker model of obesity which includes a circadian rhythms variable.

IV. Materials and methods

IV. 1. Study description

IV. 1.1. Study design

The present research is a **transversal and analytical study** on children who attended the Paediatric Outpatient Office of the Hospital Universitario del Doctor Peset de Valencia, Spain, approved by the ethics committee of this hospital (**Annex 1**).

IV. 1.2. Study population

The children were divided in two groups based on their body mass index (BMI), calculated as the weight in kilograms divided by the squared height in meter (kg/m^2), and using the criterions of Cole et al., 2000.[223] The children presenting a BMI higher or equal to 1 standard deviation (SD) above the mean for age and sex are considered to present overweight, and higher than 2 SD to present obesity, according to the World Health Organization (WHO).[2] The Carrascosa table of Spanish paediatric population developed from 1995 to 2017 provides a reference for BMI distribution from birth until majority.[224] On Carrascosa table, 1 SD above the mean for age and sex correspond to the 84.1th percentile of BMI, and 2SD to the 97.7th percentile. The participants presenting a BMI inferior to one standard deviation were assigned to the control group, and above this cut-off to the overweight and obesity group. The nutritional application of the Sociedad Española de Gastroenterología, Hepatología y Nutrición Pediátrica[225] was used to calculate the percentile and equivalent SD for each participant. All participants were informed in detail about the study and ethical measures and signed the informed consent (**Annex Q1**) by themselves if they are at least 12 years old, or, in the opposite case, their legal tutor.

IV. 1.3. Sample size estimation

The **sample size** was calculated according to the following formula $n = (2 (Z\alpha + Z\beta)^2 \times S^2) / d^2$. The association level for $Z\alpha$ and $Z\beta$ is 99% α, β =0.01. As our aim is to characterize the circadian rhythms in the children, we used melatonin as marker. Therefore, as S we used 11.4, corresponding to the standard deviation of melatonin levels in children[226]. The desired d is 7, corresponding to the minimum difference of expression of melatonin levels after one hour of sleep between the two previously defined groups: normal weight and obesity. With these data a total of 64 patients needed per group is obtained. Estimating a loss of 15%, we need 74 patients per group, thus a minimum of 148 participants in total are needed.

IV. 1.4. Inclusion criteria

Patients between 7 and 16 years old attending the paediatric consultation and who completed the informed consent were included in the study, to the exception of children presenting a particularity that may affect their metabolic and inflammatory profile such as medical treatment, inflammatory or metabolic pathology (other than obesity and comorbidities), or elite athletes.

IV. 1.5. Exclusion criteria

A crucial element of the present work are the saliva samples collected at home by the participants and used for melatonin determination. The participants who lacked providing the saliva samples were excluded from the present analysis.

IV. 1.6. Final sample size

Initially 263 children were recruited, from which 51 were excluded because they did not provide the saliva samples at all or the samples did not meet the standard criteria (minimum volume, conservation in complete tightness of the tube and in darkness). The 212 participants were subdivided between an obesity group composed of 123 of them, and a control group counting 89 children. The obesity group presenting a higher average age, 6 participants of 14 years old and 3 participants of 15 years old were excluded from the obesity group to restore an age balance. The study finally counted 127 participants in the obesity group (61 of female and 66 of male sex) of a mean age ± standard deviation (SD) of 11.47 ± 2.35 years, 76 in the control group (39 of female and 37 of male sex) of 10.8 ± 2.38 years (**Figure 12**). Of the 127 children of the overweight and obesity group, 114 present a BMI higher than two standard deviations for age and sex and can, the criteria for obesity, and 13 present a BMI between 1 and two standard deviations, criteria for overweight. The recruitment started before the covid-19 pandemic, 86.8% of the participants of the overweight and obesity group and 80.3% in the control participants were recruited before, which was not significantly different ($p = 0.318$).

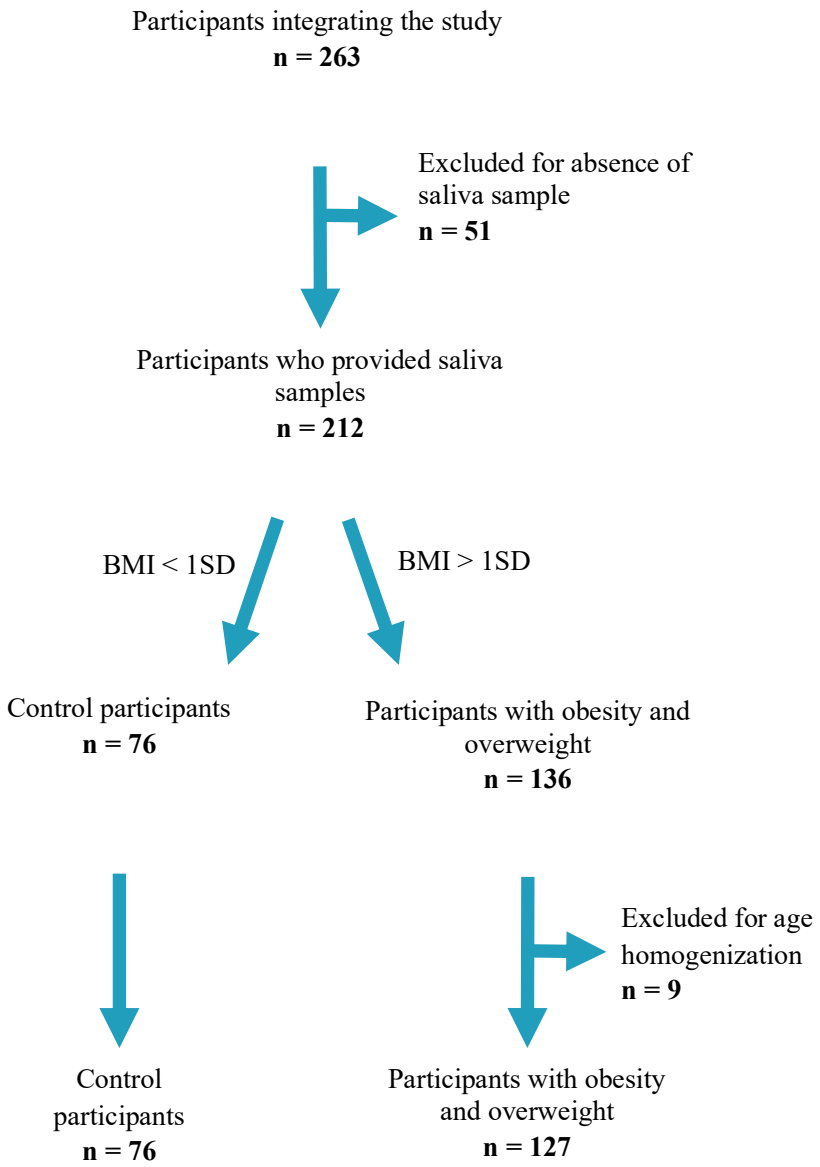


***Figure 12**. Study population and participants exclusion*

IV. 2. Study variables

The variables of the study are grouped in the following categories:

- **Anthropometry and clinical data**: height (cm), weight (kg), BMI (kg/m^2), z-score BMI circumference of hips, waist, and right arm (cm), percentage of fat mass (%), lean mass (kg), systolic and diastolic blood pressure (mmHg), heart rate (BPM). In addition, the percentile for age and sex was obtained for all these parameters.
- **Habits, environment and chronotype**: Sleep times, paediatric sleep disturbance tests of Bruni, Paediatric Sleep Questionnaire, physical activity frequency, presence of screens in the bedroom, sociodemographic familiar context, chronotype, 3-days nutritional intake analysed on the DIAL software, time of meals
- **Metabolic and immune markers**: Glucose, Urea, Creatinine, Uric acid, Total cholesterol, High Density Lipoprotein cholesterol, Low Density Lipoprotein cholesterol, Very Low Density Lipoprotein cholesterol, Triglycerides, Aspartate aminotransferase, Alanine aminotransferase, γ-Glutamyl transpeptidase, Serum total proteins, Albumin, Calcium, Phosphorus, Iron, Transferrin, Transferrin saturation, Calcium corrected for albumin, Creatinine, Microalbuminuria (Urine), Microalbumin creatinine ratio (Urine), Retinol Binding Protein (RBP), Apolipoprotein A1, Apolipoprotein B, high-sensitivity C-Reactive Protein, Cystatin C, C3 ,Thyroid-Stimulating Hormone, insulin, Leptin, Ghrelin, MCP1, TNF-alpha, IL-6, PAI-1, Adiponectin, Resistin, Omentin
- **Melatonin** concentration and total antioxidant capacity in saliva
- **Circadian rhythms of clock genes and clock-controlled genes** BMAL1, CRY, PER2, MT2, PPARγ, C/EBPα, LPL, aP2, LEP, GBP-28 and PAI-1 transcriptional expression relative to β-actin, with and without melatonin stimulation.

IV. 3. Global development of the study

The patients and tutors received the information on the study either by the investigator or the dietician of the team, together with the paediatrician. Data such as age, sex and anthropometric measures were collected, and the patient and its tutor answered the questionnaires. At home, the children had to complete, together with their referent adult, a 3-days nutritional intake record, listing when, what and how much food and drinks they consumed during 2 school days and 1 weekend day. Plus, the evening prior blood extraction, the participant helped by the adult, collected three samples of saliva at different time points: four hours before bedtime, two hours before bedtime, and after one hour of sleep. The participants and families collect the saliva at these times in the tubes provided for this purpose, properly close the tubes, store them in the fridge protected from the light, and to bring them to the hospital the following day, blood extraction day. This day was scheduled during the school week on Tuesdays or Wednesdays to avoid circadian rhythms changes associated to holidays and weekends. The patients came to the hospital on an empty stomach for the blood extraction, bringing the nutritional record and the saliva samples. The nurse in charge of the blood extraction collected the tubes necessary for the basal biochemical analysis, plus two additional tubes, one containing EDTA for the plasma (5ml EDTA BD vacutainer Mississauga ON Canada) and one with gelose for the serum (5ml serum BD vacutainer Mississauga ON Canada). The basal biochemical analysis was performed in the central laboratory of the hospital under the standardized conditions, whereas the two extra tubes of blood and the three samples of saliva were brought to the laboratory of Paediatrics, Obstetrics and Gynaecology of the University of Valencia by the investigator for further hormonal, immune and antioxidant analysis. The data from the questionnaires, anthropometrics measures, biochemical analysis from blood and saliva were anonymized and identified by a general lab code. Data under that code were used for subsequent statistical analysis.

IV. 3.1. Anthropometry and clinical assessment

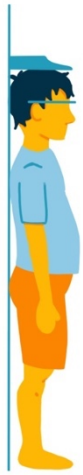


Figure 13. *Measurement of height*

The same conditions and the same material were employed with all the participants. To record the **height**, the stadiometer Holtain (Holtain Ltd., Dyfed, UK) with a precision of 0,1cm was used. The measurement was realized without shoes, joined feet, glutei, back and head in contact with the wall, and a straight head with the ear and eye form a horizontal line (**Figure 13**).

The participant wearing only underwear took place on the bioelectric impedance and electronic balance BC-418 MA Tanita Segmental Body Composition analyser (Tanita Europe BV, Hoofddorp, The Netherlands, **Figure 14**). It functions as an electronic balance and measures the **body weight**. With the input of the age and height, this device displays the **BMI**, and via its impedancemeter function, it recorded the **percentage of fat** and **lean mass** of the body. The **percentiles** were calculated via the application of the Spanish Society of Gastroenterology, Hepatology and Paediatric Nutrition (SEPHNP)[225], based on Carrascosa tables as a reference.[227]

Figure 14*. Bioeletric impedance*

Waist, **hips,** and **right arm circumference** is also measured manually using a non-extensible, flexible, and calibrated steel tape in centimetres with millimetre graduation and following the standardized protocols of the International Society for the Advancement of Kinanthropometry (ISAK). The **waist/hips index** and the **waist/height index** were calculated.

Systolic blood pressure, **diastolic blood pressure** and **heart rate** were assessed in sitting position with an automated sphygmomanometer (Dinamap DPC101X - SP; GE medical Systems Information Technologies, Inc., Milwaukee, WI, USA). The qualitative assessment of blood pressure was obtained using the body composition laboratory online application of the Baylor College of Medicine.[228]

IV. 3.2. Habits, environment and chronotype

The categories of parameters which assessed are the following: chronotype, nutritional intake, physical activity, sleep, exposure to technological devices in the bedroom, and familiar socio-demographic context.

IV. 3.2.1. Chronotype

The investigator asks to the participant the questions designed by Horne and Östberg to assess the **chronotype** (**Annex Q7**).[134] The questionnaire is designed so that each answer gives a score. The higher the total, the earlier the chronotype and inversely. A chronotype is this way assigned to each participant: extreme evening chronotype for scores below 30, moderate evening chronotype between 31 and 41, intermediate between 42 and 58, moderate morning chronotype between 59 and 69, and extreme morning chronotype above 70.

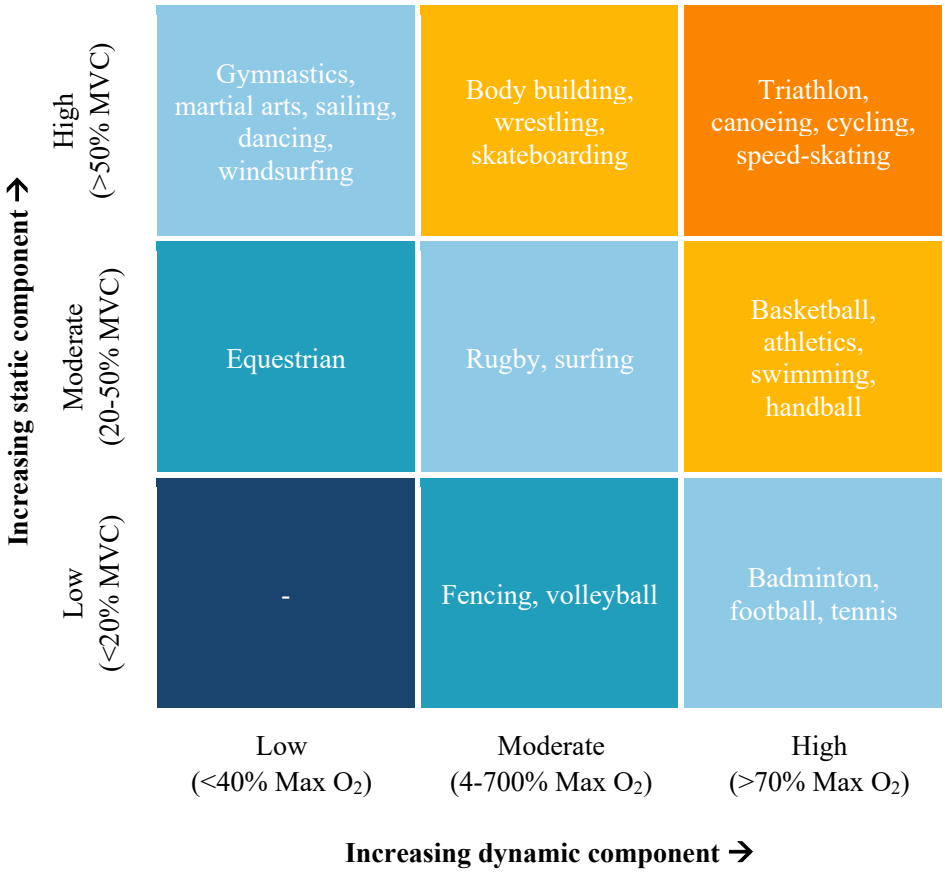
IV. 3.2.2. Nutritional intake

The investigator finally provided instructions and a template (**Annex Q5**) for the participants to record at home its **nutritional intake** for three days, with the support of the adult. Every time they were drinking or eating, they had report it as accurately as possible in regards of type of food, cooking, quantity, time. Of the three days of the recording, two were weekdays and one weekend day. The document was brought back to the investigator on the day of the blood extraction, who would input all the nutritional items for analysis in the software DIAL (Alce Ingeniería SA, Madrid, España).[229] From this analysis, the following data are obtained: energy consumed (kcal), energy distribution in the main macronutrients (%): carbohydrates, proteins and lipids to know the most relevant characteristics of the patients diet. The values of these parameters for each meal are also collected, and ratios are calculated to assess for each individual which meal conveys a higher nutritional intake compared to the others. The time of each meal was measure during the week and the weekend, and the time amplitude of nutritional intake was calculated as the time difference between the first meal (generally breakfast), and the last meal (generally dinner). From the nutritional 3-days diary was also recorded the mentions of "homemade" and "processed" foods, the snacking habits, the number of mentions of sodas and sweets in the diary. The quality of the report was quantified on a scale from 1 to 5 (5 being the best quality) as follows: 5/5 when each meal was reported in detail with the times, foods types, cooking type, brands, and quantities. 4/5 when all meals are reported but with a lower level of details for example brands or type of cooking is missing. 3/5 when all the meals are reported but in little details and elements such as quantities are incomplete. 2/5 little information is reported for each meal content, or quantities are missing. 1/5. Very incomplete report, barely any information and lacking meals.

IV. 3.2.3. Physical activity

The researcher reports on the adapted questionnaire (**Annex Q2**) the information on **physical activity** transmitted by the patient. They were asked whether they practice a physical activity out of school and if so which one, at what time and at which frequency. The physical activities were classified in three categories according to the level of cardiovascular demand.[230,62] The mechanical action of a muscle can be measured on two scales: dynamic (isotonic) and static (isometric). Static component is measured in regards with the percent of maximal voluntary contraction (MVC), and dynamic component in percentage of the maximal oxygen uptake (Max O_2). Each of these components stimulates cardiovascular adaptation to a level that is scaled from "low" to "high" as presented in **Table 1**.

***Table 1**. Physical activity cardiovascular demand**



**The level of cardiac demand is symbolized by the colours of the table cells: orange for the highest cardiovascular demand (cardiac output and blood pressure), followed by low moderate in yellow, moderate in sky-blue, low moderate in sapphire and low in marine blue. Maximal voluntary contraction (MVC), maximal oxygen uptake (Max O2). Adapted from the classification of sports[62] with agreement licence n°5310101250242 from the publisher.*

IV. 3.2.4. Sleep

The researcher asked the patient at what **time** he or she uses to go to **sleep** and **wake-up**, during the school **week** and during the **weekend**, and reports it on the document shown in annex Q2. From these data, the **sleep duration** and **difference in sleep duration during week and weekend** are calculated. In the meantime, the present parent was asked to complete several questionnaires on the child sleep quality. The Bruni questionnaire validated in Spanish (**Annex Q3**) was used to detect **sleep disturbances**.[231] In this test, a figure is associated to each answer, and the sum of these numbers are calculated, and a total outranking 39 suggests a sleep disturbance. Paediatric sleep questionnaire validated in Spanish is a paediatric questionnaire employed to assess **sleep quality**.[232] The short version oriented towards sleep apnoea diagnostic has been used (**Annex Q4**). To each item, the parent answered "yes", "no", or "unknown" and the number of positive answers was quantified afterwards. Above 33% of positive results sleep is considered of poor quality.

Sleep duration was considered **adequate** when complying the recommendations of the Spanish sleep society: for children from 6 to 13 years old, 9 to 11 hours of sleep recommended per night, and 8 to 10 hours from teenagers after 14 years old.[233]

IV. 3.2.5. Exposure to technological devices in the bedroom

The investigator enumerates the following list of devices with screens: mobile phone, television, console (Wii, Xbox, PS4…), portable console (DS, PSP, switch…), computer, tablet; and the participants are asked which are present in their bedroom at night. The questionnaire (**Annex Q2**) is used to assess the **presence** and **sum of electronic devices in the bedroom**.

IV. 3.2.6. Familiar socio-demographic context

The questionnaire Annex Q6 is completed by the parent of the participant, the following information is collected:

- **Education level of the mother** (1 = no studies; 2 = primary education; 3 = secondary education; 4 = high school; 5 = professional formation; 6 = university studies; 7 = master's degree; 8 = PhD)
- **Education level of the father** (1 = no studies; 2 = primary education; 3 = secondary education; 4 = high school; 5 = professional formation; 6 = university studies; 7 = master's degree; 8 = PhD)
- **Working situation of the mother** (1 = unemployed; 2 = temporal contracts; 3 = stable contract)
- **Working situation of the father** (1 = unemployed; 2 = temporal contracts; 3 = stable contract)
- Familial context in regards of the **parental situation** (living together, separated, and single, separated and with someone else, other)

IV. 3.3. Routine biochemical parameters

The basal biochemical assessment is performed on patient's blood. The blood sample was collected from the patient after twelve hours fasting, processed immediately after collection (centrifuged at 3000 g at 4°C for 5 minutes), and the determinations were performed under standardized conditions. **Glucose, total cholesterol, HDL, triglycerides, uric acid, urea, creatinine, apolipoprotein A1 and B** were measured using automated direct methods (Aeroset System® Abbott Chemical Clinic, Wiesbaden, Germany). **LDL** and **VLDL** cholesterol levels were calculated using the Friedewald formula and the triglyceride/5 ratio, respectively. **Insulin** and **homocysteine** were determined using automated electrochemiluminescence immunoassay (c8000® Architect, Abbott Clinic-Chemistry). **Cystatin C** levels were analysed by immunonephelometry with Behring 2 (Dade Behring, Marbung, Germany) nephelometer. The **high sensitivity C-reactive protein (hs-CRP)** was analysed by immunonephelometry with a Behring2 nephelometer (Dade Behring, Marbung, Germany). **Γ-GT** was measured by enzymatic colorimetry at 37℃ on a Roche / Hitachi automated analyser (Mannheim, Germany). **Vitamin D** (25-hydroxyvitamin D form) were quantified by electrochemiluminescent immunoassay on the Roche COBAS 6000 autoanalyzer (Roche Diagnostics GmbH, Mannheim, Germany). To determine **TSH**, a direct immunoradiometric assay was used (Beckman Coulter UniceDxl 800, Beckman Coulter, L´Hospitalet de Llobregat, Barcelona). The **HOMA** index was calculated according to the following formula: HOMA = fasting insulin (μUi / ml) × fasting glucose (mg / dL) / 405. **The atherogenic index** was calculated using the following formula: triglycerides (mg / dL) / HDL (mg / dL). **Haematological parameters** were obtained in venous blood obtained in tubes with EDTA anticoagulant, using the Coulter LH750 Analyzer autoanalyzer from Beckman Coulter.

***Figure 15**. Laboratory of Paediatrics, Obstetrics and Gynaecology facilities at the Universitat de Valencia*

IV. 3.4. Experimental parameters

The participant gave the saliva samples to the investigator on the day of the blood extraction during which two blood samples were collected in vacutainer for the basal biochemical assessments: one with gelose and one with EDTA. Those tubes, together with the samples of saliva are transported under refrigerated and darkness conditions to the laboratory of Paediatrics, Obstetrics and Gynaecology of the Facultat de Medicina of the University of Valencia (**Figure 15**). Immediately after arrival, the samples of saliva are transferred to cryotubes and stored at -80°C. The blood samples are centrifuged at 1500rpm during 10 minutes at 4°C, separating plasma, serum, and cells. The samples were aliquoted and stored at -80°C until the tests were performed (**Figure 16**).

Figure 16. Samples from the hospital are centrifuged, aliquoted and frozen until the experiments.

IV. 3.4.1. Metabolic and inflammation markers

Several interesting parameters in obesity investigation are not included in the routine biochemical blood analysis, so we have assessed them separately in the samples we collected. The adipokines **Leptin, Resistin, Adiponectin, Ghrelin**, and the immune markers **MCP-1, PAI-1, IL-6, TNFα** and **INF-γ** were determined via multiplex immunoassay (Labscan 100 Luminex©, Merck Millipore Merck KGaA, Darmstadt, Germany) with Luminex technology using the specific software 3.1 (Luminex Corporation. Austin, TX, USA.) (**Figure 17**).

Figure 17*. Material necessary for experimental metabolic and immune markers.*

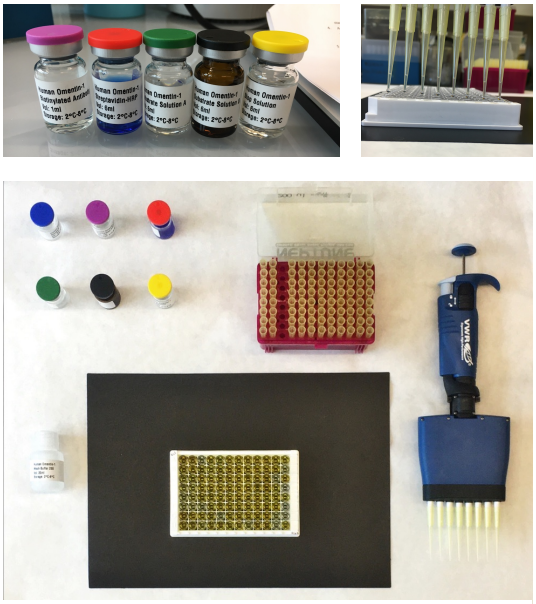


Figure 18. *Material necessary for omentin quantification.*

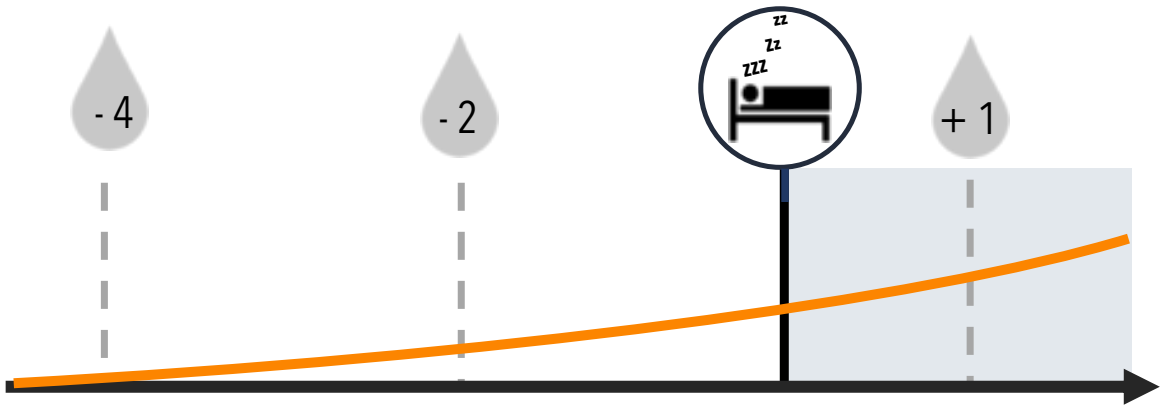
Omentin was quantified with enzyme-linked immunosorbent assay (ELISA) (Human Omentin-1 ELISA, Merck-Millipore, Darmstadt, Germany) (**Figure 18**) with the VICTORTM X3 2030 multilabel plate reader (Perkin Elmer, Waltham, MA, USA).

IV. 3.4.2. Melatonin and antioxidant capacity

***Figure 19**. Saliva collection*

The participants were provided 30ml plastic tubes usually used for urine collection, in which the children were instructed to let their saliva fall until collecting around 300µl (**Figure 19**). They were asked to avoid screen exposure and physical activity in the 15-30 minutes before saliva collections. Three saliva samples were collected from each patient the evening before the blood extraction: 4h before sleep time, 2h before sleep time, and after 1h of sleep (**Figure 20**).

***Figure 20**. Saliva collection 4h before sleep, 2h before sleep and after 1h of sleep*



Before analysis, saliva was centrifuged at 5000RPM during 2 minutes at 4°C. It was verified that all the samples were conditioned in darkness, the tubes in complete tightness and that the minimum volume necessary for subsequent analysis was present. In each sample, the **melatonin** level is quantified via immunoassay using a salivary kit from Salimetrics (Salimetrics, LLC Carlsbad, CA USA), following the instructions available online at https://salimetrics.com/wp-content/uploads/2018/03/melatonin-saliva-elisa-kit.pdf. The more melatonin in the sample, the more intense is the yellow coloration at the end of the reaction, which quantification is performed using a VICTOR TM X3 2030 multilabel plate reader (Perkin Elmer, Waltham, MA, USA) (**Figure 21**).

The hourly rate of melatonin concentration increase over time was calculated between the different time points:

- **Melatonin increase rate over evening** was calculated as the difference between melatonin concentration at the last time point (+1h after sleep onset) and the first time point (-4h before sleep onset) divided by the number of hours between them (5 hours).
- **Melatonin increase rate before sleep** was calculated as the difference between melatonin concentration at the middle time point (-2h after sleep onset) and the first time point (-4h before sleep onset) divided by the number of hours between them (2 hours).

- **Melatonin increase rate around sleep** was calculated as the difference between melatonin concentration at the last time point (+1h after sleep onset) and the middle time point (-2h before sleep onset) divided by the number of hours between them (3 hours).
-

***Figure 21**. Material necessary for melatonin quantification.*

The total **antioxidant capacity** of these saliva samples at three time points was assessed using the colorimetric reaction of 2,2-diphenyl-1-picrylhydrazyl (DPPH). This molecule is a stable free radical and has a strong purple colour absorbing at 517nm, which disappear when it is scavenged by an antioxidant. This colour change can be measured with a spectrophotometer, this method has been described and improved numerous times, and recently our investigation group has adapted this method to have the possibility to use it on very small samples of biological fluids.[234] We used this adapted method as described in the publication, the DPPH was purchased from Aldrich (Chemical Company, USA) and applied it in a 96-wells plate with flat bottom. For the quantification of the colorimetric reaction, a standard curve of Trolox equivalent was used as reference for the calculation of the antioxidant capacity of saliva samples. After 30 minutes of biochemical reaction, a picture of the plate was taken with homogeneous light exposure of the wells using a retroluminescent background. The colour was quantified using the PlateReader 3.0 plugin on the imageJ software (https://imagej.nih.gov/ij/plugins/readplate/index.html) analysing RGB images separately (**Figure 22**). As for melatonin quantification, the measurement of antioxidant capacity was performed saliva samples from three time point: 4h before sleep, 2h before sleep and after 1h of sleep. And as for melatonin, increase rates were calculated from the three measurements as follows:

- **Antioxidant capacity increase rate over evening** was calculated as the difference between melatonin concentration at the last time point (+1h after sleep onset) and the first time point (-4h before sleep onset) divided by the number of hours between them (5 hours).

- **Antioxidant capacity increase rate before sleep** was calculated as the difference between melatonin concentration at the middle time point (-2h after sleep onset) and the first time point (-4h before sleep onset) divided by the number of hours between them (2 hours).
- **Antioxidant capacity increase rate around sleep** was calculated as the difference between melatonin concentration at the last time point (+1h after sleep onset) and the middle time point (-2h before sleep onset) divided by the number of hours between them (3 hours).

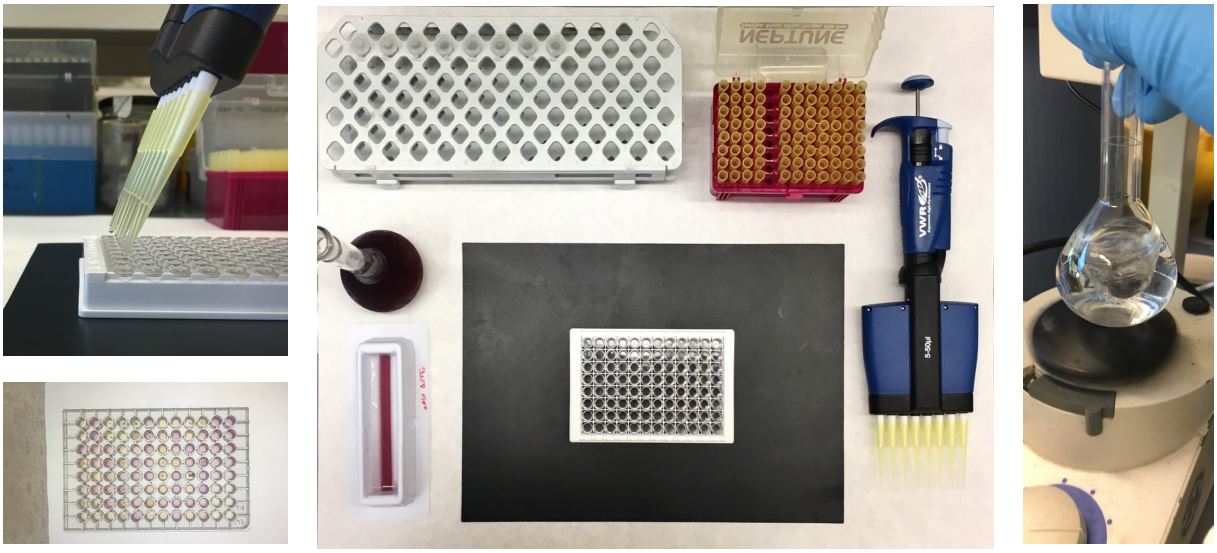


Figure 22. Material necessary for the antioxidant capacity DPPH assay.

IV. 3.5. *In vitro* study of the circadian rhythms of adipocyte genes: the impact of melatonin

The *in vitro* study protocol is summarized in **Figure 23**.

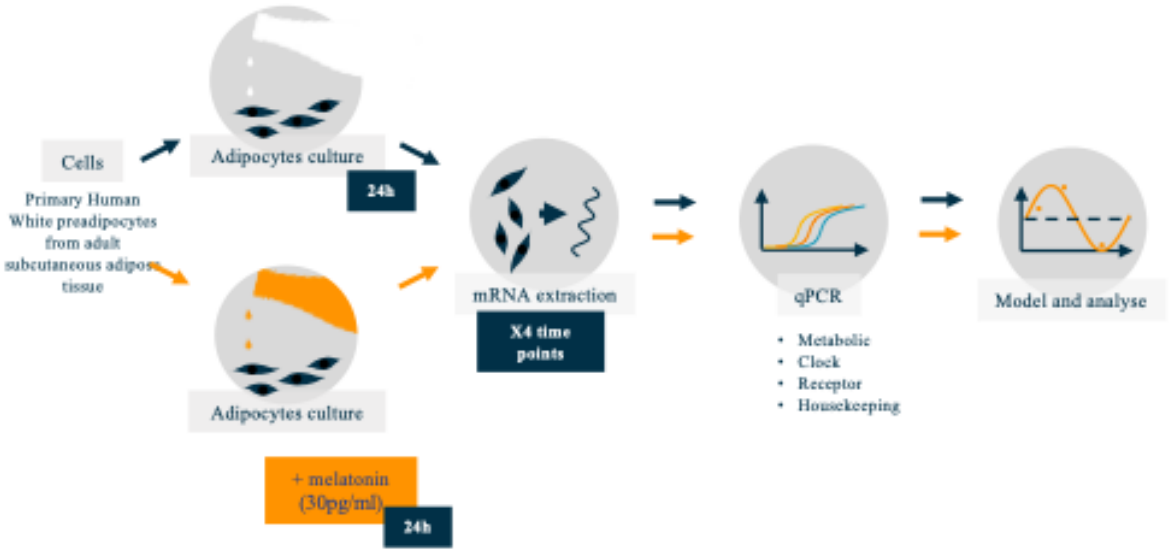


Figure 23. In vitro *study protocol*

IV. 3.5.1. Cell culture

The experiment was performed on Primary Human White Preadipocytes isolated from adult subcutaneous adipose tissue (Promocell, Heidelberg, Germany) after abdominal liposuction in a Caucasian woman aged 52. Cells were seeded at 10^5 cells/ml in six-well plates, grown to confluence in growth medium (Promocell, Heidelberg, Germany) at 37°C in a humidified atmosphere containing 5% CO2. They were then placed in differentiation medium (Promocell, Heidelberg, Germany). In half of the cell cultures, the differentiation medium was supplemented with 30pg/ml of melatonin (N-Acetyl-5-methoxytryptamine). A day after, the cell cultures were collected at 4 time points: 8:00 AM, 10:45 AM, 6:30PM, 11:00PM. All cultures were performed in triplicates.

IV. 3.5.2. Transcript quantification

The mRNA was extracted using a Qiagen RNA extraction kit retro-transcripted qScript microRNA cDNA Synthesis Kit. The **quantitative PCR** was performed using PerfeCTa SYBR Green Super Mix Low ROXTM (QIAGEN Beverly, Inc.) in 384-wells plates using a thermocycler QuantStudio 3 (Applied Biosystems Waltham, Massachusetts). Cycle Threshold (CT) values were obtained in triplicate for the genes β-actin, BMAL1, CRY, PER2, MT2, PPARγ, C/EBPα, LPL, LEP, aP2, Adiponectin (GBP-28) and PAI-1 using the probes presented in **Table 2**. The β-actin gene was used as reference, results are presented in ΔCT.

***Table 2**. qPCR probes*

Gene	Reverse probe	Forward probe
β-actin	GCCTCGTCGCCCACATAG	GCCGTCTTCCCCTCCATC
BMAL1	TGCCTCGTCGCAATTGG	ACCCTGATTTCCCCGTTCA
CRY	AGTGGGCTGAGGGCAAGAC	CAGCCCTCCTGCCTCAGTT
PER2	GTCCAGCCCCCACCTTTC	GGGAAGGAATAACTGGGTAGCA
PPARγ	GGCGGTCTCCACTGAGAATA	GAGCCCAAGTTTGAGTTTGC
C/EBPα	TTTAGCAGAGACGCGCACATTCAC	ATTGCCTAGGAACACGAAGCACGA
LPL	CTGGCATTGCAGGAAGTCTG	GCATCATCAGGAGAAAGACGA
AP2	TCTCTTTATGGTGGTTGATTTT	CAGTGTGAATGGGGATGTG
MT2	AGCCAGATGAGGCAGATGTGCAGA	TCCTGGTGATCCTCTCCGTGCTCA
GBP-28	TGAATGCTGAGGGCTAT	CATGACCAGGAAACCACGACT
PAI-1	ACAGCATTTTGGTGGTGACTT	TGCTGGTGAATGCCCTCTACT
LEP	CCATGCAATGCTCTTCAATCCTGGAG ATACC	CCATCCAAAAAGTCCAAGATGACACC AAAA

IV. 4. Statistical analysis

R software (version 3.5.0 R Core Team, 2018, Vienna, Austria) was used for the entire analysis[235], and the tidyverse package was employed for the statistical work and the representations.[236] Other packages were used along the data analysis: readxl[237], psych[238], RcmdrMisc[239], ggsignif.[240] The sjPlot package was used to build the tables.[241]

Regarding outliers, each variable was manually reviewed and points distant from more than 6 standard deviations to the mean were excluded from the analysis. No individual was identified as an outlier using Mahalanobis distance. For age matching, the three 14 years old participants and two 15 years old participants from the obesity group which had the highest number of missing values were excluded from the study. A Shapiro-Wilk test was used to assess for normality for each quantitative variable.[242] In our study population, the variables do not follow a normal distribution so the statistic tests were chosen accordingly. Quantitative data are expressed as median and interquartile range, categorical data as absolute number or percentage. Statistical significance is established on the basis of a $p < 0.05$. Wilcoxon tests were used for group comparisons of quantitative variables, unpaired for inter group comparisons and paired for comparisons of a same group at several timepoints. χ^2 test was used for group comparisons of qualitative variables.

Odds-ratios were calculated using the following for risk factor estimation:

	Outcome = yes	Outcome = no
Predictor = yes	a	b
Predictor = no	c	d

$$OR = \frac{\frac{a}{c}}{\frac{b}{d}}$$

A fisher test was performed to analyse the significance of the odds-ratios. The parameters involved in the odds ratio analysis and cut-offs points are shown in the following **Table 3**. Cut-off values for odds-ratio calculations. Part of them are traditional metabolic risk factors which cut-off values were previously published. In the case of the leptin, the normality limits for these variables were calculated as the 97th percentile of the control group. Regarding schedules, the median time of the meals in the control group was used to define early and late.

***Table 3**. Cut-off values for odds-ratio calculations*

Variables	*Limit*	*Background*
Percentage of fat mass	Age and sex referenced	Freedman et al. 2009[82]
Waist/height index	> 0.5	Bacopoulou et al. 2015[243]
HOMA index	> 3.16	Keskin et al. 2004[244]
Leptin (ng/mL)	> 7.46	97th percentile of control group
Bedtime during schooldays	> 22h25	Median of control group
Waking hour during weekend	> 10h00	Median of control group
Dinner time during the week	> 21h00	Median of control group
Lunch time during the weekend	> 14h30	Median of control group
Time amplitude of nutritional intake	> 12h45	Median of control group

For the modelling of the circadian rhythms of clock genes, the packages Cosinor (version 1.1)[245], ggplot2 and dplyr were used. The transcript expression in ΔCT was plotted according to time, and a cosinor model was built for each condition (with and without melatonin) applying a partial least squares regression of a period of 24h. The Wald-test integrated in the cosinor R package was applied to estimate the difference between the two conditions in terms of amplitude, acrophase and mesor of the rhythm (**Figure 24**).

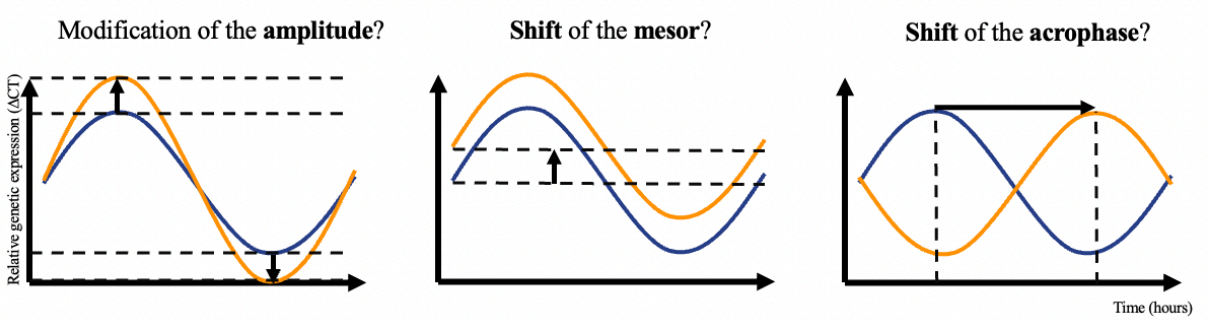


***Figure 24**. Hypothetical changes induced by melatonin (orange curve) on the circadian rhythms of clock and metabolic genes of adipocytes*

Correlations were assessed using the pcor.test function of the ppcor package (R package version 1.1.; 2015)[246] to calculate Spearman's rho coefficient and the associated p-value. The spider chart was performed using the package fmsb version 0.7.3, 2022.[247] All correlations were adjusted for sex, age and puberty index. Prior to Principal Component Analysis (PCA), the variables correlated with melatonin increase around sleep rime were selected, the Kaiser – Mayer – Olkin index, known as KMO index, was calculated and Barlett sphericity test was performed to assess sample adequation. The package Factoshiny (R package version 2.4.; 2021) was used for PCA and the subsequent Hierarchical classification.[248] For each multivariate analysis, missing values were imputed with a model at k-dimensions selected in the Factoshiny app including only the variables concerned by the analysis. Finally, the caret package was used to build random forest models using as metrics the receiver operating characteristic (ROC), which integrates both the sensitivity (true positive rate) and specificity (false positive rate) of the model.[249]

IV. 4.1. Confidentiality, information, and ethics

All patients and their relatives were informed about the study: description of the study, benefits and possible risks, steps to be followed in sample collection and the treatment to be given to the results obtained by the researcher or the dietician of the team, together with the paediatrician. After informing the patients and acceptance by the parents of the minors, the informed consent form was signed by the parents and by the participant from 12 years of age (**Annex Q1**).

All the information collected from the participants were treated with confidentiality and all database followed the pseudo-anonymization process in accordance with the legal regulations in force in Spain (Organic Law 7/2021, de May 26th on Personal Data Protection and Law (UE) 2016/679 European parliament modified on May 23rd, 2018, Ley Orgánica 3/2018, de 5 de diciembre, de Protección de Datos Personales y garantía de los derechos digitales).

The present research project was approved by the Ethic Committee of the Hospital Universitario Dr. Peset de Valencia on March 29th, 2018 (**Annex 1**).

V. Results

The result section is organized in four parts.

- First, the overweight and obesity group and the control group are **described and compared** to each other for all study variables.
- Second, correlations and odds-ratios among the variables related to **circadian rhythms**, **life environment**, and **metabolic health**.
- Third is presented the *in vitro* experiment on the impact of **melatonin** on the **circadian rhythms** of **adipocytes genes**.
- The results section ends with the **multivariate analysis and models**.

V. 1. Description and comparisons

The two study groups are first compared in terms of **age, sex, and puberty stage**, then by **anthropometrics and clinical characteristics**, followed by **metabolic, inflammatory, and circadian markers**, and finally in respect with **life history, habits, and environment.**

V. 1.1. Age, sex, and puberty stage

The median **age** of the participants from the control group is 11 years old (9-12.75) and 12 years old (10-13) in the participants from the overweight and obesity group, a difference which is not statistically significant (p = 0.057, **Figure 25**). **Sex** distribution is also homogeneous between the groups with 51.4 % girls in the control group and 47.7 % in the obesity and overweight group (p = 0.721, **Figure 26**). The **puberty stage** is more advanced in the obesity and overweight group, with only a third of the participants not presenting yet signs of puberty and therefore scored as 1 on Tanner scale, and more than 15% presenting advanced signs of puberty, scored 4 and 5. On the other hand in the control group, puberty is started in only half of the participants and is advanced in less than 5% of them (**Figure 27**). This difference is statistically significant (p = 0.011). Plus, the analysis narrowed by sex indicates that the advance in puberty is strongly present in girls with obesity (p = 0.009), but not in boys with obesity (p = 0.108).

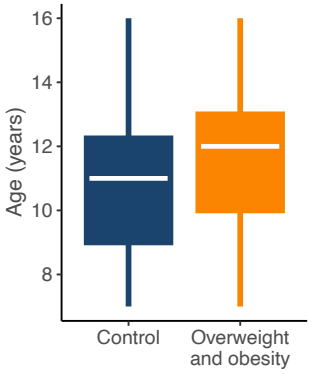


***Figure 25**. Age distribution*

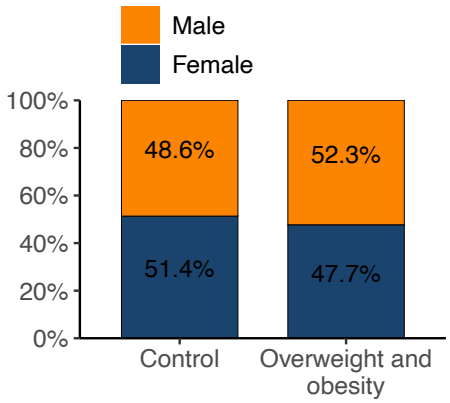


***Figure 26**. Sex distribution*

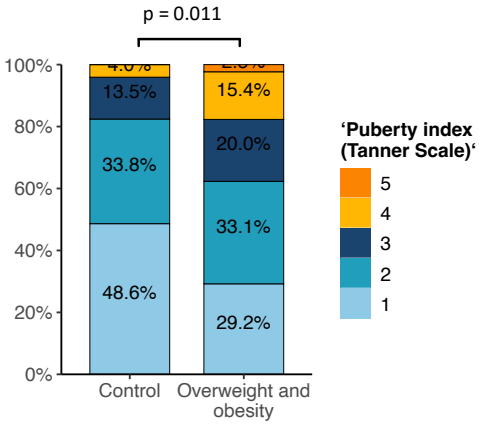


***Figure 27**. Puberty index distribution*

V. 1.2. Anthropometry and clinical characteristics

The **anthropometric characteristics** are presented in **Table 4** and the clinical characteristics in **Table 5**. The median and interquartile range is presented for each group and the total population. The p-values of the differences between the two groups figure in the last column. **Figure 28** and **Erreur ! Source du renvoi introuvable.** illustrate some of the differences observed between the groups.

***Table 4**. Anthropometric characteristics**

Variables	Control	Overweight and obesity	Total	p-values
BMI (kg/m2)	17.1 (15.9-18.32)	27.5 (24.5-30.77)	24.15 (18-28.8)	**< 0.001**
BMI percentile	28.5 (13-57.25)	99 (99-99)	99 (48.75-99)	**< 0.001**
BMI z-score	-0.56 (-1.21-0.19)	3.22 (2.32-4.53)	2.3 (-0.07-3.52)	**< 0.001**
Weight (kg)	33.1 (28.5-45.3)	65.9 (52.85-78.45)	53.65 (36.95-69.92)	**< 0.001**
Height (cm)	142 (130-158.2)	155.8 (146-161.4)	153.3 (139-160.7)	**< 0.001**
Weight z-score	-0.74 (-1.43-0)	3.12 (2.05-4.54)	2.01 (-0.25-3.79)	**< 0.001**
Height z-score	-0.6 (-1.98-0.7)	0.39 (-0.5-1.28)	0.18 (-0.87-1.12)	**< 0.001**
Arm circumference (cm)	21 (19.2-23.9)	30 (27.9-32.45)	27.75 (22.95-31.12)	**< 0.001**
Hips circumference (cm)	74 (69.45-83.65)	99.2 (90.25-108)	90.55 (77-103)	**< 0.001**
Waist circumference (cm)	61 (56.9-67)	87 (80-96.75)	79.4 (64.55-91)	**< 0.001**
Arm z-score	-0.68 (-1.15-0.18)	1.91 (1.36-2.65)	1.33 (0.04-2.23)	**< 0.001**
Hips z-score	-0.74 (-1.35--0.06)	1.78 (1.2-2.48)	1.14 (-0.32-2.13)	**< 0.001**
Waist z-score	-0.74 (-1.43-0.08)	2.17 (1.38-2.65)	1.29 (-0.23-2.34)	**< 0.001**
Waist/hips index	0.82 (0.77-0.86)	0.88 (0.84-0.94)	0.86 (0.81-0.91)	**< 0.001**
Waist/height index	0.44 (0.41-0.46)	0.58 (0.53-0.61)	0.53 (0.45-0.59)	**< 0.001**
Percentage of fat mass	21.05 (17.88-23.52)	35.9 (32.1-40.3)	31.6 (22.25-37.4)	**< 0.001**
Lean mass (kg)	26.25 (22.2-36.08)	41.35 (34.12-49.4)	37.25 (27.32-44.65)	**< 0.001**

*Data are presented in median (interquartile range). Comparisons between the two groups were made by Wilcoxon test.

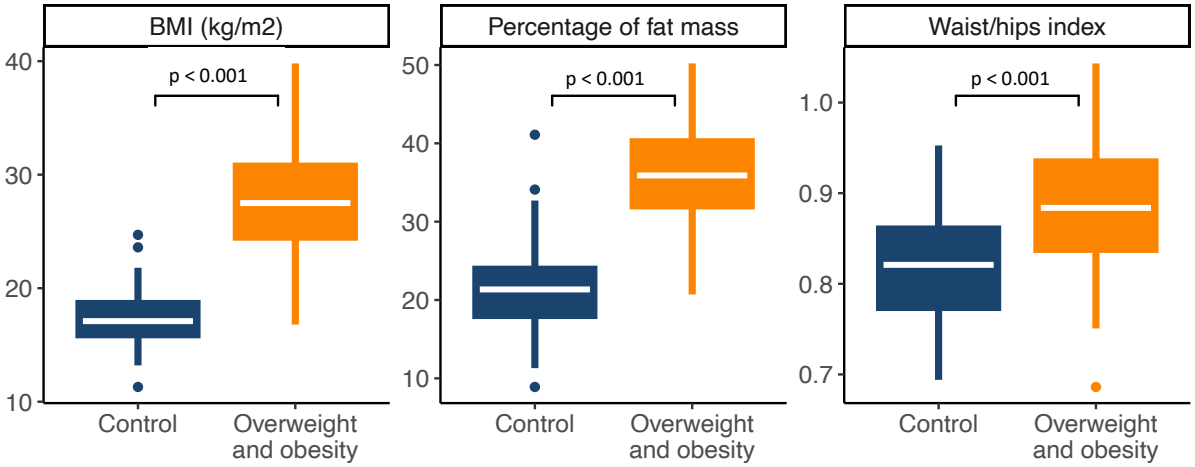


***Figure 28**. Comparison of the BMI, fat mass percentage and waist/hips index.*

The participants from the overweight and obesity group present significantly increased values for all the anthropometric variables compared to the controls (**Figure 28**, **Table 4**).

***Table 5**. Clinical characteristics**

Variables	*Control*	*Overweight and obesity*	*Total*	*p-values*
Systolic blood pressure (mmHg)	106 (96.25-114.75)	114 (109-124.5)	113 (103-120)	**< 0.001**
Diastolic blood pressure (mmHg)	58 (53-64)	62.5 (58-68)	61 (56-66)	**< 0.001**
Systolic blood pressure percentile	67 (32.5-84.5)	85.5 (62-94.25)	77 (52-93)	**< 0.001**
Diastolic blood pressure percentile	38 (23-66)	48 (30.5-68)	45 (27-67.75)	**0.015**
Heart rate (BPM)	78 (65-88)	83 (73-93.5)	82 (72-90)	**0.002**

**Data are presented in median (interquartile range). Comparisons between the two groups were made by Wilcoxon test.*

Children with overweight and obesity present higher **blood pressure** and heart rate compared to the control group participants (**Table 5**). In addition, blood pressure was classified for age and sex in three categories: Normal, elevated and hypertension. A difference in the percentage of participants in each category was encountered between both groups regarding systolic blood pressure ($p = 0.001$), but not for diastolic blood pressure ($p = 0.29$).

V. 1.3. Metabolic and inflammatory markers in blood

The **metabolic variables** are presented in **Table 6** and the inflammation markers in **Table 7**.

Table 6. *Metabolic characteristics**

Variables	*Control*	*Overweight and obesity*	*Total*	*p-values*
Glucose (mg/dL)	88.5 (84-93)	92 (86-95.75)	90 (85-95)	**0.008**
Insulin (µUI/mL)	7.4 (5.97-9.5)	15.6 (11-21.7)	11.5 (7.8-17)	**0.000**
HOMA index	1.6 (1.29-2.09)	3.44 (2.43-4.95)	2.61 (1.72-3.97)	**0.000**
HDL cholesterol (mg/dL)	56 (47-64)	45 (41-50)	47 (42-57)	**0.000**
VLDL cholesterol (mg/dL)	11.5 (10-14)	15 (11-22)	13 (11-18)	**0.000**
LDL cholesterol (mg/dL)	93 (80-109)	96 (82-112)	94 (81-112)	0.374
Triglycerides (mg/dL)	58 (50.25-69.75)	76 (57-112)	67 (53-90)	**0.000**
Leptin (ng/mL)	0.93 (0.43-1.86)	9.33 (4.93-17.83)	4.82 (1.23-12.34)	**0.000**
Ghrelin (pg/mL)	5.91 (3.29-8.92)	7.29 (1.38-10.35)	6.78 (3.29-9.94)	0.705
Omentin (pg/mL)	38.61 (29.61-60.67)	54.79 (29.08-77)	45.07 (29.54-68.64)	0.279
Resistin (ng/mL)	17.26 (8.61-27.67)	23.06 (16.78-33.03)	21.3 (14.04-30.35)	**0.002**
Adiponectin (µg/ml)	44.74 (29.2-76.5)	38.89 (17.69-70.89)	41.4 (24.48-74.24)	0.108
Retinol Binding Protein (mg/dL)	1.7 (1.38-1.9)	1.9 (1.6-2.1)	1.8 (1.5-2)	**0.000**
ALT (UI/L)	15 (11-19)	18 (14-24.75)	17 (13-23)	**0.000**
AST (UI/L)	23 (20-27)	21 (19-24)	22 (19-26)	**0.037**
γ-GT (UI/L)	12 (10-14)	15 (13-19)	14 (11-18)	**0.000**
Creatinine (mg/dL)	0.62 (0.58-0.69)	0.65 (0.6-0.71)	0.64 (0.59-0.7)	**0.021**
Apolipoprotein A1 (mg/dL)	135 (125-165.5)	128 (117-140.2)	130 (118.5-145)	**0.002**
Apolipoprotein B (mg/dL)	69 (56-82)	73 (63.75-85)	72 (60-84)	0.090
Microalbumin mg/L	9 (5-13.5)	7 (5-12)	8 (5-12)	0.153
Microalbumin creatinine coefficient	7.5 (6-11.25)	7 (5-9.45)	7 (5-10)	0.092

**Data are presented in median (interquartile range). Comparisons between the two groups were made by Wilcoxon test.*

Variables	*Control*	*Overweight and obesity*	*Total*	*p-values*
Plasmatic Homocysteine (µmol/L)	6.1 (5.1-7.1)	7.15 (6.18-8.7)	6.8 (5.7-8.4)	**0.000**
Atherogenic index	1.02 (0.78-1.37)	1.71 (1.24-2.38)	1.38 (1.01-2.05)	**0.000**
Folic acid (ng/mL)	7.35 (5.38-10.9)	6.1 (4.55-7.8)	6.4 (4.8-9)	**0.003**
Vitamin D (ng/mL)	25 (22-28.75)	23 (18.5-28)	24 (20-28)	**0.028**
Transferrin saturation index	20 (15-24)	17 (14-23)	18 (14-23)	**0.026**
Iron (µg/dL)	86 (64.25-99)	75 (57.5-92)	76.5 (60.75-96)	0.058
Ferritin (ng/mL)	31 (21-45)	38 (23-55.5)	35 (22-53)	0.082
Transferrin (mg/dL)	279 (256-303.8)	299 (279-317.5)	293 (273-315)	**0.001**

**Data are presented in median (interquartile range). Comparisons between the two groups were made by Wilcoxon test.*

The children of the overweight and obesity groups present higher levels of glucose, insulin, HOMA index, VLDL cholesterol, triglycerides, leptin, resistin, Retinol Binding Protein, ALT, AST, γ-GT, creatinine, plasmatic homocysteine. In addition, HDL cholesterol, apolipoprotein A1, folic acid, and vitamin D, which are protective metabolic markers, are decreased in this group (**Table 6**, **Figure 29**). Plus, iron metabolism markers appear also modified in the children with overweight and obesity: transferrin saturation index was decreased, and transferrin level was increased.

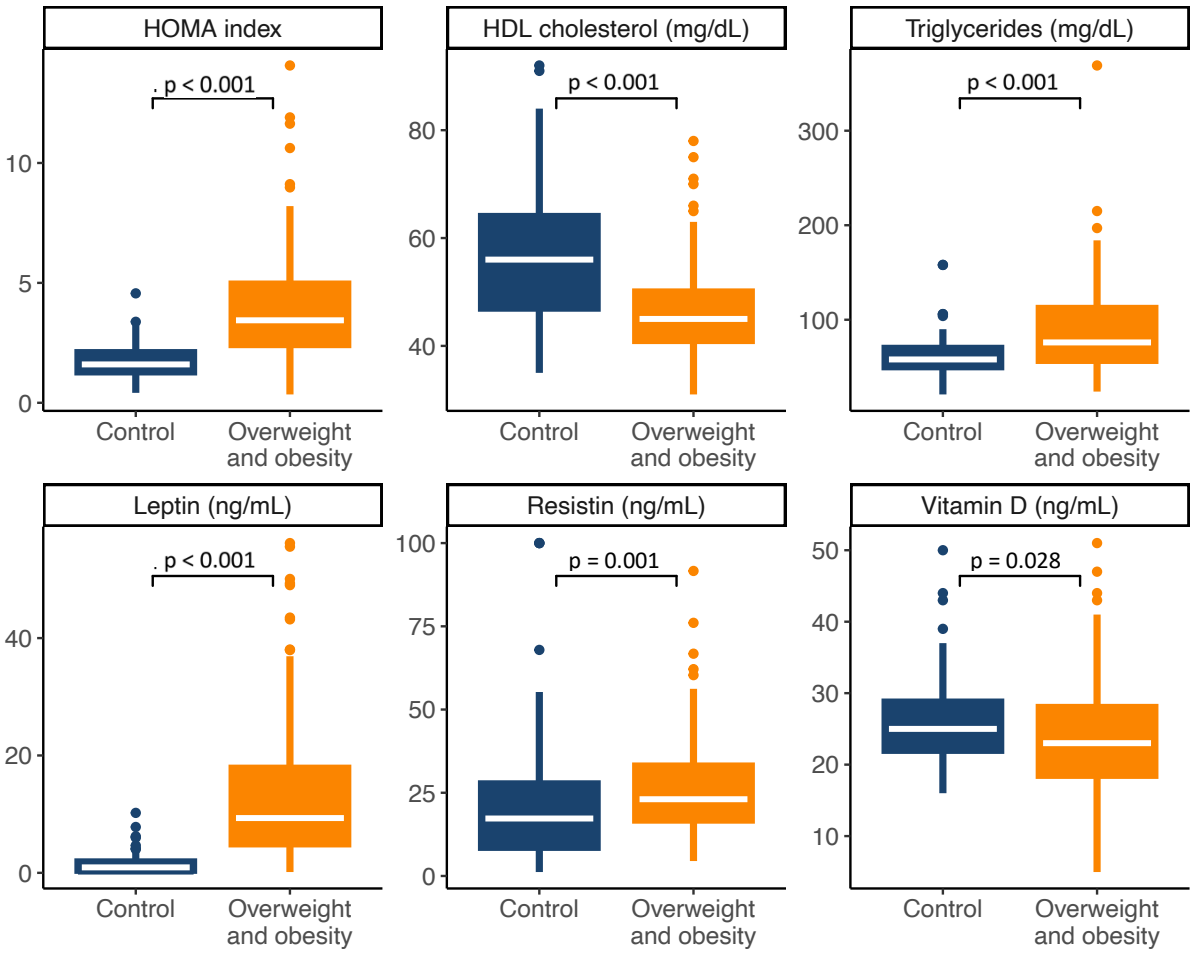


Figure 29. *Metabolic characteristics*

Immune factors are presented in **Table 7** and illustrated in **Figure 30**.

Table 7. Inflammation characteristics*

Variables	*Control*	*Overweight and obesity*	*Total*	*p-values*
hs-CRP (mg/L)	0.27 (0.2-0.53)	1.7 (0.8-4.37)	0.95 (0.28-2.99)	**0.000**
C3 (mg/dL)	111.5 (101.8-124)	143 (131-159.2)	132 (114-149)	**0.000**
Uric acid (mg/dL)	3.9 (3.38-4.62)	5 (4.3-5.75)	4.6 (3.9-5.4)	**0.000**
Cystatin C (mg/L)	0.82 (0.74-0.92)	0.83 (0.7-0.95)	0.82 (0.71-0.94)	0.745
Interferon-γ (pg/mL)	12.74 (8.42-17.37)	11.14 (8.2-15.22)	11.4 (8.24-15.94)	0.315
Tumour Necrosis Factor-α (pg/mL)	3.93 (2.77-4.97)	1.76 (1.11-5.22)	2.93 (1.16-5.03)	**0.003**
Interleukin-6 (pg/mL)	2.11 (1.24-3.51)	2.13 (0.61-3.82)	2.12 (0.93-3.71)	0.564
Plasminogen activator inhibitor-1 (ng/mL)	10.84 (6.94-21.57)	35.46 (20.77-52.12)	25.57 (12.28-42.95)	**0.000**
Monocyte Chemoattractant Protein-1 (pg/mL)	44.23 (32.62-74.26)	53.94 (39.67-85.4)	51.08 (35.7-81.64)	**0.042**
Absolute monocyte count (x10e9/L)	0.5 (0.4-0.68)	0.6 (0.4-0.75)	0.5 (0.4-0.7)	**0.033**
Erythrocytes (x10e12/L)	4.78 (4.64-4.97)	4.86 (4.68-5.08)	4.82 (4.66-5.06)	0.101
Eosinophils	4.9 (2.88-8.07)	3.7 (2.3-6.3)	3.9 (2.4-7.15)	0.058
Basophils	0.6 (0.5-0.9)	0.6 (0.4-0.8)	0.6 (0.4-0.8)	0.114
Lymphocytes	40.6 (36.1-45.75)	36.55 (31.07-42.48)	37.8 (32.25-43.05)	**0.003**
Platelets (x10e9/L)	254 (218.8-294.2)	279 (240.5-319)	267 (232-310.5)	**0.005**
Absolute neutrophil count (x10e9/L)	2.6 (2.3-3.42)	3.4 (2.9-4.38)	3.2 (2.5-4.1)	**0.000**
Leucocytes (x10e9/L)	6.1 (5.3-7.72)	6.95 (6.1-8.65)	6.8 (5.7-8.32)	**0.003**
Fibrinogen (mg/dL)	348 (319.2-381.8)	437 (392-477.5)	400.5 (349.2-460)	**0.000**

**Data are presented in median (interquartile range). Comparisons between the two groups were made by Wilcoxon test*

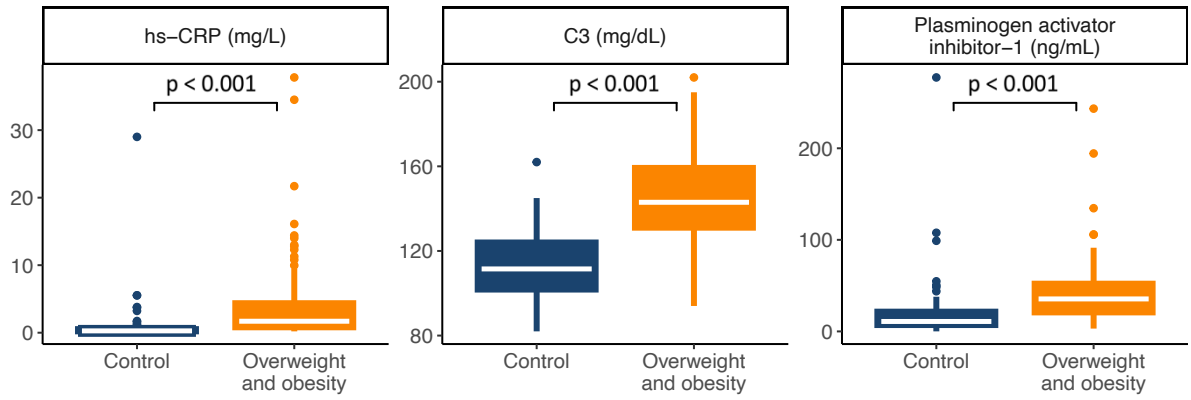


Figure 30. Inflammation characteristics

In children with overweight and obesity, various inflammation markers were found increased, such as hs-CRP, C3, uric acid, PAI-1, MCP-1, monocytes, neutrophils, platelets, leucocytes, fibrinogen and the atherogenic index. On the other hand, the TNF-a and lymphocytes are decreased in this group (**Table 7**, **Figure 30**).

V. 1.4. Circadian rhythms and chronotype

V. 1.4.1. Melatonin

***Table 8**. Salivary melatonin levels and increase rates over evening**

Variables	*Control*	*Overweight and obesity*	*Total*	*p-values*
Salivary melatonin 4h before sleep (pg/mL)	3.72 (0.92-10.16)	3.84 (0.9-10.75)	3.83 (0.92-10.74)	0.898
Salivary melatonin 2h before sleep (pg/mL)	5.31 (1.28-10)	6.05 (1.73-17.73)	5.36 (1.6-14.12)	0.200
Salivary melatonin after 1h of sleep (pg/mL)	25.93 (15.48-43.77)	25.48 (14.78-40.8)	25.85 (14.97-42.08)	0.380
melatonin increase measured over evening (pg/ml per hour)	4.26 (1.8-7.25)	3.55 (1.4-6.16)	3.89 (1.53-6.48)	0.262
melatonin increase measured before sleep (pg/ml per hour)	0 (-1.76-1.41)	0.38 (-1.21-2.92)	0 (-1.29-2.64)	0.054
melatonin increases around sleep time (pg/ml per hour)	7.35 (3.11-12.93)	4.66 (1.75-9.05)	5.53 (2.33-9.89)	**0.016**

**Data are presented in median (interquartile range). Comparisons between the two groups were made by Wilcoxon test.*

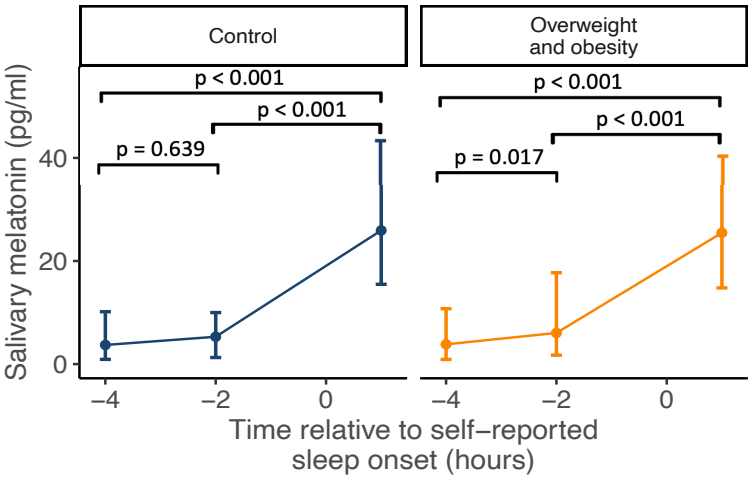


***Figure 31**. Median melatonin levels over evening*

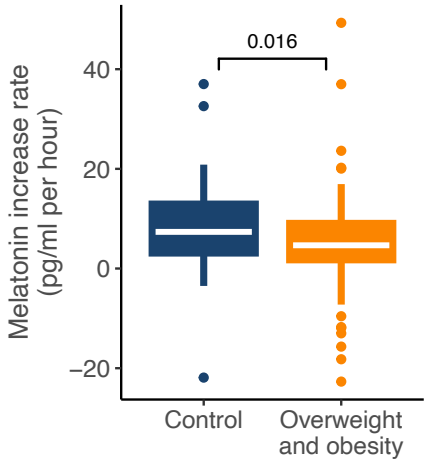


***Figure 32**. Melatonin increase rate around sleep time*

Melatonin levels are not significantly different between groups at any of the three time points studied (**Table 8**). In both groups, melatonin levels increase significantly between the first and the last measurement, with the most important increase between 2 hours before sleep onset, and 1h after sleep onset. Nevertheless, only in the obesity group the melatonin already show a significant increase 2h before sleep compared to 4h before sleep (**Figure 31**). Plus, the increase rate of melatonin around sleep onset is lower in the overweight and obesity participants compared to the control (**Figure 32**).

V. 1.4.2. Antioxidant capacity

***Table 9**. Salivary antioxidant capacity levels and increase rates over evening**

Variables	*Control*	*Overweight and obesity*	*Total*	*p-values*
Salivary antioxidant capacity 4h before sleep	82.02 (23.09-145.17)	123.47 (61.31-201.37)	107 (52.59-178.31)	**0.007**
Salivary antioxidant capacity 2h before sleep	85.86 (33.69-154.24)	137.22 (70.93-214.7)	117.53 (46.97-196.12)	**0.002**
Salivary antioxidant capacity after 1h of sleep	149.4 (0-233.5)	142.29 (35.98-224.31)	147 (23.3-228.4)	0.553
Antioxidant capacity increase around sleep time (Eq. Trolox per hour)	0 (-13.12-42.54)	0 (-19.46-18)	0 (-16.94-24.02)	0.085
Antioxidant capacity increase measured before sleep (Eq. Trolox per hour)	-2.9 (-25.32-24.49)	6.16 (-14.18-22.26)	2.04 (-17.97-23.16)	0. 161
Antioxidant capacity increase measured over evening (Eq. Trolox per hour)	4.35 (-4.45-31.83)	1.11 (-9.4-17.46)	1.93 (-7.98-20.47)	0.073

**Data are presented in median (interquartile range). Comparisons between the two groups were made by Wilcoxon test.*

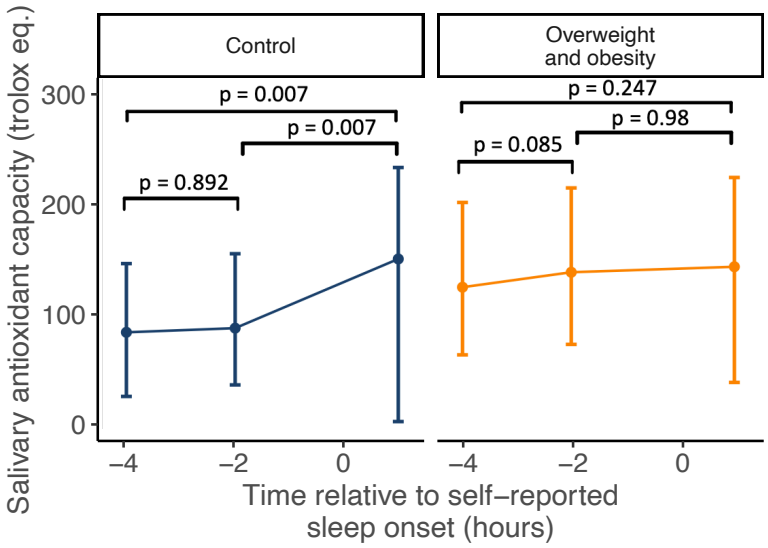


***Figure 33**. Median salivary antioxidant capacity over evening*

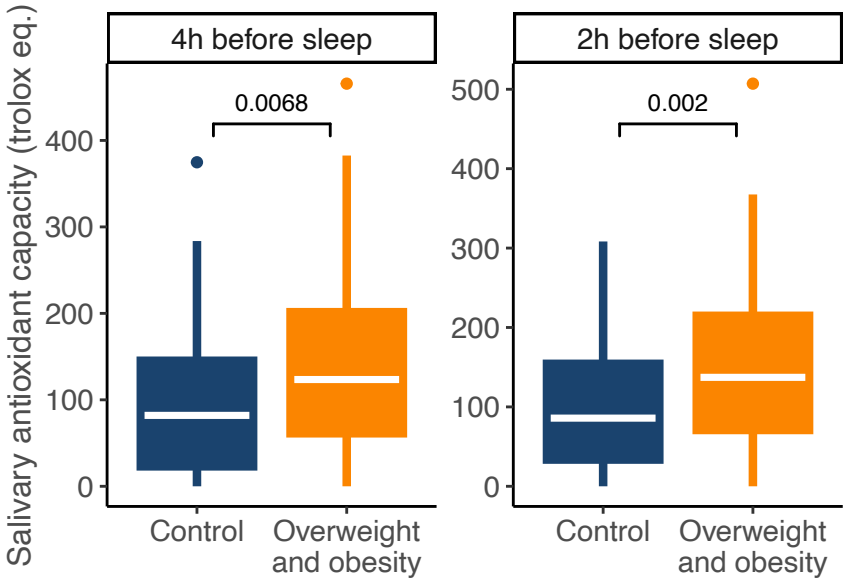


***Figure 34**. Salivary antioxidant capacity 4h and 2h before sleep*

The antioxidant capacity in saliva and the evolution over evening are reported in **Table 9**, and important interindividual differences were found. In the children with overweight and obesity, the antioxidant capacity is higher before sleep, whereas in the control group, it is relatively low at the beginning of the evening and increase significantly to reach the levels encountered in the obesity group at the last time point (**Figure 33**). In consequence, during the two first time points, the antioxidant capacity levels in overweight and obesity participants are significantly higher than the controls as illustrated in **Figure 34**.

V. 1.4.3. Chronotype

The chronotype score obtained by the Horne and Östberg questionnaire was similar in both group, with a median score of 49 (44-56) in the control group and of 50 (43-56) in the overweight and obesity group ($p = 0.809$). No difference was encountered either when the scores were translated into a chronotype category ($p = 0.996$, **Figure 35**).

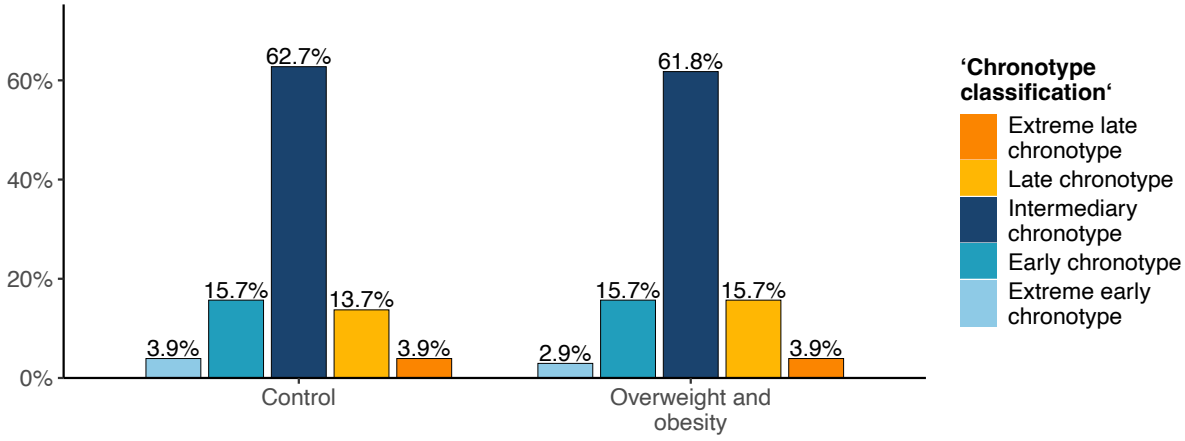


***Figure 35**. Chronotypes distribution*

V. 1.5. Life habits, history, and environment

V. 1.5.1. Nutrition

A 3-days nutritional record was completed by the participants, and the software DIAL was used to measure the quantity and type of nutrients consumed by the participants.

***Table 10**. Nutritional intake content**

Variables	*Control*	*Overweight and obesity*	*Total*	*p-values*
Calories	2000 (1734-2567)	1763 (1439-2022)	1798 (1576-2194)	**0.000**
Lipids	93.9 (79.3-114)	84.9 (65.6-102)	88.8 (71.6-106.5)	**0.003**
Proteins	77.1 (63.77-97.35)	68.3 (60.9-81.2)	71.2 (61.45-85.45)	**0.033**
Carbohydrates	200.5 (171.2-280)	160 (137-195)	173 (147-214.5)	**0.000**

**Data are presented in median (interquartile range). Comparisons between the two groups were made by Wilcoxon test.*

The record from the participants of the control group reported a higher level of calories, lipids, proteins, and carbohydrates (**Table 10**).

***Table 11**. Caloric intake of each meal**

Variables	*Control*	*Overweight and obesity*	*Total*	*p-values*
Breakfast	327.1 (222.1-445.5)	285.5 (214.1-382.2)	310.3 (215.2-399.8)	0.183
Morning snack	313.3 (236.3-412.1)	261 (167.4-325.5)	287.9 (172.5-346.8)	**0.016**
Lunch	496.5 (419.1-668.5)	447.3 (387.7-526)	455.4 (397.8-554.6)	**0.007**
Afternoon snack	338 (291.7-419.6)	274.2 (196-375.2)	305 (214.9-394.2)	**0.006**
Dinner	498.5 (407.3-585)	479.4 (368.7-587.1)	497.6 (389-587)	0.486
Other (% of yes)	28.6%	31.3%	30.4%	0.880

**Continuous data are presented in median (interquartile range) and Comparisons between the two groups were made by Wilcoxon test. Binary variables are presented in percentage of positive answer and comparisons were made by Chi^2 test.*

The caloric content for each meal was quantified and found significantly higher in the controls for the morning snack, lunch, and afternoon snack (**Table 11**).

***Table 12**. Elements mentioned in the nutritional report**

Variables	*Control*	*Overweight and obesity*	*Total*	*p-values*
Sodas	32%	25%	27.3%	0.476
Sweets	44%	36%	38.7	0.441
Fruit juice	52%	32%	38.7%	**0.028**
Industrial sauces mentioned (ketchup,…)	28%	19%	22%	0.296
Transformed products mentioned	84%	79%	80.7%	0.609
Homemade products mentioned	78%	76%	76.7%	0.946
First price brand	52%	21%	31.3	**0.000**
Branded product mentioned	52%	34%	40	0.052

**Data are presented in percentage of positive answer and comparisons were made by Chi² test*

In addition to DIAL software data used for quantifying the nutritional intake, more information was extracted from the 3-days nutritional record, such as the mentioned elements like sodas, industrial sauces, transformed or homemade products, but also the mention of first price or branded products (**Table 12**). The participants from the control group were more numerous to mention fruit juice and first price brand products.

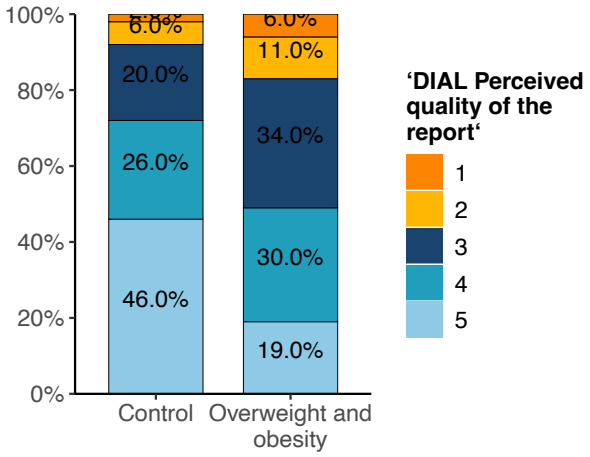


***Figure 36**. Report quality*

The quality of the report was quantified by the investigator from 1, poor quality to 5, perfectly well completed. The grade reflects the level of details provided, for the quantity, the type of foods and cooking, the brands consumed. This score was found significantly higher in reports provided by the control participants ($p = 0.006$, **Figure 36**).

This element constitutes a problem in terms of the reliability of the results obtained through this report. Indeed, for an equivalent meal, a report completed in detail will tend to have a higher caloric content than a report which has omitted to mention part of the nutritional intake.

Because of this bias, which adds to other probable bias described further in discussion, the row data from the nutritional intake content will not be included in further analysis. Nevertheless, for a same individual, the ratio of caloric content between the different meals were calculated, which should limit the effect of the bias.

***Table 13**. Proportion of nutritional intake for each meal compared with the others**

Variables	*Control*	*Overweight and obesity*	*Total*	*p-values*
Breakfast/morning snack calories	1.05 (0.7-1.54)	1.09 (0.75-1.73)	1.06 (0.75-1.69)	0.703
Breakfast/lunch calories	0.64 (0.42-0.93)	0.64 (0.45-0.81)	0.64 (0.44-0.89)	0.741
Breakfast/afternoon snack calories	1.01 (0.68-1.27)	1.03 (0.72-1.56)	1.01 (0.71-1.42)	0.347
Breakfast/dinner calories	0.63 (0.4-0.93)	0.59 (0.37-0.92)	0.62 (0.39-0.93)	0.515
Morning snack/lunch calories	0.59 (0.35-0.86)	0.55 (0.37-0.73)	0.56 (0.36-0.77)	0.495
Morning snack /afternoon snack calories	0.99 (0.61-1.17)	0.84 (0.55-1.21)	0.88 (0.55-1.2)	0.663
Morning snack/dinner calories	0.58 (0.34-0.88)	0.52 (0.31-0.7)	0.54 (0.31-0.76)	0.085
Lunch/afternoon snack calories	1.53 (1.2-2.03)	1.62 (1.2-2.41)	1.59 (1.2-2.23)	0.361
Lunch/dinner calories	0.95 (0.79-1.45)	0.91 (0.77-1.13)	0.93 (0.78-1.22)	0.186
Afternoon snack/dinner calories	0.68 (0.51-0.81)	0.56 (0.41-0.75)	0.6 (0.42-0.79)	**0.017**

**Data are presented in median (interquartile range). Comparisons between the two groups were made by Wilcoxon test.*

Only the afternoon snack/dinner calories ratio is different between groups, found in average lower in the participants with overweight and obesity. This means that in these children, the afternoon snack was only about 55% as caloric as dinner, whereas in controls, it was about 67% as caloric as dinner (**Table 13**).

***Table 14**. Meal timings**

Variables	*Control*	*Overweight and obesity*	*Total*	*p-values*
Breakfast time during the week	8 (7:30-8:25)	7:50 (7:30-8:15)	8 (7:30-8:15)	0.180
Morning snack time during the week	10:45 (10:30-11)	11 (10:45-11)	11 (10:30-11)	0.409
Lunch time during the week	14 (13:30-14:30)	14 (13:30-14:30)	14 (13:30-14:30)	0.298
Afternoon snack time during the week	17:10 (17-17:30)	17:30 (17-18)	17:15 (17-18)	0.173
Dinner time during the week	21 (20:30-21:25)	21 (20:30-21:30)	21 (20:30-21:30)	0.617
Breakfast time during the weekend	10:30 (10-11)	10 (9-10:30)	10 (9:30-10:30)	0.020
Morning snack time during the weekend	12 (11-12:30)	11 (11-12)	11:30 (11-12)	0.183
Lunch time during the weekend	14:30 (14-14:30)	14:30 (14-15)	14:30 (14-14:55)	0.836
Afternoon snack time during the weekend	18 (17:30-18)	18 (17:15-18:30)	18 (17:20-18:30)	0.796
Dinner time during the weekend	21:30 (21-21:30)	21:30 (21-21:50)	21:30 (21-21:50)	0.360
Time amplitude of the nutritional intake	13 (12:30-13:30)	13:05 (12:45-13:50)	13 (12:30-13:50)	0.198

**Data are presented in median of hours: minutes (interquartile range). Comparisons between the two groups were made by Wilcoxon test.*

The schedules of nutritional intakes collected from the 3-days nutritional report were not significantly different between the two groups during the school week nor the weekend (**Table 14**).

***Table 15**. Breakfast context**

Variables	*Control*	*Overweight and obesity*	*Total*	*p-values*
Breakfast duration (min)	10 (10-15)	10 (5-15)	10 (5-15)	0.119
Sit down to have breakfast	90.7 %	88.9 %	89.5 %	0.987
Breakfast with screen devices	51.6%	68.3 %	63.6 %	0.155

**Continuous data is presented in median (interquartile range) and comparisons was made by Wilcoxon test. Binary variables are presented in percentage of positive answer and comparisons were made by Chi^2 test.*

The context of the breakfast was also assessed through a self-reported estimation of its usual duration, whether the children usually take the time to sit for this first nutritional intake of the day, and the presence of screens. No difference was found between the groups regarding breakfast context (**Table 15**).

V. 1.5.2. Physical activity

The participants were asked whether they exercise or not out of school, and if so, which activity they practice. The activities were then classified in groups according to the static component and dynamic components of the sport practice. The time at which the physical activity was performed was also asked, as was the weekly frequency.

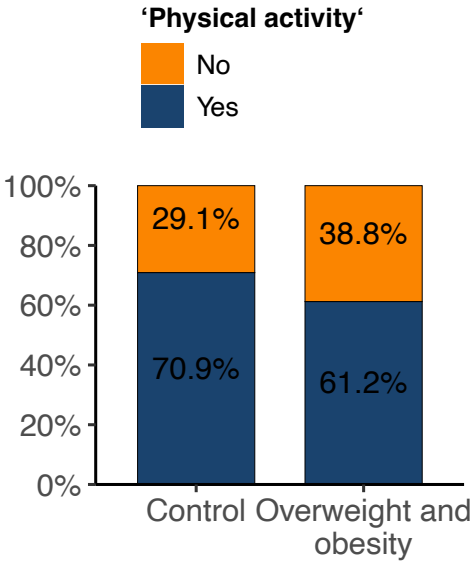


***Figure 37**. Physical activity practice*

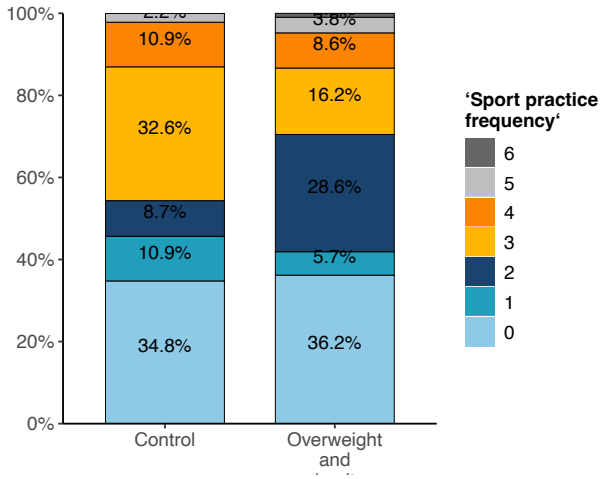


***Figure 38**. Physical activity frequency*

The proportion of participants practicing sport was not significantly different between the two groups ($p = 0.439$, **Figure 37**), nor was the frequency of sport practice ($p = 0.135$, **Figure 38**).

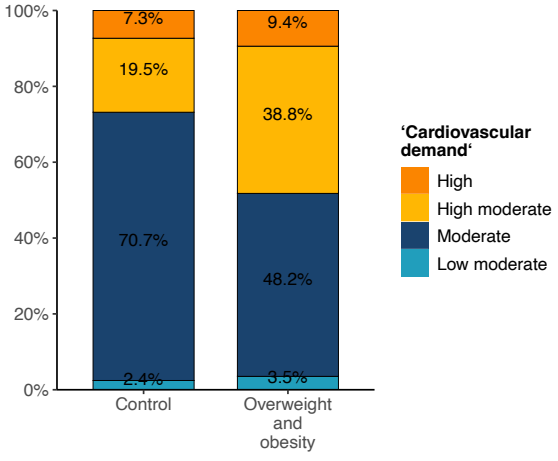


***Figure 39**. Cardiovascular demand of physical activity*

Each sport implies a different dynamic and static component, from which can be deduced the level of cardiovascular demand during the effort. The difference in cardiovascular demand levels was not significantly different between the two groups (p = 0.115, **Figure 39**).

Regarding the **sport schedule**, it was found that the children from the obesity group had a slightly but significantly (p = 0.045) later time of physical activity start compared to the controls (**Figure 40**).

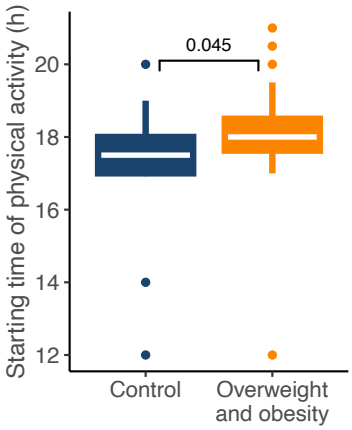


***Figure 40**. Sport start time*

V. 1.5.3. Sleep

***Table 16**. Sleep schedules**

Variables	*Control*	*Overweight and obesity*	*Total*	*p-values*
Bedtime during schooldays	22:30 (22-23)	22:30 (22-23)	22:30 (22-23)	0.163
Waking hour during schooldays	7:45 (7:15-8)	7:20 (7-7:58)	7:30 (7-8)	**0.002**
Sleep duration during schooldays (hours)	9:30 (8:30-10)	8:45 (8-9:30)	9 (8:12-9:59)	**0.005**
Bedtime during weekend	00:00 (23-0)	00:00 (23-00:30)	00:00 (23-00:30)	0.420
Waking hour during weekend	10 (9-10:30)	9:30 (8:30-10:30)	9:30 (8:30-10:30)	0.241
Sleep duration during weekend	10 (9:30-10:30)	10 (8:30-10)	10 (9-10:30)	**0.045**
Difference in sleep duration schooldays and weekends (hours)	0:30 (-0:30-1:30)	0:30 (-0:03-2)	0:30 (-0:20-2)	0.774

**Data are presented in median of hours: minutes (interquartile range). Comparisons between the two groups were made by Wilcoxon test.*

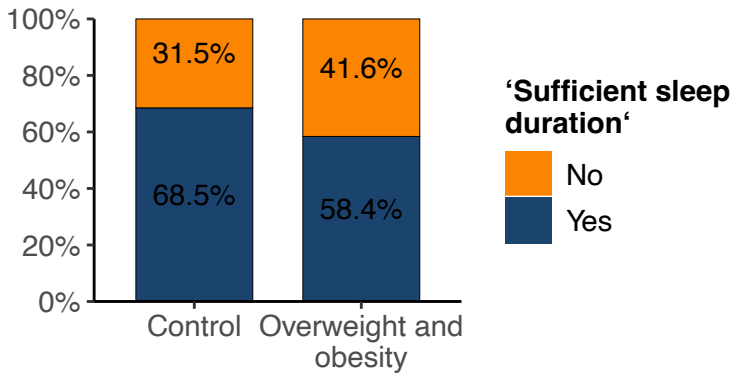


***Figure 41**. Sufficient sleep duration*

The children with overweight and obesity sleep in average for a shorter duration during the night, and they wake up significantly earlier (**Table 16**). When reported to the Spanish sleep society criteria of adequate sleep duration, no significant difference was found between the two groups (p=0.227, **Figure 41**).

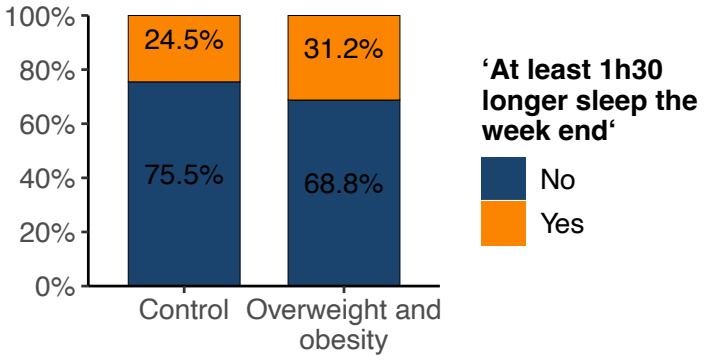


***Figure 42**. Longer sleep duration during the weekend*

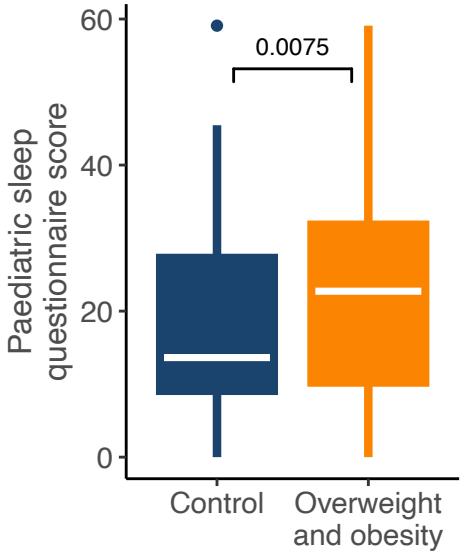


***Figure 43**. Paediatric sleep questionnaire score*

The weekend sleep recovery, quantified here as a weekend sleep duration more than 1h30 longer than the sleep duration during the school week, was not significantly different between the groups (p=0.481, **Figure 42**). Among the study participants, 1 out of 10 have the habit to take a nap, indifferently from the groups (p = 0.364). Participants presenting overweight and obesity have higher scores at the sleep questionnaire detecting sleep disturbances (**Figure 43**).

V. 1.5.4. Exposure to technological devices with screens

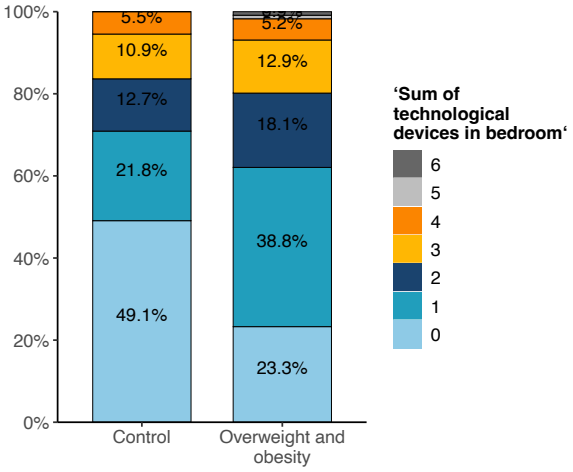


***Figure 44**. Number of screen devices in the bedroom at night*

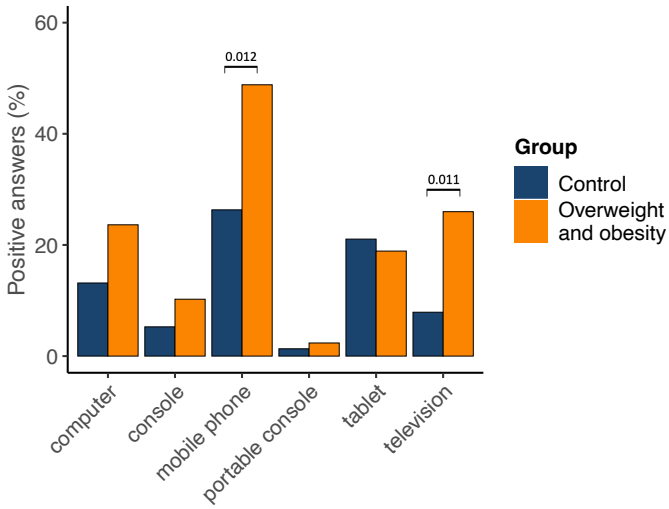


***Figure 45**. Types of screen devices in the bedroom at night*

About half of the participants from the control group have screen devices in their bedroom at night against three quarters of the participants from the overweight and obesity group (p = 0.002, **Figure 44**). In the overweight and obesity group, the presence of mobile phone and television was more frequently reported than in controls (**Figure 45**).

V. 1.5.5. Delivery and early nutrition

In the control group, 38.5% were born by c-section and 34.6% in the overweight and obesity group (p = 0.726). In the control group, 71.9% had breastmilk as early nutrition and 69.4% in the overweight and obesity group (p = 0.853).

V. 1.5.6. Sociodemographic context

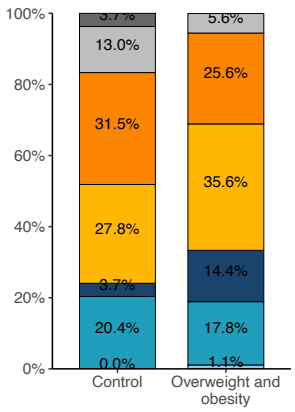


***Figure 46**. Mother education level*

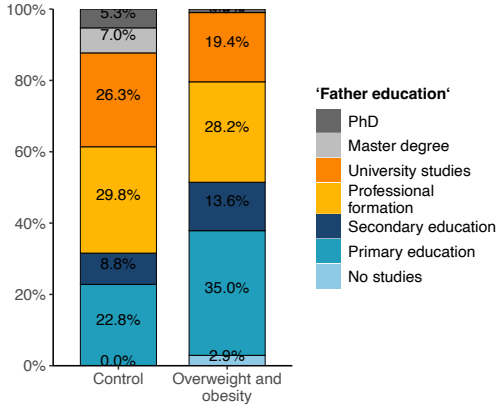


***Figure 47**. Father education level*

In the control group, the proportion of fathers with higher education levels is significantly higher (p = 0.021, **Figure 47**). It was not found significantly higher in the mother (p = 0.080, **Figure 46**).

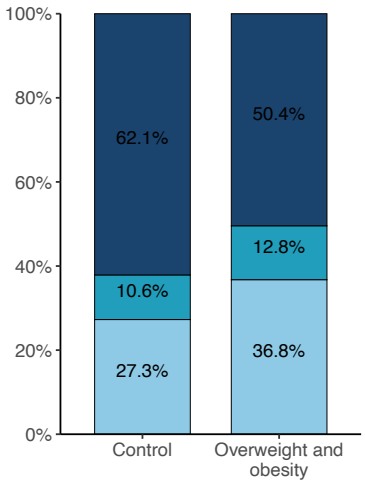


***Figure 48**. Mother employment status*

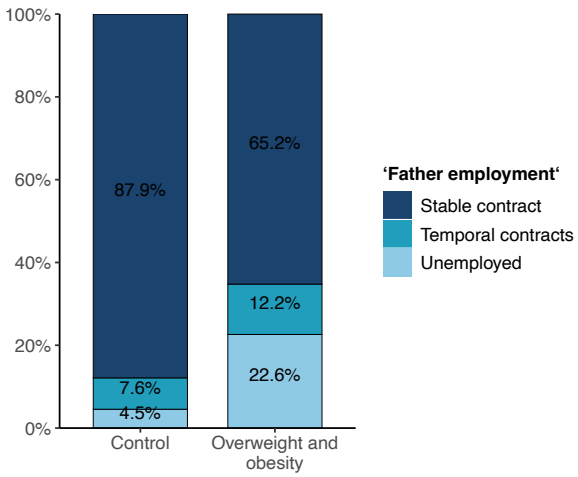


***Figure 49**. Father employment status*

Regarding the mother employment status, it was not different between groups (p = 0.306, **Figure 48**), on the other hand, in the control group, the proportion of fathers with stable contracts is higher (0.002, **Figure 49**).

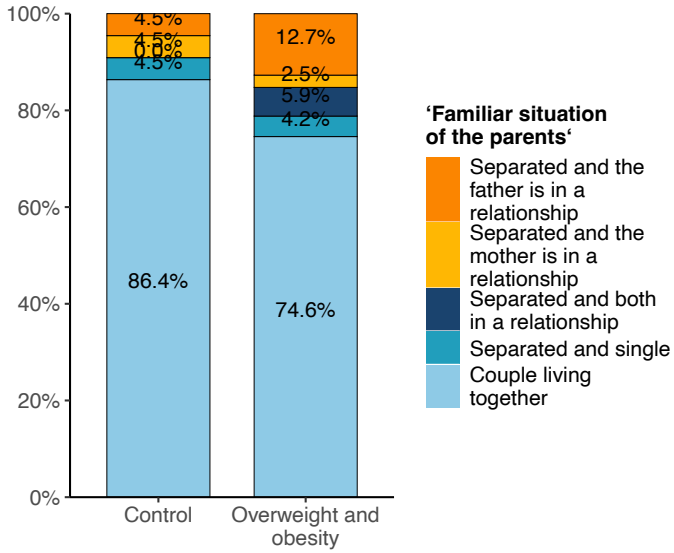


***Figure 50**. Parental situation*

No significant difference was found between the two groups regarding the parental situation (p = 0.071, **Figure 50**).

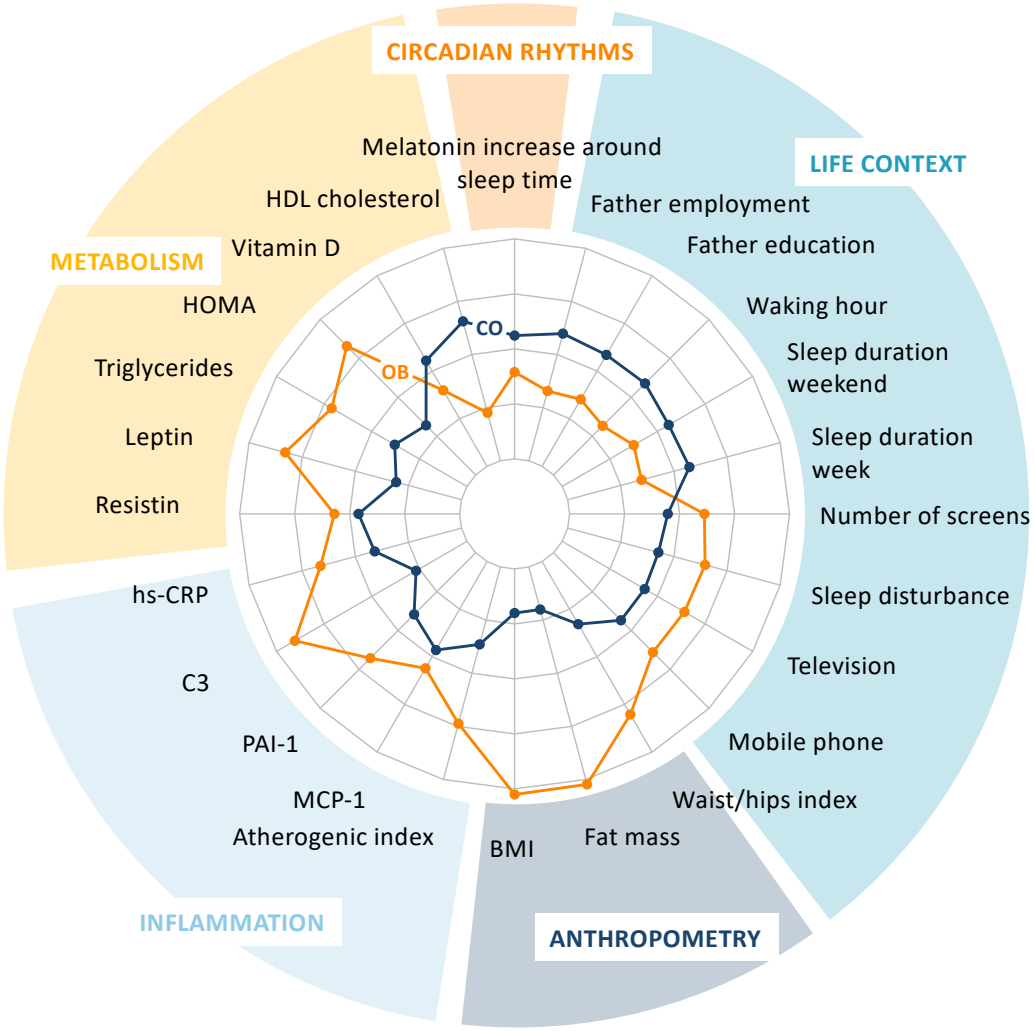


***Figure 51**. Summary of the differences between the two groups*

The spider chart was performed using the package fmsb version 0.7.3, 202227 on R.

In summary, in the present study the children with overweight and obesity present significant differences compared to children with normal BMI for equivalent age and sex. Differences were encountered in all the domains that we investigated: metabolism, inflammation, anthropometry, circadian rhythms, and life context (**Figure 51**).

V. 2. Relationships among study variables

In this part, Spearman correlations and odds-ratios with Fisher's tests are calculated to identify to which parameters are related melatonin increase rate around sleep, chronotype, sleep duration, mealtime, screen exposure, and sociodemographic context.

V. 2.1. Melatonin

Melatonin increase rate around sleep time consists in the difference in melatonin measured two hours before sleep, and one hour after sleep onset, divided by 3, the number of hours between these two measurements. This rate was found significantly lower in the children presenting overweight or obesity (**Table 8**). The correlation between this melatonin rate and other parameters is now studied.

***Table 17**. Correlations with melatonin increase rate**

Variables	*rho*	*P value*
Percentage of fat mass	-0.16	**0.03**
BMI (kg/m2)	-0.17	**0.023**
BMI z-score	-0.17	**0.024**
Weight z-score	-0.16	**0.035**
Arm z-score	-0.16	**0.037**
Waist/height index	-0.17	**0.026**
HOMA index	-0.15	**0.037**
Uric acid (mg/dL)	-0.16	**0.034**
γ-GT (UI/L)	-0.21	**0.004**
Retinol Binding Protein (mg/dL)	-0.18	**0.014**
Transferrin (mg/dL)	-0.19	**0.014**
Plasmatic Homocysteine (¬µmol/L)	-0.17	**0.026**
Leucocytes (x10e9/L)	-0.18	**0.018**
Absolute neutrophil count (x10e9/L)	-0.19	**0.013**
Absolute lymphocyte count (x10e9/L)	-0.22	**0.002**
Platelets (x10e9/L)	-0.25	**0.001**
Ghrelin (pg/mL)	0.15	**0.04**
Leptin (ng/mL)	-0.24	**0.001**
Chronotype	0.26	**0.002**
In bedroom at night: mobile phone	-0.18	**0.022**
Number of technological devices in bedroom	-0.18	**0.026**
Nap habit	-0.21	**0.009**
Bedtime during schooldays	-0.23	**0.004**
Sleep duration during schooldays (hours)	0.2	**0.014**
Bedtime during weekend	-0.26	**0.001**
Breakfast/morning snack calories	0.2	**0.03**
Breakfast/lunch calories	0.18	**0.038**
Breakfast/dinner calories	0.26	**0.003**

**Spearman correlations adjusted for age, sex and puberty index were performed between the variables, Spearman's rho is coloured by strength and sign of correlation and is presented with the associated p-value.*

The elements in **Table 17** were found significantly correlated with melatonin increase rate around sleep, independently from the relationship with age, sex, and puberty stage.

Regarding anthropometric characteristics, a higher melatonin rate is correlated with a lower BMI, percentage of fat mass, waist/height index, weight, and arm z-scores. It is also negatively correlated with metabolic markers such as the HOMA index-ex, uric acid, γ-GT, RBP, Transferrin, Plasmatic homocysteine, Leucocytes, neutrophils, lymphocytes, platelets, and leptin. It is positively correlated with the hunger hormone ghrelin and with a higher the chronotype score which indicate morningness. The higher the melatonin rate also correlates with lower number of screens in the bedroom at night, in particular the mobile phone. Higher melatonin rate finally correlates with life habits, such as lower nap habit, earlier bedtime and longer sleep duration during schooldays, and with higher ratio of caloric intake during breakfast with other meals.

V. 2.2. Chronotype

The Horne and Östberg questionnaire was used to obtain for each participant a morningness-eveningness score. The higher the score, the more a person has the tendency to be active during the earlier part of the day. This score was found correlated to various parameters presented in **Table 18**.

***Table 18**. Correlations with chronotypes**

Variables	*rho*	*p-value*
Salivary melatonin after 1h of sleep (pg/mL)	0.18	**0.033**
melatonin increase measured over evening (pg/ml per hour)	0.24	**0.005**
melatonin increase around sleep time (pg/ml per hour)	0.26	**0.002**
Creatinine (mg/dL)	0.18	**0.026**
Albumin (g/dL)	0.17	**0.039**
Transferrin (mg/dL)	-0.18	**0.047**
Leucocytes (x10e9/L)	-0.19	**0.024**
Absolute lymphocyte count (x10e9/L)	-0.24	**0.003**
Absolute monocyte count (x10e9/L)	-0.22	**0.009**
Absolute basophil count (x10e9/L)	-0.26	**0.002**
Platelets (x10e9/L)	-0.2	**0.015**
Interferon-γ (pg/mL)	-0.21	**0.012**
Tumour Necrosis Factor-α (pg/mL)	-0.25	**0.003**
Bruni sleep questionnaire score	-0.28	**0.001**
SAHS sleep questionnaire	-0.17	**0.049**
In bedroom at night: television	-0.24	**0.004**
In bedroom at night: console	-0.19	**0.023**
In bedroom at night: tablet	-0.26	**0.002**
Number of technological devices in bedroom	-0.3	**<0.001**
Bedtime during schooldays	-0.25	**0.002**
Bedtime during weekend	-0.28	**0.001**
Waking hour during weekend	-0.37	**<0.001**
Sleep duration during weekend	-0.22	**0.011**
Difference in sleep duration schooldays and weekends (hours)	-0.29	**0.001**
Breakfast time during the weekend	-0.29	**0.005**
Father employment	0.25	**0.003**
Breakfast/lunch calories	0.19	**0.036**
Breakfast/afternoon snack calories	0.21	**0.021**
Breakfast/dinner calories	0.24	**0.01**

**Spearman correlations adjusted for age, sex and puberty index were performed between the variables, Spearman's rho is coloured by strength and sign of correlation and is presented with the associated p-value.*

Independently from sex, age, and puberty index, the chronotype score was correlated with higher melatonin levels after 1h of sleep, with a higher melatonin increase rate around sleep time and over the entire evening. It was also correlated with higher creatine and albumin, lower transferrin leucocytes, lymphocytes, monocytes, basophils, platelets, ING-g, TNF-a. Regarding sleep, chronotype score is correlated with lower sleep disturbances scores, earlier bedtimes during both the school days and the weekend, and an earlier wake-up time during the weekend, resulting in less difference in terms of sleep duration between the week and the weekend. The chronotype score is also inversely correlated with the number of screen devices present in the bedroom at night, in particular television, console, and tablet. As was melatonin, the chronotype score is correlated with the caloric ratio between breakfast and the other meals. This suggests that the earlier the chronotype, the higher nutritional intake in the breakfast compared to the other meals.

V. 2.3. Dinnertime

The dinner time was self-reported on the 3-days nutritional report. The correlations between later dinner time and variables of the study are listed in the **Table 19**.

***Table 19**. Correlation with dinnertime during the week**

Variables	*rho*	*p-value*
Glucose (mg/dL)	0.29	**0.003**
Urea (mg/dL)	0.26	**0.008**
Albumin (g/dL)	0.2	**0.036**
Iron (µg/dL)	-0.21	**0.029**
Plasmatic Homocysteine (µmol/L)	0.21	**0.035**
Bedtime during schooldays	0.26	**0.007**
Sleep duration during schooldays (hours)	-0.28	**0.004**
Time amplitude of nutritional intake	0.76	**< 0.001**
Afternoon snack time during the week	0.3	**0.003**
Lunch time during the weekend	0.22	**0.028**
Afternoon snack time during the weekend	0.27	**0.02**
Dinner time during the weekend	0.57	**< 0.001**
Breakfast/lunch calories	-0.24	**0.018**
Breakfast/afternoon snack calories	-0.32	**0.001**
Morning snack/afternoon snack calories	-0.29	**0.004**

**Spearman correlations adjusted for age, sex and puberty index were performed between the variables, Spearman's rho is coloured by strength and sign of correlation and is presented with the associated p-value.*

Dinnertime is positively correlated with glucose level, urea, albumin, and plasmatic homocysteine, and negatively correlated with iron. It is also correlated positively with bedtime during school days, time amplitude of nutritional intake over the day, dinner, afternoon snack and dinner time during the weekend, and negatively correlated with sleep duration during the week. Finally, it is inversely correlated with the ratios of breakfast/afternoon snack calories, breakfast/lunch calories and morning snack/afternoon snack calories.

V. 2.4. Sleep duration during the school days

Sleep duration was calculated from the self-reported bedtime and wake-up time during the school week. The statistically significant correlations measured between sleep duration and the study variables are gathered in **Table 20**.

***Table 20**. Correlations with sleep duration**

Variables	*rho*	*p-value*
Salivary melatonin after 1h of sleep (pg/mL)	0.17	**0.031**
melatonin increase around sleep time (pg/ml per hour)	0.2	**0.014**
Waist circumference (cm)	-0.19	**0.016**
Waist/hips index	-0.16	**0.049**
Percentage of fat mass	-0.19	**0.015**
BMI (kg/m2)	-0.17	**0.025**
Waist/height index	-0.25	**0.001**
Diastolic blood pressure level	-0.2	**0.011**
Glucose (mg/dL)	-0.17	**0.029**
Atherogenic index	-0.2	**0.009**
VLDL cholesterol (mg/dL)	-0.17	**0.028**
Triglycerides (mg/dL)	-0.17	**0.026**
AST (UI/L)	0.17	**0.028**
Plasmatic Homocysteine (µmol/L)	-0.17	**0.035**
Plasminogen activator inhibitor-1 (ng/mL)	-0.22	**0.006**
Monocyte Chemoattractant Protein-1 (pg/mL)	-0.16	**0.047**
In bedroom at night: mobile phone	-0.28	**< 0.001**
Number of technological devices in bedroom	-0.26	**0.001**
Bedtime during weekend	-0.49	**< 0.001**
Difference in sleep duration school days and weekends (hours)	-0.4	**< 0.001**
Breakfast duration (min)	0.22	**0.026**
Time amplitude of nutritional intake	-0.35	**< 0.001**
Afternoon snack time during the week	-0.26	**0.012**
Dinner time during the week	-0.28	**0.004**
Breakfast/lunch calories	0.21	**0.017**

**Spearman correlations adjusted for age, sex and puberty index were performed between the variables, Spearman's rho is coloured by strength and sign of correlation and is presented with the associated p-value.*

Sleep duration was found correlated positively with the salivary melatonin level after one hour of sleep, and with melatonin increase rate around sleep, independently from the effect of age, sex, and puberty index. It was additionally found negatively correlated with anthropometrics characteristics associated to obesity, such as BMI, percentage of fat mass, waist/hips index and waist circumference. In addition, sleep duration was negatively correlated with diastolic blood pressure and metabolic risk markers such as glucose, VLDL cholesterol, triglycerides, plasmatic homocysteine, PAI-1, atherogenic index, MCP-1, and positively correlated with AST. In addition, sleep duration is negatively correlated with the number of technological devices present in the room at night, and in particular the mobile phone. Plus, it is negatively correlated with bedtime during the weekend, and with difference in sleep duration between the sleep and weekend. In the participants with longer sleep duration, the duration of the breakfast and the breakfast/lunch calory ratio is higher, whereas the dinner time and amplitude of the nutritional intake over a day are shorter.

V. 2.5. Screen exposure

The participants were asked to answer whether a mobile phone, console (Wii, PS4…), portable console (switch, DS, PSP…), computer, TV or tablet was present in their bedroom during the night. The presence of at least one of these technological devices with screens is associated with higher odds of presenting anthropometrics and biochemical markers of obesity, and also other risk factors of life habits and environment, as shown in **Figure 52**.

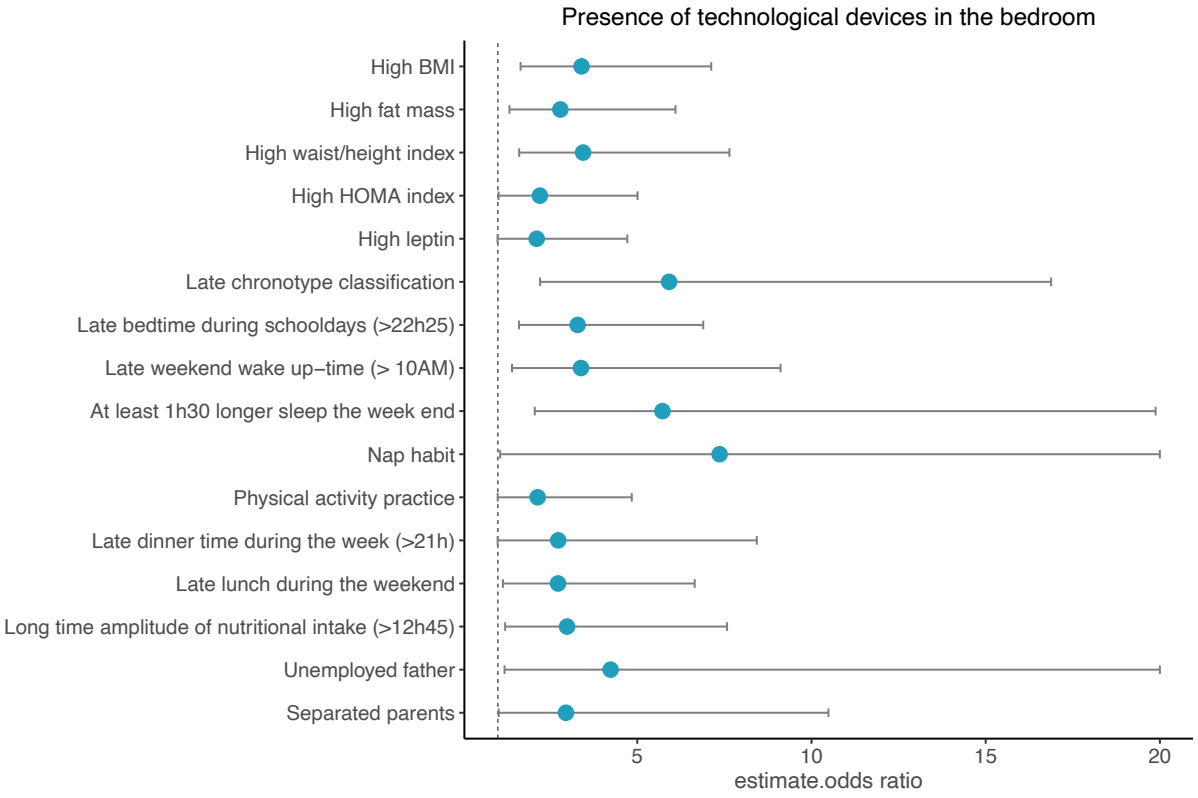


***Figure 52**. Odds-ratios for presence of screens in the bedroom*

The presence of screens in the bedroom at night is associated with 3.4 (1.65-7.12) more probability of presenting a BMI >2 SD for age and sex, in other words to present obesity (p = 0.001). In addition, it is also multiplying by 2.62 (1.26-5.48) the probability of presenting a high fat mass for age and sex (p = 0.004), by 3.44 (1.39-6.32) the likelihood to present a waist/height index > 0.5 (p = 0.001), these limits were set as the 97th percentile in the control group. Regarding metabolic factors, the presence of screens in the bedroom multiplies by 2.2 (1.02-5.01) the odds for a HOMA higher than 3.16 (p = 0.038), and by 2.11 (0.99-4.71) for a high leptin level, superior to 9.11 (p = 0.041). It is associated with an increase of 5.91 (2.21-16.88) times of probability to present a moderately or extremely late chronotype (p < 0.001), of 3.29 (1.6-6.89) to go to bed later than 22h25 during the week (p < 0.001), of 3.38 (1.4-9.11) to wake up later than 10AM in the weekend (p = 0.004), of 5.72 (2.06-19.88) to sleep at least 1h30 longer during a weekend night than a week

night ($p < 0.001$), of 7.36 (1.06-20) to report the habits of taking naps ($p = 0.039$). Surprisingly, the physical activity practice is also 2.14 (0.99-4.84) more reported in the participants who have screens in their bedroom at night ($p = 0.04$). Regarding meal timings, having screens in the bedroom is associated with 2.73 (0.99-8.43) more dinner time later than 21h during the week ($p = 0.049$), 2.72 (1.14-6.65) more likelihood to have lunch later than 14h30 during the weekend ($p = 0.016$), and of 2.98 (1.21-7.58) the odds to have a nutritional intake amplitude over the course of the day longer than 12h45 ($p = 0.012$). Finally, the fathers of children who have screens in their bedroom are 4.23 (1.19-20) more likely to be unemployed, and the parents 2.95 (1.03-10.49) times more probabilities to be separated.

V. 2.6. Parental characteristics

Table 21. *Correlations with the level of education in the mother**

Variables	*rho*	*p-value*
Weight (kg)	-0.17	**0.025**
Weight z-score	-0.17	**0.021**
Hips circumference (cm)	-0.15	**0.047**
Waist circumference (cm)	-0.17	**0.025**
Arm z-score	-0.18	**0.022**
Hips z-score	-0.17	**0.023**
Waist z-score	-0.21	**0.006**
Waist/hips index	-0.19	**0.011**
Percentage of fat mass	-0.21	**0.005**
BMI (kg/m2)	-0.22	**0.004**
BMI percentile	-0.17	**0.021**
Waist/height index	-0.22	**0.003**
Atherogenic index	-0.16	**0.037**
VLDL cholesterol (mg/dL)	-0.15	**0.04**
Triglycerides (mg/dL)	-0.15	**0.044**
Leucocytes (x10e9/L)	-0.25	**0.001**
Absolute neutrophil count (x10e9/L)	-0.25	**0.001**
Absolute monocyte count (x10e9/L)	-0.22	**0.003**
Lymphocytes	0.16	**0.029**
Leptin (ng/mL)	-0.18	**0.019**
Early nutrition (0 = breastmilk; 1 = formula)	-0.27	**0.001**
SAHS sleep questionnaire (% positive answers)	-0.2	**0.011**
Physical activity (0 = no; 1 = yes)	0.18	**0.029**
Waking hour during weekend	-0.23	**0.004**
Sleep duration during weekend	-0.2	**0.015**
Difference in sleep duration schooldays and weekends (hours)	-0.17	**0.033**
Father education	0.53	**< 0.001**
Mother employment	0.17	**0.025**
Breakfast/morning snack calories	0.19	**0.031**
Breakfast/lunch calories	0.24	**0.006**
Breakfast/afternoon snack calories	0.27	**0.002**
Breakfast/dinner calories	0.27	**0.002**

**Spearman correlations adjusted for age, sex and puberty index were performed between the variables, Spearman's rho is coloured by strength and sign of correlation and presented with the associated p-value.*

The education level of the mother was negatively correlated with anthropometric characteristics of obesity. In addition, it was negatively correlated to eight metabolic and inflammatory markers, sleep disturbance scores, late sleep schedules and weekend sleep recovery. I was found positively correlated to physical activity practice, father education, mother employment, and to caloric ratios between breakfast and all the other meals of the day (**Table 21**).

***Table 22**. Correlations between maternal study level and nutritional report results**

Variables	*rho*	*p-value*
Perceived quality of the report	0.25	**0.004**
Calories	0.22	**0.01**
Lipids	0.19	**0.027**
Carbohydrates	0.24	**0.006**
Homemade products mentioned	0.25	**0.004**
First price brand	0.21	**0.016**
Branded product mentioned	0.26	**0.002**

**Spearman correlations adjusted for age, sex and puberty index were performed between the variables, Spearman's rho is coloured by strength and sign of correlation and is presented with the associated p-value.*

As explained previously, the nutritional report was found unreliable to measure the differences between the two groups because it was significantly better completed in the case of the participants from the control group. It seems that this bias may be related to the mother education level, as the quality of the report as well as all the nutritional parameters collected from the report were significantly correlated with the mother study level (**Table 22**). Two parameters from this list were also correlated with the study level of the father: the carbohydrates (rho = 0.23; p = 0.009) and the number of branded products (rho = 0.19; p = 0.031).

The odds ratios for risk factors when having and mother with an unstable job were calculated. The only significant association is that mothers who went to university were 3.9 (1.65-10.39) times more likely to have a stable job ($p < 0.001$).

***Table 23**. Correlations with the education level of the father**

Variables	*rho*	*p-value*
Weight (kg)	-0.23	**0.002**
Weight z-score	-0.24	**0.001**
Arm circumference (cm)	-0.21	**0.006**
Hips circumference (cm)	-0.21	**0.004**
Waist circumference (cm)	-0.27	**< 0.001**
Arm z-score	-0.26	**0.001**
Hips z-score	-0.25	**0.001**
Waist z-score	-0.29	**< 0.001**
Waist/hips index	-0.24	**0.001**
Percentage of fat mass	-0.28	**< 0.001**
BMI (kg/m2)	-0.28	**< 0.001**
BMI percentile	-0.25	**0.001**
Waist/height index	-0.31	**< 0.001**
Systolic blood pressure percentile	-0.15	**0.044**
Systolic blood pressure level (0 = normal; 1 = elevated; 2 = hypertension)	-0.17	**0.025**
Diastolic blood pressure level (0 = normal; 1 = elevated; 2 = hypertension)	-0.18	**0.019**
HOMA index	-0.25	**0.001**
Creatinine (mg/dL)	0.18	**0.016**
Apolipoprotein A1 (mg/dL)	0.16	**0.034**
C3 (mg/dL)	-0.18	**0.026**
Insulin (µUI/mL)	-0.24	**0.001**
Folic acid (ng/mL)	0.22	**0.004**
Plasmatic Homocysteine (µmol/L)	-0.16	**0.031**
Absolute neutrophil count (x10e9/L)	-0.18	**0.015**
Neutrophils	-0.16	**0.03**
Lymphocytes	0.15	**0.046**
Plasminogen activator inhibitor-1 (ng/mL)	-0.16	**0.039**
Leptin (ng/mL)	-0.21	**0.006**
Salivary antioxidant capacity 4h before sleep	-0.17	**0.04**
Salivary antioxidant capacity 2h before sleep	-0.21	**0.007**
Early nutrition (0 = breastmilk; 1 = formula)	-0.23	**0.005**
SAHS sleep questionnaire (% positive answers)	-0.18	**0.022**
In bedroom at night: television	-0.22	**0.006**
Number of technological devices in bedroom	-0.17	**0.039**
Physical activity (0 = no; 1 = yes)	0.16	**0.043**
Waking hour during weekend	-0.25	**0.002**

Variables	*rho*	*p-value*
Sleep duration during weekend	-0.19	**0.018**
Difference in sleep duration schooldays and weekends (hours)	-0.22	**0.008**
Afternoon snack time during the weekend	0.29	**0.008**
Mother education	0.53	**< 0.001**
Father employment	0.2	**0.006**
Breakfast/lunch calories	0.22	**0.013**
Breakfast/afternoon snack calories	0.17	**0.047**
Breakfast/dinner calories	0.23	**0.009**

**Spearman correlations adjusted for age, sex and puberty index were performed between the variables, Spearman's rho is coloured by intensity and sign of correlation and is presented with the associated p-value.*

The study level of the father was also negatively correlated with anthropometric characteristics of obesity, in addition to clinical variables such as systolic and diastolic blood pressure. Nine metabolic and inflammatory markers are improved in the participants in which the father has studied more. It was negatively correlated with sleep disturbance score, technological devices presence in the bedroom at night, difference in sleep duration during school nights and weekend nights. Father education level is related to the mother education level and father employment stability, and finally to the ratio between ratio of calories ingested during breakfast compared to other meals of the day (**Table 23**).

The odds-ratios were also calculated for having a father with unstable or no job, the results are gathered in **Figure 53**. They show that the children whose father has an unstable or no job have a 7.43 (2.13-20) times higher probability to present an obese BMI ($p < 0.001$), 3.76 (1.5-10.14) times to have a high fat mass for age and sex ($p = 0.003$), 3.66 (1.46-9.85) times to have a high heart rate ($p = 0.003$). Regarding metabolism, the children whose father has an unstable or no job have a 3.33 (1.35-8.53) more odds to have a HOMA index higher than 3.16 ($p = 0.005$), 3.23 (1.31-8.18) times more to have a high leptin level ($p = 0.008$), 4.46 (1.7-13.23) more times to present high insulin ($p = 0.001$), 2.9 (1.18-7.31) times the probability to present a high VLDL cholesterol ($p = 0.016$). Finally, having a father with an unstable or no job increases of 4.23 (1.19-20) the odds of having screens in the bedroom ($p = 0.021$), 3.71 (1.45-9.74) the odds of sleeping at least 1h30 longer during the weekend night compared to school nights ($p = 0.003$), and 3.96 (1.51-10.28) the odds to having separated parents ($p = 0.003$).

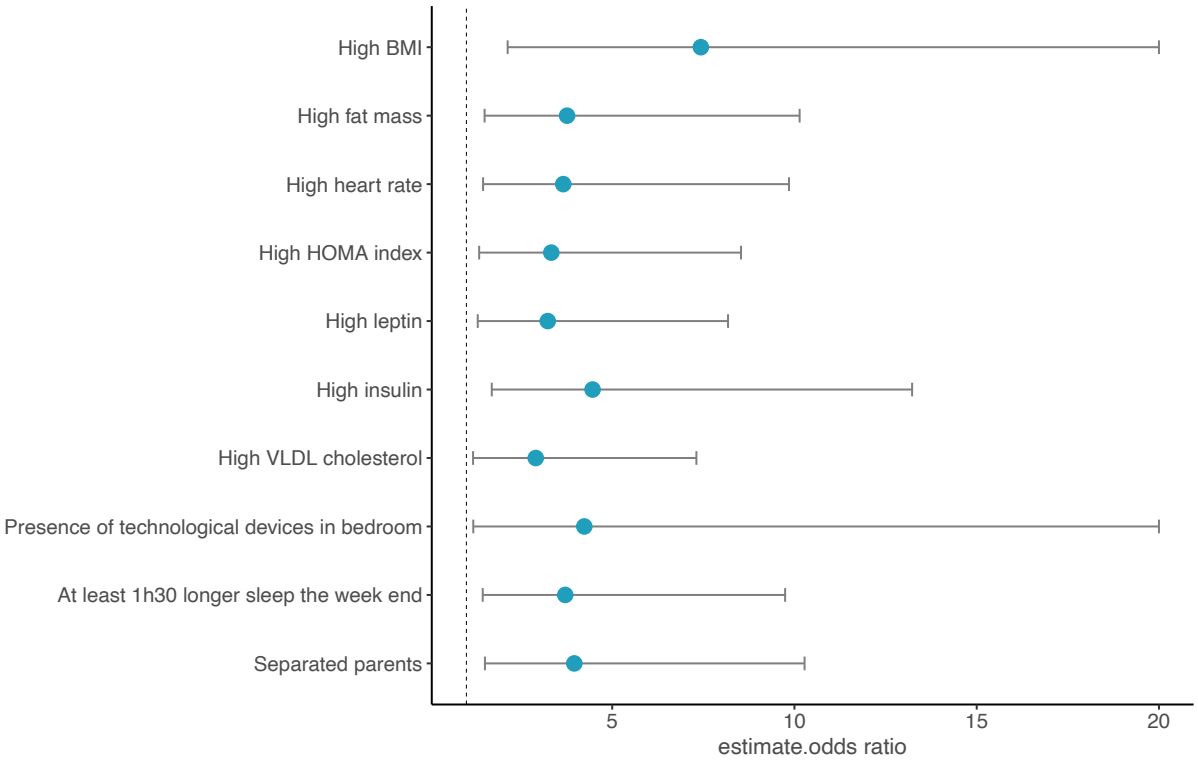


Figure 53. *Odds ratios for father with unstable or no job*

V. 3. *In vitro* study of the circadian rhythms of adipocyte genes: the impact of melatonin

In the present part are presented our results regarding the impact of melatonin on the circadian rhythms of clock genes, melatonin receptor gene and metabolic genes in human subcutaneous adipocytes. The relative expression of each gene relative to β-actin is plotted according to the time of the day, and a cosinor model is built for each condition, as presented in **Figure 54**.

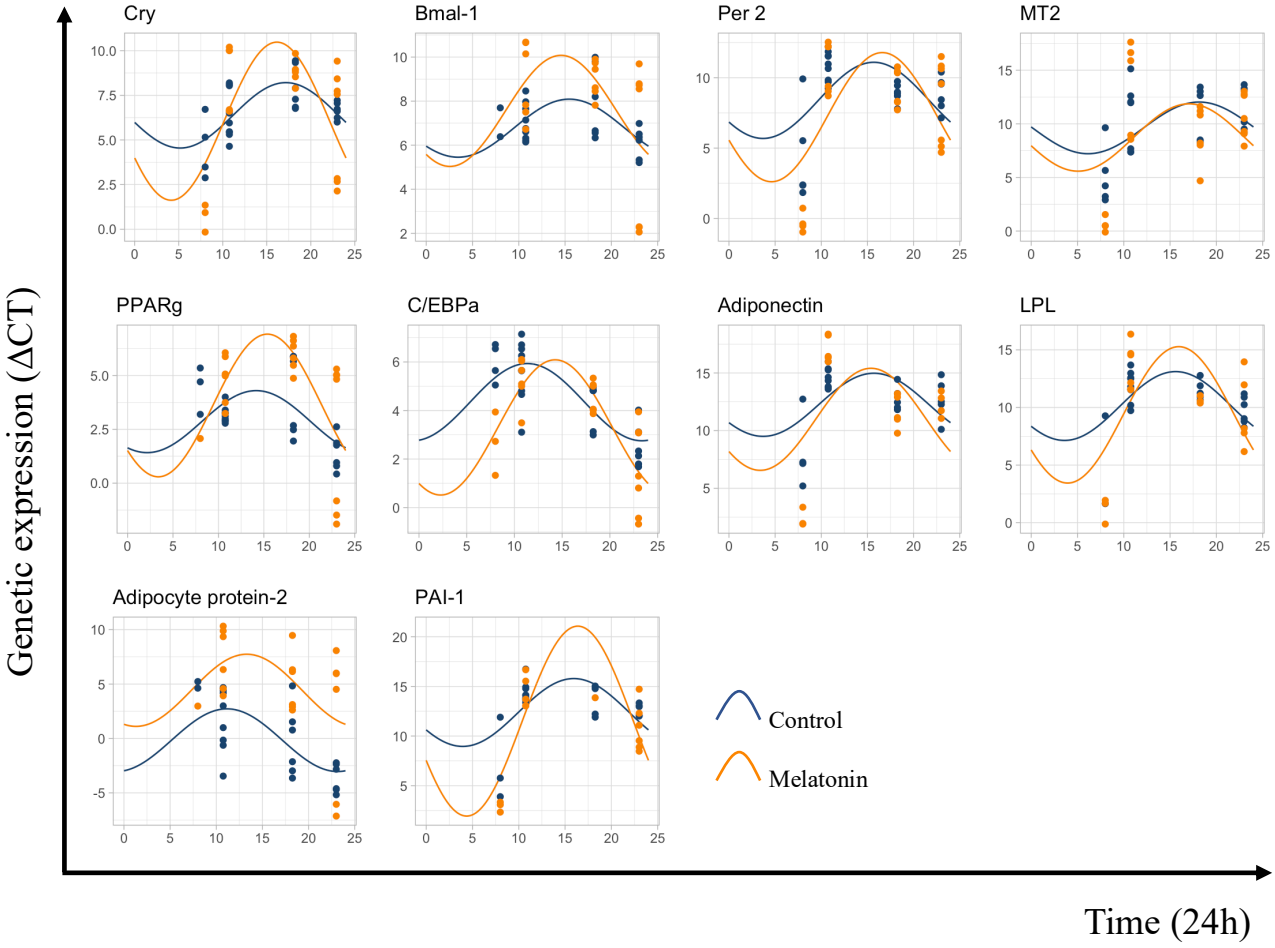


***Figure 54**. Circadian rhythms of clock genes, melatonin receptor gene and metabolic genes in adipocytes, with and without melatonin*

In a second time, the amplitude, the acrophase, and the mesor were compared for each gene in adipocytes without melatonin (control), and with melatonin stimulation (**Figure 55**).

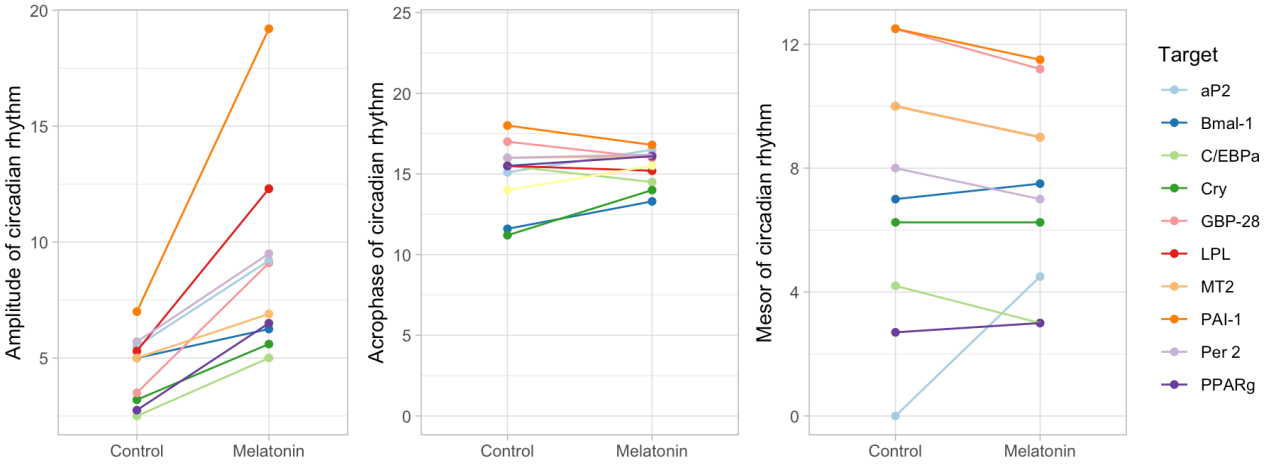


***Figure 55**. Amplitude, acrophase, and mesor of the circadian rhythms of adipocytes, without and with melatonin*

The **amplitude** was significantly increased in four of the genes: two of the three clock genes studied, **PER2** ($p = 0.005$) and **CRY** ($p = 0.002$), as well as the hormone **adiponectin** ($p < 0.001$) and the enzyme **LPL** ($p < 0.001$). As a general tendency, all the genes studied presented an increased amplitude with melatonin.

No general tendency was found regarding an impact of melatonin on the **acrophase** or the **mesor** of the circadian rhythm of adipocytes genes. In addition, no significant difference was observed.

V. 4. Multivariate analysis

Multiple variables have been observed to vary between the two study groups, from anthropometry, metabolism, circadian rhythms, life habits and environment. Number of these variables also appear to be interconnected. Multivariate analysis is employed to deepen the understanding of these interconnections, identify clusters of individuals sharing similar characteristics, measure how much a combination of external variables may favour obesity, and illustrate the metabolic footprint of obesity integrating circadian rhythms.

V. 4.1. From the interconnections between variables correlated with melatonin to the apparition of clusters of participants

To represent data of interest according to two variables, a two dimensions-plot can be used, and the data can be represented according to the value they take for the x and y axis. For three variables at a time, using a 3-D plot is still possible although not very functional and rarely used, but more is impossible with usual techniques. The principal component analysis (PCA) is a technique which allows to represent multiple variables in a reduced number of dimensions.

In the present work, we focus on the relationship between melatonin and metabolic health, and we have observed that melatonin increase rate around sleep onset was correlated with several variables from anthropometry, metabolism, and life habits. The variables that are calculated from one another are left out of the analysis (for example, HOMA, glucose and insulin should not be included all together, so only HOMA was selected), the Kaiser-Meyer-Olkin factor adequacy test was ran and indicated that the leucocytes count should be left out of the study. In order to identify how many variables should be left out of the study, the inertia of each dimension is represented (**Figure 56**).

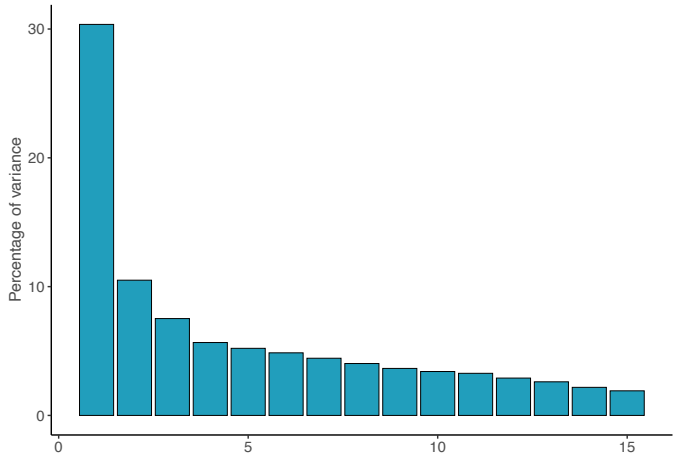


Figure 56. *Decomposition of the total inertia*

The selection of the first three axis appears to be appropriate for further analysis. The amount of inertia of these three-axis combined is higher than the one obtained by the 0.95-quantile of random distributions (48.38% against 20.53%), which suggests these three axes contain real information. Consequently, the description will stand to these three axes.

The variables projections are represented in the two first dimensions in **Figure 57**.

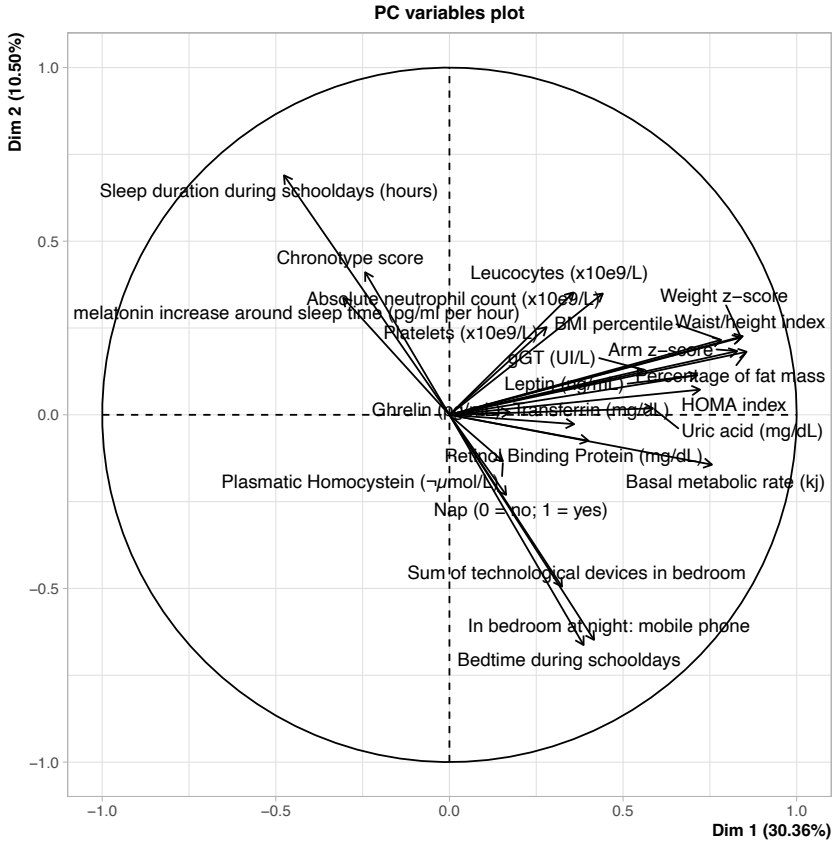


***Figure 57**. PCA variables plot*

On this figure each variable is represented as an arrow. The variables which are correlated positively go in the same direction, such as sleep duration, melatonin increase around sleep, and chronotype score. The negatively correlated go in opposite direction, such as bedtime during schooldays or the presence of mobile phone in the bedroom. We also see an important number of variables correlated together and with the first dimension, for instance the HOMA index, waist/height index, BMI-percentile, etc.

The participants of the study can also be represented on the same plan (**Figure 58**). The dots that are the most on the right of the graph correspond to the individuals who take higher values for the parameters which arrows point in this direction: HOMA index, waist/height index, BMI-percentile, etc.

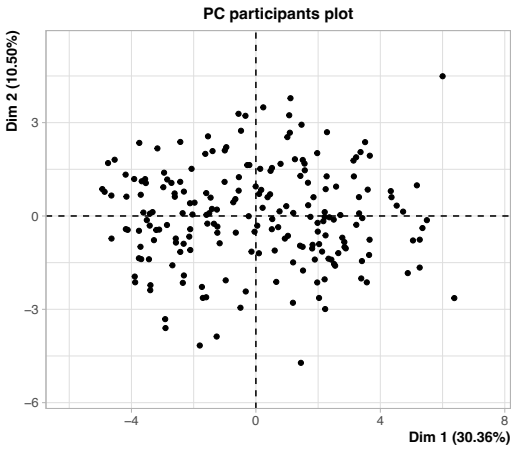


***Figure 58**. PCA plot of the participants*

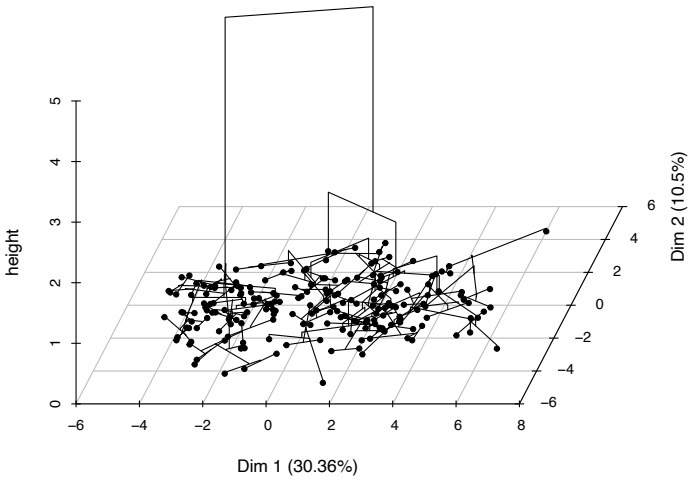


***Figure 59**. Ascending classification tree in three dimensions built from PCA results*

From this plot, an ascending hierarchical tree is built by connecting the dots by order of distance until connecting all the dots on the graph (**Figure 59**). This tree can be represented in two dimensions as in **Figure 59**, where three big branches appear, each one formed by a cluster of individuals sharing similar values for different variables.

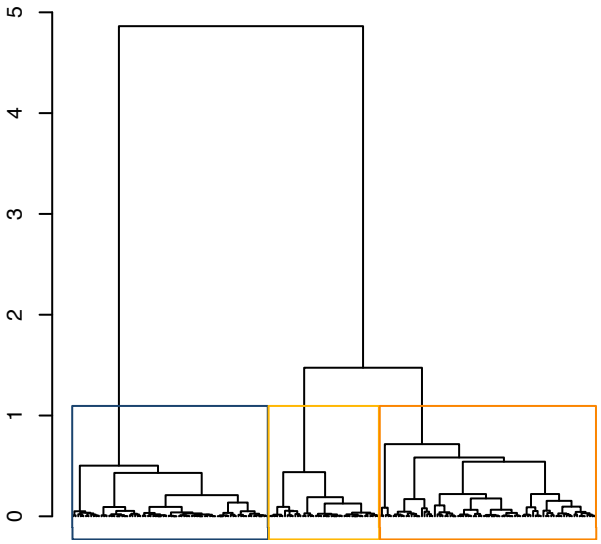


***Figure 60**. Ascending classification tree from PCA results in two dimensions*

The dots representing the participants can be coloured by cluster (**Figure 61**), and the average scaled centred values for each cluster are reported in **Table 24**. The cluster 1, in dark blue on the graph, is composed of individuals who present low values for variables like BMI percentile, percentage of fat mass, arm z-score, waist/height index, weight z-score, leptin, HOMA index, uric acid and γ-GT, and high values for the variables sleep duration during schooldays and melatonin increase rate around sleep time. This cluster corresponds to the control group.

The two other clusters take high values for anthropometric measures, corresponding to the overweight and obesity group, although slightly less high in the cluster 2. Nevertheless, what truly differentiate the two clusters lays in the sleep characteristics such as bedtime and sleep duration during schooldays, nap habit, and screens exposure, which are clearly opposed in the cluster 2 than in the cluster 3. Interestingly, the cluster 2 participants, which present high anthropometric markers of obesity but protective sleep characteristic, also present better metabolic characteristics than the cluster 3 members, although not as much as the controls.

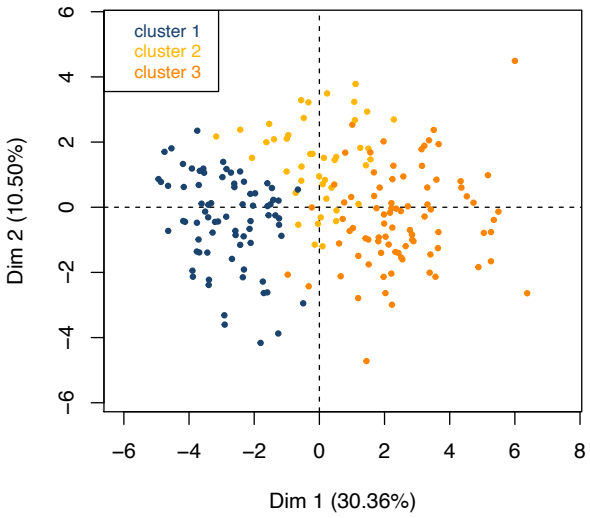


***Figure 61**. Three clusters identified by ascending hierarchical clustering and PCA*

***Table 24**. Scaled variables levels for each cluster*

Variables	*Cluster 1*	*Cluster 2*	*Cluster 3*
BMI percentile	-10.4	4.98	6.41
Percentage of fat mass	-10.7	2.97	8.58
Waist/height index	-10.4	3.16	8.08
Arm z-score	-10.1	4.01	7.02
Weight z-score	-10.1	2.87	8.01
Leptin	-7.92	0.947	7.46
Uric acid	-7.87	2.72	5.8
HOMA index	-7.85	0.546	7.74
Retinol Binding Protein	-5.91	2.86	3.62
γ-GT	-5.65	-0.343	6.23
Absolute neutrophil count	-5.39	1.13	4.63
Leucocytes	-4.11	0.46	3.89
Transferrin	-3.64	0.371	3.48
Platelets	-2.79	0.694	2.3
Plasmatic Homocysteine	-2.47	2.25	0.561
Ghrelin	-1.18	-1.23	2.34
melatonin increase around sleep time	4.48	-1.52	-3.33
Chronotype score	1.33	2.92	-4.03
Bedtime during schooldays	-3.35	-4.15	7.25
Nap	-1.79	-2.27	3.92
Sleep duration during schooldays	4.75	3.44	-8.08
In bedroom at night: mobile phone	-4.61	-1.57	6.25
Number of technological devices in bedroom	-3.44	-1.01	4.51

V. 4.2. Predicting obesity from life environment

Life history, habits and environment have been associated to overweight and obesity in numerous studies including the present one. These variables are now used together to develop a model that can predict whether a child presents a normal weight or overweight/obesity, based only on life habits, history, and environment.

A repeated cross validation technique was applied to successively train and test a random forest algorithm to build the model. The random forest algorithm builds 500 different decision trees, each composed of some of the predictive parameters, which allows it to perform classification and prediction, but also to rank the parameters by order of importance for the prediction. The receiver operator curve (ROC) was used as metrics to set the algorithm.

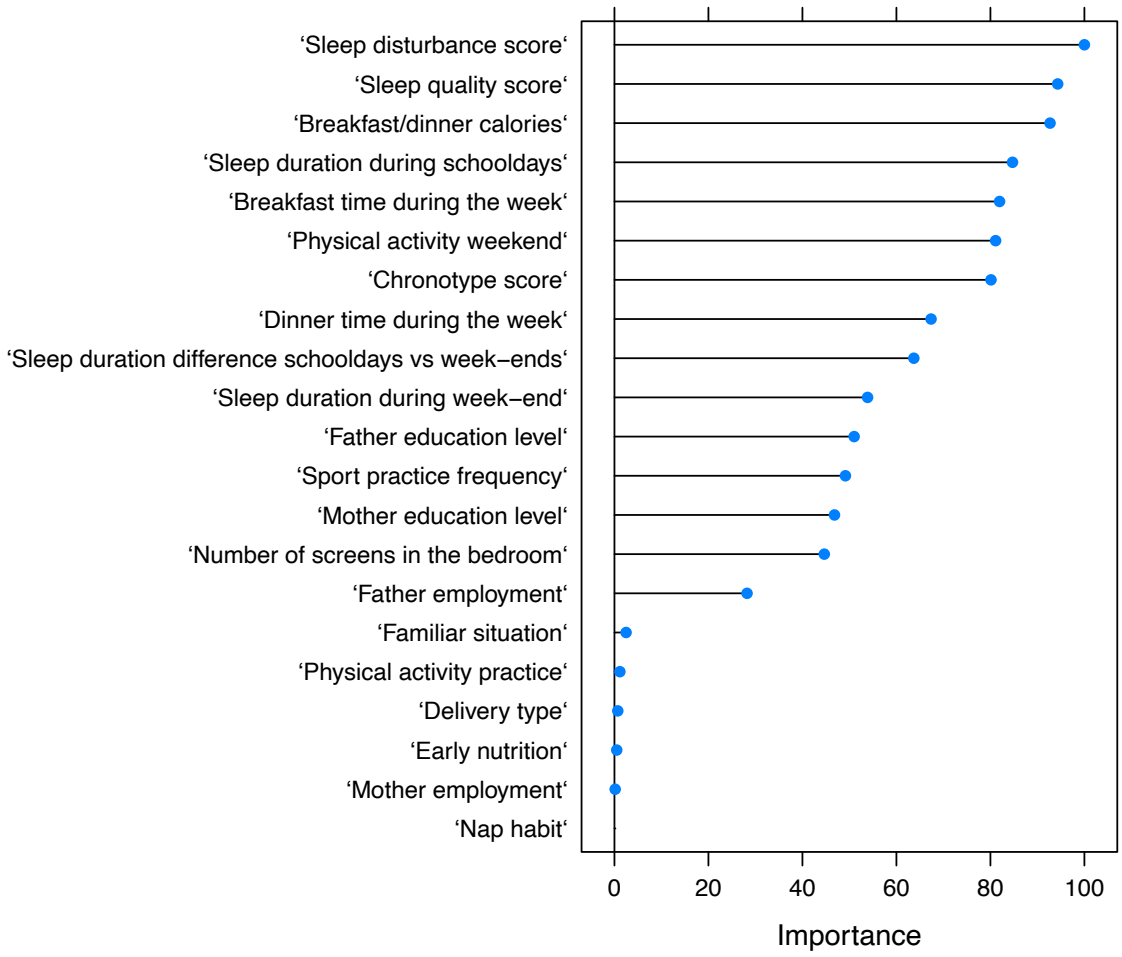


***Figure 62**. Scales importance variables for obesity prediction based on life habits*

The accuracy of the model using these variables is of 0.7, meaning that these environmental parameters allow to predict correctly whether a child from this study has a BMI in the overweight/obesity or the normal range, in average 7 times out of 10.

The sensitivity of the model is of 0.82, and the specificity of 0.39, in the present case it means that the algorithm is efficient at correctly classifying children with overweight/obesity but tends to incorrectly classify the children with normal weight. The variables ranked by importance are shown in **Figure 62**. The importances are calculated as the sum of the decrease in error when split by a variable. The relative importance corresponds to a variable importance divided by the highest variable importance value. Consequently, they take values from 100% for the variable contributing most to the model, to 0% for variables which do not contribute.

Here, the most important variable is the sleep disturbance score obtained by the Bruni sleep questionnaire, followed by the sleep quality score obtained from the short version of the paediatric sleep questionnaire for sleep apnoea diagnostic. Then comes the breakfast/dinner calories ratio followed by different life habits times and sleep durations, the chronotype score, physical activity, education levels of parents, screen devices presence in the bedroom, and father employment status. Finally come the last parameter which contribute less to the present model: parental situation, physical activity practice, delivery type, early nutrition, mother employment, and habit of taking naps.

V. 4.3. Metabolic and circadian biomarker footprint of obesity

In addition to the relationship between life environment and obesity, here have been quantified the metabolic, inflammatory, and circadian modifications associated to obesity. A final model is now built to integrate these variables modifications together as a metabolic and circadian biomarker footprint of overweight and obesity. Fifty metabolites quantified in the participants either in blood or saliva were included in the model. As for the previous part, a random forest algorithm was built, and the same settings and metrics were applied. The accuracy obtained was of 0.88 with a sensitivity of 0.81 and a specificity of 0.92. The variables and their relative importance are illustrated in **Figure 63**. Leptin is the variable which most strongly characterises a sample from a child with overweight/obesity compared to a normal weight child in our study group. Various classic metabolic and inflammatory markers figure in the upper part of the list, and melatonin appears around the middle of the list with other obesity parameters such as adiponectin, omentin, MCP-1.

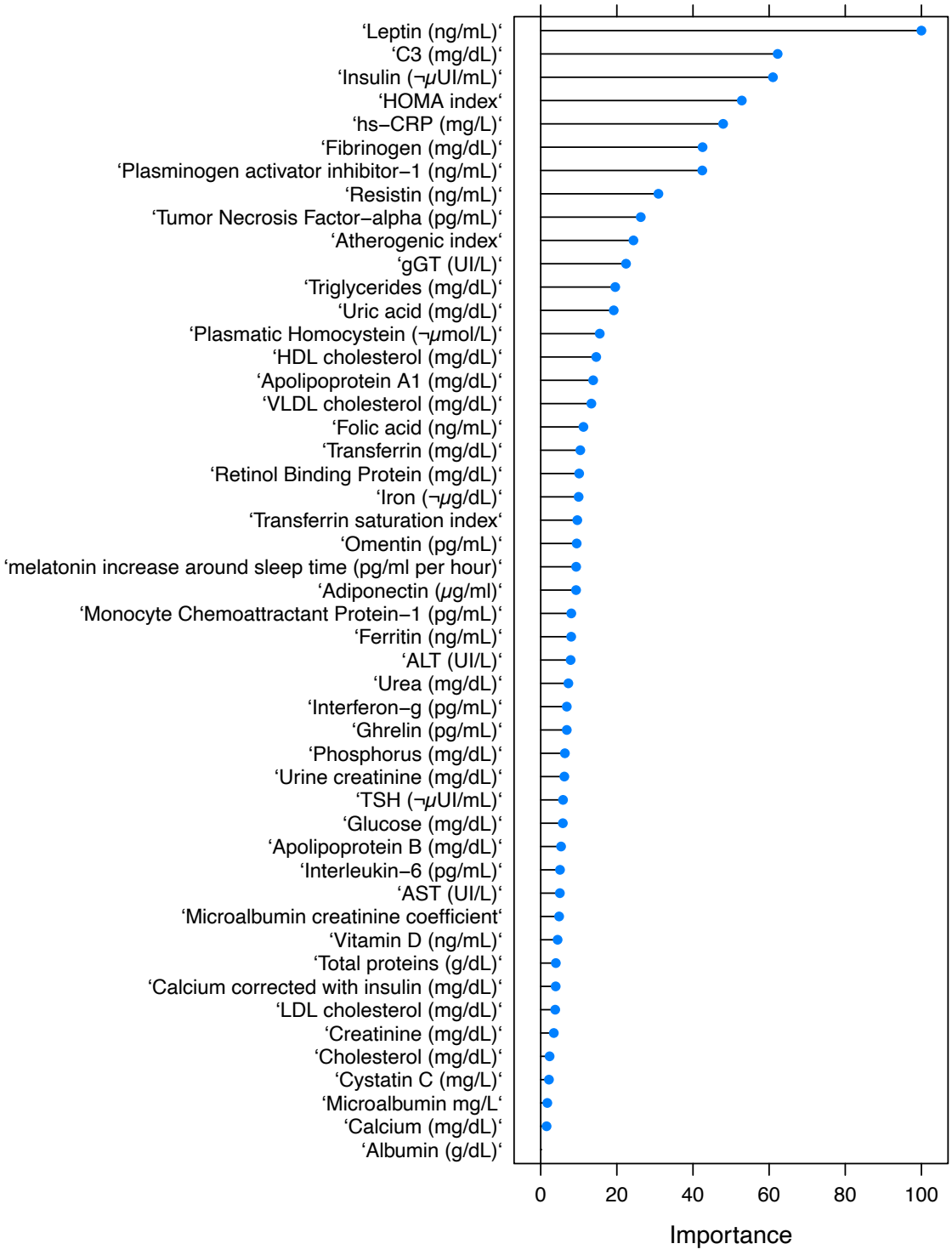


Figure 63. *Scaled variables importance of obesity metabolic footprint*

VI. Discussion

In the present study, we have investigated the relationships between life habits, circadian rhythms of melatonin and metabolism in childhood obesity.

VI. 1. Melatonin and characteristics of obesity

a) Melatonin and obesity characteristics in childhood

The primary objective was the analysis of melatonin expression over evening in childhood, comparing children with and without obesity and overweight. Our observation is that the kinetic of the circadian hormone is different. Indeed, in participants with obesity, an increase in melatonin levels which was not present in the control group was observed in the first period of the evening, and a slower increase of melatonin levels around sleep time was also measured. Consistently, we encountered melatonin increase rate around sleep to be inversely correlated with anthropometric characteristics of obesity as the percentage of fat mass or the waist/height ratio. Interestingly, vitamin D levels, associated with healthy light exposure, are significantly lower in children with obesity. Therefore, a hypothesis could be that children with overweight and obesity do not spend enough time outside. It is nevertheless to note that no significant correlation was found between vitamin D and melatonin increase in our study.

However, melatonin increase rate around sleep was also found inversely correlated with metabolic markers such as the HOMA index of insulin resistance, RBP and uric acid associated with diabetes risk, and γ-GT, associated with hepatic disease. The slower melatonin rate around sleep may result for the organs in a lower overnight exposure to melatonin, reducing in turn activation of melatonin receptor's pathways and inducing insulin resistance as observed on rodents.[250] This would be consistent with the available bibliography on the subject, as cumulative nocturnal melatonin secretion measured in the first morning urine of 149 adolescents with obesity has been found significantly lower in the individuals with insulin resistance.[222] In addition, the increase rate of melatonin around sleep was found inversely correlated with leptin levels. Although some inconsistencies have been found in the studies linking melatonin and leptin, rodent studies have shown that pinealectomy induces an increase in leptin level, which are lowered consequently to melatonin supplementation. On the other hand, in *in vitro* studies, melatonin has been found to be downregulated by leptin treatment. Nevertheless, the associations in human between melatonin and leptin are variable, which may be explained by the diversity of mechanisms involved in their mutual regulations.[143]

Other associations have been found with the increase rate of melatonin around sleep, including immune factors such as the levels of leucocytes, lymphocytes, neutrophils, platelets, the atherosclerotic risk factor homocysteine, but not with the studied cytokines (IL-6, TNF-α, IFN-γ). Surprisingly, melatonin was found positively correlated to ghrelin in the studied children, contrary to what was previously reported on animal models.[251,252]

The present associations encountered between melatonin and characteristics of obesity do not provide indication on which element is the cause and which is the consequence, but reinforces the idea that melatonin is tightly linked to parameters involved in obesity development. In the aim of investigating whether it is melatonin which causes modifications of the factors implicated in obesity, we designed an *in vitro* study on human adipocytes.

b) Melatonin and circadian rhythms of adipose tissue transcript: *in vitro* study

In the adipose tissue, various key metabolic factors display circadian rhythms of expression, such as enzymes of the lipid metabolism, hormones regulating insulin sensitivity, nutrient transporters, and transcription factors regulating adipogenesis.[138,143,141,142] These rhythmic expressions depend on the clock genes and the melatonin rhythmical stimulation, but how melatonin impacts clock genes and clock controlled genes is only partially understood. On rodents, it was showed that the amplitude of the circadian expression of clock genes and metabolic gene is altered secondary to pinealectomy.[144] In addition, when experiencing jetlag consequently to travelling to a different time zone, the internal clocks of travellers progressively align to the new solar time, shifting of about one hour each day.[114] These elements suggest that melatonin have the capacity to influence the circadian rhythm of the peripheral clocks in term of amplitude, and to shift the acrophase. In the present work, we have investigated the nature of the impact of melatonin on the circadian genetic expression, in human adipocytes. We cultivated human subcutaneous adipocytes in a medium supplemented with a concentration of melatonin corresponding to that measured at night in our control group of children. In these adipocytes compared to the controls, we measured a global increase in the amplitude of the rhythm in all the genes tested, which was significant for four of the genes: two of the three clock genes tested, PER2 and CRY, as well as the hormone adiponectin and the enzyme LPL.

A hypothesis is that melatonin may influence the levels of the clock genes through regulating post-translational components of the clock loop. Indeed, degradation level of Cry protein by the proteasome depends on a cycle of ubiquitination and deubiquitylation, which consequently regulated the quantity of protein available for feedback. Researchers have hypothesized that melatonin inhibits the proteasome and this way interferes with the negative feedback loops involving CRY and PER[105], which is consistent with our findings.

Regarding adiponectin, the amplitude increase caused by melatonin may explain the discrepancies observed in previous studies. Indeed, some studies report that melatonin upregulate significantly adiponectin levels, while other studies report the contrary, or no effects.[143] It is therefore possible that a melatonin-induced increase in adiponectin amplitude of expression would result, according to the time of the day at which adiponectin is measured, in an increased, decreased, or unchanged expression. The increase in LPL amplitude is also consistent with literature, which states that nocturnal LPL activity reflects the insulin

sensitivity of the adipocytes in that period of the day in which cholesterol synthesis is regulated by insulin, in association with melatonin activity.[253]

As the amplitude increase in the circadian expression of the leptin gene was not found significant, the present *in vitro* study provides no information on the cause/consequence mechanism underlying the association observed between these two parameters in childhood obesity.

In parallel, we observed no modifications of the acrophase nor the mesor. In other words, no temporal shift of the rhythm was measured in the rhythm of expression of our adipocytes cultivated with melatonin. Further studies investigating the effect of punctual melatonin stimulations, at different times, durations, and concentration, are indicated to complete the understanding of the influence of melatonin on peripheral oscillating genes. This would constitute strategic advances in chronomedicine. In future studies, it may be strategic to apply a glucocorticoid shot to the cells before cultivating them with or without melatonin in order to synchronize the all the cells together[254], and therefore limit the potential bias of loss of synchrony of cells after several days in culture. Plus, new studies comparing the effects of melatonin with the one of other antioxidants may be interesting to identify whether the observed increase in the amplitude of transcript is linked with melatonin's specific pathway, or to its general antioxidant activity. Finally, melatonin has been previously shown to inhibit the proliferation and differentiation of adipocytes through inhibiting PPARγ. Here, no clear effect of melatonin on PPARγ and C/EBP was observed, indicating that a longer period of contact with melatonin may be needed to encounter these effects, and measure whether the impact of melatonin on these factors consists in fact of a modification of their rhythm of expression.

In summary, the present *in vitro* study comes in complement of the study in children by bringing an indication that melatonin at the concentration measured in children can have an impact on physiological factors. The nature of the impact measured consists in an increase in the amplitude of the rhythms (**Figure 64**), which constitutes a reinforcement of the rhythmic variations in the adipose tissue. This strengthens the idea that the integrity of melatonin expression is necessary to maintain homeostasis. In this sense, it appears relevant to identify the behaviours and environmental parameters which are associated with a healthy melatonin expression.

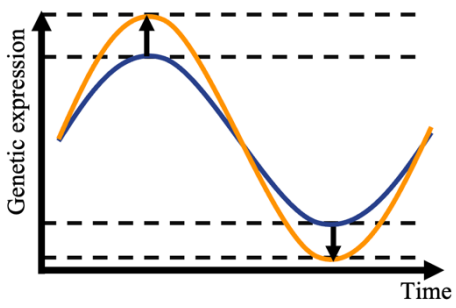


***Figure 64**. Increased amplitude of genetic expression in adipocytes cultivated with melatonin*

c) Melatonin and life habits and environment

In our paediatric cohort, we have also observed that the expression of melatonin is linked to life habits and sleep environment. To begin, we analysed the relationship between melatonin and the light exposure during the night associated to screen devices. As other investigators[255], we chose to ask only the presence of screens in the bedroom, estimating the participants were more likely to tell the truth than if they had been asked about their nocturnal use of these devices. It was conclusive, as melatonin increase rate around sleep was found inversely correlated with the number of screen devices in the bedroom at night. As previously published, light exposure even a light pulse around bedtime inhibits melatonin expression.[124,256] Consequently, it is logical that screen exposure around sleep can induce a delay and flatten the melatonin curve (**Figure 65**), resulting in a lower melatonin increase rate around sleep as we observed, and an overall decrease in melatonin nocturnal production as previously reported.[257]

***Figure 65**. Nocturnal exposure to light alters melatonin expression*

Among the electronic devices in the bedroom, only the mobile phone was found significantly associated with lower melatonin. This association can be explained by the normalized automatic and frequent behaviour of notification checking[258], which is particularly associated to moments of inoccupation, boredom or loneliness feeling[259], which are typically encountered around bedtime. When the mobile phone is present in the room, it is classically on the nightstand, making accessible an instantaneous gratification[260] at minimal movement cost at hand reach even while staying in bed. Among the screen devices present in the room, the mobile phone is therefore probably the most systematically turned on at night, and consequently the most directly related to melatonin inhibition.

The relationship between sleep and melatonin has evolved in the modern era. Ancestrally, the sun was the only blue light zeitgeber. Melatonin expression started raising at dusk, which prepared the organism for sleep. Nowadays, it is frequent for humans to be exposed continuously to blue light until closing eyes to sleep. This short-circuit makes sleep not only an output, but also an input of the clock.[261] Consequently, later

sleep onset delays and reduces the exposure of the organs to melatonin, as previously observed.[257] In our present study, we also found the increase rate in melatonin around sleep to be negatively correlated with bedtime during both the week and the weekend. A positive correlation was found with sleep duration, and a positive correlation with the nap habit, which suggests that not only sleep duration is shorter, but insufficient compared to the children and adolescents needs in the children with low melatonin rate.[262]

In the present work, we did not find statistically significant correlation between melatonin increase around sleep and the timing of meals. However, we did encounter that the more the children eat during breakfast compared to later meals (morning snack, lunch, dinner), the stronger is their nocturnal melatonin increase rate. This may be explained by an increased hunger feeling in the morning in the children with higher melatonin increase rate around sleep onset. Indeed, among the questions of the Horne and Östberg chronotype questionnaire[134], several questions concern the hunger feeling in the first thirty minutes after waking up, and we also found this chronotype score associated with melatonin increase rate around sleep. It appears therefore coherent that children with an early chronotype also have an earlier melatonin onset, as previously observed in adults in sleep laboratory[263], and in the morning, feel hungrier than their late chronotype friends.

Melatonin, as most of the health parameters and habits studied, evolve with growth. For example, the melatonin peak of expression lowers with age, and in parallel, children go to bed later when they get older. Consequently, without adjusting the correlations as we did here for age and puberty index, the association between melatonin and bedtime may have been just the reflect of the age variability in our cohort. The fact that even once adjusted, we still encounter that the children with obesity present circadian characteristics of individuals older than their age may therefore be of interest. Indeed, it is considered that numerous tissues of individuals with obesity present an accelerated aging.[264] Therefore, it would be interesting to analyse whether this process also applies to the circadian system, and verify whether even during childhood, melatonin levels in obesity indeed correspond to those of older individuals.

Finally, the correlation encountered here with melatonin are statistically significant, with diverse and numerous parameters, but are of a relatively low strength. A possible interpretation is that melatonin may at the interface between external and internal parameters forming together a multivariate network of regulation of the organism's health. In addition to melatonin's links in this network of interactions, we also analysed the characteristics of obesity that were associated with chronotype, life habits, and life environment.

VI. 2. Chronotypes, habits, environment, and characteristics of obesity

a) Chronotype

Unlike melatonin, the chronotype score obtained from the Horne and Östberg morningness and eveningness questionnaire[134] was not found correlated with any of the anthropometric parameters analysed in this study, and the chronotype classification distribution was similar in the control and obesity groups. Similarly, a recent meta-analysis gathered different studies investigating chronotype and obesity in adolescence, and although a trend between eveningness and overweight was observed, it was not found significant.[265] Nevertheless, we did encounter positive correlations between chronotype with kidney's health markers, and negative correlations with inflammation markers. In parallel, various parameters of life habits characteristics were found associated with chronotypes. For instance, the earlier the chronotype, the lower the sleep disturbance score, an association which had been previously been reported in childhood.[266] In our cohort, earlier chronotype also correlated with earlier bedtimes during both the school days and the weekend, and an earlier wake-up time during the weekend. The participants with late chronotypes on the other hand presented stronger social jetlag. They also take later breakfast during the weekend, and globally a smaller proportion of the nutritional intake during this first meal compared to the rest of the meals. Finally, late chronotype is also correlated with the number of screen devices present in the bedroom at night, in particular television, console, and tablet. In childhood, many habits are strongly influenced by the home context, parental own habits, and decisions, leaving to the children a relatively small room for manoeuvre in terms of schedules. Therefore, on one hand, the associations we encountered may reflect that the children's chronotypes are influenced and delayed by the later timing of activities at home and the presence of screen devices in the bedroom. On the other hand, these links may illustrate that the late chronotype of the children drives them towards later behaviours regarding the aspects that they decide themselves. For instance, they are more likely to be able to influence weekend sleep timings or whether they will eat a lot for breakfast, rather than the lunch time or wake up time during the week. A combination of these two aspects may result favouring social jetlag, a repeated misalignment between the inner time of the organs and the time at which they are stimulated. This may trigger a rise in inflammation like the one measured here, and possibly in the longer term, more general metabolic and anthropometric effects, which could explain why they were observed in adulthood but not here.

b) Dinnertime

In our study, late dinnertime is associated with metabolic alterations including elevated glucose and decreased iron levels, two elements classically encountered in obesity. Although the timing of nutritional intake is known to regulate the digestive clocks, in the present study dinnertime was not found linked with melatonin, with chronotype score, with BMI nor with the anthropometric characteristics. Although, as the

children with obesity studied in this work are currently undergoing an interventional program with a nutritionist, it will be interesting to see if, as previously reported in adults[129], the individuals with earlier meal times lose weight more efficiently. In parallel, we unsurprisingly measured that the later the individuals have dinner the greater is their time amplitude of nutritional intake. Lately, studies tend to show that the opposite, early time-restricted feeding, is associated with healthier metabolic markers and lower weight.[267–269] In parallel, the later the individuals ate dinner, the less they would eat during the early meals (breakfast or morning snack) in regards with the other meals. The late dinner time during the school week was also encountered associated with later afternoon snack, lunch time, and dinner times during the weekend. This suggests that in certain families, for each meal which is not pressured by the work and school schedule, the timing will be consistently later than in other families who eat consistently earlier over the different meals. Finally, we also could observe that individuals with later dinnertime also have later bedtime during schooldays, and logically, with shorter sleep duration.

c) Sleep duration

In addition to the mentioned relationship with melatonin levels, we also encountered a tight link between sleep duration and the characteristics of obesity. Indeed, BMI, waist/height index, the percentage of fat mass, but also the diastolic blood pressure, are found lower in individuals with longer sleep duration. In addition, the metabolic characteristics such as glucose, atherogenic index, VLDL cholesterol and triglycerides, but also inflammatory characteristics as PAI-1 and MCP-1 are better in individuals with longer sleep. These findings are in line with previously published works[210], and come complementing the paediatric bibliography on this subject. As already mentioned, the sleep duration is linked with other life habits such as the timing of nutritional intakes. In addition, the shorter the sleep duration during the school week, the stronger the social jetlag, measured as the number of hours of difference in sleep duration between school days and weekend. At epidemiological levels, social jetlag has been shown associated with obesity.[199] Finally, sleep duration was found shorter when the number of technological devices with screens were present in the bedroom, an element which has already been published.[255,270]

d) Screen exposure

Indeed, we observed that the presence of one or more screen device, including mobile phone, TV, tablet, console, or computer, was a risk factor for obesity, insulin resistance, and high leptinaemia, consistently with what previously published.[270] It also multiplies by almost 6 the probability to be a late chronotype and have late life habits timings of sleep and nutrition. Children with screens in their bedroom also present signs of insufficient sleep such as a higher prevalence of nap and longer weekend sleep recovery. Although weekend sleep recovery allows to partially recover from sleep insufficiency in regards of metabolic and inflammation markers[271–275] compared to individuals who do not even recover during the weekend, alterations still remain visible compared to individuals with adequate sleep.[276–279] These elements, together with the present work, comfort the necessity to increase the prevention towards excessive screen device use in childhood, and more particularly in the times and spaces dedicated to sleep. Previous studies showed the

parental education level to be related with the children's screen devices use.[280,281] In the present study, no significant association was found between the mother nor the father's education level with the presence of screen devices in the child's bedroom. Nevertheless, the presence of screen devices in the children's bedroom was found significantly associated with the father being unemployed and the parents being separated, in line with what has been previously published on a Portuguese epidemiological study.[282] The link between screen exposure and parental separation may be explained by at least three different mechanisms. Indeed, parents which themselves have a high technology use are more likely to be permissive with their children, yet high screen use can increase the probability to get divorced[283]. In addition, "telepresence", or the technological communication with the parent which does not have the custody after the divorce also increases the time in contact with screen devices in the family.[284] Finally, divorced parents and helping grandparents may be more permissive and more inclined to authorize and buy technological devices to the children.[285]

e) Familiar context

Studies show that in 2020 in western countries, the repartition of household's tasks is still unequally reparteed between the parents, with higher percentages of fathers with stable employments and higher pays than mothers. Consequently, divorce or separation results in a lower income in the mother's household and a higher probability to cross the poverty line, in particular in women with a low education level.[286,287] The mothers are usually in charge of the children and their health, as we could observe in the recruitment and follow up of the study participants. Indeed, the nutritionist of our team, who performs a longitudinal study on 121 children from the obesity group, estimates that about 10% of the children come accompanied by the father, 5% by the grandmother and 85% by the mother. When asked for a contact number, in 100% of the cases the mother's number was given, which underlines that they are in charge of this aspect of their lives. Plus, in our study, no relationship was found between the mother's employment status and the child's health characteristics, whereas the father's unemployment situation constitutes a risk factor to the child's BMI, fat mass percentage, high heart rate, high insulin resistance, leptinaemia and VLDL cholesterol. It also multiplies by 4 the odds to have screens in the children's bedroom, to present social jetlag, and finally to be separated from the mother. These results support the idea that the fathers are more often in charge of the family incomes. Their precarious work situation is associated with lower socioeconomic status, in which obesity and metabolic disorders are more prevalent even during childhood.[288] In addition to the economic context, the education levels of the parents and health awareness is known to be linked to the children's health.[289,290] Here, both maternal and paternal high education levels appear as protective factors regarding the anthropometrics and metabolic characteristics of obesity. Consistently with literature, the children of highly educated mothers were more likely to have been breastfed[291], an element which is protective of obesity. In addition, our findings suggest that high education levels in the father as well as the mother have a positive influence on life habits and their timing, being associated with physical activity practice and higher nutritional intake during breakfast compared to the later meals. Regarding sleep, children whose parents have higher education levels have a better sleep quality and less need for weekend recovery sleep. Father's high education level and stable employment status appear to reduce the probability of the children to have screens in their

bedroom. Together, these results are in line with the previously published works linking socioeconomic context with sleep and metabolic health. Therefore, our findings support the relevance of sensibilization initiatives promoting parental awareness around sleep.[215]

In summary, the present work show that numerous associations exist between elements from life habits, circadian rhythms, and metabolic health. Far from being isolated associations, they appear to cross with one another, and to form a network of interconnected parameters.

VI. 3. Network of elements linking habits, circadian rhythms, and metabolism in obesity

While a few decades ago the norm in medical research was to focus mainly on one marker, technical and statistical advances now allow to integrate massive amounts of data and to include multiple variables in a same analysis. Multivariate analysis and machine learning are now more and more frequently used in the fields of obesity and sleep research.[292,293]

In the present work, principal component analysis has been performed to represent all together the different variables correlated with nocturnal melatonin increase rate. Thanks to this method, the study participants were clustered in three groups of individuals sharing similar characteristics. Three clusters appear, among which one takes low values for the anthropometric characteristics of obesity, and two subgroups of individuals presenting higher values for these parameters. Among these last two clusters, one was composed of individuals with better metabolic and inflammation outcomes than the other group. Interestingly, in this metabolically healthier group, the participants also presented earlier bedtime, less nap habit, a longer sleep duration during schooldays, less technological devices in the bedroom and an earlier chronotype compared to the other group. This supports the hypothesis that among individuals with obesity, earlier chronotype and better sleep quality and duration may prevent comorbidities development. Plus, over the three groups, the lean individuals shared healthier metabolic characteristics in addition to higher melatonin increase rate around sleep, longer sleep duration and lower exposure to screens. Although the correlations between these elements were previously mentioned individually, the clustering allows to represent the data in a manner that evidences the interrelationships existing between the studied variables.

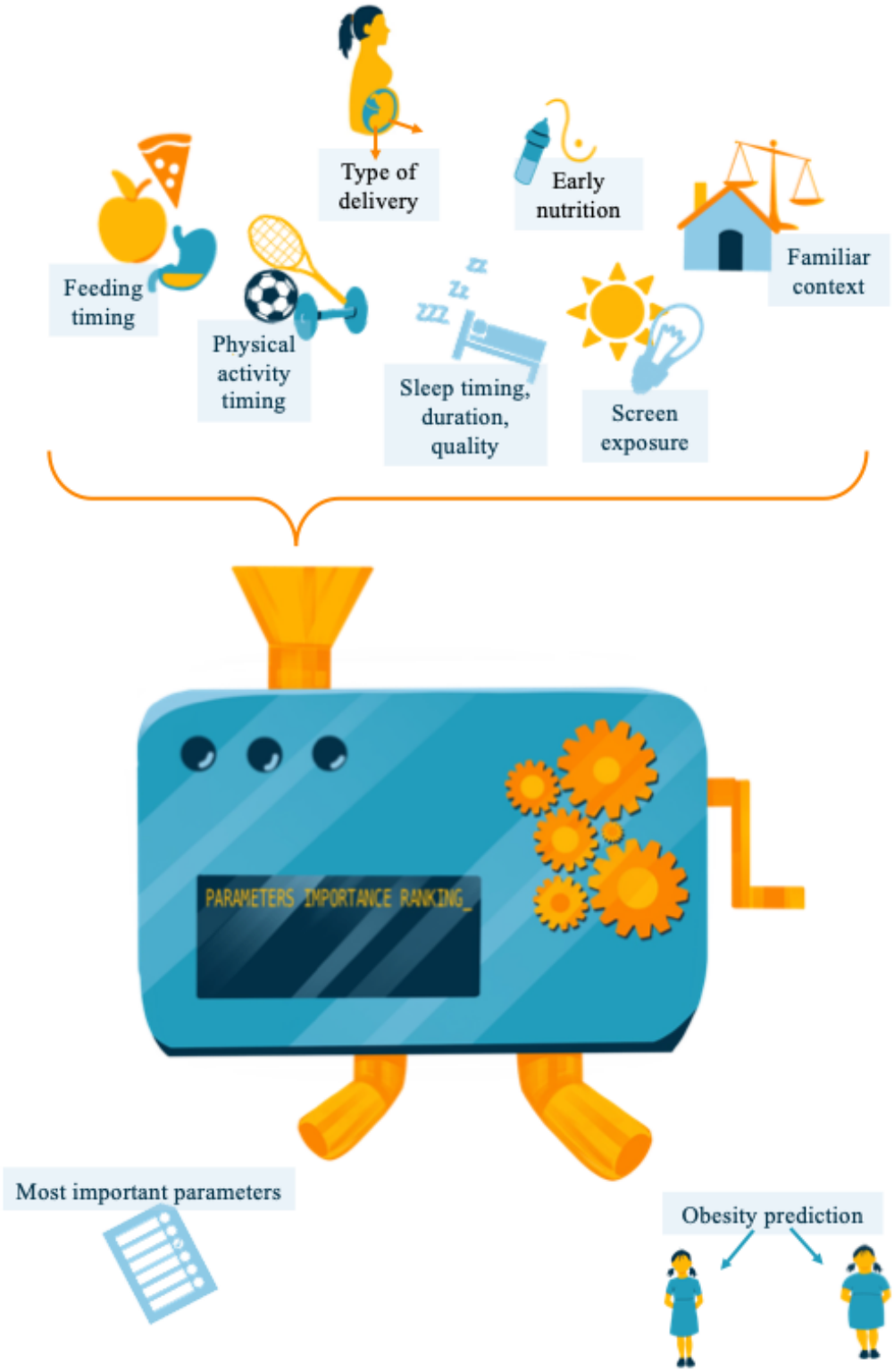


Figure 66. *Algorithm predicting obesity based on life habits, personal history, and environment*

In order to deepen the comprehension of this network of parameters of life habits, circadian rhythms, and metabolism, in obesity development, we employed another modern tool: the machine learning algorithm called random forest. As shown previously, classifying algorithms including this one can be used to predict obesity based on life and health variables such as estimated energy intake and expenditure, parental BMI, cholesterol...[294–296] In the present work, we have chosen to create a similar model integrating only external elements such as life habits quantity, quality, and timing, and sociodemographic context (**Figure 66**). This narrower selection of predictive variables could be relevant in the perspective of identifying which groups of elements are the most likely to promote BMI increase, and therefore should be prevented in priority. Our model has a regular accuracy but does to many false positives to be satisfying. In theory, if in a similar model, someone was able to integrate perfectly accurate measurements of life behaviour and context, as well as all the genetic, epigenetic, and microbiotic characteristics associated to obesity, he may reach 100% of accuracy. Even without reaching this level, the integration of objectively assessed life characteristics may provide a more accurate model, more reliable to estimate the relative importance of the different factors. Therefore, the present algorithm constitutes more a proof of concept rather than a strong source of information on the importance of life characteristics. Nevertheless, tendencies appear, such as a very low importance of early childhood characteristics such as early nutrition and delivery type. More surprisingly, the physical activity practice was also found among the poor predictors of obesity in this analysis. Among the analysed factor, father employment and both parental education were among the factors that contributed moderately to the model. At a similar level of contribution were encountered the screens in the bedroom, the social jetlag, sport practice frequency and dinner time. Other parameters contributed more, including chronotype, sleep duration during the school week and the greater food intake during an early meal than a late meal. Interestingly, the two variables that were found the most helpful for the algorithm to classify the participants, are the scores of sleep quality and disturbance. Although they may be the consequence of obesity and not its cause, sleep can quality and duration can be improved by increasing the awareness on the importance of sleep for health. Therefore, this finding supports the importance of promoting sleep sensibilization.
Even at its modest scale, the present model shows that the integration of self-reported timing of life habits as well as sleep characteristics influence the organism's health to the extent that they are useful, although insufficient to predict if the individual with these characteristics presents obesity.

As we saw, random forest algorithms can be used to predict a condition from risk factors and return the ranking of importance of the risk factors. They can also be used to identify the biomarkers of a condition, as well as a similar ranking.

The biomarkers of obesity are numerous, new ones are frequently discovered, often among metabolic or inflammatory factors either promoting or affected by the disease. In this study, we have measured that melatonin increase around sleep is modified in children with obesity, therefore we are interested to know whether it could be considered as one of its biomarkers. We therefore developed another algorithm classifying obesity, which this time integrated the fifty biochemical parameters measured in the plasma of

the participants, as well as the parameter melatonin increase rate. The obtained ranking of metabolites importance showed that our melatonin parameter was not among the top elements as leptin, classic low-grade inflammatory markers, or insulin resistance. Nevertheless, it was found around the half of the list, at the same levels as omentin and adiponectin, key metabolic markers produced by the adipose tissue, and above other classical markers such as LDL cholesterol, total cholesterol, glucose, or apolipoprotein B. This finding supports the hypothesis that nocturnal melatonin increase may constitute a biomarker of obesity in childhood.

Overall, these different multivariate approaches strengthen the idea that life habits, circadian rhythms and metabolic health parameters are interconnected. The present work proposes an original and integrative approach of the study of childhood obesity, nevertheless, several limitations need to be underlined, from which future directions of studies will rise.

VI. 4. Limitations of the study and future study directions

To begin, the **study design** being transversal, the results can only be associations and correlation, but no causality relationships can be shown between the study variables. In particular, whether the evolution of nocturnal melatonin expression is a cause or a consequence of obesity, or if both are consequences of a same origin cannot be concluded from the present work. Obviously, in human compared to animal models, there are more limited possibilities of studies which can demonstrate the role of an element by removing it (pinealectomy) and replacing it (melatonin supplementation). Therefore, many of the important discoveries of the field of circadian rhythms and metabolism have been made studying rodent models. Nevertheless, when it comes to the influence of life habits, the animal models are of limited support, hence the relevance of performing studies in humans, even of transversal design. Harder to perform on large groups and using numerous indicators, the longitudinal studies are however very informative. In our group, we are currently investigating the effect of life habits changes on melatonin levels and metabolic health, through a nutritionist's intervention aiming to accompany the patients with obesity and their families towards engaging in healthier nutritional habits, increased physical activity, and improved sleep context and preparation. In complement, ***in vitro*** **studies** as the one presented here can be performed to understand better the interrelationships between melatonin and circadian variations of clock and metabolic genes. Although a significant and redundant increase was observed in the amplitude of rhythmic expression of clock genes and metabolic genes, our study does not bring information on the metabolic pathway involved in this modification. It should be replicated analysing the activation status of possible elements involved in this regulation such as the proteasome pathway, as previously suggested.[105] Plus, as mentioned, melatonin effect may be analysed after a longer incubation period, with only punctual melatonin stimulations at different times of the day and different concentrations, but also controlling with other antioxidant molecules.

Regarding **melatonin measurement** in children, the present work confirms that the nocturnal increase can be assessed thanks to an autonomous sample collection at home by the child helped by a caregiver. This method permitted to quantify a significant increase of melatonin salivary levels in both control and obesity groups over evening, and to identify a difference between the groups. Regarding the application of the method, it is to note that some participants did not provide a sufficient volume, not well sealed and light-protected sample, did not collect saliva, or forgot to bring it. Although the investigators contacted the caregivers of these participants to schedule a new saliva collection, only part of them complied so in the end, 19% (51 out of 263) could not be included in the analysis because of the absence of melatonin measurement. This constitutes a relatively high proportion of the participants, which should be aimed to be reduced in future studies. For instance, providing an infographic document explaining the process by illustrations, as well as a little container to transport the samples in the dark, may improve the adherence to the sample collection. In addition, other types of tubes may be more appropriate for the collection of saliva. Finally, if this saliva-collection practice becomes more common, the patients may get used and adhere more easily.

Another limitation of the present saliva measurement method is that it does not provide the time of melatonin onset, which is the most widely used characteristic of melatonin in literature. More frequent measurements would be necessary to obtain this information, for example at 30minutes of interval from the afternoon to one hour after sleep, as done previously.[297] In addition, if we aimed to measure all the peak, including the time and quantity reached at the acrophase, measurements would have to be done all night long. This would be more laborious and uncomfortable for the participant, impact negatively sleep and risk to decrease more the adherence. In addition, it would make the experiment considerably more expensive. Nevertheless, if methodological advances allow to perform this kind of experiment with reduced negative impact on the patient, it will be an ideal way to analyse melatonin variations.

It is to note that the continuous measurement of peripheral temperature constitutes another manner to monitor circadian rhythm reliably and conveniently. Indeed, it does not implies sample collection so it is handier for the participants, nor laboratory work which makes it cheaper.[298] In addition, this kind of wearable device can also be used to collect other relevant data for studies on circadian rhythms and obesity. For instance, environmental light exposure as well as actigraphy, a technique that records the movements of the individual wearing the device. It has shown useful to measure physical activity but also sleep quality and even sleep stages, although not yet as accurately as the gold standard: polysomnography. Nevertheless, they constitute a promising lead considering the benefits of wearing a wrist or finger device in daily life context, instead of doing a polysomnography in a sleep laboratory. In addition, heart rate measurement over day, and even electrocardiograms can be performed by wearables. These technologies are now integrated in smartwatches used by the public and researchers, as the Fitbit, Apple watch, or Withings.[299–301] Even smart rings exist and are used in investigation projects, as the Oura ring which can measure the menstrual cycle.[302] These devices provide access to objective measures continuously collected during days, months, or even years. These measures concern external factors and behaviours such as light exposure, sleep, physical

activity, but also internal measurements such as skin temperature and cardiac activity. Plus, smartphone applications now can be used to obtain the time and duration of use, which can be monitored for personal awareness and for research purpose. These elements constitute a revolution compared to the traditional questionnaires on sleep, physical activity, or screen exposure (in annexes) and single-time measurements of cardiac activity as we performed them in the present study. Therefore, in our group's next study, wearable devices are being used.

Despite these recent advances, one element remains harder to monitor accurately: the nutritional intake. In our work, the three days recording of mealtimes and content was often done inaccurately, writing a posteriori the menus of the last three days instead of during or just after a meal, and the results were dependant on the parental (and in particular the maternal) education level. Other studies have employed similar nutrition diaries but on smartphones and adding pictures of every food consumed. In addition to providing accurate mealtimes, these smart diaries may increase adherence and reliability of the information provided on food intake, as applications developed for this purpose encounter a great success. Nevertheless, contrary to a wearable which automatically records movement, completing a nutrition diary is still laborious and the accuracy still depends on how well the user completes it.

Besides the quality and quantity of data obtainable on an individual with these new materials, the quantity of individuals on which these data are generated is also compelling. Indeed, since their creation a few years ago, wearable's market has exploded, reaching in 2021 more than 150 millions of shipping units all over the world, of which one third are Apple watches.[303] In parallel, MyFitnessPal, one of the most popular apps for nutritional follow-up, has received 30 638 reviews to date on the AppStore, which implies number of downloads over 200 000.[304] It is to note that the apps associated to the wearables or monitoring nutritional intake usually integrate other self-reported information such as height and weight. Plus, among the companies gathering these enormous amounts of information on human life habits and health, some as Apple claim to be leaders in data protection and never sharing them for marketing purposes[305], whereas others like MyFitnessPal clearly informs that the data will be collected, used, and transferred for commercial purposes.[306] This high volume of high quality data can subsequently be integrated in machine learning models like the ones presented here, but in the aim of accurately identifying marketing targets. By comparison, the number of individuals included in the present study is extremely small.

A high number of individuals permits to build more accurate machine learning models, and to refine the analysis by subgroups. For instance, in the present study, the age range is wide, and many parameters evolve during this life period. In a bigger cohort, the relationships between life habits, circadian, and metabolic markers could be analysed for each age-range and puberty index. This would help building more personalized recommendations for health prevention. A sub-analysis for which we already have a sufficient amount of data is the influence of sex on the network of parameters measured in this study. It will be proximately performed by our team.

As another limitation of the present study, some important parameters in the regulation of behaviours influencing metabolism were not investigated. For instance, no psychological characteristic was measured, despite its importance in the regulation of physical activity practice, sleep, and more particularly nutritional intake. Interestingly, it appears that when biologists propose models of body weight regulation, they include metabolic and mechanic parameters[307,308], whereas when psychologists do the same exercise, the parameters that they underline are the self-observation (including self-weighing), and the healthy-lifestyle awareness.[309,310] Both of these approaches appear relevant and complementary, underlying the importance to promote multidisciplinary research. In addition, numerous studies show the link between disruption of circadian rhythms and/or insufficient sleep with anxiety, bipolar disorders, and depression.[311] In parallel, the individuals with obesity are also more at risk for these psychiatric outcomes, which supports the relevance of integrating the psychological status in the network of parameters interconnected and evolving in obesity.

Finally, only limited information was collected regarding the familiar context and socioeconomic environment in which the children were growing. In previous studies which used machine learning to predict obesity, it was observed that the parental BMI was among the most important predictors.[294–296] Considering the links we encountered between life activities timing and obesity, and that in childhood the schedules are mostly driven by the parents, it would be interesting to test the link between parental chronotype, life habits timing at home, and relationship with the children's metabolic health. Regarding the familiar context we included education and work stability but did not have more information regarding the household incomes. No data was collected either regarding the socioeconomic characteristic context neighbourhood in which they live, nor whether it is located in an urban, peri-urban or rural area. Although it was not quantified, the present study was conducted on groups mainly composed of Caucasian individuals. Studies integrating a larger number of individuals of diverse ethnic origins may be useful to assess possible variations and improve prevention efficacy.

In summary, from the molecular to the societal scale, all the elements described are interconnected. They form a network, which in the context of obesity, is modified. Some studies investigate in detail the relationship between specific elements of health. Other works connect numerous elements together and build a model to aim to globally understand health. Together, these complementary approaches allow the generation of accurate and integrative models. This study reinforces the multidimensional model associated with obesity and underline the importance of the timing in health.

VII. Conclusions

- **The melatonin** increase rate around sleep time was found significantly lower in the group presenting childhood obesity. This modification was found associated with different risk factors as anthropometric (higher body mass index, fat mass percentage, and waist/height index), metabolic (increased HOMA index, ghrelin, leptin), later chronotype, life habits (later bedtime, shorter sleep duration, later repartition of caloric intakes over meals), and environmental factors (more screen devices in the bedroom).
- In human adipocytes stimulated with melatonin, an increase was observed in the amplitude of the **circadian expression of clock genes and metabolic genes**.
- The melatonin measurement **method** using three saliva samples over evening showed functional for the assessment of melatonin increase rate around sleep time.
- A correlation was found between the **chronotype** and numerous factors: the earlier the chronotypes, the healthier the metabolism (lower creatine and albumin, higher transferrin) and lower inflammation (IFN-γ, TNF-α), healthier life habits (earlier bedtime, longer sleep duration, less social jetlag), and environmental factors (less screen devices in the bedroom, more stable work situation of the father).
- **The sleep duration** was found negatively correlated to obesity through anthropometric (lower BMI, fat mass percentage, and waist/height index), clinical (lower diastolic blood pressure), metabolic (lower glucose, triglycerides), inflammatory factors (lower PAI-1, MCP-1), life habits (less social jetlag, earlier bedtime, shorter time amplitude of nutritional intake, earlier meals times and repartition of the nutritional intake over meals) and environmental factors (less screens in the bedroom).
- **The dinnertime** was found positively correlated to obesity through metabolic (higher glucose and albumin, lower iron), life habits (shorter sleep duration, and longer time amplitude of nutritional intake, later meals times and repartition of the nutritional intake over the meals).
- **The nocturnal screen exposure** was found positively correlated to obesity through anthropometric (higher BMI, fat mass percentage, and waist/height ratio), metabolic (higher HOMA index, leptin), chronotype, life habits (more napping, social jetlag, and late bedtime) and environmental factors (unemployed father, separated parents).
- **The parental sociodemographic characteristics** were found linked with obesity, as lower study levels of the parents, unstable employment of the father were correlated with anthropometric (higher BMI, fat mass percentage, and waist/height ratio), clinical (higher systolic and diastolic blood pressure levels), metabolic (higher HOMA, triglycerides, leptin, vLDL), inflammatory (higher C3, PAI-1), life habits factors (poorer sleep quality, more social jetlag, lower physical activity, more formula milk as early nutrition, later repartition of the nutritional intake over the meals), and environmental factors (more screen devices in the bedroom).

- A **clustering** analysis revealed that the group of children with obesity and overweight was composed of two subgroups. In one of them compared to the other, the children with obesity and overweight presented better metabolic health characteristics in parallel of earlier bedtime, less nap habit, a longer sleep duration during schooldays, less technological devices in the bedroom and an earlier chronotype.
- A **prediction model of obesity** based on life habits and environmental factors underlined that obesity is associated with sleep quality, life habits timing, and the repartition of the caloric intakes over meals.
- A **biomarker model** showed that melatonin increase rate around sleep was not among the stronger markers of obesity (leptin, C3, HOMA index, hs-CRP), but that it was one on the same level as classic markers such as adiponectin, omentin, ghrelin, or glucose.

As a general conclusion, in the present study, a relationship was found between life habits, melatonin variations, and metabolic disorders in childhood obesity (Figure 67).

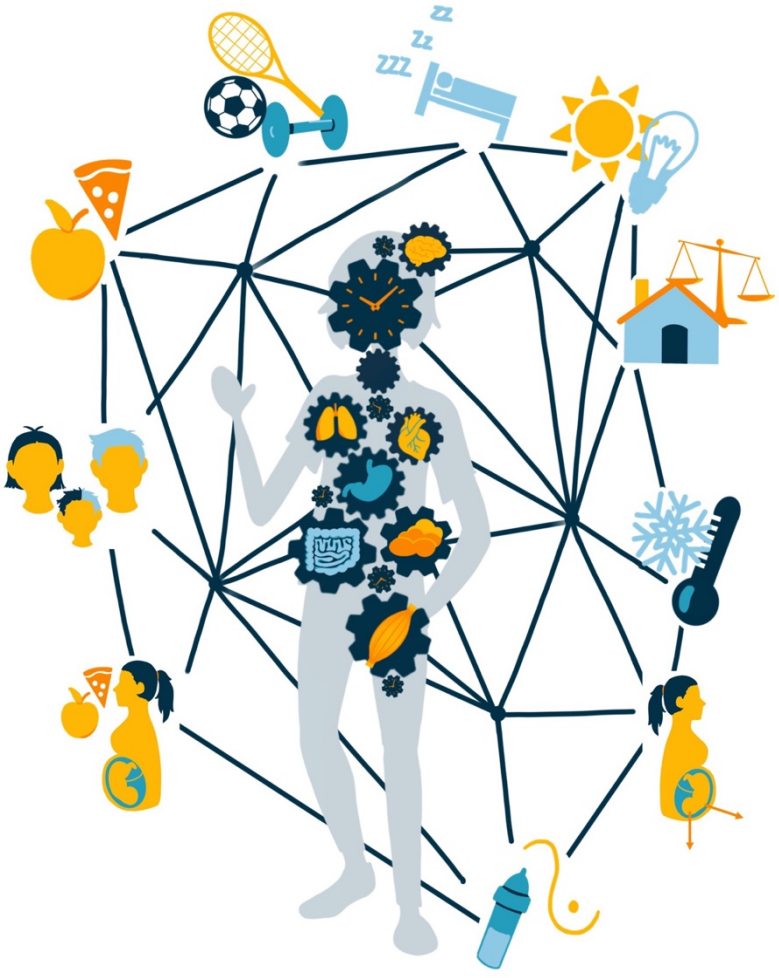


***Figure 67**. Network of relationships between life habits, melatonin variations, and metabolism*

Conclusiones en castellano

- La tasa de aumento de **melatonina** alrededor de la hora de dormir se encontró significativamente más baja en el grupo presentando obesidad infantil. Esta modificación se encontró asociada a diferentes factores de riesgo como antropométricos (mayor índice de masa corporal, porcentaje de masa grasa e índice cintura/talla), metabólicos (aumento del índice HOMA, grelina, leptina), cronotipo más tardío, hábitos de vida (hora de acostarse más tarde, menor tiempo de sueño). duración, reparto posterior de la ingesta calórica durante las comidas) y factores ambientales (más dispositivos de pantalla en el dormitorio).
- En adipocitos humanos estimulados con melatonina se observó un aumento en la **amplitud de la expresión circadiana de genes reloj y genes metabólicos**.
- El **método de medición de melatonina** utilizando tres muestras de saliva durante la noche resultó funcional para la evaluación de la tasa de aumento de melatonina alrededor de la hora de dormir.
- Se encontró una correlación entre el **cronotipo** y numerosos factores: cuanto más tempranos los cronotipos, más saludable el metabolismo (menor creatina y albúmina, más transferrina) y menor inflamación (IFN-γ, TNF-α), hábitos de vida más saludables (acostarse más temprano, mayor duración del sueño, menos jetlag social) y factores ambientales (menos dispositivos con pantalla en el dormitorio, situación laboral más estable del padre).
- La **duración del sueño** se correlacionó negativamente con la obesidad a través de factores antropométricos (menor IMC, porcentaje de masa grasa e índice cintura/talla), clínicos (menor presión arterial diastólica), metabólicos (menor glucosa, triglicéridos), inflamatorios (menor PAI-1, MCP-1), hábitos de vida (menos desfase horario social, acostarse más temprano, menor amplitud temporal de la ingesta nutricional, comidas más tempranas y reparto de la ingesta nutricional entre las comidas) y factores ambientales (menos pantallas en el dormitorio).
- Se encontró que **la hora de la cena** se correlacionó positivamente con la obesidad a través de hábitos metabólicos (glucosa y albúmina más altos, hierro más bajo), hábitos de vida (duración del sueño más corta y mayor amplitud de tiempo de la ingesta nutricional, más tardías comidas y reparto de la ingesta nutricional entre las comidas).
- La **exposición nocturna a las pantallas** se correlacionó positivamente con la obesidad a través de factores antropométricos (mayor IMC, porcentaje de masa grasa y relación cintura/estatura), metabólicos (mayor índice HOMA, leptina), cronotipo, hábitos de vida (más siestas, jetlag social y hora de acostarse) y factores ambientales (padre desempleado, padres separados).
- Las **características sociodemográficas** de los padres se relacionaron con la obesidad, ya que menor nivel de estudio e inestabilidad laboral del padre se correlacionaron con factores antropométricos (mayor IMC, porcentaje de masa grasa y relación cintura/talla), clínicos (mayor presión arterial sistólica y diastólica). hipertensivos), metabólicos (mayor HOMA, triglicéridos, leptina, vLDL),

inflamatorios (mayor C3, PAI-1), factores de hábitos de vida (peor calidad del sueño, más jetlag social, menor actividad física, más leche de fórmula como nutrición temprana, posterior reparto de la ingesta nutricional a lo largo de las comidas), y factores ambientales (más dispositivos con pantalla en el dormitorio).

- Un **análisis de conglomerados** reveló que el grupo de participantes con obesidad y sobrepeso estaba compuesto por dos subgrupos. En uno de ellos en comparación con el otro, los participantes presentaban mejores características de salud metabólica en paralelo a acostarse más temprano, menor hábito de siesta, mayor duración del sueño durante la jornada escolar, menos dispositivos tecnológicos en el dormitorio y un cronotipo más temprano.
- Un **modelo de predicción de la obesidad** basado en hábitos de vida y factores ambientales subrayó que la obesidad está asociada con la calidad del sueño, el momento de los hábitos de vida y el reparto de las ingestas calóricas entre las comidas.
- Un **modelo de biomarcadores** mostró que la tasa de aumento de la melatonina durante el sueño no estaba entre los marcadores más fuertes de obesidad (leptina, C3, índice HOMA, hs-CRP), pero estaba al mismo nivel que los marcadores clásicos como adiponectina, omentina, grelina o glucosa.

Como conclusión general, en el presente estudio se encontró una relación entre los hábitos de vida, las variaciones de melatonina y los trastornos metabólicos en la obesidad infantil.

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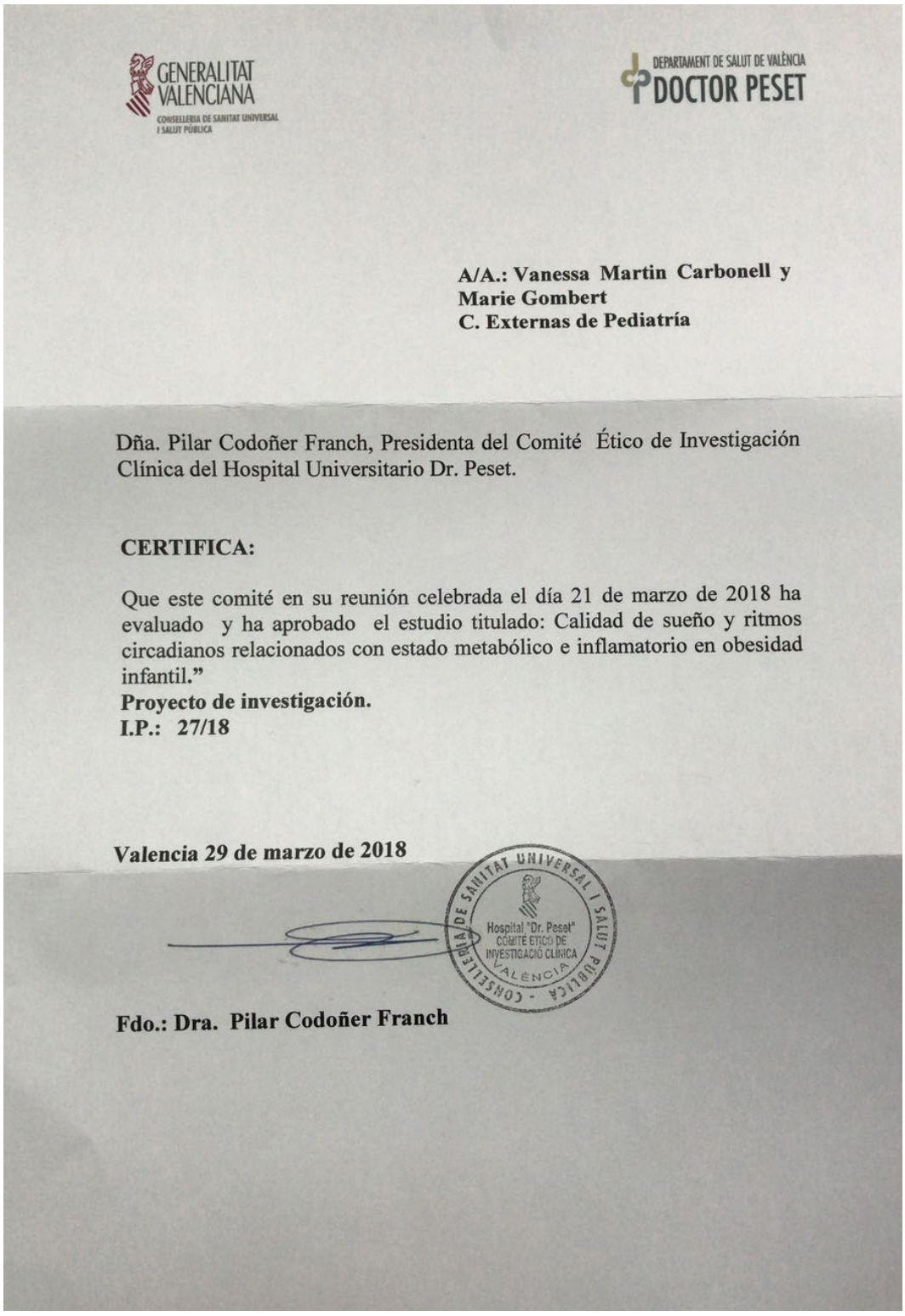
IX. Annexes

Annex 1. Ethical committee approval



GENERALITAT VALENCIANA
CONSELLERIA DE SANITAT UNIVERSAL I SALUT PÚBLICA

DEPARTAMENT DE SALUT DE VALÈNCIA
DOCTOR PESET

A/A.: Vanessa Martin Carbonell y Marie Gombert
C. Externas de Pediatría

Dña. Pilar Codoñer Franch, Presidenta del Comité Ético de Investigación Clínica del Hospital Universitario Dr. Peset.

CERTIFICA:

Que este comité en su reunión celebrada el día 21 de marzo de 2018 ha evaluado y ha aprobado el estudio titulado: Calidad de sueño y ritmos circadianos relacionados con estado metabólico e inflamatorio en obesidad infantil."
Proyecto de investigación.
I.P.: 27/18

Valencia 29 de marzo de 2018

CONSELLERIA DE SANITAT UNIVERSAL I SALUT PÚBLICA - VALÈNCIA
Hospital "Dr. Peset"
COMITÉ ÉTICO DE INVESTIGACIÓ CLÍNICA

Fdo.: Dra. Pilar Codoñer Franch

Annex Q1. Informed consent

Hospital Universitari Doctor Peset

CONSENTIMIENTO INFORMADO

ESTUDIO OBESIDAD Y SUEÑO HOSPITAL PESET/UV

Investigador Principal: Vanessa Martín Carbonell

D. /Dña.,…… (*Nombre y apellidos del padre/madre/tutor*), con DNI…….. en nombre y representación del paciente …… (N*ombre y apellidos del niño/a*) con DNI …….. declara que:

- He recibido y comprendido la hoja de información que se me ha entregado.
- He podido hacer preguntas y resolver las dudas correspondientes sobre el estudio.
- He recibido respuestas satisfactorias a mis preguntas.
- He recibido suficiente información sobre el estudio.
- Comprendo que la participación es voluntaria.
- Comprendo que puedo retirarme del estudio cuando desee.
- Comprendo que no recibiré ninguna compensación económica por la participación en dicho estudio.

De igual modo, declaro haber sido informado de las medidas que serán adoptadas, para garantizar la confidencialidad y disociación de cuanta información sobre mi persona pudiera recogerse durante el desarrollo del estudio, así como de la posibilidad de ejercitar mis derechos de acceso, rectificación, cancelación y oposición a través de una petición formal realizada ante el responsable del estudio.

Presto libremente mi conformidad para participar en el estudio.

_____ Fecha __ / __ / ____

(*Firma del participante*)

_____ Fecha __ / __ / ____

(*Firma del investigador*)

Annex Q2. Screen and sleep questionnaire

Cuestionario hábitos sueño y uso de nuevas tecnologías

Tecnologia
En tu habitacion, tienes :
❑Movil
❑TV
❑Consola (wii, PS4, Xbox…)
❑Consola portatil (DS, PSP…)
❑Ordenador
❑Tableta

Uso tecnologia al levantarse:
□Si
□No

Uso tecnologia en las dos horas antes de dormir:
□Si
□No

Deporte
Haces deporte ? __
Cuál ? _____
Cuántas veces a la semana ? _____
Y fines de semana ?_____
A que hora ? _____

Sueño
Haces siesta ?_____
Semana :
Hora de dormir : __:__
Hora de despertar : __:__

Fin de semana :
Hora de dormir : __:__
Hora de despertar : __:__

Condiciones de desayuno
Desayuno sentado?
□Si
□No

Duración del desayuno?
□< 5min
□> 5min

Annex Q3. Sleep disturbances Bruni questionnaire

SDSC[104]: Sleep disturbance Scale for Children. Escala de alteraciones del sueño en la infancia de Bruni (modificado)

1. ¿Cuántas horas duerme la mayoría de las noches?				
1 9-11 h	2 8-9 h	3 7-8 h	4 5-7 h	5 < 5 h
2. ¿Cuánto tarda en dormirse?				
1 < 15 m	2 15-30 m	3 30-45 m	4 45-60 m	5 > 60 m
En las siguientes respuestas, valore: 1 = nunca; 2 = ocasionalmente (1-2 veces al mes); 3 = algunas veces (1-2 por semana); 4 = a menudo (3-5 veces/semana); 5 = siempre (diariamente)				
3. Se va a la cama de mal humor				
4. Tiene dificultad para coger el sueño por la noche				
5. Parece ansioso o miedoso cuando "cae" dormido				
6. Sacude o agita partes del cuerpo al dormirse				
7. Realiza acciones repetitivas tales como rotación de la cabeza para dormirse				
8. Tiene escenas de "sueños" al dormirse				
9. Suda excesivamente al dormirse				
10. Se despierta más de dos veces cada noche				
11. Después de despertarse por la noche tiene dificultades para dormirse				
12. Tiene tirones o sacudidasde las piernas mientras duerme, cambia a menudo de posición o da "patadas" a la ropa de cama				
13. Tiene dificultades para respirar durante la noche				
14. Da boqueadas para respirar durante el sueño				
15. Ronca				
16. Suda excesivamente durante la noche				
17. Usted ha observado que camina dormido				
18. Usted ha observado que habla dormido				
19. Rechina los dientes dormido				
20. Se despierta con un chillido				
21. Tiene pesadillas que no recuerda al día siguiente				
22. Es difícil despertarlo por la mañana				
23. Al despertarse por la mañana parece cansado				
24. Parece que no se pueda mover al despertarse por la mañana				
25. Tiene somnolencia diurna				
26. Se duerme de repente en determinadas situaciones				
				Total

Annex Q4. SAHS questionnaire for sleep apnoea diagnostic

Cuestionario Abreviado de Sueño Pediátrico. SAHS

Instrucciones
Por favor responda las preguntas siguientes relacionadas con el comportamiento del niño o niña, tanto durante el sueño como cuando esta despierto. Las preguntas hacen referencia al comportamiento **habitual**, no necesariamente al observado en los últimos días porque puede que no sea representativo si no se ha encontrado bien. Si no esta seguro de cómo responder a alguna pregunta consulte con nosotros. Cuando se usa la palabra habitualmente significa que ocurre la mayor parte del tiempo o más de la mitad de las noches. Usamos el término niño para referirnos tanto a niñas como a niños.

Nombre del niño:			Fecha de nacimiento	
Edad:	Curso Escolar		Fecha de la encuesta	
Encuesta hecha por	Madre	□	Observaciones:	
	Padre	□		
	Ambos	□		

NS: significa NO SABE

Comportamiento nocturno y durante el sueño	**SI**	**NO**	**NS**
MIENTRAS DUERME SU NIÑO			
1. Ronca más de la mitad del tiempo?			
2. Siempre ronca?			
3. Ronca con fuerza?			
4. Tiene una respiración agitada o movida?			
5. Tiene problemas para respirar o lucha para respirar?			
6. Alguna vez ha visto a su hijo parar de respirar durante la noche?			
7. Durante el día su hijo suele respirar con la boca abierta?			
8. Se levanta con la boca seca?			
9. Se orina de manera ocasional en la cama?			
10. Su hijo se levanta como si no hubiese descansado?			
11. Tiene problemas de excesivo sueño (somnolencia) durante el día?			
12. Le ha comentado algún profesor que su hijo parezca dormido o adormilado durante el día?			
13. Le cuesta despertarle por las mañanas?			
14. Se levanta a la mañana con dolor de cabeza?			
15. Su hijo no ha tenido un crecimiento normal en algún momento desde que nació?			
16. Tiene sobrepeso?			
17. Su hijo a menudo parece que no escucha cuando se le habla directamente?			
18. Tiene dificultades en tareas organizadas?			
19. Se distrae fácilmente con estímulos ajenos?			
20. Mueve continuamente sus manos o pies o no para en la silla?			
21. A menudo actúa como si tuviera un motor?			
22. Interrumpe o se entromete con otros (por ejemplo en conversaciones o juegos?			

Annex Q5. Nutrition diary

ESTUDIO OBESIDAD Y SUEÑO HOSPITAL PESET/UV

NOMBRE:

SIP:

Nº ASIGNADO:

REGISTRO DIETETICO 3 DIAS:

Es muy importante que rellenen la encuesta con la máxima atención. Por ello, les rogamos que lean detalladamente las instrucciones, antes de empezar. Si tienen alguna duda, ya saben que pueden consultarnos en cualquier momento.

Les recordamos no olviden traer la encuesta ya completa a la consulta. En ese momento repasaremos con ustedes sus respuestas, para comprobar que toda la información es correcta.

Normas para la correcta utilización del cuestionario

- Hay que anotar todos los alimentos y bebidas consumidos por el niño/a en 3 días, uno de los cuales debe ser sábado o domingo, sin olvidar los que haya tomado entre horas (refrescos, aperitivos, caramelos...). No olvidar tampoco los vasos de agua o de otras bebidas tomados en las comidas o fuera de ellas.
- Cada día está dividido en 6 apartados: Desayuno, Media mañana, Comida, Merienda, Cena y Otros (en este último se incluyen los alimentos y bebidas que se hayan tomado entre horas, refrescos, chucherías...).
- En la primera columna del cuestionario hay que apuntar la hora del día a la que se hizo la comida y el lugar (casa, guardería, calle,...).
- En la segunda columna se detallará el nombre de cada plato de cada comida y todos los ingredientes que incluye, indicando sus características y si lo sabemos la marca comercial.
- En la tercera columna hay que indicar la cantidad de los alimentos consumidos, es decir lo que de verdad se toma el niño, no lo que se le prepara; calculándolo con la mayor aproximación posible. Para ello:
- Si el niño ha tomado un plato de comida: Anotar si el plato era hondo, de postre, pequeño, etc. También podemos calcular cuantas raciones como la del niño nos salen con la cantidad de comida total que hemos hecho, aunque para eso tenemos que anotar antes las cantidades de todos los ingredientes usados para hacer el guiso, sin olvidar el aceite y el agua.
- Para alimentos sólidos: anotar el tamaño aproximado de la porción y cómo se ha preparado. Por ejemplo: 1 lenguado pequeño enharinado y frito (a la plancha, etc). No olvidar las guarniciones (arroz, patatas, verduras).
- En los alimentos elaborados, indicar la marca, el peso de la porción y la composición (si se puede).
- En observaciones pueden escribir todo aquello que consideren oportuno añadir en relación a lo que el niño ha comido. Por ejemplo: si vomita, si se lo toma a la fuerza, etc.

Ejemplo:

HORAS	ALIMENTOS, INGREDIENTES	CANTIDAD
DESAYUNO		
8,30H en casa	leche semidesnatada Carrefour	1 vaso grande lleno
	Cola-cao marca Lidl	1 cucharada sopera
	Galletas María con mantequilla Hacendado	3 galletas untadas con una capa fina
MEDIA MAÑANA		
11,00H en recreo	manzana y pera en trozos	1 pera pequeña y media manzana normal
	Agua	1 vaso pequeño
COMIDA		
13,30 en el cole	Lentejas con arroz (para 5 personas): 5 puñados de lentejas Hacendado, 3 patatas medianas, 1 cebolla mediana, 1 pimiento verde, 1 tomate pequeño, 1 diente de ajo, especias, 1 chorrito aceite girasol, 1 litro de agua	1 plato hondo pequeño (en puré)
	Pan blanco	1 rebanada pequeña
	Yogur de fresa Hacendado	1 entero menos 2 o 3 cucharadas
	Agua	1 vaso normal lleno
MERIENDA		
17H en casa	Zumo piña Don simón	1 entero (pequeño)
CENA		
20,30H en casa	1 rodaja pan blanco	de 1 dedo de grueso
	Ensalada de tomate aliñada con aceite de oliva	1 tomate mediano y 1 chorrito de aceite
	Tortilla francesa con aceite de oliva	1 huevo y 1 chorrito de aceite de oliva
	1 plátano	tamaño mediano
	Agua	1/2 vaso tamaño normal

HORAS	ALIMENTOS, INGREDIENTES	CANTIDAD
DESAYUNO		
MEDIA MAÑANA		
COMIDA		
MERIENDA		
CENA		
OTROS		
OBSERVACIONES		

Annex Q6. Sociodemographic questionnaire

CUADERNO DE RECOGIDA DE DATOS PARA PARTICIPAR EN EL ESTUDIO

ESTADO DE SALUD BUCODENTAL DE LOS PACIENTES CON OBESIDAD INFANTIL

CUESTIONARIO A CUMPLIMENTAR POR LOS PADRES *Nº identificación:*

DATOS DE FILIACIÓN Y DEL ENTORNO FAMILIAR

Nombre y apellidos del niño/a	**Sexo** ☐ Masculino ☐ Femenino
Fecha de nacimiento	**Edad**
Nivel de estudios del padre ☐ Sin estudios ☐ Educación primaria/EGB ☐ Educación secundaria ☐ Bachiller ☐ Formación Profesional ☐ Estudios universitarios ☐ Máster ☐ Doctorado	**Nivel de estudios de la madre** ☐ Sin estudios ☐ Educación primaria/EGB ☐ Educación secundaria ☐ Bachiller ☐ Formación Profesional ☐ Estudios universitarios ☐ Máster ☐ Doctorado
Situación laboral del padre ☐ Desempleado ☐ Trabajo hogar no remunerado ☐ Contratos temporales ☐ Trabajo estable	**Situación laboral de la madre** ☐ Desempleada ☐ Trabajo hogar no remunerado ☐ Contratos temporales ☐ Trabajo estable
Trabajo del padre (describir)	**Trabajo de la madre** (describir)
Situación familiar de los padres ☐ Viven juntos en pareja o casados ☐ Están separados, pero no tienen otra pareja ☐ Están separados y los dos tienen otra pareja ☐ Están separados y la madre tiene otra pareja ☐ Están separados y el padre tiene otra pareja ☐ Otros	
Número total de personas que conviven en el domicilio familiar	
Menores de 14 años que conviven en el domicilio familiar	

Annex Q7. Horne and Östberg morningness eveningness questionnaire

VERSIÓN CASTELLANA DEL CUESTIONARIO DE MATUTINIDAD-VESPERTINIDAD DE HORNE Y ÖSTBERG (revisado)[1]

Nombre: _____ Fecha: _____

Por favor, para cada pregunta seleccione la respuesta que mejor se ajuste a su caso marcándola con una cruz en el cuadrado correspondiente. Responda en función de cómo se ha sentido en las últimas semanas.

1. Si sólo pensaras en cuando te sentirías mejor y fueras totalmente libre de planificarte el día. ¿A qué hora te levantarías?

   5 ☐ Entre las 05:00 *(5 AM)* y 06: 30 *(6:30 AM)* de la mañana
   4 ☐ Ente las 06:30 *(6:30 AM)* y las 07:45 *(7:45 AM)* de la mañana
   3 ☐ Entre las 07:45 *(7:45 AM*) y las 09:45 *(9:45 AM)* de la mañana
   2 ☐ Entre las 09:45 *(9:45 AM)* y las 11:00 *(11 AM)* de la mañana
   1 ☐ Entre las 11 *(11 AM)* de la mañana y las 12 de la tarde *(12 noon)*

2. Si sólo pensaras en cuando te sentirías mejor y fueras totalmente libre de planificarte el día. ¿A qué hora te acostarías?

   5 ☐ A las 20:00 *(8 PM)* – 21:00 *(9 PM)*
   4 ☐ A las 21:00 *(9 PM)* – 22:15 *(10:15 PM)*
   3 ☐ A las 22:15 *(10:15 PM)* – 00:30 *(12:30 AM)*
   2 ☐ A las 00:30 *(12:30 AM)* – 01:45 *(1:45 AM)*
   1 ☐ A las 01:45 *(1:45 AM)* – 03:00 *(3 AM)*

3. Para levantarte por la mañana a una hora específica. ¿Hasta qué punto necesitas que te avise el despertador?

   4 ☐ No lo necesito
   3 ☐ Lo necesito poco
   2 ☐ Lo necesito bastante
   1 ☐ Lo necesito mucho

4. ¿Te resulta fácil levantarte por las mañanas? (cuando no te despiertan de forma inesperada)

   1 ☐ Nada fácil
   2 ☐ No muy fácil
   3 ☐ Bastante fácil
   4 ☐ Muy fácil

[1] Algunas preguntas y algunas de las opciones de las posibles respuesta se han reescrito a partir del test original (Horne y Östberg, 1976) para adaptarlo al español. Las opciones que suponían categorías discretas se han substituido por escalas gráficas continuas. Preparado por Terman M, Rifkin JB, Jacobs J, and White TM. New York State Psychiatric Institute, New York, NY USA. Ver también la versión automatizada (AutoMEQ) en www.cet.org. La traducción del inglés fue realizada por el Dr. Mª Angeles Rol de Lama, Dr. Beatriz Baño Otálora, Dr. Maria Teresa, Mondéjar Abenza, y Dr. Juan Antonio Sarabia Carazo. Para las preguntas en España, entre en contacto con por favor a Juan Antonio Madrid, Dr. en Fisiología, Especialista en Cronobiología, Universidad de Murcia, Campus de Espinardo, Murcia, España, jamadrid@um.es.

Horne JA and Östberg O. *A self-assessment questionnaire to determine morningness-eveningness in human circadian rhythms.* International Journal of Chronobiology, 1976: 4, 97-100.

5. Una vez levantado por las mañanas. ¿Qué tal te encuentras durante la primera media hora?

   1 ☐ Nada alerta
   2 ☐ Poco alerta
   3 ☐ Bastante alerta
   4 ☐ Muy alerta

6. Una vez levantado por las mañanas. ¿Cómo es tu apetito durante la primera media hora?

   1 ☐ Muy escaso
   2 ☐ Bastante escaso
   3 ☐ Bastante bueno
   4 ☐ Muy bueno

7. Una vez levantado por las mañanas. ¿Qué tal te sientes durante la primera media hora?

   1 ☐ Muy cansado
   2 ☐ Bastante cansado
   3 ☐ Bastante descansado
   4 ☐ Muy descansado

8. Cuando no tienes compromisos al día siguiente. ¿A qué hora te acuestas en relación con tu hora habitual?

   4 ☐ Nunca o raramente o más tarde
   3 ☐ Menos de 1 hora más tarde
   2 ☐ De 1 a 2 horas más tarde
   1 ☐ Más de 2 horas más tarde

9. Has decidido hacer un poco de ejercicio físico. Un amigo te propone hacerlo una hora dos veces por semana y según él, la mejor hora sería de 7 a 8 de la mañana. No teniendo nada más encuenta salvo tu propio reloj "interno", ¿cómo crees que te encontrarías?

   4 ☐ Estaría en buena forma
   3 ☐ Estaría en una forma aceptable
   2 ☐ Me resultaría difícil
   1 ☐ Me resultaría muy difícil

10. ¿A qué hora aproximada de la noche te sientes cansado y como consecuencia necesitas dormir?

   5 ☐ A las 20:00 *(8 PM)* – 21:00 *(9 PM)*
   4 ☐ A las 21:00 *(9 PM)* – 22:15 *(10:15 PM)*
   3 ☐ A las 22:15 *(10:15 PM)* – 00:45 *(12:45 AM)*
   2 ☐ A las 00:45 *(12:45 AM)* - 02:00 *(2 AM)*
   1 ☐ A las 02:00 *(2 AM)* – 03:00 *(3 AM)*

11. Quieres estar en tu punto máximo de rendimiento para una prueba de dos horas que va a ser mentalmente agotadora. Siendo totalmente libre de planificar el día y pensando sólo en cuando te sentirías mejor. ¿Qué horario elegirías?

6 ☐ De 08:00 *(8 AM)* a 10:00 *(10 AM)*
4 ☐ De 11:00 *(11 AM)* a 13:00 *(1 PM)*
2 ☐ De 13:00 *(1 PM)* a 17:00 *(5 PM)*
0 ☐ De 19:00 *(7 PM)* a 21:00 *(9 PM)*

12. Si te acostaras a las 11 de la noche. ¿Qué nivel de cansancio notarías?

0 ☐ Ningún cansancio
2 ☐ Algún cansancio
3 ☐ Bastante cansancio
5 ☐ Mucho cansancio

13. Por algún motivo te has acostado varias horas más tarde de lo habitual, aunque al día siguiente no has de levantarte a ninguna hora en particular. ¿Cuando crees que te despertarías?

4 ☐ A la hora habitual y ya no dormiría más
3 ☐ A la hora habitual y luego dormitaría
2 ☐ A la hora habitual y volvería a dormirme
1 ☐ Más tarde de lo habitual

14. Una noche tienes que permanecer despierto de 4 a 6 de la madrugada debido a una guardia nocturna. Sin tener ningún compromiso al día siguiente, ¿qué preferirías?

1 ☐ No acostarme hasta pasada la guardia
2 ☐ Echar una siesta antes y dormir después
3 ☐ Echar un buen sueño antes y una siesta después
4 ☐ Sólo dormirías antes de la guardia

15. Tienes que hacer dos horas de trabajo físico pesado. Eres totalmente libre para planificarte el día. Pensando sólo en cuando te sentirías mejor, ¿qué horario escogerías?

4 ☐ De 08:00 *(8 AM)* a 10:00 *(10 AM)*
3 ☐ De 11:00 *(11 AM)* a 13:00 *(1 PM)*
2 ☐ De 13:00 *(1 PM) a* 17:00 *(5 PM)*
1 ☐ De 19:00 *(7 PM)* a 21:00 *(9 PM)*

16. Has decidido hacer ejercicio físico intenso. Un amigo te sugiere practicar una hora dos veces por semana de 10 a 11 de la noche. Pensando sólo en cuando te sentirías mejor, ¿Cómo crees que te sentaría?

    1 ☐ Estaría en buena forma
    2 ☐ Estaría en una forma aceptable
    3 ☐ Me resultaría difícil
    4 ☐ Me resultaría muy difícil

17. Imagínate que puedes escoger tu horario de trabajo. Supón que tu jornada es de CINCO horas al día (incluyendo los descansos) y que tu actividad es interesante y remunerada según tu rendimiento. ¿Qué CINCO HORAS CONSECUTIVAS seleccionarías? ¿Empezando en qué hora? Considera la casilla marcada más a la derecha para escoger entre los siguientes rangos:

    5 ☐ Entre las 04:00 *(4 AM)* y las 08:00 *(8 AM)*
    4 ☐ Entre las 08:00 *(8 AM)* y las 09:00 *(9 AM)*
    3 ☐ Entre las 09:00 *(9 AM)* y las 14:00 *(2 PM)*
    2 ☐ Entre las 14:00 *(2 PM)* y las 17:00 *(5 PM)*
    1 ☐ Entre las 17:00 *(5 PM)* y las 04:00 *(4 AM)*

18. ¿A qué hora del día crees que alcanzas tu máximo bienestar?

    5 ☐ Entre las 05:00 *(5 AM)* y las 08:00 *(8 AM)*
    4 ☐ Entre las 08:00 *(8 AM)* y las 10:00 *(10 AM)*
    3 ☐ Entre las 10:00 *(10 AM)* y las 17:00 *(5 PM)*
    2 ☐ Entre las 17:00 *(5 PM)* y las 22:00 *(10 PM)*
    1 ☐ Entre las 22:00 *(10 PM)* y las 05:00 *(5 AM)*

19. Se habla de personas de tipo matutino y vespertino. ¿Cuál de estos tipos te consideras ser?

    6 ☐ Un tipo claramente matutino.
    4 ☐ Un tipo más matutino que vespertino.
    2 ☐ Un tipo más vespertino que matutino.
    0 ☐ Un tipo claramente vespertino.

Suma los puntos que figuran al lado de la casilla.

La puntuación obtenida ha sido: _____ puntos.

INTERPRETA Y UTILIZA TU PUNTUACIÓN DE MATUTINIDAD-VESPERTINIDAD

Este cuestionario tiene 19 preguntas, cada una con un número de puntos. Primero, suma los puntos e introduce tu puntuación de matutinidad- vespertinidad aquí:

Las puntuaciones pueden ir de 16 86 puntos. Puntuaciones de 41 o menos indican "tipo vespertino". Puntuaciones de 59 o más indican "tipo matutino". Puntuaciones entre 42-58 indican "tipo intermedio."

16-30	31-41	42-58	59-69	70-86
Vespertino extremo	Vespertino moderado	Intermedio	Matutino moderado	Matutino extremo

Ocasionalmente algunas personas pueden tener problemas con el cuestionario. Por ejemplo, algunas de las preguntas son difíciles de contestar si has sido un trabajador a turnos, si no trabajas, o si te vas a la cama inusualmente tarde. Tus respuestas pueden estar influidas por estar pasando una enfermedad o estar tomando medicamentos. Si no estás seguro de tus respuestas, tampoco deberías seguir ciegamente los siguientes consejos.

Una forma de estar más seguros es comprobar si tu puntuación de matutinidad- vespertinidad coincide aproximadamente con las horas de inicio y final del sueño que aparecen a continuación:

Puntuación	16-30	31-41	42-58	59-69	70-86
Inicio del sueño	02:00-03:00 *2:00-3:00 AM*	00:45-02:00 *12:45 PM-2:00 AM*	22:00-00:45 *10:45 PM-12:45 AM*	21:30-22:45 *9:30-10:45 PM*	21:00-21:30 *9:00-9:30 PM*
Final del sueño	10:00-11:30 *10:00-11:30 AM*	08:30-10:00 *8:30-10:00 AM*	06:30-08:30 *6:30-8:30 AM*	05:00-06:30 *5:00-6:30 AM*	04:00-06:30 *4:00-5:00 AM*

Si sueles irte a dormir antes de las 21:00 (9:00 PM) o más tarde de las 03:00 (3:00 AM), o si tu hora de despertar es anterior a las 04:00 (4:00 AM) o más tarde de las 11:30 (11:30 AM), deberías consultar a un experto clínico en luminoterapia para someterte a un tratamiento efectivo.

Nosotros usamos la puntuación de matutinidad-vespertinidad para mejorar el efecto antidepresivo de la luminoterapia. Aunque la mayoría de las personas experimentan una buena respuesta antidepresiva a la luminoterapia cuando reciben una sesión matinal de 10,000 lux durante 30 minutos con un dispositivo de luz adecuado (ver recomendaciones en www.cet.org), podríamos no obtener la mejor respuesta posible. Si tu reloj interno está desplazado con respecto a la hora ambiental (cuya medida indirecta es la puntuación de matutinidad-vespertinidad), necesitaremos ajustar la hora de aplicación de la luminoterapia.

La tabla que se incluye más abajo muestra la hora recomendada para comenzar la luminoterapia para un amplio rango de matutinidad –vespertinidad. Si tu puntuación se sale de este rango (ya sea por muy alto o muy bajo), deberías buscar consejo en una clínica de luminoterapia para buscar un tratamiento efectivo.

Puntuación de Matutinidad- Vespertinidad	Hora de inicio de los 30 minutos de luminoterapia
23-26	8:15 AM
27-30	8:00 AM
31-34	7:45 AM
35-38	7:30 AM
39-41	7:15 AM
42-45	7:00 AM
46-49	6:45 AM
50-53	6:30 AM
54-57	6:15 AM
58-61	6:00 AM
62-65	5:45 AM
66-68	5:30 AM
69-72	5:15 AM
73-76	5:00 AM

Si normalmente duermes más de 7 horas por noche, necesitarás despertarte algo antes de lo normal para conseguir el efecto – pero deberías sentirte por ello mejor. Algunas personas compensan acostándose antes, mientras que otras se sienten bien durmiendo menos. Si, normalmente, duermes menos de 7 horas por noche deberías poder mantener tu hora actual de despertar. Si automáticamente, puedes despertarte por ti mismo con más de 30 minutos antes del inicio de tu sesión, deberías retrasar tu sesión. Evita tomar sesiones antes de la hora recomendada, pero si te quedas dormido a pesar de que suene la alarma del reloj, es mejor tomar la sesión tarde que saltártela.

Nuestra recomendación para las personas vespertinas – es decir, las 08:00 (8:00 AM) para una puntuación de matutinidad vespertinidad de 30 – puede dificultar que lleguemos a tiempo al trabajo, pero recibir la sesión de la luz antes no sería eficaz. Una vez que notes mejoría con la hora recomendada, puedes ir adelantando poco a poco la sesión de luminoterapia, en unos 15 minutos al día, permitiendo que tu reloj interno se sincronice con el ciclo sueño vigilia y horario de trabajo que deseas.

El consejo personalizado que te ofrecemos aquí, está basado en un amplio ensayo clínico con pacientes que sufren desorden afectivo estacional (SAD, *seasonal affective disorder*) realizado en el Centro Médico de la Universidad de Columbia de New York. Los pacientes que recibieron la sesión de luz demasiado tarde por la mañana sólo mejoraron la mitad que aquellos que la recibieron aproximadamente a la hora indicada. Estas normas no se aplican sólo al SAD, sino que también pueden ser de ayuda en el tratamiento de la depresión no estacional, reduciendo el insomnio y las ganas de quedarse durmiendo por las mañanas.

Nuestros consejos sólo constituyen una guía general para los nuevos usuarios de la luminoterapia. Hay muchos factores individuales que pueden requerir diferentes programas o dosis (intensidad, duración) de luz. Ninguna persona con depresión clínica debería someterse a luminoterapia a menos que sea de forma controlada y bajo supervisión médica.

Bibliografía: Terman M, Terman JS. *Light therapy for seasonal and nonseasonal depression: efficacy, protocol, safety, and side effects.* CNS Spectrums, 2005;10:647-663 (descargable en www.cet.org).

X. Resumen en castellano

X. 1. INTRODUCCIÓN

La obesidad infantil constituye uno de los principales retos de salud pública de nuestro siglo. Para construir estrategias de prevención eficaces contra esta acumulación patológica de grasa corporal, es necesario comprender los elementos que favorecen su desarrollo, así como los mecanismos metabólicos subyacentes.

Esta acumulación de grasa corporal está relacionada con una ingesta energética excesiva en comparación con el gasto energético, lo que da lugar a un mayor almacenamiento de energía. La ingesta de energía corresponde a la porción de nutrientes que no sólo ha sido ingerida sino también absorbida por el organismo. Estos procesos están influenciados por factores biológicos, psicológicos y ambientales. Por otro lado, el gasto energético suma los gastos de energía vinculados al metabolismo basal, la homeostasis de la temperatura, el proceso de digestión, la actividad física y mental, y el crecimiento. La ingesta de energía es puntual, por lo que la energía se almacena convenientemente para cubrir los gastos energéticos incesantes. La energía se almacena principalmente en forma de triglicéridos, moléculas muy energéticas que se acumulan en los adipocitos del tejido adiposo blanco. Como la nutrición, la actividad física, el sueño, la actividad mental y la exposición a la temperatura y a la luz desempeñan papeles reguladores de la ingesta y el gasto de energía, están estrechamente vinculados a la salud metabólica. Estos hábitos de vida son rítmicos y, en consecuencia, también lo son los mecanismos metabólicos que se activan posteriormente. Sin embargo, esta activación rítmica del metabolismo no es sólo una consecuencia de los comportamientos y exposiciones rítmicas, sino que es intrínseca al organismo, asegurada por mecanismos reunidos bajo el nombre de ritmos circadianos.

Los ritmos circadianos se estructuran y sincronizan gracias a dos mecanismos principales: los genes reloj y la actividad de la melatonina. Los genes reloj son un grupo de genes que forman un doble bucle de regulación cuyo periodo dura aproximadamente 24 horas. Se expresan de forma ubicua en las células nucleadas humanas. Bajo la regulación de estos genes también figuran genes del metabolismo y de la inflamación como factores de transcripción, transportadores, portadores, hormonas y citoquinas. Estos llamados relojes periféricos inducen un ritmo en la fisiología del órgano. Paralelamente, el tiempo externo es percibido por el organismo principalmente a través de la percepción de la luz azul. Esta señal fótica es traducida en una señal nerviosa por la retina del ojo, y posteriormente en una señal endocrina por la glándula pineal, que sintetiza melatonina en ausencia de señal luminosa. Una vez producida, la melatonina fluye por todo el organismo a través de los fluidos corporales, y luego se une a sus receptores en la superficie celular y en la célula o actúa directamente como antioxidante al eliminar los radicales libres. Esta hormona circadiana actúa globalmente para preparar los órganos para entrar en la fase de sueño. Se considera que la señal rítmica de la melatonina sincroniza los relojes periféricos entre sí y con el tiempo externo. Juntos, los genes del reloj y la expresión rítmica de la melatonina participan para conferir un ritmo a la fisiología y la actividad de los órganos implicados en la ingesta, el gasto y el almacenamiento de energía, incluido el tejido adiposo. Sin

embargo, el impacto preciso de la melatonina en los ritmos circadianos de la expresión genética en el tejido adiposo aún no se conoce del todo.

Además de la luz externa, el horario de los hábitos de vida, como la ingesta nutricional, la actividad física y el sueño, también parece participar activamente en la regulación de los ritmos circadianos. La tendencia de un individuo a tener comportamientos tempranos o tardíos se denomina cronotipo. Está influenciado por la genética y el entorno. De hecho, aunque uno presente predisposición a un cronotipo temprano o tardío, la participación repetida en comportamientos tempranos o tardíos puede inducir lentamente el cambio de cronotipo.

Los ritmos circadianos internos del organismo confieren a los órganos una capacidad de anticipación que les permite activarse de forma óptima en determinados momentos del día. Por el contrario, cuando los órganos son solicitados en horarios absurdos como ocurre en el trabajo nocturno, la masa grasa corporal aumenta y los marcadores de riesgo metabólico se elevan. En estos individuos, el ritmo de la melatonina está alterado. Incluso sin llegar a la cronodisrupción extrema que se da en los trabajadores del turno de noche, los estudios tienden a mostrar que los hábitos de vida tardíos se asocian con resultados metabólicos negativos y con un aumento del peso corporal. En los últimos años, múltiples estudios han mostrado asociaciones entre la insuficiencia de sueño y el aumento de peso. Paralelamente, la alteración de la señalización de la melatonina se asocia a la obesidad, incluso en la infancia.

Los cronotipos tardíos se asocian a un mayor jetlag social, definido por la diferencia en la sincronización de las fases del sueño entre los días de trabajo y los días libres, lo que también se asocia a mayores riesgos metabólicos. Aunque todos estos elementos ponen de manifiesto la relación entre los ritmos circadianos y la salud metabólica, la importancia de esta relación en la infancia, y la influencia de la cronología de los hábitos de vida, aún no está caracterizada con precisión. Por ello, hemos diseñado un estudio con los siguientes objetivos:

X. 2. Objetivos

El objetivo principal del presente proyecto es evaluar las relaciones entre los hábitos de vida, los ritmos circadianos y la salud metabólica durante la infancia.

Con el fin de alcanzar este objetivo principal, se establecen los siguientes objetivos secundarios:

- Cuantificar la diferencia en la expresión de melatonina vespertina entre participantes con obesidad y con peso normal, probando un nuevo enfoque de medición de los ritmos circadianos mediante un método de recogida de saliva en casa para la detección de melatonina en tres momentos de la noche.

- Medir el efecto de la melatonina en la expresión circadiana de los genes del reloj y del metabolismo en los adipocitos humanos.
- Evaluar la relación entre la expresión de la melatonina y los factores clínicos, metabólicos, inflamatorios, circadianos y ambientales relacionados con la obesidad en la infancia.
- Evaluar la relación entre el cronotipo y los factores clínicos, metabólicos, inflamatorios, circadianos y ambientales relacionados con la obesidad y el sobrepeso en la infancia.
- Medir la relación entre la hora de la cena durante la semana escolar y los factores clínicos, metabólicos, inflamatorios, circadianos y ambientales relacionados con la obesidad y el sobrepeso en la infancia.
- Medir la relación entre la duración del sueño y los factores clínicos, metabólicos, inflamatorios, circadianos y ambientales relacionados con la obesidad y el sobrepeso en la infancia.
- Medir la relación entre la exposición nocturna a las pantallas y los factores clínicos, metabólicos, inflamatorios, circadianos y ambientales relacionados con la obesidad y el sobrepeso en la infancia.
- Identificar subgrupos de participantes que comparten características metabólicas, circadianas, hábitos de vida y ambientales similares.
- Construir un modelo de predicción de la obesidad basado en los hábitos de vida y el entorno.
- Construir un modelo de biomarcadores de obesidad que incluya una variable de ritmos circadianos.

X. 3. Material y métodos

X. 3.1. Diseño del estudio

La presente investigación es un estudio transversal y analítico en participantes que acudieron a las consultas externas de Pediatría del Hospital Universitario del Doctor Peset de Valencia, España, aprobado por el comité de ética de dicho hospital.

X. 3.1.1. Población de estudio

Los y las participantes fueron divididos en dos grupos en función de su índice de masa corporal (IMC), calculado como el peso en kilogramos dividido por la altura en metros al cuadrado (kg/m2), y utilizando los criterios de Cole et al, 2000. Se considera que los individuos que presentan un IMC superior o igual a 1 desviación estándar (DE) por encima de la media para la edad y el sexo presentan sobrepeso, y superior a 2 DE presentan obesidad, según la Organización Mundial de la Salud (OMS)2. La tabla de Carrascosa de la población pediátrica española elaborada desde 1995 hasta 2017 proporciona una referencia para la distribución del IMC desde el nacimiento hasta la mayoría de edad. En la tabla de Carrascosa, 1 DE por encima de la media para la edad y el sexo corresponde al percentil 84,1 del IMC, y 2 DE al percentil

97,7. Los individuos que presentaron un IMC inferior a una desviación estándar fueron asignados al grupo control, y por encima de este corte al grupo de sobrepeso y obesidad. Se utilizó la aplicación nutricional de la Sociedad Española de Gastroenterología, Hepatología y Nutrición Pediátrica para calcular el percentil y la DE equivalente de cada participante. Todos fueron informados detalladamente sobre el estudio y las medidas éticas y firmaron el consentimiento informado por ellos mismos si tienen al menos 12 años, o, en el caso contrario, su tutor legal.

X. 3.1.2. Estimación del tamaño de la muestra

El tamaño de la muestra se calculó según la siguiente fórmula $(2\ (Z\alpha + Z\beta)^2 \times S^2) / d^2$. El nivel de asociación $Z\alpha$ y $Z\beta$ es 99% α, β =0.01. Como nuestro objetivo es caracterizar los ritmos circadianos, utilizamos la melatonina como marcador. Por lo tanto, como S utilizamos 11,4, correspondiente a la desviación estándar de los niveles de melatonina. La d deseada es 7, correspondiente a la diferencia mínima de expresión de los niveles de melatonina tras una hora de sueño entre los dos grupos previamente definidos: peso normal y obesidad. Con estos datos se obtiene un total de 64 pacientes necesarios por grupo. Estimando una pérdida del 15%, necesitamos 74 pacientes por grupo, por lo que se necesita un mínimo de 148 participantes en total.

X. 3.1.3. Criterios de inclusión

Se incluyeron en el estudio los pacientes de entre 7 y 16 años que acudieron a la consulta de pediatría y que completaron el consentimiento informado, a excepción de los y las participantes que presentaban alguna particularidad que pudiera afectar a su perfil metabólico e inflamatorio como tratamiento médico, patología inflamatoria o metabólica (distinta de la obesidad y comorbilidades), o deportistas de élite.

X. 3.1.4. Criterios de exclusión

Un elemento crucial del presente trabajo son las muestras de saliva recogidas en casa por los y las participantes y utilizadas para la determinación de melatonina. Los y las participantes que no proporcionaron las muestras de saliva fueron excluidos del presente análisis.

X. 3.1.5. Tamaño final de la muestra

Inicialmente se reclutaron 263 participantes, de los cuales se excluyeron 51 porque no proporcionaron las muestras de saliva en absoluto o las muestras no cumplían los criterios estándar (volumen mínimo, conservación en la estanqueidad completa del tubo y en la oscuridad). Los 212 participantes se subdividieron entre un grupo de obesidad compuesto por 123 de ellos, y un grupo de control que contaba con

89 participantes. En el grupo de obesidad, que presentaba una media de edad más elevada, se excluyeron del grupo de obesidad 6 participantes de 14 años y 3 de 15 años para restablecer un equilibrio de edad. El estudio contó finalmente con 127 participantes en el grupo de obesidad (61 de sexo femenino y 66 de sexo masculino) de una edad media ± desviación estándar (DE) de 11,47 ± 2,35 años, 76 en el grupo de control (39 de sexo femenino y 37 de sexo masculino) de 10,8 ± 2,38 años. De los 127 participantes del grupo de sobrepeso y obesidad, 114 presentan un IMC superior a dos desviaciones estándar para la edad y el sexo y pueden, los criterios de obesidad, y 13 presentan un IMC entre 1 y dos desviaciones estándar, criterios de sobrepeso. El reclutamiento comenzó antes de la pandemia de covid-19, el 86,8% del grupo de sobrepeso y obesidad y el 80,3% del grupo control fueron reclutados antes, lo que no fue significativamente diferente (p = 0,318).

X. 3.2. Variables del estudio

Las variables del estudio se agrupan en las siguientes categorías:

Datos antropométricos y clínicos: altura (cm), peso (kg), IMC (kg/m2), IMC z-score, circunferencia de cadera, cintura y brazo derecho (cm), porcentaje de masa grasa (%), masa magra (kg), presión arterial sistólica y diastólica (mmHg), frecuencia cardíaca (BPM). Además, se obtuvo el percentil de edad y sexo para todos estos parámetros.

Hábitos, entorno y cronotipo Horas de sueño, test de alteración del sueño pediátrico de Bruni, cuestionario de sueño pediátrico, frecuencia de actividad física, presencia de pantallas en el dormitorio, contexto familiar sociodemográfico, cronotipo, ingesta nutricional de 3 días analizada en el programa informático DIAL, hora de las comidas.

Marcadores metabólicos e inmunológicos: Glucosa, urea, creatina, ácido úrico, colesterol total, colesterol de lipoproteínas de alta densidad, colesterol de lipoproteínas de baja densidad, colesterol de lipoproteínas de muy baja densidad, triglicéridos, aspartato aminotransferasa, alanina aminotransferasa, γ-glutamil transpeptidasa, proteínas séricas totales, albúmina, calcio, fósforo, hierro, transferrina, saturación de transferrina, Calcio corregido por albúmina, Creatinina, Microalbuminuria (orina), Relación microalbúmina creatinina (orina), Proteína de unión al retinol (RBP), Apolipoproteína A1, Apolipoproteína B, Proteína C-Reactiva de alta sensibilidad, Cistatina C, C3 ,Hormona estimulante de la tiroides, insulina, Leptina, Grelina, MCP1, TNF-alfa, IL-6, PAI-1, Adiponectina, Resistina, Omentina

Concentración de melatonina y capacidad antioxidante total en la saliva

Ritmos circadianos de los genes del reloj y de los genes controlados por el reloj BMAL1, CRY, PER2, MT2, PPARγ, C/EBPα, LPL, aP2, LEP, GBP-28 y expresión transcripcional PAI-1 en relación con la β-actina, con y sin estimulación de melatonina.

X. 3.2.1. Desarrollo global del estudio

Los pacientes y los tutores recibieron la información sobre el estudio por parte del investigador o del dietista del equipo, junto con el pediatra. Se recogieron datos como la edad, el sexo y las medidas antropométricas, y el paciente y su tutor respondieron a los cuestionarios. En casa, todo participante debía rellenar, junto con su adulto de referencia, un registro de ingesta nutricional de 3 días, en el que se enumeraba cuándo, qué y cuántos alimentos y bebidas habían consumido durante 2 días escolares y 1 día de fin de semana. Además, la noche anterior a la extracción de sangre, el participante, ayudado por el adulto, recogió tres muestras de saliva en diferentes momentos: cuatro horas antes de acostarse, dos horas antes de acostarse y después de una hora de sueño. Los y las participantes con sus familias recogen la saliva en estos momentos en los tubos previstos para ello, los cierran adecuadamente, los guardan en la nevera protegidos de la luz y los llevan al hospital al día siguiente, día de la extracción de sangre. Este día se programó durante la semana escolar, los martes o los miércoles, para evitar los cambios del ritmo circadiano asociados a las vacaciones y los fines de semana. Los pacientes acudían al hospital en ayunas para la extracción de sangre, llevando la ficha nutricional y las muestras de saliva. La enfermera encargada de la extracción de sangre recogió los tubos necesarios para el análisis bioquímico basal, más dos tubos adicionales, uno con EDTA para el plasma (5ml EDTA BD vacutainer Mississauga ON Canada) y otro con gelosa para el suero (5ml serum BD vacutainer Mississauga ON Canada). El análisis bioquímico basal se realizó en el laboratorio central del hospital bajo las condiciones estandarizadas, mientras que los dos tubos extra de sangre y las tres muestras de saliva fueron llevados al laboratorio de Pediatría, Obstetricia y Ginecología de la Universidad de Valencia por el investigador para su posterior análisis hormonal, inmunológico y antioxidante. Los datos de los cuestionarios, las medidas antropométricas y los análisis bioquímicos de sangre y saliva se anonimizaron y se identificaron con un código general de laboratorio. Los datos bajo ese código se utilizaron para el posterior análisis estadístico.

X. 3.2.2. Antropometría y evaluación clínica

Se emplearon las mismas condiciones y el mismo material con todos los y las participantes. Para registrar la altura se utilizó el estadiómetro Holtain (Holtain Ltd., Dyfed, Reino Unido) con una precisión de 0,1cm. La medición se realizó sin zapatos, con los pies unidos, los glúteos, la espalda y la cabeza en contacto con la pared, y la cabeza recta con la oreja y el ojo formando una línea horizontal.

El participante, que sólo llevaba ropa interior, se colocó en el analizador de impedancia bioeléctrica y balanza electrónica BC-418 MA Tanita Segmental Body Composition (Tanita Europe BV, Hoofddorp, Países Bajos). Funciona como una balanza electrónica y mide el peso corporal. Con la introducción de la edad y la altura, este aparato muestra el IMC y, mediante su función de impedanciómetro, registra el porcentaje de grasa y masa magra del cuerpo. Los percentiles se calcularon mediante la aplicación de la

Sociedad Española de Gastroenterología, Hepatología y Nutrición Pediátrica (SEPHNP), basándose en las tablas de Carrascosa como.

El perímetro de cintura, cadera y brazo derecho también se midió manualmente utilizando una cinta de acero no extensible, flexible y calibrada en centímetros con graduación milimétrica y siguiendo los protocolos estandarizados de la International Society for the Advancement of Kinanthropometry (ISAK). Se calculó el índice cintura/cadera y el índice cintura/altura.

La presión arterial sistólica, la presión arterial diastólica y la frecuencia cardíaca se evaluaron en posición sentada con un esfigmomanómetro automatizado (Dinamap DPC101X - SP; GE medical Systems Information Technologies, Inc., Milwaukee, WI, USA). La evaluación cualitativa de la presión arterial se obtuvo utilizando la aplicación en línea del laboratorio de composición corporal del Baylor College of Medicine.

X. 3.2.3. Hábitos, entorno y cronotipo

Las categorías de parámetros que se evaluaron son las siguientes: cronotipo, ingesta nutricional, actividad física, sueño, exposición a dispositivos tecnológicos en el dormitorio y contexto sociodemográfico familiar.

X. 3.2.4. Cronotipo

El investigador formula al participante las preguntas diseñadas por Horne y Östberg para evaluar el cronotipo.134 El cuestionario está diseñado de forma que cada respuesta da una puntuación. Cuanto más alto sea el total, más temprano será el cronotipo e inversamente. De este modo, se asigna un cronotipo a cada participante: cronotipo vespertino extremo para las puntuaciones inferiores a 30, cronotipo vespertino moderado entre 31 y 41, intermedio entre 42 y 58, cronotipo matutino moderado entre 59 y 69, y cronotipo matutino extremo por encima de 70.

X. 3.2.5. Ingesta nutricional

Finalmente, el investigador proporcionó instrucciones y una plantilla para que los y las participantes registraran en casa su ingesta nutricional durante tres días, con el apoyo del adulto. Cada vez que bebían o comían, debían informarlo con la mayor precisión posible en cuanto al tipo de alimento, la cocción, la cantidad y el tiempo. De los tres días de registro, dos eran días laborables y uno de fin de semana. El documento se llevaba al investigador el día de la extracción de sangre, quien introducía todos los elementos nutricionales para su análisis en el programa informático DIAL (Alce Ingeniería SA, Madrid, España). De este análisis se obtienen los siguientes datos: energía consumida (kcal), distribución de la energía en los

principales macronutrientes (%): hidratos de carbono, proteínas y lípidos para conocer las características más relevantes de la dieta de los pacientes. También se recogen los valores de estos parámetros para cada comida y se calculan los ratios para valorar para cada individuo qué comida supone un mayor aporte nutricional respecto a las demás. La hora de cada comida se midió durante la semana y el fin de semana, y la amplitud temporal de la ingesta nutricional se calculó como la diferencia de tiempo entre la primera comida (generalmente el desayuno), y la última (generalmente la cena). Del diario nutricional de 3 días también se registraron las menciones de alimentos "caseros" y "procesados", los hábitos de picoteo, el número de menciones de refrescos y dulces en el diario. La calidad del informe se cuantificó en una escala de 1 a 5 (siendo 5 la mejor calidad) de la siguiente manera 5/5 cuando se informaba detalladamente de cada comida con los horarios, tipos de alimentos, tipo de cocción, marcas y cantidades. 4/5 cuando se informa de todas las comidas pero con un nivel de detalle menor, por ejemplo, faltan las marcas o el tipo de cocción. 3/5 cuando se informa de todas las comidas pero con poco detalle y elementos como las cantidades están incompletos. 2/5 cuando se comunica poca información sobre el contenido de cada comida o faltan las cantidades. 1/5. Informe muy incompleto, apenas hay información y faltan comidas.

X. 3.2.6. Actividad física

El investigador recoge en el cuestionario adaptado la información sobre la actividad física transmitida por el paciente. Se les preguntó si practicaban alguna actividad física fuera de la escuela y, en caso afirmativo, cuál, a qué hora y con qué frecuencia. Las actividades físicas se clasificaron en tres categorías según el nivel de exigencia cardiovascular. La acción mecánica de un músculo puede medirse en dos escalas: dinámica (isotónica) y estática (isométrica). El componente estático se mide en relación con el porcentaje de contracción voluntaria máxima (MVC), y el componente dinámico en porcentaje de la captación máxima de oxígeno (Max O2). Cada uno de estos componentes estimula la adaptación cardiovascular a un nivel que se escala de "bajo" a "alto".

X. 3.2.7. Sueño

El investigador preguntó al paciente a qué hora suele irse a dormir y despertarse, durante la semana escolar y durante el fin de semana. A partir de estos datos, se calcula la duración del sueño y la diferencia en la duración del sueño durante la semana y el fin de semana. Mientras tanto, se pidió a los padres presentes que rellenaran varios cuestionarios sobre la calidad del sueño del niño. Para detectar las alteraciones del sueño se utilizó el cuestionario de Bruni validado en español. En esta prueba se asocia una cifra a cada respuesta y se calcula la suma de estos números, y un total superior a 39 sugiere una alteración del sueño. El cuestionario pediátrico del sueño validado en español es un cuestionario pediátrico empleado para evaluar la calidad del sueño. Se ha utilizado la versión corta orientada al diagnóstico de la apnea del sueño. A cada ítem, los padres respondían "sí", "no" o "desconocido" y se cuantificaba después el número de respuestas positivas. Por encima del 33% de resultados positivos se considera que el sueño es de mala calidad.

La duración del sueño se consideró adecuada al cumplir las recomendaciones de la sociedad española del sueño: para los y las participantes de 6 a 13 años, se recomiendan de 9 a 11 horas de sueño por noche, y de 8 a 10 horas a partir de los adolescentes de 14 años.

X. 3.2.8. Exposición a dispositivos tecnológicos en el dormitorio

El investigador enumera la siguiente lista de dispositivos con pantalla: teléfono móvil, televisión, consola (Wii, Xbox, PS4...), consola portátil (DS, PSP, switch...), ordenador, tablet; y se pregunta a los y las participantes cuáles están presentes en su dormitorio por la noche. El cuestionario se utiliza para evaluar la presencia y la suma de dispositivos electrónicos en el dormitorio.

X. 3.2.9. Contexto sociodemográfico familiar

El cuestionario lo rellena el padre del participante, se recoge la siguiente información

Nivel educativo de la madre (1 = sin estudios; 2 = educación primaria; 3 = educación secundaria; 4 = bachillerato; 5 = formación profesional; 6 = estudios universitarios; 7 = máster; 8 = doctorado)

Nivel de estudios del padre (1 = sin estudios; 2 = educación primaria; 3 = educación secundaria; 4 = bachillerato; 5 = formación profesional; 6 = estudios universitarios; 7 = máster; 8 = doctorado)

Situación laboral de la madre (1 = desempleada; 2 = contratos temporales; 3 = contrato estable)

Situación laboral del padre (1 = desempleado; 2 = contratos temporales; 3 = contrato estable)

Contexto familiar en cuanto a la situación de los padres (conviviendo, separados y solteros, separados y con otra persona, otros)

X. 3.2.10. Parámetros bioquímicos de rutina

La evaluación bioquímica basal se realiza en la sangre del paciente. La muestra de sangre se recogió del paciente tras doce horas de ayuno, se procesó inmediatamente después de la recogida (centrifugado a 3000 g a 4°C durante 5 minutos) y las determinaciones se realizaron en condiciones estandarizadas. La glucosa, el colesterol total, el HDL, los triglicéridos, el ácido úrico, la urea, la creatinina y la apolipoproteína A1 y B se midieron con métodos directos automatizados (Aeroset System® Abbott Chemical Clinic, Wiesbaden, Alemania). Los niveles de colesterol LDL y VLDL se calcularon mediante la fórmula de Friedewald y la relación triglicéridos/5, respectivamente. La insulina y la homocisteína se determinaron mediante un inmunoensayo de electroquimioluminiscencia automatizado (c8000® Architect, Abbott Clinic-Chemistry). Los niveles de cistatina C se analizaron mediante inmunonefelometría con el nefelómetro Behring 2 (Dade Behring, Marbung, Alemania). La proteína C reactiva de alta sensibilidad (hs-CRP) se analizó por inmunonefelometría con un nefelómetro Behring2 (Dade Behring, Marbung, Alemania). La Γ-GT se midió por colorimetría enzimática a 37℃ en un analizador automatizado Roche / Hitachi (Mannheim,

Alemania). La vitamina D (forma 25-hidroxivitamina D) se cuantificó por inmunoensayo electroquimioluminiscente en el autoanalizador Roche COBAS 6000 (Roche Diagnostics GmbH, Mannheim, Alemania). Para determinar la TSH se utilizó un ensayo inmunoradiométrico directo (Beckman Coulter UniceDxl 800, Beckman Coulter, L´Hospitalet de Llobregat, Barcelona). El índice HOMA se calculó según la siguiente fórmula: HOMA = insulina en ayunas (µUi / ml) × glucosa en ayunas (mg / dL) / 405. El índice aterogénico se calculó mediante la siguiente fórmula: triglicéridos (mg / dL) / HDL (mg / dL). Los parámetros hematológicos se obtuvieron en sangre venosa obtenida en tubos con anticoagulante EDTA, utilizando el autoanalizador Coulter LH750 Analyzer de Beckman Coulter.

X. 3.2.11. Parámetros experimentales

El participante entregó las muestras de saliva al investigador el día de la extracción de sangre durante la cual se recogieron dos muestras de sangre en vacutainer para las evaluaciones bioquímicas basales: una con gelosa y otra con EDTA. Dichos tubos, junto con las muestras de saliva son transportados en condiciones de refrigeración y oscuridad al laboratorio de Pediatría, Obstetricia y Ginecología de la Facultat de Medicina de la Universidad de Valencia. Inmediatamente después de su llegada, las muestras de saliva se transfieren a criotubos y se almacenan a -80°C. Las muestras de sangre se centrifugan a 1500rpm durante 10 minutos a 4°C, separando el plasma, el suero y las células. Las muestras se alicuotan y se almacenan a -80°C hasta la realización de las pruebas.

X. 3.2.12. Marcadores metabólicos y de inflamación

Varios parámetros interesantes en la investigación de la obesidad no están incluidos en los análisis bioquímicos de sangre rutinarios, por lo que los hemos evaluado por separado en las muestras que recogimos. Las adipocinas Leptina, Resistina, Adiponectina, Ghrelina, y los marcadores inmunológicos MCP-1, PAI-1, IL-6, TNFα e INF-γ se determinaron mediante inmunoensayo múltiplex (Labscan 100 Luminex©, Merck Millipore Merck KGaA, Darmstadt, Alemania) con tecnología Luminex utilizando el software específico 3.1 (Luminex Corporation. Austin, TX, USA.).

La omentina se cuantificó con un ensayo inmunoenzimático (ELISA) (Human Omentin-1 ELISA, Merck-Millipore, Darmstadt, Alemania) con el lector de placas multilabel VICTORTM X3 2030 (Perkin Elmer, Waltham, MA, USA).

X. 3.2.13. Melatonina y capacidad antioxidante

A los y las participantes se les proporcionaron tubos de plástico de 30 ml utilizados habitualmente para la recogida de orina, en los que se instruyó a los y las participantes para que dejaran caer su saliva hasta recoger unos 300µl. Se les pidió que evitaran la exposición a pantallas y la actividad física en los 15-30 minutos anteriores a la recogida de saliva. Se recogieron tres muestras de saliva de cada paciente la noche anterior a la extracción de sangre: 4h antes de la hora de dormir, 2h antes de la hora de dormir y después de 1h de sueño.

Antes del análisis, la saliva se centrifugó a 5000RPM durante 2 minutos a 4°C. Se verificó que todas las muestras estuvieran acondicionadas en la oscuridad, los tubos en completa hermeticidad y que se contara con el volumen mínimo necesario para el análisis posterior. En cada muestra se cuantificó el nivel de melatonina mediante inmunoensayo utilizando un kit salival de Salimetrics (Salimetrics, LLC Carlsbad, CA USA), siguiendo las instrucciones disponibles en línea en https://salimetrics.com/wp-content/uploads/2018/03/melatonin-saliva-elisa-kit.pdf. Cuanta más melatonina haya en la muestra, más intensa será la coloración amarilla al final de la reacción, cuya cuantificación se realiza utilizando un lector de placas multimarcador VICTOR TM X3 2030 (Perkin Elmer, Waltham, MA, USA).

Se calculó la tasa horaria de aumento de la concentración de melatonina a lo largo del tiempo entre los diferentes puntos temporales:

La tasa de aumento de melatonina a lo largo de la noche se calculó como la diferencia entre la concentración de melatonina en el último punto temporal (+1h después del inicio del sueño) y el primer punto temporal (-4h antes del inicio del sueño) dividida por el número de horas entre ellos (5 horas).

La tasa de aumento de melatonina antes de dormir se calculó como la diferencia entre la concentración de melatonina en el punto temporal medio (-2h después del inicio del sueño) y el primer punto temporal (-4h antes del inicio del sueño) dividida por el número de horas entre ellos (2 horas).

La tasa de aumento de melatonina alrededor del sueño se calculó como la diferencia entre la concentración de melatonina en el último punto temporal (+1h después del inicio del sueño) y el punto temporal medio (-2h antes del inicio del sueño) dividida por el número de horas entre ellos (3 horas).

La capacidad antioxidante total de estas muestras de saliva en los tres puntos temporales se evaluó mediante la reacción colorimétrica del 2,2-difenil-1-picrilhidrazilo (DPPH). Esta molécula es un radical libre estable y tiene un fuerte color púrpura que absorbe a 517 nm, que desaparece cuando es eliminado por un antioxidante. Este cambio de color puede ser medido con un espectrofotómetro, este método ha sido descrito y mejorado en numerosas ocasiones, y recientemente nuestro grupo de investigación ha adaptado este método para tener la posibilidad de utilizarlo en muestras muy pequeñas de fluidos biológicos.234 Nosotros utilizamos este método adaptado tal y como se describe en la publicación, el DPPH fue adquirido en Aldrich (Chemical Company, USA) y aplicado en una placa de 96 pocillos con fondo plano. Para la cuantificación

de la reacción colorimétrica, se utilizó una curva estándar de Trolox equivalente como referencia para el cálculo de la capacidad antioxidante de las muestras de saliva. Tras 30 minutos de reacción bioquímica, se tomó una fotografía de la placa con una exposición lumínica homogénea de los pocillos utilizando un fondo retroluminiscente. El color se cuantificó utilizando el plugin PlateReader 3.0 en el software imageJ (https://imagej.nih.gov/ij/plugins/readplate/index.html) analizando las imágenes RGB por separado. En cuanto a la cuantificación de la melatonina, la medición de la capacidad antioxidante se realizó con muestras de saliva de tres puntos temporales: 4h antes de dormir, 2h antes de dormir y después de 1h de dormir. Y en cuanto a la melatonina, los índices de aumento se calcularon a partir de las tres mediciones de la siguiente manera

La tasa de aumento de la capacidad antioxidante a lo largo de la noche se calculó como la diferencia entre la concentración de melatonina en el último punto temporal (+1h después del inicio del sueño) y el primer punto temporal (-4h antes del inicio del sueño) dividida por el número de horas entre ambos (5 horas).

La tasa de aumento de la capacidad antioxidante antes del sueño se calculó como la diferencia entre la concentración de melatonina en el punto de tiempo medio (-2h después del inicio del sueño) y el primer punto de tiempo (-4h antes del inicio del sueño) dividida por el número de horas entre ellos (2 horas).

La tasa de aumento de la capacidad antioxidante en torno al sueño se calculó como la diferencia entre la concentración de melatonina en el último punto temporal (+1h después del inicio del sueño) y el punto temporal medio (-2h antes del inicio del sueño) dividida por el número de horas entre ambos (3 horas).

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X. 3.2.14. Cultivo celular

El experimento se realizó con preadipocitos blancos humanos primarios aislados de tejido adiposo subcutáneo adulto (Promocell, Heidelberg, Alemania) tras una liposucción abdominal en una mujer caucásica de 52 años. Las células se sembraron a 105 células/ml en placas de seis pocillos y se cultivaron hasta la confluencia en medio de crecimiento (Promocell, Heidelberg, Alemania) a 37°C en una atmósfera humidificada con un 5% de CO2. A continuación, se colocaron en medio de diferenciación (Promocell, Heidelberg, Alemania). En la mitad de los cultivos celulares, el medio de diferenciación se complementó con 30pg/ml de melatonina (N-acetil-5-metoxitriptamina). Un día después, se recogieron los cultivos celulares en 4 puntos temporales 8:00 AM, 10:45 AM, 6:30PM, 11:00PM. Todos los cultivos se realizaron por triplicado.

a) Cuantificación del transcrito

El ARNm se extrajo utilizando un kit de extracción de ARN de Qiagen retrotranscrito con el kit de síntesis de microARNc qScript. La PCR cuantitativa se realizó utilizando PerfeCTa SYBR Green Super Mix Low ROXTM (QIAGEN Beverly, Inc.) en placas de 384 pocillos utilizando un termociclador QuantStudio 3 (Applied Biosystems Waltham, Massachusetts). Los valores del umbral de ciclo (CT) se obtuvieron por triplicado para los genes β-actina, BMAL1, CRY, PER2, MT2, PPARγ, C/EBPα, LPL, LEP, aP2, Adiponectina (GBP-28) y PAI-1. Se utilizó el gen β-actina como referencia, los resultados se presentan en ΔCT.

X. 3.3. Análisis estadístico

Se utilizó el software R (versión 3.5.0 R Core Team, 2018, Viena, Austria) para todo el análisis235, y se empleó el paquete tidyverse para el trabajo estadístico y las representaciones. Se utilizaron otros paquetes a lo largo del análisis de los datos: readxl, psych, RcmdrMisc, ggsignif. Se utilizó el paquete sjPlot para construir las tablas.

En cuanto a los valores atípicos, se revisó manualmente cada variable y se excluyeron del análisis los puntos distantes de más de 6 desviaciones estándar respecto a la media. No se identificó ningún individuo como atípico utilizando la distancia de Mahalanobis. Para el cotejo de edades, se excluyeron del estudio los tres participantes de 14 años y los dos de 15 años del grupo de obesidad que tenían el mayor número de valores perdidos. Se utilizó una prueba de Shapiro-Wilk para evaluar la normalidad de cada variable cuantitativa. En nuestra población de estudio, las variables no siguen una distribución normal, por lo que las pruebas estadísticas se eligieron en consecuencia. Los datos cuantitativos se expresan como mediana y rango intercuartil, y los datos categóricos como número absoluto o porcentaje. La significación estadística se establece sobre la base de una $p < 0,05$. Se utilizaron las pruebas de Wilcoxon para las comparaciones de grupos de variables cuantitativas, no apareadas para las comparaciones entre grupos y apareadas para las comparaciones de un mismo grupo en varios puntos temporales. Se realizó una prueba de Fisher para analizar la significación de los odds-ratios. Los parámetros implicados en el análisis de odds ratio y los puntos de corte se muestran a continuación. Una parte de ellos son factores de riesgo metabólico tradicionales cuyos valores de corte fueron publicados previamente. En el caso de la leptina, los límites de normalidad para estas variables se calcularon como el percentil 97 del grupo de control. En cuanto a los horarios, se utilizó la mediana de la hora de las comidas en el grupo de control para definir temprano y tarde.

Para la modelización de los ritmos circadianos de los genes reloj, se utilizaron los paquetes Cosinor (versión 1.1), ggplot2 y dplyr. Se graficó la expresión de los transcritos en ΔCT en función del tiempo, y se construyó un modelo cosinor para cada condición (con y sin melatonina) aplicando una regresión de mínimos cuadrados parciales de un periodo de 24h. Se aplicó el test de Wald integrado en el paquete R cosinor para estimar la diferencia entre las dos condiciones en términos de amplitud, acrofase y mesor del ritmo. Las

correlaciones se evaluaron utilizando la función pcor.test del paquete ppcor (paquete R versión 1.1.; 2015) para calcular el coeficiente rho de Spearman y el valor p asociado. El gráfico de araña se realizó con el paquete fmsb versión 0.7.3, 2022. Todas las correlaciones se ajustaron por sexo, edad e índice de pubertad. Previo al Análisis de Componentes Principales (ACP), se seleccionaron las variables correlacionadas con el aumento de melatonina alrededor de la hora del sueño, se calculó el índice Kaiser - Mayer - Olkin, conocido como índice KMO, y se realizó la prueba de esfericidad de Barlett para evaluar la adecuación de la muestra. Se utilizó el paquete Factoshiny (paquete R versión 2.4.; 2021) para el ACP y la posterior clasificación jerárquica. Para cada análisis multivariante, los valores perdidos se imputaron con un modelo en k dimensiones seleccionado en la aplicación Factoshiny que incluía sólo las variables afectadas por el análisis. Por último, se utilizó el paquete caret para construir modelos de bosque aleatorio utilizando como métrica la característica operativa del receptor (ROC), que integra tanto la sensibilidad (tasa de verdaderos positivos) como la especificidad (tasa de falsos positivos) del modelo.

X. 3.4. Confidencialidad, información y ética

Todos los pacientes y sus familiares fueron informados sobre el estudio: descripción del estudio, beneficios y posibles riesgos, pasos a seguir en la toma de muestras y tratamiento a dar a los resultados obtenidos por el investigador o el dietista del equipo, junto con el pediatra. Tras informar a los pacientes y la aceptación por parte de los padres de los menores, se firmó el consentimiento informado por parte de los padres y del participante a partir de los 12 años.

Toda la información recogida de participantes fue tratada con confidencialidad y toda la base de datos siguió el proceso de pseudoanonimización de acuerdo con la normativa legal vigente en España (Ley Orgánica 7/2021, de 26 de mayo de Protección de Datos de Carácter Personal y Ley (UE) 2016/679 Parlamento Europeo modificada el 23 de mayo de 2018, Ley Orgánica 3/2018, de 5 de diciembre, de Protección de Datos Personales y garantía de los derechos digitales).

El presente proyecto de investigación fue aprobado por el Comité Ético del Hospital Universitario Dr. Peset de Valencia el 29 de marzo de 2018.

X. 4. RESULTADOS Y CONCLUSIONES

Se describieron y compararon los dos grupos del estudio. La tasa de aumento de melatonina en torno al sueño se encontró mayor en el grupo de control en comparación con el grupo de obesidad.

Nos propusimos comprender mejor el efecto de la melatonina en los ritmos del metabolismo. Estudiamos in vitro los ritmos circadianos de los genes de los adipocitos y el efecto de la melatonina sobre estos ritmos. Observamos que los adipocitos cultivados en contacto con la melatonina mostraban una mayor amplitud en todos los genes estudiados, aumento que fue significativo para dos de los tres genes del reloj

estudiados (PER2 y CRY), y dos genes metabólicos (Adiponectina y LPL). Para completar la comprensión de la influencia de la melatonina sobre los genes oscilantes periféricos, están indicados otros estudios que investiguen el efecto de estimulaciones puntuales de melatonina, a diferentes horas, duraciones y concentraciones.

En nuestro estudio en la infancia, encontramos que la tasa de aumento de melatonina entre -2h antes de dormir y +1h después del inicio del sueño, se encontró correlacionada con los marcadores antropométricos y metabólicos, el cronotipo, los hábitos de vida y las características del entorno del sueño. El cronotipo también se correlacionó con numerosas variables características de la obesidad, incluyendo factores metabólicos, inflamatorios, hábitos de vida y ambientales. Además, la duración del sueño, la hora de la cena, la presencia de dispositivos de pantalla en el dormitorio por la noche y el contexto sociodemográfico familiar, también se correlacionaron con características antropométricas, clínicas, metabólicas e inflamatorias asociadas a la obesidad. Este enfoque de correlaciones múltiples nos mostró que muchas variables del estudio están vinculadas individualmente con otras. Para visualizar las relaciones entre múltiples variables al mismo tiempo, se realizó un análisis de componentes principales (ACP) incluyendo todas las variables correlacionadas con la melatonina. La jerarquía basada en este PCA mostró la existencia de tres grupos entre los que los individuos compartían características similares. En particular, reveló que el grupo de obesidad/sobrepeso podía en realidad separarse en dos subgrupos, uno compuesto por individuos con mejores resultados metabólicos y de inflamación que el otro subgrupo. Curiosamente, en este subgrupo metabólicamente más sano, los y las participantes también se acostaban más temprano, con menor hábito de siesta, una mayor duración del sueño durante la jornada escolar, menos dispositivos tecnológicos en el dormitorio y un cronotipo más temprano en comparación con el otro grupo. Esto sugiere que entre los individuos con obesidad, un cronotipo más temprano y una mejor calidad y duración del sueño pueden limitar el desarrollo de comorbilidades y, por el contrario, los comportamientos más tardíos y el sueño corto pueden empeorar los riesgos metabólicos asociados a la obesidad. Para seguir construyendo una comprensión multidimensional de los parámetros asociados a la obesidad y visualizar la fuerza de su vínculo con esta enfermedad, desarrollamos un bosque aleatorio. Gracias a este algoritmo de aprendizaje automático, obtuvimos un modelo predictivo de la obesidad basado en las características de los hábitos de vida, el sueño y el entorno. Aunque la precisión fue relativamente baja, esta prueba de concepto demuestra que estos parámetros se asocian conjuntamente con la obesidad. Paralelamente, desarrollamos un segundo algoritmo para ilustrar la huella de los biomarcadores de la obesidad, incluyendo todas las características metabólicas e inflamatorias, así como la tasa de aumento de la melatonina en torno al tiempo de sueño. El análisis de la importancia relativa de los metabolitos incluidos reveló que la melatonina estaba al mismo nivel que los marcadores clásicos como la adiponectina, la omentina, la grelina o la glucosa. En conjunto, estos análisis ilustran el aspecto multidimensional de la obesidad y respaldan la importancia de tener en cuenta los ritmos circadianos, pero también los factores externos, como el sueño, el entorno y los hábitos de vida, a la hora de investigar, prevenir y tratar la obesidad.

- La tasa de aumento de melatonina en torno a la hora de dormir se encontró significativamente menor en el grupo que presentaba obesidad infantil. Esta modificación se encontró asociada a diferentes factores de riesgo como antropométricos (mayor índice de masa corporal, porcentaje de masa grasa e índice de cintura/altura), metabólicos (aumento del índice HOMA, grelina, leptina), cronotipo más tardío, hábitos de vida (acostarse más tarde, menor duración del sueño, reparto más tardío de la ingesta calórica en las comidas) y factores ambientales (más dispositivos de pantalla en el dormitorio).
- En los adipocitos humanos estimulados con melatonina, se observó un aumento de la amplitud de la expresión circadiana de los genes del reloj y de los genes metabólicos.
- El método de medición de la melatonina mediante tres muestras de saliva durante la noche resultó funcional para la evaluación de la tasa de aumento de la melatonina en torno a la hora de dormir.
- Se encontró una correlación entre el cronotipo y numerosos factores: cuanto más precoces son los cronotipos, más saludable es el metabolismo (menor creatina y albúmina, mayor transferrina) y menor inflamación (IFN-γ, TNF-α), hábitos de vida más saludables (acostarse antes, mayor duración del sueño, menos jetlag social) y factores ambientales (menos dispositivos de pantalla en el dormitorio, situación laboral más estable del padre).
- La duración del sueño se correlacionó negativamente con la obesidad a través de factores antropométricos (menor IMC, porcentaje de masa grasa e índice de cintura/altura), clínicos (menor presión arterial diastólica), metabólicos (menor glucosa, triglicéridos), inflamatorios (menor PAI-1, MCP-1), hábitos de vida (menor jetlag social, acostarse más temprano, menor amplitud temporal de la ingesta nutricional, horarios de comidas más tempranos y reparto de la ingesta nutricional en las comidas) y factores ambientales (menos pantallas en el dormitorio).
- La hora de la cena se correlacionó positivamente con la obesidad a través de factores metabólicos (mayor glucosa y albúmina, menor hierro), hábitos de vida (menor duración del sueño y mayor amplitud temporal de la ingesta nutricional, horarios de comidas más tardíos y reparto de la ingesta nutricional en las comidas).
- La exposición nocturna a la pantalla se correlacionó positivamente con la obesidad a través de factores antropométricos (mayor IMC, porcentaje de masa grasa y relación cintura/altura), metabólicos (mayor índice HOMA, leptina), cronotipo, hábitos de vida (más siesta, jetlag social y acostarse tarde) y ambientales (padre desempleado, padres separados).
- Las características sociodemográficas de los padres se relacionaron con la obesidad, ya que los niveles de estudio más bajos de los padres y el empleo inestable del padre se correlacionaron con factores antropométricos (mayor IMC, porcentaje de masa grasa y relación cintura/altura), clínicos (mayores niveles de presión arterial sistólica y diastólica), metabólicos (mayor índice HOMA triglicéridos, leptina, vLDL), inflamatorios (mayor C3, PAI-1), factores de hábitos de vida (peor calidad del sueño, más jetlag social, menor actividad física, más leche de fórmula como nutrición temprana, reparto posterior de la ingesta nutricional en las comidas) y factores ambientales (más dispositivos de pantalla en el dormitorio).

- Un análisis de agrupación reveló que el grupo con obesidad y sobrepeso estaba compuesto por dos subgrupos. En uno de ellos, en comparación con el otro, el grupo de participantes con obesidad y sobrepeso presentaban mejores características de salud metabólica en paralelo a una hora de acostarse más temprana, un menor hábito de siesta, una mayor duración del sueño durante la jornada escolar, menos dispositivos tecnológicos en el dormitorio y un cronotipo más temprano.
- Un modelo de predicción de la obesidad basado en los hábitos de vida y los factores ambientales subrayó que la obesidad está asociada a la calidad del sueño, el horario de los hábitos de vida y el reparto de la ingesta calórica en las comidas.
- Un modelo de biomarcadores mostró que la tasa de aumento de la melatonina en torno al sueño no se encontraba entre los marcadores más potentes de la obesidad (leptina, C3, índice HOMA, hs-CRP), sino que estaba al mismo nivel que los marcadores clásicos como la adiponectina, la omentina, la grelina o la glucosa.

Como conclusión general, en el presente estudio se encontró una relación entre los hábitos de vida, las variaciones de la melatonina y los trastornos metabólicos en la obesidad infantil.

Abstract

Introduction: Numerous studies on nightshift workers evidence a link between circadian rhythms disruption and metabolic disorders. In parallel, other investigations mainly carried out on animal models or in human adults, show a link between the timing of life habits, insufficient duration and poor quality of sleep, and the development of obesity and its comorbidities. Nevertheless, little is known about the role played by these mechanisms in the current epidemic of childhood obesity. Therefore, we developed a study of the relationship between life habits, circadian rhythms of melatonin, and metabolism, in the context of childhood obesity. In complement, we also studied *in vitro* the impact of melatonin on the circadian rhythm of the genetic expression of adipocytes.

M&M: A transversal and analytical study was performed on 203 children between 7 and 16 years old, assigned to the control group or the overweight and obesity group. Anthropometric and clinical characteristics were collected, metabolic and inflammatory markers were measured in the plasma. Melatonin was assessed by immunoassay in saliva that was collected by the participants at home at three time points: 4h before sleep time, 2h before sleep time and after 1h of sleep. Questionnaires were used to collect information about life habits, chronotype, and life environment. The *in vitro* study was performed on human subcutaneous adipocytes, after 24h in culture with or without melatonin supplementation, RNA was extracted at four time points and quantified by rtqPCR for clock genes and metabolic genes.

Results: A lower increase rate of melatonin around sleep time was observed in children with overweight and obesity. In parallel, the *in vitro* study showed that adipocytes stimulated with melatonin present a greater amplitude in the circadian expression of clock genes and metabolic genes. In children, correlations and multivariate analysis showed interrelationships between variables from all the different categories: anthropometry, clinic, metabolism, inflammation, circadian rhythms, chronotype, life habits, and environment. A subsequent clustering analysis showed that among the individuals from the obesity group, a subgroup of individuals presented a better metabolic health in parallel of earlier life habits and a longer sleep duration. An algorithm showed that, among the parameters studied, poor sleep quality and duration and late meal timing were the strongest predictors of obesity. Another algorithm showed that melatonin nocturnal increase rate was as much a biomarker of obesity as classic markers such as adiponectin, omentin, ghrelin, or glucose.

Discussion: The present findings support that there is, in childhood obesity, a relationship between life habits, circadian rhythms of melatonin and metabolism. We observed that late chronotypes and life habits, short sleep duration, poor sleep quality, and eating more in the later part of the day, are associated to poorer metabolic health outcomes, in parallel of an altered nocturnal melatonin rise. Plus, the presence of screen devices in the sleep environment, as well as low education levels of the parents and precarious work situation of the father, appear as risk factors for the children in term of social jetlag, short sleep, late life habit, but also obesity and its metabolic alterations. These new findings emphasize the importance to address sleep, life habits timing, and life environment, in the development of measures of prevention and treatment of obesity.