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Evaluation of gut microbiota in melanoma and vitiligo patients

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A Vittorio,

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e alla mia famiglia.

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1. Human gut microbiota

1.1 Composition of a healthy gut microbiota

The human microbiome is the collection of the microrganisms inhabiting the human body. This community is composed by thousands of trillions of microbes including eukaryotes, archaea, bacteria and viruses [1]. Bacteria in an average human body are ten times more numerous than human cells, for a total of about 1000 more genes than those pertaining to human genome [1,2]. A theory defined as *symbiogenesis* the result of the permanent coexistence of various bionts to form the *holobiont* (namely, the host and its microbiota) [3]. Accordingly, the *holobiome* is the totality of the genomes inhabiting a eukaryotic organism, which includes the genome of a specific member of a given taxon (the host genome, for example human) and the microbiome (the genomes of the symbiotic microbiota). Microbiome is composed by the genes of different microbial communities which survived and were not eliminated by natural selection [3].

So far, the knowledge about the adult human microbiota depended on culture-based methods [1]. However, the intensity and the duration of the culture methods and the difficulty to cultivate and identify some microorganisms growth medium made the definition human microbiome composition difficult [1].

The advent of culture-independent approaches such as high-throughput and low-cost sequencing methods changed such scenario, enabling to elucidate the microbial composition of several body areas [1]. Hence, metagenomics disclosed millions of microbes, and allowed to extract sequence data from microbial communities exactly as they exist in nature.

The NIH Common Fund Human Microbiome Project (HMP) was established in 2008, with the mission of generating resources that would enable the comprehensive characterization of the human microbiome and the analysis of its role in human health and disease. Its repository is the Data Analysis and Coordination Center (DACC) website [2]. The Metagenomic of the Human Intestinal Tract (MetaHit) Consortium Europe is a project financed by the European Commission which started in 2008 with the central objective to establish associations between the genes of the human intestinal microbiota and human health and disease [4].

The above-mentioned projects together have provided a comprehensive view of the human-associated microbial repertoire, and combined data from these studies identified

2172 species isolated from humans, classified into 12 different phyla mainly belonging to Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes. Only three of the 12 identified phyla contained species isolated from humans, and among them intestinal species, *Akkermansia muciniphila*, representative of the Verrucomicrobia phyla, was found. In humans, some of the identified species are anaerobic and hence are usually found in oral cavity and the gastrointestinal (GI) tract [5,6].

The GI tract is an important and abundant interface between the host, exogenous factors and in the human body [5].

The totality of bacteria, archaea and eukarya colonizing the GI tract is called the 'gut microbiota'. Human co-evolved with microbes, finally tolerating them and eventually developing a beneficial relationship [1,5].

Human healthy gastrointestinal tract contains a number of microbes reaching approximately 10^{14} cells in the entire gut [1,4].

As regards to bacteria over 1000 gut bacterial species have now been characterized. Overall, a healthy gut microbiota, as characterized by sequencing, is dominated by bacteria belonging to main two phyla—*Bacteroidetes* and *Firmicutes* [1,7-9]. *Bacterioides*, which have been further classified into 5 additional genera (*Alistipes*, *Prevotella*, *Paraprevotella*, *Parabacteroides*, and *Odoribacter)* [1,10] are Gram-negative bacteria able to digest carbohydrates [9,11]. Firmicutes on the contrary are mostly Gram-positive bacteria able to digest vegetal fibers, such as cellulose, and less involved in carbohydrate digestion [9,12].

Even if mainly predominated by bacteria, gut microbiota is also composed by Archaea, Eukaryotes and Viruses.

Among archaea, the *Methanobrevibacter* genus are frequently found in the gut [1,13]. They collaborate with other microbes to digest dietary polysaccharides [1,14] and to synthesize methane from H_2 produced by bacterial catabolism [1,15,16].

Candida, *Malassezia*, and *Saccharomyces* are constant inhabitants of the human gut, being other distinct fungi usually found under pathological conditions [1,17]. Interestingly, fungi seem to have constituted a beneficial relationship with humans, as shown in the case of the protective role of the probiotic yeast *Saccharomyces boulardii* toward cholera. Interestingly, the opportunistic pathogen *C. albicans* can pass in a

specific and beneficial regulated commensal state called GUT (gastrointestinally induced transition) in the host intestine [17]. Candida albicans in the GUT state develops a specific benign phenotype, in which virulence-associated genes, such as the white-opaque switching and hyphal formation genes, are downregulated, enabling fungal adaptation for long-term survival in the large intestine [17,19]. In case of an impairment of the host immunity, *C. albicans* cells in the GUT state can modify their phenotype and become pathogens [17].

As regards to *Saccharomyces*, a specific microorganism called *S. Cerevisiae* has been identified in human gut and has been considered so far as a transient gut inhabitant [17]. Its role and presence in the human gut at birth is still under investigation, being possibly just introduced in GI tract later in life through commonly assumed food and beverages (wine, beer, bread) [17]. Recent studies on patients affected by Crohn's disease however found elevated serum autoantibodies directed toward *S. Cerevisiae* [17,20], thus suggesting a possible more active role of this yeast in modulating inflammatory and immune responses.

Some protozoa are even common inhabitants of healthy microbiomes [1,21,22], with even greater interpersonal variability as compared to bacteria [1,21]. Some of them, such as the *Blastocystis*, have been associated with a protective role toward gastrointestinal disease. Also, Helmints can be occasionally found in gut [1,23], where they might have an immunomodulatory role, but their presence is nowadays limited due to the western dietary habits [1,23].

Even if it has been estimated that the human virome might be particularly extensive, dataa regarding viruses colonizing the human gut microbiota are still lacking, due to the difficulties in the methodologies needed for their characterization. [1,24].

It has been postulated that each human has a specific and hypervariable virome [1,25,26], consisting primarily of *bacteriophages* [1,24,27].

Overall, more than a list of specific microorganisms, a healthy gut microbiota should have a collection of microbial species collectively sharing the ability to perform metabolic functions necessary for microbial survival or implicated in host-microbe interaction, such as the production of several short-chain fatty acids, vitamins and essential amino acids ("functional core") [1]. Moreover, a heathy gut microbiota should have high richness and diversity of species, it should counteract exogenous perturbation and it should return to a healthy state after damages (resilience) [1].

In general, according to the above-mentioned features, a healthy gut microbiota is expected to be mainly composed by *Bacteroidaceae*, *Clostridiaceae*, *Prevotellaceae*, *Eubacteriaceae*, *Ruminococcaceae*, *Bifidobacteriaceae*, *Lactobacillaceae*, *Enterobacteriaceae*, *Saccharomycetaceae* and *Methanobacteriaceae*.

1.2 Features of gut microbiota according to different gastro-intestinal districts

Besides the dominant phyla, *Bacterioides* and *Firmicutes*, microbiota composition in the GI tract is variable, according to specific anatomic, physical and chemical properties of the different GI regions.

The upper digestive tract stomach and small intestine is characterized by high levels of acids (Ph 4-5), oxygen and antimicrobials, and a short time of transit [5,9,28]. Hence, only rapidly growing, aerobic or facultative anaerobes such as *Lactobacillaceae* and *Helicobacteriacee*, can resist [5,28-31]. Overall, in the small intestine the most frequently found species are *Enterococcaceae*, *Streptococcacee*, and *Lactobacillacee*.

On the contrary, the gut microbiota of the large intestine is more stable and mainly composed by anaerobes, which can use in the colon (pH=7) the amount of complex carbohydrates previously undigested in the small intestine [29]. Hence, in this district the community of bacteria has an increased diversity and is mainly composed by *Clostridiacee* (SFB, segmented filamentous Bacteria), *Prevotellaceae*, *Bacterioidacee*, *Lachnospiraceae* and *Rikenellaceae* is predominant. [5, 28-30].

In addition, microbial composition is variable among the faecal/luminal and mucosal regions. [5,32,33].

Bacteroidetes appears to be higher in faecal/luminal samples than in the mucosa [5,32,34], while *Firmicutes*, specifically *Clostridium* cluster XIVa, are most frequently found in the mucus layer, as compared to the lumen [5,34].

1.3 Changes in gut microbiota across life

The composition of gut microbiota changes during life and the process of colonization starts at birth.

The intrauterine environment has been considered so far sterile [5,35,36] and the occurrence of any microbe in uterus is largely considered as a harmful and worrying

event [35]. Recently, some investigations changed such credence, since they reported the presence of a diversified microbiota in the placenta [37], umbilical cord blood [38], amniotic fluid [39], fetal membranes [40] and in the spontaneously released meconium of pre-term infants [41].

Following birth, gut is characterized by low diversity and predominance of the phyla *Proteobacteria* and *Actinobacteria* [35,36]. During the growth of the neonate, the presence of *Firmicutes* and *Bacteroides* increases [35,36], finally reaching an adult-like gut microbiota by the 2.5 years of age [5,42-44].

The adult gut microbiota is overall stable, while it tends toward a change with elderly [5,35,45-47]. It has been demonstrated that over the age of 65 there is an increase in *Bacteroidetes* phyla and *Clostridium* cluster IV (while in younger people cluster XIVa is more present) [5,35,45-47]. Gut microbiota in centenarians shows further reduced diversity, with an increase in facultative anaerobes such as *Escherichia coli* and decrease in butyrate producers (e.g. *Faecalibacterium prausnitzii*) [5,35,46,48]. Such changes imply increased inflammatory status, reduced immune competence, reduced short chain fatty acids (SCFA) production and increased proteolysis [5,47,49,50].

1.4 Factors shaping gut microbiota composition

Some factors have been reported to affect the composition of gut microbiota.

Delivery mode can affect early gut microbiota [1,5,35,51], since caesarean section leads to an increase of microbes usually found in the skin or in the hospital, such as *Haemophilus spp*., *Enterobacter cancerogenus*/*E. hormaechei, Veillonella dispar/V. parvula* [51,52-56], and *Staphylococcus* [57], while vaginal delivery on the contrary implies to the colonization by maternal vaginal and fecal microbes, such as *Lactobacillus* and *Bifidobacterium* spp [58-60].

Furthermore, **preterm birth** implies a reduced diversity and number of *Bifidobacterium* and *Bacteroides* [61], compared to full-term infants, while the use of perinatal short-term antibiotics leads to an increase in *Proteobacteria* [41,62].

Infant diet is another shaping factor, since breast feeding implies an exposure to more than 700 different kinds of bacteria contained in human milk [35,63]. Moreover, mother' milk holds complex oligosaccharides [63,64] with prebiotic activity, able to facilitate the growth of beneficial bacterial groups [35,63,64]. Accordingly, formula-

fed infants have been reported to have a different gut microbiota composition than breastfed neonates. [5,36,65,66].

Since the introduction of solid food progressively increases the ratio of butyrate producers such as *Bacteroides* and *Clostridium* species, the **age of weaning** can affect gut microbiota composition too.

Diet is another essential factor able to modify gut microbiota, both in children and in adults.

Gut microbiota composition depends on the presence of microbiota-accessible carbohydrates (MACs) contained in fibers assumed by diet [5]. Totally 'animal-based' or 'plant-based' diets lead to changes in gut microbiota composition [5].

In case of normal dietary carbohydrates intake and in physiological conditions, there is an equilibrium between bacteria able to ferment carbohydrates and those able to utilize and modify the protective mucus overlying gut epithelial cells. A depletion of dietary carbohydrates leads to a condition of dysbiosis and mucus layer thinning, because of a shift of the functions of bacteria which become from usually carbohydrates fermenting to mucus utilizing [29,67,68].

Infants from rural Africa have a diet mainly composed by starch, fibers and plant polysaccharides. Hence their microbiota has been found to be abundant in the *Actinobacteria* and *Bacteroidetes* phyla [69]. On the contrary, in European children, that have a diet rich in sugar, starch and animal protein, the presence of the abovementioned groups is lower [69]. Some short chain fatty acids (SCFAs) producers, such as *Prevotella*, can be only recognized in the gut microbiota of African children [69].

The abundance of carbohydrates introduced with diet is reduced in the Westernized populations, both in children and in adults. Hence, there might be a consequent decreased production of SCFAs, with potential harmful effects of human wellbeing and possibly leading to the onset of the diseases.

Also, the **mucus layer** overlying the intestinal epithelium in the whole GI tract strongly affects gut microbiota composition [35,70-75]. Namely, as previously mentioned as regards to diet, the thickness and glycosylation pattern of the mucus layer influences the kind of resident microbes, which in turn affect and regulate the mucus layer status [35,70].

Obviously, the use of **antibiotics** affects the composition of human gut microbiota, leading to a reduction in richness and diversity of microbial species [76].

It is possible that some **host genetic factors** could affect gut microbiota composition too. Although results by different studies are quite contrasting and further studies are needed, an interesting study reported a similarity between gut microbiota in monozygotic twins, as compared to controls [35,77]. More convincing evidences regarding a possible, yet to be elucidated, role of genetics in shaping gut microbiota, come from mouse models in which the biases related to diet, environment and genetic can be experimentally overpassed [35].

Host immune system also affect gut microbiota composition, due to the ability of some cells in the GI tract, such as Paneth cells, to produce antimicrobial molecules which limit pathogen microbes' growth [78]. Experimental models of mice lacking NOD2 or TLR5 (specific kinds of pattern recognition receptors), display pathogenic bacterial colonization and a condition of dysbiosis [79-81]. Further evidences of the role of an active and competent host immune system in shaping a proper and healthy gut microbiota come from investigations on patients affected by HIV infection, in which have been found high levels of pro-inflammatory pathobionts such as *Erysipelotrichaceae* and *Enterobacteriaceae* (which include *Salmonella*, *Escherichia*, *Serratia*, *Shigella* and *Klebsiella*), together with reduced abundance of genera of *Bacteroides* and *Alistipes* [82,83].

1.5 Role of the gut microbiota in human health

Evidences for the role of the microbiota in human health derive from comparative studies of mice under germ-free versus conventional microbiota conditions, from in vitro studies using human faecal incubations, or complex culture gut models and from clinical studies comparing the gut microbiota in healthy patients and in patients affected by several disorders. Main roles of gut microbiota are to metabolize foods and provide nutrients, to protect against pathogens, to maintain the integrity of the mucosal barrier and to regulate both local and systemic immune functions [5,79].

The GI microbiota synthetizes autonomously vitamin B12, lactic acid bacteria, folate (*Bifidobacteria*), vitamin K, riboflavin, biotin, nicotinic acid, panthotenic acid, pyridoxine and thiamine [5,9,84,85]. Colonic bacteria can also metabolize bile acids eventually not previously reabsorbed [5,85,86] and express carbohydrate-active enzymes needed to ferment carbohydrates, whose degradation leads to the generation of metabolites such as SCFAs [5,79,85]. The main SCFAs in colon are propionate,

butyrate and acetate, which are respectively mainly produced by *Bacteroides*, *Firmicutes* and several different gut anaerobes [1,5,79,85]. These fatty acids are absorbed by GI tract epithelial cells and exert several actions, such as production of energy sources [79,87], as shown by the conversion of propionate to glucose in intestinal gluconeogenesis [79,88,89], stimulation of the proliferation and turnover of colonocytes (butyrate) [90], improvement of the gut barrier function [88], regulation of the tight-junction assembly and stimulation of the mucin synthesis (butyrate) [88,91], activation of liver gluconeogenesis (oropionate) and lipogenesis (acetate and butyrate) [88].

Moreover, they regulate inflammatory and immune response [79], [79,92-94] and are able to epigenetically control gene expression through their capacity to act as histone deacetylase inhibitors [79,88].

As to the regulation of the epithelium turnover, besides the indirect above-mentioned role exerted by butyrate, gut microbes can directly modulate epithelial cell and mucus layer proliferation, thus modulating the ability of other pathogens or commensals to colonize and proliferate. For example, *Lactobacilli rhamnosus* GG [95] *A. muciniphila* [96] and *Lactobacillus plantarum* [97] have been reported to modulate cell turnover, while *A. muciniphila, B. thetaiotaomicron* and *F. prausnitzii* regulate mucus layer [98-100].

The colonic microbiota can also convert ingested dietary protein and endogenous proteins (enzymes, mucin, and death intestinal cells) into shorter peptides, amino acids and derivatives, short and branched-chain fatty acids, and gases, including ammonia, H_2, CO_2, and H_2S [85,101].

Interestingly, gut microbiota is also involved in the metabolism of several polyphenols, including flavonoids, isoflavones, lignans, hydroxycinnamic acids, ellagotannins, and anthocyanins, which are usually poorly absorbed in the small intestine and in the colon [85,102], hence affecting their bioactivity [85,103,104]. Human dietary supplementation trials and in vitro evaluations regarding the faecal metabolism of dietary plant polyphenols pointed out large inter-individual variations in absorption, metabolism, and excretion, which could be related to differences in the species composing the gut microbiota [105-109].

Among the roles of gut microbiota, it is noteworthy and crucial for human health its ability to modulate bot immune and adaptive immunity, both at local and systemic level. Overall, gut microbiota is able to regulate immune homeostasis leading

to immune-stimulatory effects, inducing T reg activation, stimulating neo angiogenesis, determining anti-inflammatory effects or strengthening the intestinal epithelium barrier functions [79]. Evidences for these functions come from in vitro studies and germ-free mice models.

As regards to **the innate immunity**, the first line response to microbes depends on the presence of a family of receptors called pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) or nucleotide-binding oligomerization domain-like (NOD-like) receptors [5,29,79,110]. Such receptors sense and recognize specific molecular effectors expressed by intestinal microbes known as pathogen-associated molecular patterns (PAMPs) [5,79,110]. The activation of such receptors by PAMPs triggers immune procedures able to discriminate between beneficial and pathogenic bacteria, modulating the activity and recruitment of specific immune cells in order to elicit tolerance or elimination [5,79,111]. One of the most recognized kind of PAMPs are the unmethylated CpG dinucleotides, present both in prokaryotes [79,112] and eukaryotes cells. Mammalian cells express mostly specifically methylated (mt) DNA [79,113,114] in case of an apoptosis, necrosis or autophagy, with a consequent prompt recognizing by immune cells [79,114]. Interestingly, gut flora DNA can at the same time elicit also immunotolerance, through the presence of immunosuppressive DNA fragments, able to suppress dendritic cells activity and to activate Treg cells [79,115]. Among other important PAMPs can be mentioned the flagellin, that is expressed by flagellated bacteria and is detected by TLR5 [29,116] and the lipopolysaccharide (LPS), which is expressed by Gram negative bacteria and is sensed by TLR4 [29,117]. The development of variants in the flagellin or LPS structures is a strategy used by microbes to escape TLRs recognition and immune cell activation. Other species-specific antigens are the polysaccharide A (PSA), from *Bacteroides Fragilis*, which activates CD11c+ dendritic cells through a mechanism involving TLR2 [118] and *Segmented Filamentous Bacteria* (SFBs), a spore forming Clostridia-related gut commensals, which induce a strong immune response [29,119-121] through the activation of the dendritic cells and the consequent release of IL-23 [79,119-121], able to further activate a Th17 response [79,119-122]. It has been shown that mice only colonized by SFB show the same immunocompetence level as compared to mice colonized by a complex and complete gut microbiota [114-120]

Hence dendritic cells represent a crucial immune element connecting microbes with adaptive immunity, since they can process and present microbial antigens to adaptive immune promoting tolerance or elimination.

The GI microbiota also stimulates human immune and epithelial colonic cells to produce specific antimicrobial compounds, such as cathelicidins, C-type lectins and pro-defensins by the host Paneth cells through a mechanism involving PRRs [5,123]. The interaction between PRRs and PAMPs triggers several signaling pathway finally leading to an enhancement of mucosal barrier function and to the release of mucins and secretory mucosal IgA [5,124]. Such events result in a protection against invading pathogens and limit the overgrowth of the commensals [5,124].

Even if poorly studies in humans, a new class of three different subsets of lymphoid cells, called innate lymphoid cells ILC1s, ILC2s and ILC3s, exists and has been recently detected on the gut mucosal surface [125-127]. Human ILCs express PRRs[127] and promote responses to various harmful events through the release of soluble factors such as cytokines and other peptides [127]. Among these there are known effector cytokines, such as IFN-γ and TNF, for ILC1s; IL-5, IL-9 and IL-13, for ILC2s; and IL-17, IL-22, GM-CSF and IFN-γ, for ILC3s [127]. The release of these mediators in turn recruits and activates several adjunctive immune cells [127].

In addition to the above mentioned recently identified cells, also intra epithelial lymphocytes such as γδ T cells and NK T cells represent a crucial defense against pathogens and can regulate gut microbiota [125]. In turn, gut microbiota composition modulates the function of innate immunity, as shown by the influence of microbes on the number and function of NK cells in the first phases of human life [125,128].

Furthermore, a population of CD103+ dendritic cells and of CD11c+ macrophages are specifically involved in immune tolerance or immune response toward gut microbes [125]. It is noteworthy that the ability of dendritic cells or macrophages to affect immunity and inflammation depends on the presence of butyrate, produced by a specific gut microbial signature [125,129], or on the presence and amount of microbial microRNA [125,130].

As regards to the immunomodulatory activity of microbial metabolites, recent findings pointed out that some molecular compounds called quorum sensing (QS) [79,131], which are usually secreted by microbes to form a protective biofilm or to trigger the expression of virulence factors [131-133], are able to impair the function of macrophages and dendritic cells [79,134,135], hence escaping immune system (*P.*

Aeruginosa, Gram negative) [79,134,136], or to downregulate the expression of pro-inflammatory mediators and to upregulate the expression of the immunosuppressive cytokines (*B. Subtilis*, Gram positive) [79,137] by human intestinal epithelial cells.
Another interesting immunomodulatory agent is indole [79,133], which physiologically reaches elevated levels in human gut and seems to regulate the interaction between microbial commensals [133]. It can reduce the expression of pro-inflammatory mediators such as TNF-α and IL-8 [79,138], or increase the expression of the immunosuppressive cytokine IL-10 [89,138].

As regards to the **adaptive immunity**, both T and B cells activities are modulated by gut microbiota and are crucially involved in tolerance or response toward microbes.
T cells function and differentiation for instance are modulated by ATP produced by *Segmented Filamentous Bacteria (*SFBs) [125,129,130], which, as already mentioned, strongly activate Th17 cells through a mechanism involving IL-6, IL-23 and TGF beta release and dendritic cells recruitment [139,140]. SFBs colonization is also able to promote the production of mucosal secretory IgA and to regulate the differentiation both of Th1 and Th2 [79,141].
Concerning T regs, such cells are abundant in the lamina propria, where they regulate microbial homeostasis and suppress inflammation [139]. Human *Clostridia* activate T regs proliferation through a process involving TGF beta [139,140]. Furthermore, in vitro studies performed on T reg cultures show that the addition to the medium of SCFAs elicits the activation of T regs and the release of the immunosuppressive cytokine [139,142]. SCFAs also inhibits histone deacetylase inhibitors in Tregs, thus inducing their hyperactivation [139,143]. Not only their metabolites, but also commensal microbes themselves are able to contribute to a homeostatic proliferation of $FOXP3^-$ CD4+ T cells and $FOXP3^+$ T regs [79,144].
Among other metabolites able to modulate T cells activity,polysaccharide A, produced by *B. Subtilis* during fermentation of soybean [79,145], regulates Th1/Th2 development, modulates Th17 activation [79,145] and lead to the differentiation and recruitment of Tregs [79,146].
The Th17 response is particularly stimulated in case of an interaction with fungi. First line defense toward fungi is the epithelium, which secretes anti-microbial peptides, and the activation of an innate immunity, in which monocytes, macrophages, neutrophils, endothelial and epithelial cells respond to fungal wall components or other

fungal products through specific PRRs, such as C-type lectin receptors, TLRS, or galectin family proteins [17,147,148]. Such a stimulation results in phagocytosis and direct cell killing. Activation of this immune response can recruit dendritic cells, which uptake fungi and trigger an antigen specific adaptive immune response, in which naïve T cells are driven toward a differentiation in effector Th1 cells releasing IFN-γ, and Th17 cellssecreting IL17A, IL22 and IL17F [17].

As regards to B cells, microbes strongly activate their IgA release in response to possible other pathogens [125,149]. IgA exerts several important roles in gut homeostasis and immune response, such as mucus anchoring by bacteria [150], opsonization [125,149,150] and the expression of pro-inflammatory structures of bacteria [125,151]. Mouse models lacking the ability to produce IgA display an increased proliferation of pathogens, such as SFBs [152]. Furthermore, in the absence of a proper microbiota, B cell function is impaired and IgA production is switched toward an IgE release [125,153], with consequent risk of allergic diseases through an activation of basophils and mast cells in mouse models [153,154].

2- Gut microbiota and cancer

Several evidences point out a possible role of gut microbiota in modulating pro-oncogenic or anti-cancer mechanisms. Such proofs derive from studies on mice selectively grown on germ free conditions and subsequently colonized by selected microorganisms, or human or mice treated with antibiotics. Recently, also clinical epidemiological or observational studies identified differences in gut microbiota composition in humans selected for the presence of a specific tumour as compared to healthy controls.

It is widely recognised that certain microbes, both viruses and bacteria, have a clear role in the aetiology of specific cancers. At present, it has been estimated that 20% of tumour arises from a specific and unique colonising pathogen [155,156] and a list of microbes designated as class 1 agents, thus carcinogens, has been drawn up by the International agency for Research on Cancer [IARC] [155,156].

Among them it can be recognised *Helicobacter Pylori*, as the etiological factor of gastric cancer [155-158], which however has been reported to have a protective role toward oesophagus cancer, possibly because of its induction of changes in the pH and reflux [155,157].

Other recognised cases of cancer specifically triggered by selected pathogens are liver cancer [HBV, HCV, *Opisthorchis viverrini, Clonorchis sinensis*], bladder cancer [*Schistosoma haematobium*], adult T-cell lymphoma [Human T-cell lymphotropic virus type 1 (HTLV-1)], nasopharynx, non-Hodgkin and Hodgkin lymphoma [Epstein-Barr Virus, (EBV)] and cervix, vagina, vulva, anus, penis and oropharynx carcinomas [human papilloma virus, (HPV)] [155,156].

Recent findings coming from the advent of metagenomics suggest some cancers might be due to an altered composition in the whole gut microbiota [155,157-159], rather than to a single pathogen, in a condition known as dysbiosis. Such a suggestion is clear and easily acceptable in consideration of the plethora of effects exerted by gut microbes and their metabolites on human biology, especially on immune regulation.

The organs in which the above-mentioned relation is more evident are those in which the bacterial density is highest, such as gut, lung and urogenital tract. There are several evidences for a decreased microbial diversity in patients affected by colorectal cancer [155,157,160-162], in which members of the phyla *Firmicutes* and *Bacteroidetes* are reduced, and the phyla *Proteobacteria*, *Actinobacteria*, *Fusobacterium*,

Verrucomicrobia and *Cyanobacteria* are increased [160,163-165]. Even among the same affected individual, healthy colonic sites adjacent to colon cancer display increased *Bacteroides* and *Firmicutes*, and reduced *Fusobacterium*, as compared to carcinomatous sites [155,165-167].

Even if the results are contrasting and the studies are lacking, due to the difficulties of sampling, it has been proved that lung harbours a unique microbiota, dominated by *Proteobacteria* [160,168,169]. Patients affected by chronic obstructive pulmonary disease [COPD], one of the recently identified risk factor for lung cancer [160,169,170], have exacerbations of COPD episodes after *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis* infections, hence suggesting a possible yet to elucidate pro-oncogenic role of lung microbiota in pulmonary cancer.

As to urogenital tract, the healthy urinary tract was considered so far as sterile [171]. However, recent next generation sequencing evaluations identified a specific urinal microbiota characterized by the main presence of *Gardnerella*, *Lactobacillus* and *Prevotella* [171-173]. In addition, *Salmonella enterica* subsp. *enterica serovar Typhi infection* is also associated with gall bladder cancer [173,174], probably through mechanisms involving the release of of β-glucuronidase, the deconjugation of bile acids and toxins and subsequent toxic carcinogenic actions on the epithelium of gall bladder [173,174,175].

From a molecular and pathogenetic point of view, microbes can modulate cancer onset and progression, or can protect against tumorigenesis through a wide range of mechanisms. Some of them have been discovered only in recent times and are yet to be fully elucidated.

Overall, microbiota composition can affect cancer course modulating immune system and inflammation, affecting cell signalling pathways, inducing genotoxic effects and releasing pro-tumorigenic or tumour-suppressing metabolites [155,157,159,160, 174,176,177].

One of the most important drivers of tumorigenesis is inflammation, and several cancers arise from causal chronic inflammatory processes [174]. Moreover, microbiota seems to be involved in pro-oncogenic inflammatory process due to a host response toward the pathogen [174,176].

Studies focusing on knockout mice for NOD and Toll like receptors and evaluating the effects of pro-carcinogenic inflammatory stimuli in the tumorigenesis of colorectal

cancer suggested a possible causal role of a specific microbiota capable of affecting immune processes and thus contributing to colorectal cancer [174,178-185]. It is noteworthy that the inflammation-induced colorectal cancer driven by an intestinal dysbiosis could be transmitted between mice [174,186,187].

Another study showed that *Enterobacteriaceae* family were highly upregulated in the colons of IL-10 knockout mice with colitis, as compared to wild-type controls without colitis [188, 159]. In this study, the presence of *E. coli* belonging to *Enterobacteriacee*, correlated with the onset of colorectal cancer after stimulation with procarcinogen azoxymethane in the IL-10 knockout mice with colitis. At the basis of the pathogenicity of *E.coli* there was the production of a genotoxin that could induce tumor formation through a DNA damage. Interestingly, E. coli strains have been found to be highly represented in the gut microbiota of patients with inflammatory bowel disease and colorectal cancer versus controls [159,188,189].

Furthermore, other studies have shown the presence of high levels of *Fusobacterium nucleatum* in human colorectal carcinoma samples [174, 190]. Such pathogen releases a toxin known as FadA which can bind E-cadherin on epithelial cells, hence triggering the activation of β-catenin signalling, finally enhancing epithelial cell proliferation [174,191, 160, 192, 157]. Another bacterium, the *Enterotoxigenic Bacteroides fragilis* [ETBF], can stimulate the development of colon cancer, through a mechanism involving a toxin called Btf which increases the production of reactive oxygen species (ROS) [160,174,192-195]. The same toxin has been found to also cause Th17-mediated colitis.

Hence, microbiota drives tumorigenesis also through the release of genotoxins, namely toxins able to damage DNA through a wide range of mechanisms including increasing ROS levels, loss of cell polarity and dsDNA damage and rupture, finally leading to a genomic instability. Other pro-tumorigenic genotoxins are AvrA [160,192,196] produced by *Salmonella Typhi*, involved in hepatobiliary cancer, and Cdt Cytolethal Distending Toxin, produced by several microbes including *Epsilonproteobacteria* and *Gammaproteobacteria*, able to induce strong genomic instability [160,197,198].

As regards to the release of pro-tumorigenic metabolites, as partly already mentioned for inflammation, an important source of oncogenic molecules comes from the metabolism of proteins, namely deriving from red meat. Amino acids from proteins are fermented by *Firmicutes* and *Bacteroides* into N-nitroso compounds, which induce DNA alkylation and mutations in the colonic cells [155, 199,200]. In addition, the

carcinogenic heterocyclic amines of charred meat are metabolized by colonic bacteria and transformed in electrophilic metabolites, able to induce DNA damage [155,201]. During this process, an increased production of bile acids conjugated to taurine or glycine can be found in the liver, which are secreted into the GI tract. An amount of such bile acids can be reabsorbed by the colon, where microbes reconvert them into secondary bile acids, such as deoxycholic acid [DCA] by *Clostridium Scindens*. Both DCA and taurine are genotoxic and damage DNA, by generating ROS and the genotoxic metabolite hydrogen sulphide respectively [155,202].

Given the ability of metabolites and immune cells to be absorbed by blood and lymphatic circulation and to reach the liver through enterohepatic circulation, and of microbes to translocate, it is reasonable that gut microbiota alteration could exert pro-tumorigenic effects also on distant organs.

The intestinal microbiota indeed has been found to contributes to the development of hepatocellular carcinoma through the induction of TLR4 signalling, which promotes the carcinogenetic hepatomitogen epiregulin in the liver [174, 203]. It is noteworthy that antibiotics limit the anti-apoptotic effect of microbiota-triggered TLR4 signalling [174, 203].

Breast cancer could be affected by gut microbiota since some intestinal microbes are involved in oestrogens metabolism [155, 204,205] and because a specific GI bacteria, *Helicobacter hepaticus*, has been reported to be involved in mouse breast cancer through a TLR5 activation. It is noteworthy that mutation on TLR5 have been reported to be associated with long-term survival in patients with ovarian cancer [155, 206].

Equols, which are protective gut derived metabolites [207-210] produced by several bacteria including bacteria *Enterococcus faecium* strain EPI1, *Lactobacillus mucosae* strain EPI2, *Finegoldia magna* strain EPI3, and *Veillonella sp*. [207, 208], have been found in breast tissue, as well as in blood, urine and prostatic fluids [155, 207-210], hence suggesting a possible protection toward cancer in these organs.

3. Gut microbiota and autoimmune diseases

Immune system is set to provide a prompt response toward pathogens and to contemporarily maintain a tolerance toward self-components.

Auto-reactive T cells are usually destructed early in life during thymic education [211]. Some of them however can by pass such process of control and reach the circulation as mature T cells [211]. In case of failure of other regulating mechanism, such as release of immunosuppressive cytokines, involvement of suppressing T regs, induction of functional anergy and induction of apoptosis, and in the presence of concomitant genetic and environmental factors, the expansion of autoreactive T might arise and an autoimmune disorder might develop [211-213].

In the light of the known role of gut microbiota in regulating adaptive immunity, several studies investigated its possible causal role in the development of autoimmunity and recognized several gut microbes as possible triggering factors of autoimmune disorders [212-217].

The most convincing and exhaustive evidences come from experimental models of mice genetically predisposed to the development of specific autoimmune disorders and from observational human sequencing studies comparing patients affected by autoimmune diseases with healthy controls.

Type 1 Diabetes (T1D) is an autoimmune disorder characterized by the selective destruction of insulin producing β cells of the pancreas by selective autoreactive T CD4+ cells and Th17 cells [218-220]. Segmented Filamentous Bacteria have been reported to be protective toward the development of Type 1 Diabetes in non-obese mice [217,221). Furthermore, the immune activation of antigen presenting cells triggered by the sensing of microbial peptides by TLRs seems to have a crucial role in the development of Type 1 Diabetes in non-obese mice [218,222-224]. Accordingly, the experimental model of mice lacking the protein MyD88, a crucial component of TLR2 which is involved in its activation [225], do not develop Type 1 Diabetes [218,223].

Microbes seem to be involved also in Rheumatoid Arthritis (RA) onset and severity, as suggested by mouse models.

RA is a chronic autoimmune disease characterized by the progressive destruction of cartilage and joints, because of the release of autoantibodies by activated T cells, of

proinflammatory cytokines by macrophages and an involvement of Th17 cells [218, 226].

IL-1 receptor antagonist deficient (IL1rn -/-) mice are a mouse model which spontaneously develop T cell mediated autoimmune arthritis and are susceptible to other autoimmune diseases such as psoriasis, diabetes, and encephalomyelitis [218,227,228]. In germ-free condition, such mice do not develop the disease, and interestingly the contemporary TLR2 deficiency in IL1rn -/- mice induces a Treg suppression and a more severe arthritis [218,227] due to a consequent Th17 activation and IL-17 release [218,227,229]. Other studies on humanized transgenic model of RA pointed out that arthritis-susceptible (DRB1*0401) and arthritis-resistant mice display different gut microbiota compositions [230,231]. Finally, other interesting experimental works showed that the autoimmune arthritis in K/BxN mice under GF conditions was less severe and was associated with less autoantibodies production and a reduction in splenic Th17 cells, which are usually responsible indeed of the pathogenesis of the disease [211, 218, 232-234]. In one of these models, the re-colonization with SFB is able to induce the disease through the differentiation of Th17 cells, which circulate toward the joints and trigger the disease [233].

Other recent studies on Il1rn -/- mice identified a crucial role of interleukin-1 receptor antagonist (IL-1Ra) in regulating gut microbiota, namely in maintaining the natural diversity and composition, and confirmed the role of TLR4 in mucosal Th17 cell induction in autoimmunity [228].

Also, multiple sclerosis (MS), an autoimmune disease in which autoreactive Th1 and Th17 cells infiltrate the central nervous system thus inducing axonal damage and demyelination [218,235], has been studied on experimental mice models. Experimental models show that gut microbes and their activation of innate system, seem to have a crucial role in the pathogenesis of the diseases. Indeed, it has been shown that gut microbiota restoring with *Bacteroides fragilis* in mice previously antibiotic-treated is able to protect against experimental autoimmune encephalomyelitis (EAE) [236], an experimental murine model of MS. Such event is due to the recruitment of IL-10-producing Treg cells [218, 236] triggered by the presence of *Bacteroides fragilis* PSA [218, 237]. On the contrary, the interaction between polysaccharides A with TLR4 stimulates in vitro differentiation of Th17 cells, with a consequent triggering action [218, 238]. Overall, the above-mentioned studies show that protective or causal role of gut peptides on MS pathogenesis depend on the

kind of TLRs involved in innate immune activation. Further, it has been reported that microbial metabolites of dietary tryptophan can modulate astrocyte activity and central nervous system inflammation in mice models of autoimmune encephalitis [239,240]

Systemic Lupus Erythematosus (SLE) is a heterogeneous autoimmune disease involving several organs with a variable clinical course. The diagnosis is based on serological parameters such as antinuclear antibodies (ANA), mainly to dsDNA, and on specific clinical findings of the skin, joints, kidneys, and the central nervous system [241].

Emerging evidences suggest a possible role of gut microbioma in SLE, even if its causal involvement is less known and in part controversial, as compared to other autoimmune disorders.

Female MRL/ Mp-Faslpr (MRL/lpr) mice, which are lupus prone, have gut microbiota alterations, with increased *Lachnospiraceae* and decreased *Lactobacillaceae* [215,242], as compared to controls [215,242]. Other studies showed that germ free GMZ mice, which spontaneously develop lupus, have higher ANA levels but lower IgG levels, while on the contrary other studies showed that the same kind of mouse model develop a less severe associated nephritis in case of germ free conditions and antigens free diet [217,243]. Further, a recent interesting investigation identified a marked depletion of *Lactobacillales* in the gut microbiota of a mice model of lupus nephritis (MRL/lpr) [244]. Interestingly, an experimental increase in *Lactobacillales* in the gut through feeding improved renal function of these mice and prolonged their survival, due to a decrease of IL-6 and an increase of IL-10 production in the gut, an increase of IL-10 in circulation and a shift in Treg-Th17 balance towards a Treg phenotype which developed directly in the kidney [244].

As regards to humans, some comparative studies evaluating gut microbiota in autoimmune patients and controls identified differences in the microbial composition and in some cases correlations with clinical features of the disease.

A study on a cohort of Finnish subjects genetically prone to Type I diabetes (high-risk HLA) [217,245] pointed out increased *Bacteroidetes* and decreased *Firmicutes* in children with autoantibodies, as compared to healthy controls, with an overall decreased bacterial diversity and reduced stability over time in Type I diabetes prone subjects [245]. Such study was partially confirmed by another subsequent investigation, which revealed an increase in the abundances of *Bacteroidetes* and

Clostridium spp. associated with a reduction in the genera *Lactobacillus* and *Bifidobacterium* [246].

Other studies reported the absence of *Bifidobacterium* species and confirmed increased levels of the genus *Bacteroides* in children with two or more islet autoantibodies [247], while a recent investigation pointed out that subjects with autoantibodies and new-onset patients had different levels of the *Firmicutes* genera *Lactobacillus* and *Staphylococcus* , as compared with healthy control subjects with no family history of autoimmunity [248].

Finally, other evaluations also found a decrease in butyrate-producing bacteria in the Type I diabetes seropositive group [249].

Concerning RA, bacteria pertaining to the *Clostridiales*, usually detected in healthy human gut, have been detected in the synovial fluid of RA patients, suggesting how gut microbes could possibly translocate towards peripheral tissues [230,250]. Intestinal microbiota of new-onset untreated rheumatoid arthritis has been found to be characterized by a strong presence of *Prevotella copri*, as compared to controls [251] Furthermore, RA patients have significantly less bifidobacteria and bacteria of the *Bacteroides-Porphyromonas-Prevotella* group, *Bacteroides fragilis* subgroup, and *Eubacterium rectale--Clostridium coccoides* group as compared to fibromyalgia group [232]. Finally, even if its anti-rheumatic effect has mainly been linked to the capacity to inhibit matrix metalloproteinases [215,252], it is noteworthy that minocycline, which is a tetracycline derived antibiotic, is able to reduce RA symptoms [215,253].

Investigations on human gut microbiota have also been performed on MS patients, in which differences in archaea, clostridial and butyrate-producing bacteria where found, as compared to healthy controls [254]. Other investigations pointed out an increased abundance of *Pseudomonas*, *Mycoplasma*, *Haemophilus*, *Blautia*, and *Dorea* genera in MS patients, while on the contrary controls showed increased abundance of *Parabacteroides*, *Adlercreutzia* and *Prevotella* genera [255] Recently, MS patients with high disease activity and increased intestinal Th17 cell frequency have been found to display a higher *Firmicutes*/*Bacteroidetes* ratio, associated with an increased relative abundance of Streptococcus and decreased *Prevotella* strains, when compared to healthy controls and MS patients with remitting disease [256]. Interestingly, the intestinal Th17 cell frequency in active MS patients was inversely related to the relative abundance of *Prevotella* [256]. An association between immune markers and a specific microbial gut signature was also found in a recent investigation in children

affected by MS [257]. At the basis of an altered microbiota in MS patients, there could be an altered intestinal permeability, as demonstrated by a recent investigation [258].

A recent study on 21 systemic lupus erythematosus patients and controls pointed out that Firmicutes to Bacteroidetes ratio and the production of short chain fatty acids were altered in SLE patients, as compared to controls [259].

Another study in SLE patients from Spain also found a depletion of Firmicutes and an enrichment of *Bacteroidetes*, together with an overrepresentation of genes associated with glycan metabolism and oxidative phosphorylation [260]. These findings were recently confirmed also in Chinese patients, in whom adjunctive species such as *Rhodococcus*, *Eggerthella*, *Klebsiella*, *Prevotella*, *Eubacterium*, *Flavonifractor* and *Incertae sedis* were significantly enriched, while genera *Dialister* and *Pseudobutyrivibrio* were significantly depleted [261]. Interestingly, a study performed on in vitro cultures showed that microbiota coming from SLE patient stool samples promoted lymphocyte activation and Th17 differentiation from naïve CD4(+) lymphocytes to a greater extent than healthy control-microbiota [262].

As regards to Behçet disease, which is an inflammatory disease characterized by muco-cutaneous and ocular manifestations, possibly involving central nervous system, vascular and/or gastro-intestinal districts, an alteration of gut microbiota was also found. Namely, a study performed on 22 patients with Behçet syndrome and that of 16 healthy co-habiting controls pointed out a depletion in the genera *Roseburia* and *Subdoligranulum* in Behçet's patients, as compared to controls Behçet's syndrome patients exhibit specific microbiome signature [263]. Of interest is the finding of this study of a significant decrease of butyrate production in Behçet subjects.

Another recent investigation found a significant increase in the genera *Bifidobacterium* and *Eggerthella* and a decrease in the genera *Megamonas* and *Prevotella* in a group of 12 Behçet disease subjects, as compared with 12 normal individuals [264].

A common feature of all the so far mentioned autoimmune disorders is a gender bias affecting their incidence.

Overall, studies on humans and mice indeed show that females are 2-10 times more susceptible to autoimmune disorders such as RA, MS, SLE, myasthenia gravis, Sjogren syndrome and Hashimoto’s thyroiditis [230].

Gut microbiota composition could also contribute to explain the gender bias in the incidence of organ and non-organ specific autoimmune disorders [216,230,265]. In

fact, both microbes and hormones indeed are able to interact and modulate each other [230,216], since some bacteria are able to produce and metabolize hormones, while in turn specific sexual hormones are able to influence immunity and hence to indirect modulate microbiota composition [216,230,265,266]. While investigations on human gut microbiota comparing male and female compositions in autoimmune disorders are still lacking and inconsistent, mice models clearly demonstrated differences in gut microbiota between males and females, which predispose to the development of autoimmunity. The most convincing evidences come from investigations of mice models of type I diabetes [230,265,266], RA [230,231], and SLE [215,242].

4. Gut microbiota and dermatological diseases

In the last decades, several experimental studies pointed out a possible role of gut microbiota in triggering dermatological diseases. Accordingly, it has been postulated and widely sustained the existence of a gut-skin-axis, meant as a continuous, massive and complex interplay between skin and intestinal microbes, able to maintain skin homeostasis and whose perturbation leads to the onset of dermatological diseases [267,268].

The first suggestion of a strict interplay between skin and microbes come from the empirical observations of D. Strachan, who pointed out that infants with a high number of siblings had a decreased risk of developing atopy, including eczema. Hence, he postulated that the increase of atopic disorders in the industrialized countries could originate from a deprivation of microbial exposure in infancy, which could finally lead to an immune dysregulation and to an aberrant response to innocuous antigens later in life (hygiene hypothesis) [268-272].

Interestingly, subsequent recent investigations on gut microbiota colonization really pointed out that infant in westernized countries are colonized later and display a reduced turnover of specific bacterial strains than children in developing countries [273-275].

As to the hygiene hypothesis, recent studies suggest that more than the development of an aberrant response to antigens, a rupture of the tolerance mechanisms happens. Namely, cells involved in immune tolerance, such as Tregs and regulatory Antigens Presenting Cells, could be subjected to an impaired maturation because of a reduced exposure to certain microbes, leading to the consequent triggering of Th1 or Th2 responses [268,276].

Interesting evidences of a possible dysbiosis in skin disorders come from investigations on stool samples of patients affected by **atopic dermatitis**, through culture-dependent methods and culture independent approaches, the latter performed using PCR, FISH, IgG serology (on serum) and metagenomic (NGS) [273].

As to culture dependent methods, investigators identified different colonization patterns when comparing patients affected by atopic dermatitis and healthy controls. Overall, a low prevalence of *Bifidobacteria* [273,277-279] and *Lactobacilli* [273,277] together with a high prevalence of *S. Aureus* [273, 277-279] and *Clostridia* [273,277,280] were found in fecal samples of atopic patients, when compared with

healthy subjects. Consecutive culture-independent studies increased the knowledge about to gut microbiota in atopy. Cross-sectional studies assessing microbes using FISH techniques pointed out no differences in concentration of specific gut microbes among atopic dermatitis patients and controls, even if a higher bacterioides count and a lower bifidobacterial levels correlated with the severity of the diseases in the disease group [281]. On the contrary, an interesting prospective study using FISH methods on stool samples of infants at high risk for allergy, pointed out a lower bifidobacterial/clostridia ratio in subjects who subsequently developed atopy [282]. In addition, PCR-DDGE methods have been applied to identify differences in gut microbiota among atopic dermatitis patients and controls. Among them, of interest is the Dutch KOALA birth study, due to its prospective design. This study examined the gut microbiota composition in 957 faeces samples of infants aged 1 month and the subsequent development of atopic manifestations and sensitization at 12 months [268]. According to this evaluation, the presence of *Escherichia coli* was associated with a higher risk of developing eczema, while infants colonised with *Clostridium difficile* were at higher risk of developing both eczema, recurrent wheeze and allergic sensitization [268].

Among *Clostridia*, the presence of *C. difficile* was associated with a higher risk of a diagnosis of atopic dermatitis [268].

The recent advent of metagenomic definitely disclosed the features of gut microbiota in atopy. An evaluation on 90 subjects with atopic dermatitis identified a dysbiosis affecting the *Faecalibacterium Prausnizii* subspecies-level. This condition results in a reduced production of butyrate and propionate, which are short chain fatty acids with known anti-inflammatory properties, and causes a consequent intestinal epithelial damage, possibly allowing the passage of toxins and pathogenic microbes in circulation, which finally reaches skin and triggers atopy [283]. Another NGS study on a lower number of patients analyzed early microbial gut composition and its correlation with IgE-associated eczema and innate immune responses, the latter assessed as cytokine production in response to microbial TLR4 and TLR2 ligands [284]. Authors pointed out a dysbiosis and an immune activation in atopic patients, since they observed an increased relative abundance of Gram-positive *Ruminococcaceae* at 1 week in infants developing IgE-associated eczema, as compared to controls. Such increase was inversely associated with TLR2-induced releases of IL-6 and TNF- α [284]. In such a group, at 1 week, was also found an

inverse association between the abundance of *Proteobacteria* (comprising Gram-negative taxa) and TLR4-induced TNF- α secretion [285]. This relationship persisted at 1 month, while at 1 year, a-diversity of *Actinobacteria* was lower in infants with IgE-associated eczema, as compared to controls [284].

Gut microbiota composition was evaluated also in **psoriasis**. Some researchers performed PCR analysis on fecal samples of patients affected by psoriasis, inflammatory bowel disease and hidradenitis suppurativa [285]. In this study, a significantly lower abundance of *F. prausnitzii* was found in psoriasis, as compared healthy controls [286]. Interestingly, authors found comparable results in the group affected by inflammatory bowel disease [285]. In addition, psoriatic patients had a significantly higher abundance of E. coli, while no significant difference in *F. prausnitzii* or *E. coli* abundance were found in hidradenitis suppurativa group [285]. Since patients affected by inflammatory bowel disease have an increased incidence of psoriasis [286], the finding of a common gut microbiota signature among these diseases strengthens the hypothesis of the existence of a gut-skin axis. Another PCR study also found a condition of dysbiosis, suggested by an altered Bacteroides/Firmicutes ratio, in psoriatic patients [287]. Interestingly, the presence of Actinobacteria, which are a group of microbes owning anti-inflammatory properties, inversely correlated with PASI (psoriasis activity severity index) [287].

More recently, studies on antibiotic treated and control mice found that antibiotic treatment led to a resistance to develop psoriasis-like skin inflammation, which was experimentally triggered by imiquimod cream application [288]. The evaluation of the stool samples of such antibiotic treated and controls mice through NGS techniques pointed out that antibiotic treatment induced an increase in *Lactobacillales* and a decrease in *Coriobacteriales* and *Clostridiales*. Since *Lactobacillacee* can suppress the IL23/Th17 axis [289], which has a crucial role in the development of psoriasis [290], their increase in antibiotic treated mice might be at the basis of their resistance to the growth of psoriasis-like skin inflammation [288].

As to humans, metagenomic evaluations of stool samples was conducted both in psoriatic and psoriatic arthritis patients. Accordingly, a recent evaluation identified a relative decrease in *Coprococcus* spp. in both groups, as compared to controls, while psoriatic arthritis samples were further characterized by a significant reduction in *Akkermansia*, *Ruminococcus*, and *Pseudobutyrivibrio* [291]. Interestingly, again the

results obtained from the psoriatic arthritis group resemble those so far reported in inflammatory bowel disease patients [292].

A metagenomic study aiming to assess gut microflora composition was performed on twelve Korean patients affected by **rosacea** [293]. Authors found a dysbiosis in the rosacea group, since they observed different compositions of enteral microbiota in roseacea patients as compared to rosacea-free controls [293]. Patients with rosacea had reduced abundance of *Peptococcaceae* and *Methanobrevibacter* and an increased abundance of *Acidaminococcus* and *Megasphaera* [293]. This study is the first assessing gut microbiota in rosacea and shows promising results, even if it should be validated by further studies in a larger group of patients.

Finally, metagenomic approaches have been recently applied to firstly identify the gut microbiota composition in experimental mice models developing **alopecia** [294]. In a recent study authors focused on the metabolism of biotin, which is a vitamin involved in hair growth and homeostasis [295]. Humans and mice are not able to autonomously synthetize it; hence biotin levels decrease unless this vitamin is introduced by diet or synthetized by specific gut microbes in condition of eubiosis. Authors found that vancomycin treatment, associated with a biotin deficient diet, induced the overgrowth of intestinal *L.Murinus,* and led to the development of a kind of alopecia in which hair follicles were retained and congested in a proliferating anagen phase. As to *L. Murinus*, it is noteworthy that this microbial specie beyond its inability to produce biotin, has the capacity to consume biotin eventually supplied with the diet. Overall, this study indicates that a gut dysbiosis could induce alopecia through a biotin deficiency mechanism, induced by the over growth of microbes unable to synthetize biotin or by species even able to consume such vitamin.

In addition, recently it has been reported the hair re-growth in two cases of alopecia areata universalis (an autoimmune non-scarring alopecia) treated with faecal transplantation for recurrent infection with *C. Difficilis* [296].

5. Gut microbiota and melanoma

Melanoma is a malignant tumour arising from melanocytes. It causes the greatest number of skin cancer-related deaths worldwide and despite the recent advances in therapeutic options, its prognosis in advanced stages remains unfair [297-299].

Hence, early detection of melanoma still represents the best means of reducing mortality. Before 2011, treatment options for patients with advanced melanoma were limited to chemotherapy, IL-2 and interferon-α 2b (IFN) [300]. Recently, new agents were demonstrated to improve the clinical course of patients with advanced melanoma: BRAF inhibitors (vemurafenib and dabrafenib), MEK inhibitors (trametinib), bcr-abl/c-kit/PDGF-R inhibitors (imatinib), and angiogenesis inhibitors (bevacizumab and aflibercept), as well as immunotherapy with anti-CTLA-4 antibodies (ipilimumab), anti-PD-1 antibodies (nivolumab and lambrolizumab) [300].

In the last years, some studies focused on the composition of gut microbiota in murine models of melanoma treated with targeted therapies or conventional chemotherapy. The most interesting aspect coming from such investigations regards a possible immune-modulating role of gut microbiota in the clinical response to anticancer drugs.

A recent study indeed compared the immune responses of antibiotic-treated and untreated mice to transplanted lymphoma, colon carcinoma and melanoma cells [301]. Authors assessed gene expression in the tumour microenvironment and found that B16 melanomas inoculated in mice treated with antibiotics (ABX, vancomycin, imipenem, neomycin) displayed a decreased expression of genes involved in inflammation, phagocytosis and adaptive immunity, while genes related to tissue development, cancer and metabolism were increased [301]. Moreover, mice were subjected to combination of intra-tumoral CpG-oligodeoxynucleotides (ODN), a ligand of Toll-like receptor 9 (TLR9), and inhibitory IL-10 receptor antibodies (anti-IL-10R) [301, 302]. This immunotherapy is applied to retard tumor growth and prolong survival and acts by rapidly inducing haemorrhagic necrosis dependent on TNF production by tumor-associated myeloid cells, followed by a CD8+T cell response able to eradicate tumor [303]. Authors found that ABX tumor-bearing mice had an impairment of the immunotherapy efficacy, showing an increased tumor growth and reduced survival. Accordingly, in antibiotics-treated or germ-free mice, tumor-infiltrating myeloid-derived cells responded poorly to therapy, with a lower production

of TNF alpha and IL12B after CpG-oligonucleotide treatment [301]. Authors were also able to identify specific bacterial species that could promote or impair the efficacy of immunotherapy. Namely, Gram-negative genera, such *Alistipes*, positively correlated with an increased TNF production, responsible of the abovementioned anticancer effects. In addition, *Lactobacillus genus*, including *L. murinum*, L. *intestinalis*, and *L. fermentum*, positively correlated with TNF expression, while on the contrary *Ruminococcus* showed a negative correlation [301]. In this group a deficient production of ROS and CD8 mediated cytotoxicity was discovered after chemotherapy [301].

Another interesting study was conducted on mice inoculated with B16F10 melanoma or MCA205 sarcomas and exposed to cyclophosphamide (CTX), an anticancer treatment able to reduce cancer growth recruiting Th1 and Th17 cells [304,305]. This study showed that CTX disrupted intestinal barrier increasing its permeability, induced a condition of dysbiosis and led to translocation of commensal bacteria (namely *Lactobacillus johnsonii*, *Lactobacillus murinus* and *Enterococcus hirae*) into the mesenteric lymph nodes and the spleen [304]. In melanoma bearing mice grown in conventional conditions, cyclophosphamide treatment and the following translocation of bacteria led to an increased the number of interferon-γ and IL-17-producing T cells in the spleen. Interestingly, these positive anticancer effects were not observed in germ-free mice or in mice that had been treated with antibiotics [304]. In addition, *Lactobacillus johnsonii* and *Enterococcus hirae* could polarize CD4+ T cells coming from conventional mice into a Th1 or Th17 cell phenotype both in vitro, while these effects were abrogated in germ-free or antibiotic treated mice. Overall, such findings confirm the primary role of an intact microbiota in triggering and modulating the immunological anticancer responses elicited by chemotherapies [304].

Additional studies were also performed on mice exposed to targeted therapies for melanoma. A recent evaluation by Vetizou et al. [306] compared the therapeutic efficacy of the anti–CTLA-4 treatment (ipilimumab) in mice models of melanoma housed in specific pathogen-free (SPF) versus germ-free (GF) or antibiotic therapy conditions. Anti–CTLA-4 therapy could regulate melanoma progression in SPF but not in GF mice or antibiotic treated mice [306]. The reduced anticancer effect found in GF or antibiotic treated mice depended on the decreased anti–CTLA-4–induced activation of splenic effector CD4+ T cells and on the reduction of tumor infiltrating

lymphocytes (TILs) in melanoma [306]. As to gut microbiota composition, anti CTLA-4 treatment induced an increase of specific Bacteroides species in the small intestine mucosa, as assessed by qPCR; the recolonization of GF or antibiotic treated mice with such species restored anti-CTLA4 anticancer effect [306]. One of the most interesting feature of this investigation is the contemporary assessment of gut microbiota of humans treated with ipilimumab. Namely, stool samples of 25 individuals with metastatic melanoma under ipilimumab therapy were recruited and analysed. During ipilimumab therapy, melanoma patients' gut was found to be mainly colonized by *Bacteroides* species. Fecal microbial transplantation of faeces harvested from metastatic melanoma bearing mice in whose intestine the *Bacteroides* species *B.Fragilis* and *B.Thetaiotaomicron* were predominant, induced a marked response to CTLA-4 blockade [306]. Hence, this study demonstrates that the anticancer effects of anti-CTLA4 treatment in mice can be modulated by a specific composition of gut microbiota.

Also, anti PD-L1 efficacy has been found to be modulated by specific gut microbes [307]. A recent investigation indeed compared melanoma growth in mice having distinct commensal microbiota (TAC and JAX mice] [307]. Authors identified differences in spontaneous antitumor immunity among JAX and TAC mice under anti-PD-L1 treatment, being such treatment more effective in JAX mice [307]. Interestingly, such therapeutic differences disappeared after fecal transfer of feces from JAX to TAC mice, or after a co-housing of the two groups. NGS techniques applied on stool samples of JAX and TAC mice identified Bifidobacterium as the most represented species in JAX mice, and was hence suspected to be associated with the antitumor effects [307]. Such suggestion was confirmed by the evidence that oral administration of *Bifidobacterium* alone to TAC mice was able control tumor growth, while combination of *Bifidobacterium* and anti-PD-L1 nearly abolished tumor, through an increased dendritic cell activation and a subsequent CD8+ T cell involvement, which can invade tumor microenvironment to elicit an anticancer effect [307]. Finally, a recent study confirmed that antibiotic treatment in mice models of melanoma (inoculated with B16F10 melanoma cells) is able to induce a dysbiosis in gut microbiota with a consequent impaired anticancer immune response [308]. Such treatment reduced the presence of infiltrated mature antigen-presenting cells in the tumor, together with lower levels of co-stimulators proteins, such as CD80, CD86 and

MHCII. Antibiotics also induced a defective release of Th1 cytokines, including IFNγ, TNFα, IL12p40, and IL12p35 [308].

In the last few years a couple of investigations addressed gut microbiota composition in humans affected by metastatic melanoma under targeted therapy.

A prospective study on 34 melanoma subjects treated with ipilimumab found that a specific microbiota composition before starting ipilimumab correlated with the subsequent development of a checkpoint-blockade-induced colitis [309]. This clinical phenomenon is a complication of the ipilimumab therapy which affects at least 1/3 of patients and leads in some cases to the treatment discontinuation [309-311]. Authors found that an increased presence of *Bacterioides* in the faeces at baseline correlated with a reduced development of colitis, thus suggesting that this group of microbes could represent a biomarker able to predict the possible development of this complication [309].

This finding was also confirmed in a recent independent study assessing gut microbiota composition [312], anticancer response and colitis development in melanoma patients treated with ipilimumab [312]. Authors found that gut microbiota of patients prone to develop colitis was enriched by *Firmicutes*, while the presence of *Bacterioides* was on the contrary associated to a decreased risk to display the colitis [312]. Authors also found that the clinical response to ipilimumab treatment depended on a specific microbial composition. They demonstrated indeed that patients whose baseline microbiota was mainly composed by with *Faecalibacterium* genus and other *Firmicutes* had longer progression-free survival and overall survival, as compared to patients whose intestinal microbiota before treatment was enriched by Bacteroides [312].

Finally, another recent correlative study assessed gut microbiota composition of four groups of melanoma patients treated with ipilimumab alone (I group), with a combination of nivolumab and ipilimumab followed by nivolumab (IN group), with nivolumab alone (N group) or with pembrolizumab alone (P group), and searched for possible correlation of gut microbiota with the efficacy of the therapy [313]. Authors found that gut microbiota of responders to all types of therapy was enriched by *B.caccae* and *S. Parasanguinis*, as compared to non-responders showing cancer progression [313]. The gut microbiome of IN group responders also showed an increased abundance of *Faecalibacterium prausnitzii*, *Bacteroides thetaiotamicron*,

and *Holdemania filiformis*, while P group responders showed a gut enrichment of *Dorea formicogenerans* [313].

These studies performed on humans, are not able to provide a causal connection between gut microbes and anticancer response of targeted therapies. However, even if they should be validated by larger follow up clinical studies, they provide novel insights into the mechanisms regulating the anticancer responses and suggest possible integrative therapeutics options (probiotics or prebiotics). In addition, these clinical investigations propose new biomarkers useful to predict both the response to treatments and the occurrence of adverse effects.

6. Gut microbiota and vitiligo

Vitiligo is an acquired dermatological disorder characterized by the appearance of circumscribed depigmented macules. Such lesions originate from the loss of functional melanocytes in the epidermis [314].

Several theories have been proposed so far to elucidate vitiligo pathogenesis, such as the autoimmune theory [315,316], the auto-cytotoxic theory [317,318], the neural theory [319], the "impaired epidermal cytokine" theory [320-322], the melanocythorragic hypothesis [323] and the recent inflammatory theory [324]. All these theories are currently considered as synergistic in determining the disease and are sustained by several clinical and experimental evidences [317]. However, among them the auto-cytotoxic and the autoimmune theories are at present the most accredited. While in the past autoimmunity and oxidative stress were considered to act in a mutually exclusive way in determining vitiligo, recent findings on the contrary suggest that these two mechanisms are contemporarily involved in the depigmentation process [325].

In autoimmune disorders such as vitiligo indeed the immune system induces a chronic inflammatory milieu, in which ROS accumulate and exert a toxic effect on surrounding cells [325].

Structural or functional melanocytic proteins therefore could be modified by acute and chronic oxidative stress, possibly becoming neoantigens able to trigger auto-reactive reactions [326]. Hence, according to this hypothesis, autoimmunity and oxidative stress interact in initiating and/or amplifying the loss of melanocytes in vitiligo.

As to a potential microbial role in vitiligo pathogenesis, this issue has been poorly evaluated so far.

One recent prospective study on 79 patients with vitiligo and 72 patients with telogen effluvium tested the prevalence of H. pylori infection in such groups [327]. Authors found significantly higher rates of H. pylori positivity and dyspepsia in the vitiligo group than in the telogen effluvium group [327]. In addition, the number of patients with dyspepsia was significantly higher in the vitiligo group [327]. No correlation was found with clinical features of the disease such as the Vitiligo Disease Activity score [327].

In the last decades, also some viruses including cytomegalovirus [328-331], herpes virus [332-333], hepatitis virus [334-338] and the human immunodeficiency virus [338-341] have been suggested as possibly responsible for vitiligo [342]. Such observations came from PCR studies on vitiligo skin [329,330], from specific antibodies detection in the serum of vitiligo subjects [332,334,335], or from anecdotal studies subjects developing vitiligo after triggering viral infections [337-341].

As regards to gut microbiota, at present no study has been performed concerning its composition in vitiligo, neither on mice models nor on humans affected by this pigmentary disorder.

The only metagenomic study performed so far on vitiligo assessed skin bacterial microbiota of lesional and non-lesional skin of vitiligo patients [343]. Such investigations pointed out a dysbiosis of microbial community in lesional skin of vitiligo subjects, which was sustained by a decrease in taxonomic richness and evenness in lesional skin, with a predominance of Firmicutes [343].

7. Aim of the study

Melanoma it has been widely demonstrated to be highly immunogenic, since it stimulates the immune system to generate a humoral (antibody-mediated) and cellular (cytotoxic lymphocyte-mediated) response to cytoplasmic or membrane antigens of melanoma cells [344-345].

Such event is clinically demonstrated by the occurrence of the vitiligo-like depigmentation in melanoma patients, a prognostically favorable event resulting from a strong anti-melanoma immunity that also targets healthy melanocytes, because of a shared expression of melanocyte differentiation antigens [345-346]. Melanoma patients affected by the vitiligo-like depigmentation display high circulating levels of antibodies against melanocytic differentiation antigens (tyrosinase, TYRP1, TYRP2, and Pmel17) and reactive CD8+ T cells (mainly against MART-1or against gp100) [345-349] and have a longer metastasis-free survival and overall survival [350-351].

Recently, the term immune-surveillance has been coined and is intended as the capacity of lymphocytes to recognize tumor-associated antigens expressed by cancer cells and to elicit a response finally leading to their elimination [352]. The activation of this immune response is crucial for controlling melanoma growth and metastasizing and has been so far intensively evaluated through clinical and experimental investigations which led to the development of toxins, immune stimulants (such as IL-2), adoptive cell transfer therapies (expanded autologous CD4+T cell clones against specific melanoma-associated antigens) and the recently discovered and approved immune targeted therapies for melanoma treatment [352,353,300].

Several investigations have disclosed the association of gut dysbiosis with cancer development [155, 157, 159] and pointed out the active role of a specific gut microbial signature in regulating anticancer immune surveillance [354]. Such beneficial immune event can be elicited by the presence of specific microbes able to hyperactivate the immune system through mechanisms involving microbial pattern recognition receptors, or through cross reactivity phenomena between microbial epitopes ant tumour antigens possibly able to shape T cell repertories [354,355]. Interestingly, specific microbiota-derived peptides/antigens have been reported to cross react with specific melanoma antigens such as Melan /MART-1 or MAGE-A6 [356-357].

Further, a specific gut microbiota composition has been found to be associated with an improved response to targeted immuno-therapies for advanced melanoma in humans

and has been reported to be predictive of the occurrence of immune-related complications such as colitis [312,313].

As regards to vitiligo, several evidences pointed out the involvement of both innate ad adaptive response in its pathogenesis [314, 358,359].

As to innate immunity, a marked immune signature was recently identified by a recent skin trascriptome analysis performed on vitiligo, which revealed a strong natural killer cells and inflammatory dendritic cells gene expression in lesional skin, as compared to non lesional skin [359-360]. Such inflammatory dendritic cells might be activated by the interaction of their PRRs with stimulating factors [359]. Among the triggering factors, epidermal or melanocyte-derived nucleic acids deriving from damaged melanocytes or keratinocytes have been recognised as able to activate PRRs [359,360]. Finally, the innate immune activation leads to an imbalance toward the release of pro-inflammatory cytokines, such as TNFα, IFNγ, CXLX10 and IL17 at both skin and systemic level [357-360]. All the above-mentioned cytokines have been found to be increased in vitiligo epidermis [320,321,322,361-367] or in the circulating blood [359,364,368-370] of affected patients and have been proposed to have a pathogenetic role in the disease.

As to adaptive immunity, both CD4+ and CD8+ T cells have been identified as involved in vitiligo pathogenesis, since they are increased in lesional and perilesional skin, as compared to healthy skin [358,359,364, 371-373]. Recently, also the involvement of Th17 cells have been disclosed, since they have been found to be increased both in the affected epidermis and in the blood of vitiligo subjects [365-367]. Furthermore, an altered proportion and impaired function of T-regs have been found in vitiligo, possibly involved in the loss of tolerance to self-melanocytic antigens [345, 371,373].

Finally, an increased oxidative stress, characterized by high epidermal levels of reactive oxygen species (ROS) [374,375] and an abnormal function of the metabolic system of biopterins [376-380], finally leading to the disease through lipid peroxidation, DNA damage, an increased production of pro-inflammatory and anti-melanogenic cytokines, and the loss of functionality of enzymes playing a key role in melanogenesis, have been demonstrated in vitiligo patients [381-382].

A plethora of evidences reveal a gut dysbiosis in patients affected by organ-specific and non-organ specific autoimmune disorders [211, 214, 215], some of which can be found in association with vitiligo [383], and suggest that microbes inhabiting the

human gastrointestinal tract can modulate both innate and adaptive systemic immune responses [78, 111, 124,125]. Recently, it has also been demonstrated that the contact between the gut epithelial cells and some groups of enteric commensal bacteria leads to the rapid generation of reactive oxygen species (ROS) within host cells [384-386], which might affect inflammatory responses both locally and systemically.

Overall, given the current knowledge on vitiligo pathogenesis and the ability of gut microbial species to modulate immunity and oxidative stress balance, it is conceivable that a specific gut microbiota composition might be involved in vitiligo pathogenesis. At present, nothing is known about gut microbiota composition in vitiligo patients, neither at bacterial nor at fungal level. As regards to melanoma, only a few studies at present assessed bacterial gut microbiota in advanced stage melanoma, subjected to immunological targeted therapies [309,310,312,313].

It is conceivable that the knowledge of gut microbial composition in melanoma patients not subjected to therapies might led to the identification of a specific bacterial and fungal gut signature, which could be associated to cancer development. The evaluation of possible correlations with clinical and prognostic features of the diseases might help to lead to new insights in the identification of possible yet unknown roles of microbes in controlling melanoma in early phases of growth.

In addition, the knowledge of gut microbiota composition in vitiligo patients might be useful to identify a specific gut microbiota signature possibly responsible of activating autoimmune pathways involved in the development of an autoimmune response toward melanocytic antigens, which could be shared by melanoma cells too and could be implicated also in the development of the beneficial vitiligo-like depigmentation in melanoma patients. In addition, the presence of beneficial and “anti-cancer” bacteria or fungi in the gut of vitiligo patients might also justify the reported decreased risk of developing melanoma in patients affected by vitiligo [387-390].

With this background, we aimed to evaluate bacterial and fungal gut microbiota composition through next generation sequencing targeting bacterial 16S rRNA and fungal ITS in a group of patients affected by melanoma and patients affected by vitiligo.

Further aim of this thesis is to correlate a specific gut or bacterial microbiota composition of melanoma and vitiligo patients with clinical and histopathological features of melanoma, and with clinical, historical and serological features of vitiligo.

Finally, given the rediscovered importance of the classical cultivation methods suggested by recent investigations [391], a cultivation approach has been followed simultaneously with the metagenomic evaluation. We decided to focus on fungi (yeasts) due to their unique ability to shape innate and adaptive immunity [17]. Hence, further aim of this investigation is to isolate and identify cultivable fungal species from the melanoma faecal samples subjected to metagenomic evaluation.

8. Materials and Methods

8.1 Study participants

The study was designed as a observational study involving the analysis of biological samples. Ten patients with a histopathological diagnosis of melanoma (M1-M10), 10 patients with clinical diagnosis of vitiligo (V1-V10). Eighteen faecal samples (C1-C18) from healthy subjects were used as controls.

Patients were recruited among subjects referring to the university-based outpatient service for melanoma prevention and follow-up of the Dermatology Clinic II, Florence (DCTM, Section of Dermatology) and among those referring to the university-based outpatient for vitiligo diagnosis and treatment of the same Dermatology Clinic.

The study was carried out in compliance with the Declaration of Helsinki principles for medical research involving human subjects. The study was approved by the local Institutional Review Board and written informed was signed by all the subjects before enrollment in the study.

Inclusion criteria for the melanoma group were:

- Age > 18 years
- A histological diagnosis of melanoma
- Excision of melanoma in the previous year

Inclusion criteria for the vitiligo group were:

- Age > 18 years
- The presence of vitiligo, possibly confirmed by histopathological examination in case of an uncertain clinical diagnosis.

Exclusion criteria

- Age < 18 years
- Presence of clinically evident inflammatory disorders, such as upper respiratory tract or urinary infection, pneumonia, stomatitis, periodontal inflammation.
- Gastro-enteric symptoms such as fever, diarrhoea or vomitus in the month preceding the collection of faecal samples

- Presence of irritable bowel syndrome, coeliac disease or autoimmune disorders such as inflammatory bowel diseases, rheumatoid arthritis, Behcet disease, multiple sclerosis, type I diabetes, systemic lupus erythematosus.
- History of colorectal cancer or colectomy in the previous three years
- Use of systemic antibiotics in the three months preceding the collection of faecal samples
- Refuse to sign informed written consent
- Systemic treatment with corticosteroids (vitiligo patients only)
- Diagnosis of segmental vitiligo (vitiligo patients only)

As to the control group, healthy adult subjects not affected by melanoma or vitiligo had been selected according to the same exclusion criteria used for the enrollment of melanoma and vitiligo patients.

8.2 Samples' handling and collection

All enrolled patients were given a photographic booklet to instruct them to collect samples autonomously.
After the consignment of samples by the patients, collected stool samples were included in 8 ml of RNA solution (RNAlater™ Stabilization Solution, ThermoFisher Scientific) and stored at -20°C until analysis.
At the moment of samples' consignment patients underwent a questionnaire aiming to collect data as regards to dietary assumption in the three days preceding the sample collection and as regards to delivery mode, weaning, breastfeeding and food allergy (Appendix 1).

8.3 Personal data collection, past medical history, medication assumption

Age, gender, height, weight and body mass index (BMI) and smoking were recorded for all enrolled patient.
Personal medical history of autoimmune disorders such as autoimmune thyroid disease, diabetes mellitus type 1, Addison's disease, systemic lupus erythematosus, autoimmune atrophic gastritis, celiac disease, alopecia areata, rheumatoid arthritis,

Sjögren's syndrome, primary biliary cirrhosis, cardiovascular diseases (coronary heart disease, cerebrovascular disease, peripheral arterial disease, rheumatic heart disease, congenital heart disease, deep vein thrombosis and pulmonary embolism) [392], not autoimmune thyroid diseases, skin cancer (melanoma and non-melanoma skin cancer for vitiligo patients, and non-melanoma skin cancer for melanoma patients), visceral cancers (intended as each kind of tumor affecting the lungs, the heart, and the organs of the digestive, excretory, reproductive, and circulatory systems) [393], or other diseases was also recorded.
Medication assumption at the moment of enrolment was also investigated.
As to melanoma patients, information as regards to previous sunburns and to sun-exposure habits (occupational exposure, recreational exposure or indoor tanning) were recorded.

8.4 Evaluation of clinical and histopathological parameters of melanoma patients

The following clinical data and histological parameters about melanoma were assessed and recorded [394, 395]: body site, histopathological subtype, Breslow thickness (recorded in millimetres and measured from the granular layer or, when present, the ulcer base, to the deepest extent of invasion by tumour cells), ulceration, mitotic index (number of mitoses per mm^2), lymphovascular invasion, microsatellite/in transit metastasis, perineural invasion/neurotrophism, growth phase, tumour infiltrating lymphocytes (TILs) and type (brisk or not brisk), regression, Clark level [394, 395].
Melanoma staging was defined according to AJCC 7th edition pathological staging of cutaneous malignant melanoma, regional lymph nodes and metastasis [396].

8.5 Evaluation of clinical, historical and serological parameters of vitiligo patients

Vitiligo diagnosis was clinically made in case of achromic depigmented macules that vary in size from a few to several centimetres in diameter, often involving both sides of the body with tendency toward symmetrical distribution [397].
The diagnosis was confirmed using Wood's lamp, a device emitting UV light which can be directed at patient's skin. UV radiation penetrates the epidermis and enters the dermis, where it stimulates fluorescence emission directed towards the surface of the skin by collagen fibres [398]. In case of absence or reduced melanin, as it occurs in

vitiligo, part of the emitted fluorescence cannot be attenuated by the epidermal melanin [398]. Hence, vitiligo skin under Wood's lamp appears as fluorescent and intensely white. This procedure enhances the epidermal pigmentation differences usually not detectable under visible light and improves the assessment of the extent of vitiligo [398].

Biopsy of the achromic lesions with histopathological examination was performed to confirm the diagnosis for doubtful cases.

Assessment and scoring of vitiligo was performed using a modified Vitiligo European Task Force (VETF) form [399, 400]. According to the above-mentioned assessment, head and neck, trunk, upper extremities and lower extremities were independently assessed for the extent of depigmentation, stage of disease and spreading. As regards to staging and spreading, the largest vitiligo patch in each body site was chosen as a reference for assessment.

The extent of the disease was calculated evaluating the amount of vitiligo patches involving the body and was expressed as a percentage, considering that the patient's palm including digits averages 1% of body surface area (BSA) [399].

The stage of the disease was assessed using Wood lamp with magnifying lens. A stage 0 was defined as a normal pigmentation, stage 1 as an incomplete pigmentation (e.g. spotty depigmentation), stage 2 as a complete depigmentation, stage 3 as a partial hair whitening (< 30%) and stage 4 as a complete hair whitening [399].

The spreading was evaluated assessing the patch limits both under natural light and using Wood's lamp. A score 0 was given in case of similar limits, a score +1 in case of progressive vitiligo and a score -1 in case of a regressive vitiligo [399].

A total score for the abovementioned parameters was then performed (0–100% for vitiligo area, 0-16 for staging and -4 to +4 for spreading).

Age at onset, duration of the disease, phototype, vitiligo subtype according to the revised classification and nomenclature of vitiligo proposed by the Vitiligo Global Issues Consensus Conference [397], Koebner phenomenon, modality of onset, growth, leukotrichia, emotional stress at onset or that worsened the disease, early hair greying (>50% white hair before the age of 40), signs of inflammation/pruritus, presence and number of. Sutton nevi, activity of the disease (active = appearance of vitiligo lesions/enlargement of the existing macules during 6 months before our clinical evaluation, borderline = 6-18 months and stable = >18 months) and previous repigmentations were also investigated.

Thyroid stimulating hormone (TSH), anti-thyroglobulin autoantibodies (Tg-Ab), anti-thyroid peroxidase antibodies (TPO-Ab), anti-thyroid stimulating hormone receptor antibodies (TSH-R Ab), anti-nuclear antibodies (ANA), anti-parietal cell autoantibodies (APCA) and 25 (OH) D level were dosed. These parameters were evaluated in different analysis laboratory which individually provided their reference ranges.

8.6 Storage of faecal samples

Stool samples were collected and divided into two rates. On portion was stored at -20°C in RNA later solution, and was designated to the metagenomic analysis. The other was immediately used for cultural examination.

8.7 DNA extraction and amplification of the V3-V4 region of bacterial 16S rDNA and ITS1 region of fungal rDNA

Total DNA extraction was performed from each faecal sample (250 mg, wet weight) using the DNeasy PowerSoil Kit (Quiagen N.V, Venlo, The Netherlands.). The extraction kit, originally designed for the extraction of total DNA from soil samples, was chosen on the basis of literature review [401] demonstrating its effectiveness for extraction of faecal microbiome.

DNA integrity and quality were checked on 1% agarose gel and quantified with QUBIT instrument (Thermo Fisher).

For each DNA sample were amplified the bacterial 16S rRNA genes using a primer set specific for V3–V4 hypervariable regions (341F: 5′-CCTACGGGNGGCWGCAG-3′and 805r: 5′-GACTACNVGGGTWTCTAATCC-3′) (reference for primers http://www.int-res.com/articles/ame_oa/a075p129.pdf also used for the Earth Microbiome Project) and the internal transcribed spacer (ITS) using a primer set specific for fungal ITS1 rDNA region (ITS1f: 5′-CTTGGTCATTTAGAGGAAGTAA-3′ and ITS2r: 5′-GCTGCGTTCTTCATCGATGC-3′) (used by the Earth Microbiome Project http://www.earthmicrobiome.org/protocols-and-standards/its/) containing adaptors, key sequence, and barcode sequences as described by the Illumina MiSeq (Illumina, San Diego, CA, USA) instruction for amplicons experimental design.

Sequencing were performed at the Fondazione Edmund Mach, Trento, Italy, following their internal protocol.

8.8 Sequencing and data analysis

Demultiplexed forward and reverse reads were downloaded an analysed. Initial quality check was performed by inspecting resulting libraries with FastQC program [402]. Low-quality end of forward and reverse reads were trimmed using Sickle software (command *sickle pe*)[403] with a quality cut-off of 20 and a length threshold after trimming of 200 (parameter –q and –l respectively). After trimming, paired-ends reads were joined using PEAR [404]) (setting parameter -p to 0.001, parameter -q to 30 and parameter -u to 0). Chimeric sequences were identified using Vsearch (v2.5) [405] against the GreenGenes (v13.8) [406] and the UNITE (v uchime reference dataset 28.06.2017) database, for 16S and ITS libraries respectively. After chimera removal, sequences where analysed with the QIIME (v 1.9.1) [407] software. Briefly, operational taxonomyc units (OTUs) picking was performed with the open reference protocol (command *pick_open_reference_otus*) against the GreenGenes (v13.8) [406] and the UNITE (sh refs qiime ver7 dynamic 10.10.2017 file) [408] for 16S and ITS respectively. OUT picking was performed in both cases using the UCLUST algorithm [409] while taxonomy assignment was performed with UCLUST for 16S and with BLAST for ITS. Low abundant OTUs (i.e. OTUs represented by low number of sequences) were removed as indicated in Bokulich et al. [410-411] (parameter c = 0.005%).

8.9 Statistical analysis

All downstream analysis was performed in R (v3.4.2). Microbiome data (i.e. biom file produced by QIIME pipeline) were imported using *phyloseq* package [412]. The same package was also used to perform alpha diversity analysis, measuring observed OTUs and Shannon index, and beta diversity analysis, performing Principal Coordinates Analysis (PCoA) and costrained based Canonical Correspondence Analysis (CCA) using the Bray-Curtis distance metric.

The bacterial and fungine taxonomic differences between melanoma and vitiligo were evaluated using the negative binomial Wald test with Benjamini–Hochberg correction

for multiple comparisons (performed with package edgeR) [413] and reported through Volcano Plots [414-415].

Multivariate analysis of variance using Bray-Curtis distance measurements (PERMANOVA analysis) was performed to correlate the identified microbial profiles with selected clinical, histological and serological features of vitiligo and melanoma patients.

As regards to melanoma, invasion, growth phase, Breslow thickness, mitotic index, presence of tumour infiltrating lymphocytes, and presence of regression were chosen as relevant parameters to use to perform the above-mentioned analysis, while autoimmune thyroid diseases, associated autoimmune disorders, ANA, 25(OH)D, Ab anti TPO, activity, onset, growth, inflammation/pruritus, leukotrichia, total extension and stress at onset were chosen for the vitiligo group.

8.10 Isolation and identification of cultivable fungal species from faeces

Stool samples were diluted in sterile water and plated on solid YPD medium (1% yeast extract, 2% bacto-peptone, 2% D-glucose and 2% agar) added with 25 U/ml of penicillin and 25 µg/ml of streptomycin to inhibit bacterial growth and incubated aerobically at 30°C for 3-5 days.

All fungal isolated grown on such selective media were then isolated, to obtain single cell pure colonies. Genomic DNA was extracted from such colonies by thermal lysis, and the ITS1-4 region was amplified using primers for ITS1 and ITS4. Fungal isolates were identified using the BLAST algorithm in the NCBI database (*nr* database) taking species level identification if sequence identity was above 97% and coverage was above 95%.

9. Results

9.1 General characteristics of the populations

Eleven patients affected with melanoma and 10 patients diagnosed with vitiligo were enrolled. As to the melanoma group, 10 patients (4 females, 6 males) finally gave the faecal samples and were involved in the metagenomic and cultural evaluation. As to the vitiligo group, all patients (7 females and 3 males) consigned the faecal samples, which were thus all analysed. As to the dietary questionnaire, all patients mainly followed a Mediterranean diet. No vegetarianism or veganism were detected. Most patients were breastfed (7/10 melanoma group, 8/10 vitiligo group) and all patients were born by vaginal delivery. Furthermore, no cases of food allergy or prebiotics/probiotics assumption were found in both groups. The complete results of the dietary questionnaire are reported in Table 1 and 2.

As to the main personal data, median age was 63.20 ± 3.51 and 55.20 ± 5.65, respectively in the melanoma group and in the vitiligo group, while median BMI was 25.34 ± 1.21 (melanoma group) and 25.41 ± 0.9 (vitiligo group). No significant difference in gender, age and BMI was found comparing melanoma and vitiligo patients (respectively p:0.178, p:0.245 and p:0.946, Table 3). About associated disorders, vitiligo patients mainly suffered from autoimmune diseases (6/10) and among them, autoimmune thyroid diseases were the most often found (5/6). No cases of cancer were detected in the vitiligo group. On the contrary, no associated autoimmune diseases were found in the melanoma group, while one patient previously had a colorectal cancer some years ago. Nine patients with melanoma reported a personal history of sunburns in childhood and adolescence and all patients reported a recreational exposure.

Other results pertaining to personal and historical data of patients are reported in Table 4 and 5.

With regards to histological parameters of melanoma, 5 patients were affected by melanoma in situ and 5 subjects by invasive melanoma, with a staging ranging from 0 to III. Breslow thickness ranged from 0.3 to 3.6 mm, while the mitotic index ranged from 0 to 7. In addition, 3 patients had TILs in the excised melanoma and 3 patients had histological signs of regression. Other histological features of the melanoma of enrolled patients can be found in Table 6.

Finally, as regards to vitiligo, all patients suffered from a non-segmental generalized vitiligo, with the exception of a case of universal vitiligo; the median total extension of achromic macules was 25.95 ± 9.01 (range 0.7 to 83 % of total body surface area). Five patients had an active vitiligo at the moment of the sample collection, while 4 patients were stable, and 1 patient had a borderline vitiligo. The complete data of the clinical features of vitiligo patients can be found in Table 7. As to serological features, in 3/10 patients the presence of Ab anti TG was found, while in 4 patients Ab anti TPO were detected. As to 25(OH) levels, 7/10 patients had impaired values of this vitamin, with a median value of 26.58 ± 1.92 ng/ml (range 18.9-37.4 ng/ml). All the results of serological evaluation of vitiligo patients are reported in Table 8.

9.2 Gut microbiota characterization by metagenomic sequencing

9.2.1 Fungal gut microbiota characterization by ITS1 sequencing

We sequenced the internal transcriber spacer 1 (ITS1) region of the fungal r DNA for the meta-taxonomic evaluation of gut mycobiota in a total of 10 faecal samples of melanoma patients and 10 faecal samples of vitiligo patients.

Two different estimators of *alpha diversity*, which is a measurement of the fungal richness within each sample, were used. Namely, the observed number of OTUs and the Shannon index were calculated. Using the estimator of the observed number of OTUs (which is a measurement of the total species present in a microbial community), the fungal gut microbiota of melanoma was found to be significantly less rich than healthy controls (Fig. 1; p= 0.00962). As well, also the fungal gut microbiota of vitiligo was found to be significantly less rich than healthy controls (Fig. 1; p= 0.000523). Fungal microbiota of vitiligo subjects was less rich than melanoma, although this difference was not significant.

However, we did not find significant differences among the three groups using measures of biodiversity that take the evenness of the species distribution into account, such as the Shannon entropy (Shannon index) (Fig.1).

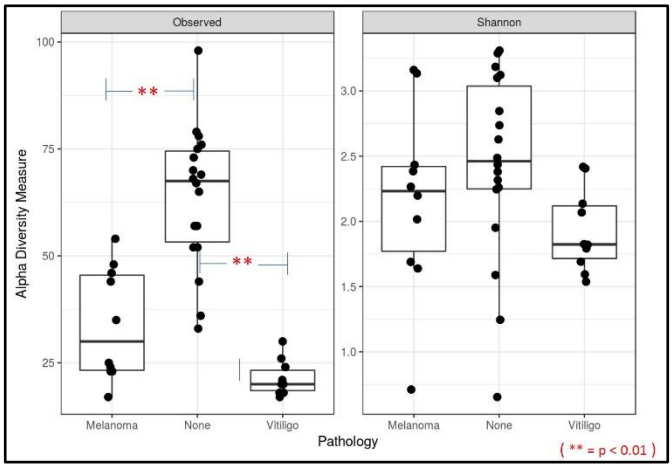

Figure 1 – α-diversity (observed number of OTUs and Shannon index); fungal microbiota

The comparison of the rarefaction curves confirmed the results of the alpha diversity index evaluation, since a reduced richness of the gut mycobiota community in vitiligo subjects was observed, as compared to melanoma subjects (Fig. 2). Overall, the shape of the rarefaction curves tends to reach the plateau, suggesting that the depth of coverage we accomplished was sufficient to capture nearly the entire biological richness within samples.

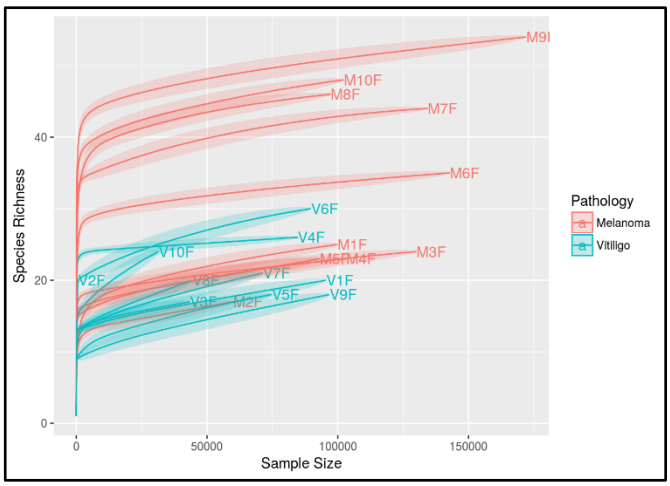

Figure 2- Rarefaction curves- fungal microbiota

The estimate of the variability of fungal communities between samples, defined as the *beta diversity*, was assessed by Principal Coordinates Analysis (PCoA) using Bray Curtis dissimilarities (Fig. 3 and 4). We found that the fungal microbiota of melanoma and vitiligo subjects clusters apart from that of healthy controls. More in details, the

fungal community of melanoma group clusters in two major groups along axis 2, while the vitiligo community does not cluster and is more homogeneously distributed (Fig. 3). As to axis 1 and 3, only healthy controls fungal community cluster in these axes, both in a major group (axis 1) and more homogeneously (axis 3) (Fig.4).

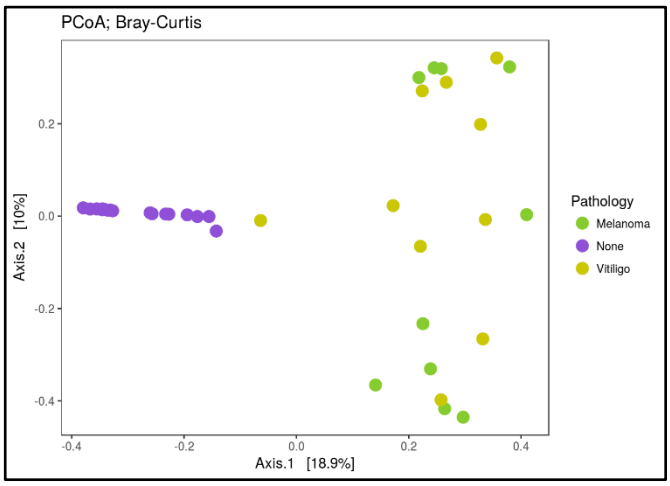

Figure 3- beta diversity PCoA; Bray-Curtis (axis 1 and 2); fungal microbiota

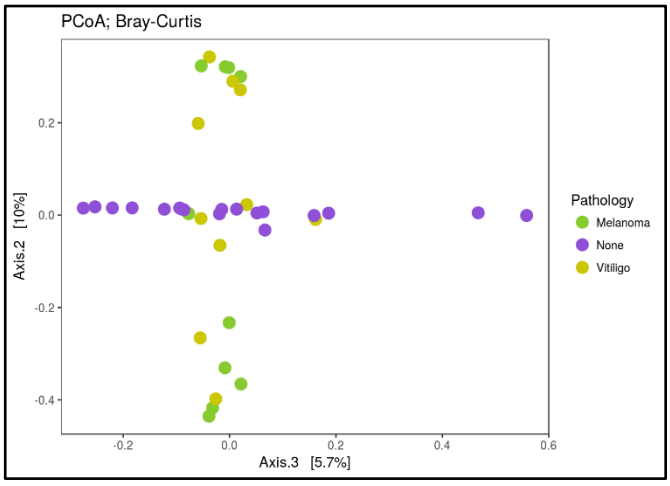

Figure 4- beta diversity PCoA; Bray-Curtis (axis 2 and 3); fungal microbiota

The analysis of the relative abundance of fungal taxonomic groups at the genus level (Fig. 5) revealed that about a half of fungi were unidentified in the control group. As for the melanoma group and vitiligo, such percentage drops to lower values. The remaining identified fungal community in controls was constituted by *Candida* and *Pichia*.

As regards to the patients' groups, the comparison among them pointed out a higher abundance of *Saccharomyces* and *Malassezia* in the vitiligo group, while on the contrary a higher abundance of *Debaromyces*, *Candida* and *Dipodascus* was found in melanoma group.

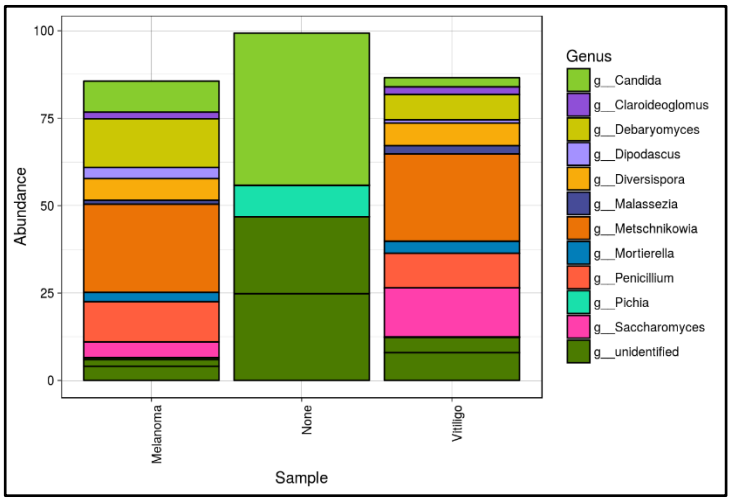

Figure 5- Bar plot relative abundance; fungal, genus

The same analysis conducted at Species level (Fig 6) confirmed the prevalence of *Candida* (%) species in the control group (mostly *C. parapsilosis* and less *C. albicans* and *C. sake*) and recognised as *Pichia kluyveri* the remaining identified fungi. Both *C. parapsilosis*, *Pichia kluyveri* were completely absent both in vitiligo and in melanoma. As to the diseases, a higher abundance of *Saccaromyces Cerevisiae*, *Malassezia restricta* and *Penicillium coccotrypicola* were observed in vitiligo, as compared to melanoma. A higher abundance of *Metschnikowia pulcherrima* was found in melanoma as compared to vitiligo, while *Penicillium roqueforti* and *Candida sake* were only identified in the melanoma group, being absent in vitiligo (Fig.6).

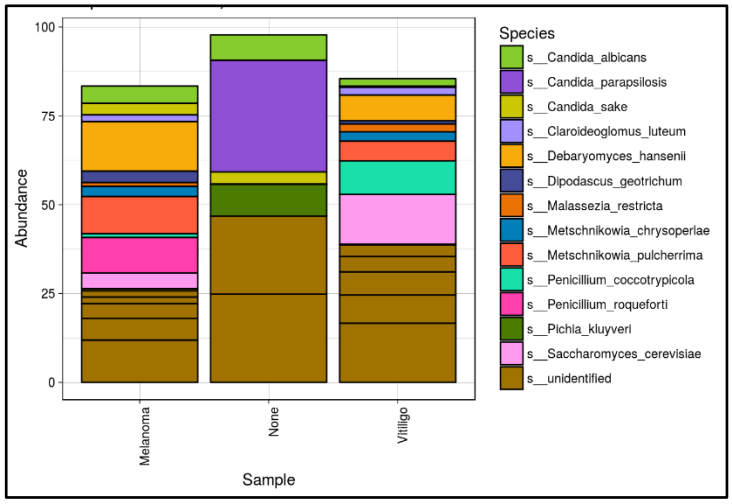

Figure 6- Bar plot relative abundance; fungal, species

Barplots showing the results of the analysis of the relative abundance of fungal taxonomic groups at Phylum, Class, Order, Family, Genus, and Species level in each faecal sample of melanoma, vitiligo and controls were also generated and are reported in Figures 7-12.

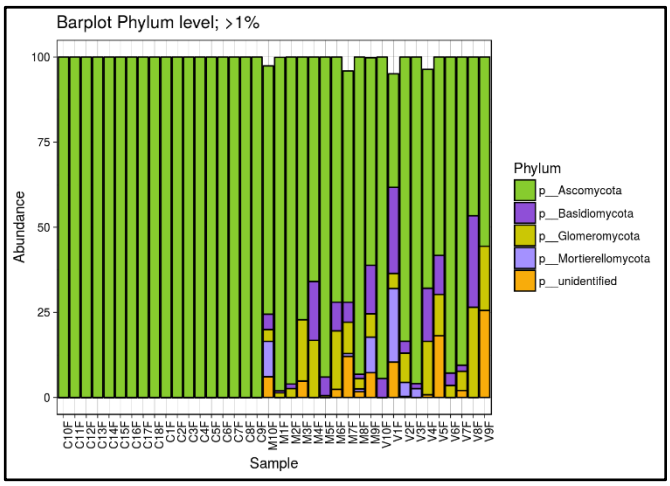

Figure 7- Bar plot, relative abundance for sample- fungal, phylum

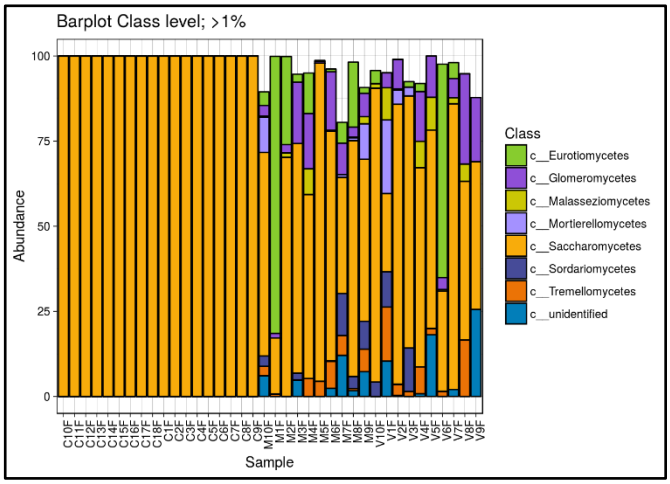

Figure 8- Bar plot, relative abundance for sample- fungal, class

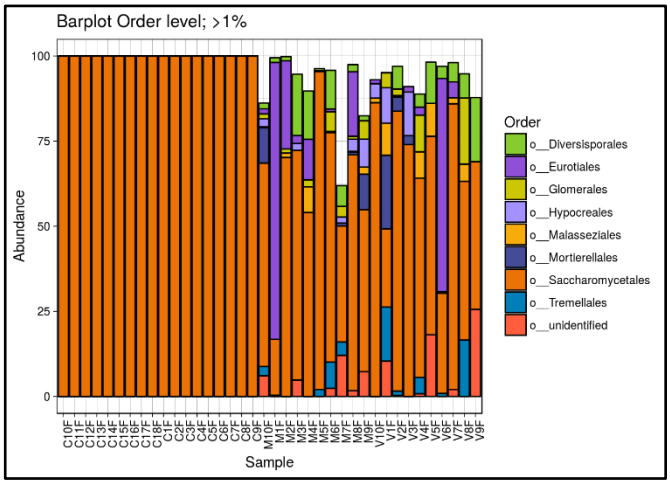

Figure 9- Bar plot, relative abundance for sample- fungal, order

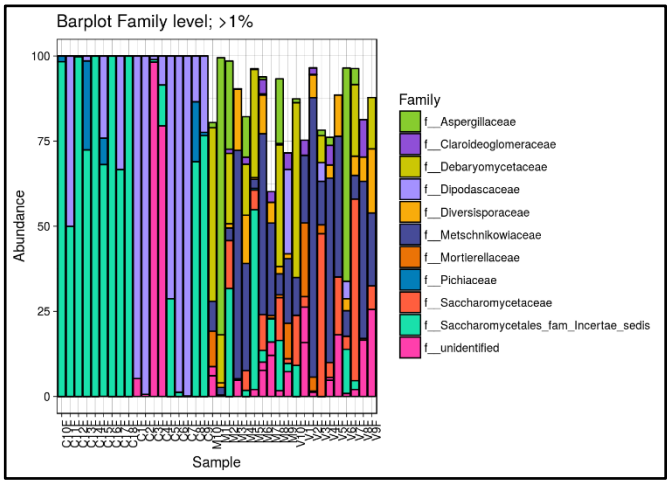

Figure 10- Bar plot, relative abundance for sample- fungal, family

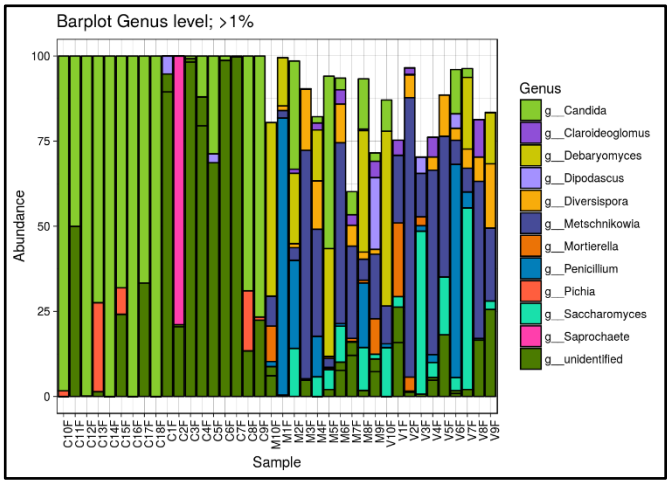

Figure 11- Bar plot, relative abundance for sample- fungal, genus

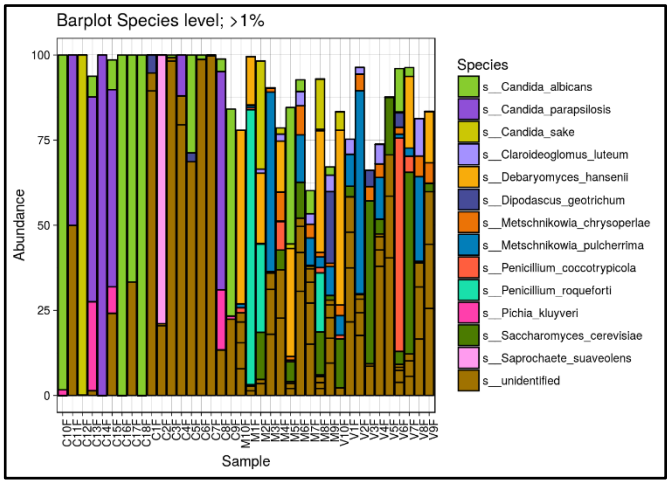

Figure 12-- Bar plot, relative abundance for sample- fungal, species

As to the statistical significance of the abovementioned fungal taxonomic differences between melanoma and vitiligo, the analysis was conducted at species and genus level and evaluated the differences of individual microbial OTUs between vitiligo and melanoma subject using the negative binomial Wald test with Benjamini–Hochberg correction for multiple comparisons. The abovementioned analysis and the relative Volcano Plot graph (Fig 13) conducted at Species level graphically showed that *Penicillium Roqueforti* ($p<0.01$), *Fusarium sp.* ($p<0.01$) and *Candida sake* ($p<0.05$) had a significantly higher relative abundance in the melanoma group as compared to vitiligo group, while *Malasseziales* ($p<0.01$), *Sporobolomyces roseus* ($p<0.01$) and *Epicoccum nigrum* ($p<0.01$) had a significantly higher relative abundance in the vitiligo group, as compared to the melanoma group. The remaining differences in the

relative abundance of fungal species among vitiligo and melanoma samples were not statistically significant.

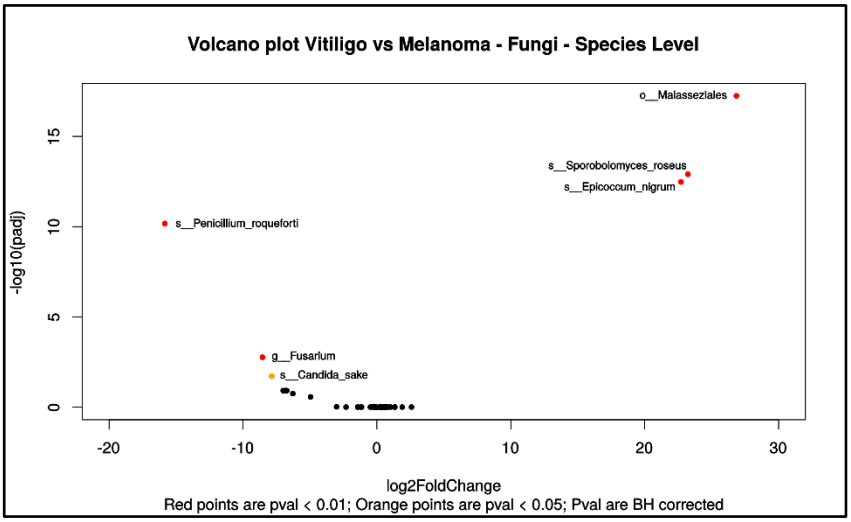

Figure 13- Volcano Plot, Vitiligo vs Melanoma, Fungal

We further sought to overcome the problem related to the elevated undetermined species and *Candida* prevalence in the control group, with the aim to elucidate possible adjunctive differences in the relative abundance of fungal taxonomic groups between controls and disease groups. Hence, we performed a meta-analysis at species level with our results and those pertaining to a published metagenomic evaluation of fungal gut microbiota of healthy subjects. The study of Hellen-Adams at al. [416] was chosen for its completeness and similarity with our study design and methods. We found comparable results to ours as regards to the relative abundance of species between diseases and the heathy controls which were evaluated by the above-mentioned study [416]. Figure 14 reports the relative bar-plot graph.

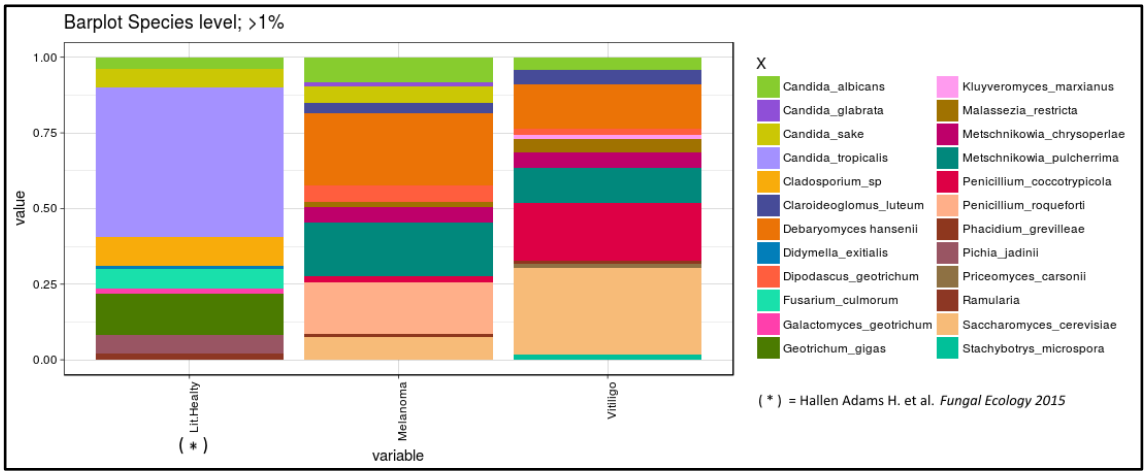

Figure 14- Bar plot, meta-analisis with literature- fungal microbiota

Correlations between fungal gut microbiota and clinical features of the diseases

Multivariate analysis of variance using Bray-Curtis distance measurements (PERMANOVA analysis) was conducted to evaluate possible influences of invasiveness, growth phase, Breslow thickness, mitotic index, presence of tumour infiltrating lymphocytes, and presence of regression on the diversity of fungal species of the melanoma group.

As to the vitiligo group, possible effects of associated autoimmune thyroid diseases, associated autoimmune disorders, ANA positivity, 25(OH)D values, Ab anti TPO positivity, activity of the disease, onset, growth, signs of inflammation/pruritus, presence of leukotrichia, total extension and reported stress at onset on the diversity of species of the vitiligo group were evaluated.

In melanoma group no correlation was found; in vitiligo group a significant association was found for total extension of vitiligo patches ($p= 0.023$; $R^2=0.198$) (Table 9). Hence, with the aim of further analysing dependence of community diversity and the total extension variable in vitiligo group, constrained ordination with Canonical Correspondence Analysis (CCA) was performed. This analysis showed that the community diversity in the vitiligo group is influenced by the total extension of the disease, event tough this correlation was only marginal significant ($p= 0.05$).

Ordination graph (Fig15) shows that samples with higher total extension of the disease tend to separate over the first ordination axis (CCA1 explaining 18.5% of total variance). In particular, sample V1, and to a lesser extent V7, having the highest

extension among vitiligo patients (83 and 73 respectively), tend to separate from the rest of the samples. All the samples with lower extension values, separated along the second ordination axis (CA1, explaining 20.3% of total variance) not correlated to the variable value. We can thus hypothesise that the diversity of fungal community is indeed influenced by the total extension of the disease, but only at very high values.

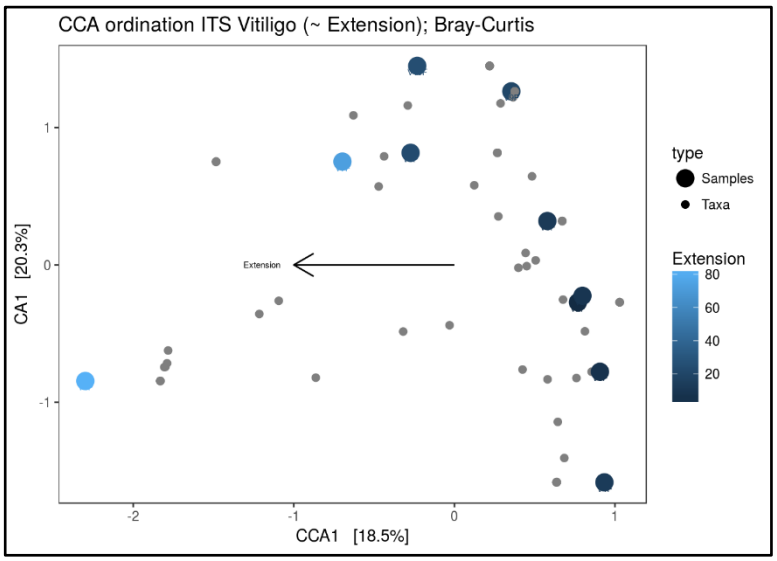

Figure 15- Ordination graph, vitiligo and extension, fungal

Furthermore, when colouring the taxa points based on the genus (Fig16.) some genera appear to be mostly associated to sample V1 (with the highest total extension of the disease), namely *Candida*, *Penicillum*, and some unidentified genera.

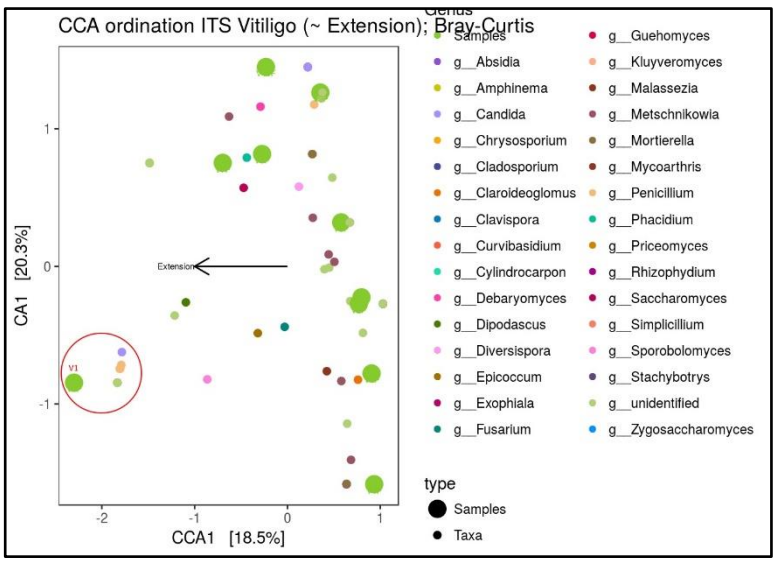

Figure 16 Ordination graph with taxa, vitiligo and extension, fungal; V1 sample is marked with a circle.

.9.2.2 Bacterial gut microbiota characterization by 16S r DNA sequencing.

We sequenced the V3-V5 regions of the prokaryotic 16S r DNA for the meta-taxonomic evaluation of bacterial gut microbiota in a total of 10 faecal samples of melanoma patients and 10 faecal samples of vitiligo patients.

As to the *alpha diversity*, the richness estimators observed number of OTUs and the Shannon index did not reveal any significant difference between the bacterial community of melanoma and vitiligo groups *versus* healthy controls, and between that pertaining to melanoma *versus* vitiligo.

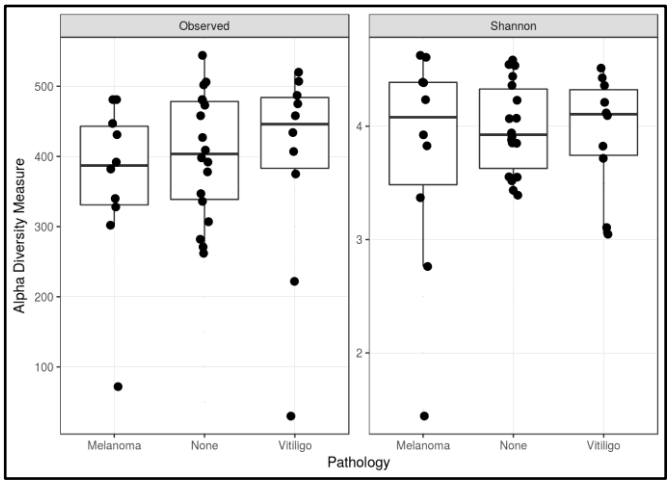

Figure 17 α-diversity (observed number of OTUs and Shannon index); bacterial microbiota

Also about beta diversity, we found that the bacterial microbiota of melanoma and vitiligo did not cluster apart from that of healthy controls. More in details, the bacterial community of melanoma group clusters in a well-defined and coincident major group along axis 1, 2 and 3 (Fig. 18 and Fig. 19).

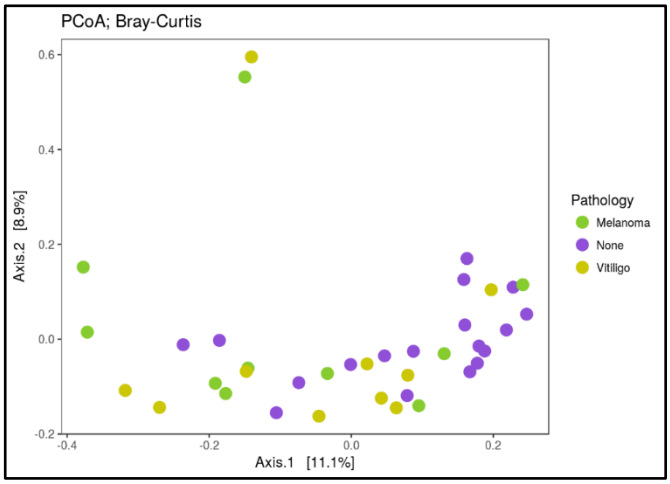

Figure 18- beta diversity PCoA; Bray-Curtis (axis 1 and 2); bacterial microbiota

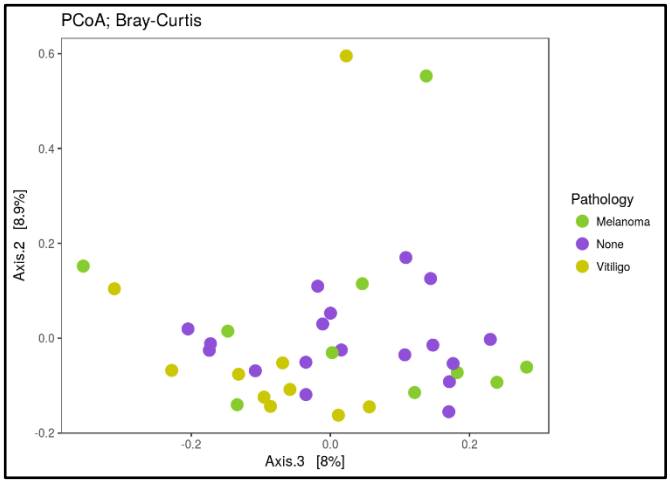

Figure 19- beta diversity PCoA; Bray-Curtis (axis 2 and 3); bacterial microbiota

The analysis of the relative abundance of bacterial taxonomic groups at the family level revealed that a very small proportion of bacteria remained undetermined in each group and that the relative abundance of the remaining determined species was similarly distributed in all groups, with only minimal differences (Fig. 20). Namely, the bacterial community of controls was mainly constituted by *Bacteroidaceae*, *Lachnospiraceae* and *Ruminococcaceae*. The relative abundance of *Lachnospiraceae* was higher in controls as compared to vitiligo and melanoma group, the abundance of *Bacteroidaceae* was lower than vitiligo but similar to that of melanoma, and the relative abundance of *Ruminococcaceae* was lower than that of vitiligo and melanoma.

As regards to the diseases groups, the comparison among them at family level principally pointed out similar relative abundance of all bacteria, with the exception of a higher abundance of *Lachnospiraceae*, *Enterobacteriaceae* and *Bifidobacteriaceae* in melanoma as compared to vitiligo, and a lower abundance of *Bacteroidaceae* in melanoma *versus* vitiligo groups (Fig. 20).

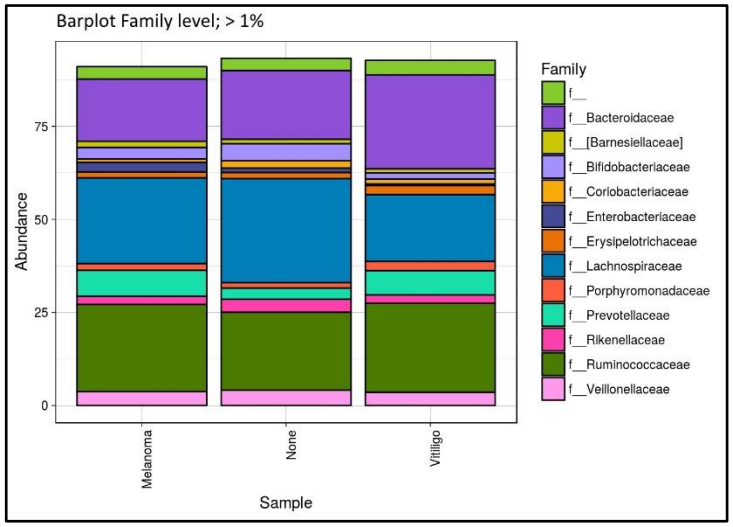

Figure 20- Bar plot relative abundance; bacterial, family.

The same analysis conducted at genus level pointed out a higher relative abundance of *Coprococcus*, *Dialister* and *Bifidobacterium* in controls, as compared to diseases; a lower relative abundance of *Prevotella*, as compared to the diseases, and a lower relative abundance of *Bacteroides*, as compared to vitiligo. A noticeable relative abundance of unidentified bacteria was observed at genus level in all three groups.

As to the diseases, a higher abundance of *Ruminococcus* and *Bifidobacterium* was found in the melanoma group, as compared to vitiligo group. On the contrary, a lower relative abundance of *Bacteroides* was observed in the melanoma samples, as compared to vitiligo samples.

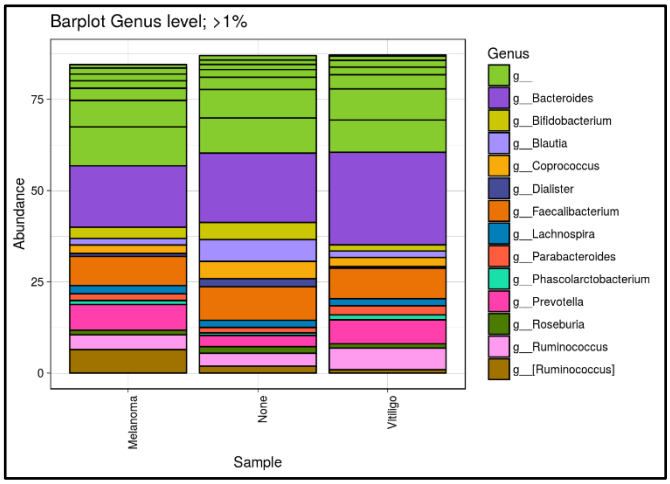

Figure 21- Bar plot relative abundance; bacterial, genus

Barplots showing the results of the analysis of the relative abundance of bacterial taxonomic groups at Phylum, Class, Order, Family, Genus, and Species level in each faecal sample of melanoma, vitiligo and controls were also generated and are reported in Figures 22-27.

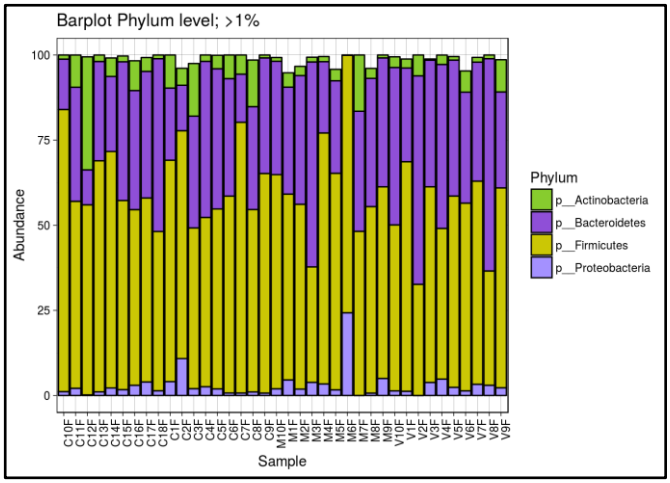

Figure 22- Bar plot, relative abundance for sample- bacterial, phylum

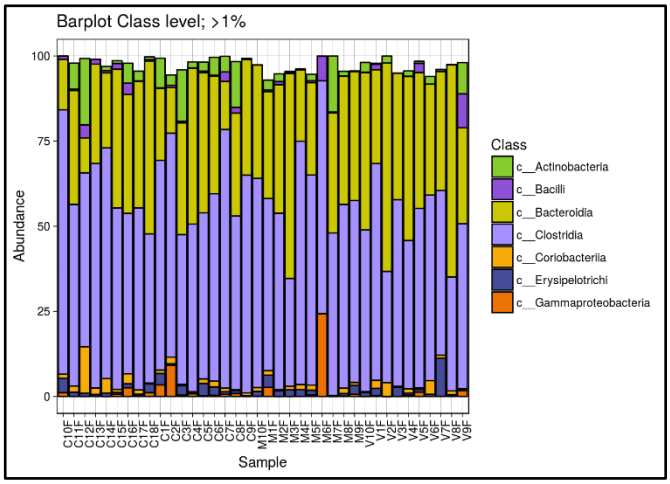

Figure 23- Bar plot, relative abundance for sample- bacterial, class

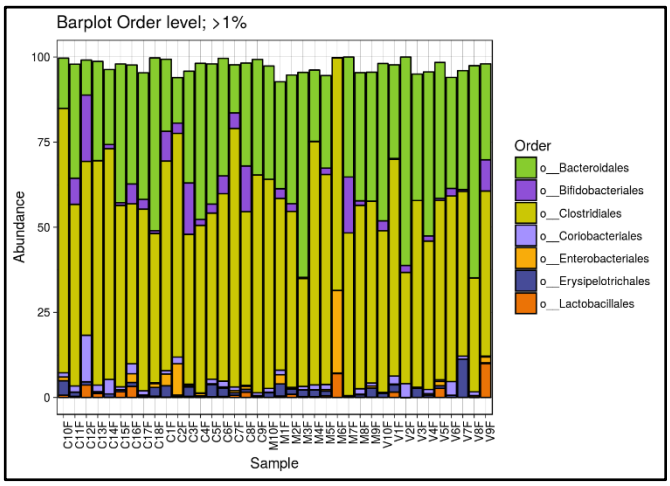

Figure 24- Bar plot, relative abundance for sample- fungal, order

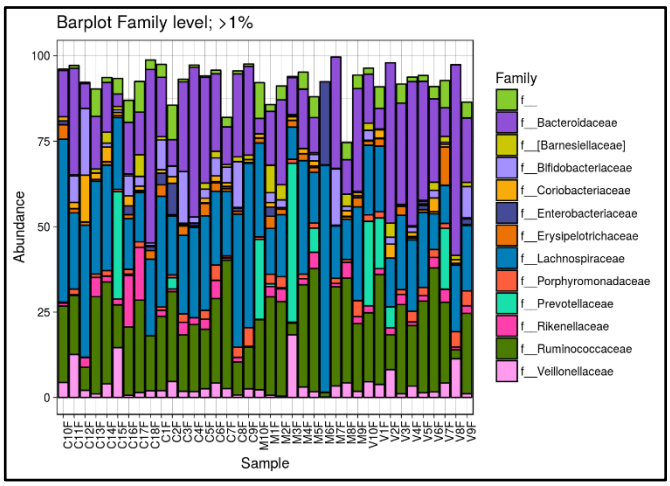

Figure 25- Bar plot, relative abundance for sample- bacterial, family

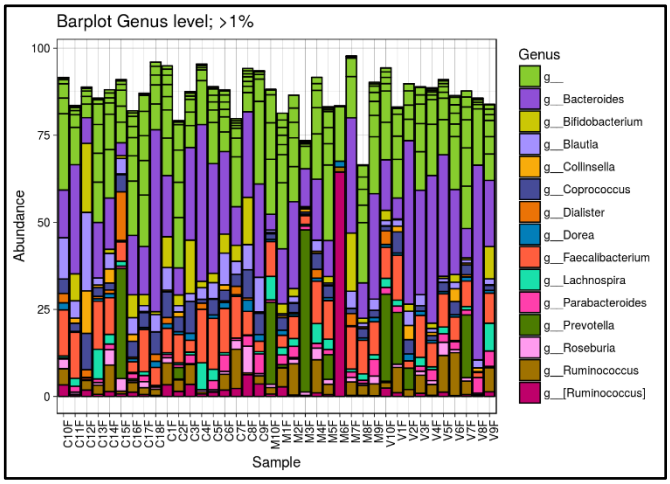

Figure 26- Bar plot, relative abundance for sample- bacterial, genus

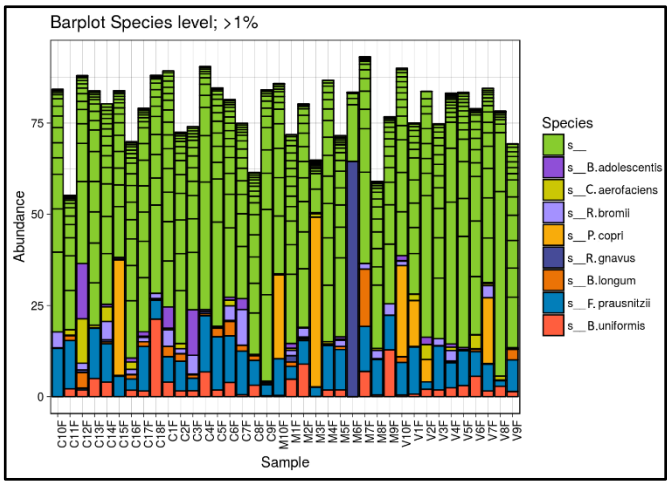

Figure 27- Bar plot, relative abundance for sample- bacterial, species

As to the statistical significance of the abovementioned bacterial taxonomic differences between melanoma and vitiligo, the analysis was conducted at phylum and genus level and evaluated the differences of individual microbial OTUs between vitiligo and melanoma subject using the negative binomial Wald test with Benjamini–Hochberg correction for multiple comparisons. The abovementioned analysis and the relative Volcano Plot graph conducted at Species and Genus level graphically showed that Firmicutes *Ruminococcus* ($p<0.05$), *Firmicutes Lactobacillus* ($P<0.05$), *Firmicutes Coprococcus* ($p<0.01$), *Firmicutes Catenibacterium* ($p<0.01$), *Bacteroidetes Prevotella* ($p<0.01$), were significantly associated with the melanoma group, while Bacteroidetes ($p<0.01$), *Firmicutes Acidaminococcus* ($p<0.01$) were associated with the vitiligo group (Fig 28). The remaining differences in the relative

abundance of bacterial species among vitiligo and melanoma samples were not statistically significant.

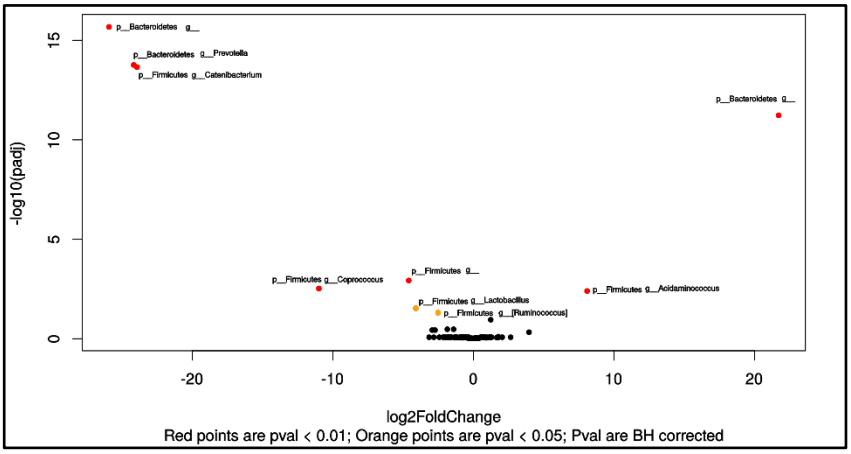

Figure 28- Volcano Plot, Vitiligo vs Melanoma, Bacterial

Correlations between bacterial gut microbiota and clinical features of the diseases

Multivariate analysis of variance using distance measurements (PERMANOVA analysis) was conducted to evaluate possible influences of invasiveness, growth phase, Breslow thickness, mitotic index, presence of tumour infiltrating lymphocytes, and presence of regression on the diversity of bacterial species of the melanoma group.

As to the vitiligo group, possible effects of associated autoimmune thyroid diseases, associated autoimmune disorders, ANA positivity, 25(OH)D values, Ab anti TPO positivity, activity of the disease, onset, growth, signs of inflammation/pruritus, presence of leukotrichia, total extension and reported stress at onset on the diversity of species of the vitiligo group were evaluated.

The above-mentioned evaluations were statistically significant (Table 9) for mitotic index in the melanoma group ($p=0.016$; $R^2=0.181$), and for 25(OH)D values ($p=0.017$; $R^2=0.192$) and association with autoimmune disorders ($p=0.008$; $R^2=0.170$) for vitiligo group (Table 9).

Hence, with the aim of further analysing dependence of community diversity and the mitotic index in melanoma group and 25(OH)D values in vitiligo group, the relative constrained ordination with CCA were performed. These analyses showed that the

bacterial community diversity in melanoma was influenced by the mitotic index, while the bacterial community diversity in vitiligo was influenced by 25(OH)D values.

Ordination graph for mitotic index showed that the samples with the highest mitotic index tend to separate over the first ordination axis (CCA1 explaining 16.8% of total variance). In particular, samples having the highest mitotic index among melanoma patients (7 mitoses/mm^2), tend to separate from the rest of the samples. Still, all the samples with lower mitotic index values, separated along the second ordination axis (CA1, explaining 20.1% of total variance), not correlated to the variable value (Fig. 29).

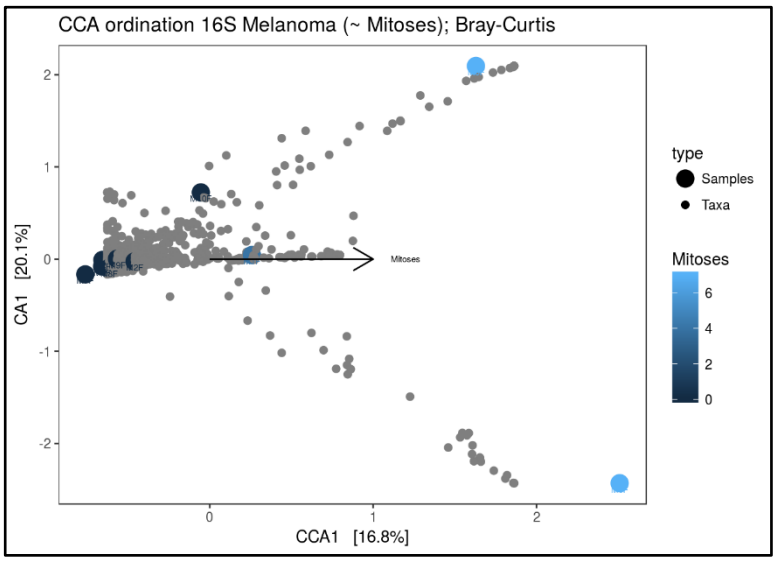

Figure 29- Ordination graph, melanoma and mitotic index, bacterial.

Furthermore, when colouring the taxa points based on the genus (Fig. 30) some genera appear to be mostly associated to sample with the highest mitotic index, namely *Firmicutes* and *Bacteroidetes.*

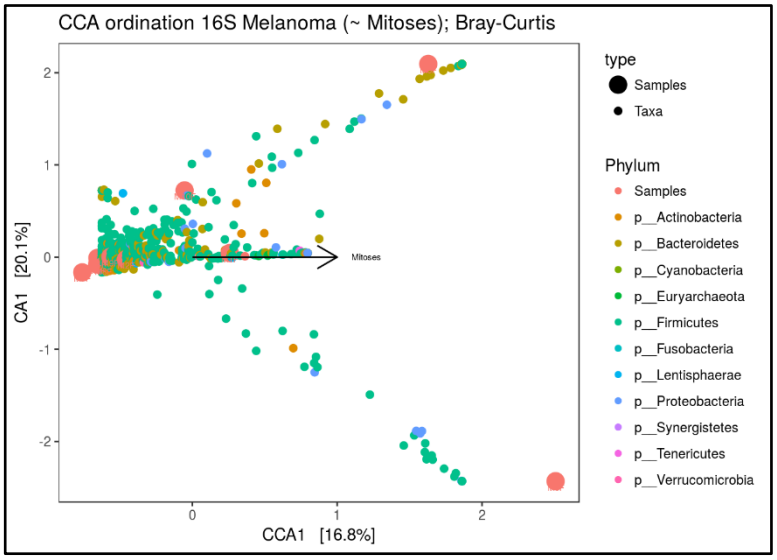

Figure 30- Ordination graph with taxa, melanoma and mitotic index, bacterial

Ordination graph for 25(OH)D values showed that the samples with the highest 25(OH)D values tend to separate over the first ordination axis (CCA1 explaining 15.8% of total variance). In particular, samples having the highest 25(OH)D values among vitiligo patients (respectively 37.4 and 34 ng/ml), tended to separate from the rest of the samples. All the samples with lower 25(OH)D values, separated along the second ordination axis (CA1, explaining 16.5% of total variance), not correlated to the variable value.

Furthermore, when colouring the taxa points based on the genus (Fig. 31) some genera appear to be mostly associated to sample with the highest 25(OH)D values, namely *Firmicutes* and *Bacteroidetes*.

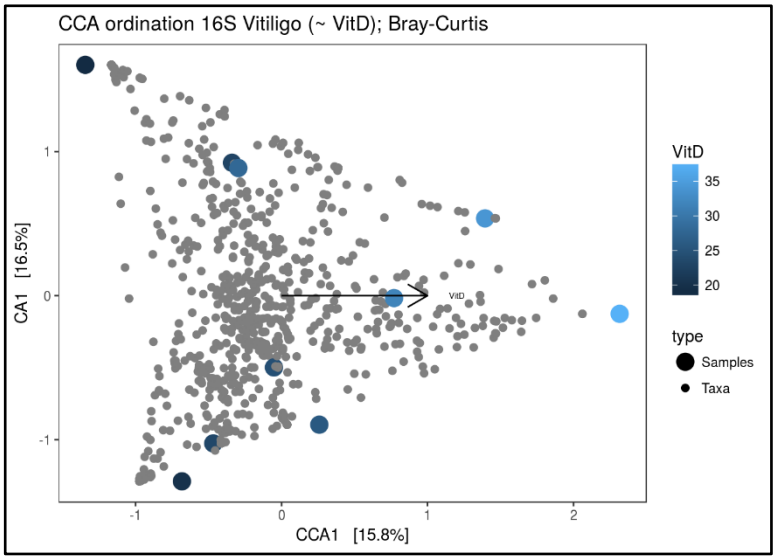

Figure 31- Ordination graph, vitiligo and vitamin D, bacterial

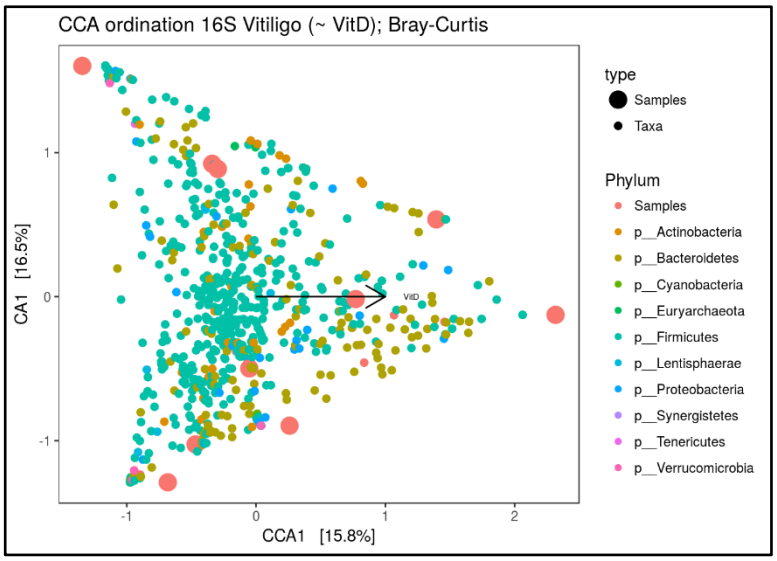

Figure 32- Ordination graph with taxa, vitiligo and vitamin D, bacterial

As to the statistical significance of the association with concomitant autoimmune disorders in the vitiligo groups, the statistical analysis and the negative binomial Wald test with Benjamini-Hochberg correction for multiple comparison was applied. Such analysis and the relative Volcano Plot graph conducted at Species level graphically showed that *Bifidobacterium* ($p<0.05$), *Bacteroides Plebeius* ($p<0.05$), and *Prevotella* ($p<0.05$) had a statistically significant increased abundance in the subgroup of vitiligo patients without associated autoimmune disorders, while *Bacteroides Barnesiae* had a

statistically significant increased abundance in vitiligo patients with associated autoimmune comorbidities ($p<0.05$) (Fig.33).

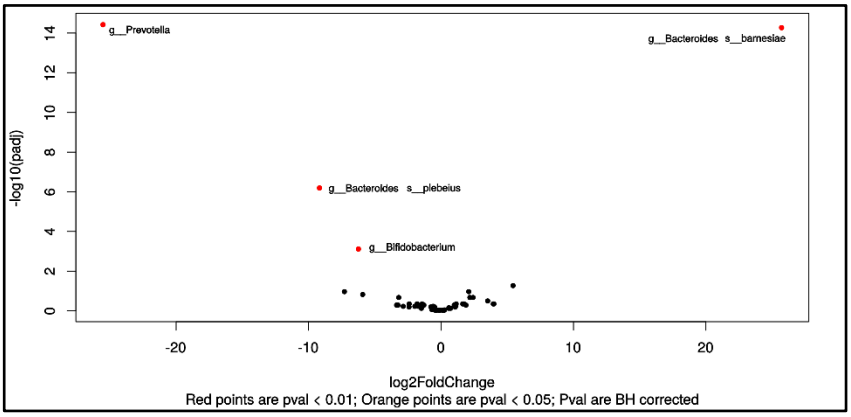

Figure 33- Volcano Plot, Vitiligo with autoimmune diseases vs Vitiligo without autoimmune diseases, Bacterial

The box plot conducted at genus level showed how the relative abundance of bacteria were distributed among the two subgroups (vitiligo with or without associated autoimmune disorders), and clearly elucidates the above mentioned statistically significant finding (Fig.34)

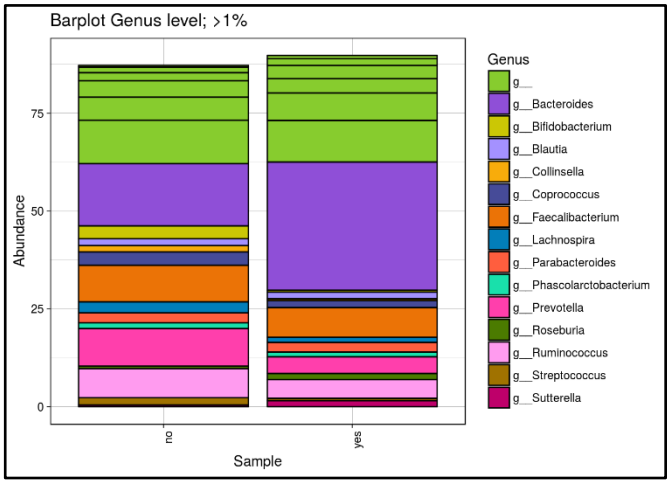

Figure 34- Box plot relative abundance:

Vitiligo with autoimmune diseases (yes) vs Vitiligo without autoimmune diseases (no),

Bacterial

9.3 Isolation and identification of cultivable fungal species from faeces

The cultivable gut mycobiota of melanoma and vitiligo patients was investigated through isolation in selective media. Fungi were detected in three melanoma faecal samples (M6, M8, M9). Among the identified fungal species there were *Candida albicans*, *Saccharomyces cerevisiae* and *Saccharomyces paradoxus*. Results are reported in Table 10.

10. Discussion

The gut microbiota is crucial for regulating immune system [214] and modulating cancer mechanisms [192].

Microbial species have been found to be able to modulate pro-oncogenic or anti-cancer mechanisms [192]. Indeed, patients affected by cancer, namely gastrointestinal tumours, have been found to harbour a specific microbiota composition [162], which is different from that pertaining to healthy subjects. Several investigations reported a condition of dysbiosis also in patients affected by autoimmune disorders [215] and identified selective microbial species possibly involved in modulating the clinical features of such diseases.

Most of studies performed so far, both on mice models or on humans, mainly focused on the metagenomic evaluations of bacterial gut microbiota and extensively assessed its features in autoimmune disorders and cancer.

At present there is a paucity of evaluations of fungal gut microbiota in patients affected by systemic autoimmune diseases or visceral cancers.

As to dermatological diseases, the most relevant evaluations of gut microbiota are restricted to a few immune-mediated diseases (psoriasis and atopic dermatitis) [271-273] and to advanced metastatic melanoma [309,310,312,313] . However, such investigations only assessed bacterial microbiota, while gut mycobiota has never been assessed so far in autoimmune/immune mediated and tumoral dermatological diseases.

Therefore, in this thesis we decided to investigate bacterial and fungal gut microbiota in a group of early stages melanoma and vitiligo subjects. The knowledge of the features of bacteria and fungi inhabiting the intestinal tract of patients affected by such melanocytic disorder indeed is completely obscure, but the current knowledge of how bacteria and fungi regulate immune system and cancer mechanisms, together with the increased data on vitiligo and melanoma pathogenetic mechanisms, suggests a possible and reasonable involvement of gut microbiota compositions in these disorders too.

Our study reports a reduction of fungal richness (alpha diversity) and variability (beta-diversity) in melanoma and vitiligo patients, as compared to healthy controls (Fig.1,3,4). In addition, vitiligo and melanoma fungal richness were different, although not significantly. These findings suggest that a specific distinctive fungal mycobiota can be observed in melanoma and vitiligo subjects. As previously indicated,

no other comparable studies so far assessed fungal microbiota in melanoma or vitiligo. Gut mycobiota has been found to be altered in colorectal cancer patients and in patients affected by polyp adenomas [417,418], and some species involved in colonic inflammation have been found to be significantly increased in colorectal carcinoma patients. Inflammation is considered as a crucial step toward cancer [174], and recent findings suggest a possible role of gut bacteria in such a pathogenetic process [161,174]. As regards to melanocytic disorders, such as melanoma, we cannot hypothesize a direct action of gut fungi on melanocytes, since such pigmentary cells are located directly in the skin. It has been demonstrated however that fungi, besides their known role in maintaining the homeostasis of bacterial gut community, might play a key role in immune regulation, since they elicit and direct strong immune and adaptive responses [419,420]. We might hence assume that an altered fungal environment might lead to a systemic immune impairment of the patho-physiological responses involved in immune surveillance, which destroys tumoral cells or limit their growth or metastatic potential. Several investigations indeed suggest an increased risk of melanoma in immune suppressed patients [421-423]. Furthermore, a dysbiotic gut mycobiota could lead to a systemic pro-inflammatory status, characterized by an increased activation of immune cells and by the release of pro-inflammatory cytokines, possibly able to reach the skin and modulate the function of immune cells at skin level. Skin inflammation is a crucial factor for neoplastic differentiation of melanocytes [424,425], as demonstrated by the effects of the pro-tumorigenic role of UV light, which is a strong inflammatory stimulus, activating pro-carcinogenetic biological pathways [426]. Furthermore, an increased expression of proteins involved in inflammasome activation, such as ATP-regulated plasma membrane channels that trigger inflammasome activation, together with the consequent constitutive activation of inflammasomes such as NALP3 and the increased levels of COX2, have been reported in melanoma [424], thus demonstrating a skin inflammatory status in this neoplasia.

Hence, a skin inflammatory status, possibly induced or modulated by a systemic inflammation related to a gut fungal dysbiosis, can occur in the epidermal melanocytic microenvironment, leading to the final accumulation of immune cells capable of releasing pro-inflammatory cytokines, or tumour growth factors possibly promoting a pro-tumorigenic milieu able to contribute to the melanocytic neoplastic shift. The crucial role of melanoma microenvironment in modulating the progression and

metastatic potential of this skin cancer has been widely demonstrated and assessed so far [427,428].

As regards to vitiligo, the finding of an altered gut fungal richness and diversity is noteworthy and needs future investigations. In case of fungal infections innate immune cells, mainly Th1 and Th17 cells, use a wide variety of membrane-bound and soluble receptors (lectin receptors, Toll-like receptors and scavenger receptor family member, mannose-binding lectin) to recognize fungi [420, 17]. These receptors trigger phagocytosis, respiratory burst (via the NADPH oxidase) and trigger intracellular signalling pathways finally leading to the activation of pro-inflammatory transcription factors and the destruction of fungi [420]. Consequently, the release of pro-inflammatory cytokines and chemokines such as IL-22 , IFN-γ, IL-17A and IL-17F can occurr [420], and might eventually reach circulation. Th17 cells and their related cytokines, together with an increased oxidative stress, are pathogenetic hallmark of vitiligo and have been reported to be increased both systemically and at epidermal level [421]. It might be possible thus that a gut fungal dysbiosis might contribute, in genetically predisposed subjects, to the development of a targeted damage toward melanocytes, through several mechanisms connected with Th17 activation and IL-17 release [429], leading to the development of vitiligo. An increase of systemic IL-17 might contribute to the melanocytic damage through the release of the chemokine CCL20, a homing molecule that can attract cytotoxic CD8+ T cells from systemic circulation into peripheral tissues such as the skin, where they could be able to directly kill melanocytes [429]. In addition, systemic IL-17 might reach epidermis and stimulates keratinocytes to release several chemokines resulting in further immune cell recruitment or indirect loss of melanocytes [429], clinically resulting in vitiligo lesions.

Interestingly, a gut fungal impairment in vitiligo patients could explain the reduced risk of melanoma in such patients and could explain the prognostic role of melanoma associated leukoderma. In fact, Th17 cells play a key role in anti-melanocyte immune response during melanoma, as reported by the evidence that melanocyte-specific T cells were able to clear melanoma in a more efficient way if they were cultured in Th17-polarizing conditions [429,430]. Such effect might be due to the ability of IL-17 to decrease expression of MITF (a survival factor for

melanocytes) and also promote melanocyte death by down-regulating BCL2, thus leading to the apoptosis of melanoma cells [429,430].

Even if not significant, our finding of a difference in fungal richness and diversity (alpha and beta diversity) between melanoma and vitiligo, together with the differences in the relative abundance of species in melanoma and vitiligo that we observed, suggests that different fungal gut composition could exert a possible pro-tumorigenic role in melanoma, and a possible anti-melanocytic and antitumorigenic role in vitiligo or in melanoma associated leukoderma.

As to the relative abundance of fungal species in controls, we found a high abundance of *Candida* species, associated with the remining group constituted by undetermined species. The elevated presence of undetermined species, as indicated by the bar plot relative to species level (Figures 5, 6), might be due to different sequencing methods, since controls were evaluated in a different and preceding sequencing run than melanoma and vitiligo samples. However, the increased richness at OTUs level, as compared to melanoma and vitiligo, indicated by alpha diversity index, suggests that the controls' mycobiota was richer in species than in the diseases' groups, and that a problem eventually possibly occurred in the taxonomic attribution of OTUs. Only a few investigations [416, 431] assessed healthy gut mycobiota, and identified *Candida,* subdivided into its major species (*Candida albicans*, *Candida glabrata, Candida dubliniensis* and *Candida parapsilosis*), as one of the most frequently found genera in human gut. Such data was also confirmed by a recent investigation on human faecal samples, which used materials and methods comparable to ours [416]. The meta-analysis we made between the data reported by this investigation and our results also confirms such an abundance and distribution of fungal species [416] (Figure 14).

With regards to the comparison of the relative abundances of fungi between melanoma and vitiligo, the analysis conducted at species level showed that *Penicillium Roqueforti* ($P<0.01$), *Fusarium sp*. ($P<0.01$) and *Candida sake* ($P<0.05$) had a statistically significant higher abundance in melanoma, as compared to vitiligo (Fig.13). Several *Candida* species were also detected by the isolation and identification process of cultivable fungal species from faeces (Table 10).

It has been reported that mice models chronically supplied with *Penicillium Roqueforti* mycotoxin (PR toxin) developed cancer, and interestingly one animal developed a skin cancer on the neck (squamous cell carcinoma) [432].

On the contrary, other studies reported that Andrastins, which are mycotoxins produced by P. *Roqueforti*, have been proposed as novel and interesting anticancer drug candidates, due to their ability to inhibit farnesyltransferase of the Ras proteins implicated in cell division control, and due to their capacity to enhance the accumulation of anticancer drugs in vincristine-resistant cancer cells [433]. Other experimental investigations also report that another *P. Roqueforti* mycotoxin, called botryodiplodiatoxin, is able to affect DNA, RNA and protein synthesis in growing cultures of mammalian cells and to induce DNA-protein cross-links [434,435].

On the contrast, *Fusarium*, a large genus of filamentous fungi, part of a group often referred to as hyphomycetes, is widely distributed in soil and associated with plants and has been demonstrated to produce different mycotoxins, able to interact with in vitro melanoma cells cultures. In particular, Sansalvamide A, isolated from a marine fungus of the *Fusarium* genus was found to inhibit proliferation and to induce differentiation and apoptosis of murine B16 melanoma cells [436]; Neosansalvamide, produced by *Fusarium solani* KCCM90040 (isolated from *Fusarium* -contaminated potato in Korea) [437], and enniatins H, I, and MK1688, isolated from Fusar*i*um *oxysporum* KFCC 11363P, were found to be cytotoxic for SK-MEL-2 melanoma cells [438]; Fusarochromanone, a toxic metabolite produced by *Fusarium equiseti*, was found to be cytotoxic for many melanoma cell lines [439]; and Fumonisin B1, a mycotoxin produced by the corn fungus *Fusarium moniliforme*, inhibits integrin-mediated cell-matrix adhesion [440], pivotal process in neoplastic dissemination.

The finding of an increased abundance of *Fusarium* in melanoma group is in contrast with the above-mentioned evidences, but, even if the best way to improve melanoma prognosis remains an early diagnosis and a prompt excision, we could theoretically hypothesize that *Fusarium* species might have limited the development of an aggressive phenotype in our melanoma patients.

Of course, given the limitations in translating mouse or in vitro models on human pathophysiology, the fact that some mycotoxins are rarely isolated and only experimentally produced, and the possibility that different strains of *P. Roqueforti* might produce different pro-tumorigenic or anti-tumorigenic mycotoxins, we absolutely cannot correlate the increased abundance of such fungus in our melanoma patients with melanoma development. As well, for the same reasons, we cannot ascribe a certain protective role of *Fusarium* sp. in melanoma. However, we think that this interesting finding is noteworthy of further evaluation.

As to the higher abundance of *Candida Sake*, given the extensive presence of such fungi also in healthy patients and the absence of known interactions of this yeast with melanocytes or cancer, we cannot speculate a possible role of this presence in melanoma.

The comparison of the relative abundances of fungi between melanoma and vitiligo at species level showed that *Sporobolomyces roseus* ($P<0.01$) and *Epicoccum nigrum* had a statistically significant higher abundance in vitiligo, as compared to melanoma (Fig.13). As to *Sporobolomyces roseus*, an experimental biochemical study showed that cell-free extracts of this fungus can degrade tyrosine [441], a key enzyme in melanogenesis.

As to *Epicoccum nigrum*, its derived bioactive compound Di-(2-ethylhexyl) phthalate (DEHP) has been demonstrated to exert a moderate toxicity against SK-MEL-28and A375P human melanoma cell lines [442].

It is not known if such fungal metabolites are produced in vivo. However, assuming that somehow this species or its metabolites might reach the epidermis, hypothetically an accumulation of *Sporobolomyces roseus* might interfere with the synthetic pathways involved in melanogenesis, since tyrosine is the substrate for melanin synthesis, while an increase in *Epiccoccum nigrum* could account for the reduced proneness to melanoma in vitiligo.

S. cerevisiae was found to have an increased higher relative abundance in vitiligo, as compared to melanoma. Although this difference did not reach a statistically significant level, we found this evidence of interest. It has been demonstrated indeed that extract from rice bran fermented with *S. cerevisiae* is able to downregulate in B16 melanoma cell lines the expression of MITF, a transcription factor regulating gene expression essential for melanin synthesis in melanocytes, thus inhibiting melanogenesis [443]. Furthermore, EP2, natural yeast extract isolated by ethanol precipitation from *Saccharomyces cerevisiae*, was found to inhibit melanogenesis and melanosome transfer when added to melanocytes/keratinocytes co-cultures [444]. Interestingly, EP2 has been demonstrated to interact with protease-activated receptor 2 (PAR-2), a key protein associated with melanosome transfer from melanocytes to keratinocytes, down-regulating this receptor [444]. It is noteworthy that PAR-2 m RNA and protein expression have been reported to be reduced in the skin tissue specimens of vitiligo patients and in cultured keratinocytes obtained from the lesional or non-lesional skin of vitiligo patients [445]. Hence, hypothesising that *S. cerevisiae*

or a possible derivative of this yeast might reach the epidermis, a possible interaction with PAR-2 receptor could justify its reported down regulation observed in vitiligo and could be responsible for the appearance of vitiligo macules. Another interesting study showed that in experimental lung metastasis of B16-BL6 melanoma cells, the prophylactic administration of β-glucan purified from mutated *S. cerevisiae* significantly inhibited lung metastasis in a dose-dependent manner [446]. The same study showed that beta glucan inhibits metastasis through the activation of an immune-surveillance mechanism involving innate immune cells such as macrophages and NK cells [446]. This study suggests a theoretical possible protective role of this yeast toward melanoma in vitiligo and could possibly justify the prognostic role of the occurrence of leukoderma in melanoma patients. *Saccharomyces cerevisiae* was also detected by the isolation and identification process of cultivable fungal species from faeces (Table10).

Such observations definitely suggest the future need for a fungal microbiota evaluation of vitiligo and melanoma skin.

Our investigation also showed that the community diversity in the vitiligo group is influenced by the total extension of the disease. This data is interesting since it proposes that fungal gut community in vitiligo not only has a distinct richness and diversity from controls and melanoma, but also could be involved in affecting the clinical features of the diseases, such as the total extension, and in other words the severity of vitiligo. We observed that some genus, namely *Candida* and *Penicillum*, and some unidentified genera appeared to be mostly associated to the sample V1 (with the highest total extension of the disease) (Fig. 16) . This data at present raises poor interest, since *Candida* has also been identified in controls and melanoma (Fig.11), and the *Penicillium* specie found in this patient might belong to *Penicillium Coccotrypicola* (Fig.12), whose interaction with immunity or melanocytes have not been studied at present, or to other unidentified *Penicillium* genera. A possible role of fungi in influencing the extension of the disease in this patient might possibly be carried out from other fungi fallen into the group of undetermined species.

Our study also aimed to characterize the bacterial microbiota of melanoma and vitiligo patients. Contrarily to what was observed for gut mycobiota, we did not find significant differences in richness and diversity of bacterial composition between melanoma, vitiligo and healthy samples (Fig. 17-19). Furthermore, the examination of

the relative abundance of different bacteria confirmed this evidence, showing an overall similar abundance of species between the three groups (Fig.20,21).

Gut microbiota of healthy subjects, melanoma and vitiligo patients overall appeared to be mainly composed at phylum level by *Bacteroides* and *Firmicutes*, and to a less extent by *Proteobacteria* and *Actinobacteria* (Fig. 21). This evidence is in accordance with similar evaluations published so far which identified similar gut microbiota compositions in humans [1,447,448].

With regards to the comparison of the relative abundances of bacteria between melanoma and vitiligo, the analysis conducted at species level pointed out a statistically significant higher abundance *Firmicutes* of different genera [*Ruminococcus* ($P<0.05$), *Lactobacillus* ($P<0.05$), *Coprococcus* ($P<0.01$) and *Catenibacterium* ($P<0.01$)] and *Bacteroidetes Prevotella* ($P<0.01$) in the melanoma group, as compared to vitiligo (Fig 28).

Contrarily to what we found in our melanoma population, bacteria relative to the Firmicutes phylum had been reported to be reduced in the gut of patients affected by colorectal cancer [449]. Accordingly, *Ruminococcus*, a genus related to *Firmicutes*, has been reported to be less abundant in the gut of colorectal cancer or colitis associated colorectal cancer patients [450]. Nothing is known at present as regards to a possible influence of *Firmicutes* genera *Ruminococcus*, *Lactobacillus*, *Coprococcus* or *Catenibacterium* in melanoma. However, it has been demonstrated that butyrate, which is produced by some *Firmicutes* and in particular by *Coprococcus* and *Ruminococcus* genera from fermentation of dietary fibers, induces the expression of annexin A1 in human melanoma cells, thus promoting invasion through the activation of the epithelial to mesenchymal transition (EMT) signalling pathway, a critical step in cancer cells metastasis [451]. Since short chain fatty acids such as butyrate can reach circulation and tissues, it might be theoretically possible that an imbalance in butyrate-producing bacteria might influence melanoma growth through an increased systemic butyrate level. It might be also conceivable however that the higher abundance of *Firmicutes* might be simply linked to a high dietary intake of fibres [452] or salt [453], that our enrolled patients with melanoma disclosed in the dietary questionnaire (Appendix 1, Table 1, 2).

As to the increased relative abundance of *Bacteroides Prevotella* in melanoma, it is unknown at present if this bacterium might interact with melanocytes to promote tumorigenesis, but this data is in accordance with an evaluation of gut microbiota in

colorectal cancer, where a higher abundance of this bacteria was found [454,455]. *Prevotella* has been demonstrated to be involved in the expression of immunoinflammatory response genes in colorectal cancer, particularly genes encoding for inflammatory cytokines and chemokines [455]. Among them, CXCL1 expression has been shown to increase the survival of cancer cells and to promote angiogenesis in colorectal cancer [456], and interestingly it has been demonstrated to be involved in melanoma growth, survival, angiogenesis and metastasis [457]. Thus, the increased abundance of *Prevotella* might lead to the development of a systemic pro-inflammatory status possibly involving the release of CXCL1 and hence theoretically modulating melanoma growth.

With regards to the comparison of the relative abundances of bacteria between melanoma and vitiligo, the analysis conducted at species level pointed out a statistically significant higher abundance of *Firmicutes Acidaminococcus* ($P<0.01$) in the vitiligo group, as compared to melanoma (Fig.28). This bacterium has been demonstrated to be have an unfavourable influence on intestinal energy metabolism, since it consumes the glutamate, an essential compound for barrier function, amino acid metabolism and nitrogen balance [458,459]. Hence, the increased abundance of this bacteria might have induced a gut dysbiosis. Accordingly, community-level changes in the gut microbiota and a linear growth faltering have been found in association with increased abundance of *F.acidaminococcus* in two cohorts of children from Malawi and Bangladesh [460]. Even if dysbiosis has been found to be associated with several autoimmune disorders, *F. acidaminococcus* has been found to be reduced in faecal samples of Type I diabetes children, as compared to controls [461]. However, although this result is partially in contrasts with our finding, it might be possible that the increased relative abundance of this bacteria in vitiligo might induce dysbiosis and hence limit the growth of beneficial and tolerogenic microbes, thus triggering autoimmunity.

Our study also found that the bacterial community diversity in the melanoma group is influenced by the mitotic index (Fig. 29,30). More in detail, samples belonging to melanoma patients with the highest mitotic index tended to separate from the rest of the samples. Some phylum appeared to be mostly associated to highest mitotic index samples, namely both *Bacteroides* and *Firmicutes*. This analysis failed to identify an association of such samples at lower taxonomic level. Hence, being *Firmicutes* and *Bacteroides* constituted by several diverse bacteria with different and

sometimes opposite effects on cancer, it is not possible at present to speculate about possible pathogenetic mechanisms which could explain this association. However, the evidence of a possible influence of mitoses on bacterial diversity shed light to an eventual role of bacteria in modulate and affect the histological features of melanoma, and hence its prognosis.

The bacterial community diversity was also found to be related to vitamin D level in vitiligo patients (Fig.31,32). More in details, samples belonging to vitiligo patients with the highest vitamin D level tended to separate from the rest of the samples. Also in this case, some phylum appeared to be mostly associated to such samples, namely both Bacteroides and Firmicutes and again we did not identify a clear association of such samples at lower taxonomic level. Hence, possible hypothesis explaining a pathogenetic link between vitamin D levels and specific bacteria cannot be provided in our study. However, vitamin D deficiency and polymorphisms in vitamin D receptor (VDRs) have been widely demonstrated in vitiligo patients [462, 463], similarly to other autoimmune diseases [464], and recently also a strict interconnection between vitamin D level and microbiota has been disclosed in autoimmunity.

In particular, vitamin D modulates the microbiome of the upper gastrointestinal tract, maintains the integrity of the gut mucosal barrier through an enrichment of intercellular junctions involved in the control of mucosal permeability, thus preventing the translocation of endotoxins into the circulation, and reduces the release of pro-inflammatory cytokines such as IL-8 [465,466].

Also, VDRs play a crucial role in gut homeostasis, since their activation modulates autophagy and elicits the production of antimicrobial peptides (mainly cathelicidin and β-defensin), which can modify gut intestinal microbiota toward a healthier composition [467]. It is noteworthy that besides vitamin D, also by-products of the microbiota such as secondary bile acids might also trigger VDR activation [467].

In the light of the abovementioned considerations, the evidence we found of a direct influence of vitamin D level in microbial diversity of vitiligo patients strengthens the importance of maintaining adequate vitamin D serum levels in vitiligo. Sufficient vitamin D levels might reduce the autoimmune and inflammatory processes associated with vitiligo not only through a direct interaction of this vitamin with immune system, but also through the maintenance of a healthy gut microbiota.

Finally, a statistically increased relative abundance of *Bifidobacterium* ($P<0.05$), *Bacteroides Plebeius* ($P<0.05$), and *Prevotella* ($P<0.05$) was significantly associated

with the subgroup of vitiligo patients without associated autoimmune disorders, while *Bacteroides Barnesiae* was associated with vitiligo with autoimmune comorbidities ($P<0.05$) (Fig.33,34).

Bifidobacterium is a ubiquitous bacterium of the gastrointestinal tract exerting potent probiotic activities. Distinct species of this bacteria have been reported to be decreased in the gut microbiota of patients affected by type 1 diabetes [468] but increased in ankylosing spondylitis [469] and enthesitis-related arthritis [470]. The supplementation of some strains of *Bifidobacterium* can exert beneficial effects on mouse models of multiple sclerosis [471] and systemic lupus erythematosus [472], due to their ability to prevent CD4(+) lymphocyte over-activation [472] in the latter disease. On the contrary, *Bifidobacterium bifidum* 791 cell-surface biopolymers interact selectively with human serum thyroid peroxidase and thyroglobulin autoantibodies [473], suggesting a possible role of *Bifidobacteria* in the pathogenesis of autoimmune thyroid diseases through molecular mimicry mechanisms.

We are not able to identify from our analysis what kind of species or specific strain of *Bifidobacterium* could account for the diversity in vitiligo patients or vitiligo patients without autoimmune disease. Hence, we reasonably hypothesize a protective role of *Bifidobacterium* in vitiligo patients toward the development of associated autoimmune disorders, but we cannot exclude the presence of associated undetermined different *Bifidobacterium* strains in the group pf patients contemporarily developing autoimmune diseases.

As regards to *Prevotella*, some species of this bacteria, namely *P. Copri*, have been found increased in patients affected by autoimmune disorders, such as rheumatoid arthritis [474]. This is in contrast with our finding of a major abundance of this bacteria in vitiligo patients without autoimmune disorders. However, no enrolled vitiligo patients however suffered from rheumatoid arthritis, being autoimmune thyroiditis the most frequently found comorbidity, and a decreased abundance of *Prevotella* has been recently demonstrated in patients affected by Hashimoto thyroiditis [475]. This evidence sustains our finding of a lower presence of *Prevotella* in patients with concomitant autoimmune disorders, most of them affecting thyroid, as compared to patients affected by vitiligo only, in which *Prevotella* had a significant higher abundance.

As to the significant increase of *B.Barnesiae* in vitiligo patients with autoimmune diseases, we cannot provide a reasonable explanation for this evidence. Indeed, this is

the only study performed on humans aimed to isolate this Gram-negative, anaerobic, rod-shaped bacterium from human faeces [476]. No other study reported so far differences in the abundance of this bacterium in health and disease.

11. Conclusions

This study has characterized, for the first time, the fungal and bacterial gut microbiota in patients affected by melanocytic disorders, such as non-metastatic melanoma and vitiligo. The most promising findings were obtained at fungal levels, suggesting how the characterization of this still obscure component of human gut microbiota deserves more attention and more in-depth investigations. Despite the limited number of enrolled subjects, we also found that the diversity of microbial community, both at fungal or bacterial level, was influenced by histological indicators of prognosis in melanoma, such as the mitotic index, and by clinical indicators of severity of vitiligo, such as disease extension. Furthermore, the isolation and identification of fungi through classical cultural techniques and advanced sequencing approaches allowed us to confirm the results of the metagenomic evaluation and to plan further physiological investigations aiming to identify pathogenicity and virulence features of the isolated fungi.

Major limitation of this study, due to its design, is the impossibility to prove a causal role of the increased/reduced relative abundance or absence of the micro-organisms in the pathogenetic process of melanoma or vitiligo. Furthermore, even if a growing body of evidences sustains the presence of a gut-skin axis, we do not know exactly at present how gut fungi or bacteria might exert biological effects at skin level. Other limitations are the restricted number of assessed patients and the possibility that unidentified species might have a possible undetectable role on the diseases.
Nonetheless, our study prompts further investigations on a larger cohort of melanoma and vitiligo patients, and suggests evaluating also the gut microbiota of subjects developing melanoma associated leukoderma.

The improved knowledge of fungal and bacterial microbiota in melanoma patients might help to define possible lifestyle habits such as a specific diet, weigh reduction or lower antibiotics assumption, which might help to recover a healthy microbiota. The restoring of eubiosis might reduce a possible genotoxicity, anti-apoptosis, anti-inflammation and anti-proliferative ability connected with gut microbes. This favourable microbial community might also stimulate an immune response toward melanoma cells, limit its progression and positively affect prognosis.

The knowledge of the fungal and bacterial gut microbiota signature in vitiligo might help to identify a specific anti-melanoma microbiota signature, possibly able, if reconstituted through lifestyle habits, diet, probiotics/prebiotics or maybe faecal transplantation, to favour the development of beneficial immune-surveillance responses in melanoma patients.

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Table 1. Results of the dietary diary of melanoma patients

TABLE 1

	Drinks	Biscuitsr usks cereals	Pasta rice potatoes	Animal proteins (meat fish)	Eggs cheese	Dressing	Spices	Bread	Vegetables	Fruit	Water (lt)	Sweets	Probiotics Prebiotics	Oral Antibiotics	Yogurt	Breast feeding	Weaning (months)	Vaginal delivery	Caesarean delivery	Food Allergy
M1	3 coffee	6rusks	3 pasta	3 fish 3 meat	no	6 olive oil	salt pepper	3	3	3	4.5	no	no	no	no	yes	7	yes	no	no
M2	2 coffee	3 cereals	3 pasta	1 meat	1 egg	3 olive oil	salt	6 brown	3 salad	3 apricot 3 banana	6	no	no	no	yes	yes	6	yes	no	no
M3	5 coffee	3 biscuits	1 pasta; 1 potatoes	3 meat	3 cheese	3 olive oil	salt	3	3 salad; 3tomato	1water melon 3peaches	6	3 ice cream	no	no	yes	yes	6	yes	no	no
M4	6 coffee; 3 soy milk	9 rusks	3 pasta	1 meat	1 cheese	3 olive oil	salt	no	3 salad	3 peaches; 3apricot	6	3 sugar	no	no	yes soy	yes	8	yes	no	no
M5	3 coffee	6 biscuits	3pasta	no	3cheese	3 olive oil	salt	3	3 tomato	6	3	3marmalade	no	no	yes	yes	6	yes	no	no
M6	1 coffee	no	3 pasta; 3 rice	1 fish	3 eggs	3 olive oil	salt	3	no	no	3	1icecream	no	no	no	yes	8	yes	no	no
M7	12 coffee	12 biscuits; 15 rusks	6 pasta	no	3 cheese	3 olive oil	no	3 brown	1 salad; 1 tomato	3 apricot 3peaches 3walnuts	4.5	no	no	no	yes	unknown	unknown	yes	no	no
M8	6 coffee	9 biscuits	3 pasta	1 meat	no	3 olive oil	salt	3	3 salad; 3 tomato	3apricot; 3peaches 3water melon	3	no	no	no	no	no	8	yes	no	no
M9	3 coffee	3 cereals	3 pasta	3 meat; 3 fish	3 cheese	6 olive oil	salt	3	3 salad; 3 chicory	3 apricot; 3peaches 3 pear	6	3 cakes	no	no	no	yes	unknown	yes	no	no
M10	6 coffee	no	no	3 meat	3 cheese	3 olive oil	salt pepper	6 brown	3 salad; 3 zucchini	3peaches 3 apricot; 3 plum	4.5	no	no	no	no	no	unknown	yes	no	no

Table 2. Results of the dietary diary of vitiligo patients

TABLE 2

	Drinks	Biscuits, rusks, cereals	Pasta, rice, potato	Animal proteins (meat, fish)	Eggs, cheese	Dressing	Spices	Bread	Vegetables	Fruit	Water (lt)	Sweets	Probiotics Prebiotics	Oral Antibiotics	Yogurt	Breast feeding	Weaning (months)	Vaginal delivery	Caesarean delivery	Food Allergy
V1	3, coffee	6biscuits 3 rusks	3, pasta	no	3,eggs	3, olive oil	salt	3, brown	3, salad	3 pear;6 strawberries	3	no	no	no	no	yes	6	yes	no	no
V2	no	no	3,pasta	no	no	6, olive oil	salt	3	9, salad; 3,broad beans	6, apples	3	no	no	no	no	yes	8	yes	no	no
V3	no	3biscuits	3,pasta	3,meat	no	3, olive oil	salt	3	3,salad	6,apples	4.5	no	no	no	no	yes	8	yes	no	no
V4	3, coffee	no	3, pasta	3,meat; 3,fish	no	3, olive oil	salt	3	3, salad	3 apples 3bananas	4.5	no	no	no	no	yes	6	yes, twins	no	no
V5	3, coffee	3biscuits	3,pasta	no	no	6, olive oil	salt	6	3,salad	6peaches	4.5	3,sugar	no	no	no	yes	6	yes	no	no
V6	6, coffee	9biscuits	3,pasta	1,meat; 1,fish	no	3, olive oil	salt	3, brown	3, salad	6, peaches	4.5	no	no	no	no	no	6	yes	no	no
V7	3,coffee	6biscuits	3,pasta	5, meat; 3 fish	3, eggs	3, olive oil	salt	6	3,salad; 3 potatoes	6, apricots	4.5	3,sugar	no	no	no	yes	6	yes	no	no
V8	9, coffee	no	no	3, meat; 3 fish	3, cheese (sheep)	3, olive oil	salt	6, brown	3, salad; 3 chard	3apricots; 3,plums	3	3, sugar; 3,jam	no	no	yes	no	6	yes	no	no
V9	3,coffee	9biscuits	3, pasta	1, meat	no	6, olive oil	salt	3	3, salad	3, apples	4.5	3, sugar	no	no	no	yes	8	yes	no	no
V10	6,coffee; 3, milk	6biscuits	6, pasta	1, meat; 1, fish	no	3, olive oil	salt	3	1, salad; 1,pepper 1zucchini	3, apricots	4.5	6, sugar	no	no	no	yes	7	yes	no	no

Table 3. Main personal and historical data of melanoma and vitiligo patients

TABLE 3										
	Age (years) (media ± DS and range)	Sex	BMI (media ± DS and range)	Smoking	Autoimmune diseases	Cardiovascular diseases	autoimmune thyroid diseases	thyroid diseases (other)	Visceral cancer	NMSK
Melanoma	63.20 ± 3.51 (Range 48-80)	4 F; 6 M	25.34 ± 1.21 (Range 21.3-31.9)	0/10 (0%)	0/10 (10%)	2/10 (20%)	0/10 (0%)	0/10 (0%)	1/10 (10%)	1/10 (10%)
Vitiligo	55.20 ± 5.65(Range 22-84)	7 F; 3 M	25.41 ± 0.9 (Range 20-30.1)	2/10 (20%)	6/10 (60%)	2/10 (20%)	5/10 (50%)	1/10 (10%)	0/10 (0%)	1/10 (10%)
P value	P: 0.245	P: 0.178	P:0.946	P: 0.136	P:0.003	P:1	P: 0.010	P:0.305	P:0.305	P:1

Table 4- Complete personal and historical data of Melanoma patients

TABLE 4

	Age	Sex	BMI	Smoking	sun exposure	previous sunburns	Autoimmune diseases	Cardiovascular diseases	Visceral cancer	NMSK	Other diseases	drugs	familial melanoma
M1	67	F	22.6	no	recreational	no	no	no	no	no	gallbladder polyps hypertension	ramipril	yes
M2	48	M	22.8	no	recreational	yes	no	yes	no	no	no	nebivolol	yes
M3	49	M	31.3	no	recreational	yes	no	No	no	no	sarcoidosis	no	no
M4	48	F	21.3	no	recreational	yes	no	No	no	no	no	no	no
M5	80	M	21.8	no	recreational	yes	no	No	no	no	no	no	no
M6	68	F	23.5	no	recreational	yes	no	No	Yes colon carcinoma	yes	no	no	no
M7	63	F	24.2	no	recreational	yes	no	No	No	no	no	no	no
M8	71	M	26.6	no	recreational	yes	no	no	No	no	no	no	no
M9	70	M	31.9	Past smoker	recreational	yes	no	yes	No	no	chronic obstructive pulmonary disease gout disease hypertension	allopurinol enalapril atenolol	yes
M10	68	M	27.4	Past smoker	recreational occupational	yes	no	no	no	no	hypercholesterolemy	no	no
TOTAL	63.20 ± 3.51	4 M; 6 F	25.34 ± 1.21	0/10 (0%)	10/10 recr. 1/10 occ.	9/10 (90%)	0/10 (0%)	2/10 (20%)	1/10(10%)	1/10(10%)	n.a.	n.a.	3/10(30%)

Table 5- Complete personal and historical data of Vitiligo patients

TABLE 5

	Age	Sex	BMI	Smoking	autoimmune thyroid diseases	thyroid diseases (other)	autoimmune diseases	Cardiovascular diseases	Cancer	NMSK	other diseases	DRUGS
V1	71	F	30.1	yes, 1/week	yes	no	thyroiditis, atrophic gastritis	yes	no	yes	hypercholesterolemy, Osteoporosis, hypertension	candesartan cilexetil, amlodipine, levothyroxine, rivaroxaban, metoprolol,colecalciferol
V2	70	F	29.1	no	no	no	no	yes	no	no	type II diabetes mellitus, hypertension, arthrosis, psoriasis	atenolol, metformin, valsartan+ hydrochlorothiazide
V3	22	F	20	no	no	no	no	no	no	no	anxiety disorder	escitalopram, trazodone chlorydrate
V4	41	M	23.9	yes, 15/day	yes	no	alopecia areata, thyroiditis	no	no	no		no
V5	42	F	27.4	no	yes	no	thyroiditis	no	no	no		no
V6	51	M	22.8	no	yes	no	thyroiditis	no	no	no	psoriasis, atopy	duloxetine, trimipramine
V7	84	M	25.2	no	no	no	no	no	no	no	gout disease, benign prostatic hyperplasia	allopurinol
V8	60	F	26.2	no	no	no	no	no	no	no	cholecystectomy	no
V9	61	F	23.9	no	no	yes	atrophic gastritis	no	no	no	Past hyperthyroidism, nephropathy	levothyroxine, sodium valproate, clonazepam
V10	50	F	25.5	no	yes	no	thyroiditis	no	no	no	epilepsy	oxcarbazepine, escitalopram
TOTAL	55.20 ± 5.65(media ± DS)	7 F; 3 M	25.41 ± 0.9 (media ± DS)	2/10 (20%)	5/10 (50%)	1/10 (10%)	6/60 (10%)	2/10 (20%)	0/10 (0%)	1/10 (10%)	n.a	n.a.

Table 6- Histopathological features of Melanoma

TABLE 6

	Body site	Invasion	Hystological subtype	Breslow thickness (mm)	Ulceration	Mitotic index (mitoses/mm^2)	Lympho vascular invasion	Satellitosis	Perineural invasion	Growth phase	TILs	Regression	Clark level	SNB	Classification- AJCC 7	Staging- AJCC 7	Therapy
M1	upper limb left	no in situ	NOS	n.a	no	0	no	no	no	radial	no	no	I	no	pTis	0	surgery
M2	upper limb left	yes invasive	SSM	0.3	no	0	no	no	no	vertical	yes not brisk	yes moderate	II	no	pT1a	IA	surgery
M3	back	yes invasive	spitzoid	1.46	no	4	no	no	no	uncertain	no	no	III	yes negative	pT2a	IB	surgery; SNB
M4	upper limb right	yes invasive	SSM	0.3	no	0	no	no	no	vertical	yes	yes moderate	II	no	pT1a	IA	surgery
M5	back	yes invasive	SSM	3.6	yes	7	no	no	not assessed	vertical	no	no	IV	yes positive	pT3b	III	surgery; SNB LND
M6	upper limb left	no in situ	SSM	n.a	no	0	no	no	no	radial	no	yes marked	I	no	pTis	0	surgery
M7	foot left	no in situ	NOS	n.a	no	0	no	no	no	radial	no	no	I	no	pTis	0	surgery
M8	abdomen	no in situ	SSM	n.a	no	0	no	no	no	radial	no	no	I	no	pTis	0	surgery
M9	face	yes invasive	SSM	0.9	no	7	no	no	no	vertical	yes not brisk	no	IV	no	pT1b	IB	surgery
M10	back	no in situ	NOS	n.a	no	0	no	no	no	radial	no	no	I	no	pTis	0	surgery
TOTAL	n.a.	5 in situ 5 invasive	6 SSM 1 spitzoid 3 NOS	0.65 ± 0.36	1 yes 9 no	1.8 ± 0.95	10 no	10 no	9 no 1 not assessed	5 radial 4 vertical 1 uncertain	7 no 3 yes	7 no 3 yes	5 I 2 II 1 III 2 IV	2 yes 8 no	5 pTis 2 pT1a 1 pT1b 1 pT2a 1 pT3b	5 st. 0 2 IA 2 IB 1 III	10 surgery 1 sur+LNB 1 surg+SNB +LND

SSM: superficial spreading melanoma
NOS: no otherwise specified
SNB: sentinel node biopsy; LND: lymph node dissection

Table 7- Clinical features of Vitiligo

TABLE 7

	% Area TOT	Staging TOT	Spreading TOT	Age at onset	Duration	Phototype	Vitiligo subtype	Koebner	Onset	Growth	Leuko-trichia	Stress at onset	Hair greying	Inflamma tion or pruritus	Sutton nevi	Activity	Past repigmen tation
V1	83	8	0	60	11	II	NS, universal	no	sudden	fast	no	yes	no	yes	no	active	no
V2	12	7	0	61	10	II	NS, generalized	yes	gradual	slow	no	yes	no	yes	no	stable	yes, UVB,sun
V3	0.7	6	0	16	6	II	NS, generalized	no	sudden	slow	no	yes	no	yes	no	stable	yes, sun
V4	19.5	8	0	18	23	II	NS, generalized	yes	sudden	fast	yes	yes	no	yes	no	stable	yes, sun
V5	8	6	0	41	1	II	NS, generalized	yes	sudden	slow	yes	yes	No	no	no	borderline	no
V6	5.7	8	4	12	39	II	NS, generalized	yes	gradual	slow	no	yes	No	yes	no	active	yes, UVB,sun
V7	73	8	4	83	1	II	NS, generalized	no	sudden	fast	no	no	No	no	no	active	no
V8	24	9	0	40	20	III	NS, generalized	yes	sudden	slow	yes	no	No	no	no	borderline	no
V9	11	6	-3	34	27	II	NS, generalized	no	gradual	slow	no	yes	No	no	no	active	yes, UVB,sun
V10	22.6	8	0	7	43	III	NS, generalized	yes	sudden	slow	yes	no	no	no	no	active	yes, UVB
TOTAL	25.95 ± 9.01 (media ± DS	7.4 ± 0.33 (media ± DS	0.5 ± 0.65 (media ± DS	37.2 ± 7.87 (media ± DS	18.1 ± 4.7 (media ± DS	8 ph. II; 2 ph. 9	9 NS generalized, 1 NS universal	6 yes, 4 no	7 sudden, 3 gradual	3 fast, 7 slow	4 yes, 6 no	7 yes, 3 no	10 no	5 yes, 5 no	10 no	5 active, 1 bord, 4 stable	6 yes, 4 no

NS= non segmental vitiligo

Table 8- Serological features of Vitiligo patients

TABLE 8								
	ANA	Ab anti TG UI/ml	Ab anti TPO UI/ml	Ab anti TSH-R UI/ml	APCA	25OH D	25OH D value ng\ml	TSH
V1	neg	Pos 59.30	Pos 24.8	neg	Pos (1:320)	not sufficient	23.2	normal
V2	neg	neg	neg	neg	neg	not sufficient	22.7	normal
V3	neg	neg	neg	neg	neg	not sufficient	27.8	normal
V4	neg	Pos 44	Pos 710	neg	neg	not sufficient	25.8	normal
V5	Pos (1:80)	Pos 176	Pos 2344	neg	Pos (1:320)	not sufficient	18.9	normal
V6	Pos (1:80)	neg	neg	Pos 2.5	neg	sufficient	34	normal
V7	neg	neg	neg	neg	neg	not sufficient	24.4	normal
V8	neg	neg	neg	neg	neg	not sufficient	20	normal
V9	Pos (1:320)	neg	Pos 3250	neg	neg	sufficient	37.4	normal
V10	neg	neg	neg	neg	neg	sufficient	31.6	normal
TOTAL	3 pos, 7 neg	3 pos, 7 neg	4 pos, 6 neg	1 pos, 9 neg	2 pos, 8 neg	7 not sufficient, 3 sufficient	26.58 ± 1.92 media ± DS	normal

A= altered N= normal

Table 9. Results of the PERMANOVA analysis related to fungal and bacterial microbiota diversity and clinical features of the diseases

PARAMETER	P value	R^2
FUNGAL		
Melanoma		
Invasiveness	0.110	0.164
Growth phase	0.114	0.273
Breslow thickness	0.203	0.142
Mitotic index	0.314	0.116
Tumour infiltrating lymphocytes	0.510	0.184
Regression	0.439	0.100
Vitiligo		
Autoimmune thyroid diseases	0.650	0.066
Associated autoimmune disorders	0.935	0.104
ANA positivity	0.228	0.155
25(OH)D values	0.871	0.072
Ab anti TPO positivity	0.803	0.082
Activity of the disease	0.494	0.202
Onset	0.174	0.151
Growth	0.600	0.088
Signs of inflammation/pruritus	0.159	0.153
Leukotrichia	0.900	0.055
Total extension	**0.023** (*)	0.198
Stress at onset	0.162	0.150
BACTERIAL		
PARAMETER	**P value**	**R^2**
Melanoma		
Invasiveness	0.161	0.125
Growth phase	0.513	0.188
Breslow thickness	0.454	0.101
Mitotic index	**0.016 (*)**	0.181
Tumour infiltrating lymphocytes	0.187	0.259
Regression	0.234	0.132
Vitiligo		
Autoimmune thyroid diseases	0.713	0.078
Associated autoimmune disorders	**0.008** (**)	0.170
ANA positivity	0.346	0.110
25(OH)D values	**0.017** (*)	0.192
Ab anti TPO positivity	0.159	0.129
Activity of the disease	0.235	0.239
Onset	0.221	0.124
Growth	0.286	0.119
Signs of inflammation/pruritus	0.243	0.122
Leukotrichia	0.248	0.116
Total extension	0.530	0.107
Stress at onset	0.624	0.084
(*) $P<0.05$; (**) $P<0.01$		

Table 10. Results of isolation and identification of fungi from melanoma faecal samples

NUMBER	ISOLATES	SPECIES
1	M6F_2Y	Candida albicans
	M6F_2Y	Candida albicans
2	M6F_4Y	Candida albicans
3	M6F_6Y	Candida albicans
4	M6F_8Y	Candida albicans
5	M6F_12Y	Candida albicans
6	M6F_13Y	Candida albicans
7	M6F_15Y	Candida albicans
8	M6F_16Y	Candida albicans
	M6F_16Y	Candida sp.
9	M8F_1Y	Candida albicans
10	M8F_2Y	Saccharomyces cerevisiae
	M8F_2Y	Saccharomyces sp.
11	M8F_3BY	Saccharomyces paradoxus
	M8F_3BY	Saccharomyces cerevisiae
	M8F_3BY	Saccharomyces cf.
	M8F_3BY	Saccharomyces sp.
12	M9F_1Y	Candida albicans

Appendix 1

Appendix 1

FOOD DIARY

PATIENT ID:		
DATE OF BIRTH:		
HEIGH (mt):	WEIGHT (kg):	BMI:
DATE OF SAMPLE COLLECTION:		

1. **Drinks (milk, coffee)**: *indicate amount (1 glass= 150 ml) and how many times a day.*

   ……

2. **Biscuits, Rusks, Cereals or other**: *indicate amount for day (or number of pieces).*

   ……

3. **Pasta, Rice and Potatoes**: *(handful of rice or pasta; number of potatoes).*

   ……

4. **Animal Proteins (meat, fish):** *indicate amount as raw food and kind (e.g. red or white meat, fish or shellfish) (1 slice of meat or fish = 120-150 gr approximately)*

   ……

5. **Eggs, Cheese:** *indicate amount; for the cheese, indicate the derivation of milk (cow, sheep, goat)*

   ……

6. **Condiment (dressing):** *indicate the amount expressed in spoons; for oil, specify the kind*

   ……

7. **Spices:** *indicate the most commonly used spices*

   ……

8. **Bread:** *indicate the kind (brown or refined, and the kind of cereal of origin) and the amount, expressed as number of slices (1 slice=40-60 gr)*

……
……

9. **Vegetables:** *indicate the kind, the daily amount (number of portions: 150-200 gr=1 portion) and accompanying condiment*

……
……..

10. **Fruit:** *indicate the kind and the daily amount (excluding scraps)*

……
……

11. **Beverages (e.g. water):** *indicate amount in ml*

……
……

12. **Sweets (sugar, marmalade, chocolate, honey):** *indicate the daily amount expressed in spoons/teaspoons*

……
……

Appendix 1

OTHER INFORMATIONS

1. Assumption of probiotics/prebiotics in the last 6 months

   YES □ NO □

2. Assumption of antibiotics in the last 6 months

   YES □ NO □

   kind……

3. Assumption of yoghurt

   YES. □ NO □

   type……

4. Feeding in the first 4-6 months

- □ Breastfeeding
- □ Formula milk

5. Weaning
   months…….

6. Delivery
   - □ Vaginal
   - □ Caesarean

7. Food allergy
   type: …….

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