A tutti coloro che mi hanno sempre sostenuta,

A tutti coloro che mi hanno dedicato un minuto o anni della loro vita per insegnarmi quello che tutt'oggi mi porto dentro.

A tutti coloro che ci hanno sempre creduto più di quanto io stessa potessi fare.

Alla biologia che è riuscita a dare luce anche ai giorni più bui.

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Interplay among microbial communities, epithelial cells, and immune system in vaginal mucosa of women with high-risk Human Papillomavirus infection

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Abstract

Persistent infection with HR-HPVs is a primary cause of cervical cancer worldwide. Among HR-HPV, subtypes HPV-16 and HPV-18 are most associated with invasive cancers and are thought to cause approximately 65-75% of cases. Emerging evidence indicates that CVM plays a substantial role in the viral persistence and subsequent disease. Accordingly, a role of CVM composition in the regression of high grade of CIN lesions has been reported. Data from clinical studies show that *Lactobacillus*-dominated cervicovaginal microbiota and *L. crispatus* particularly was positively associated with the clearance of the virus and negatively associated with the neoplastic progression of intraepithelial lesions. In contrast, vaginal-dysbiosis associated bacteria were correlated with the persistence of HR-HPVs infection and with increasing cancer risk. The molecular mechanisms based on such effects are not elucidated yet.

Aim of this study was to investigate whether each vaginal bacterial species, commonly found in cervicovaginal microbial communities, may affect the expression of viral oncogenes and accelerate the neoplastic transformation of HR-HPV transformed cells. For this purpose, the HPV-16 transformed SiHa epithelial cells (with ~1,2 copies of integrated viral genome) were selected as experimental model of CIN-1 and bacterial strains, representatives of the 5 CSTs, were evaluated for their effect on the expression of viral oncogenic proteins E6 and E7, the fate of cellular oncosuppressor protein p53, the effects on cell survival/growth of HPV-transformed SiHa cells, and the synthesis of matrix-degrading enzymes.

Our data show that *G. vaginalis* and *M. micronuciformis* directly induce the expression of the viral E6 and E7 oncogenes and the synthesis of the respective products by SiHa cells. These bacteria also induce the expression of E6-AP, a ubiquitin protein ligase that binds to E6 and induce p53 degradation. As consequence of E6 activation and p53 degradation, cell proliferation is increased. *L. crispatus* never induces the expression of viral oncogenes or proteins, while *L. iners* and *L. jensenii* slightly induce the viral E7 gene

expression but not E6. In contrast to *G. vaginalis* or *M. micronuciformis*, vaginal *Lactobacilli* did not affect the cell cycle of cervical epithelial cells.

G. vaginalis and *M. micronuciformis* also induce high expression of MMP-9, a cellular protein which overexpression has been observed in different malignant tumors.

All these data were obtained by using live bacterial cells, supernatants, or bacterial lysates. Supernatants from bacterial culture are not effective in the system suggesting the responsivity of very labile or not secreted products. Although not all strains of *G. vaginalis* have the same property to induce the expression of viral oncogenes and oncoproteins, our data strongly suggest that this species together with *M. micronuciformis* may play an important role in neoplastic transformation of HR-HPV infected cell.

According with data from clinical data, the presence of *G. vaginalis* or *M. micronuciformis* in the vaginal fluid of women with persistent HR-HPV infection or CIN-1 should direct these groups to a close follow-up or to surgical excision respectively.

Abbreviations: HR-HPV (High-Risk Human Papillomavirus), CVM (cervicovaginal microbiota), CST (community state type), CIN (cervical intraepithelial neoplasia), CIN-1 (low grade of cervical intraepithelial neoplasia), E6-AP (E6-Associated Protein), L. (*Lactobacillus*), G. (*Gardnerella*), M. (*Megasphaera*), MMP-9 (Matrix Metalloproteinase-9).

1. Introduction

1.1 Cervicovaginal microbiota (CVM)

Over time, humans have coexisted with bacterial communities, which are unique to specific niches. The *Human Microbiome Project* has extensively examinate the vaginal microbiota and its role in women health and reproductive function [1].

The vaginal tract is colonized by various microorganisms that constitute the cervicovaginal microbiota (CVM). Unlike other body sites, where the variety of microbial communities is considered a health signature, the healthy premenopausal vaginal bacterial communities are usually populated by *Lactobacillus* spp., essential to ensure a low pH in vaginal environment which provide the first line of defense against pathogenic agents [2, 3]. Additionally, these bacteria can produce numerous antimicrobial peptides and metabolic products, such as lactic acid and other acidic compounds that can inhibit pathogenic bacteria adhesion, growth, and disruption of biofilms [4-6]. In addition, bacteriocin-like compounds, such as bio-surfactants and hydrogen peroxide, play an important role in *Lactobacillus* spp. persistence in the vagina [6]. During evolution of humans, *Lactobacillus* species that are extraordinary producers of all these anti-microbial factors have been selected as inhabitants of the cervicovaginal environment [7].

However, vaginal *Lactobacilli* are not necessarily stable or "healthy". A dominance of *L. iners* has been correlated with the development of vaginal "dysbiosis", better known as bacterial vaginosis (BV) [8, 9]. In contrast, *L. crispatus* dominance appears protective against the development of BV [10, 11].

Ravel *et al* [12] were the first to define the composition of the CVM using bacterial 16S RNA sequencing technology. They defined the vaginal microbiota into five community state types (CSTs) (I, II, III, IV, and V) based on the presence or absence of a particular *Lactobacillus* spp. CST I, II, III, and V are characterized by *L. crispatus, L. gasseri, L. iners,*

and *L. jessenii*, respectively. On the other hand, CST-IV is the results of a depletion of *Lactobacillus* spp. subsequently promoting the colonization by strictly anaerobic species such as *Gardnerella, Prevotella, Sneathia, Atopobium, Megasphaera* [12, 13].

While a relatively low microbial diversity is associated to "health status" in the majority of women [14], a CVM with increased indexes of microbial diversity has been associated to BV, the most frequent infection of female genital tract. Prevalence of the BV condition is ranging from 12% in Australian women [15, 16], up to 50% in sub-Saharan African women [17], making it the most prevalent vaginal disorder of women of reproductive age. This has important public health implications because BV has been associated with serious and significant reproductive morbidity including pelvic inflammatory disease [18], miscarriage [19] and may increase the risk of pre-term birth [20].

Moreover, BV has been associated with High-risk (HR) Human Papillomavirus (HPV) infection and cervical cancer in HR-HPV positive patients [21, 22].

Studies of chronic wounds showed that *Lactobacillus* spp. supernatants contain antimicrobials (mevalonolactone, benzoic acid, and 5-methyl-hydantoin), surfactants (distearin, dipalmitin, and 1,5-monolinolein), anesthetics (bituric acid derivatives), and autoinducer type 2 precursors (4,5-dihydroxy-2,3-pentanedioneand2methyl-2,3,3,4-tetra- hydroxytetrahydrofurane).

In addition to lactic acid, *Lactobacillus* spp. supernatant also contains phenolics, hydrogen peroxide, sodium, calcium, potassium, magnesium, and DNAases [23].

As microbes struggle for nutrients and adherence to the vaginal epithelium, the local immune system may activate the production of host antibacterial substances to further control microbial growth [24].

1.1.2 Cervicovaginal microbiota in women in reproductive age

The composition of the CVM is dynamic and undergoes changes corresponding to hormonal ones during the reproductive life of the woman [25].

At puberty, with follicular development, estrogen production occurs. High levels of estrogens favor the increase in the thickness of the vaginal epithelium and mucous layer and the intracellular production of glycogen, which will be fermented by *Lactobacilli* into lactic acid with consequent vaginal acidification [25-27]. This acidic pH environment (3.5–4.5) promotes the growth of *Lactobacilli* at the expense of other anaerobic bacterial species [25, 28].

In reproductive age, the menstrual cycle can play a certain role in converting one CST into another. In a study conducted on women of reproductive age it was shown that in 81% of menstrual cycles, the levels of *G. vaginalis* along with *L. iners* significantly increase with menstruation and decrease towards the end. On the contrary, the levels of *L. crispatus* and *L. jensenii* decrease during menstruation and increase at its end [28]. This can be attributed to the availability of iron during menstruation that improves the growth of *G. vaginalis* and *L. iners* [29]. In the cervicovaginal environment in reproductive age glycogen levels are kept high and then decrease steadily once menopause is reached. In postmenopausal women, the reduced production of estrogens and the consequent vaginal atrophy causes the decrease of *Lactobacilli*, with the consequent increase in intravaginal pH, and the greater possibility of colonization by harmful species [30].

Several physiological and behavioral factors may increase the risk of BV and inflammation, including sexual intercourse, reproductive and contraceptive hormones, use of antibiotics, hygiene, vaginal insertion practice, and lifestyle habits [3].

During the menstrual cycle estrogen and progesterone levels are at a minimum and this affects the diversity and instability of the microbiota [29]. The CVM fluctuate rapidly throughout the menstrual cycle, with generally a decrease of *L. crispatus* and *L. jensenii*

and increase of *L. iners,* or *G. vaginalis*. In the estrogenic phase of the menstrual cycle, is observed the maximum stability of the composition of the CVM [28].

It has been shown that the widespread use of synthetic hormones for contraceptive purposes has an impact on the composition of the CVM. A recent meta-analysis showed that the use of hormonal contraceptives is associated with an 18% reduction in the incidence of BV and a 31-32% reduction in recurrent BV [31].

In addition to the use of hormonal contraceptives, other environmental factors such as smoking and sexual intercourse affect the composition of the microbiota and are both associated with a loss of *L. crispatus* and increased diversity of microbial species [32]. Vaginal washes, especially after menstruation, significantly increase the risk of BV, and cessation of practice can reduce its the risk [33, 34].

1.1.3 Cervicovaginal microbiota in pregnancy

The pregnant woman has a CVM predominantly consisting of *Lactobacillus* spp. and is less prone to fluctuations in microbial composition due to the level of estrogen maintained high throughout pregnancy [5, 22]. However, factors such as gestational age, multiparity and ethnicity can affect the diversity of the vaginal microbiota during pregnancy [35-37]. As pregnancy progresses, the vaginal microbiota stabilizes and there is typically an increase in the relative abundance of *Lactobacilli*, especially *L. crispatus*, and a decrease in narrow anaerobic or anaerobic species, up to about 36 weeks of gestation. After 36 weeks of gestation the number of anaerobic species may increase [38, 39].

Microbiota changes during pregnancy may favor ascending infections in the uterus, often due to preterm delivery [40].

It has been observed that preterm births are often associated with the prevalence of *L. iners* or bacterial species characteristic of CST-IV with high abundance of *Gardnerella* or

Ureaplasma species [41]. Indeed, the ascending infections favored by this microbial structure can activate the innate immune system with consequent remodeling and shortening of the cervix, destruction of the amniotic membrane and ultimately preterm birth [39, 42]. Changes in the CVM were also observed immediately after delivery and include a decrease in *Lactobacilli* and an increase in anaerobic species, regardless of the vaginal communities that were present during pregnancy and ethnicity [37].

1.2 Interplay among cervicovaginal microbiota and local immunity in the female tract

The vaginal mucosal barrier has specialized features to protect against infectious and noxious environmental damage, at the same time maintaining symbiotic mutualism with commensal microbes. Microbes, immune regulatory actions, and host genes interact closely to govern the homeostasis of the vaginal environment [3]. The cervicovaginal mucosa is a critical site for protection against urogenital pathogens. The female genital tract consists of two types of mucosal surface. The lower genital tract (vagina and ectocervix) represents the type II mucosal surface, lined by stratified squamous epithelia. Defining features include the presence of mucus-secreting cells and the expression of polymeric immune-globulin R (pIgR) on the basolateral surface of the epithelia. pIgR binds to polymeric IgA (pIgA) secreted by plasmacells and exports pIgA trans-epithelial [24, 43]. Plasmacells also release secretory IgA (SIgA) into the lumen. In contrast, endocervix and endometrium (the upper genital tract) represent the type I mucosal surface, which is covered by a monolayer of columnar epithelial cells, with tight junctions and SIgA presenting in the microenvironment. The boundary between type I and type II mucosa, also known as the cervical transformation zone, is the most vulnerable region invaded by pathogens and heavily populated with T cells and antigen-presenting cells (APCs) [44].

In the human vaginal mucosa, four unique myeloid-derived APC subsets have been characterized including cervicovaginal Langerhans cells (cvLCs), CD14-dendritic cells (DCs), $CD14^+$, DCs, and $CD14^+$ macrophages [24, 45]. Langerhans cells are also found in

the epidermis (epLCs) and express the C-type lectin molecule. cvLCs are found in the human cervicovaginal epithelium, whereas CD14-DCs, $CD14^+$, DCs, and $CD14^+$ macrophages are found in the subepithelial lamina propria region [45]. Transcriptional profiling showed that CD14-cells (cvLCs and CD14-DCs) displayed T_H2-inducing and regulatory properties, whereas the $CD14^+$-APCs ($CD14^+$-DCs and macrophages) exhibited signatures of innate immune defense and proinflammatory responses [46]. Resident memory T cells in the vaginal mucosa mediate rapid protection upon infection. $CD4^+$ T-cell entry into the cervicovaginal region facilitates the entry of circulating memory $CD8^+$ T cells via the production of IFN-γ. Innate immune sensing and surveillance of commensal and pathogenic microbes in the female genital tract is achieved by microbial motif pattern recognition via pattern recognition receptors (PRRs), such as toll-like receptors (TLRs) [47], dectin-1 receptor, and nucleotide-binding oligomerization domain receptors present in and on both squamous epithelial cells [24]. PRRs stimulation in the vaginal environment initiates various cytokine and chemokine signaling cascades, including secretion of interleukin-1β, IL-6, IL-8 [48], and tumor necrosis factor-α (TNF-α), to activate or recruit macrophages, natural killer cells, $CD4^+$- and $CD8^+$T-cell lymphocytes, and B lymphocytes [25]. Other factors that strength vaginal defense system include IgA, IgG, vaginal anti-microbial peptides (AMPs) and mannose binding lectin (MBL). N-acetyl glucosamine and MBL bind mannose, which is present on the cell surface of microbes [49]. IgG and IgA may assist to avert vaginal epithelial cell adherence and uptake, playing a role in neutralizing and clearing infectious microbes from host sites [50]. Similarly, there are various classes of vaginal AMPs which normally engage immune cells via chemotaxis or has anti-endotoxin activity. The cervicovaginal mucosa is a gateway to several sexually transmitted pathogenic microorganisms. The interaction between the vaginal microbiota and the mucosal immunity system is necessary to the protection of the cervicovaginal environment from pathogenic species [24, 51]. Cells and mediators of innate immunity and adaptive immunity cooperate to prevent and treat viral infections.

Innate immunity is the first line of defense against pathogens, while adaptive immunity acts later and eliminates or prevents reinfection. Following the entry of a virus into cells, some receptors recognizing specific parts of the viral genome are immediately alerted.

In DNA viruses, areas of the CpG-rich genome are recognized by endosolic receptors primarily represented by TLR-9. Viral RNA is recognized by endosolic receptors such as TLR-3, TLR-7, TLR-8 and the cytosolic rig1 and MDA5 [52]. Stimulation of these receptors induces the production of type I IFN and other inflammatory cytokines, through the induction of molecular Pathways that converge in the activation of nuclear factor kB (NF-kB) and the MAP-kinase system (MAPK) [52-54]. Virus-infected cells and often membrane viral antigens and are recognized as foreign by the immune system. They can be killed by natural Killer-lymphocytes or endocytosed by macrophages and DCs that have the very important function of presenting the antigen and activating the T lymphocytes, the adaptive immunity cells [52].

Once activated by antigen presenting DCs, T cells differentiate towards at least 4 different types of effectors: T_H1 (T helper 1), T_H2 (T helper 2), T_H17 and T_{reg}. T_H1 cells are the most important effectors in defense against viral infections because they produce IFNγ, which boosts the activity of cytotoxic $CD8^+$ lymphocytes that kill virus-infected cells. T_H2 lymphocytes stimulate the humoral response allowing B lymphocytes to produce antibodies [44].

Sex hormones also regulate the release of pro-inflammatory cytokines, chemokines and antimicrobial peptides that provide additional protection from external agents and contribute to the selection of vaginal microbial species [3]. The production of estrogens is a determining factor in the selection of *Lactobacilli* in the vaginal microenvironment. Indeed, estradiol and progesterone induce epithelial cells to synthesize glycogen and alpha-amylase, this helps to select the *Lactobacilli* and to provide their nourishment [3, 7]. This triggers a "positive feedback protection" in which *Lactobacilli* continue to produce lactic acid and maintain a low pH and an inhospitable environment for potentially pathogenic species. The presence of lactic acid affects the immune responses of the host by modulating the production of pro-inflammatory mediators such as IL-6 and IL-8, TNF-α and pro-inflammatory macrophages [7]. A vaginal microbiota no longer dominated by *Lactobacilli* causes a change in immune response with increased production of cytokines and chemokines pro-inflammatory [53, 54], increased

permeability of the mucosal barrier and reduced viscosity of CVF due to the presence of enzymes degrading the mucin [54, 55].

Other host-microbiota interactions occur at the epithelial cell interface; many of them are not well understood. For example, bacterial strains produce a range of metabolites, some of which could cross the epithelial barrier and affect cellular function in other sites, which are influenced by host factors such as hormones and mucins. Therefore, a complex interaction and synergy between host and microorganism to maintain the homeostasis in the vaginal epithelia exists and it is important to understand the interplay between the microbes and potential pathogens[40, 56].

1.3 The Human Papillomavirus (HPV) infection and the cervicovaginal microenvironment

The vaginal microbial population continually changes throughout life, but it may be consistent and stable in a certain period in healthy women. However, the vaginal dysbiosis with loss of *Lactobacillus* spp. and the overgrowth of anaerobic bacteria is a condition that is associated with the higher incidence, prevalence, and persistence of HR-HPV infection, known to be cause of one of the most frequent female neoplastic diseases, the cervical cancer. It is believed that the presence of micro-abrasions in the squamous epithelium allows the exposure of proliferative cell layers that can be infected by HPV virions [57, 58].

Vaginal dysbiosis also increases the risk of other STI as HIV (Human Immunodeficiency Virus), HSV (Herpes Simplex Virus), Neisseria gonorrhoeae, Chlamydia trachomatis, and Trichomonas vaginalis [59-62].

HPVs are viruses belonging to the family of *Papillomaviridae* consisting of an icosahedral capsid of about 60 nm in diameter containing circular double-stranded DNA of about 8,000 pairs of bases and first of envelopes [63].

More than 200 types of HPVs have been described in humans and are classified as high- and low-risk HPV in relation to the presence/absence of oncogenic potential. Low-risk HPVs (LR) include types 6, 11, 42, 43 and 44 and are mostly found in non-malignant lesions in various skin districts. High-Risk HPVs (HR) strains include genotypes 16, 18, 31, 33, 34, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68 and 70. Among these, the most widespread worldwide are the types 16, 18, 31, 33, and 35 that have a dominant role in the onset of cervical cancer [64], one of the most frequent neoplastic diseases of the female sex still widespread in all continents. HPV-16 is the most common type of HPV found in invasive cervical carcinoma (ICC) (55%), followed by HPV-18 (12.8%), and together these two types cause 70% or more of the ICC regardless of geographical location. HPV 33, 45 and 31 are the next most frequently detected HPV all over the world, except in Asia where HPV 58, 33 and 52 are the most common types [64]

HPVs have trophism prevalent for stratified squamous epithelia including the cutaneous epidermis and the mucosa of the oropharyngeal and uro-ano-genital pathways. When the virion of HPV reaches the basal or proliferative layers of epithelium, which have been exposed because of micro-abrasions, the binding of the capsid protein L2 with the heparan-sulfate surface receptors of the epithelial cell takes place [65]. Following ligand-receptor interaction, viruses are internalized through clathrin-mediated cellular endocytosis or caveolae-mediated cellular endocytosis [66]. The viral genome is maintained both in episomal form, as a circular extra-chromosomic element, and integrated into the host cell genome. In the episomic form, the virus encodes for a limited number of proteins, only those necessary for its replication and maintenance of viral particles. The replicative cycle of the virus continues along the various epithelial layers, until the viral particles are assembled and released by the normal desquamative process of the superficial layers of the epithelium. HPV infection, including that mediated by viral strains at high oncogenic risk, is eliminated in more than 90% of infected women within 6-18 months. In 10% of these, however, the virus persists in the cervical epithelium giving rise to a persistent or latent infection where the virus is maintained in episomal form. At this stage, can be eliminated by a vigorous activity of the immune

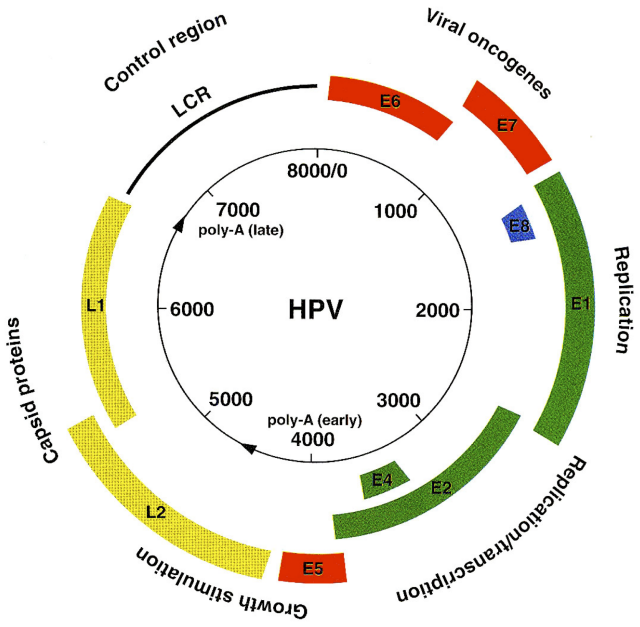
system. In 10% of persistent infections the HPV escapes the immune system and integrates its genome into the epithelial cell [64, 67].

Integration is facilitated by the elimination of the E2 gene and the consequent expression of the E6 and E7 genes [68]. If expressed inappropriately, these viral genes can interfere with the processes of cell growth and differentiation, which under normal conditions are finely controlled, favoring the neoplastic transformation of the cervical epithelium [58].

1.3.1 HPV-genome

The genome organization of all HPVs is very similar and contains three functional regions reported in Table 1 [67]:

- region of early genes, encoding the proteins E1, E2, E4, E5, E6 and E7;
- region of late genes, which includes L1 and L2, viral capsid proteins;
- long-term control region (LCR), also known as NCR or URR, located between the L1 and E6 ORFs, which controls the expression of viral genes.



Viral Protein	Protein Functions
E1	Viral DNA replication and transcription
E2	Viral DNA replication, apoptosis, transcription repressor of E6/E7
E4	Viral replicatiom
E5	Immune recognition (major histocompatibility complex, MHC)
E6	p53 degradation, alteration of cell cycle regulation, apoptosis resistance
E7	retinoblastoma protein (pRb) degradation, re-entry into S phase cell cyclem p16 overexpression
L1	Major viral capsid protein
L2	Minor viral capsid protein

Table 1: High-Risk Human Papillomavirus (HR-HPV) proteins and functions [67].

L1 is the capsid protein that allows the binding of viral particles to epithelial receptors, represented by heparan-sulfates and proteoglycans, typical of the cellular matrix of the epithelium. The L1 gene sequence is the most conserved nucleotide sequence in the HPV genome. The L2 capsid protein is responsible for packing viral DNA. L1 and L2 are expressed in the last stages of the virus replication cycle [69].

Among the early genes, the E1 protein is necessary for *Papillomavirus* replication, and it is the second most preserved sequence of the HPV genome. E1 contains three functional domains: a terminal-N-domain, a central domain, and a terminal-C-domain. The terminal-N-domain carries the sequence for nuclear localization and nuclear export, the

central domain is responsible for the formation of the complex with the E2 protein. A part of the terminal-C-domain acts as a helicase by binding and stretching viral DNA, making it accessible to the cell replication complex. All three domains are critical for viral replication [70].

E1 and E2 form a complex that binds to the site of initiation of replication; after binding, E2 is expelled from the complex and E1 forms a dimer which recruits several enzymes implicated in HPV replication, including topoisomerase I, DNA polymerase α, and replication protein.

In addition, the E1 protein also contributes to the breakdown of the DNA of the host cell, facilitating viral integration [71].

The E2 protein acts as a transcriptional regulator for the oncogenes E6 and E7 because it can bind to the promoter by preventing it from binding to RNA polymerase II [72]. Therefore, E2 acts as a transcriptional repressor of the oncogenes E6 and E7 as it inhibits their protein expression [71, 73].

The E4 protein binds to the cytoskeleton proteins, facilitating the release and spread of HPV virions [74]. E5 is a transmembrane protein defined as virioporin, it induces the formation of pores on the cell membrane by modulating ionic homeostasis, vesicle transfer, virion production and penetration of the viral genome into the cell. In addition, E5 is involved in the control of cell survival, growth and differentiation and can inhibit apoptosis induced by the tumor necrosis factor ligand (TNFL) and the CD95 ligand (CD95L) because enhances the activity of E6 and E7 [73, 75, 76].

The main viral oncogenes of HR-HPV are E6 and E7 and encode two oncoproteins whose expression depends on the genomic status of HR-HPV. If the virus is in episomal form, E6 and E7 are blocked by E2, while once the viral genome integrates into the genome of the host cell, the E2 coding sequence is disrupted and the two oncoproteins are expressed. E6 and E7 directly affect cell cycle checkpoint by both the degradation of p53 (tumor

suppressor protein) and pRb (retinoblastoma protein), and the inhibition of cyclin dependent kinase (CDK) inhibitors (p21, p27, p16) [77].

Under physiological conditions, p53 activity ensure the integrity of the cellular genome, preventing cell division after DNA damage or delaying it until the damage has been repaired. Physiologically, it is inhibited by the ubiquitin ligase Mdm2, which by binding to it induces ubiquitination and degradation via proteasome. When in HPV positive cancer cells Mdm2-dependent p53 regulation is completely deactivated, p53 is regulated solely by E6 [78].

E6 recruits E6-AP (ubiquitin-protein ligase associated with E6 protein) forming the E6/E6-AP complex, which induces the degradation of p53 ubiquitin-proteasome-dependent allowing cell proliferation [79, 80]. In this way E6 protein induces cell cycle arrests in the S phase of the cycle. In addition, E6 also induces the degradation of other factors involved in cellular apoptosis such as Bak, Bax and the tumor necrosis factor 1 (TNFR1) receptor and can induce aberrant cell proliferation by inducing the synthesis of a telomerase (hTERT), which prevents telomeres from shortening [69, 81].

E7 is an oncoprotein constituted by three domains: conserved regions 1-3 (CR1-CR3). The oncogenicity of this protein is restricted to the CR2. This has a LXCXE domain that recognizes and binds pRb and its related proteins p107 and p130 and leads the pRb degradation[79, 80].

Physiologically, during G1 phase, non-phosphorylated pRb interacts with the transcription factor E2F inducing the checkpoint. E7 protein binds pRb and the E7-pRb complex triggers the ubiquitination and subsequent degradation of pRb in the proteasome. The absence of pRb induces the release of the transcription factor E2F and the consequent synthesis of cdk2 dependent kinase and cyclins A and E, implicated in the G1-S transition (cell proliferation) [69, 80].

The alteration of the cell cycle checkpoints triggering the replication machinery are thus exploited by HR-HPVs to increase their replication rate [80].

1.3.2 HPV replication cycle

The HPV replication cycle is closely linked to the differentiation of keratinocytes in the host. In stratified squamous epithelium, the cells in the basal layer act as stem cells and undergo cell division when they replace the cells released from the surface layer. The infection starts from the basal layer through micro-abrasion. Virions enter in the host cell interacting with cellular receptors (e.g., alpha-integrin 6). Once inside, HPV duplicates its own genome (50-100 copies/cell) following the cell differentiation [77]. Early genes E1 and E2 are implicated in the viral genome replication, while E5-E7 promotes cell proliferation disrupting the cell cycle checkpoints [80]. Once the basal cell differentiates, late genes are transcribed. L1 and L2 induce the vegetative stage, which serve as booster for viral genome amplification [82].

Viral particles are endocytosed via caveolae or clathrin, and the translocation of the HPV genome into the nucleus occurs through disruptions of the nuclear membrane during cell mitosis [83].

At the end of the differentiation process, the cell is exposed in the lumen. Here late genes L1 and L2, implicated in the virion formation, are expressed. The assembled viral particle is released and the HPV life cycle re-starts [84].

HPVs do not induce complete lysis in host cells, but new virions are deposited in scales that are continuously released [85]. Immunogenic virions are assembled only in the outer portions of the epithelium, in this way the virus manages to escape the control of the host's immune system. In addition, the viral proteins E6 and E7 act to ensure that the infection remains asymptomatic by deactivating the regulatory pathways of interferon [82]. Squamous epithelial cells infected with HPV undergo koilocytosis to become cells called koilocytes. Compared to normal cells, these cells have a larger, darker, and

asymmetrically delineated nucleus enclosed by an area of transparent space, called the perinuclear halo, and appear to be vacuolated. This alteration suggests minor cellular dysplasia and is indicative of a highly replicative viral state [86]. HR-HPV infection is the major risk of factor to develop cervical cancer, however the presence of episomes is not sufficient to transform the infected cell. The crucial step is represented by the integration of the HR-HPV genome into the host one. The episome is linearized by a double strand break in the E2 coding sequence [87]. In rare cases, in conjunction with damage in cellular DNA, viral nucleic acid can be integrated thanks to host DNA repair systems. E2 suppress the transcription of other early genes (e.g., E6 and E7). Once E2 is suppressed, viral oncogenes are expressed. E5 modulate immune system and acts as an evasion mechanism; E6 and E7 directly confer to the cell two tumor phenotypes: the block of the apoptosis program (E6 leads p53 degradation), and the increase of proliferation rate (pRb the degradation). The E2 protein is recognized as having the ability to repress the transcription of E6 and E7, and thus its interruption causes dysregulated expression of these oncoproteins [69]. Combined, these proteins can transform epithelial cells that retain their mitotic ability and do not experience terminal differentiation. These cells generate clones with immortalized phenotype [88]. The immune response is a key factor in the fight against HPV infection and cervical carcinogenesis. However, HPV can promote immune evasion through the expression of the E5 oncogene, which is responsible for modulation of several immune mechanisms, including antigen presentation and inflammatory pathways [89].

1.4 Cervical carcinoma

It is well established that cervical cancer development is the final stage of a sequence of cellular and molecular changes induced by HR-HPV infection. Host immune response and evasion mechanisms involve a termination host response against tumor antigens. An increasing number of literatures suggests the association of natural HPV clearance and cervical intraepithelial neoplasia (CIN) regression with CVM composition. This modulates a finely tuned immune responses balancing reproductive tolerance with protection against STIs [90].

Cervical cancer is the second most common malignant cancer among women worldwide, after the most frequent breast cancer. The age of onset of is around 35-50 years, but there are rare cases when it occurs before 20 years of age [91].

HPVs infect the basal cells of the epithelium and exploit cellular mechanisms to replicate their genetic heritage by following the cell cycle. However, given that the basal epithelial cells are protected by several layers of differentiated cells and are not easily accessible to the virus, such infection occurs in conjunction with micro-wounds that lead the lower epithelial layers to be exposed in the lumen [63].

HR-HPV infection is a risk factor for the development of CIN and cervical cancer. Among the various HR-HPV, genotypes 16 and 18 are responsible for about 70% of CIN cases [67].

In 60-90% of cases HR-HPV infections are transient and the virus is cleared by the immune system within 2 years. In the remaining cases, there is a persistent infection that drives the onset of the tumor lesion. The persistence of the virus within the host cell increases the likelihood that the HR-HPV genome can integrate into the host cell genome, promoting oncogenesis.

Pre-malignant changes or dysplasia of squamous cells in the cervical epithelium are known collectively as CIN and are histologically classified into CIN-1, CIN-2 and CIN-3 based on epithelial cell atypia and the extent of the lesion from the lower basal layers of the squamous epithelium [92]. CIN-1 and CIN-2 may progress to *in situ* carcinoma and invasive carcinoma if they are not treated during the early stage or if the virus is able to interfere with the host's cellular functions [93]. CIN-1, also known as low-grade CIN, denotes mild dysplasia where the lower one-third of the epithelium shows dysplasia. When two-thirds of the epithelium is affected, it is referred to as CIN-2 or moderate dysplasia. CIN-3 is severe dysplasia or carcinoma *in situ* and it is classified when more than two-thirds of the total thickness of the epithelium is affected. CIN-2 and CIN-3 lesions are collectively classified as high-grade lesions [92, 93]. The progression from

HPV-infected epithelial cells to invasive cancer is a very long process that is accompanied by accumulation of mutations in the DNA of host cells, genetic and epigenetic changes with activation of oncogenes and inactivation of tumor suppressors.

Furthermore, the ability to metastasize is essential characteristic for tumor progression. Studies focus on tumor invasion and metastases declared that metalloprotease (MMPs) have a crucial role in tumor progression. MMP-9 is one of the most investigated MMP for key roles in cancer cell invasion and tumor metastasis [94]. Together with MMP-2, MMP-9 belongs to gelatinase subgroup of the MMPs family, and its biological processes include proteolytic degradation of extracellular matrix (ECM), changes in cell-cell and cell-ECM interactions, cleavage of cell surfaces proteins in extracellular environment. Their overexpression is often observed in different malignant tumors [94-98].

In cervical pre- and cancer lesion many authors observed a degradation of the extracellular matrix and endothelial cell basement membrane and highlight the central role of MMPs in the degradation of the ECM. MMP-9 is expressed in 83-100% of HSIL (High grade Squamous Intraepithelial Lesion), is less expressed in LSIL (Low grade Squamous Intraepithelial Lesions) and normal squamous epithelium (13%)[99].

Others different molecular processes are involved in cancer cell invasion such as the production of MMPs natural inhibitors TIMP-1 and TIMP-2 and their activation. Substantial evidence suggests the importance of the MMPs/TIMPs ratio in tumor tissues [100]. There are increasing data on their contribution to the tumor angiogenesis [101] and their impact on cytokines regulation [102]. The process of angiogenesis requires degradation and remodeling of ECM, cell migration and proliferation, and tube formation [96, 101] and it can explain the relevant role played by MMPs in tumor growth and progression. Overexpression of MMP-2 and MMP-9 has been observed in pre-cancer and cancer lesions of the cervical uterine. The potential significance of MMP-2 and MMP-9 in the progression of cervical uterine cancer suggests their prognostic value [95, 96]. Many lines of evidence, in fact, show that the activity of MMP-9 tends to increase from normal cervix to HSIL and CC, and more advanced stages [97]. HPV infection is an

essential step in the development of cervical cancer and transfection with the E6 and E7 HPV ORFs is sufficient to induce malignant transformation in normal squamous cells *in vitro*. Recent findings suggest that HPV E6 and E7 transcription correlates with MMPs and TIMPs transcription [103, 104, 105] and show a strong correlation of MMP-9 and TIMP-1 with *Gardnerella, Atopobium* and *Prevotella* and with inflammatory cytokines, IL-1β, IL-8 and TNF-α, in cervicovaginal fluid of HIV^+ women [106].

In conclusion, microbial communities, crosstalk between host microenvironment and microbiota, immune regulatory actions, and gene expression interact closely to govern the homeostasis of the vaginal environment and the host's health.

2. Cervicovaginal microbiota, persistent HR-HPV infection and cervical dysplasia or cancer: state of art

High-Risk Human papillomavirus (HR-HPV) strains are deeply involved in the onset of premalignant precursor cervical intraepithelial neoplasia (CIN) and cervical cancer (CC) [107], the most common infection-associated neoplasm [108]. HPVs-16 and -18 represent the most prevalent HR-HPV strains and are endowed with the highest oncogenic potential. These HPV-strains are responsible for around 70% of cases of CC [109]. Persistence of the virus is essential for development of high-grade CIN and CC. Emerging evidence indicates that cervicovaginal microbiota (CVM) plays a substantial role in the viral persistence and subsequent disease. Accordingly, a role of CVM composition in the regression of high-grade lesions has been reported [109].

Vaginal *Lactobacillus spp.* are known to provide a broad-spectrum protection against invading pathogens through their production of lactic acid, bacteriocins, antagonistic bacteriocin-like substances, and biosurfactants. *Lactobacilli* strongly adhere to epithelial cells as well as to mucus, which form barriers against pathogens [110] and disrupt biofilm [7].

Further, Motevaseli *et al* demonstrated that *Lactobacilli* exert cytotoxic effects on cervical tumor cells in vitro, independent of pH and lactic acid [111].

Low numbers of *Lactobacilli* and high bacterial diversity indicate a perturbation of homeostasis and favor inflammation processes in the cervicovaginal environment [21].

Many factors are reported to affect the balance of microbiome and among them the host's factors cannot be ignored. Eating habits, contraception, smoking and sex life, are the factors linked to the social environment (health and socio-economic conditions) that influence the composition of the microbiome [58, 21].

Lactobacillus-dominated microbiota is not always present in vaginal environment of healthy women. As for example, a non-*Lactobacillus* dominated vaginal microbiota is more common in healthy Hispanic women and women of African descendent [112-114]. A recent study reported that a vaginal microbiota, dominated by lactic acid bacteria, is more common in women of Japanese and Caucasians ancestry then in women of African descent [115].

Interestingly, the prevalence of CC is higher in areas where vaginal microbiota is not dominated by *Lactobacilli* than in areas where *Lactobacillus* is the dominant specie [5], suggesting that this is the bacterial genus most involved in defending the vaginal microenvironment and protecting the female reproductive tract [21].

The differences observed among ethnic groups could be due to genetic factors as well as to differences in innate and adaptive immune system, the composition and quantity of vaginal secretions, and ligands on epithelial cell surfaces, among others [116].

It was reported that selected composition of the CVM with the presence of *Ralstonia pickettii, Streptococcus anginosus, Prevotella bivia, Prevotella timonensis* [117] and *Sneathia* are more represented in HPV-positive women than in HPV-negative women [13, 114,146].

All these cross-sectional studies indicate that a depletion of Lactobacilli is associated with a greater persistence of HPV infection. However, the role of *L. iners* remains unclear. *L. iners* is present in the vaginal ecosystem of healthy women, but it represents the dominant *Lactobacillus* species in the transitional stage from healthy vaginal environment to BV state [8,9] or even is enriched in HPV^+ women [114, 118] and in women with Low and High-grade of dysplasia [114].

Longitudinal studies represent a more suitable modality to define a microbiota at high risk of HR-HPV infection. Several longitudinal studies recruiting healthy women revealed that women that exhibit a vaginal microbiota with a low relative abundance of *Lactobacillus* spp. (CST IV) or dominated by *L. iners* (CST III) are more infected by HR-HPV. These studies thus agree with the results from cross-sectional studies and suggest that CST-III and CST-IV represent microbial community that increases the risk of HPV acquisition and/or persistence [58, 113, 119, 120].

Moreover, a longitudinal study performed in our laboratory in collaboration with ISPRO recruiting large numbers of HPV^+ and HPV^- women, revealed a significant enrichment of *G. vaginalis*, *Sneathia, Megasphaera, Atopobium, Prevotella, Dialister* in women that did not clear the infection over one year of observation [13]. Among *Lactobacillus* species, a correlation between relative abundance of *L. gasseri* and HPV clearance has been reported by Brotman [119], Shannon *et al* [121] and McKee *et al* [122]. Higher abundance of *L. crispatus* has been shown to be associated with lower HR-HPV prevalence and increase detection of normal cytology [113] [123] [120, 124].

The role of *L. iners* remains controversial yet. In fact, some longitudinal studies reported *L. iners* as the taxon most positively associated with the clearance of HR-HPV infection [124].

The vaginal microbial diversity and decreasing relative abundance of L*actobacillus* spp. are associated not only with HPV infection, but also with advancing CIN severity [120].

Two recent meta-analysis and systematic reviews revealed that vaginal dysbiosis (CST-IV) is associated with persistence of HPV cervical infection and squamous intra-epithelial lesions (SIL) [116, 125]. More specifically, vaginal microbiota dominated by non-*Lactobacilli* species (CST-IV) or vaginal microbiota dominated by *L. iners* (CST-III) represent a risk factor for HR-HPV infection/persistence and progression to dysplasia/cancer compared to vaginal microbiome dominated by *L. crispatus* (CST-I) [116].

Cross-sectional studies revealed a marked decrease of *L. crispatus* abundance in women with CIN-1, CIN-2 or CIN-3, and CC compared with healthy women. Accordingly, vaginal microbial diversity was higher in cervical disease group than in normal group [114, 126]. *L. iners* was found significantly enriched in women with Low grade and High grade of dysplasia [114]. Other studies found *Gardnerella* spp. [129] and *Sneathia spp.* [58] as the most abundant species in the cervix of women with SIL.

Gardenerella vaginalis was identified as a key biomarker in women with CIN-2 [124]and *G. vaginalis, A. vaginae, P. timonensis* were reported to significantly increase the species-specific risk for CIN-2 or CIN-3 and CC [122, 126].

Mitra *et al* [127] investigated temporal relationship between vaginal microbiome and the natural history of CIN. They found that women with a *Lactobacillus*-dominant microbiota are more likely to have regressive disease at 12 months. *Lactobacillus spp.* depletion and presence of specific anaerobic taxa including *Megasphaera, Prevotella, Atopobium,* and *G. vaginalis* are associated with CIN-2 persistence and slower regression or non-regression [127, 128].

The metabolomic asset of the cervicovaginal microenvironment, which includes either metabolite produced by the host and those of microbial origin, cannot be overlooked since it can affect some clinical endpoints. As for example, vaginal metabolome and specially selected metabolites have been associated with important pathological conditions such as BV, pre-term delivery and pre-eclampsia [129] [22].

Borgogna and colleagues [130] associated the vaginal metabolome to HPV detection status and vaginal CST, identifying unique metabolites in clinical isolates tested for the most common BV species. The vaginal metabolome of women with CST IV microbiota (here defined as molecular-BV) clustered distinctly from those with other CSTs. Higher biogenic amine and phospholipid concentrations were observed in HPV^+ women compared with HPV^-women, and significant differences in metabolomic profiles of HPV^+ and HPV^- women were evident within each stratum of CST. Within CST III, HPV^+ women had higher concentrations of biogenic amines compared to HPV^- women. Within CST IV, HPV^+ women had lower concentrations of glutathione, glycogen, and phospholipid-related metabolites than HPV^- women [130]. *A. vaginae* and *S. amnii* create metabolic microenvironment promoting immune response of host [114]. Sphingolipids, plasmalogens, and linoleate positively correlated with genital inflammation, an increase in the *Lactobacillus* abundance was positively associated with the levels of anti-inflammatory nucleotides [131].

Given the cancer is a metabolic disease, metabolic profiling of the cervicovaginal microenvironment has the potential to reveal the functional interplay between the host and microbes in HPV persistence and progression to cancer. There are metabolic signatures that effectively distinguish HPV-infected individuals, women with cervical dysplasia and cancer from HPV^- women. Three-hydroxybutyrate, eicosenoate, and oleate/vaccenate discriminated (with excellent capacity) between cancer patients versus the healthy participants. In low-grade (LSIL) and principally high-grade (HSIL) dysplasia groups, polymicrobial communities perturb amino acid and nucleotide metabolism in a similar manner to bacterial vaginosis [131]. Lipids mainly involved in carnitine metabolism such as decanoylcarnitine, hexanoylcarnitine, and acetylcarnitine were depleted in the Ctrl HPV (+), LSIL, and HSIL groups compared to Ctrl HPV^- and CC groups. A higher abundance of lipid metabolites in CC patients compared to healthy individuals can be explained by an increase in cell proliferation and cell membrane synthesis through activation of oncogenic pathways in the tumor microenvironment [131, 132].

In addition, *G. vaginalis* and *P. bivia* principally affect the physicochemical properties of the epithelial barrier. In contrast, *L. crispatus* did not affect metalloproteases (MMPs), mucins, or proteins involved in cellular stress, strongly suggesting that colonization of cervical cells with *L. crispatus* guarantees the epithelial barrier integrity [132].

Collectively, both cross-sectional and longitudinal studies agree that the composition of vaginal microbiota profoundly impact on HR-HPV acquisition, genital inflammation, and metabolic changes of the CVM, having a decisive role in the regression or persistence of HPV infection and on the consequent severity of intraepithelial lesions up to cervical cancer. Specifically, everyone agrees that a microbiota dominated by *L. crispatus* is associated with a state of health and correlates positively with the regression of HPV and negatively with the advancement of intraepithelial lesions; while a microbiota characterized by a high microbial diversity (CTS-IV) and with prevalence of *Gardenerella, Prevotella, Atopobium, Sneathia* and *Megasphaera* correlates with the persistence of infection and neoplastic progression.

However, several biases and discrepancies on the actual role of some *Lactobacilli*, primarily *L. iners* and *L. gasseri* need to be elucidated yet.

In addition, a non-negligible factor is that the ability to derive a causal link between vaginal microbiota and HPV infection and CIN/CC is limited by the cross-sectional nature of most studies in this area and by lack of longitudinal studies able to differentiate the impact of the microbiome on clinical outcomes and disease status. In fact, the few longitudinal studies currently present in the literature also have limitations: the results depend on the size and the features of the analyzed cohort. Contradictory results also are strictly dependent on the size and the features of the cohorts; there is currently no standardized method of sample collection and analysis; adequate and standardized adjustment models for potential confounding factors (age, smoking, co-infections, ethnicity, vaginal intercourse without the use of barrier contraception) are not used and, finally, metabolomic studies identify the association of specific metabolites with the different stages of HPV and SIL infection, but are unable to provide clear data on which

metabolites to use as biomarkers before the onset of cancer. Most metabolome changes in fact are evident only when cancer is advanced.

For this reason, the aim of our study was to investigate how the single bacterial species of vaginal microbial communities affect the trophism of epithelial cells, the expression of viral oncogenic proteins, the amounts of key cellular oncosuppressors, as p53 and pRb, crucial in the control of cellular apoptosis and proliferation, and expression of one of the metalloproteases most involved in the progression of cervical cancer.

3.Materials and Methods

3.1 Bacterial strains

A collection of 12 bacterial reference strains were included in the study and related features are reported in Table 2. *L. crispatus* (JV-V01), *L. gasseri* (SV-16A), *L. iners* (UPII-60-B) and *L. jensenii* (JV-V16) were used as representative of CST-I, II, III and V, respectively. *Gardnerella vaginalis* was selected as representative of CST-IV and four strains isolated respectively from healthy woman (315-A) or from women with bacterial vaginosis with Nugent Score 5 (49145/JCP-7276), 8 (14019/JCP-7659), 10 (14018/JCP-7275) were selected. *A. vaginae* (DSM-15829), *M. micronuciformis* (DNF00954), *P. bivia* (DNF 00188 and DNF-00650) were also used as representative of CST-IV.

CST	Family and Genus	Specie	ATCC code/strain/ abbrevetion
I	*Lactobacillaceae, Lactobacillus*	*Lactobacillus crispatus*	*JV -V01*
II	*Lactobacillaceae, Lactobacillus*	*Lactobacillus gasseri*	*SV -16°*
III	*Lactobacillaceae, Lactobacillus*	*Lactobacillus iners*	*UPII -60 -B*
V	*Lactobacillaceae, Lactobacillus*	*Lactobacillus jensenii*	*JV -V16*
IV	*Bifidobacteriaceae, Gardnerella*	*Gardnerella vaginalis*	*315-A/normal flora/ G.vag n.f.*
IV	*Bifidobacteriaceae, Gardnerella*	*Gardnerella vaginalis*	*49145/JCP-7276/ G.vag. sc 5*
IV	*Bifidobacteriaceae, Gardnerella*	*Gardnerella vaginalis*	*14019/JCP-7659/ G.vag. sc 8*
IV	*Bifidobacteriaceae, Gardnerella*	*Gardnerella vaginalis*	*14018/JCP-7275/ G.vag. sc 10*
IV	*Atopobiaceae, Atopobium*	*Atopobium vaginalis*	*DMS-15829*
IV	*Prevotellaceae, Prevotella*	*Prevotella bivia*	*DNF-00188/P. bivia 1*
IV	*Prevotellaceae, Prevotella*	*Prevotella bivia*	*DNF-00650/P. bivia 2*
IV	*Veillonellaceae, Megasphaera*	*Megasphaera micronuciformis*	*DNF-00954/M. micron*

Table 2. ATCC reference bacterial strains and the abbreviations used in this work.

3.2 Comparative genomic analysis of *G. vaginalis* strains

Lists of genomic sequences whose functions are noted on ATCC were taken and a comparative analysis was carried out to assess whether among these there were one or more specific genes for each strain coding for the Antitoxin/Toxin system.

The total number of genes considered and the specific genes for each strain are shown in **Table 3**. For the representation of the data of the comparative genomic analysis, the online tool http://bioinformatics.psb.ugent.be/webtools/Venn/ was used.

Strain	Total genes	Specific - strain gene	Anti-toxin	Toxin	unknown
G. vaginalis n.f.	50	10	3	5	2
G. vaginalis score 5	50	6	2	3	1
G. vaginalis score 8	61	11	0	4	7
G. vaginalis score 10	53	10	6	3	1

Table 3. In table are reported the total number of genomic sequences noted on ATCC depository, the genomic sequences strain-specific, and the coding gene for Antitoxin/Toxin for each G. vaginalis strain.

3.3 Bacterial cultures

Anaerobic bacteria were grown in Tryptic Soy Agar (TSA), composed by Tryptic Soy Broth (Oxoid, Basingstoke, United Kingdom) and 15mg/L of Bacto-Agar (Sigma Aldrich, US),

with the addition of 5% Horse whole blood lysed (Oxoid, Basingstoke, UK). The plates were incubated at 37°C for 72 hours in anaerobic conditions inside a jar (AnaeroGen™, Thermo Fisher Scientific, Waltham, USA) to create ideal growth conditions (CO_2: 9-13%; O_2<0,01%)

Bacterial strains were also grown in liquid cultures using TSB with 5% horse lysed blood (Oxoid, Basingstoke, United Kingdom). The turbidity of the culture broth of each individual strain was measured by using the DensiCHECK densitometer after centrifuging 1 mL of culture at 4,000 g for 5 minutes and resuspending the pellet in 1 mL of physiological solution.

Bacterial concentration was calculated according to the following formula:

$$[bacterial] = [McFarland \times 1.5/0.5] \times 10^8$$

Optical Density assessment (OD) was performed using DensiCHECK™ densitometer (bioMérieux, Marcy l'Étoile, France).

To obtain supernatants and heat-inactivated bacteria cultures were centrifuged at 4,000 g for 5 minutes and suspended in RPMI-1640 medium or DMEM with the addition of 10% FBS (Fetal bovine serum) and 1% of L-glutamine (Euroclone, Pero, Italy) and incubated for 1 hour at 37°C. After centrifugation at 6,000 g for 10 minutes supernatants were collected. Bacterial inactivation was performed by heating at 95°C for 15 minutes.

The bacterial lysates were obtained after incubation of bacterial cells with PBS-0.1% TRITON X-100 at 37°C for 15 minutes. Before this step Gram-positive bacteria were incubated with Lysozyme (10mg/mL) for 1 hour at 37°C. Each sample was heated at 95°C and immediately frozen in liquid nitrogen for at least three times. Protein concentration has been quantified by the BCA (Bicinchoninic Acid) method and each sample was used as stimulus at the concentration of 1µg/mL.

3.4 Epithelial cell culture

SiHa cell line, isolated from squamous cell carcinoma and containing HPV-16 genome (1 to 2 copies per cell) was obtained from ATCC® (ATCC® HTB35™). SiHa cells were cultured in DMEM medium (Euroclone, Pero, Italy) supplemented with 10% FBS (Fetal bovine serum), 1% L-glutamine, 1% penicillin and streptomycin (Euroclone, Pero, Italy) at 37°C in presence of 5% of CO_2.

3.5 Viability test

SiHa cells were cultured in 96-multiwells at the concentration of $2.5x10^3$cells/mL with bacterial cell supernatants (10-20-30% v/v) or bacterial lysates (1µg/mL) or live bacteria (50 MOI/cell). Intracellular ATP was measured as index of metabolic activity using CellTiter-Glo® LuminescentCell Viability Assay (Promega Corporation, Medison) after 6 and 24 hours of culture.

3.6 SCFAs (Short Chain Fatty Acids) profile of bacterial strains

Supernatants of bacterial strains were prepared from liquid cultures in broth containing TSB and 5% Horse lysed blood) (Oxoid, Basingstoke, United Kingdom) following OD determination and ultracentrifugation.

The SCFAs were assessed through an isotope dilution [9] quantitative method that use a gas-chromatography coupled with mass spectrometry (GC-MS) system.

Briefly, the SCFAs were recovered from the samples by liquid-liquid extraction and then analyzed, as free acid form, by GC-MS instrument equipped with a Supelco Nukol column 30 m length, 0.25 mm internal diameter and 0.25 µm of film thickness. The SCFAs separation was carried out by the temperatures program as follows: initial temperature of 40 °C was held for 1 min, then it was increased to 150 °C at 30 °C/min, finally grow up to 220 °C at 20 °C/min. A 1 µl aliquot of extracted sample was injected in spitless mode

(spitless time 1 min) at 250 °C, while the transfer line temperature was 280 °C. The carrier flow rate was maintained at 1 mL/min.

The quantitative SCFAs' evaluation was carried out by ratios between the area abundances of the analytes with the area abundances of respective labelled internal standards (isotopic dilution method). The ionic signals and the reference internal standard, used for the quantitation of each SCFA, were reported in Table 4.

In 15 mL centrifuge tube,3 mL of pre-fermented medium sample was added of 50µL of internal standards (ISTD) mixture, 1 mL of tert-butyl methyl ether and 50 µL of 1 M HCl solution. Then, each tube was shaken in vortex apparatus for 2 minutes, centrifuged at 10,000 rpm for 5 minutes, and finally the solvent layer was transferred in auto sampler vial and analyzed by GC-MS method. Each sample has been prepared and processed three times by the previously described method.

SCFAs	Quan. Ion	Qual. Ion	ISTD
Acetic acid	60	-	[^{2}H3] Acetic
Propionic acid	74	73	[^{2}H3] Propionic
Butyric acid	60	73	[^{2}H3] Propionic
Valeric acid	60	73	[^{2}H9] iso-Valeric

Table 4. Ionic signal used for quali-quantitation and relative ISTD of each Short Chain Fatty Acids (SCFAs) acquired by the ID-GC-MS method.

3.7 Gene expression

RNA extraction was performed starting from 10^6 cells for each sample using Total RNA Extraction Kit (RBCBioscience,Real Genomics, New Taipei City,Taiwan cat. #YRB100). Extracted RNAs were quantified using Nanodrop (Thermo Fisher, Waltham, MA, USA) and stored at -80ºC.

From each sample 2 ug of RNA were reverse-transcribed using PrimeScript RT-PCR (TaKara, Kusatsu, Japan, cat. #RR047A). Real-Time PCR was performed by using QuantiNova SYBR Green PCR kit (Qiagen, Hilden, Germany, cat. #208056). 50 ng of cDNA were amplified using Rotor-GeneQ (Qiagen, Hilden, German) from each sample. 18S gene was used as housekeeping. The primers used in this work are reported in Table 5.

Gene	Forward 5'- 3'	Reverse 3'- 5'
18S	ATTAAGGGTGTGGGCCGAAG	GGTGATCACACGTTCCACCT
E6	CGACCCAGAAAGTTACCA	AGCAAAGTCATATACCTCACG
E7	GCCACCATGCATGGAGATACACCTACA	GATCAGCCATGGTACATTATGG

Table 5. Real Time-PCR primers for HPV-16 oncogenes expression.

3.8 Western Blot analysis

A total of $1x10^6$ SiHa cells were cultured in DMEM for 6 hours in the presence or absence of live bacterial cells (50 MOI/cell).

After the incubation time, cells were lysed with RIPA buffer in the presence of phosphatase/protease inhibitor cocktail inhibitor cocktail (Sigma-Aldrich, Saint Louis, MO, USA) and centrifuged at 12,000 g for 10 minutes. Protein concentration was determined by BCA (Bicinchoninic acid) assay (Quantum Protein Assay Kit, EuroClone Pero, Italy, cat. #EMP014250). 40 µg of proteins/lane were loaded onto Stain Free gel

(Bio-Rad Hercules, CA, USA) SDS-PAGE and blotted onto PVDF filters, Millipore Immobilon Transfer membrane (Millipore- Sigma-Aldrich, Saint Louis, MO, USA).

Membranes were stained with mouse mAb anti-HPV16 E6/18 E6 (cat. #C1P5: sc-460, Santa Cruz Biotechnology, Inc., Europe), mouse mAb anti-HPV16 E7 (cat. #NM2:sc-65711, Santa Cruz Biotechnology, Inc., Europe), mouse mAb anti-E6-AP (cat. #E-4:sc-166689 Santa Cruz Biotechnology, Inc., Europe), mouse mAb anti-MMP-9 (cat. #2C-sc:-2172 Santa Cruz Biotechnology, Inc., Europe), mouse mAb anti-p53 (D0-7) (cat. # 48818, Cell Signaling Technology, Danvers, MA, USA), rabbit mAb anti-Rb (D20) (cat. #9313, Cell Signaling Technology, Danvers, MA, USA), mouse mAb anti-beta-actin (C4) (cat. #sc:-47778 Santa Cruz Biotechnology, Inc., Europe) or mouse anti-alpha-tubulin (B-5-1-2) (cat. #sc:-23948, Santa Cruz Biotechnology, Inc., Europe) antibodies at 1:1,000 final dilution. Goat anti-mouse IgG (H+L)-HRP Conjugate or Goat anti-rabbit IgG (H+L) (Human IgG Adsorbed) Horseradish Peroxidase Conjugate (cat. #170-6516; cat. #170-6515, Bio-Rad Hercules, CA, USA,) were used as secondary antibodies at 2,000 final dilutions. The reactions were visualized by the ECL detection system as recommended by the manufacturer (Bio-Rad Hercules, CA, USA). The intensity of proteins of interest was acquired by ChemiDocTouch System (Bio-Rad Hercules, CA, USA). The densitometric analysis was expressed as the ratio between interest protein and total proteins by ImageLab software (Bio-Rad Hercules, CA, USA).

3.9 Cellular cycle analysis

A total of $10x10^4$ SiHa cells were cultured in DMEM for 24 hours in the presence or absence of live bacterial cells (50 MOI/cell). Cell cycle phase distribution (propidium iodide staining) was determined as previously reported [133] using a FACS Canto (Beckton & Dickinson, San Josè, CA, USA).

3.10 Statistical analysis

Real time-PCR and viability data were analyzed by analysis of variance (ANOVA) from three different experiments. Bonferroni P value adjustment method for multiple comparisons was used. Probability value of $p < 0.05$ was considered significant. Statistical analysis was performed using R software version 3.6.1 [134]. Western blot statistical analysis was performed by paired T-test.

4.Results

4.1 Bacterial strains: genomic analysis and metabolic profiles

Strains of *L. crispatus, L. gasseri, L. iners* and *L. jensenii* were used as representative of CST-I, II, III and V, respectively. *G. vaginalis* was selected as representative of dominant species of CST-IV. Four different strains of *G. vaginalis,* isolated from vaginal fluids of healthy woman (*G. vaginalis* normal flora) or from woman with different degrees of bacterial vaginosis (Nugent inflammation score from 5 to 10) were used in all of experiments. The virulence and the ability to degrade mucin of each strain were described [135, 136]. Strains of other bacterial species enriched in CST-IV vaginal microbiota as *Atopobium vaginae, Megasphaera micronuciformis, Prevotella bivia* were used in selected experiments.

Short Chain Fatty Acids (SCFAs) have been reported to affect epithelial cell metabolism and innate inflammatory response [137, 7]. Acetate, propionate, butyrate and isovalerate are usually found in the vaginal environment [138].

As first step of our study, we assessed the production of SCFAs in culture supernatants of all bacterial strains by gas-chromatography coupled with mass spectrometry (GC-MS) system.

Acetic acid turned out to be the dominant SCFA produced by all vaginal bacteria. However, relevant differences in acetic acid production by dominant species of vaginal microbiome were revealed by GC-MS. Figure 1 Panel A shows that all vaginal Lactobacilli produced acetic acid in lower amount compared to *G. vaginalis*.

On the other hand, *Lactobacilli* produced butyric and valeric acid amounts higher than *G. vaginalis* strains. The metabolic profiles of *A. vaginae* and *P. bivia,* that are usually associated to *G. vaginalis* in vaginal dysbiosis, were largely superimposable to that of *G. vaginalis* (Figure1 Panel B).

M. micronuciformis showed a metabolic profile intermediate between *Lactobacilli* and *G. vaginalis* strains, with lower amounts of both acetic and butyric acid compared to either *G. vaginalis, A. vaginae,* and *P. bivia.*

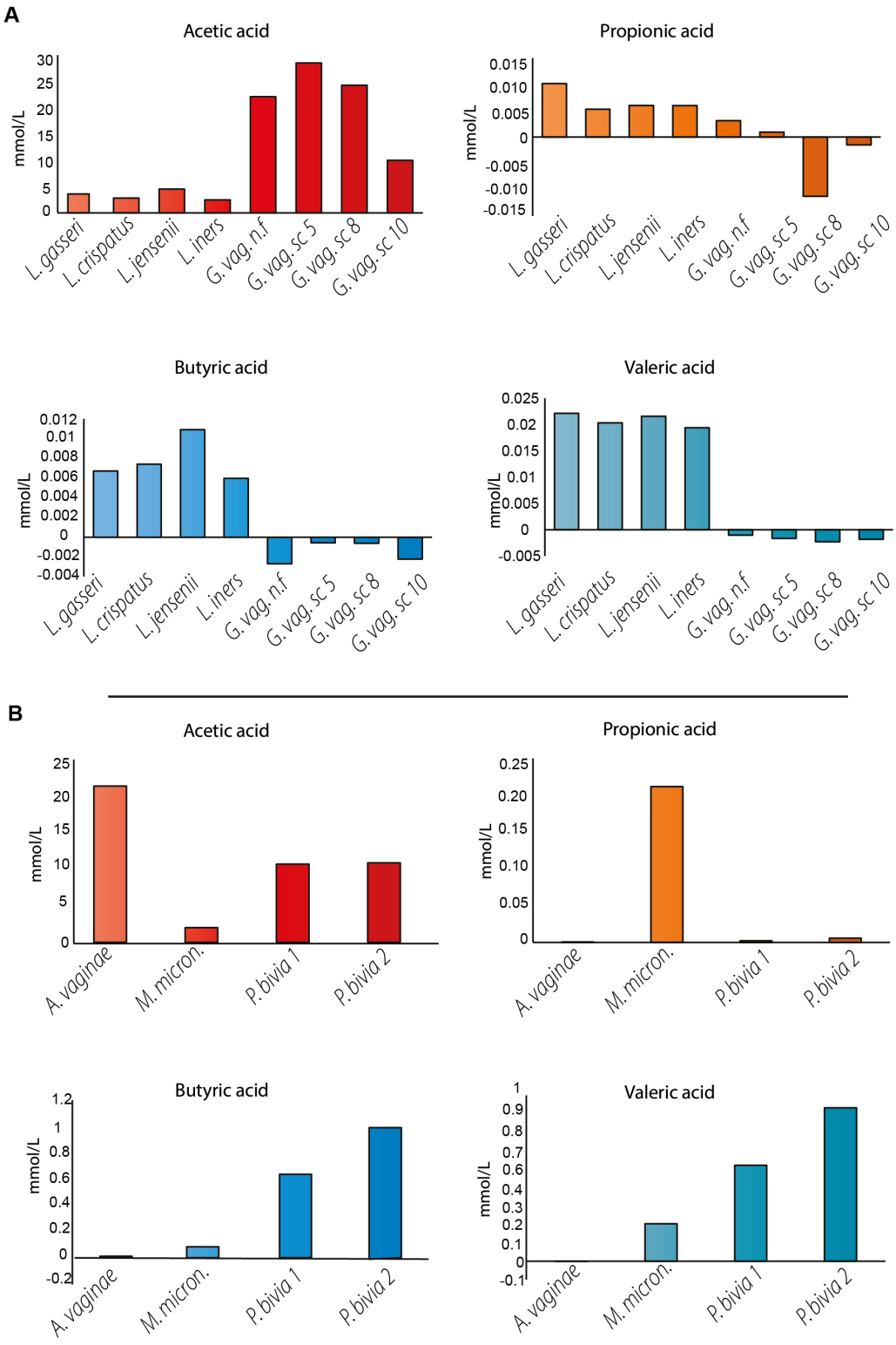


Figure 1. Short Chain Fatty Acids (SCFAs) profile. Supernatants of **A)** *Lactobacillus* spp., *Gardnerella* spp. and of **B)** others vaginal dysbiosis bacteria were analyzed and the qualitative and quantitative determination of acetic, propionic, butyric and valeric acids

was performed using gas-chromatography coupled with mass spectrometry (GC-MS) system. The quantitative SCFAs' evaluation was carried out by ratios between the area abundances of the analytes with the area abundances of respective labelled internal standards (isotopic dilution method).

We also observed that the selected strains of *G. vaginalis* differ in the production of SCFAs and particularly of acetic acid and propionic acid.

A comparative genomic analysis performed on *G. vaginalis* sequences in ATCC depositary revealed differences in the expression of toxin/antitoxin system genes among our *G. vaginalis* strains (**Table 1** and **Diagram 1**).

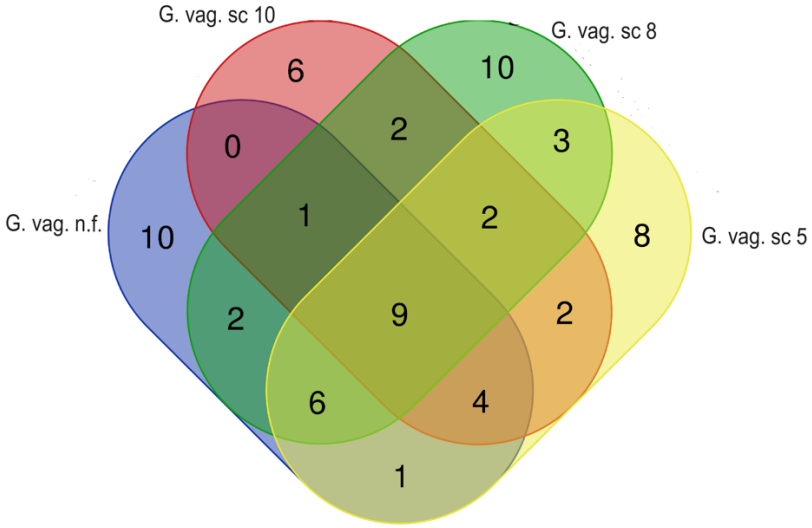


Diagram 1. Venn's diagrams of common and unique genes among *G. vaginalis* strains.

4.2 Cervical epithelial cell line features

For this study we used SiHa cells as experimental model of CIN-1. This cell line, containing ~ 1,2 copies of integrated HPV 16 genome, well represents a cervicovaginal epithelium infected with HR-HPV 16 in the early stages of neoplastic transformation [139].

4.3 Effects of supernatants, bacterial lysates, and live bacteria on viability of cervical epithelial cell line

To evaluate the effects of vaginal microbiota bacteria or their products on cervicovaginal epithelium trophism we performed a viability assay on SiHa cells by culturing them with the different bacterial stimuli.

We cultured SiHa cells in the presence or absence of different amount of bacterial culture supernatants or bacterial lysates for 24 hours or with live bacteria for 6 and 24 hours. Intracellular ATP and ADP/ATP ratio as index of metabolically active cells is reported in Figure 2.

Figure 2 panel A shows that supernatants from all *Lactobacilli* cultures induced a significant increase of ATP production by SiHa cells. In contrast Figure 2 panel B shows that supernatants from most vaginal dysbiosis bacteria affected cell viability in a dose-dependent manner. We observe the greatest decrease in SiHa cell viability with the supernatants from *A. vaginae* culture. A cytotoxic effect of this strain is markedly evident already at the lowest concentration (10% v/v). All other lysates from vaginal dysbiosis bacteria induce a decrease in the viability of SiHa cells ranging from 20% to 50%.

In contrast to vaginal dysbiosis bacteria, Figure 2 panel C shows that supernatants or lysates from *Lactobacilli* did not significantly affect the viability of SiHa cells, except for lysates from *L. iners*.

Figure 3 shows the results obtained by measuring the ADP/ATP ratio: most of *Lactobacillus spp.* live cells induced an increase in ADP. *L. jensenii* only induced an increase in ATP of about 70% after 6 hours of incubation.

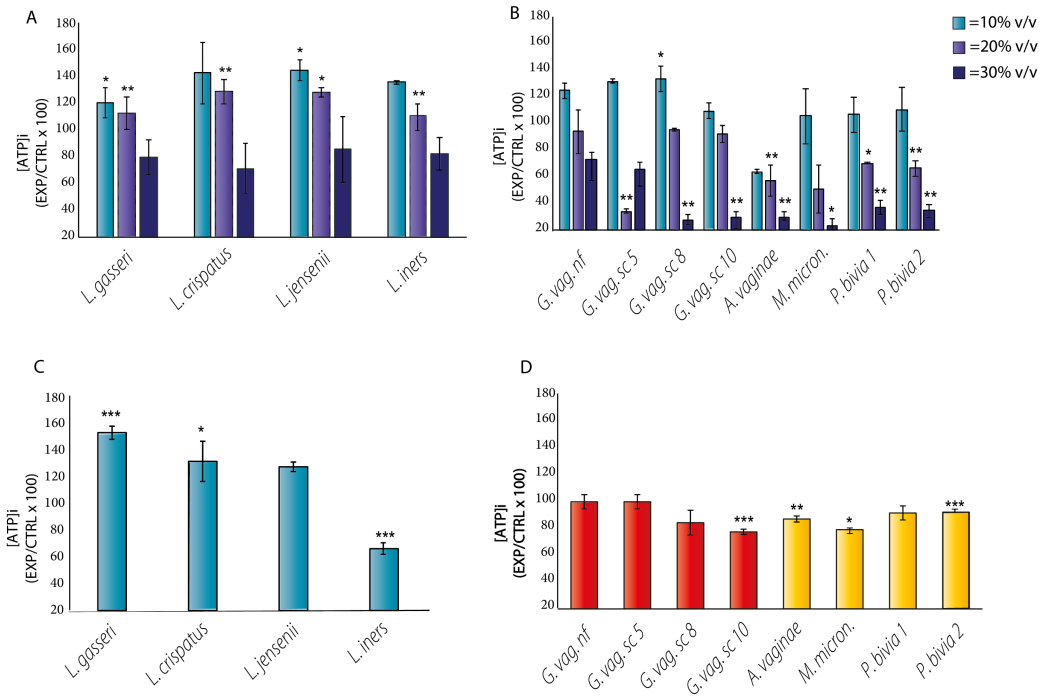


Figure 2. Effects of bacterial products on SiHa cell viability. ***A-B)*** ATP production by cells cultured with 10%; 20% or 30% (v/v) of bacterial supernatants. **C-D)** ATP production by cells cultured with bacterial lysates. The bar graph shows results from one representative experiment out of three performed. Data are expressed as ATP produced by stimulated cultures/unstimulated control ± standard deviation (EXP/CTRL×100). Statistically significant differences among stimulated/unstimulated cultures were reported * = p-value < 0.05; **= p-value < 0.01; ***= p-value < 0.001.

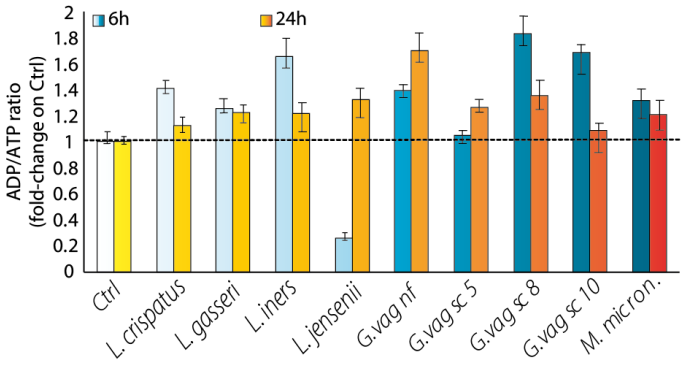


Figure 3. ADP/ATP ratio in SiHa cells with live bacteria. Data are expressed as ADP/ATP ratio produced by stimulated cultures/unstimulated control ± standard deviation. The

bar graph shows results from one representative experiment out of three performed. Statistical analysis did not reveal significant differences among stimuli.

4.4 Effects of vaginal bacteria on the expression of HPV oncogenes E6 and E7 by SiHa cells

The HPV-16 E2 protein regulates the replication and transcription of viral DNA, acting as a repressor of the E6 and E7 genes [72]. E2 gene is expressed only when the viral DNA is in episomal form. Following integration of viral genome in that of the host cell, lack of the E2 gene facilitates the expression of the viral oncogenes E6 and E7. This event represents a crucial step for both the increase of the viral fitness and manifestation of malignant cell phenotype [80]. Just a single copy of the viral genome is enough for the maintenance of malignant phenotype and neoplastic progression [140, 80].

To evaluate whether bacteria of vaginal microbiota or their relative products affect the expression of viral oncogenes, we cultured SiHa cells with live or heat-inactivated bacteria or with their lysates and the results are reported in Figure 4.

By using live bacteria, among *Lactobacilli* we observed that *L. crispatus* and *L. iners* did not modulate the expression of viral oncogenes, while *L. jensenii* induced an increase of E6 gene expression, but not of E7. Among vaginal dysbiosis bacteria, *M. micronuciformis* was the most powerful inducer of the expression of E6 and E7 gene and *G. vaginalis* (score 10) induced a 1.5-fold increase in E6 gene expression (Figure 4 panel A and panel B).

Figure 4 panel C and panel D show the results obtained by using bacterial lysates or heat-inactivated bacteria: we confirmed the ability of *M. micronuciformis* and *G. vaginalis* score 10 to induce a significant increase in the expression of E6 and E7 genes (1.5-3 fold increase compared to the unstimulated control). Among *Lactobacilli*, lysates from *L. jensenii* only induced a double fold increase in the expression of E7 gene compared to control cultures.

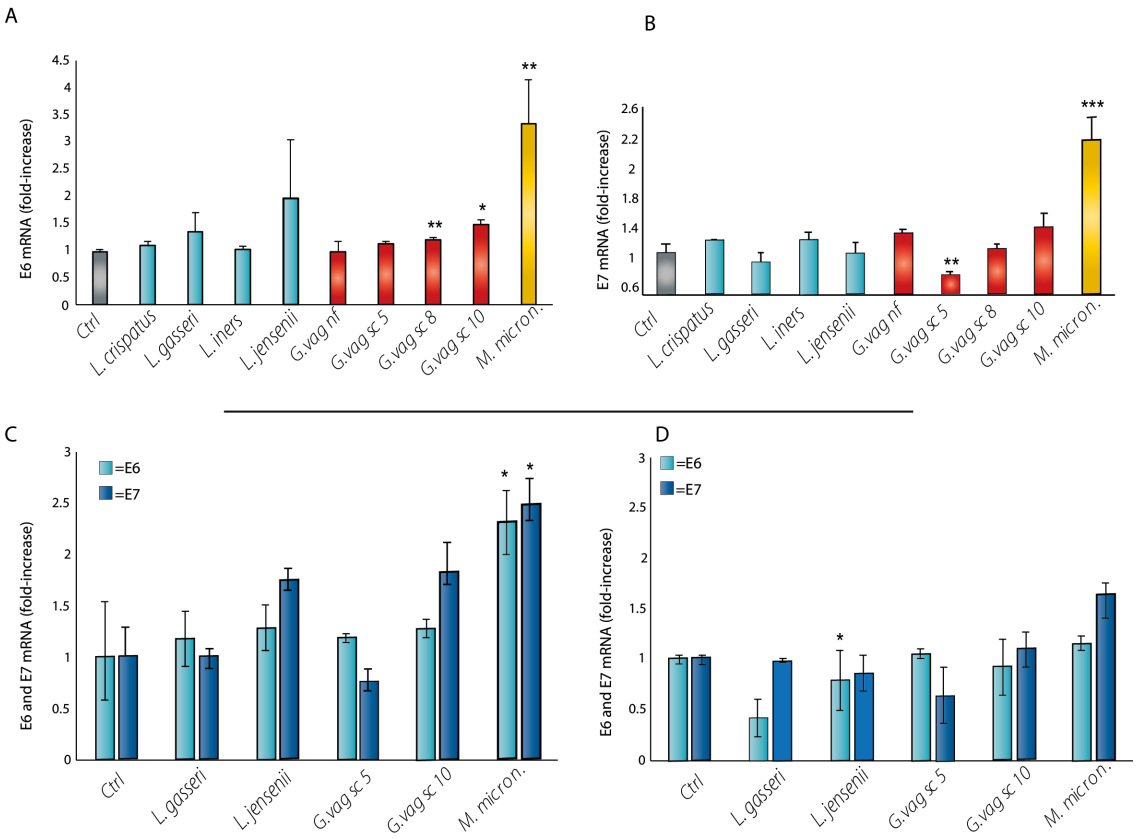


Figure 4. Effects of bacterial strains on the expression of HPV 16 oncogenes in SiHa cells. The expression of genes encoding for E6 and E7 in SiHa cells cultured with (**A)** and **B)**) 50 MOI/cell of live bacteria or **C**) 100µg/mL of bacterial lysates or **D)** 50MOI/cell of heat-inactivated bacteria. Data are expressed as fold-change in mRNA relative amount respect to unstimulated cultures (Ctrl). Bar graph shows data (mean ± SE) of three different experiments. (*=*p-value* < 0.5; **= *p-value* < 0.002)

4.5 Effects of vaginal bacteria on the synthesis of HPV-16 E6 and E7 proteins by SiHa cells

The products of the viral early genes, E6 and E7, play a key role in HPV persistence and in neoplastic progression of transformed cells [69]. Indeed, these proteins target key check points of cells with different mechanisms and affect cell cycle and apoptosis control [80]. The inhibition of the apoptotic program with the concomitant increase in cell proliferation allow increasing in mutational rate and give cells neoplastic features [141]. In addition, the continuous expression of E6 and E7 gene is necessary for the maintenance of the malignant phenotype [80].

To add further evidence about the effects of vaginal bacteria on the expression of viral oncoproteins, we investigated the expression of E6 and E7 proteins following stimulation of SiHa cells with the dominant species of CVM, mainly *Lactobacillus* ssp., *G. vaginalis* and *M. micronuciformis*.

Figure 5 shows the results obtained by culturing SiHa cells with live bacteria. Figure 5 panel A shows that *G. vaginalis* strains induced an increase in the expression of the E6 protein. The maximum increase (1.5- fold compared to unstimulated control) was evident in cultures with strains of *G. vaginalis* isolated from vaginal fluid of women with diagnosed bacterial vaginosis (inflammatory score of 5-10). *M. micronuciformis* also induced ~2-fold increase of the E6 protein expression, while vaginal *Lactobacilli* did not affect its expression. We evaluated the expression of E7 oncoprotein in the same SiHa cell cultures. Figure 5 panel B shows that 3/4 strains of *G. vaginalis* induced a significant increase (2-fold-change) in the expression of E7 protein. *M. micronuciformis* induced a milder increase (>1.5-fold-change) in E7 protein expression. Among Lactobacilli, *L. iners* surprisingly induced a 2-fold increase in E7 protein expression.

In summary *Lactobacilli spp.* never induced E6, while only *L. iners* induced E7 protein. In contrast, *G. vaginalis* and *M. micronuciformis* induced the expression of both E6 and E7 proteins. Figure 5 panel C and panel D also shows data of protein expression are in accordance with those of gene expression.

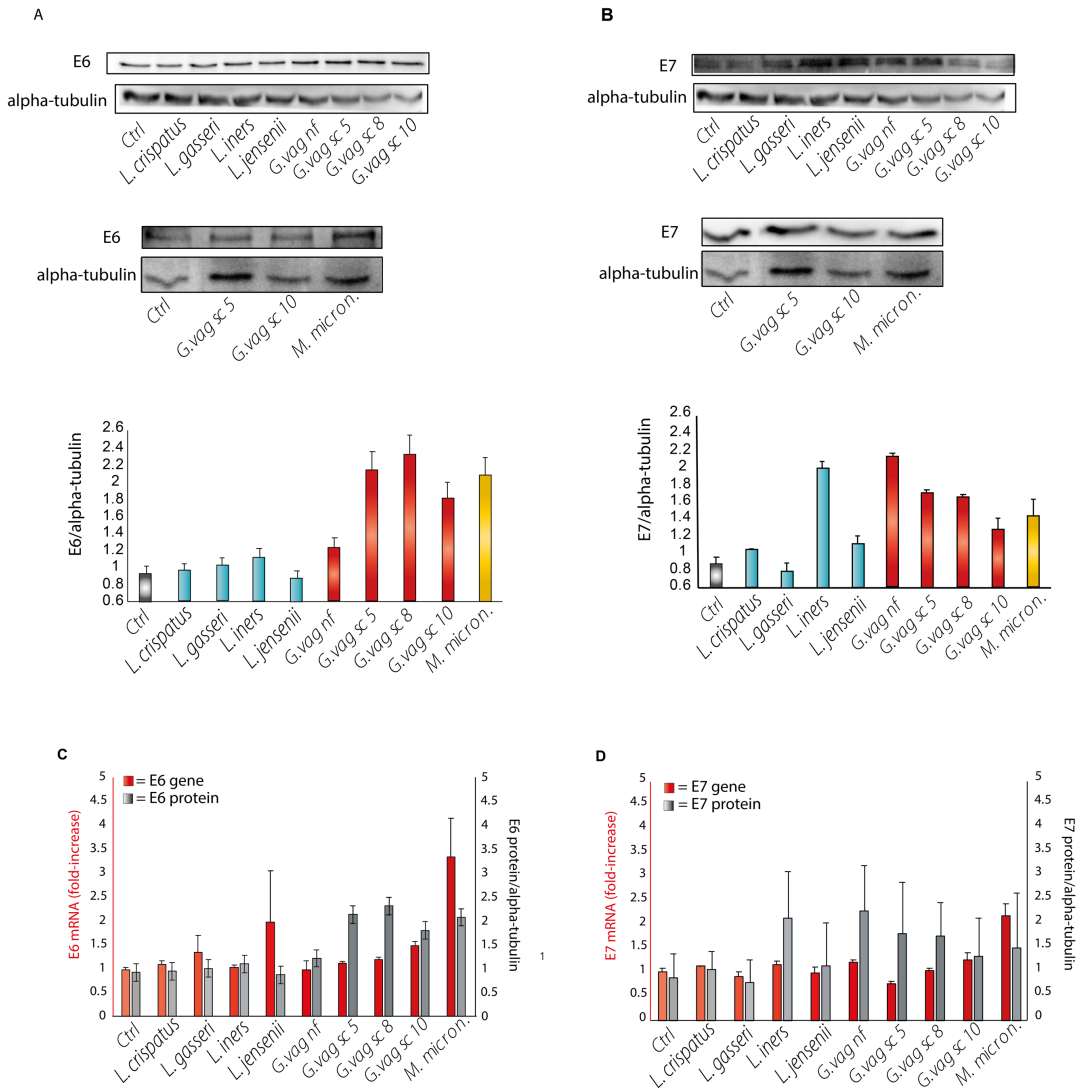


Figure 5. E6 and E7 expression. **5A)** and **5B)** show respectively E6 and E7 protein in SiHa cells cultured with vaginal-microbiota live bacteria. **5C)** and **5D)** show E6 and E7 gene expression compared to respective products expression. Data are expressed as fold-change respect to unstimulated cultures (Ctrl). Bar graph shows data (mean ± SE) of three different experiments. Statistical analysis did not reveal significant differences among stimuli.

4.6 Effects of vaginal bacteria on the production of E6-Associated Protein (E6-AP) by SiHa cells

E6-associated protein (E6-AP) mediates the binding between E6 and p53 and plays a major role in p53 degradation. The viral protein induces an increase of activated E6-AP, functioning as allosteric activator of the enzyme [142].

Thus, we evaluated the amounts of E6-AP protein in SiHa cells cultured with dominant species of vaginal microbiota. **Figure 6** shows that E6-AP expression was significantly induced by all *G. vaginalis* strains (2.5/3.5-fold) and again *G. vaginalis* strains, isolated from vaginal fluids with high inflammatory score, were the most powerful inducers. We also observed that, apart *L. crispatus*, a mild increase in E6-AP amount was revealed also in SiHa cells cultured with *Lactobacilli*.

Overall, these results indicate *G. vaginalis* as the vaginal bacterial species that better induces E6, E6-AP and E7 proteins and suggest that differences related to the genetic features of the strain may interfere with this property.

Among Lactobacilli, *L. crispatus* was the only species that never interferes with the expression of HPV-oncoproteins or with the E6-AP and thus appears to be the most protective *Lactobacillus* species against the neoplastic progression.

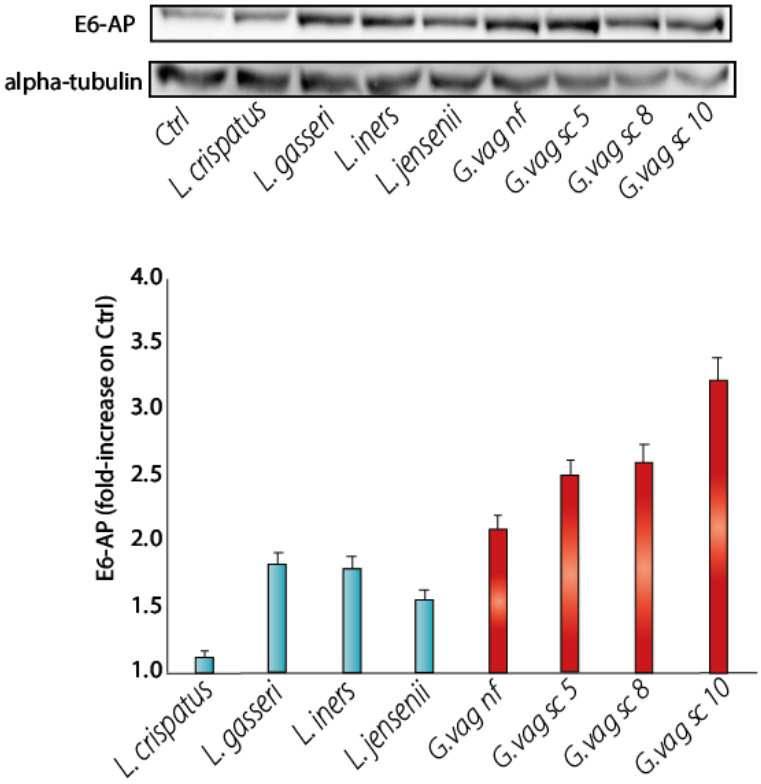


Figure 6. E6-Associated Protein (E6-AP) expression in SiHa cells. Protein expression was evaluated in cells cultured in absence or presence of each vaginal microbiota strain. Data are expressed as fold-change respect to unstimulated cultures. Bar graph shows one representative of three performed experiments. Statistical analysis did not reveal significant differences among stimuli.

4.7 Effects of vaginal bacteria on the expression of p53 oncosuppressor protein by SiHa cells

In the next phase of our study, we analyzed the effects of vaginal *Lactobacilli*, *Gardnerella* spp., and *M. micronuciformis* on the expression of the tumor suppressor p53. This protein is responsible to control the cellular apoptotic program and it is main target of the viral oncoprotein E6.

Figure 7 panel A shows that all *Lactobacilli* induced an increased expression of p53 protein compared to unstimulated cultures. In contrast, *G. vaginalis* score 5 e score 10 leave unaffected or reduced the expression of p53 protein. In **Figure 7 panel B**, the trend

line, calculated through the linear regression equation, underscores the opposite control of the viral E6 protein compared to cellular p53 production by vaginal bacteria.

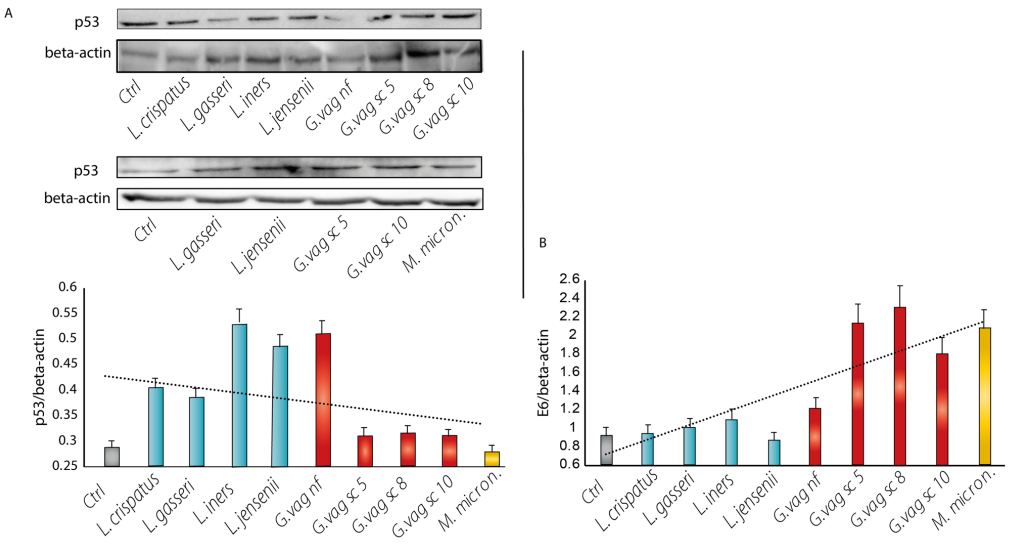


Figure 7. p53 oncosuppressor expression and E6 protein expression in SiHa cells **7A)** Protein expression was evaluated in cells cultured in absence or presence of each vaginal microbiota strain. Data are expressed as fold-change respect to unstimulated cultures. Bar graph shows one representative of three performed experiments. **7B)** E6 protein expression compared to p53 protein expression. The dotted line is the trend line calculated through the linear regression equation in Excel.

4.8 Effects of vaginal bacteria on SiHa cell proliferation

Given the ability of E6 and E7 to interfere directly or indirectly with cell cycle and apoptosis control, we completed the study by evaluating the effects of vaginal bacteria on cell proliferation. We cultured SiHa cells with live bacterial cells for 24 hours and we recorded cell proliferation by FACS, using CSFE fluorescent staining.

Among vaginal dysbiosis bacteria, *M. micronuciformis* significantly affected the S-phase of the cell cycle compared to unstimulated controls (**Figure 8**). Among *Gardnerella* strains*, G. vaginalis normal flora* also induced an increase in the S-phase of the cell cycle. In contrast, *Lactobacilli* did not affect any phase of cell cycle.

According with previously reported results, *M. micronuciformis* and *G. vaginalis* confirm the species that most interfere with the integrity of normal cellular functions.

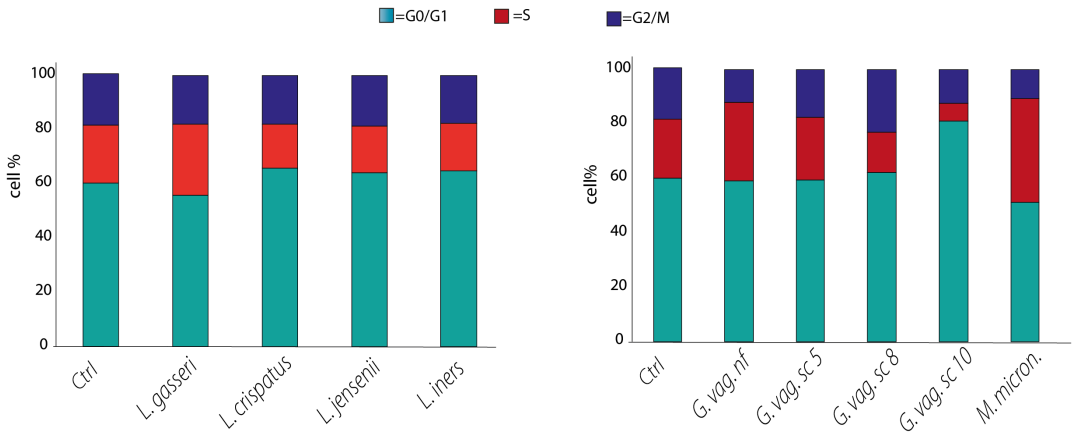


Figure 8 Cell-cycle analysis. G0/G1, S, and M cycle-phases of SiHa cells were analyzed by FACS. Data are expressed as percentage of cells in G0/G1, S and M phase respectively.

4. 9 Effects of vaginal bacteria on the expression of metalloproteinase MMP-9

In the second part of this study, we evaluated the effect of vaginal bacteria on the expression of proteins involved in the destruction of the basement membrane. This phenomenon is considered an essential step in the tumor development since it supports cancer cell invasion and metastasis [94, 95].

Matrix Metalloproteinase-9 (MMP-9) plays an important role in ECM remodeling and membrane cleavage. It is one of the most investigated MMPs since its overexpression was found in various cancers and is considered a potential biomarker also in cervical carcinoma [94].

Thus, we evaluated the ability of vaginal bacteria to modulate MMPs activity as measure of their potential role in tumor progression from stage CIN-1.

Figure 9 shows that *G. vaginalis* strains, isolated from vaginal fluid with BV at high inflammatory score, had a marked effect on the synthesis of MMP-9 that is enhanced by 3 to 5-fold-increases compared to the not stimulated SiHa cells. On the other hand, none of the L*actobacillus spp.* induced a significant increase in the expression of MMP-9.

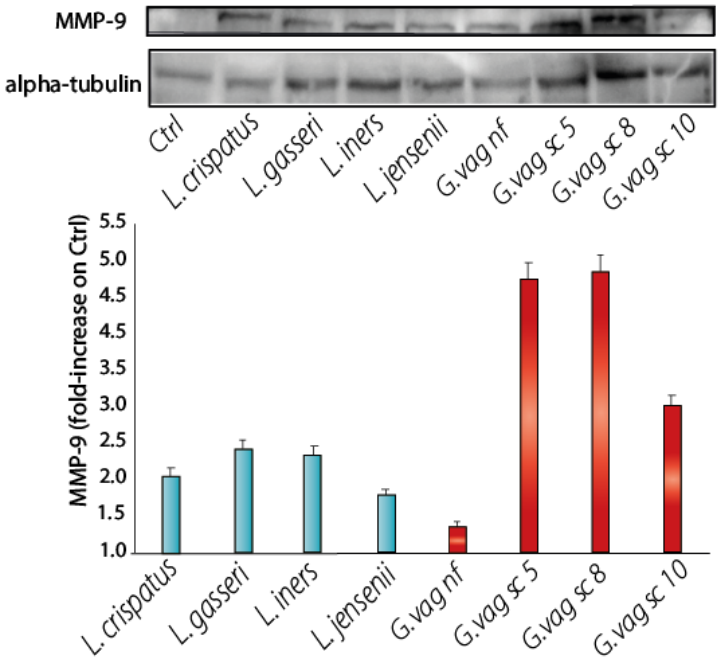


Figure 9. Metallo-proteinase 9 (MMP-9) expression in SiHa cells. Protein expression was evaluated in cells cultured in absence or presence of each vaginal microbiota strain. Data are expressed as fold-change respect to unstimulated cultures. Bar graph shows one representative of three performed experiments. Statistical analysis did not reveal significant differences among stimuli.

5. Discussion

The loss of the eubiotic state, caused by a depletion in vaginal *Lactobacilli* and by the prevalence of anaerobic bacteria mainly from genera *Gardnerella, Megasphaera, Atopobium, Sneathia* and *Prevotella*, defines the condition of vaginal dysbiosis, usually identified as CST-IV [13, 120]. This condition is frequently associated with symptomatic Bacterial Vaginosis (BV), one of the most common causes of vaginal infections in women of reproductive age [12, 14]. Vaginal dysbiosis is closely related to the increased chance to infect with sexually transmitted pathogens including HR-HPV [3].

Many cross-sectional and longitudinal studies agree that the composition of the cervicovaginal microbiota has a key role not only in the acquisition of HR-HPV infection but also in infection persistence and surge of cervical cancer. Metabolic changes of the cervicovaginal microenvironment are determined mainly by vaginal microbiota composition and are strictly driven by community state types (CSTs). Apart the stage of cancer, there are no appreciable metabolic changes of vaginal environment not attributable to the microbial community state types [132]. Signs of genital inflammation as well as an increased catabolism of lipids are evident only in cancer stages and are typically considered as marker of neoplasia [127].

Despite the lack of specificity of metabolic changes in vaginal environment from CIN-1, CIN-2 lesions, data about the impact of cervicovaginal microbiota composition on the progression/regression of advanced lesions are quite consolidated [127].

We know that, among the *Lactobacilli*-dominated CST, CST-I with dominance of *L. crispatus* is the community most positively associated with the virus clearance and most negatively associated with the neoplastic progression of intraepithelial lesions, thus representing a predictive factor of the evolution of disease [116, 120, 143].

Several biases and discrepancies on the actual role of other vaginal *Lactobacilli*, primarily *L. iners* and *L. gasseri,* need to be elucidated yet. A non-negligible factor is represented by the cross-sectional nature of most of studies in this area, that do not allow to clearly trace a causal link between CST and stages of HPV disease. However, longitudinal studies [109, 144] clearly identified CST-IV or CST-III dominated by *L. iners* as cervico-types more associated to HR-HPV infection and cancer progression. Among CST-IV, *G. vaginalis, P. timonensis,* and *M. micronuciformis* may represent microbial markers of the disease's progression [145].

The aim of this study has been to investigate how each single bacterial species may affect factors involved in the neoplastic transformation of cervical epithelial cells, following HR-HPV infection. Thus, we studied the effects of common vaginal bacteria on the expression of viral oncogenic proteins known to neutralize key cellular oncosuppressors.

SiHa cells is a widely accepted experimental model of CIN-1, because has ~ 1,2 copies of integrated HPV-16 genome and well represents an infected cervicovaginal epithelium in the early stages of neoplastic transformation [139].

We obtained innovative data about the ability of some bacterial species to directly interfere on the expression of the viral oncogenes E6 and E7, as well as on the synthesis of the respective oncoproteins. As known from a long time, E6 and E7 viral gene expression is modulated by E2 gene. Lack of the E2 gene, usually occurring as consequence of viral integration in the host genome [69, 80] facilitates the expression of the viral oncogenes E6 and E7 and thus represents a crucial step for both the increase of the viral fitness and the neoplastic progression[69].

The products of the viral early genes, E6 and E7, play a key role in HPV persistence and in accumulation of chromosomal rearrangements [80]. Indeed, these proteins target peculiar cellular pathways involved in control of cell cycle and of apoptosis [68], increase the cellular proliferation as well as the cellular rate of mutation giving to the cells typical properties of a neoplastic cell [58].

The effects of *Gardnerella vaginalis* and *Megasphaera micronuciformis* on E6 and E7 gene expression and synthesis of the respective proteins define for the first time a direct role of vaginal bacteria in the neoplastic progression of the epithelium infected with HR-HPV. Moreover, unlike most other field studies that relate microbial communities only indirectly to neoplastic progression as a result of an inflammatory state, ours defines for the first time a direct role of the microbial communities in the neoplastic progression up to cervical cancer.and distinguish our study from most studies in the field that generally stress the direct role of microbial communities on inflammation and their indirect, inflammation-related, role in the neoplastic progression [58, 146].

The mechanisms involved in the induction of viral oncogenes by *G. vaginalis* and *M. micronuciformis* are not known yet. Our data show that live bacterial cells but also heat-inactivated bacteria and bacterial lysates significantly modulate the expression of viral oncogenes, while conditioned media of bacterial cultures were ineffective. Therefore, it is highly likely that metabolic products such as SCFAs are not involved in the phenomenon.

We observed differences in the induction of E6 and E7 genes and their products depending on the strain of *G. vaginalis*.

G. vaginalis strains have numerous genes encoding products that enhance the ability to compete with other vaginal bacteria and include toxin-antitoxin systems as well as antitoxins lacking cognate toxins [136]. Toxin–antitoxin (TA) [107] systems are composed of a toxin gene and its cognate antitoxin. Typically, TA systems act on crucial cellular processes including translation, replication, cytoskeleton formation, membrane integrity, and cell wall biosynthesis. The toxins of all characterized bacterial TA systems are proteins, while the antitoxins are either proteins or small RNAs (sRNAs) [147].

A comparative genomic analysis of the strains used in this work also suggested strain-related differences in toxin/antitoxin systems. Further experiments however are needed to assess whether the products of these systems may affect the physiology of HPV-

infected cervical epithelial cells or may activate the expression of histone-deacetylase complex (H-DAC) [148] or the production of specific transcriptional factors.

Although not all strains of *G. vaginalis* have the same properties to induce the expression of viral oncogenes and oncoproteins, our data strongly suggest that this species, together with *M. micronuciformis*, plays an important role in neoplastic transformation of HR-HPV infected cell.

In addition to viral oncogenes and their products, we found that *G. vaginalis score 5, score 8* and *score 10*, and *M. micronuciformis* also induce an increased expression of E6-associated protein (E6-AP). This protein is crucial in E6-related oncogenic functions since it functions as a ubiquitin protein ligase and binds to E6. The E6/E6-AP complex targets the *core dominus* of p53 inducing its ubiquitination and degradation [79, 80]. In our experimental assay, the functional activity of E6/E6-AP complex is suggested by data showing an opposite trend for E6 and p53. This indicates that E6 and E6/E6-AP complexes are present proportionally to p53 protein degradation.

In addition to the pattern E6/E6-AP/p53, we found two strains of *G. vaginalis (n.f.* and score 10) and *M. micronuciformis* may significantly affect the expression the E7 gene expression compared to unstimulated cultures. The expression of the E7 oncoprotein was not associated to gene expression, except for *G. vaginalis* score 10, suggesting again the involvement of post-transcriptional mechanisms of regulation. As for E6 gene, E7 protein was significantly induced by *G. vaginalis* strains, but not by *M. micronuciformis* suggesting the possibility that the two species may interfere with different activation pathways. Unfortunately, we could not be able at this time to associate the expression of bacterial induced E7 protein with Rb phosphorylation and degradation. As expected, total Rb protein expression is increased by *L. crispatus, L. gasseri, L. iners* as well as by strains of *G. vaginalis* (data not shown).

Overall, our results show *Lactobacilli,* and in particular *L. crispatus*, maintain or increase the expression of p53 and pRb by SiHa cells. In contrast, *G. vaginalis* strains or *M. Micronuciformis* significantly induces the degradation of p53.

According with these results, we observed the highest percentage of cells entering in S-phase in SiHa cells cultured with *M. Micronuciformis* or with selected strains of *G. vaginalis.*

Since the coordinate inactivation of p53 tumor suppressor pathways facilitates cellular transformation [149] we can propose the search of these species as biomarkers for neoplastic progression in women with CIN-1/CIN-2 lesions.

Further investigations are required to define the bacterial factors involved in oncogenic pathways. The genomic sequence of many strains isolated from vaginal fluid of women with CIN-1 and CIN-2 would greatly help to identify strain-specific factors that associate to the neoplastic progression of HR-HPV infected women, whose role need to be investigated in *in vitro* systems.

The activation of oncogene expression by *G. vaginalis* and *M. micronuciformis* represents a direct interference of bacterial cells in the oncogenic process. However, our data revealed that these bacteria strongly increase the expression of MMP-9. Strains of *G. vaginalis*, isolated from vaginal fluids with high inflammatory score, shown the strongest activity of this protein.

As reported in the introduction, MMP-9 is a metallo-proteinase that has been repeatedly associated with the progression of cervical cancer [96, 97]. MMP-9 degrades many proteins of basement membrane of epithelial cells, affects cell-cell and cell-ECM interaction through the cleavage of surface proteins and of extracellular environment. In this way, MMP-9 facilitates the *in-situ* progression of cancerous cells as well as their neoplastic dissemination [95, 98].

Finally, our study considered some metabolic aspects of SiHa cells exposed to products from bacteria of vaginal microbiota CSTs. In agree with Salliss *et al* [145], our data show that soluble factors, present in culture media of *G. vaginalis, A. vaginae,* and *M. micronuciformis* affect the viability of the cells even at low concentrations. Since this phenomenon is not evident with cell-associated bacterial products the responsivity of secreted, soluble metabolic products, such as SCFAs, is strongly suggested.

Lactobacillus-dominated CST produce lower amounts of pro-inflammatory acetate and high amounts of the anti-inflammatory butyric and valeric acid [107], metabolites that together with lactic acid are endowed with anti-inflammatory activity [7].

This metabolic assessment is completely reverted in culture of vaginal dysbiosis bacteria suggesting that the abundance of pro-inflammatory products can have repercussions on vaginal homeostasis, epithelial cell trophism and the integrity of epithelial cell barrier. Although indirectly all these phenomena can affect the process of neoplastic transformation occurring in the infected epithelial basal layers:

- Perturbation of epithelial barrier facilitates the entry of pathogens that promote inflammation by recalling neutrophils/macrophages and inducing autophagy of infected cells. The tissue reparative processes may have repercussions on the proliferation of epithelial cells.

- *Lactobacillus*-dominated CST and vaginal dysbiosis bacteria induce different adaptive immune response.

A recent paper of our group [107] showed that *Lactobacilli*, and especially *L. gasseri* and *L. jensenii*, induce a marked T_H1 response in lymphocytes from peripheral blood mononuclear cells and are supposed to better assist immune cells with clearing HPV infection, bypasses the viral escape and restores immune homeostasis. In contrast, vaginal dysbiosis bacteria induces the differentiation of high pro-inflammatory T_H1/T_H17 clones that are not efficient in supporting viral clearance and strongly contribute to

inflammation, a condition repeatedly associated with the surge and the progression of cervical cancer [150, 151, 152].

The experimental model used in this work may represent a weak point, since in most experiments we observed the effects of a direct interaction between live bacterial cells and infected epithelial cells. However, data in literature report that *Lactobacilli* and *G. vaginalis* directly interact with epithelial cells bypassing the vaginal mucous layer [153] [128]. The culture medium (DMEM) used for SiHa cells does not contain many nutrients necessary for bacterial growth *in vitro*. Moreover, most of the bacteria used are strictly anaerobes. Thus, these experimental conditions represent a disadvantage for bacterial cells. Nevertheless, they are much closer to the real conditions of the cervicovaginal microenvironment.

Based on our experimental data we can affirm that:

- *L. crispatus* is confirmed as the most protective species against neoplastic progression. Indeed, SiHa cells exposed to *L. crispatus* neither increase the expression of viral oncoproteins or related ubiquitin-ligase (E6-AP), nor decrease the expression of cellular tumor suppressor (p53), nor an increase of inactive form of pRb oncosuppressor. Accordingly, *L. crispatus* does not affect any phase of cellular cycle

- *L. gasseri* induces effects largely superposable to that of *L. crispatus*.

- *L. jensenii* and *L. iners* can induce or modulate one or more processes involved in oncogenesis but, unlike strains of *G. vaginalis*, do not have unidirectional effects on the observed processes. Indeed, *L. iners* and *L. jensenii* induce the expression of the oncoprotein E7 but not that of E6, and at the same time they provide to increase the pool of pRb and p53. Accordingly, vaginal *Lactobacilli* do not affect the cell cycle.

- *G. vaginalis* and *M. micronuciformis* increase the production of viral oncoprotein, affect the expression of cellular oncosuppressors, and induce more cells to entry in the S-phase of cell cycle.

- Our data show that bacterial taxa act on oncogenic process either directly on the infected epithelial cells either indirectly by promoting inflammation and matrix degradation. Inflammation may be induced by bacterial metabolic products [145], as consequence of epithelial barrier damage and entry of dangerous pathogens that can influence the neoplastic transformation and, finally, by inducing MMP-9 synthesis with consequent dissemination of neoplastic cells.

  We think that our data provides an experimental basis for the results obtained in prospective studies that have identified *G. vaginalis* and *M. micronuciformis* as the most represented species in women with CIN-2/-3 that do not regress [4, 120].

  Although we need more in-depth studies to define the molecular mechanisms underlying each observed effect, our data strongly suggest considering the presence of *M. micronuciformis* (and perhaps other members of *Veillonacee* family) and *G. vaginalis* as microbial marker associated to the neoplastic progression of HR-HPV transformed epithelium.

  The presence of *G. vaginalis* or *M. micronuciformis* in the vaginal fluid of women with persistent HR-HPV infection should direct this group to a close follow-up. The administration of probiotics and prebiotics before the surge of intraepithelial lesions might be taken in consideration.

  Most of all, the presence of specific microbial markers can strongly influence the medical choice to extend the observation period of CIN-1/-2 lesions (in women with *Lactobacillus*-dominated microbiota) or to decide for surgical intervention (in women with vaginal dysbiosis).

6. Conclusions

In conclusion, many studies have shown the association between CST-IV and persistent infection and/or tumor progression. Our study is the first that examines the direct effect of many of the dominant species of the vaginal bacterial microbiota on cervical cells transformed by HPV-16.

Our data suggest that *G. vaginalis* and *M. micronuciformis* are likely involved in the neoplastic transformation of cervical epithelial cells. They also suggest that a more detailed analysis of bacterial strains, isolated from vaginal fluids of HPV^{+} women with early cervical lesions, may help to identify the factor/s able to affect the oncogenic process.

A weak point of the study is that not all the bacterial species described in the vaginal microbiota have been studied. Therefore, the study panel should be extended to other species such as *Prevotella (bivia* and *timonensis)*, *Atopobium* and *Sneathia* and in the same way the intraspecies genomic diversity of *G. vaginalis* must be considered.

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Additional section

During the PhD course, collaborations with other research groups and collateral projects have made it possible to produce the following attached works:

International Journal of
Molecular Sciences

MDPI

Article

Vaginal Lactobacilli and Vaginal Dysbiosis-Associated Bacteria Differently Affect Cervical Epithelial and Immune Homeostasis and Anti-Viral Defenses

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Abstract: Persistent infection with High Risk-Human Papilloma Viruses (HR-HPVs) is a primary cause of cervical cancer worldwide. Vaginal-dysbiosis-associated bacteria were correlated with the persistence of HR-HPVs infection and with increased cancer risk. We obtained strains of the most represented bacterial species in vaginal microbiota and evaluated their effects on the survival of cervical epithelial cells and immune homeostasis. The contribution of each species to supporting the antiviral response was also studied. Epithelial cell viability was affected by culture supernatants of most vaginal-dysbiosis bacteria, whereas *Lactobacillus gasseri* or *Lactobacillus jensenii* resulted in the best stimulus to induce interferon-γ (IFN-γ) production by human mononuclear cells from peripheral blood (PBMCs). Although vaginal-dysbiosis-associated bacteria induced the IFN-γ production, they were also optimal stimuli to interleukin-17 (IL-17) production. A positive correlation between IL-17 and IFN-γ secretion was observed in cultures of PBMCs with all vaginal-dysbiosis-associated bacteria suggesting that the adaptive immune response induced by these strains is not dominated by T_H1 differentiation with reduced availability of IFN-γ, cytokine most effective in supporting virus clearance. Based on these results, we suggest that a vaginal microbiota dominated by lactobacilli, especially by *L. gasseri* or *L. jensenii*, may be able to assist immune cells with clearing HPV infection, bypasses the viral escape and restores immune homeostasis.

Keywords: vaginal microbiota; *Lactobacillus*; *Gardnerella vaginalis*; IFN-γ; IL-17

1. Introduction

Microorganisms of the "cervico-vaginal microbiota" establish mutual relationships with the host and strongly contribute to defending the mucosal barrier against the invasion of sexually transmitted pathogens, including the Human Papilloma Virus (HPV). *Lactobacillus* species, evolutionally selected in the vaginal environment, are mainly represented by *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus iners* and *Lactobacillus jensenii* and contribute to the host's defenses against invading pathogens by lowering the pH (through the production of lactic acid) and secreting huge amounts of antimicrobial peptides (AMPs) [1]. *Lactobacillus* species dominate the vaginal microbiota in a large majority of women and define specific vaginal microbial communities [2,3]. In detail, *L. crispatus* is representative of community state type (CST)-I, *L. gasseri* of CST-II, *L. iners* of CST-III

and *L. jensenii* of CST-V, respectively [2–4]. Loss of the *Lactobacillus* dominance and the colonization by anaerobic and aerobic species define the CST-IV vaginal microbiota. Strains of the genera *Gardnerella*, *Atopobium*, *Prevotella*, *Megasphaera*, *Mobiluncus*, *Streptococcus* and *Ureaplasma* are highly represented in CST-IV, with *Gardnerella vaginalis* usually representing the dominant species. This bacterial community produces lower amounts of AMPs and lactic acid and represents a condition of vaginal dysbiosis highly associated with bacterial vaginosis (BV), the most common bacterial infection of the lower female genital tract [5–7].

Vaginal-dysbiosis-associated bacteria often produce mucin-degrading enzymes [3,8] and induce a pro-inflammatory response [8] with impairment of mucosal barrier that facilitates invasion by sexually transmitted pathogens, including high-risk (HR) Human Papilloma Virus (HPV) [3,4]. Persistent infection with HR-HPVs occurs in 10% of infected women and is a primary cause of cervical cancer worldwide [9].

The antiviral-specific immune response is crucial to the eradication of HPV infection and requires the cooperation of $CD4^+$ T-helper (T_H) and cytotoxic $CD8^+$ T cells (CTLs) [10]. In fact, the high levels of IFN-γ secreted by T_H1 cells potentiate the cytotoxic activity of CTLs that specifically recognize and kill cells expressing viral antigens linked to MHC I molecules [11]. Clinical studies confirmed the strong association between the T_H1 pattern and the clearance of HR-HPV infection [12]. In contrast, IL-17 has been shown to suppress the effectors of the immune response in HPV-associated diseases [13], and the role of T_H17 and IL-17 seems more crucial in immune enhancement and disease progression but not in the eradication of HR-HPV infection [14].

HR-HPVs evolved different mechanisms of escape from host adaptive response, secreting lower amounts of proteins and manipulating the antigen processing machinery [15]. Despite this, infection clearance is not a rare event and is often associated with the composition of vaginal microbiota [3,16].

Vaginal microbiota composition is essential in preserving the integrity of the cervical epithelium and the functions of the cervical barrier against the invasion of sexually transmitted pathogens [3]. The production of lactate by *Lactobacillus* species maintains the vaginal pH at 3.5–4.5, preventing the overgrowth of opportunistic pathogens and maintaining low concentrations of Short Chain Fatty Acids (SCFAs). Differently than the gastrointestinal tract, an increase in vaginal SCFAs and a concomitant decrease in lactate is always a marker of dysbiosis, a condition that allows pathogens to propagate ascending intrauterine infection with adverse reproductive outcomes, including preterm birth [17]. Due to the high concentration of lactate, the concentration of SCFAs is usually low in *Lactobacillus*-dominated microbiota. An increase in vaginal SCFAs is always a marker of dysbiosis, a condition that allows infections by pathogenic microorganisms [1].

Vaginal bacteria also regulate the functions of antigen-presenting cells (APCs) and the activation of memory T_H1, T_H17 and regulatory T (T_{reg}) lymphocytes in the submucosal compartment [18]. Activation of these cells may result in high concentrations of IFN-γ, IL-17 and IL-10 that can support or repress the host's specific response against invading pathogens, including HR-HPV [12,13]. Vaginal-dysbiosis-associated bacteria were repeatedly linked with persistent HR-HPV infection and with cervical cancer [19,20]. However, the contribution of each species in epithelial cell damage and the antiviral response has not been fully defined.

Here, we selected strains of bacterial species representative of each vaginal CST and evaluated their effects on the production of inflammatory cytokines by HPV-transformed cervical epithelial cells and peripheral blood mononuclear cells (PBMCs) from healthy donors.

2. Results

2.1. Bacterial Strains and Analysis of Short Chain Fatty Acids (SCFAs) Production

Strains of *L. crispatus*, *L. gasseri*, *L. iners* and *L. jensenii* were used as representatives of CST-I, II, III and V, respectively. *G. vaginalis*, *Atopobium vaginae*, *Megasphaera micronuciformis* and *Prevotella bivia* were used as representatives of CST-IV. Table 1 shows the related features of the used strains.

Table 1. Reference bacterial strains.

CST	Family and Genus	Specie	Strain's Name
I	*Lactobacillaceae*, *Lactobacillus*	*L. crispatus*	JV-V01
II	*Lactobacillaceae*, *Lactobacillus*	*L. gasseri*	SV-16A
III	*Lactobacillaceae*, *Lactobacillus*	*L. iners*	UPII-60-B
V	*Lactobacillaceae*, *Lactobacillus*	*L. jenseni*	JV-V16
IV	*Bifidobacteriaceae*, *Gardnerella*	*G. vaginalis*	315-A
IV	*Bifidobacteriaceae*, *Gardnerella*	*G. vaginalis*	49145/JCP-7276
IV	*Bifidobacteriaceae*, *Gardnerella*	*G. vaginalis*	14019/JCP-7659
IV	*Bifidobacteriaceae*, *Gardnerella*	*G. vaginalis*	14018/JCP-7275
IV	*Atopobiaceae*, *Atopobium*	*A. vaginalis*	DSM-15829
IV	*Prevotellaceae*, *Prevotella*	*P. bivia*	DNF-00188
IV	*Prevotellaceae*, *Prevotella*	*P. bivia*	DNF-00650
IV	*Veillonellaceae*, *Megasphaera*	*M. micronuciformis*	DNF-00954

SCFAs have been reported to affect epithelial cell metabolism and innate inflammatory response [20,21]. Acetate, propionate, butyrate and isovalerate are usually found in the vaginal environment [22]. As the first step of our study, we assessed the production of SCFAs in culture supernatants of all bacterial strains by gas-chromatography coupled with a mass spectrometry (GC-MS) system.

Acetic acid turned out to be the dominant SCFA produced by all vaginal bacteria. Figure 1A shows that all vaginal lactobacilli produced a lower acetic acid amount than *G. vaginalis* that usually represents the dominant species of vaginal dysbiosis.

On the other hand, vaginal lactobacilli produced butyric and valeric acid amounts higher than *G. vaginalis* strains. Figure 1B shows that the metabolic profile of *A. vaginae* and *P. bivia* is largely superimposable to that of *G. vaginalis*. In contrast, *M. micronuciformis* produced low amounts of either acetic and butyric acid, and its metabolic profile was intermediate between lactobacilli and *G. vaginalis* strains.

2.2. Effects of Supernatants and Bacterial Lysates on Viability of Cervical Epithelial Cell Lines

The cervical epithelial cell lines SiHa and CaSki were cultured in the presence or absence of different amounts of bacterial culture supernatants or bacterial lysates for 24 h. Intracellular ATP was measured as an index of metabolically active cells.

Figure 2A shows that supernatants from all lactobacilli cultures induced a significant increase in ATP production by SiHa cells. In contrast, supernatants from most vaginal dysbiosis bacteria affected cell viability in a dose-dependent manner. The greatest decrease in cell viability was observed in cultures with supernatants from *A. vaginae* culture. Lysates from vaginal dysbiosis bacteria and from *L. iners* induced a decrease in the viability of SiHa cells (20–50%).

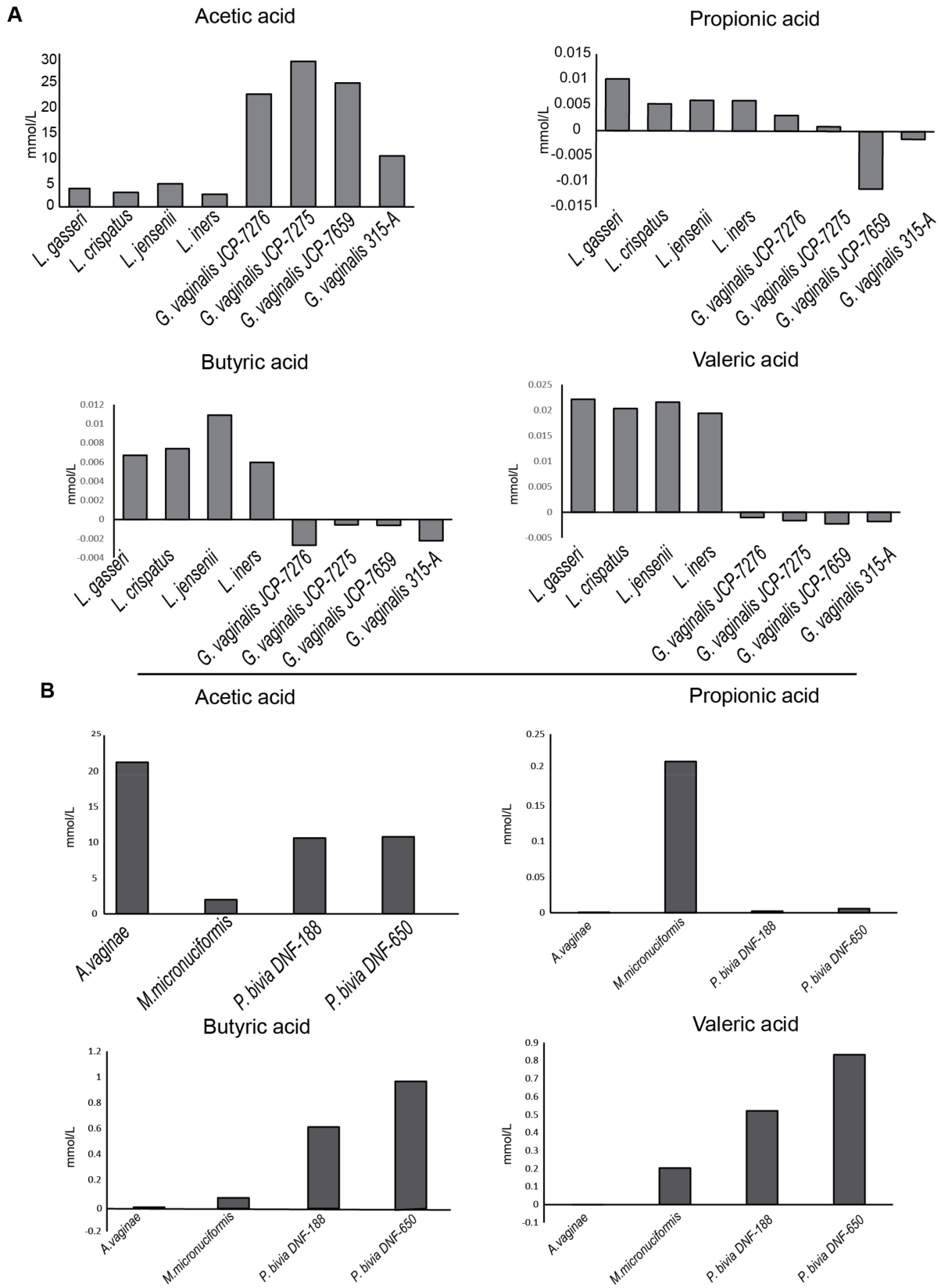

Figure 1. Short Chain Fatty Acids (SCFAs) profile. Supernatants of (**A**) *Lactobacillus* spp., *Gardnerella* spp. and (**B**) other vaginal dysbiosis bacteria were analyzed to the qualitative and quantitative determination of acetic, propionic, butyric and valeric acids using gas-chromatography coupled with mass spectrometry (GC-MS) system. The quantitative SCFAs' evaluation was carried out by ratios between the area abundances of the analytes with the area abundances of respective labelled internal standards (isotopic dilution method).

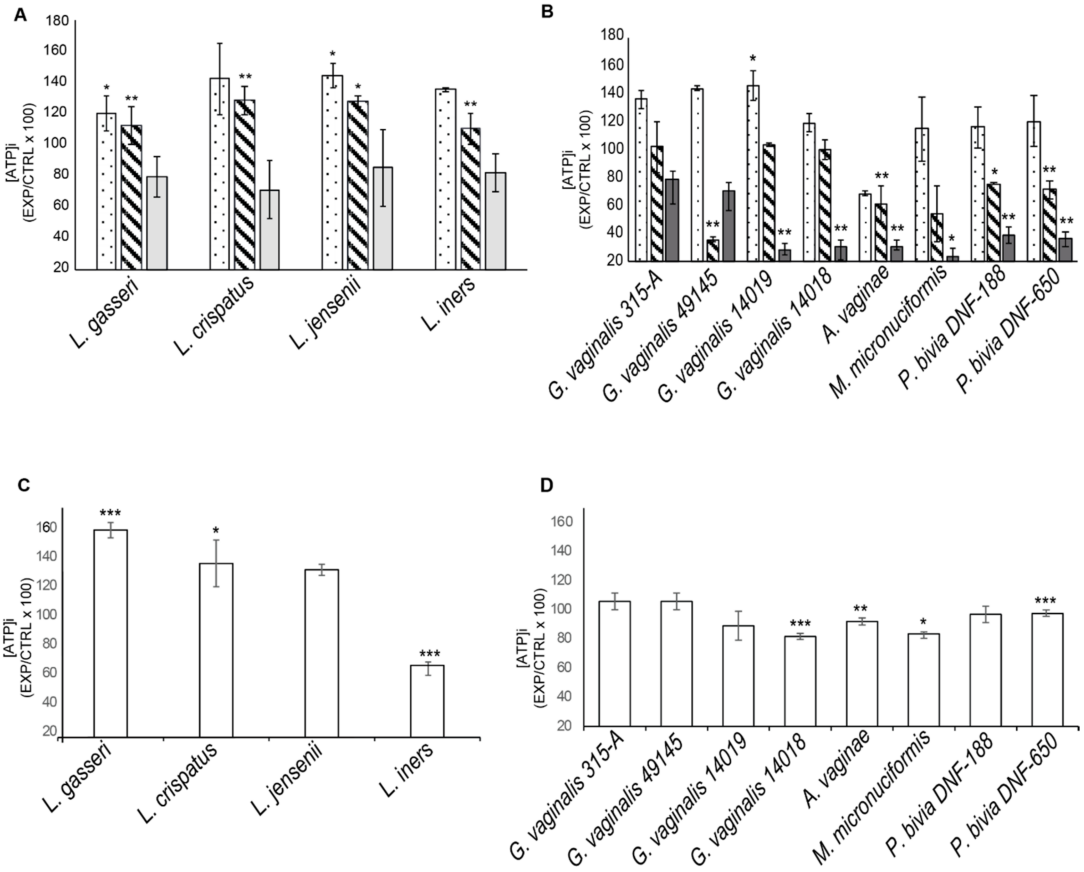

Figure 2. Effects of bacterial products on SiHa cell viability. (**A**,**B**) ATP production by cells were cultured with 10% (dotted-bar), 20% (ruled-bar), 30% (grey-bar) (*v/v*) of bacterial supernatants for 24 h. (**C**,**D**) ATP production by cells cultured with bacterial lysates (1 µg/mL) for 24 h. The bar-graph shows results from one representative experiment out of three performed. Data are expressed as ATP produced by stimulated cultures/unstimulated control ± standard deviation (EXP/CTRL × 100). Statistical analysis was performed by ANOVA and Student t-test. Significant differences among stimulated/ unstimulated cultures were reported * p-value < 0.05; ** p-value < 0.01; *** p-value < 0.001.

The effects of bacterial supernatants on CaSki cell viability were comparable to those induced in SiHa cells (data not shown). Bacterial lysates from *G. vaginalis*, *P. bivia* and from *L. jensenii* significantly affected CaSki cell viability (Supplementary Figure S1).

The whole data suggest that most vaginal lactobacilli best support the viability and metabolic activity of cervical epithelial cells. In contrast, vaginal dysbiosis bacteria produce factors that affect the viability of epithelial cells and potentially compromise the cervical barrier.

2.3. Cytokine's Production by CaSki and SiHa Cells Cultured with Vaginal Bacteria

SiHa and CaSki cells were cultured with heat-inactivated bacteria (50 MOI/cell) from each species representative of the vaginal microbiota, and the concentration of TNF-α, IL-1β and IL-8 was assessed after 5 days of stimulation. The data obtained have shown that no significant increase in these cytokines compared to unstimulated control was evident in bacterial stimulated cultures (Supplementary Table S1). We noted, however, that, despite the huge amounts of IL-8 that were spontaneously produced by CaSki cells, vaginal dysbiosis bacteria slightly increase the production of this cytokine, while lactobacilli always led to a decrease (12–30%) (Supplementary Table S1).

2.4. Cytokine's Production by PBMCs Stimulated with Dominant Species of Vaginal Microbiota

As T_H1 differentiation and IFN-γ production are essential to eradicate viral infections [12], we assessed whether vaginal *Lactobacillus* species induce the production of IFN-γ differently than vaginal dysbiosis bacteria. We cultured PBMCs from healthy donors ($n = 9$) with heat-inactivated bacteria (50 MOI/cell) from all strains reported in Table 1, and cytokine concentration was assessed in supernatants after 5 days of culture. In addition to IFN-γ, the production of IL-4, IL-17, IL-10, IL-6, IFN-γ and IP-10 was analyzed. Figure 3 shows that almost all species of vaginal microbiota were able to induce the production of a significant amount of IFN-γ, IL-17, IL-6 and IL-10 compared to unstimulated controls. In contrast, they did not induce significant production of IFN-α, IP-10 and IL-4 (Supplementary Figure S2).

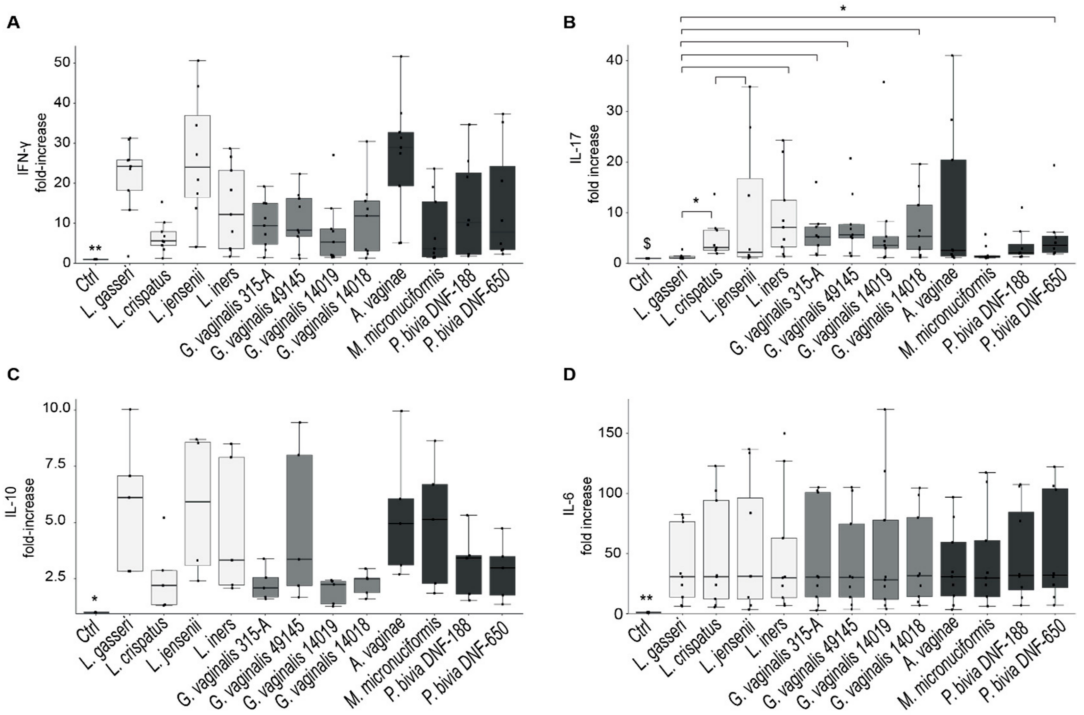

Figure 3. Cytokine's production by PBMCs stimulated with dominant species of the vaginal microbiota. PBMCs were stimulated with 50 MOI/cell of heat-inactivated bacteria for 5 days. (**A**) IFN-γ, (**B**) IL-17, (**C**) IL-10 and (**D**) IL-6 concentration was measured in culture supernatants of bacterial-stimulated cultures. Results are expressed as a fold increase in cytokine concentration with respect to unstimulated cultures. The bar graph shows the median, and the whisker is calculated on the formula IQR × 1.5. Differences in cytokine concentrations among cultures were evaluated by Kruskal–Wallis test with Holm–Bonferroni p-value adjustment. $^{\$}$ *p*-value referred to unstimulated cultures; * $p < 0.05$; ** $p < 0.02$.

Among lactobacilli, *L. gasseri* was the species that induced the highest amounts of IFN-γ and the lowest amounts of IL-17; *L. iners*, in contrast, was an optimal stimulus for either IFN-γ and IL-17 production.

G. vaginalis strains induced more IL-17 production compared to lactobacilli, and significant differences emerged, especially from the amounts induced by *G. vaginalis* strains and those induced by *L. gasseri*.

The anti-inflammatory cytokine IL-10 was significantly induced by all bacterial strains compared to unstimulated control, and in particular, *Lactobacillus* species induced amounts of IL-10 highest than *G. vaginalis* or *P. bivia*. However, statistical analysis did not reveal significant differences among the bacterial-stimulated cultures.

To further confirm the differences in cytokine's production between lactobacilli and vaginal dysbiosis bacteria, we obtained pools of bacterial lysates from (1) *Lactobacillus* species (*L. crispatus*, *L. jensenii*, *L. gasseri* and *L. iners*); (2) *G. vaginalis* strains (315-A, 49145, 14019 and 14018); (3) other vaginal dysbiosis bacteria (*A. vaginae*, *P. bivia* (DNF-188, DNF-

650) and *M. micronuciformis*) and used them to stimulate PBMCs as reported above. Culture supernatants were collected after 5 days of stimulation, and the production of IFN-γ, IL-17 and IL-10 was assessed.

Figure 4 shows that IFN-γ production induced by vaginal lactobacilli was significantly higher than those induced by *G. vaginalis*. In contrast, *G. vaginalis* promoted higher production of IL-17 and lower amounts of IL-10 compared to lactobacilli as well as to other species of vaginal dysbiosis.

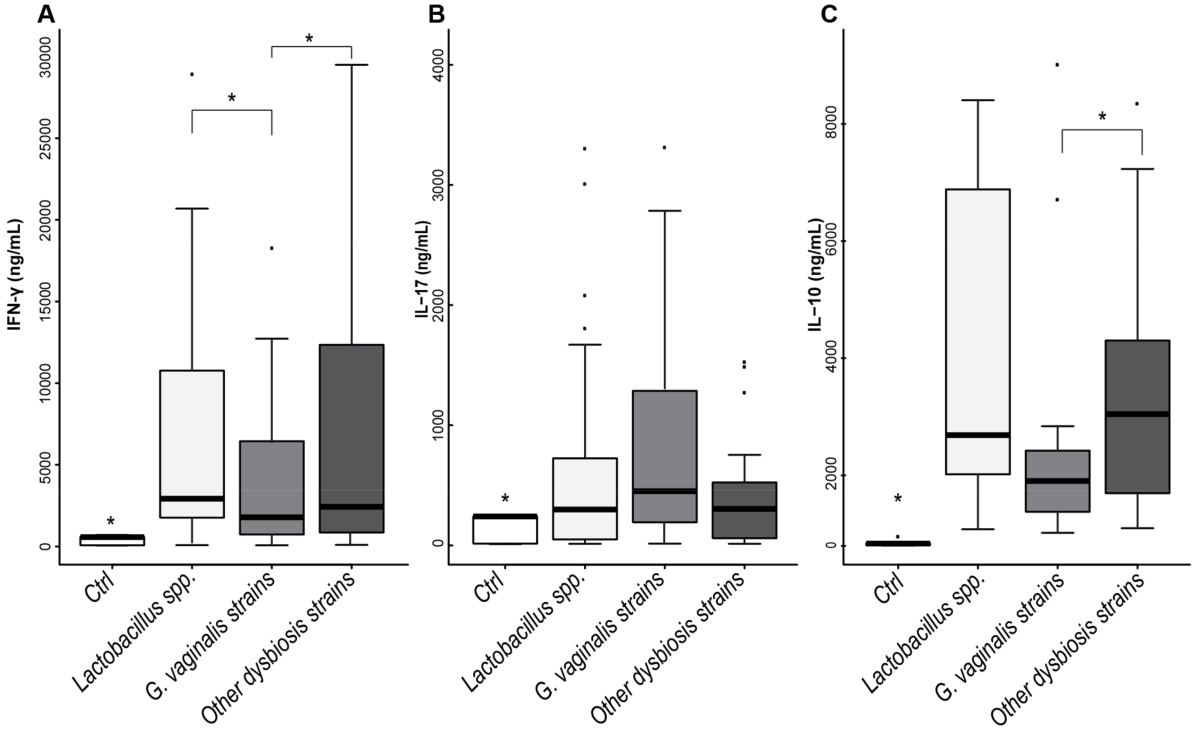

Figure 4. Cytokine production induced by lactobacilli or vaginal dysbiosis bacteria. Pooled bacterial lysates obtained by (1) *Lactobacillus* strains (*L. crispatus*, *L. jensenii*, *L. gasseri* and *L. iners*), (2) *G. vaginalis* strains (315-A, 49145, 14019 and 14018); (3) others vaginal dysbiosis bacteria (*A. vaginae*, *M. micronuciformis*, *P. bivia* DNF-188 and *P. bivia* DNF-650) were used as a stimulus for PBMCs ($n = 9$). (**A**) IFN-γ, (**B**) IL-17 and (**C**) IL-10 concentration was measured in culture supernatants of bacterial-stimulated cultures. The bar graph shows the median, and the whisker is calculated on the formula IQR × 1.5. Statistical analysis was performed by Kruskal–Wallis test, * $p \leq 0.01$. Bonferroni p-value adjustment was used for IFN-γ and IL-10. Steel p-value adjustment was used for IL-17. Student t-test paired was used for IFN-γ and IL-10, $p < 0.02$.

Finally, Spearman's correlation analysis among the cytokine levels in cultures with vaginal dysbiosis bacteria revealed a significant correlation between the production of IFN-γ and that of IL-17 (Figure 5 and Supplementary Figure S3). Notably, these data suggest that vaginal dysbiosis bacteria may compromise the anti-viral T_H1 response by increasing the differentiation of an increased number of T_H17 cells and IL-17 concentration in the vaginal environment.

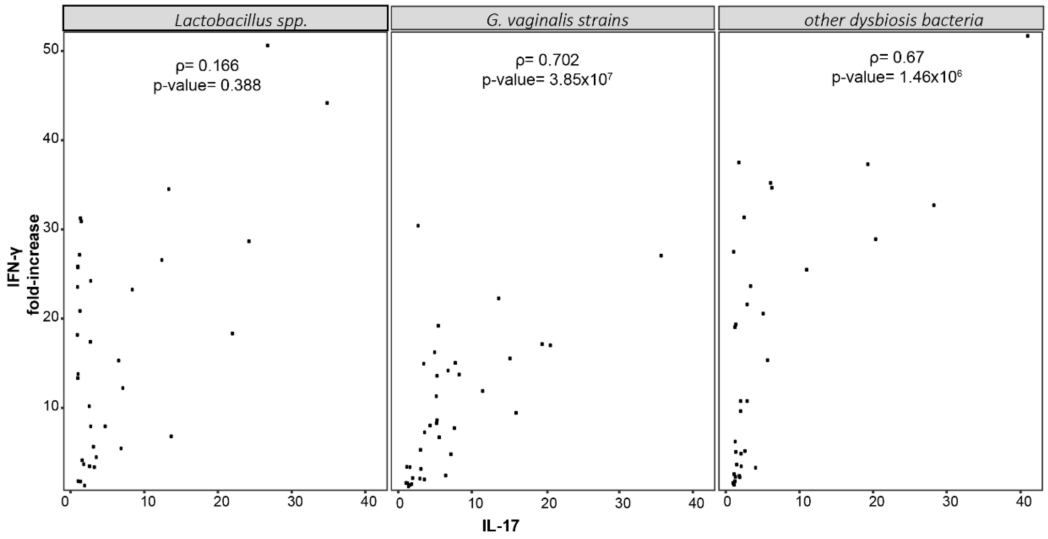

Figure 5. Spearman's correlation analysis among cytokines produced in cultures with *Lactobacillus* spp., *G. vaginalis* strains, other vaginal dysbiosis bacteria. Correlation among cytokines produced under different stimulation was evaluated by Spearman rank correlation analysis.

3. Discussion

Epithelial and immune homeostasis in the vaginal microenvironment is crucial for host defenses against sexually transmitted pathogens [21–25]. Microbial and the host metabolites in the host microenvironment may affect the course of sexually transmitted infections [26].

We observed that products from most vaginal-dysbiosis-bacteria affected the cell viability of cervical epithelial cells in a dose-dependent manner while products from all lactobacilli (either secreted or not secreted) are able to maintain or increase cell viability. A single exception was observed with lysates from *L. iners* that induced a decrease in the viability of SiHa cells. Although we are aware that in vitro culture of cervico-vaginal epithelial cells is not representative of the complexity of the vaginal microenvironment, our data support the hypothesis that vaginal dysbiosis bacteria and, to a lesser extent, *L. iners* compromise the cervical epithelial barrier. Similar data were reported by Anton L. et al. [27], Randis T. et al. [28] and Lopez-Moreno et al. [29].

Following bacterial stimulation, the cervical epithelial cells produce IL-8, a potent chemoattractant and activator of polymorphonuclear leukocytes [30,31]. Huge amounts of this chemokine are spontaneously produced by HPV-transformed cervical epithelial cell lines [32]. We found that *L. crispatus* and *L. gasseri* were able to negatively interfere with the molecular pathways leading to the high constitutive production of IL-8. In contrast, the *G. vaginalis* increased chemokine production.

The high production of lactate and low production of SCFAs by lactobacilli could be responsible for the modulation of pro-inflammatory properties of cervicovaginal epithelial cells [17].

In addition, the results from the SCFAs profile, performed in supernatants from bacterial cultures, show that *Lactobacillus* species produced a much lower amount of the pro-inflammatory acetic acid compared to all vaginal-dysbiosis-associated bacteria. In contrast, and according to other reports, lactobacilli produced higher amounts of butyric and valeric acid, metabolites with anti-inflammatory activity [33].

A metabolomic analysis performed on vaginal samples from HPV+ and HPV- women ascertained that the metabolome of vaginal-dysbiosis-bacteria clustered differently from *Lactobacillus*-dominated microbiota [26].

To summarize, our data are in accordance with previous reports showing that most of the vaginal dysbiosis bacteria affect the viability and the inflammatory properties of

cervical epithelial cells [34,35] and may therefore contribute to increasing the risk of sexually transmitted viral infections, including HR-HPV infection.

HR-HPVs are spontaneously cleared by the immune response in most infections, but viral persistence occurs in 10% of infected women and may induce carcinogenesis [9,36]. Impairment of the vaginal epithelial barrier, chronic inflammation, alterations of the metabolic signaling and of the immune response are all involved in carcinogenesis [37].

The clearance of HPV infections is associated with an optimal level of IFN-γ produced by the T_H cells [38]. The importance of immune homeostasis is highlighted by immunosuppressed women who display the increase in the incidence and persistence of HR-HPVs infections [39]. Moreover, prolonged inflammatory response and high secretion of exosomes in the vaginal environment may promote the progress of intraepithelial lesions [37].

The vaginal microbiota compositions affect the rate of infection as well as its outcome, and the CST-IV microbiota profile, dominated by common vaginal dysbiosis bacteria, has emerged as a risk factor for persistent HPV infection [3,16]. However, women who recovered from HPV infection show a significant reduction of CST-IV and an increase in CST-I compared to the time of recruitment [40]. On the other hand, *L. gasseri* (CST-II) has been associated with the complete clearance of the virus [41]. Although the number of enrolled patients was rather limited in both reports, these data suggest that vaginal *Lactobacillus* species do not have comparable protective effects against HR-HPV infections.

In addition, it is not known whether and how vaginal bacteria affect the adaptive immune response to HR-HPV infection and so the viral clearance.

In fact, in literature, most of the studies are focused on the pro-inflammatory or immunomodulating activity induced by vaginal bacteria interacting with cervical epithelial cells or with cells of the innate immunity [34,35]. These important studies have established a significant correlation between vaginal dysbiosis bacteria (CST-IV) and the progression of HPV infections to preneoplastic (CIN1/2) or neoplastic (CIN 3) stages [19,33].

The connection between the persistence of infection and the neoplastic progression was represented by the inflammatory potential of vaginal bacteria [42,43]. Bacterial species of genera *Ureaplasma*, *Atopobium*, *Prevotella*, *Gardnerella*, *Sneathia*, and especially, *Fusobacteria* have been associated with an increased oncogenic risk [41,44]. Among lactobacilli, *L. iners* can promote the progression of infection [45,46].

A more in-depth investigation of the relationship between vaginal bacteria, immune response and persistent infection and, in detail, how some vaginal bacteria can affect the adaptive immune response was performed by van Teijlingen et al. [47]. The authors studied the effect of two species often reported in vaginal dysbiosis CST-IV, *Megasphaera eldsenii* and *Prevotella timonensis*, on the activation of dendritic cells (DCs) and compared their effects with those induced by *L. crispatus*. They found that *P. timonensis* induces a strong T_H1 response while *L. crispatus* and *M. elsdenii* did not affect T_H polarization. A different study reports that *L. crispatus* confers an anti-inflammatory phenotype to DCs through up-regulation of anti-inflammatory/regulatory IL-10 cytokine production and induction of T_{regs} at optimal dosage [48]. Finally, Mitra et al. reported that the depletion of *Lactobacillus* species and the presence of anaerobic taxa of genus *Gardnerella*, *Megasphaera* and *Prevotella* are associated with persistence and slower regression of CIN2 lesions [49].

Previous studies observed that the production of IFN-γ following HPV 16 peptide stimulation is higher in recovered or HPV-negative women compared to those identified in cervical intraepithelial lesions (CIN) [50]. Ondondo and co-workers recently reported that men with HPV clearance had significantly higher IFN-γ levels than those with persistent HPV infection [51]. These data underline the relevance of T_H1 cell-mediated cytokine response in HPV clearance, but they do not define the role of each bacterial species in supporting the antiviral response.

Our data show that all vaginal bacteria induce the production of IFN-γ, with *L. gasseri* being the best inducer of the cytokine. The differences among *Lactobacillus* and vaginal dysbiosis species mostly reside in the bacteria's ability to stimulate T_H17 differentiation

and the production of IL-17 at the same time. In this scenario, *G. vaginalis* strains induce the production of greater amounts of IL-17 compared to lactobacilli suggesting that the adaptive immune response induced by these bacteria is not dominated by T_H1 differentiation and that the combined effects of lower IFN- γ availability and higher IL-17 concentration does not appropriately support the specific antiviral response. In support of this concept, a significant correlation between the production of IFN-γ and IL-17 emerged for all CST-IV species but not for lactobacilli. This suggests that T_H17 differentiation is induced as well as that of T_H1 cells and potentially compromises the antiviral response, which does not benefit from T_H17 effectors [12–14,52]. Moreover, IL-17 may be crucial in immune enhancement and disease progression.

In accordance with our results, Gosmann et al. observed an increase in the numbers of activated mucosal T_H cells in the concentrations of IL-17 and IL-17-inducing cytokines (IL-23 and IL-1β) in the cervicovaginal lavage obtained from women with CST-IV dominated microbiota [13].

4. Materials and Methods

4.1. Bacterial Strains

A collection of 12 bacterial reference strains were included in the study, and related features are reported in Table 1. *L. crispatus* (JV-V01), *L. gasseri* (SV-16A), *L. iners* (UPII-60-B) and *L. jensenii* (JV-V16) were used as representative of CST-I, II, III and V, respectively. *G. vaginalis* was selected as representative of CST-IV and four strains isolated respectively from healthy women (315-A) or from women with bacterial vaginosis with Nugent Score 5 (49145/JCP-7276), 8 (14019/JCP-7659), 10 (14018/JCP-7275) were selected. *A. vaginae* (DSM-15829), *M. micronuciformis* (DNF00954) and *P. bivia* (DNF 00188 and DNF-00650) were also used as representative of CST-IV.

4.2. Bacterial Cultures

Anaerobic bacteria were grown in Tryptic Soy Agar (TSA), composed by Tryptic Soy Broth (Oxoid, Basingstoke, UK) and 15mg/L of Bacto-Agar (Sigma Aldrich, St. Louis, MO, USA), with the addition of 5% Horse lysed whole-blood (Oxoid, Basingstoke, UK). The plates were incubated at 37 °C for 72 h in anaerobic conditions inside a jar (AnaeroGen™, Thermo Fisher Scientific, Waltham, MA, USA) to create ideal growth conditions (CO_2: 9–13.0%).

Bacterial strains were also grown in liquid cultures using TSB with 5% horse lysed blood (Oxoid, Basingstoke, UK). The turbidity of the culture broth of each individual strain was measured by using the DensiCHECK densitometer after centrifuging 1 mL of culture at 4000× *g* for 5 min and resuspending the pellet in 1 mL of physiological solution.

Bacterial concentration was calculated according to the following formula:

$$[bacterial] = [McFarland * 1.5/0.5] * 10^8;$$

Optical Density assessment (OD) was performed using DensiCHECK™ densitometer (bioMérieux, Marcy l'Étoile, France).

To obtain supernatants and heat-inactivated bacteria, cultures were centrifuged at 4000× *g* for 5 min and suspended in RPMI-1640 medium or DMEM with the addition of 10% FBS (Fetal bovine serum) and 1% of L-glutamine (Euroclone, Pero, Italy) and incubated for 1 h at 37 °C. After centrifugation at 6000× *g* for 10 min, supernatants were collected. Bacterial inactivation was performed by heating at 95 °C for 15 min.

The bacterial lysates were obtained after incubation of bacterial cells with PBS 0.1% TRITON X-100 at 37 °C for 15 min. Before this step, Gram-positive bacteria were incubated with Lysozyme (10 mg/mL) for 1 h at 37 °C. Each sample was heated at 95 °C and immediately frozen in liquid nitrogen for at least three times. Protein concentration has been quantified by the BCA (Bicinchoninic Acid) method, and each sample was used as a stimulus at the concentration of 1 µg/mL.

4.3. Epithelial Cell Culture

SiHa cell line, isolated from squamous cell carcinoma and containing HPV-16 genome (1–2 copies per cell), was obtained from ATCC® (ATCC® HTB35™). SiHa cells were cultured in DMEM medium (Euroclone, Pero, Italy) supplemented with 10% FBS (Fetal bovine serum), 1% L-glutamine, 1% penicillin and streptomycin (Euroclone, Pero, Italy).

CaSki cell line, originally isolated from a cervical carcinoma and containing 600 copies of integrated HPV-16, was obtained from BEI-Resource. CaSki cells were cultured in RPMI-1640 medium (Euroclone, Pero, Italy) supplemented with 10% FBS (Fetal bovine serum), 1% L-glutamine, 1% penicillin and streptomycin (Euroclone, Pero, Italy).

4.4. PBMCs Isolation and Culture

Buffy coats from healthy donors (n = 9) were supplied by the Transfusional Center of Azienda Ospedaliera Careggi (Firenze, Italy). PBMCs were isolated by Ficoll-Paque density gradient (Cedarlane Labs, Burlington, Ontario, Canada) according to Paccosi et al. [53] and cultured in 6-well plates at the concentration of 10^6 cells/mL in RPMI-1640 medium (Euroclone, Pero, Italy) supplemented with 10% FBS, 1% L-glutamine and 1% penicillin and streptomycin (Euroclone, Pero, Italy). After 1 h at 37 °C, cells were stimulated with heat-inactivated bacteria (50 MOI/cell) and cultured for additional 5 days at 37 °C and 5% di CO_2. Culture supernatants from unstimulated or bacteria-stimulated cultures were collected, centrifuged at 4000 rpm for 10 min and stored at −80 °C.

4.5. Viability Test

SiHa and CaSki cells were cultured in 96-multiwells at the concentration of 2.5×10^3 cells/mL with bacterial cell supernatants (10–20–30% *v/v*) or bacterial lysates (1 µg/mL). Intracellular ATP was measured as an index of metabolic activity using CellTiter-Glo® LuminescentCell Viability Assay (Promega Corporation, Medison, WI, USA) after 24 h of culture.

4.6. Cytokines' Evaluation

IFN-γ, IL-1β, IL-4, IL-6, IL-10, IL-17A, IP-10 and TNF-α cytokine's concentration was measured using Milliplex® Map kit Human Cytokine/Chemokine/Growth Factor Panel A Magnetic Bead Panel (Merck Kgaa, Darmstadt, Germany) and Luminex apparatus following manufacturer instruction (Luminex 200 MAGPIX).

IL-8 was measured by IL-8 Human ELISA (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions.

4.7. SCFAs (Short Chain Fait Acids) Profile of Bacterial Strains

Supernatants of bacterial strains were prepared from liquid cultures in broth containing TSB and 5% Horse lysed blood) (Oxoid, Basingstoke, UK) following OD determination and ultracentrifugation.

The SCFAs were assessed through an isotope dilution (ID) quantitative method [54] that uses gas chromatography coupled with a mass spectrometry (GC-MS) system.

Briefly, the SCFAs were recovered from the samples by liquid-liquid extraction and then analyzed, as free acid form, by GC-MS instrument equipped with a Supelco Nukol column 30 m length, 0.25 mm internal diameter and 0.25 µm of film thickness. The SCFAs separation was carried out by the temperatures program as follows: initial temperature of 40 °C was held for 1 min, then it was increased to 150 °C at 30 °C/min, finally grow up to 220 °C at 20 °C/min. A 1 µL aliquot of extracted sample was injected in spitless mode (spitless time 1 min) at 250 °C, while the transfer line temperature was 280 °C. The carrier flow rate was maintained at 1 mL/min.

The quantitative SCFAs' evaluation was carried out by ratios between the area abundances of the analytes with the area abundances of respective labelled internal standards (isotopic dilution method). The ionic signals and the reference internal standard, used for the quantitation of each SCFA, were reported in Table 2. 3 mL of prefermented medium

sample was added of 50µL of internal standards (ISTD) mixture, 1 mL of tert-butyl methyl ether and 50 µL of 1 M HCl solution in 15 mL centrifuge tube. Then, each tube was shaken in vortex apparatus for 2 min, centrifuged at 10,000 rpm for 5 min, and finally, the solvent layer was transferred in autosampler vial and analyzed by GC-MS method. Each sample has been prepared and processed three times by the previously described method.

Table 2. The ionic signal used for quali/quantitation and relative ISTD of each Short Chain Fat Acids (SCFAs) acquired by the ID-GC-MS method.

SCFAs	Quan. Ion	Qual. Ion	ISTD
Acetic acid	60	-	[^{2}H3] Acetic
Propionic acid	74	73	[^{2}H3] Propionic
Butyric acid	60	73	[^{2}H3] Propionic
Valeric acid	60	73	[^{2}H9] iso-Valeric

4.8. Statistical Analysis

Numerical data were expressed as Mean ± standard deviation (SD) if they were in a normal distribution, or median and interquartile range (IQR) if they were not in Gaussian distribution. Mann–Whitney U test, Wilcoxon rank sum test, or Student t-test for two-group comparison was used, whereas ANOVA or Kruskal–Wallis with Bonferroni and Holm–Bonferroni p-value correction was used in case of multiple groups comparisons. Spearman rank correlation coefficient was used to examine the relationship between two continuous variables. Statistical significance was defined as a p-value < 0.05. Statistical analysis was performed using R software version 4.0.5. R Core Team (2021). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/ (accessed on 31 March 2021).

5. Conclusions

Our results defined in more detail the contribution of each individual species of vaginal microbiota to the host's defense against viral infection and revealed that not all vaginal lactobacilli have comparable properties of stimulating an adequate immune response. Based on the support of IFN-γ and lack of T_H17 differentiation, *L. gasseri* appears to be the species that better assist the host's defenses against HR-HPV infection.

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Hypervirulent *Klebsiella pneumoniae* Strains Modulate Human Dendritic Cell Functions and Affect T_H1/T_H17 Response

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Abstract: Hypervirulent *Klebsiella pneumoniae* (Hv-Kp) strains have emerged as pathogens causing life-threatening, invasive disease even in immunocompetent hosts. Systemic dissemination usually occurs following perturbations of the gut microbiota and is facilitated by Hv-Kp resistance to phagocytosis and complement activity. Hv-Kp are usually associated with K1 or K2 capsular types, produce several iron uptake systems (e.g., aerobactin and salmochelin) and are often but not invariably, capsular material hyper-producers (hypermucoviscous phenotype: HMV). Whether Hv-Kp escape the immune response at mucosal site is unknown. In this work, we studied the effects of Hv-Kp on human dendritic cells (DCs), central players of the IL-23/IL-17 and IL-12/IFN-γ axis at mucosal sites, essential for pathogen clearance. Four Hv-Kp and HMV strains were selected and their activity on DC maturation and cytokine production was compared to that of non-virulent Kp strains with classic or HMV phenotypes. While the maturation process was equally induced by all Kp strains, significant differences between virulent and non-virulent strains were found in the expression of genes for cytokines involved in T-cell activation and differentiation. The non-virulent KP04C62 and the classic Kp, KPC157 induced high expression of T_H1 (IL-12p70 and TNFα) and T_H17 cytokines (IL-23, IL-1β and IL-6), while Hv-Kp poorly activated these cytokine genes. Moreover, conditioned media from DCs cultured with non-virulent Kp, either classical or hypercapsulated, induced the activation of IL-17 and IFN-γ genes in preactivated $CD4^+$-cells suggesting their T_H17/T_H1 differentiation. Conditioned media from Hv-Kp poorly activated IL-17 and IFN-γ genes. In summary, our data indicate that Hv-Kp interfere with DC functions and T-cell differentiation and suggest that the escape from the IL-23/IL-17 and IL-12/IFN-γ axes may contribute to pathogen dissemination in immunocompetent hosts.

Keywords: hypervirulent; hypermucoviscous *K. pneumoniae*; dendritic cells (DCs); T_H differentiation; inflammatory cytokines; immune response

1. Introduction

Klebsiella pneumoniae (Kp), known as an opportunistic pathogen with a high propensity to acquire resistance genes, is a frequent cause of severe nosocomial infections in immuno-

compromised patients, mainly pneumonia, urinary tract infections and bacteremia [1,2]. In hospitalized patients, *K. pneumoniae* colonizes the intestinal microbiota, and its establishment in this environment is considered a fundamental step for the development of subsequent infections at distant body sites [3–5]. Since the mid-1980s, peculiar Kp lineages, denominated hypervirulent Kp (Hv-Kp), have emerged worldwide and are responsible for pyogenic infections with metastatic dissemination (e.g., liver abscesses, osteomyelitis, and endophthalmitis) [6], even in immunocompetent hosts [7,8].

Although we are still far from a universally accepted definition of Hv-Kp, some genetic features are considered hallmarks of virulent strains (Table 1) and typically consist of accessory virulence factors coding for (i) regulators of the mucoid phenotype, including *rmpA* and/or *rmpA2,* and the recently described *rmpD* gene necessary for hypermucoviscosity and virulence [9]; (ii) siderophore systems, including aerobactin (*iucABCD*), allantoin (*allABCDRS*), colibactin (*clbA-QS*), yersiniabactin (*ybtAEPQSTUX-irp1/2-fyuA*) and salmochelin (*iroBCDEN*) biosynthesis loci [10]. Additional virulence factors may consist of genes coding for proteins involved in iron metabolism (*cobW*) and transport (*fecI-fecA*), the hemin and lysine transport system (*shiF*), metabolic transporter (*peg-344*), and transcriptional regulations of virulence gene expression (*luxR*) [11].

Interestingly, all *K. pneumoniae* also harbor an array of core chromosomally located pathogenicity factors, including the siderophore enterobactin (*entABCDEF-fepABCDG*), as well as genetic loci encoding type 1 (*fim*) and type 3 (*mrk*) fimbriae and the variable capsular (cps) polysaccharide (K antigen) [10,12,13]. Different from "classical" Kp strains, Hv-Kp, show high virulence potential in animal experimental models (particularly those with K1 and K2 cps types and accessory siderophore systems) and usually belong to specific clonal lineages, such as clonal groups (CGs) 23, CG86 and CG65, retaining susceptibility against most of available antibiotics [13,14].

Hv-Kp, usually colonize the gut microbiota [15] and gain access to other sterile sites in the host following the impairment of microbiota resistance to colonization and dysbiosis-induced leakage of the epithelial barrier [16].

Some hypermucoviscous phenotype (HMV) Kp strains that are negative for *rmpA*/*rmpA2* and other genotypic markers of virulence were also reported to be able to cause disseminated infections in immunocompromised patients [13,14,16,17]. These strains, however, do not show pathogenic potential in experimental models of infections suggesting that, despite the hyperproduction of capsular polysaccharides, they are efficiently cleared by healthy immune-competent human hosts at mucosal sites.

Recent evidence shows that the HMV phenotype is not essential for the persistence of Hv strains in the gut [18], suggesting that mechanisms of immune escape that are not dependent on phagocytosis resistance, cooperate for pathogen survival at the mucosal level.

Dendritic cells (DCs) at mucosal sites capture and process antigens of microbiota components and, when necessary, orchestrate the activation of T cells and innate lymphoid cells (ILCs). An efficient adaptive response against Kp requires the integrity of the IL-23a/IL-17 and IL-12a/IFN-γ axes [19,20] and thus the full functionality of DCs.

In this paper, we selected four HMV strains originally isolated from disseminated infections and studied their effects on DC functions and T-cell differentiation. The hypervirulent HMV CIP 52.145 and a "classical" multi-resistant Kp strain were used as reference strains.

2. Materials and Methods

2.1. Bacterial Strains

CIP 52.145 is an Hv/HMV-Kp, multi-susceptible, well-characterized strain, isolated from a human specimen in Indonesia. It has a K2 cps type and belongs to ST66 [21,22].

RM1628 is an Hv/HMV-Kp, multi-susceptible, clinical isolate obtained in Italy from the blood culture of a patient with a liver abscess [23]. It has a K1 cps type and belongs to an ST related to the well-characterized Hv-Kp ST23.

HMV-1 and HMV-2 are two Hv/HMV-Kp, multi-susceptible, clinical isolates obtained from bloodstream infections in Italy. They belong to ST86 and ST65, respectively, and have a K2 cps type [24].

KP04C62 is an HMV-Kp, carbapenem-resistant, clinical isolate, obtained in Italy from the blood culture of a severely immunocompromised patient with a liver abscess. It belongs to ST512 and is associated with the KL107 cps type [17].

KPC157 is a "classical", non-HMV, carbapenem-resistant, Kp strain obtained from a rectal swab of a colonized patient, in Italy. It belongs to ST512 and is associated with the KL107 cps type [24] (Table 1).

The genomic sequences of studied strains were previously deposited and are accessible at the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/, accessed on 14 December 2021).

Screening for known *K. pneumoniae* virulence genes, including the recently described *rmpD* was performed using the Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 14 December 2021) and the Virulence Factor Database (VFDB, [9,11,22,25]).

Data on virulence in the animal model were reported accordingly to previously published results [17,23,24]. The string test was performed as previously described [17]. A summary of the genetic, phenotypic, and virulence features of the studied strains is shown in Table 1.

2.2. Ethical Approval

The use of buffy coats from donated blood, not usable for therapeutic purposes, was approved by the Ethics Committee of the Azienda Ospedaliera Universitaria Careggi (AOUC, Firenze, Italy) in agreement with the D.M. of the Italian Ministry of Health (15A09709) G.U., n. 300 12.28. 2015).

2.3. Cell Isolation Procedures

Peripheral blood mononuclear cells (PBMCs) were isolated by buffy coats through gradient centrifugation using Ficoll-Paque (GE Healthcare Italia, cat #45-001-750), according to the manufacturer's recommendations. $CD14^+$-cells were isolated using anti-CD14 conjugated microbeads (Miltenyi Biotec, cat #130-050-201) [26]. Monocyte-derived dendritic cells (DCs) were obtained by stimulating $CD14^+$ cells with recombinant IL-4 (50 ng/mL) and recombinant GM-CSF (100 ng/mL) [27].

$CD4^+$ T-cells were isolated from a non-adherent fraction of PBMCs by using a $CD4^+$ T-cells separation kit (Miltenyi, cat #130-096-533) according to the manufacturer's recommendations. The purity of the populations was checked using cytofluorimetric analysis with specific antibodies and was always >90%.

2.4. T_H1 and T_H17 Differentiation

Purified $CD4^+$T-cells from different donors were pre-activated by anti-CD3/CD28 antibodies coupled to beads at a 1:1 bead/cell ratio (Gibco, cat #11131D).

Preactivated $CD4^+$ T-cells (10^6/mL) were then incubated with 1 mL of conditioned medium obtained from cultures of DCs with Kp strains following the methods of Santini et al. [28]. Cytokine gene expression was evaluated by real -time (RT) PCR.

2.5. Cell Culture Conditions

In all experiments DCs and T-cells were cultured with live bacteria (50 MOI/cell) in an RPMI medium supplemented with 10% fetal bovine serum (Celbio, cat # 26140) and 5% L-glutamine (complete medium, CM), at 37 °C in a humidified chamber with 5% CO_2.

Table 1. Characteristics of Kp strains included in this work.

	Source	Mucoid Phenotype (String Test)	Capsular Type (O Locus)	Sequence Type (ST)	Antibiotic Resistance	Virulence in Animal Model	Aerobactin (*iucABCD*)	Allantoin (*allABCDRS*)	Colibactin (*clbA-QS*)	Enterobactin (*entABCDEF-fepABCDG*)	Yiersiniabactin (*ybtAEPQSTUX* -irp1/2-fyuA)	Salmochelin (*iroBCDEN*)	Reg. Mucoid Phenotype (*rmpADC-rmpA2*)	*manBC*	*shiF*	*fecI-fecA*	*peg-344*	*luxR*	pK2044-like Plasmid	Genome Accession Number	Ref.
CIP 52.145	Human	Positive	K2 (O2v2)	66	Multi-susceptible	++			#				¤						*	GCA_000968155.1	[21,22]
RM1628	Human, BSI	Positive	K1 (O1v2)	1861	Multi-susceptible	++														JAALJC000000000.1	[23]
HMV-1	Human, BSI	Positive	K2 (O1v1)	86	Multi-susceptible	++							¤							JAALCW000000000	[24]
HMV-2	Human, BSI	Positive	K2 (O1v2)	65	Multi-susceptible	++														JAALCV000000000	[24]
KP04C62	Human, BSI	Positive	KL107 (O2v2)	512	Carbapenem-resistant	+/−						§								MIFX00000000.1	[17]
KPC157	Human, RS	Negative	KL107 (O2v2)	512	Carbapenem-resistant	+/−						§								JAALCU000000000.1	[24]

Legend: ++: high virulence comparable to that NTUH-K2044 reference strain; +/−: low virulence comparable to that of other classical *K. pneumoniae* strains; the presence of virulence genes is highlighted in green, while their absence in red; BSI, bloodstream infection; RS, rectal swab; # absence of *clbK*; § presence of *iroE* only; ¤ absence of *rmpA2*; * coverage 33%, identity 98.95%.

2.6. Western Blot Analysis

A quantity of 10^6 DCs was cultured in CM for 30 min in the presence or absence of live bacterial cells (50 MOI/cell) or 200 ng/mL of pure LPS (Sigma-Aldrich, Saint Louis, MO, USA, cat. #L4391). Cells were lysed with RIPA buffer in the presence of phosphatase/protease inhibitor cocktail (Sigma-Aldrich, Saint Louis, MO, USA) and centrifuged at 12,000× *g* for 15 min, and the protein concentration was determined by BCA assay (Quantum Protein Assay Kit, EuroClone Pero, Italy). Subsequently, 40 µg of proteins/lane were loaded onto Stain Free gel (Bio-Rad Hercules, CA, USA) SDS-PAGE and blotted onto nitrocellulose filters (Bio-Rad, Hercules, CA, USA).

Membranes were stained with rabbit anti-phospho-NF-κB (p65) and anti phospho-p38 MAPK (Cell Signaling Technology, Danvers, MA, USA) antibodies at a 1:1.000 final dilution. Anti-rabbit IgG (H+L) DyLight800 were used as secondary antibodies at a1:10.000 final dilution. The reactions were visualized with the ECL detection system as recommended by the manufacturer (Bio-Rad Hercules, CA, USA). The intensity of the total proteins on the membrane was acquired by stain-free technology (Bio-Rad Hercules, CA, USA) using the ChemiDoc Touch System (Bio-Rad Hercules, CA, USA). The densitometric analysis was expressed as the ratio between the protein of interest and the total proteins by Image Lab software (Bio-Rad Hercules, CA, USA). [29]

2.7. Cytofluorimetric Analysis

Live bacterial cells were incubated with 5×10^5 DCs as reported above, for 16 h. At the end of the incubation cells were washed and stained with a mixture of anti-CD83-FITC, anti-CD86-APC and anti-HLA-DR-PE antibodies (BD Biosciences-Pharmingen, cat. #560929; #555660, and #555812) for 30 min. Cells were analyzed using the ACCURI instrument (BD Biosciences, Franklin Lakes, NJ, USA) using Cflow Plus software (BD Biosciences, Franklin Lakes, NJ, USA). Ten thousand events for each sample were acquired.

2.8. Real-Time PCR

T-cells and DCs cells (10^6) were cultured in presence of Kp strains for 3 h and 3 days, respectively. RNA extraction was performed using TRIzol™ (Invitrogen, cat. #15596-018). Extracted RNAs were quantified using Nanodrop (Thermo Fisher, Waltham, MA, USA) and stored at −80 °C.

A total of 2 µg of RNA from each sample was reverse-transcribed using EuroScript M-MLV Reverse Transcriptase (RNase H-) (EuroClone, cat. #EMR437050). RT-PCR was performed by using the QuantiNova SYBR Green PCR kit (Qiagen, cat. #208056) and the 7900HT Fast Real-Time PCR System (Applied Biosystems™). A total of 50 ng of cDNA from each sample was amplified. The β-actin gene was used as housekeeping gene. The primers used in this work are reported in Table 2.

Table 2. Primers used in this work.

Gene	Forward 5′-3′	Reverse 5′-3′
B-actin	GAAACTACCTTCAACTCCATCATG	AGGAGGAGCAATGATCTTGATC
IL-23a	CTCAGGGACAACAGTCAGTTC	ACAGGGCTATCAGGGAGCA
IL-12a	CCTTGCACTTCTGAAGAGATTGA	ACAGGGCCATCATAAAAGAGGT
IL-1β	AGCTACGAATCTCCGACCAC	CGTTATCCCATGTGTCGAAGAA
TNF-α	CCTCTCTCTAATCAGCCCTCTG	GAGGACCTGGGAGTAGATGAG
IL-6	ACTCACCTCTTCAGAACGAATTG	CCATCTTTGGAAGGTTCAGGTTG
IL-10	TCAAGGCGCATGTGAACTCC	GATGTCAAACTCACTCATGGCT
IL-17	AGATTACTACAACCGATCCACCT	GGGGACAGAGTTCATGTGGTA
IFN-γ	TCGGTAACTGACTTGAATGTCCA	TCGCTTCCCTGTTTTAGCTGC

2.9. Cytokine Production

We measured the IL-12p70 and IL-1β concentrations in the supernatant derived from cultures of 1×10^6 DCs incubated with Kp strains for 16 h. These data were produced

using the Milliplex® Map Human Cytokine kit and Luminex apparatus following the manufacturer's instructions (Luminex 200 MAGPIX).

2.10. Statistical Analysis

RT-PCR data were analyzed using the Kruskal-Wallis test and analysis of variance (ANOVA) from three different experiments. Bonferroni p-value adjustment method for multiple comparisons was used. A probability value of $p < 0.05$ was considered significant. Statistical analysis was performed using R software version 3.6.1 [30]. Western blot statistical analysis was performed by paired t-test.

3. Results

3.1. Expression of Maturation Markers

The cytofluorimetric analysis of CD83, CD86 and HLA-DR expression on DCs showed that all Kp strains induced a robust expression of CD83 and CD86 determinants on the membranes of DCs (Figure 1).

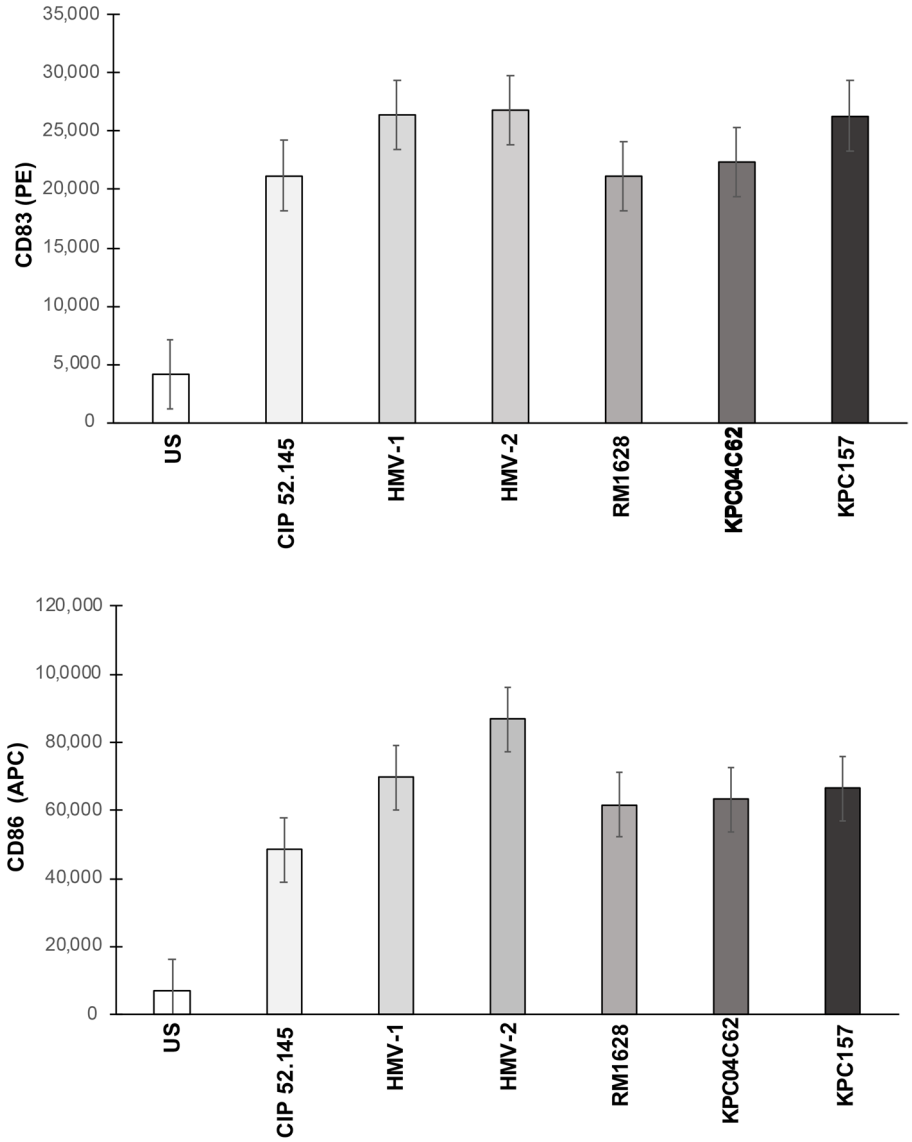

Figure 1. *Cont.*

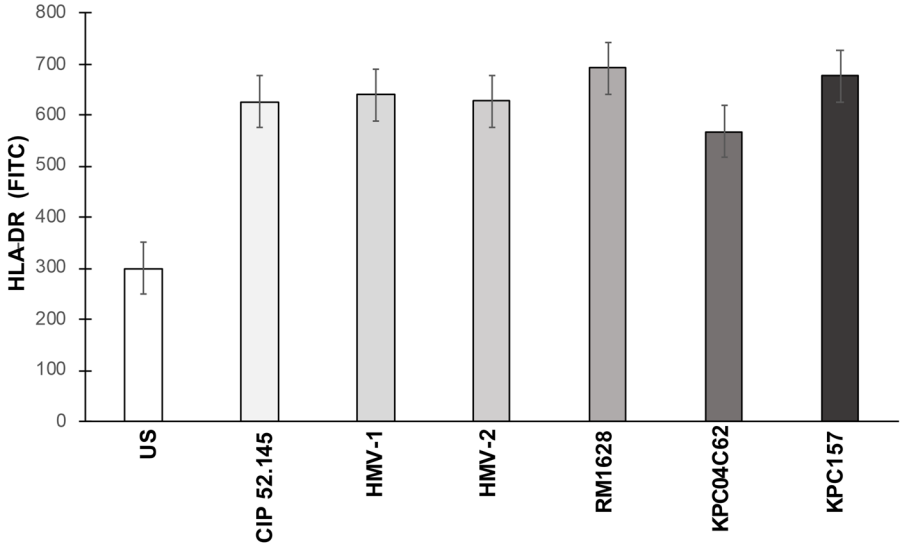

Figure 1. Maturation of DCs induced by *K. pneumoniae* strains. DCs were cultured with medium alone (unstimulated, US) or with live bacterial cells. Data were collected with a cytofluorimeter and are expressed as median fluorescence intensity (± IQR ×1.5) of three different experiments. The maturation level of each sample was compared (with the exception of US) and no significant statistical differences were observed.

The expression of HLA-DR was also induced by all Kp strains compared with their uninfected counterparts. Statistical analysis did not reveal significant differences in the expression of these markers on DCs (valuated as median fluorescence expression) among DCs cultured with different Kp strains. Histograms of one experiment out of the three performed are shown in Supplementary Figure S1.

3.2. Cytokine Gene Expression by DCs Infected with Kp Strains

The expression of cytokines involved in T_H17 (IL-1β, IL-23a, and IL-6) and T_H1 differentiation (IL-12a, and TNF-α) and the anti-inflammatory response (IL-10) was investigated. Figure 2 and Figure S2 show the cytokine gene expression induced by Kp strains in DCs. Two main clusters were detectable on the basis of cytokine gene activation. The first included RM1628, HMV-1, HMV-2, and the reference Hv-Kp CIP 52.145. All these strains induced very low or no expression of all cytokine genes evaluated, with selected cytokine genes activated less than control, unstimulated cultures. The second cluster included KP04C62 and the classical KPC157. These two Kp strains activated all cytokine genes included in the study.

The histograms presented in Figure 2 show a more detailed graphical representation of the effects of each Kp strain on cytokine gene activation. It should be noted that the HMV non-virulent KP04C62 strain had similar behavior to the classical Kp strain, and both strains activated DCs to express cytokine genes involved in the T_H17 (IL-23, IL-6, and IL-1β) and T_H1 (IL-12 and TNF-α) responses (results of post hoc tests are reported in Supplementary Table S1).

In contrast, the clinical isolates HMV1 HMV2 RM1628 and the reference virulent CIP 52.145 only slightly activated these genes. The statistical analysis revealed significant differences between Kp strains, particularly when comparing the results of virulent strains with those obtained from classical KPC157 or hypercapsulated KP04C62 strains.

Moreover, Figure 2 shows that the reference CIP 52.145 was the most potent inducer of the anti-inflammatory IL-10 gene expression. Apart from CIP 52.145, the expression of the IL-10 gene was not significantly induced by Kp strains, virulent or not, during 6 h of stimulation, suggesting that the activation of this immunomodulatory pathway may not be included in the escape strategy of virulent Kp.

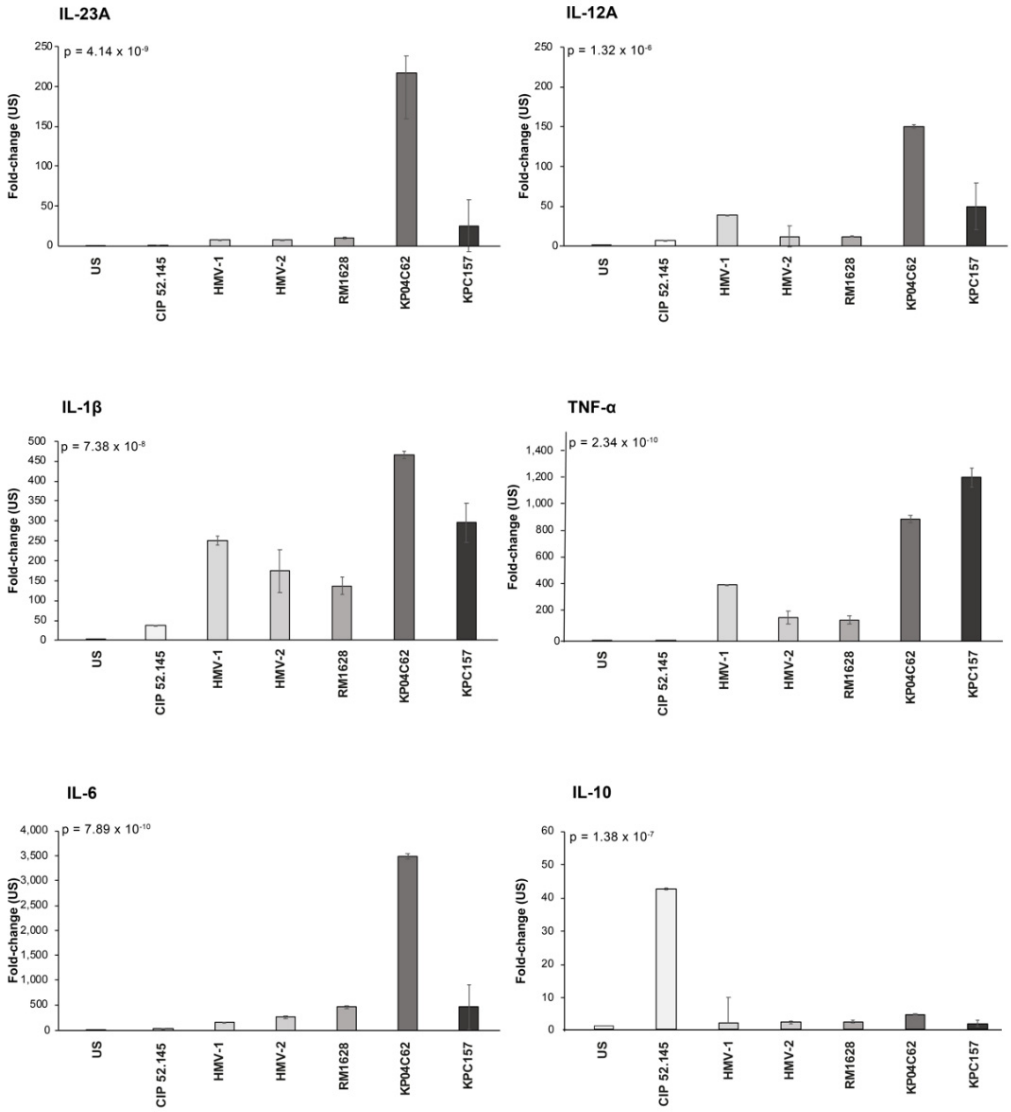

Figure 2. Cytokine expression by DCs cultured in presence of live *K. pneumoniae* strains; the global *p*-value obtained by ANOVA is reported.

We measured the concentrations of IL-1β and IL-12p70 in the supernatants from DCs cultured with *K. pneumoniae* strains. Supplementary Table S2 shows that despite the prolonged time of stimulation, IL-12 concentrations were in accordance with data from gene expression, while IL-1β was produced in high amounts by one of the Hv-Kp strains.

3.3. T_H1 and T_H17 Differentiation Induced by Kp-Conditioned Media of DCs Cultures

To add further evidence of the inhibitory effects of HMV-Kp strains on DC functions, we assessed conditioned media from Kp-DC cultures for the ability to induce the differentiation of pre-activated $CD4^+$ T-lymphocytes. Figure 3 and Supplementary Table S3 show that the conditioned media from DCs cultured with HMV-Kp strains were ineffective at inducing IL-17 gene expression of pre-activated $CD4^+$ cells. By contrast, the conditioned media from DCs cultured in presence of non-virulent Kp strains, either classical (KPC157), or hypercapsulated (KP04C62), strongly induced IL-17 gene expression by pre-activated $CD4^+$ T-cells. The medium of DCs cultured with classical Kp was also able to induce a significant amount of IFN-γ gene expression. The data suggest that virulent strains of Kp may affect DC functionality and compromise the differentiation of T_H17 and T_H1 effector T cells.

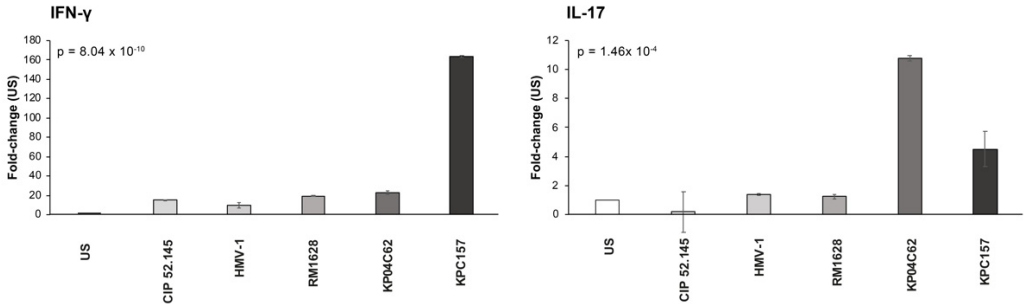

Figure 3. IFN-γ and IL-17 gene expression by pre-activated $CD4^+$ T-cells. The bar-graph shows data (mean ± SE) of three different experiments. Data are expressed as fold-change with respect to unstimulated (US) cultures; the global *p*-value obtained by ANOVA is reported.

3.4. Molecular Mechanisms of Inhibition of Cytokine Gene Expression

The nuclear translocation of AP-1 and NF-kB (p65) following phosphorylation mediated by p38-MAPK and TRAF6 respectively [31] represents a crucial step for the transcription of cytokine genes.

To investigate whether Hv-HMV Kp strains affect these pathways, DCs were cultured with live bacterial cells for 30 min and the activation of NF-kB and p38MAPK was measured using Western blot analysis. Figure 4 shows that the activation of p38-MAPK was significantly reduced in cells challenged with HMV-Kp compared with those challenged with KP04C62 and KPC157, suggesting that the HMV phenotype may interfere with the activation of pro-inflammatory pathways in DCs.

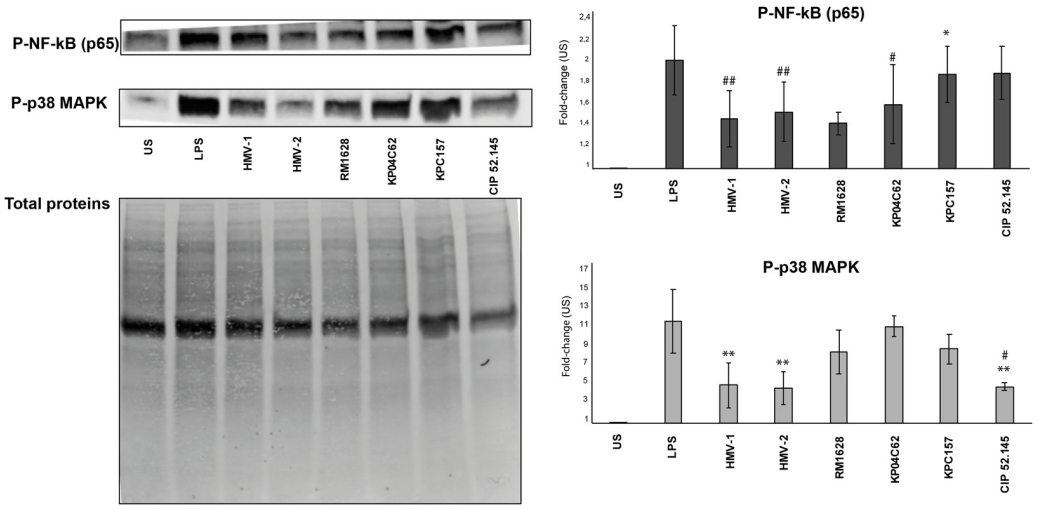

Figure 4. NF-kB (p65) activation and p38-MAPK phosphorylation by DCs. Dendritic cells were cultured in the absence (US) or presence of *K. pneumoniae* strains or LPS as a positive control. Cells were analyzed by Western blot analysis. Data from one representative experiment out of three performed are shown. Data are expressed as the fold increase of each experimental point over unstimulated control. A *t*-test was performed comparing KP04C62 (*) and KPC157 (#) (non-Hv-HMV strains) with the Hv-HMV Kp strains. We considered statistical significance as a *p*-value < 0.05 (*, #); *p*-value < 0.01, (**, ##).

4. Discussion

K. pneumoniae is considered a stealth pathogen because it fails to stimulate the innate immune response [24]. These bacteria, in fact, have evolved numerous mechanisms to avoid recognition from host pattern recognition receptors (PRRs). Much evidence, however, indicates that *Klebsiella* also actively subverts host defenses. For example they are able to manipulate phagosome maturation or modify lipid A decoration [19,20].

Hv-Kp shares many of these mechanisms with classical Kp. The HMV phenotype allows pathogens to escape neutrophil phagocytosis and complement-mediated activity and is crucial for their systemic dissemination.

Hv-Kp usually colonize the gastrointestinal (GI) tract [15], and, under certain circumstances (as for example, antibiotic-induced dysbiosis), they may spread to other organs and apparatus [16,32,33].

The persistence of Hv-Kp in the gut, however, is not strictly associated with capsule hyperproduction and the HMV phenotype [18], and it is reasonable that Hv-Kp escapes other mechanisms of innate and adaptive immunity.

It is well known that DCs are important players of mucosal immunity, as they sense the external environment, capture living or dead bacterial cells or single antigens released after cell death, and present antigens to T cells, supporting their activation [34]. Bacterial ligands interacting with PRRs localized in the cytoplasmic membrane, endosomes or cytosol of DCs promptly activate the expression of membrane determinants involved in immunological synapsis as well as the production of cytokines responsible for Th differentiation [35]. Numerous TLRs and both TRIF- and MyD88-dependent signaling contribute to host defense against *K. pneumoniae* infection [36].

In addition, capsular polysaccharides from either classical or HMV Kp activate the NLRP3 inflammasome pathway, leading to mature IL-1 beta and IL-18 production [37,38]. These cytokines activate and potentiate IL-12 and IL-23 functions, driving the differentiation of T_H precursors T_H1 and T_H17 activated T-cells [39].

DCs also activate innate group 3 lymphoid cells (ILC3) during infection sustained by members of the *Enterobacteriaceae* family [40], producing a variety of cytokines, including IL-22 and IL-17A, and providing crucial protection against enteric pathogen infections.

The main results of our study show that Hv-Kp strains interfere with DC expression of IL-12 and IL-23 and, as a result, DCs lose their ability to induce the differentiation of T-cells to T_H1 or T_H17 effectors. This behavior can potentially compromise the adaptive immune response and the clearance of these pathogens. All Kp strains, virulent or not, were able to induce the expression of surface MHC class II and other accessory molecules (CD83 and CD86), suggesting their ability to activate toll like receptor (TLR) 4 and other membrane or cytosolic PRRs. Moreover, preliminary data suggest that DCs cultured with Hv-Kp do not undergo apoptosis, suggesting that TLR pathways are activated and maintain DC viability. As such, the abundance of capsular polysaccharides is not the only cause of reduced cytokine gene activation. Consistent with this hypothesis, the non virulent HMV included in this study, KP04C62, was found to not interfere with the activation of cytokine genes.

Interestingly, it has been recently reported that Hv-Kp strains are able to resist thekilling activity of human macrophages and survive within the cells [23]. Rather than low interaction with TLRs, the molecular mechanisms of the inhibitory activity exerted by Kp more likely reside in interference with the NF-kB and MAPK signaling pathways. Our data indeed show that the activation of p38MAPK and NFkB pathways is significantly lower in DCs cultured with the Hv-Kp strains compared with that in DCs cultured with not virulent Kp, suggesting that Hv-Kp produce factors that interfere with these signaling pathways. Such interference was reported in different experimental systems [41,42] and was attributed to bacterial components, such as the LPS O-polysaccharide and PulA type 2 secretion system [43].

Genetic analysis of the strains included in the study revealed a heterogeneous content of core and accessory genes linked to virulence, which might contribute to the different behavior shown in activation of p38MAPK and NF-kB pathways by Hv-Kp strains.

Further studies are needed to clarify which factors are responsible for DC inhibition.

In accordance with the low amount of IL-12/IL-23, we observed that conditioned media from DCs cultured with Hv-Kp do not induce the T_H1/T_H17 differentiation of preactivated CD4+ cells, suggesting that the activation of the above mechanisms may not take place in vivo.

As reported above, an efficient adaptive response against Kp requires the integrity of the IL-23a/IL-17 and IL-12a/IFN-γ axes [19,41,42] and the full functionality of DCs. T_H1 and T_H17 cells support the clearance of Kp by inducing neutrophil recruitment and activation, the production of neutrophil extracellular traps (NETs) and the IFN-γ mediated potentiation of macrophage activity [41]. Along with the crucial role in the activation of the IL-23a/IL-17 and IL-12a/IFN-γ axes, DCs also stimulate innate lymphoid cells at mucosal sites to produce IL-22, a cytokine promoting gut homeostasis through its functional effect on the epithelial barrier [43]. The functional inactivation of DCs by Hv-Kp is suggested as a major cause of systemic dissemination of these pathogens even in healthy, non immune-compromised patients.

However, further experiments are needed to define the factor(s) involved in the inhibition of p38/NF-kB activated pathways. Our data underscore the necessity to formulate vaccines against proteinaceous (non-capsular) Kp antigens, which, by expanding the population of memory-resident T_H17 lymphocytes in the mucosal districts, also guarantee an optimal adaptive response to infections by antibiotic-resistant strains.

5. Conclusions

The results of this study indicate that the interference of Hv-Kp with the functions of DCs affects the differentiation of T-cells to T_H17 and T_H1 effectors. These findings provide evidence for the ability of Hv-Kp to escape the host adaptive response at mucosal sites and potentially disseminate in immunocompetent hosts.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/microorganisms10020384/s1, Table S1: ANOVA test with Holm *p*-value adjustment for each *Klebsiella pneumoniae* stimulus. Table S2: Evaluation of Cytokine's production by DCs under bacteria stimulation. The concentration reported refers to the amounts from three different cultures pooled together. US indicates the control, unstimulated cultures. Figure S1: DCs maturation under bacteria stimuli was evaluated by FACS analysis. DCs were stained with a mixture of anti-HLRA-DR-PE (upper lane), anti-CD86-APC (middle lane) and anti-CD83-FITC (lower lane) antibodies. Red gate indicates P1 population (live cells), blue gate to P2 population (we exclude doublets). The gate for the analysis was based on the US (vertical black line). Figure S2: Heat-map of the expression of cytokine in stimulated DCs. Genes are reported on x-axis and Kp stimuli on the y-axis.

Author Contributions: Conceptualization M.G.T. and F.A.; methodology, S.N., G.M., I.B., A.M.C., G.C. and A.A.; software and statistical analysis, M.T.; formal analysis, M.T., V.D.P. and T.G.; data curation, G.M.; resources, V.D.P. and T.G.; writing—original draft preparation, S.N. and M.G.T.; writing—review and editing, A.A., F.A. and M.G.T.; supervision, G.M.R. and F.C.; funding acquisition, M.G.T. and G.M.R. All authors have read and agreed to the published version of the manuscript.

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Effects of common Gram-negative pathogens causing male genitourinary-tract infections on human sperm functions

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Male genitourinary tract (MGT) bacterial infections are considered responsible for 15% of male infertility, but the mechanisms underlying decreased semen quality are poorly known. We evaluated in vitro the effect of strains of Gram-negative uropathogenic species (two *E.coli* strains, three *K. pneumoniae* strains, *P. aeruginosa* and *E. cloacae*) on motility, viability, mitochondrial oxidative status, DNA fragmentation and caspase activity of human spermatozoa. All strains, except *P. aeruginosa*, reduced significantly sperm motility, with variable effects. Sperm Immobilizing Factor (SIF) was largely responsible for deteriorating effects on sperm motility of *E. coli* strains since they were completely reverted by knockout of SIF coding *recX* gene. Sequence alignment for RecX showed the presence of high homologous sequences in *K. pneumoniae* and *E. cloacae* but not in *P. aeruginosa*. These results suggest that, in addition to *E.coli*, other common uropathogenic Gram-negative bacteria affect sperm motility through RecX products. In addition to sperm motility, the *E. coli* strain ATCC 35218 also affected sperm viability, and induced caspase activity, oxidative stress and DNA fragmentation suggesting an interspecies variability in the amount and/or type of the produced spermatotoxic factors. In general, our results highlight the need for a careful evaluation of semen infections in the diagnostic process of the infertile man.

Male factor is responsible of 40–50% of couple infertility, and it is estimated that male infertility affects up to 15% of the couples[1]. The most common cause of male infertility is poor semen quality, which may be due to alterations of testicular function or may originate during sperm transit in the male genital tract. Acute and chronic inflammation and infections are believed to be responsible for approximately 15% of cases of male infertility likely because of a detrimental effect on spermatozoa, although the association between inflammation and infections and poor semen quality has not been clearly defined[2]. *Enterobacterales* spp. are common pathogens of the urogenital tract and may interfere with male fertility[3,4]. As reported in the study by Boeri et al.[5], *Enterobacteriaceae* represent the second most frequent pathogens responsible for semen infections in a cohort of 1689 European male partners of primary infertility couples. Similar frequencies were found in subfertile men attending the outpatient clinic of the University hospital of Florence[6].

Escherichia coli is one of the most frequent species found in human semen[6,7] and in genitourinary infections[8], in particular epididymitis[9]. *E. coli* rapidly adheres to human spermatozoa in vitro, resulting in agglutination of spermatozoa. A profound decline in motility of spermatozoa is evident over time caused by severe alterations in sperm morphology[7] and by the release of soluble spermatotoxic factors such as sperm immobilizing factor (SIF,[2]). An association with oligoasthenozoospermia and male infertility was however reported also with other *Enterobacteriaceae* as *Klebsiella pneumoniae* and *Klebsiella aerogenes*[10] and with *Pseudomonas aeruginosa*[5].

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Evaluation of the direct effects of bacteria on sperm functions in vitro is of great help in understanding the role of infections in male infertility. So far, most in vitro studies evaluating the effects of *Enterobacteriaceae* on human spermatozoa, employed *E. coli* strains as pathogen (for review see[2,8]). In addition, most studies were limited to evaluate the effect of *E. coli* on human sperm motility and viability, and have been performed on highly motile selected sperm populations[11–15], which are poorly representative of the real environment where bacteria, present in the male genital tract, may produce the damage. Whether other bacterial species as *K. pneumoniae*, *K. aerogenes*, *Enterobacter cloacae*, *P. aeruginosa,* commonly causing genitourinary tract infections (GUTI), affect human sperm motility or other sperm functions is not yet known. Although progressive motility and sperm viability are of fundamental importance both in natural and assisted reproduction, other sperm characteristics are necessary for fertilization and embryo development. In particular, spermatozoa must deliver an intact DNA to the oocyte. Oxidative and apoptotic pathways may cause sperm DNA fragmentation (sDF;[16,17]), the most common type of DNA damage found in human spermatozoa[18], which has a negative impact on both natural and assisted reproduction[19]. The effects of bacteria on sperm oxidative and apoptotic pathways have been poorly investigated. Besides damaging sperm DNA by inducing fragmentation, base oxidation and mutations, oxidative stress, when present in high levels, can cause lipid peroxidation in the plasma membrane, produce modifications of sperm proteins impairing their functions, alter mitochondrial function and induce apoptosis[20]. In turn, activation of apoptotic pathways may impact on other sperm functions and, ultimately, leading to cell death[21].

Inhibitory effect of *E. coli* on sperm motility have been attributed to release of SIF[2,8], however, whether SIF is involved in the inhibitory effect of other bacterial species is presently less clear, nor it is known the role of this factor in other sperm alterations due to bacterial infections.

In the present study we selected bacterial strains belonging to potential uropathogenic species (*E. coli*, *K. pneumoniae*, *K. aerogenes* and *E. cloacae)* that express the *rec-X* SIF-coding gene or highly homologous genomic sequences and evaluated their effects on sperm motility, viability, mitochondrial oxidative status, DNA fragmentation and caspase activity in whole semen.

P. aeruginosa, a less frequent pathogen of male urogenital apparatus which was found with a prevalence of 10% in infertile couples[22] was also included in the study.

In addition, we further explored the role of SIF on sperm motility by using an *E. coli* strain KO for SIF coding gene.

Results

Effect of bacterial strains on sperm motility. Whole semen samples were incubated with live bacterial cells from *E. coli* (ATCC 29522 and ATCC 35218), *K. pneumoniae*, *P. aeruginosa*, *E. cloacae* and *K. aerogenes* strains at sperm/bacteria ratio of 1:10[23] and progressive and total motility was recorded after 1 (n = 13) and 3 h (n = 16). All bacteria strains, except *P. aeruginosa* ATCC 27853, determined a significant decrease of total and progressive motility both at 1 (Fig. 1, panels A and B) and 3 (Fig. 1, panels C and D) hours incubations. Among the bacteria tested, *E. cloacae* ATCC 13047 and *E. coli* ATCC 35218 strain were the most potent in reducing sperm progressive motility at both time points. A significant increase in the percentage of non-progressive motility of spermatozoa was detected in cultures with *K. quasipneumoniae* ATCC 700603, *K. aerogenes* ATCC 13048 and *E. cloacae* ATCC 13047 strains (data not shown). A direct adhesion of bacterial cells to the sperm tails as well as sperm agglutination was observed in cultures with *E. cloacae* ATCC 13047 (Supplemental Figure S1 and video S1), whereas such effects were not observed with the other bacterial strains (data not shown).

Role of SIF on sperm motility. The 56 kDa Sperm Immobilizing Factor (SIF), is considered one of the responsible factors of the detrimental effects of *E. coli* on sperm motility[24].

To further confirm the role of SIF in sperm motility, we performed experiments by using the *E. coli* MG1655 as reference strain and its mutated counterpart JW2668 knockout (KO) for *recX* gene[25]. JW2668 *E. coli* strain did not affect either progressive (Fig. 2A) and total (Fig. 2B) sperm motility after 3 h incubation whereas the wild type MG1655 strain decreased both motilities.

To investigate whether also the other strains affecting sperm motility were able to produce SIF homologous proteins, we performed tBLASTn and BLASTp sequence alignments using *recX* from *E. coli* K12 MG1655 as reference sequence. The results of this analysis, shown in Fig. 3, clearly indicate a high homology sequence in *recX* gene and SIF protein among the strains of *E. coli* (both strains), *K. pneumoniae* (both strains), *K. aerogenes* and *E. cloacae*. Conversely, sequence homology with RecX was not detected in the genome of *P. aeruginosa* ATCC 27853 (not shown), an *Enterobacteriales* species which belongs to the *Pseudomonacee* family.

Effect of bacterial strains on sperm viability. To investigate whether changes in motility were due to a decrease of sperm viability, we evaluated the percentage of viable spermatozoa after 3 h of incubation with the different bacteria by two different techniques (eosin staining and Live/Dead Fixable Green Dead Cell Stain coupled to flow cytometry). Figure 4 shows that, with the exception of *E. coli* ATCC 35218, none of the bacterial strains induced significant reduction in cell viability with both methods (A, B). Similar results were obtained after 1 h incubation (not shown). Neither the reference *E. coli* MG1655 strain (wild-type strain), nor the *E. coli* JW2668 strain (KO for *recX* gene), affected significantly sperm viability as evaluated by eosin staining (Fig. 4C). Since SIF was reported to reduce sperm viability at higher concentrations respect to those affecting motility[24], we collected supernatants from *E. coli* ATCC 25922 cultured at 100 and 300×10^6/ml, and purified the fractions containing proteins with molecular weights (MW) ≥ 30KDa, thus including the 56 kDa SIF. After incubation for 3 h with such fractions, a decrease in sperm viability was observed with the fractions obtained from 300 million bacteria (Fig. 4D), suggesting that the toxic effect depends on the rate of secretion of spermatotoxic factors and that such rate is variable within strains of the same species.

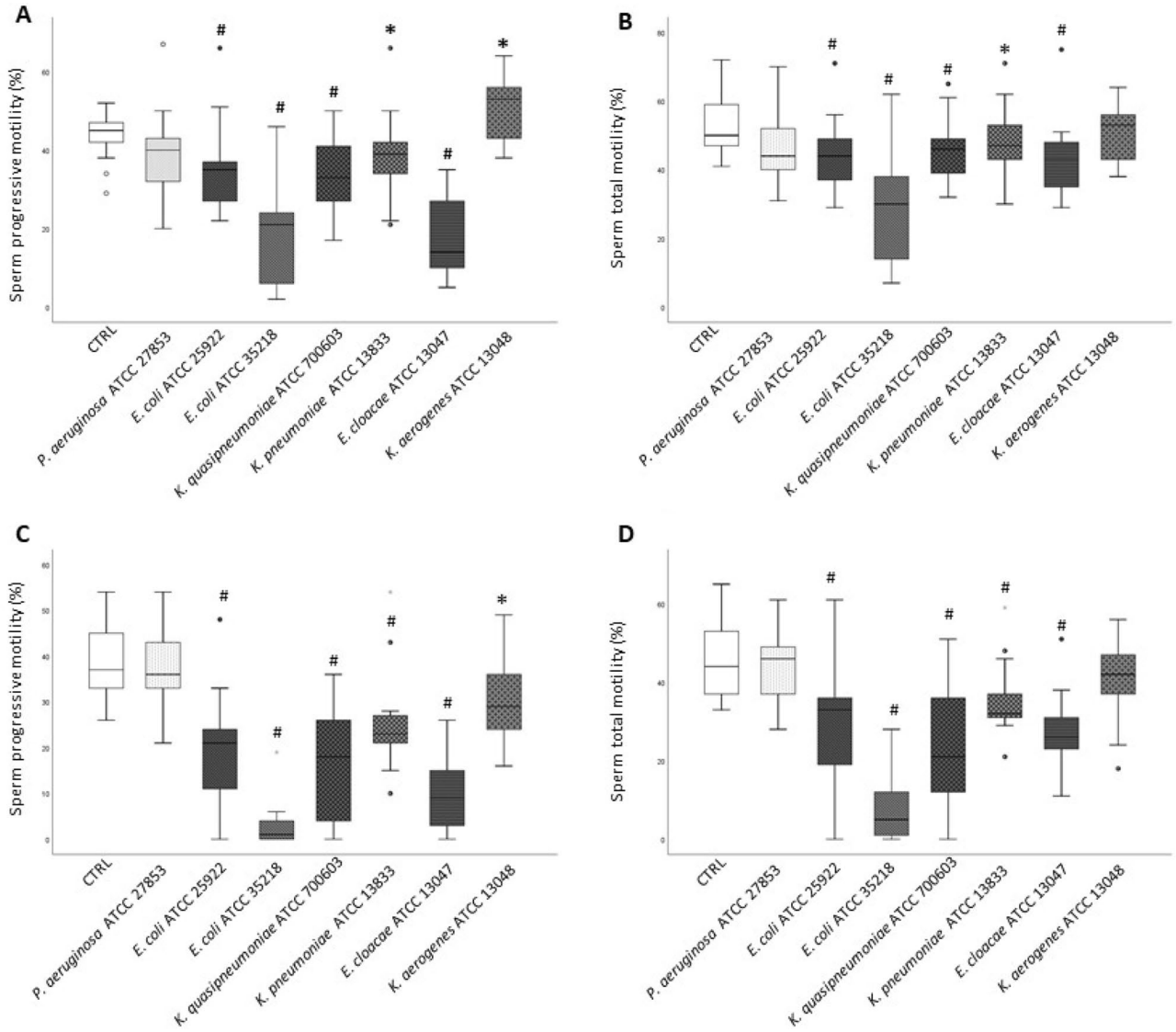

Figure 1. Box-plots representing the effect of 1 (n = 13, panels **A** and **B**) and 3 (n = 16, panels **C** and **D**) hours in vitro incubation (37 °C, 5%CO_2) of whole semen samples with various bacterial strains (*P. aeruginosa* ATCC 27853, *E. coli* ATCC 25922, *E. coli* ATCC 35218, *K. quasipneumoniae* ATCC 700603, *K. pneumoniae* ATCC 13883, *E. cloacae* ATCC 13047 and *K. aerogenes* ATCC 13048) on sperm progressive (**A** and **C**) and total motility (**B** and **D**). Wilcoxon test, *$p < 0.05$; #$p < 0.01$ versus CTRL.

Effect of bacterial strains on sperm oxidative stress, caspase activity and DNA fragmentation. Pathogenic strains of *Enterobacteriaceae* family are known to produce toxins or metabolites that induce oxidative stress in infected cells[26]. In order to understand whether the bacterial strains tested in our study could determine an increase in ROS production and sperm oxidative status, we evaluated mitochondrial oxidation by using the fluorescent probe MitoSOX™ Red. With the exception of *E. coli* ATCC 35218, none of tested bacterial strains affected sperm mitochondrial ROS generation (Fig. 5A).

Interestingly, *E. coli* ATCC 35218 also affected sperm viability (Fig. 4 A and B), suggesting that generation of oxidative stress could be involved in inducing sperm death through activation of an apoptotic pathway[16]. To support this hypothesis, the activity of caspase 3 and caspase 7, an established marker of sperm apoptosis[27], was measured. As shown in Fig. 5B, E. *coli* ATCC 35218 increased significantly the percentage of spermatozoa expressing caspase 3 and 7 activity. *E. coli* ATCC 25922 and *K. aerogenes*, used as control strain that did not affect sperm viability (Fig. 4 A and B) and mitochondrial oxidation (Fig. 5A), did not induce caspase activation. *E. coli* ATCC 35218 also induced a significant increase of total and PI brighter sDF[28], whereas the other tested bacterial strains did not affect these parameters (Fig. 5C).

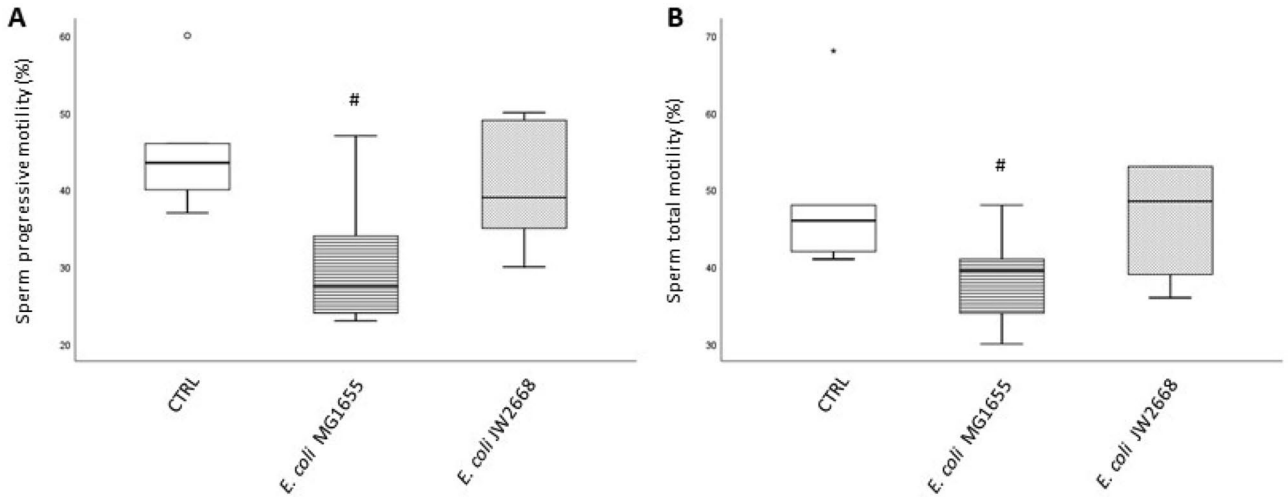

Figure 2. Box-plots representing the effect of 3 h in vitro incubation (37 °C, 5%CO_2) of whole semen samples (n = 6) with *E. coli* MG1655 (wild-type strain) and *E. coli* JW2668 (knockout for SIF) on sperm progressive (A) and total (B) motility. Wilcoxon test, *p < 0.05; #p < 0.01 versus CTRL.

Figure 3. Amino acid sequence alignment of homologous RecX proteins. Conserved amino acidic regions are shaded in grey. Percentage of aminoacidic sequence identity of SIF homologous among *Enterobacterales* strains using *E. coli* MG1655 as reference strain was also reported.

Discussion

Although bacterial pathogens are frequently found in semen samples of infertile men, there is no consistent epidemiological link between pathogens and male infertility or altered semen parameters. However, a recent study[2] reported that bacteriospermia was directly related to 15% of infertility in men treated with assisted reproduction. In our knowledge, the present study is the first one where the in vitro effect of a wide panel of bacteria belonging to the *Enterobacteriaceae* family commonly found in infected semen[5], was evaluated at the same time and in the same semen samples. We found that almost all bacterial strains directly affect human sperm motility, whereas, only the *E. coli* strain ATCC 35218 impaired sperm viability, induced mitochondrial oxidative stress, DNA fragmentation and activate the apoptotic pathway. Even if the strains included in the study were not isolated from infertile males, *K. quasipneumoniae* ATCC 700603 (ST489), has been isolated from a urine sample, and *E. coli* ST73 and ST127 (the clones of ATCC 25922 and ATCC 35218, respectively) were recently associated to hospital and community acquired urinary tract infections[29,30]. In agreement with previous studies[11–14,31,32] the reduction of sperm motility was observed following incubation in vitro with SIF-producing strains of *E. coli*. Our study extends these findings to other *Enterobacteriaceae*, whose in vitro effects on human spermatozoa have been less investigated. However, the detrimental effects on motility were highly variable and dependent on the strain.

We demonstrated the presence of high homologous sequences to *recX* gene and SIF protein from *E. coli* MG1655 in the *K. pneumoniae*, *K. aerogenes* and *E. cloacae* strains, suggesting that the secretion of this toxin may act as a common molecular mechanism used by *Enterobacteriaceae* to immobilize sperm. This conclusion is reinforced by the use of an *E. coli* strain KO for the *recX* gene that did not affect sperm motility compared to the wild type. In addition, *P. aeruginosa,* which is found in about 10% of infertile couples[22] and belongs to a different taxonomic order (*Pseudomonadales*), does not express a homologous *recX* gene and does not have the property to immobilize sperm in vitro.

We noted that, despite the culture with *K. pneumoniae*, *K. aerogenes*, *E. cloacae* strains significantly decreased sperm progressive motility, such strains did not completely immobilize spermatozoa but increased the percentage of in situ motility. This result suggests that complete immobilization is influenced by the quality or the quantity of SIF released by bacterial strains. In addition, the *E. coli* strains used in our study did not determine sperm agglutination, which was observed only after incubation with *E. cloacae*.

The effects on sperm progressive and total motility were present already after 1 h incubation and did not vary substantially after 3 h for most bacterial strains, indicating that the effect on motility may be quite rapid. This result suggests that waiting long times before sperm manipulation in assisted reproduction laboratories or

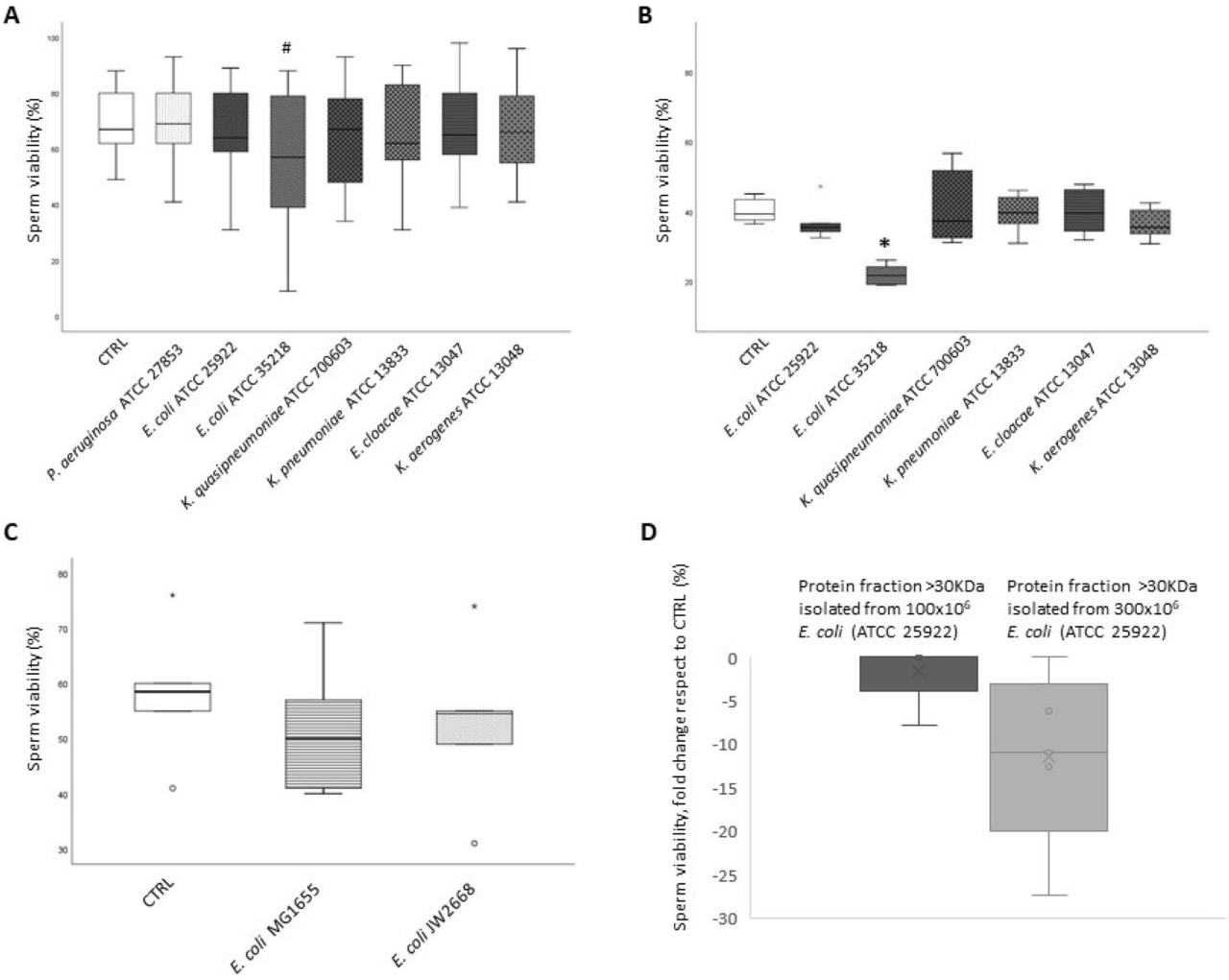

Figure 4. Box-plots representing the effect of 3 h in vitro incubation (37 °C, 5%CO_2) of whole semen samples with various bacterial strains (*P. aeruginosa* ATCC 27853, *E. coli* ATCC 25922, *E. coli* ATCC 35218, *K. quasipneumoniae* ATCC 700603, *K. pneumoniae* ATCC 13883, *E. cloacae* ATCC 13047 and *K. aerogenes* ATCC 13048) on sperm viability determined by eosin staining (n = 16, panels **A** and **C**) and by the LIVE/DEAD™ Fixable Green Dead Cell Stain (n = 6, panel **B**). In panel **C**, box-plots representing the effect of 3 h in vitro incubation (37 °C, 5%CO_2) of whole semen samples (n = 6) with *E. coli MG1655* (wild-type strain) and *E. coli JW2668* (knockout for SIF) on sperm viability. In panel **D**, box plots representing sperm viability fold change respect to CTRL of 5 subjects after incubation with supernatants of *E. coli* ATCC 25922 obtained from a different number of cultured bacteria (100 and 300 × 10^6), after isolating protein fractions ≥ 30 KDa. Wilcoxon test, *p < 0.05; #p < 0.01 versus CTRL.

during routine semen analysis, may result in a decrease of sperm motility and of the number of sperm recovered after selection procedures when strains of *E. coli*, *K. pneumoniae*, *K. aerogenes*, *E. cloacae* are present in semen.

We show that most of the *Enterobacteriaceae* tested here reduce motility without affecting sperm viability, with the exception of the *E. coli* strain ATCC 35218. This result was confirmed by using two different methods to assess sperm viability, a subjective one (eosin staining) according to indications of WHO[33] and an objective one (staining with LIVE/DEAD™ Fixable Green Dead Cell Stain coupled to flow cytometric detection). The results were qualitatively similar, although viability evaluated after staining with LIVE/DEAD™ Fixable Green Dead Cell Stain was found lower in all samples. It is possible that LIVE/DEAD™ Fixable Green Dead Cell Stain is more efficient than eosin in detecting unviable spermatozoa or is able to stain also apoptotic cells committed to die. We should also consider that the subjective analysis is done on 200 spermatozoa whereas the flow cytometric analysis regards 8.000 events, and thus, likely, more precise.

Unlike motility, the effect of bacteria on viability appears to be dependent from the amount of SIF released in culture. In fact, when spermatozoa were incubated with the SIF-containing purified fraction from 300 millions *E. coli* ATCC 25922 culture supernatants, a partial spermicidal effect was observed, in agreement with previous studies[24]. However, we cannot exclude that factors different from SIF, produced by *E. coli* ATCC 35218, could be also involved in viability impairment. Of note, our analysis revealed that different *E. coli* strains could have a spectrum of different effects on sperm functions going from decrease of motility to induction of oxidative stress,

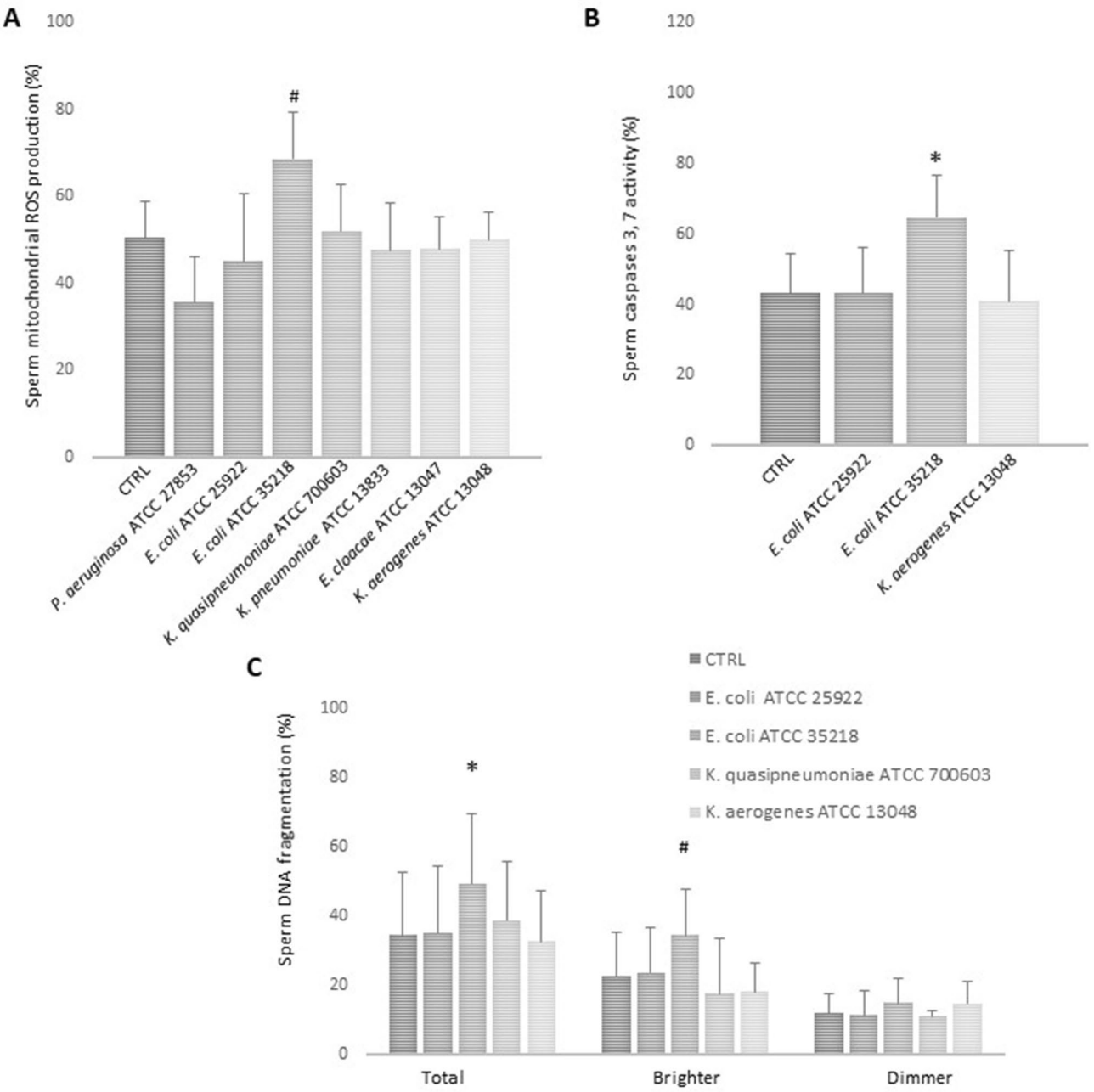

Figure 5. Histograms representing the effect of 3 h in vitro incubation (37 °C, 5%CO_2) of whole semen samples on sperm mitochondrial ROS production, measured by Mitosox Red, with all tested bacterial strains (*P. aeruginosa* ATCC 27853, *E. coli* ATCC 25922, *E. coli* ATCC 35218, *K. quasipneumoniae* ATCC 700603, *K. pneumoniae* ATCC 13883, *E. cloacae* ATCC 13047 and *K. aerogenes* ATCC 13048) (n = 6, panel **A**), sperm caspases 3 and 7 activity, measured by FLICA, with *E. coli* ATCC 25922, *E. coli* ATCC 35218 and *K. aerogenes* ATCC 13048 (n = 5, panel **B**) and sperm DNA fragmentation, measured by TUNEL/PI, with *E. coli* ATCC 25922, *E. coli* ATCC 35218, *K. quasipneumoniae* ATCC 700603 and *K. aerogenes* ATCC 13048 (n = 5, panel **C**). Student t-test, *$p < 0.05$; #$p < 0.01$.

DNA damage and cell death. In particular, the *E. coli* ATCC 35218 was the only strain, among those tested, able to increase mitochondrial ROS production, activate apoptotic pathways and induce sperm DNA fragmentation.

Oxidative stress may be both a consequence and an inducer of sperm apoptosis[16,34], and may be also involved in increasing DNA fragmentation[16,35]. In particular, oxidative stress appears to be the main inducer of sDF after spermiation and during in vitro incubations[16,36–38].

The inducing effects of *E. coli* isolates on oxidative status and apoptosis were reported previously using different experimental conditions compared to our study[14,15,39]. In particular, Boguen et al.[15], by comparing three *E. coli* strains demonstrated that the hemoliytic strain shows a greater detrimental effect on spermatozoa respect to non-haemolytic ones, including the *E. coli* strain ATCC 25922. A comparative genomic analysis of *E. coli* strains

used in our study revealed the unique presence in *E. coli* ATCC 35218 of the chromosomal HlyE gene coding for hemolysinE (data not shown), a toxin with a short half-life that is known to impair membrane integrity in other cell types[40]. Therefore, it is possible that the detrimental effects of *E. coli* strain ATCC 35218 are mediated by more than one spermotoxic factor[41].

Reduction of sperm viability and motility, as it may occur in the case of semen infections, may highly affect the reproductive performance both in natural and assisted conception, as progressive motility is the necessary pre-requisite to reach the oocyte and to penetrate its vestments, whereas viability is of fundamental importance for a correct fertilization. In particular, motility is the primary sign used to determine sperm viability during intracytoplasmic sperm injection (ICSI). If no motility is present in a sample, techniques to identify viable spermatozoa can be used by embryologists[42]. In case of *E. coli* ATCC 35218, where reduction of motility may exceed reduction of viability (89% vs 45% according to viability evaluated by LIVE/DEAD™ Fixable Green Dead Cell Stain), viable spermatozoa may show increased oxidative stress and/or activation of apoptotic pathway and/or fragmented DNA, likely compromising the outcome of reproduction. In particular, the *E. coli* ATCC 35218 induces DNA fragmentation within the PI brighter sperm population, which is unrelated to semen quality and may contain viable DNA fragmented spermatozoa[28]. sDF is associated with a reduced performance in ART affecting implantation and increasing the probability of miscarriage[43–46]. The effect of bacterial contamination in semen on the outcomes of ARTs is controversial. Some studies indicated poor outcomes because of oocyte degeneration[47,48] whereas others did not report significant effects on ART outcomes[49]. Incubation in vitro with *E. coli* reduces sperm ability to penetrate hamster oocytes, suggesting a negative effect on fertilization ability[32].

A strength of our study is testing the effect of strains of the most frequent *Enterobacteriaceae* infecting male reproductive tract, evidencing differences in their effects on sperm characteristics. In addition, we evaluated the effect of bacteria in the natural environment where they may alter sperm functions. In contrast, most previous studies have been performed on highly motile selected sperm[11–15] or washed semen samples[39], where the effect of bacteria is tested in a medium where they never act, and that does not contain substances that can limit or enhance their effects. For instance, it has been shown lactobacilli[14] may prevent the effect of *E. coli* on sperm motility. In addition, fragmented semenogelins generated after liquefaction[50] and enzymes present in semen[51] show antibacterial activity. A limitation of our study is represented by the use of commercially available bacterial strains and not those isolated from semen samples. Moreover, we chose to use strains of *Enterobacteriaceae* with known genome to allow the sequence alignment shown in Fig. 3. Such alignment allowed us to reveal that other *Enterobacteriaceae* contain *recX* homologous sequences in their genome.

In conclusion, our data indicate that common uropathogenic Gram-negative bacteria induce an impairment of sperm motility through *recX* products and suggest that an increased secretion of SIF or other factors, produced by selected strains, may be involved in impairing other sperm functions. Since the effects of bacteria on human spermatozoa may be variable and dependent on the strain, a careful evaluation of semen infections in the diagnostic process of the infertile man is warranted. Further experiments performed on bacterial samples isolated from semen cultures will be necessary in order to reinforce experimental proofs of SIF homologous activity secreted by *Enterobacteriaceae* strains derived from their natural site of infection.

Materials and methods

Ethic statement. The study was approved by the local Ethical Committee Comitato Etico Area Vasta Centro (CEAVC, protocol n. 16764_bio). All research was performed in accordance with the Declaration of Helsinki. Patients were informed about the aim of the study and signed an informed consent to use the remaining semen after routine analysis.

Reagents and bacteria. Human tubal fluid (HTF) medium was purchased from Biocare Europe (Rome, Italy). MitoSOX™ Red, LIVE/DEAD™ Fixable Green Dead Cell Stain and Vybrant FAM Caspase 3 and 7 Assay Kit were purchased from Thermo Fisher Scientific (Waltham, MA, USA). In Situ Cell Death Detection Kit, fluorescein was obtained from Sigma Aldrich (St. Louis, MO, USA).

Seven bacterial reference strains were included in the study as follows: two isolates of *E. coli* (ATCC 29522 and ATCC 35218), one isolate of *K. pneumoniae* (ATCC 13883), one isolate of K. *quasipneumoniae* (ATCC 700603), one isolate of *P. aeruginosa* (ATCC 27853), one *E. cloacae* (ATCC 13047) and one *K. aerogenes* (ATCC 13048). An *E. coli* K12 strain MG1655 and its derivative (with the knockout *recX* gene), from the Keio collection, were also added to the study collection[52]. Most of the selected reference strains were isolated from clinical human samples, except for *K. pneumoniae* ATCC 13883 and *E. coli* ATCC 25922 whose source is unknown (Supplemental Table S1). All bacterial strains were seeded on CHROMID® CPS® Elite agar (bioMérieux, Marcy l'Etoile, France) and incubated for 18 h at 35 ± 1 °C. Bacterial suspensions were prepared in 2 ml of sterile water and optical density was measured by DensiCHEK™ spectrophotometer (bioMérieux).

Extraction of proteins from bacterial supernatants. 100 or 300×10^6 *E. coli* cells from ATCC 25922 strain were cultured in Mueller Hinton broth at 37 °C overnight. Culture supernatants were collected and centrifuged at 4000 g for 10 min. The protein fraction with MW ≥ 30 kDa was purified using Centrifugal Filter Units (cut off 30,000 NWML) (Amicon® Ultra-4 and -15 Centrifugal Filter Units – 30,000 NMWL, Merk, Darmstadt, Germany) according to manufacturing recommendations. Protein amount in the purified fraction was quantified by BCA (Bicinchoninic Acid) method and used at 15 and 45 µg/µL.

Sequence alignment. Reference strains genome were downloaded from LGC website (www.lgcstandards-atcc.org), database homology searches of proteins were carried out using tBLASTn and BLASTp (https://

blast.ncbi.nlm.nih.gov/Blast.cgi) software and amino acidic sequences alignments were performed by AlignX (Invitrogen, Carlsbad, USA) using *E. coli* K12 MG1655 as consensus sequence.

Sperm samples and processing. Semen samples were obtained by masturbation from patients undergoing routine semen analysis for couple infertility, in the Andrology laboratory of the University of Florence, Italy. Semen analysis was carried out according to World Health Organization (WHO) guidelines[33]. Semen samples with leukocytes and/or evident bacteria were excluded from the study. For the study purpose, spermatozoa from n = 32 normozoospermic subjects (see Supplemental Table S2 for semen characteristics) were included. After counting of spermatozoa, whole semen samples were divided in 9 equal aliquots and seeded in 96-well plates at concentration range between 1×10^6 and 10×10^6 cells per well in a final volume of 100 µl. The bacteria infection assay was performed by incubating spermatozoa in presence of bacterial strains at 1, 10 and 100 MOI/cell for 1 and 3 h at 37 °C in a humidified chamber with 5% CO_2. The maximum effect was reached at 10 MOI/cell (data not shown). 10 MOI/cell was then used for all the experiments shown. An equal volume of sterile water was added to control aliquots.

Evaluation of sperm motility. After incubation with different bacterial strains, the percentage of progressive and total sperm motility where checked at the optical microscopy, according to WHO criteria[33], evaluating at least 200 spermatozoa for each experimental point. The analysis was conducted in the Laboratory of Andrology of the Florence Careggi University Hospital that participates in the UK-NEQAS (United Kingdom National External Quality Assessment Service) external quality control program for semen analysis since 2005. The mean (± sd) percent biases of the laboratory for the years 2019 were 7.0 (± 15.6) and 1.1 (± 11.2), respectively, for total and progressive motility and 9.2 (± 6.7) for sperm concentration (n = 16, data from UK-NEQAS).

Evaluation of sperm viability. Sperm viability was evaluated by using eosin staining[33] and by LIVE/DEAD™ Fixable Green Dead Cell Stain Kit. For eosin staining, sperm suspension and eosin (1%) (1:1) were mixed and then evaluated by optical microscopy assessing at least 200 spermatozoa for each aliquot. For staining with LIVE/DEAD™ Fixable Green Dead Cell Stain, after washing with HTF medium, semen samples were incubated for 1 h at room temperature, in the dark, in 500 µL of phosphate-buffered saline (PBS) with Live/Dead Fixable Green Dead Cell Stain Kit (diluted 1:50,000). Then samples were washed twice in PBS and acquired by flow cytometry (see below).

Assessment of mitochondrial ROS generation. Mitochondrial ROS generation was evaluated using MitoSOX Red[14,53] which shows distinct specificities toward superoxide[53]. After incubation with the different bacterial strains, spermatozoa were washed in PBS and then divided into two aliquots, one aliquot was re-suspended in 100 µL PBS (negative control) and one in 100 µL PBS containing Mitosox Red at a final concentration of 2 µM (test sample), and incubated for 15 min at room temperature. After wash in PBS, sperm samples were analysed by flow cytometry (see below).

Evaluation of caspase activity. Caspases activity was evaluated by using Vybrant FAM Caspase-3 and -7 Assay Kit based on a fluorescent inhibitor of caspases (FLICA™) according to Marchiani et al.[54]. After incubation with bacteria, each sample was splitted into two aliquots: a test sample re-suspended in 300 µL of PBS added with 10 µL of 30X FLICA working solution and a negative control incubated only with PBS. After 1 h incubation at 37 °C, samples were washed with Wash Buffer 1X and fixed with 40 µL of 10% formaldehyde for 10 min at room temperature. Wash and fixative solutions were supplied by the kit. Sperm samples were washed again twice and re-suspended in 400 µL of Wash Buffer 1X containing 6 µL of Propidium Iodide solution (PI, 50 µg/mL in PBS) and acquired by flow cytometry (see below).

Evaluation of sperm DNA fragmentation. Sperm DNA fragmentation was evaluated by Tunel/PI method[28]. Briefly, after incubation with bacteria and washing twice with HTF medium, each aliquot was fixed with 200 µL of paraformaldehyde (4% in PBS, pH 7.4) for 30 min at room temperature. Semen samples were washed twice with 200 µL of PBS/1% bovine serum albumin (BSA), and then permeabilized with 100 µL of 0.1% sodium citrate/0.1% Triton X-100 (4 min in ice). Each sperm sample was divided into two aliquots and labelled with 50 µL of labelling solution (supplied by the kit) containing (test sample) or not (negative control) the terminal deoxynucleotidyl transferase (TdT) enzyme and incubated for 1 h at 37 °C in the dark. Samples were then washed twice, re-suspended in 500 µL of PBS and stained with 7.5 µL of PI (50 mg/mL, 10 min at room temperature in the dark) and acquired by flow cytometry.

Flow cytometric analysis. Samples were acquired by a FACScan flow cytometer equipped with a 15-mW argon-ion laser for excitation. FL-1 (515–555-nm wavelength band) and FL-2 (563–607-nm wavelength band) detectors revealed green fluorescence of LIVE/DEAD™ Fixable Green Dead Cell Stain, caspases and Tunel and red fluorescence of Mitosox Red and PI, respectively. In the characteristic forward scatter/side scatter region of spermatozoa[28], 8000 events were acquired. In the dot plot of fluorescence distribution of the negative sample, a marker, including 99% of total events, was established and translated in the corresponding test sample and all the events beyond the marker were considered positive. sDF was evaluated in the two sperm populations denominated PI brighter and PI dimmer[28] and reported as percentage of sDF in the two populations and in total sperm. For acquisition and analysis, CellQuest-Pro software program (Becton–Dickinson) was used.

Statistical analysis. Statistical analysis was performed using the Statistical package for the Social Sciences version 26.0 (SPSS, Chicago, IL, USA) for Windows. Data distribution was verified by using the Kolmogorov–Smirnov test. Data normally distributed were expressed as mean (± SD), whereas, data non-normally distributed as median (interquartile, IQR). Differences between groups were evaluated by paired two-sided Student's t-test for normally distributed parameters, or by Wilcoxon signed-rank test for non-normally distributed parameters. A P-value of 0.05 was considered significant.

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Author contributions
S.M. coordinated the experiments, performed statistical analysis, interpreted the results, and drafted the article; I.B. prepared bacteria and performed sequence alignment; L.T. performed experiments and interpreted the results; G.M. interpreted the results and performed data curation; S.N. prepared bacteria; C.B. performed sequence alignment; C.P. performed experiments; L.V. and G.R. contributed to the final review of the manuscript; A.A. interpreted the results and revised the manuscript; M.T. and E.B. conceived and designed the study, drafted and revised critically the manuscript. All the Authors approved the final version to be submitted.

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ORIGINAL ARTICLE *Andrology*

Semen impairment and occurrence of SARS-CoV-2 virus in semen after recovery from COVID-19

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STUDY QUESTION: How is the semen quality of sexually active men following recovery from severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection?

SUMMARY ANSWER: Twenty-five percent of the men with recent SARS-Cov-2 infections and proven healing were oligo-crypto-azoospermic, despite the absence of virus RNA in semen.

WHAT IS KNOWN ALREADY: The presence of SARS-CoV-2 in human semen and its role in virus contagion and semen quality after recovery from coronavirus disease 2019 (COVID-19) is still unclear. So far, studies evaluating semen quality and the occurrence of SARS-CoV-2 in semen of infected or proven recovered men are scarce and included a limited number of participants.

STUDY DESIGN, SIZE, DURATION: A prospective cross-sectional study on 43 sexually active men who were known to have recovered from SARS-CoV2 was performed. Four biological fluid samples, namely saliva, pre-ejaculation urine, semen, and post-ejaculation urine, were tested for the SARS-CoV-2 genome. Female partners were retested if any specimen was found to be SARS-CoV-2 positive. Routine semen analysis and quantification of semen leukocytes and interleukin-8 (IL-8) levels were performed.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Questionnaires including International Index of Erectile Function and Male Sexual Health Questionnaire Short Form were administered to all subjects. The occurrence of virus RNA was evaluated in all the biological fluids collected by RT-PCR. Semen parameters were evaluated according to the World Health Organization manual edition V. Semen IL-8 levels were evaluated by a two-step ELISA method.

MAIN RESULTS AND THE ROLE OF CHANCE: After recovery from COVID-19, 25% of the men studied were oligo-crypto-azoospermic. Of the 11 men with semen impairment, 8 were azoospermic and 3 were oligospermic. A total of 33 patients (76.7%) showed pathological levels of IL-8 in semen. Oligo-crypto-azoospermia was significantly related to COVID-19 severity ($P < 0.001$). Three patients (7%) tested positive for at least one sample (one saliva; one pre-ejaculation urine; one semen and one post-ejaculation urine), so the next day new nasopharyngeal swabs were collected. The results from these three patients and their partners were all negative for SARS-CoV-2.

LIMITATIONS, REASONS FOR CAUTION: Although crypto-azoospermia was found in a high percentage of men who had recovered from COVID-19, clearly exceeding the percentage found in the general population, the previous semen quality of these men was unknown nor is it known whether a recovery of testicular function was occurring. The low number of enrolled patients may limit the statistical power of study.

WIDER IMPLICATIONS OF THE FINDINGS: SARS-CoV-2 can be detected in saliva, urine, and semen in a small percentage of men who recovered from COVID-19. One-quarter of men who recovered from COVID-19 demonstrated oligo-crypto-azoospermia indicating that an assessment of semen quality should be recommended for men of reproductive age who are affected by COVID-19.

STUDY FUNDING/COMPETING INTEREST(S): None.

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Key words: COVID-19 / coronavirus disease 2019 / semen / oligo-crypto-azoospermia / fertility / sexual transmission / SARS-CoV-2 / severe acute respiratory syndrome coronavirus 2

Introduction

The world is currently experiencing the outbreak of coronavirus disease (COVID-19) infection caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The virus exhibits a strong infectivity with a low virulence compared to previous coronavirus strains, and a higher fatality rate in men than in women (COVID-19 Dashboard by the Center for Systems Science and Engineering (CSSE) at Johns Hopkins University (JHU), 2020). Widespread contact tracing followed by hospital isolation or quarantine is recommended to contain the outbreak (Swerdlow and Finelli, 2020).

Human-to-human transmission of SARS-CoV-2 usually occurs within households, including relatives and friends who are in intimate contact with symptomatic or incubating patients (Pan *et al.*, 2020). In some patients, blood samples showed positive RT-PCR test results, suggesting that infection may be occasionally systemic (Wang *et al.*, 2020). Respiratory droplets and physical contact of contaminated surfaces are the confirmed transmission modality of SARS-CoV-2 (Rothe *et al.*, 2020), even if the virus has also been detected in saliva (To *et al.*, 2020), teardrops, urine, and stool (Peng *et al.*, 2020).

The occurrence of viral genomes in semen of infected men, previously not identified as being sexually transmitted, has been reported for Ebola and Zika (Feldmann, 2018). The high expression of angiotensin-converting enzyme (ACE) and transmembrane serine protease 2 (TMPRSS2) in somatic and germ cells of the testis suggests that SARS-CoV-2 virus may localize in the gonad (Wang and Xu, 2020). A recent study (Rastrelli *et al.*, 2020) demonstrated the development of hypergonadotrophic hypogonadism in patients with active COVID-19 infection, suggesting an impairment of Leydig cell function, although whether this impairment is related to localization of the virus in the testis is unknown. However, in view of the essential role played by testosterone in spermatogenesis (Smith and Walker, 2014), alterations of semen quality in patients with COVID-19 can be hypothesized. In addition, semen parameters may be compromised by the inflammatory condition occurring in men with COVID-19.

Few studies have evaluated the presence of the virus in human semen. Paoli *et al.* reported the absence of SARS-CoV-2 RNA in semen and urine samples 13 days after COVID-19 diagnosis and 4 days after the second negative nasopharyngeal (NP) swab in a single patient who tested positive for SARS-CoV-2 through a NP-swab (PaoLi *et al.*, 2020). Li *et al.* (2020) demonstrated that the semen of six patients tested positive for SARS-CoV-2, with four of these patients at the acute stage of infection and two at 2 and 3 days after clinical recovery, respectively. Pan *et al.* (2020) did not find viral RNA in any of 34 semen samples from males with active infection. Similarly, Song *et al.* (2020) demonstrated no detectable SARS-CoV-2 RNA in semen samples collected from 12 patients during the recovery phase: after 1–16 days from the second negative NP swab and 14–42 days after acute infection (Song *et al.*, 2020). Only two studies (Guo *et al.*, 2020; Ma *et al.*, 2020) evaluated semen quality in a small cohort of men during infection or in the recovery phase of COVID-19.

In the present study, we investigated the direct (RNA) presence of SARS-CoV-2 in saliva, urine, and semen; evaluated semen parameters; and investigated semen IL-8 levels as a surrogate marker of the male genital tract inflammatory condition (Lotti *et al.*, 2011) of 43 sexually active men with laboratory evidence of recovery from COVID-19.

Materials and methods

Study population and design

This prospective cohort study was conducted following the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines. All male patients with SARS-CoV-2 infection, confirmed using molecular methods on respiratory specimens, were identified. Sexually active men aged 18–65 years with proven recovery from SARS-CoV2 infection (two consecutive negative NP-swabs for SARS-CoV2 RNA) were eligible for the study (World Health Organization, 2020). Men without sexual activity or with ejaculatory disorders, being treated with prostatic surgery or alpha-blockers and those with an inability to express informed consent were excluded from the study. All the partners of the enrolled patients were tested for SARS-CoV-2 positivity (NP swabs) at the time of the original diagnosis of the men and retested according to National Health Care System criteria until NP-swab double negativity, as suggested by World Health Organization (WHO) on clinical management of COVID-19 (WHO, 2020).

Moreover, if one or more specimens collected from the patients enrolled during the study were positive, partners were retested.

Ethics

The study was carried out with the approval of the local Ethics Committee (Rif: CEAVC17104) and registered on clinicaltrial.gov (Rif: NCT04446169), in compliance with the Declaration of Helsinki. All enrolled men provided written informed consent.

Clinical data and specimen collection

Clinical data included patient demographics, comorbidities, medications, hospitalization time and features (including intensive care need), and laboratory tests and treatments (including oxygen therapy). Moreover, patients were asked to fill out questionnaires, including

International Index of Erectile Function (IIEF-5) and Male Sexual Health Questionnaire Short Form (MSHQ-SF), in order to assess a comprehensive urological, sexual and reproductive anamnesis. Data on COVID-19 status were recorded for patients' partners.

Four biological fluid samples (saliva, pre-ejaculation urine, sperm obtained with masturbation, and first fraction post-ejaculation urine) were collected in four sterile jars to be tested for SARS-CoV-2. After liquefaction of semen and assessment of volume, semen samples were divided into two aliquots, one for evaluation of the presence of SARS-CoV-2 virus by RT-PCR and one for semen analysis.

Detection of SARS-CoV-2 in biological specimens

All samples were processed on the same day as collection or stored at −80°C until further analysis. Nucleic acids from samples were extracted with the Microlab Nimbus IVD system (Seegene Inc, Seoul, South Korea) using the Starmag Universal Cartridge and amplified with the multiplex RT-PCR Allplex™ 2019-nCoV assay (Seegene Inc), targeting RdrP, E and N genes, according to the manufacturers' instructions.

Semen analysis and IL-8 evaluation

Semen analysis was carried out according to WHO guidelines (WHO, 2010). Quantification of leukocytes in semen was performed by counting the number of round cells per milliliter using an improved Neubauer hemocytometer and evaluating the percentage of leukocytes and immature germ cells after May-Grunwald staining of the sample.

For IL-8 evaluation, semen plasma aliquots were stored frozen and IL-8 levels were quantified by conventional two-step ELISA using a human IL-8 ELISA kit (BD Bioscience, San Diego, CA, USA), according to the manufacturer's instructions.

Statistics

Patients were divided into different groups for comparisons according to hospitalization, intensity of treatment, and semen parameters. Differences were tested with Independent Sample Student's *t*-test, Mann–Whitney u-test, and univariate analysis of variance (ANOVA) for continuous variables, and with χ^2 and Fisher's Exact Test for categorical variables according to sample size. A logistic regression was carried out including significant factors to better define the risk of sexual transmission and to identify the main determinants of impairment of semen quality. A value of $P < 0.05$ was considered to be significant. All statistical analyses were performed using IBM SPSS version 20.0 (SPSS Inc, Chicago, IL, USA).

Results

Patient characteristics and clinical features

From 326 male patients with a positive NP-swab, 179 were between 18 and 65 years of age and were eligible for inclusion, among these, 43 agreed to participate in the study (Fig. 1). All enrolled patients previously affected by COVID-19 had a negative result from at least two consecutive NP swabs before sample collection. Clinical features, including urological and andrological medical history, are summarized in Table I.

Timelines from the first positive NP swab to sample collection are reported in Fig. 2. In particular, median proven healing time (time from first positive to second consecutive negative NP-swab) was 31 days (range: 3–65), whereas median SARS-CoV-2-free time (time between second negative NP-swab and sample collection) was 35 days (r: 24–43).

Twelve patients (27.9%) were not hospitalized, 26 (60.5%) were hospitalized in internal medicine unit, and 5 (11.6%) were admitted to intensive care unit (Table II). Overall 19 patients (44.2%) did not require oxygen therapy, 10 (23.2%) were treated with low flow O_2 therapy, whereas 14 (32.6%) necessitated high flow/invasive O_2 therapy.

The sexual habits of enrolled men are reported in Table II. Six patients (13.9%) were without a fixed partner, while 37 (86.1%) had a stable relationship. Fourteen out of 37 stable female partners (37.8%) presented a positive NP swab for SARS-CoV-2 at the time of partner diagnosis (Table II) and four were positive at the time of enrollment of men, with two subsequent negative results for the two NP swabs performed according to National guidelines (WHO, 2020). Five of the 11 stable partners of nonhospitalized (45.5%), 8 of the 22 of the hospitalized men (34.8%) and one of the four stable partners of men requiring intensive care support (20%) tested positive at the time of partner diagnosis.

Twenty-nine male patients with a stable partner (78.4%) did not use condom: 10 of these female partners (34.5%) had a positive NP swab. In this subpopulation of stable partners having sex without condom, multivariate analysis did not show a difference in the number of sexual intercourse events per month between female partners with positive versus negative NP swab (median: 5 vs. 4, $P = 0.470$; data not shown).

SARS-CoV-2 detection in collected specimens

A total of 170 samples collected from 43 enrolled patients were tested by RT-PCR, namely saliva, urine sample collected before ejaculation, and semen and urine sample collected after ejaculation. Two samples from two different patients yielded inconclusive results. Forty patients (93%) were negative for SARS-CoV-2 RNA in any tested sample. Three patients (7%) tested positive in at least one sample: one (patient A) in saliva (collected 62 days after the second negative swab), which was positive for all target genes; one (patient B) in pre-ejaculation urines (44 days after the second negative swab), which were positive only for the N-gene (Threshold Cycle (Ct)= 37.04); one (patient C) in semen, where all target genes were detected, and in post-ejaculation urines where only the E- and N-genes were detected (Ct = 34.99 and 37.16, respectively) (21 days after the second negative swab). Patients A and C needed intensive care support during COVID-19 infection. These three patients and their partners were retested for SARS-CoV-2 RNA with NP-swabs and all samples tested negative.

Patient A was rehospitalized for sepsis resulting from bacterial infection and was treated with antibiotics, with subsequent healing. Patient B was asymptomatic, with no signs or symptoms of recurrence of COVID-19 and decided independently to undergo self-quarantine. Patient C reported having unprotected oral, vaginal, and anal sex with his stable partner after recovering from COVID-19. Therefore, they were further investigated using a urethral swab (patient) and pharyngeal, vaginal, and rectal swabs (partner): all samples tested negative.

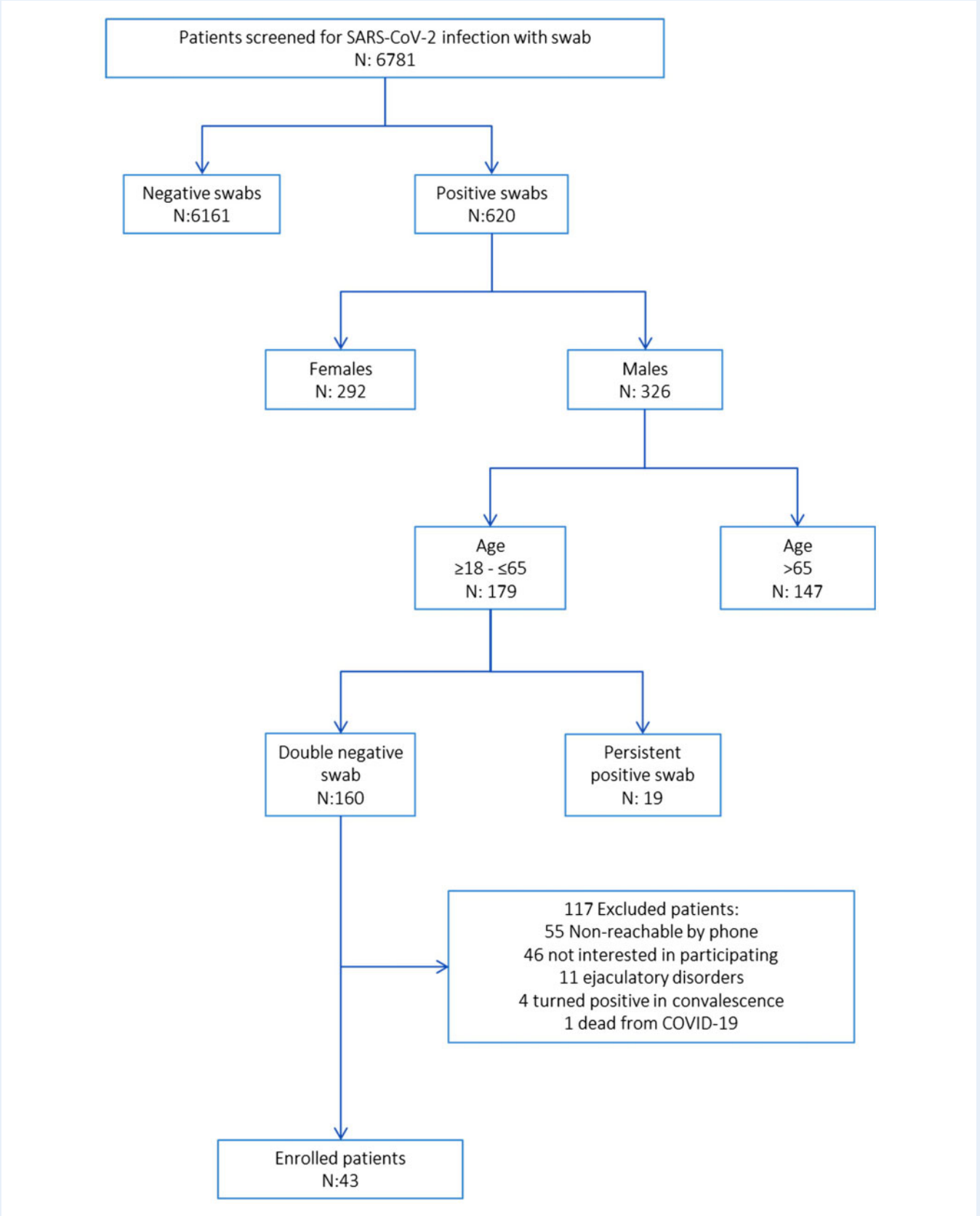

Figure 1. Flowchart of screening, eligibility, and the inclusion process for the study sample of men who recovered from COVID-19. Age is in years. CODIV-19: coronavirus disease 2019, SARS-CoV-2: severe acute respiratory syndrome coronavirus.

Table I Comparison of clinical, social, and uro-andrological characteristics of the study sample (N = 43) stratified by clinical management of COVID-19.

		Nonhospitalized (N = 12; 27.9%)	Hospitalized (without ICU) (N = 26; 60.5%)	ICU (N = 5; 11.6%)	*P* value[a]
DEMOGRAPHICS					
Age (years) (median, IQR)		44 (33–49)	52 (48–58)	59 (56–59)	**0.003**
BMI (kg/m^2) (median, IQR)		26.1 (23.7–27.9)	26.9 (23.6–31.0)	27.4 (26.3–28.7)	0.380
Smoking status	Never (n, %)	9 (75.0%)	22 (84.6%)	4 (80.0%)	0.850
	Current (n, %)	2 (16.7%)	1 (3.8%)	0 (0.0%)	0.157
	Former (n, %)	1 (8.3%)	3 (11.5%)	1 (20.0%)	0.401
Period between last negative oropharyngeal swab and sample collection (days) (median, IQR)		30 (23–39)	37 (26–49)	24 (23–32)	0.558
ANDROLOGICAL FEATURES					
Previous orchitis/genital infection (n, %)		2 (16.7%)	3 (11.5%)	0 (0.0%)	0.814
Previous parotitis (n, %)		4 (33.3%)	3 (11.5%)	1 (20.0%)	0.209
Previous surgically treated varicocele (n, %)		2 (16.7%)	2 (7.7%)	0 (0.0%)	0.747
IIEF-5[b] (median, IQR)		25 (24–25)	24 (21–25)	22 (22–23)	0.389
MSHQ[c] erection scale ([1–4]) (median, IQR)		19 (17–20)	19 (17–20)	19 (17–20)	0.487
MSHQ[c] ejaculation scale ([5–12]) (median, IQR)		37 (33–38)	36 (35–38)	34 (32–39)	0.165
MSHQ[c] satisfaction scale ([13–18]) (median, IQR)		25 (23–25)	26 (24–30)	28 (26–29)	0.433
MSHQ[c] sexual activity (19) (median, IQR)		3 (2–4)	3 (2–4)	4 (3–5)	0.554
MSHQ[c] sexual activity (20, 21) (median, IQR)		8 (6–8)	8 (7–8)	8 (8–8)	0.179
MSHQ[c] sexual desire (22–25) (median, IQR)		16 (14–16)	16 (16–17)	17 (15–18)	0.353

[a]Calculated using Chi Quadro or Fischer test for categorical variables or ANOVA test for continuous variables.
[b]International Index of Erectile Function Questionnaire.
[c]Male Sexual Health Questionnaire.
COVID-19: coronavirus disease 2019; IQR: interquartile range, ICU: intensive care unit.

Semen parameters

Data for parental and fertility status of the included patients are reported in Table II. Five of the 12 (41.6%) nonhospitalized, 5 of the 26 (19.2%) of the hospitalized and one of the five men (20%) requiring intensive care support had no children, with no significant difference between groups ($P = 0.152$).

Semen analysis demonstrated that eight patients (18.6%) were azoospermic and three were oligospermic with less than 2 million/mL (7.0%) spermatozoa: overall, 25.6% of patients were oligo-cryptoazoospermic. The occurrence of azoospermia was highly related to the severity of the illness: the condition was found in four out of five patients admitted to the intensive care unit, in three of the 26 hospitalized in the medicine department and only in one among the 12 nonhospitalized ($P < 0.001$). No relation was found between occurrence of azoospermia and severity of oxygen therapy ($P = 0.417$). Semen parameters are reported in Table III.

The presence of leukocytes in semen was found in 16 patients (37.2%) and their occurrence was higher in men admitted to the intensive care unit as compared to non-hospitalized patients or those admitted to the medicine department, but was not related to the intensity of oxygen therapy (36.8% in men without O_2 vs. 30.0% with low flow O_2 and 42.9% with high flow/invasive O_2).

Thirty-three patients (76.7%) showed pathological levels of semen IL-8 (sIL-8) (i.e., >3.8 ng/mL) (Hofny *et al.*, 2011): among them, 7 out of 12, (58.3%) were not hospitalized during the illness, 21 of 26 (80.8%) were hospitalized in the medicine department, and 5 (100.0%) were admitted to the intensive care unit ($P = 0.142$) (Fig. 3A). Pathological levels of sIL-8 were related to the severity of oxygen therapy ($r = 0.356$; $P = 0.050$). As shown in Fig. 3B, high levels of sIL-8 were found in 12 out of 19 (63.2%) men without oxygen therapy, 9 out of 10 (90.0%) with low flow O_2-therapy and 12 out of 14 (85.7%) necessitating high flow/invasive O_2 therapy.

In univariate analysis, age, hospitalization and sIL-8 were all significant items for azoospermia (Supplementary Table SI). In multivariate analysis, hospitalization (not recovered vs. hospitalized vs. intensive care unit) was the main determinant of crypto-azoospermia ($P = 0.039$) (data not shown).

Discussion

In our sample, the age range was from 30 to 64 years. This study demonstrates that young and sexually active men with proven recovery

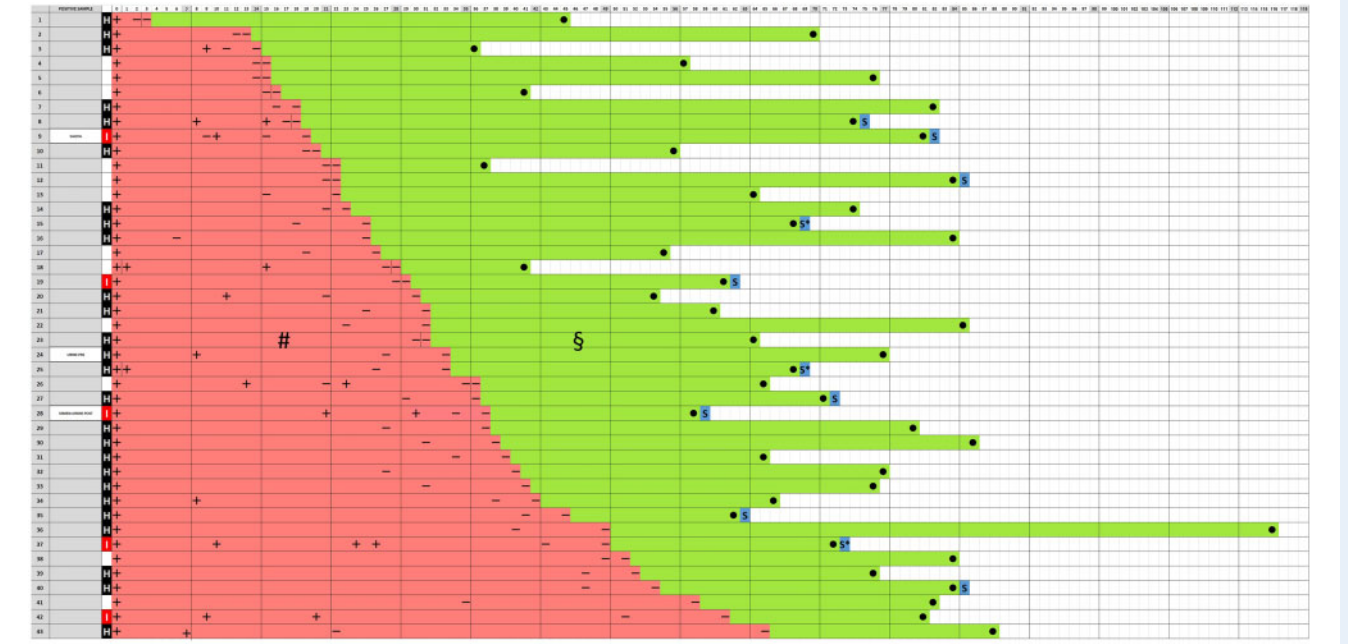

Figure 2. Timeline in days of the period between the first positive nasopharyngeal swab for COVID-19 and sample collection. Urine pre: urine collected before semen collection; urine post: urine collected after semen collection. Timeline (days) from first positive nasopharyngeal swab for COVID-19 and the sample collection for each patient. In the first column, the origin of the samples is reported. White box: not hospitalized patient; 'H': patient hospitalized in medicine units; 'I': patient hospitalized in intensive care unit. Red squares indicate the period between the first positive nasopharyngeal swab for COVID-19 and the second negative nasopharyngeal swab, while the green ones represent the period between the second negative nasopharyngeal swab and the date of sample collection. The symbols '+' and '−' indicate a positive or a negative nasopharyngeal swab result for SARS-CoV-2 RNA. 'S': azoospermic; 'S*': oligospermic; ●: sample collection time; #: median proven healing time: 31 days (range 3–65); §: median SARS-CoV-2-free time: 35 days (range: 24–43).

from COVID-19 have a significant risk of developing oligo-crypto-azoospermia.

Prolonged viral shedding from the nasopharynx in convalescent patients, up to at least 3 weeks after recovery from symptoms, has been previously reported (Young *et al.*, 2020). According to the European Centre for Disease Prevention and Control (ECDC) we considered as healed from SARS-CoV-2 infection those men with two upper respiratory tract samples that were negative for SARS-CoV-2 RNA, and showing clinical resolution of symptoms (https://www.ecdc.europa.eu/sites/default/files/documents/COVID-19-Discharge-criteria.pdf). However, in our population, one patient died from COVID-19 after clinical relapse and four tested positive again for SARS-CoV-2 infection by NP swab during the screening phase (from March 2020 to May 2020). Moreover, three men presented with persistence of SARS-COV-2 RNA in biological fluids 3–9 weeks after double negative NP swabs.

The occurrence of the virus in saliva, urine, and semen of asymptomatic patients several weeks after double negative NP swabs was demonstrated in a very small percentage of men in our cohort; nevertheless previous studies (Guo *et al.*, 2020; Ma *et al.*, 2020; Pan *et al.*, 2020) did not report occurrence of virus in semen of healed patients or in their recovery phase. Interestingly, none of the partners of these three newly positive patients tested positive for SARS-CoV-2 including the patient with a positive test result for semen. Overall, these studies suggest that the occurrence of virus in semen is a rare event. Further studies will be required to determine the risk of SARS-CoV-2 transmission by sexual intercourse.

The occurrence in the male reproductive system, in particular testis, has been described for several viral strains. The HIV and mumps virus might lead to male infertility as a result of direct damage to the testicular structure (Masarani *et al.*, 2006; Garolla *et al.*, 2013). Abnormal sperm parameters have been observed in other viral infections such as herpes simplex virus, hepatitis virus B, or hepatitis virus C (Garolla *et al.*, 2013). Recent findings seem to support an influence of SARS-CoV-2 infection on male sex hormones, with an increase in plasma levels of LH and a marked decrease in testosterone and FSH (Ma *et al.*, 2020).

In our study, we found that one out of four (11/43, 25.5%) of COVID-19 healed patients were oligo-crypto-azoospermic, a percentage clearly exceeding that reported for the general population (about 1% for azoospermia, Jarow *et al.*, 1989; 3% for oligozoospermia, Ombelet *et al.*, 2009). Interestingly, all azoospermic patients reported a previous unimpaired fertility status (five had one child, two had two children, and one had three children), and only one out of three oligo-crypto-azoospermic men reported no parenthood (while the other two each had two children). Occurrence of azoospermia could be related to antibiotics or other drugs used for the treatment of patients during COVID-19. Indeed, antibiotics, antiviral drugs, chloroquine,

Table II Comparison of relationship and parental status of the study sample (N = 43) stratified by clinical management of COVID-19.

		Nonhospitalized (N = 12; 27.9%)	Hospitalized (without ICU) (N = 26; 60.5%)	ICU (N = 5; 11.6%)	*P* value[a]
RELATIONSHIPS AND PARENTHOOD					
Long-term female partner (n, %)		11 (91.7%)	22 (84.6%)	4 (80.0%)	0.838
Long-term partner	Past COVID-19 infection at time of partner diagnosis (n, %)	5 (45.5%)	8 (34.8%)	1 (20.0%)	0.714
	Positive for COVID-19 at enrollment (n, %)	2 (18.2%)	2 (9.0%)	0	0.710
Patients without children		5 (41.6%)	5 (19.2%)	1 (20.0%)	0.152
Number of children (median, IQR)		2 (0–2)	2 (1–2)	1 (1–1)	0.472
Number of sexual relationships during the last 3 months	None (n, %)	2 (16.7%)	6 (23.1%)	1 (20.0%)	0.979
	1–4 per month (n, %)	9 (75.0%)	16 (61.5%)	3 (60.0%)	0.648
	4–8 per month (n, %)	1 (8.3%)	3 (11.5%)	1 (20.0%)	0.635
	>8 per month (n, %)	0 (0.0%)	1 (3.9%)	0 (0.0%)	0.958
Problems having a baby (n, %)		3 (25.0%)	4 (16.0%)	1 (20.0%)	0.851
Abortion by the long-term partner (n, %)		3 (25.0%)	6 (23.1%)	0 (0.0%)	0.447

[a]Calculated using Chi Quadro or Fischer test for categorical variables or ANOVA test for continuous variables.

Table III Comparison of semen analysis of the study sample (N = 43) stratified by clinical management of COVID-19.

	Nonhospitalized (N = 12; 27.9%)	Hospitalized (without ICU) (N = 26; 60.5%)	ICU (N = 5; 11.6%)	*P* value[a]
SEMEN ANALYSIS				
Collected semen volume (mL) (median, IQR)	2.5 (1.5–3.5)	2.0 (0.8–2.5)	1.5 (1–2)	0.778
Total sperm number (millions) (median, IQR)	133.25 (50.5–244.1)	38.1 (5.5–123.8)	0.0 (0.0–7.0)	**0.021**
Sperm concentration (millions/mL) (median, IQR)	65.8 (23.8–71.0)	17.8 (5.5–70.0)	0.0 (0.0–3.5)	0.215
Progressive motility (%) (median, IQR)	36.0 (26.0–58.0)	25.0 (12.0–42.0)	27.0 (27.0–27.0)	0.154
Time from collection to analysis[b] (minutes) (median, IQR)	210 (168–270)	240 (180–300)	340 (120–270)	0.558
Vitality (live sperm, %) (median, IQR)	59 (47–81)	49 (31–67)	0 (0–9)	**0.003**
Sperm morphology (%) (median, IQR)	2 (2–5)	3 (1–4)	0 (0–0)	0.332
pH (median, IQR)	7.6 (7.4–7.6)	7.3 (7.2–7.6)	7.6 (7.6–7.6)	0.598
Leucocytes (10^6/mL) (median, IQR)	0.0 (0.0–0.24)	0.0 (0.0–0.1)	0.3 (0.1–1.7)	0.324
Interleukin-8 (ng/mL) (median, IQR)	6 (3–10)	7 (5–11)	37 (13–78)	**<0.001**

[a]Calculated using Chi Quadro or Fischer test for categorical variables or ANOVA test for continuous variables.
[b]Time needed to carry the samples to laboratory.

corticosteroids, and immunomodulators might affect male fertility status (Semet *et al.*, 2017). However, a contribution to impairment of testicular function from SARS-COV-2 infection cannot be excluded, considering that, on average, the time from recovery was around 30 days, which is below the length of a complete cycle of human spermatogenesis. The virus may localize in the testis owing to the elevated expression of ACE and TMPRSS2 in the organ, and COVID-19 patients with active infection show low testosterone levels (Rastrelli *et al.*, 2020; Wang *et al.*, 2020). Moreover, although antibiotics have long been suspected of contributing to male infertility (Semet *et al.*, 2017), limited data support this hypothesis (Samplaski and Nangia, 2015). Semen quality could also be affected by febrile illness, even if limited data are currently available. In particular, semen concentration was significantly influenced by fever occurring during the meiosis and postmeiotic period, with a mean decrease of 32.6% and 35%, respectively (Carlsen *et al.*, 2003). Moreover, a large variation in sperm concentration was observed, and semen recovery was obtained almost 60 days after temperature normalization (MacLeod, 1951).

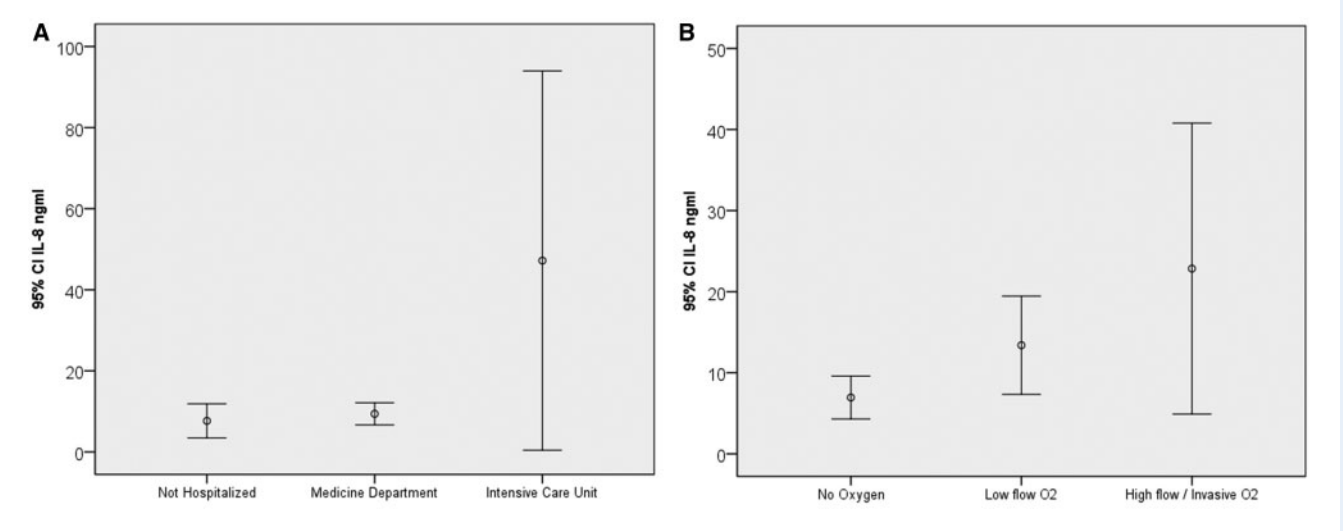

Figure 3. The 95% CIs for mean interleukin-8 concentration in semen of the enrolled patients (N = 43). Data were stratified by clinical management (not hospitalized N = 12, medicine department N = 26, intensive care unit N = 5) (**A**) and need of oxygen support (no oxygen N = 19, low flow O_2 N = 10, high flow O_2 N = 14) (**B**) for COVID-19. The time of collection is shown in Fig. 2. IL-8: interleukin-8. [a]Calculated using ANOVA test. Low flow O_2 Therapy includes nasal cannula, simple face mask, and partial rebreather mask. High flow oxygen/invasive O_2 includes trans tracheal catheters, venturi mask, aerosol mask, tracheostomy collars, non-rebreathing mask with reservoir and one way valve and high humidity face tents.

In our cohort of patients, one crypto-azoospermic semen was analyzed 4 weeks after temperature normalization and one oligospermic after 6 weeks, while the other nine oligo-crypto-azoospermic patients were tested at least 8 weeks after COVID-19 clinical healing. Moreover, 8 out of 11 men with semen impairment were crypto-azoospermics and three were oligospermics. Thus, the impact of fever on semen quality seems negligible.

Furthermore, the virus can have direct (viral replication and dissemination in the testis) and indirect (fever and immunopathology) effects on testicular function (Carlsen *et al.*, 2003). Leukocytospermia is associated with male accessory gland infection (MAGI). However, the pathophysiology of leukocytes in semen is unclear and the diagnostic significance is still debated (Grande *et al.*, 2018). New seminal biomarkers, including some cytokines, have been reported as the most promising putative markers of infection (Grande *et al.*, 2018). IL-8 is a chemokine involved in several inflammatory diseases, comprising inflammation of the prostate, seminal vesicles, and epididymis (Penna *et al.*, 2007; Lotti and *et al.*, 2012; Lotti and Maggi, 2013). Therefore, IL-8 has been proposed as a specific marker for silent MAGI (Lotti *et al.*, 2011). The high levels of sIL-8 found in a high percentage of our patients support the persistence of an inflammatory condition within the male genitourinary tract (Penna *et al.*, 2007) after healing. In addition, reanimation treatments (e.g., invasive ventilation, administration of sedative drugs, specific organ support therapy) and body distress can affect testicular function (Vishvkarma and Rajender, 2020). Clearly, in order to understand whether these men can recover from poor testicular function, they should be reassessed at least 3 months after complete healing from COVID-19.

Our study has some limitations. First, the number of enrolled patients (43) may limit the statistical power of our study regarding the cause-effect relationship between COVID-19 infection, semen quality, and sexual transmission. However, ours is the largest among studies published to date reporting on semen quality and occurrence of virus genome in the semen of sexually active men who were previously infected and who recovered from COVID-19 (12 patients in Ma *et al.*, 2020; 23 in Guo *et al.*, 2020). Another limitation of our study is that semen quality before infection of the men that demonstrated severe oligo-crypto-azoospermia after COVID-19 was not known. However, all patients who were crypto-azoospermic had previously had children.

The strength of our study is that that the SARS-CoV-2 genome has been analyzed in four different biological samples, from different anatomical sites, collected simultaneously. Finally, the study was performed over a short time frame, and in a single center: this allows us to minimize biases related to virus modifications or analytic procedures.

Conclusion

The SARS-CoV-2 genome can be detected in biological fluids such as saliva, urine, and semen in a small percentage of sexually active men who have recovered from COVID-19. Even if our evidence suggests that the risk of SARS-CoV-2 sexual transmission after recovery in stable partners seems negligible, caution should be taken when managing the semen of healed COVID-19 patients during assisted reproduction and cryopreservation.

Most importantly, one-quarter of men who recovered from COVID-19 demonstrated oligo-crypto-azoospermia and signs of male genital tract inflammation, strictly related to disease severity. Therefore, our study suggests that COVID-19 patients of reproductive age should undergo a careful follow up for reproductive function and semen parameters.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

Authors' roles

Conception and Design: M.G., S.S., E.B., F.A., M.M., L.V., A.B. and G.M.R. Acquisition of data: A.M., A.P., R.N., C.B., A.L. and L.G. Analysis and interpretation of data: A.S., M.G., E.B., S.M., S.M., S.D. and S.D. Drafting the manuscript: M.G. and A.S. Critical revision of the manuscript: all the Authors. Statistical analysis: S.M., M.G., E.B. and S.P. Administrative, technical or material support: A.A., S.P., F.L. and M.C. Supervision: S.G. Ethical approval: S.M. and C.Z.

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Conflict of interest

None.

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human reproduction LETTER TO THE EDITOR

Reply: COVID-19: semen impairment may not be related to the virus

Sir,

We read the letter by Bendayan and Boitrelle, regarding our recent paper reporting the impact of COVID-19 on male fertility, with interest (Bendayan and Boitrelle, 2021).

As correctly stated in the letter, we found a high proportion of men showing oligo-crypto-azoo-spermia about 1 month after recovery from the disease (Gacci *et al.*, 2021). In our paper, we evidenced the need for a careful evaluation of the fertility status of men recovering from COVID-19.

The official website of government of Hubei Province posted a bulletin suggesting that men infected with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) should undergo fertility checks (Meng *et al.*, 2021). On the other hand, SARS-Cov-2 infection can damage several organs besides lungs, including the testis. Moreover, COVID-19 predominantly affects male patients: therefore, the possible impact on male fertility should not be ignored (Ding *et al.*, 2004).

In particular, several evidences demonstrated that SARS-CoV-2 uses the angiotensin-converting-enzyme 2 (ACE2) receptor to enter cells and this could cause pathological injuries in multiple organs, including testes which show a high expression of ACE2 receptor(Wang and Xu, 2020). Therefore, it seems mandatory to assess whether the virus can infect the human reproductive tract and affect male fertility (Fu *et al.*, 2020; Stanley *et al.*, 2020).

In our manuscript, we demonstrated that semen impairment (oligo/crypto/azoospermia) and signs of genital tract inflammation (elevated semen levels of IL-8 and leukocytospermia, both signs of male genital tract inflammation) were related to Covid-19 severity (Gacci *et al.*, 2021).

Overall, our data are in agreement with those reported in a recent systematic review based on 70 studies (23 quantitative 47 qualitative) (Tur-Kaspa *et al.*, 2021). The Authors tested the male and female reproductive tracts of 404 adult COVID-19 patients with the aim to determine if COVID-19 is an STD or not, and to evaluate its possible effect on fertility. They concluded that COVID-19 may cause inflammation of the testes, in 5–10% of male patients of reproductive age and that this orchitis is highly correlated to the severity of the disease. They also conclude that there is no evidence to support that COVID-19 can be considered as a STD.

Several viral infections including HPV, HSV, HBV, HCV challenges reproductive health and must be considered as a risk factor for male infertility. All these viruses have been detected in semen and can impair testicular function (Batiha *et al.*, 2020). Some viruses such as MuV, HIV and SARS-CoV can affect testicular cells, resulting in severe orchitis, which can result in male infertility (Xu *et al.*, 2006).

Bendayan and Boitrelle, in their letter, suggest that even fever alone—a symptom observed in over 80% of patients infected by COVID-19—could have a negative impact on the physiological scrotal heat regulation, with the consequent semen impairment (Boitrelle and Bendayan, 2021). Actually, COVID-19 patients, such as those affected by influenza, suffer from fever, which may affect sperm production. It is well demonstrated that febrile status can have a negative impact on semen quality (Batiha *et al.*, 2020), including an induction of DNA damage (Xu *et al.*, 2006). However, it should be noted that both sperm count and motility were temporarily reduced more than one month after fever episode, before going back to normal several weeks after fever (Sergerie *et al.*, 2007).

In addition to fever, COVID-19 patients underwent severe cycles of medications, were hospitalized and may have a prolonged abstinence period, as correctly indicated in the letter by Bendayan and Boitrelle (2021). All these conditions may be involved in producing testicular damage. However, at present, whether testicular damage is produced by virus infection in the testes or is due to the associated pathological condition, medications, etc. remains to be defined. Similarly, it is not known, at present, whether testicular damage may persist for long time.

In such a situation, we fully agree with Bendayan and Boitrelle (2021) regarding the need for a re-evaluation of men that have been affected by COVID-19 at least 3 months following complete healing, which is presently under investigation in our laboratory.

Conflict of interest

The authors have nothing to disclose.

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HOST RESPONSE AND INFLAMMATION

mcr-1 Gene Expression Modulates the Inflammatory Response of Human Macrophages to *Escherichia coli*

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ABSTRACT MCR-1 is a plasmid-encoded phosphoethanolamine transferase able to modify the lipid A structure. It confers resistance to colistin and was isolated from human, animal, and environmental strains of *Enterobacteriaceae*, raising serious global health concerns. In this paper, we used recombinant *mcr-1*-expressing *Escherichia coli* to study the impact of MCR-1 products on *E. coli*-induced activation of inflammatory pathways in activated THP-1 cells, which was used as a model of human macrophages. We found that infection with recombinant *mcr-1*-expressing *E. coli* significantly modulated p38-MAPK and Jun N-terminal protein kinase (JNK) activation and pNF-κB nuclear translocation as well as the expression of genes for the relevant proinflammatory cytokines tumor necrosis factor alpha (TNF-α), interleukin-12 (IL-12), and IL-1β compared with *mcr-1*-negative strains. Caspase-1 activity and IL-1β secretion were significantly less activated by *mcr-1*-positive *E. coli* strains than the *mcr-1*-negative parental strain. Similar results were obtained with clinical isolates of *mcr-1*-positive *E. coli*, suggesting that, in addition to colistin resistance, the expression of *mcr-1* allows the escape of early host innate defenses and may promote bacterial survival.

KEYWORDS MCR-1, macrophages, cytokines, inflammation

Biochemical modification of the lipid A component of the lipopolysaccharide (LPS) of Gram-negative bacteria may serve as a strategy for promoting bacterial survival inside the host by providing resistance to components of the innate immune system (e.g., antimicrobial peptides) and helping to evade recognition by Toll-like receptor 4 (TLR4) (1). The most frequent modification of LPS is the addition of phosphoethanolamine (pET) or 4-deoxyaminoarabinose residues to the phosphate moieties of the lipid A molecule, which decrease the negative charge of lipid A and prevent the binding of cationic antimicrobial peptides, including colistin, interfering with their bactericidal activity (1). In some cases, these changes may also help Gram-negative pathogens to circumvent detection and clearance from the host organism by evading the detection by TLR4 (2, 3).

In *Escherichia coli*, modification of lipid A can occur by the upregulation of endogenous LPS modification systems (e.g., *pmrHFIJKLM*) following activation of two-component regulatory systems which control their expression (e.g., PmrA-PmrB) (4, 5) or by the acquisition of exogenous lipid A-modifying enzymes, such as those encoded by transferable *mcr*-type genes via horizontal gene transfer (6). The *mcr*-type genes, encoding pET transferases, have been recently identified in human, animal, and environmental isolates of *Enterobacteriaceae* and have attracted remarkable attention since

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they can be responsible for transferable resistance to colistin, a last-resort antibiotic for multidrug-resistant Gram-negative infections. Among *mcr* genes, *mcr-1* and related variants are the most diffused worldwide (6).

The colonization by MCR-1-producing *Enterobacteriaceae* of healthy individuals and patients has been reported in many countries where *mcr-1*-positive *E. coli* are largely disseminated (6–8), and in some cases, colonization is unrelated to the clinical use of colistin (9). This resistance determinant is also emerging in high-risk clones of *E. coli* as sequence type 131 (ST131) (6, 10), further increasing global health concerns.

The functional consequences of *mcr-1* expression on microbe-host interactions have not yet been clarified. High-level *mcr-1* mutants showed reduced fitness and attenuated virulence in a *Galleria mellonella* infection model compared with their parent strains (11), and pure modified LPS from MCR-1 strains is less active than wild-type LPS in inducing the production of tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) in human macrophages (11), suggesting that *mcr-1*-related lipid A modification may interfere with host defenses. Besides the activation of surface Toll-like receptors (TLRs), bacterial ligands are able to activate inflammatory pathways even in the cytosolic compartment. In particular, the activation of caspase-1 in the context of an inflammasome complex induces the production of procytokines (IL-1β and IL-18) and of gasdermin D that, once cleaved, forms membrane pores that lead to cytokine release (12).

Therefore, this study aims to compare the interaction of a *mcr-1*-positive *E. coli* MG1655 (MCRPEC) in PMA/THP-1 cells with respect to a *mcr-1*-negative MG1655 strain (MCRNEC). Additionally, the phosphorylation of NF-κB (p65), p38-MAPK, and stressed-activated protein kinase/Jun N-terminal protein kinase (SAPK/JNK); the production of inflammatory cytokines; and the caspase-1 activity of infected macrophages were studied. Three clinical isolates of MCR-1-producing *E. coli* were used in the same experimental system, and their impact on macrophage functions was assessed (see Table S1 in the supplemental material).

RESULTS

Cloning and expression of the *mcr-1* gene. The *E. coli* MG1655 reference strain, representative of K-12 and being one of the first bacteria for which the whole-genome sequence has been obtained (13), was chosen as the bacterial host. The expression of the *mcr-1* gene in pLBII_*mcr-1*-carrying MG1655 conferred colistin resistance (MIC, 4 μg/ml), compared with the colistin susceptible recipient strain carrying the empty vector pBCSk (MIC, $\leq$0.125 μg/ml).

Endocytosis of MCRPEC and MCRNEC by PMA/THP-1 cells. PMA/THP-1 cells were cultured with MCRPEC or MCRNEC at different concentrations, and CFU counts in supernatants and cell lysates were recorded at different times. The results showed that $>$90% of bacterial cells of each strain were internalized after 1 hour. Table S2 in the supplemental material shows that the majority of internalized bacterial cells with MCRPEC or MCRNEC were killed after 2 hours ($>$99%). A slight increase in cells that survived MCRPEC compared to MCRNEC was observed after 2 and 4 hours ($\sim$1.6-fold).

Modulation of cytokine production by MCRPEC and MCRNEC in PMA/THP-1 cells. The expression of proinflammatory cytokine genes, such as TNF-α, IL-1β, and IL-12 and of the anti-inflammatory cytokine IL-10 was evaluated by reverse transcription-quantitative PCR (RT-PCR). PMA/THP-1 cells were stimulated for 3 hours with MCRPEC or MCRNEC (50 multiplicity of infection [MOI]/cell) and afterward were incubated overnight in growth medium supplemented with antibiotics. PMA/THP-1 cells challenged with MCRPEC expressed smaller amounts of TNF-α, IL-1β, and IL-12 mRNA than cells challenged with the isogenic MCRNEC strain (Figure 1a). The expression of the IL-10 gene was upregulated in cells with MCRPEC compared with those challenged with MCRNEC, but the differences did not reach statistical significance (Figure 1b). We also measured the concentrations of TNF-α, IL-1β, IL-12, and IL-10 in the culture supernatants by immunoplex assay. Despite the short time of stimulation with bacterial cells (3 hours), TNF-α, IL-1β, and IL-10 were produced in measurable amounts and the results

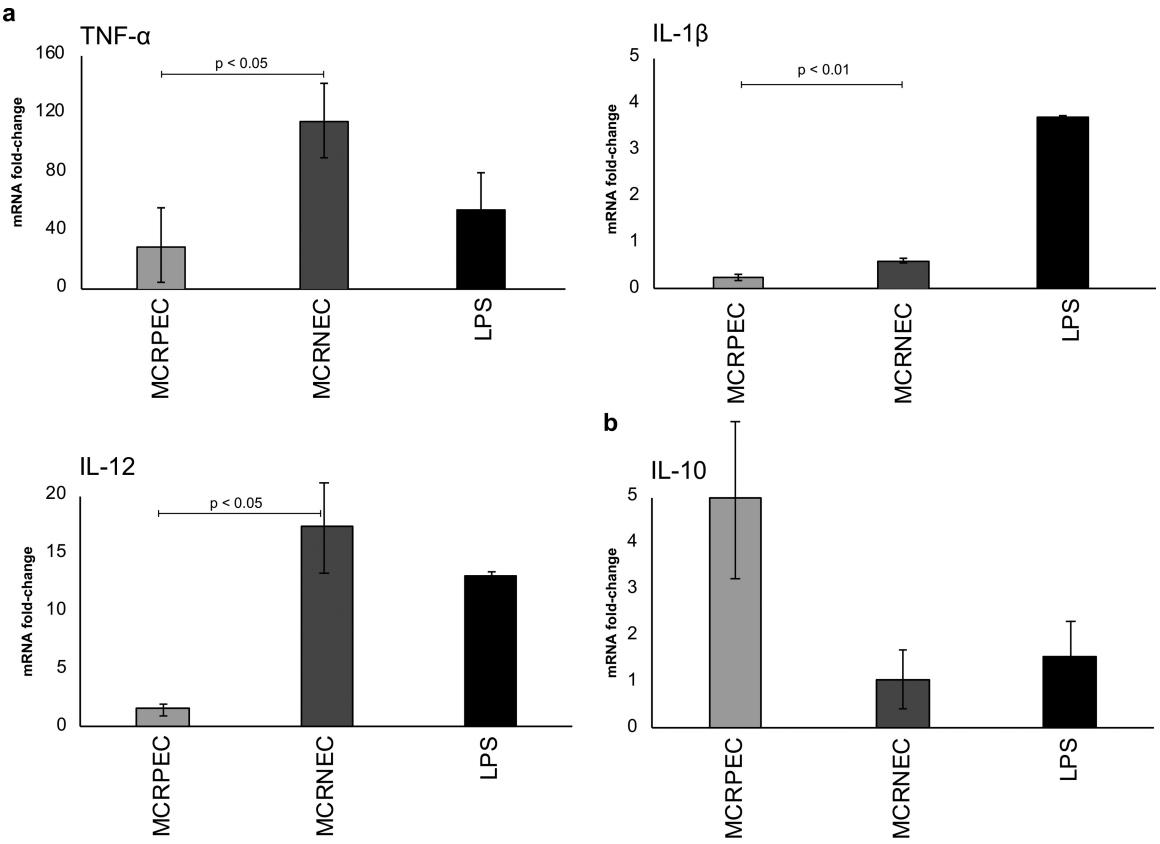

FIG 1 Expression of proinflammatory cytokine genes by PMA/THP-1 cells. PMA/THP-1 cells were cultured in the presence or absence of MCRPEC, MCRNEC, or LPS as a positive control. Results represent the fold change mRNA compared with unstimulated cultures (mean ± SE of triplicate cultures). Data from three representative experiments are shown. Statistical analysis was performed with the Student's *t* test. A *P* value of <0.05 was considered statistically significant.

confirmed that PMA/THP-1 cultured with MCRPEC produced TNF-α and IL-1β in smaller amounts than cells cultured with MCRNEC. In contrast, MCRPEC induced an increase in the production of IL-10 compared with MCRNEC (Figure 2b). These results suggested that the expression of the *mcr-1* gene is involved in the modulation of relevant inflammatory pathways in PMA/THP-1 cells.

To add further evidence to this hypothesis, three clinical isolates of *mcr-1*-positive *E. coli* isolates (FI-4551, FI-4531, and LC-17/15) were tested with PMA/THP-1 cells in the same experimental conditions used above. Figure 2 shows that all clinical isolates induced a lower production of IL-1β and a higher production of IL-10 by PMA/THP-1 cells than MCRNEC. Interestingly, similar to MCRNEC, the clinical sample LC-17/15 had a higher production of TNF-α than MCRPEC and the other clinical samples. These results reinforce the hypothesis that *mcr-1* is involved in the escape of inflammatory pathways.

Regulation of NF-κB, p38-MAPK, and SAPK/JNK phosphorylation by MCRPEC and MCRNEC in PMA/THP-1 cells. The nuclear translocation of phosphorylated NF-κB (p65) and AP-1 through the coordinate functions of TRAF6 on NF-κB and p38-MAPK and SAPK/JNK phosphorylation on AP-1 (14, 15) represent a convergence point of many activated pattern recognition receptor (PRR) pathways and a crucial step for the transcription of cytokine genes.

To investigate whether MCRPEC differs from MCRNEC in the activation of these pathways, PMA/THP-1 cells were cultured in the presence of live MCRPEC or MCRNEC and the phosphorylation NF-κB (p65), p38-MAPK, and SAPK/JNK was assessed by Western blot analysis.

Results of these experiments showed that phosphorylated-NF-κB (P-NF-κB; p65), P-p38-MAPK, and P-SAPK/JNK were significantly reduced in cells tested with MCRPEC

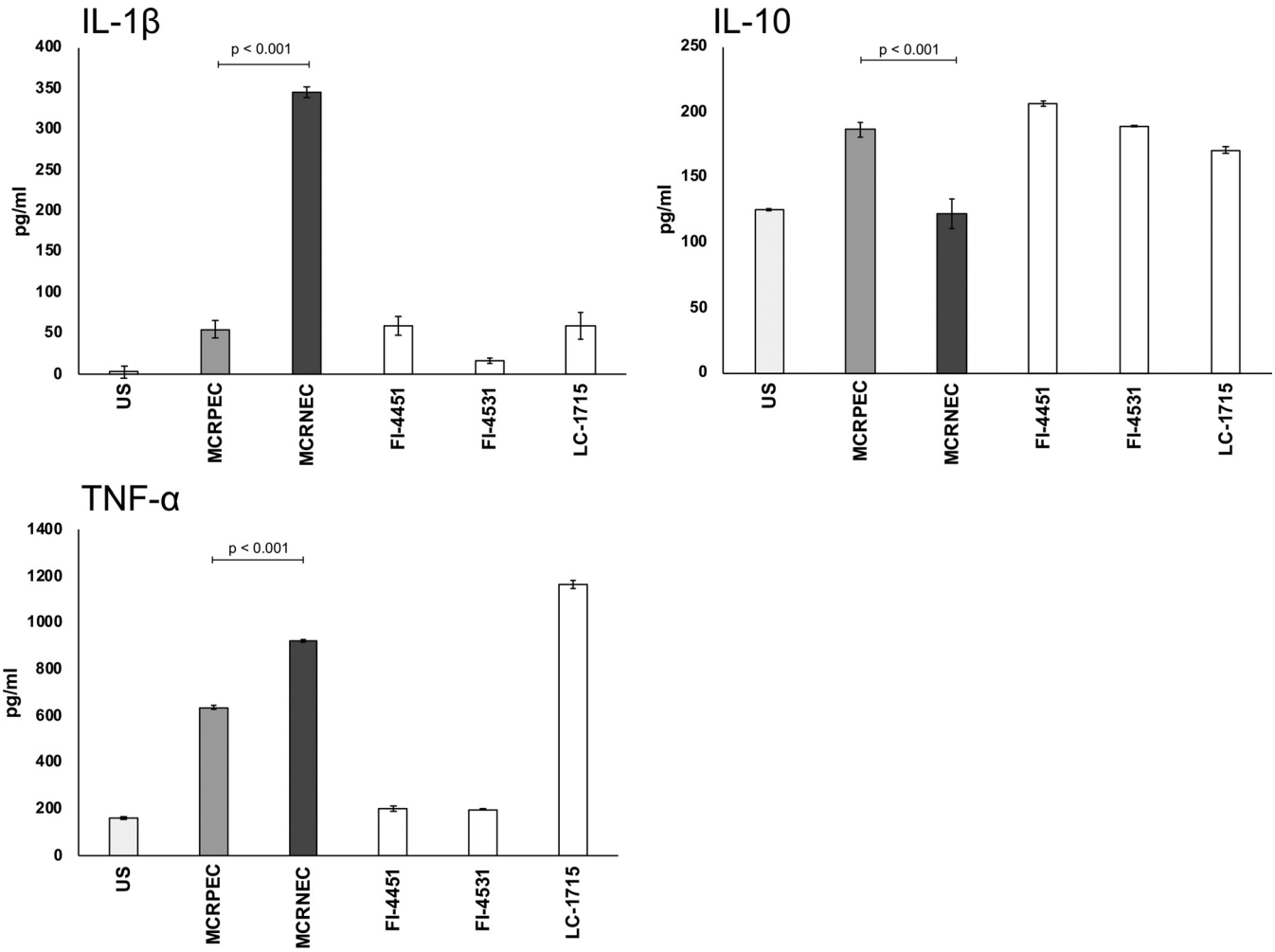

FIG 2 Production of TNF-α, IL-1β, and IL-10 by PMA/THP-1 cells. PMA/THP-1 cells were cultured in the presence of live bacteria (50 MOI/cell) for 3 hours. Supernatants were collected after 16 hours and analyzed by the immunoplex assay. ANOVA one-way analysis revealed significant differences among cultures with the different strains. The Student's *t* test was used to compare cytokine production in cultures with MCRPEC and MCRNEC. A *P* value of $\leq$0.05 was considered statistically significant.

compared with those incubated with MCRNEC (Fig. 3), suggesting that the expression of the *mcr-1* gene may interfere with the ability of bacterial cells to activate proinflammatory pathways in PMA/THP-1 cells.

Modulation of Caspase-1 activation by MCRPEC and MCRNEC in PMA/THP-1 cells. To investigate whether MCRPEC activates caspase-1 differently from MCRNEC, we cultured PMA/THP-1 cells with live bacterial cells for 6 hours and measured the caspase-1 activation through a Caspase-Glo 1 inflammasome assay.

Results of these experiments showed that caspase-1 activation was significantly lower in cultures of PMA/THP-1 with MCRPEC than cultures with MCRNEC (Figure 4a), indicating that MCRPEC affects inflammasome activation. Since caspase-1 is involved in gasdermin D activation, membrane damage, and osmotic lysis of the cell (12), we assessed whether the presence of *mcr-1* in endocytosed bacteria may attenuate these phenomena. For this reason, THP-1 cells were cultured with MCRPEC or MCRNEC (as reported above), and we measured lactate dehydrogenase (LDH) activity in culture supernatants and the incorporation of a DNA fluorescent dye in order to detect the cellular membrane damage.

Figure 4b shows that LDH activity in supernatants of PMA/THP-1 cultured with MCRPEC was significantly lower than that released in supernatants of the cells cultured with MCRNEC. These results suggest that PMA/THP-1 cultured with MCRPEC incor-

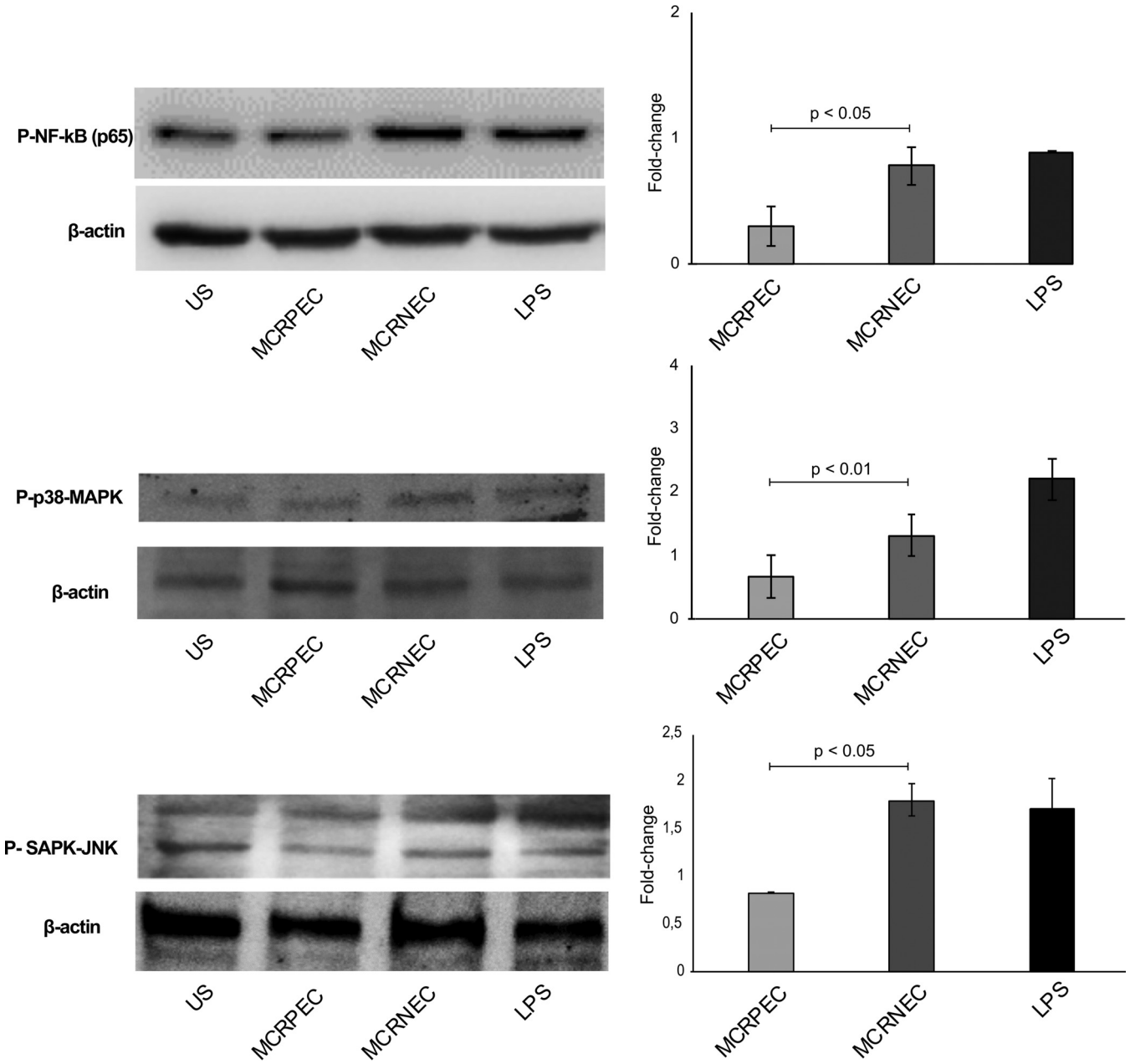

FIG 3 NF-κB, p38-MAPK, and SAPK/JNK phosphorylation by PMA/THP-1 cells. PMA/THP-1 cells were cultured for 20 minutes in the absence (US) or presence of MCRPEC, MCRNEC, or LPS as a positive control. Cells were analyzed by Western blot analysis. Data from one representative experiment out of three performed are shown. The bar graph shows the results of densitometric analysis of three different experiments. Data are expressed as mean fold increase ± SE of stimulated cultures over the unstimulated control. Statistical analysis was performed by Student's *t* test. A *P* value of ≤0.05 was considered statistically significant.

porated smaller amounts of DNA-binding dye than PMA/THP-1 cells infected with MCRNEC (see Fig. S1 in the supplemental material).

Caspase-1 activation and LDH release were finally measured in cultures of PMA/THP-1 cells with clinical isolates of *mcr-1*-positive *E. coli* (Figure 4c and d). The activation of caspase-1 and the release of LDH were reduced in recombinant strains compared with MCRNEC as well as compared with the ATCC 25922 *E. coli* strain.

DISCUSSION

The dissemination of *mcr-1*-positive strains of *Enterobacteriaceae* suggests that *mcr-1*-mediated phenotypic changes might confer selective advantages to the bacterial

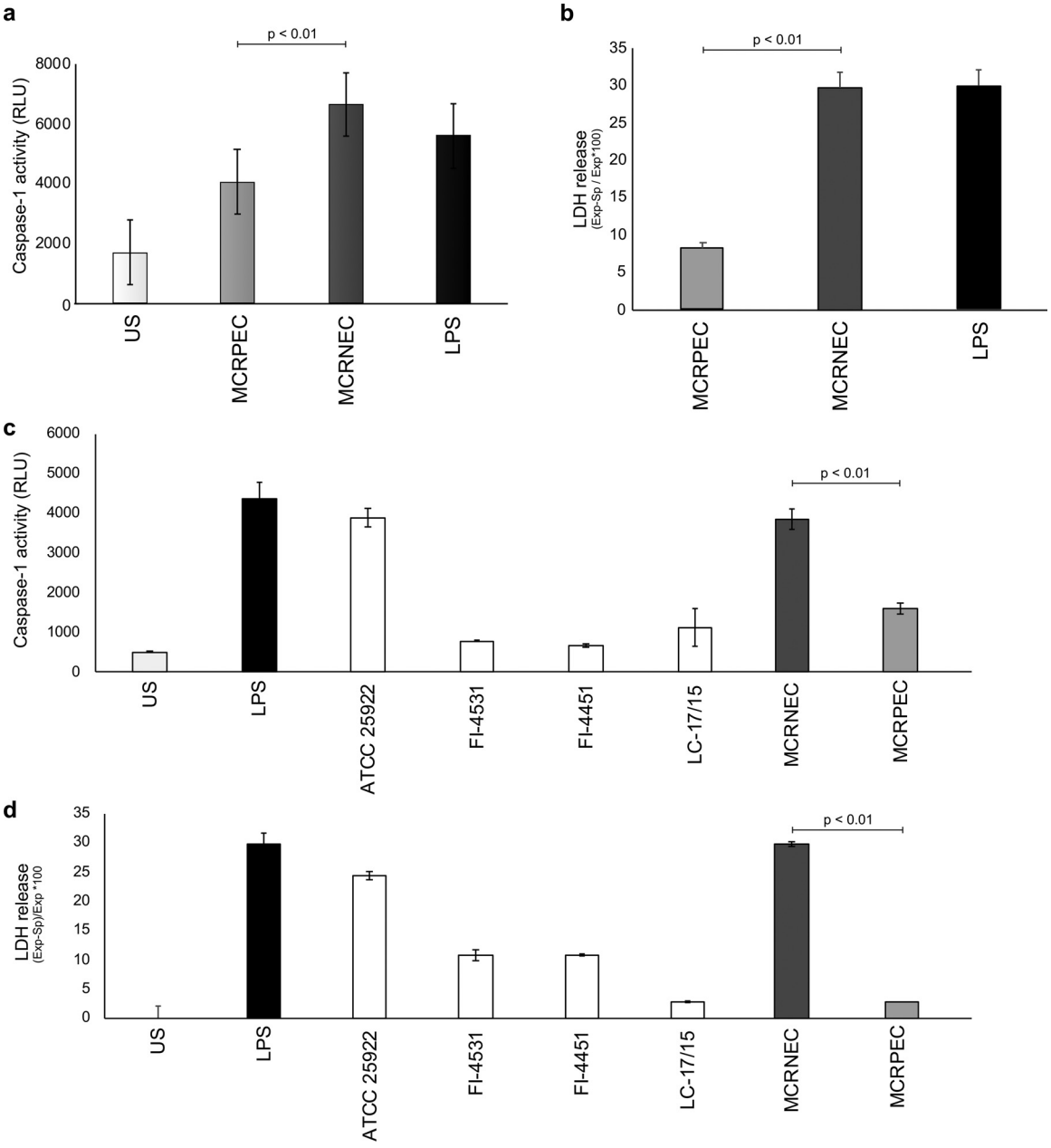

FIG 4 Caspase-1 activation and LDH release of PMA/THP-1 cells. (a) PMA/THP-1 cells were cultured in the presence of MCRPEC, MCRNEC, or LPS as a positive control for 6 hours. Caspase-1 activity is expressed as luminescence units (RLUs), as revealed by spectrophotometric analysis. (b) LDH activity in culture supernatants of PMA/THP-1 cells incubated with MCRPEC, MCRNEC, or LPS; data represent mean ± SE of triplicate cultures. (c and d) Caspase-1 activity and LDH production analysis in PMA/THP-1 cells cultured with isolated clinical samples. ANOVA one-way analysis revealed significant differences among cultures. Statistical analysis was performed by Student's *t* test. A *P* value of ≤0.05 was considered significant.

cells that go beyond the resistance to human cationic antimicrobial peptides (CAMPs) (1). Lipid A modifications can affect the stimulatory properties of LPS and the recognition of bacterial cells by cells of innate immunity. For example, the modification of pET to lipid A of *Neisseria gonorrhoeae* decreases the autophagy process activation in human macrophages, suggesting that it could represent a mechanism to escape the host immune system (16). The same modification in *Campylobacter jejuni* increases LPS recognition by human TLR-4 and also improves pathogen colonization, at least in a murine infection model (17). Modification in the lipid A structure in *Salmonella* strains did not cause a significant change in virulence (18). Therefore, it seems that the

functional consequences of pET addition to lipid A, in the context of host-pathogen interactions, depend on the bacterial species, the virulence of the strain, and its intrinsic ability to colonize the host.

For these reasons, the use of live bacterial cells, engineered to express the lipid A-modifying enzyme MCR-1, can provide information on host-pathogen interactions closer to reality than the use of single purified components. By using live bacterial cells to infect human macrophages, we observed that the expression of the *mcr-1* gene induces an immunomodulatory phenotype with a reduced expression of genes for the proinflammatory cytokines TNF-α, IL-1β, and IL-12 which suggests the inhibition of M1 differentiation of macrophage cells (19) and the delay of pathogen clearance, as reported for different pathogens (20, 21).

Signaling activated by TLR usually converges in the nuclear translocation of NF-κB and AP-1 following their phosphorylation by IRAK and p38-MAPK/JNK, respectively (14, 15, 22, 23). Interference with the activation of NF-κB and/or p38-MAPK and/or SAPK/JNK represents a common mechanism for modulating the production of proinflammatory cytokines. Our data showed that infection with *mcr-1*-positive *E. coli* is able to modulate all of these pathways and therefore escape from innate recognition of its surface molecules.

The interference with cytosolic recognition of bacterial ligands, which leads to caspase-1 activation (24) is a further strategy used by pathogens to subvert innate immunity. Caspase-1 activation allows the release of mature IL-1β and IL-18 (23, 25) through gasdermin-induced membrane pores. Activated caspase-1 can induce, through the same pathway, the osmotic lysis of the cell with the result of enhancing the inflammatory events at the infection site through the recruitment of phagocytes and other activated immune cells. Moreover, it has been reported that caspase-1 activity contributes to an increase in TLR-2 and TLR-4 signaling (26).

Our data suggest that the expression of the *mcr-1* gene also gives to *E. coli* cells the ability to escape recognition by cytosolic receptors and inflammasome activation. The recombinant *E. coli* strain (MCRPEC) and the 3 clinical isolates of *mcr-1*-positive *E. coli* modulate the inflammatory potential of THP-1 cells and limit the caspase-1 activation, the cellular membrane damage, and the massive release of inflammatory molecules compared with *E. coli* strains which do not express the *mcr-1* gene.

The alteration of these inflammatory pathways may therefore represent a common mechanism used by MCR-1-producing bacteria to prolong their survival within human hosts. A proper question is finally whether *mcr-1*-mediated lipid modifications are responsible for all the observed phenomena. Yang et al. used purified LPS extracted from *mcr-1* strains (11) to activate PMA/THP-1 cells and found a reduced production of TNF-α and IL-6, suggesting a reduced ability of modified LPS to activate inflammatory pathways. However, a recent proteomics and metabolomic profile of *mcr-1*-positive *E. coli* strains revealed the functional reduction of relevant metabolic processes, including the lipopolysaccharide biosynthesis (27). We could not rule out that, apart from lipid A modifications, the reduced synthesis of LPS could play a role in the observed modulation of proinflammatory events.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *E. coli* K-12 strain MG1655 from the Keio collection (28) was used for *mcr-1* expression, while *E. coli* DH10B was used as intermediate host to propagate the recombinant plasmid pLBII_*mcr-1*. Three different clinical isolates of MCR-1-producing *E. coli* and *E. coli* ATCC 25922 were also included for comparison (Table S1) (7).

Bacteria were grown for 18 to 22 hours at 35 $\pm$ 1°C in cation-adjusted Mueller-Hinton (Becton, Dickinson and Company, MD, USA) broth under selective conditions (85 μg/ml chloramphenicol) before incubation with cells.

Recombinant DNA methodology. The plasmid pLBII_*mcr-1*, which was used for *mcr-1* expression experiments, was a pBC-SK(−) derivative (Agilent technologies, Santa Clara, CA) in which a Shine-Dalgarno sequence flanked by an NdeI site, separated by 4 bp, has been inserted downstream of the P_{LAC} promoter at the extremity of the vector polylinker (29). The pLBII_mcr-1 plasmid was constructed by amplification of the open reading frame *mcr-1* gene from strain FI-4451 (7), using the primers NdeI_MCR-1-F (5′-GGAATTCCATATGATGCAGCATACTTCTGTGTGG-3′) and BamHI_MCR-1-R (5′-CGCGGATCCTCAGCG GATGAATGCGGTGC-3′) (restriction sites are underlined). The resulting amplicon (1,642 bp) was digested with NdeI and BamHI and cloned into pLBII digested with the same enzymes. The authenticity of the

cloned fragments was confirmed by sequencing both strands at an external sequencing facility (Eurofins Genomics, Germany). The recombinant plasmid pLBII_*mcr-1* was electroporated into competent cells as previously described (7, 30), and transformants were selected with chloramphenicol (85 μg/ml).

Endocytosis assay. The endocytosis assay was performed using *E. coli* strains MCRPEC and MCRNEC grown overnight in cation-adjusted Mueller-Hinton broth as described above. PMA/THP-1 cells were plated in 6 wells at 5×10^5 cells in 2 ml of medium and cultured with live bacteria (50 MOI/cell). Supernatant and lysates were collected at different times (1, 2, and 4 hours) and plated at different dilutions in selective medium for CFU determination. The lysates were obtained by treating the cells with 0.15% of Triton X-100 (Sigma-Aldrich, Milan, Italy) for 15 minutes at room temperature (RT).

Cell cultures and bacterial infection. The THP-1 cell line, derived from a human promonocytic leukemia patient, was obtained by ATCC (number TIB-202). Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 5% L-glutamine at 37°C and 5% CO_2. For all the experiments, THP-1 cells were seeded in 24- to 96-well plates (5×10^5 cells/ml) in the presence of 20 ng/ml of phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, Milan, Italy) for 72 hours to allow differentiation. Then, the cells were washed and cultured for an additional 24 hours in fresh culture medium without PMA. These cells are referred as PMA/THP-1 cells.

The bacterial infection assay was performed using PMA/THP-1 cells cultured in the presence of live *E. coli* strains (20 or 50 MOI/cell) in medium without antibiotics (3 hours). Afterward, bacteria were removed, and the cells were incubated for additional times in medium supplemented with 1% of penicillin and 1% streptomycin. LPS was used as internal standard control in all the experiments at the concentration of 200 ng/ml. Additionally, in order to evaluate the possible fitness reduction of MCRPEC strains, the growth curves of both MCRNEC and MCRPEC bacterial strains in RPMI medium were evaluated and no significant differences were detected (data not shown).

RNA extraction and RT-PCR. RNA from each stimulated sample was obtained through TRIzol extraction (TRIzol reagent; Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions and quantified using Nanodrop (Thermo Fisher, Waltham, MA, USA). A total of 2 μg of RNA was retrotranscribed using EuroScript Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (RNase H−) (EuroClone Pero, Italy). A total of 25 ng of cDNA from each sample was amplified by using the QuantiNova SYBR green PCR kit (Qiagen, Hilden, Germany), and the reaction was performed with the 7900HT fast real-time PCR system (Applied Biosystems, Foster City, CA, USA). The expression of genes for relevant proinflammatory cytokines was quantified using primers reported in Table S3 in the supplemental material.

Western blot analysis. To investigate the activation of NF-κB, p38-MAPK, and SAPK/JNK, 1×10^6 PMA/THP-1 cells were cultured for 20 minutes with live MCRPEC or MCRNEC at 50 MOI/cell. Cells were then lysed with RIPA buffer in the presence of phosphatase inhibitor cocktail 2 and 3, protease inhibitor cocktail (Sigma-Aldrich, Milan, Italy), and centrifuged at 12,000 $\times$ *g* for 15 minutes. The concentration of the proteins was determined by bicinchoninic acid (BCA) assay (EuroClone Pero, Italy). For each line, 40 μg of proteins was loaded on SDS-PAGE and blotted on nitrocellulose filters (Bio-Rad, Hercules, CA, USA). Membranes were stained with rabbit anti-caspase-1 (Cell Signaling Technology, Danvers, MA, USA), rabbit anti-IL-1β, rabbit anti-phosphorylated NF-κB (p65), rabbit anti-phospho-p38-MAPK, rabbit anti-phosphorylated SAPK/JNK (Cell Signaling Technology), and mouse anti-actin, (Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by staining with anti-rabbit IgG (H+L) DyLight800 or anti-mouse IgG (H+L) DyLight 650 (Thermo Fisher, Waltham, MA, USA) as indicated (31). The reactions were visualized and acquired by the Odyssey infrared imaging system (Li-Cor Biosciences, Lincoln, NE, USA).

Immunoplex assay. The concentrations of IL-1β, IL-10, IL-12 (p70), and TNF-α were determined in culture supernatants using the human cytokine/chemokine magnetic bead panel (Millipore, Bedford, MA, USA) and Luminex MagPix apparatus according to the manufacturer's recommendations.

DNA-binding dye assay. DNA damage was analyzed exploiting the RealTime-Glo annexin V apoptosis and necrosis assay (catalog number JA1011; Promega, Madison, WI, USA). Briefly, 5×10^4 PMA/THP-1 cells were seeded in 96 wells in 50 μl of medium without antibiotics and incubated with live bacteria (50 MOI/cell) for 24 hours at 37°C with 5% of CO_2. The incorporation of DNA-binding dye was recorded at different times (0, 3, 6, and 24 hours) using a Victor instrument to measure the fluorescence (relative fluorescence unit [RFU]).

Caspase-1 assay. Caspase-1 activity was quantified using Caspase-Glo 1 inflammasome assay (catalog number G9951; Promega, Madison, WI, USA) following the manufacturer's instructions. For this assay, 4×10^4 PMA/THP-1 cells were plated in 50 μl of growth medium without antibiotics and incubated for 6 hours with live bacteria of MCRPEC and MCRNEC (50 MOI/cell) at 37°C with 5% of CO_2. The amount of caspase-1 produced in PMA/THP-1 cells was measured in terms of luminescence (RLU) recorded using a Victor instrument.

LDH assay. LDH activity released from damaged cells was revealed in culture supernatants using the cytotoxicity detection kit plus (LDH) (Sigma-Aldrich, Milan, Italy) according to manufacturer's instructions.

Statistical analysis. Numerical data were expressed as mean and standard error, as indicated in the legend. We applied one-way analysis of variance (ANOVA) for multiple comparisons and the Student's *t* test for paired samples. The statistical significance was defined as a *P* value of <0.05. For the statistical analysis, we used R software version 3.6.2.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

ACKNOWLEDGMENTS

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ORIGINAL ARTICLE

Male reproductive system inflammation after healing from coronavirus disease 2019

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Abstract

Background: There is evidence that, after severe acute respiratory syndrome coronavirus 2 infection, male reproductive function and semen quality may be damaged

Objectives: To evaluate a panel of inflammatory mediators in semen in patients recovered from coronavirus disease 2019.

Material and methods: Sexually active men with previous severe acute respiratory syndrome coronavirus 2 infection and proven recovery from coronavirus disease 2019 were enrolled in a prospective cohort study. Clinical, uro-andrological data and semen specimens were prospectively collected. For previously hospitalized coronavirus disease 2019 patients, data on serum inflammatory markers were retrospectively collected.

Results: A total of 43 men were enrolled in the study. Of these, 32 men were normozoospermic, three were oligozoospermic, and eight were crypto-azoospermic. Serum inflammatory markers (procalcitonin and C-reactive protein) were analyzed in previously hospitalized patients both at admission and at peak of infection. Levels at admission were statistically significantly higher in patients resulting in crypto-azoospermic with respect to those resulting in normozoospermic ($p = 0.05$; $p = 0.03$ and $p = 0.02$,

respectively) after healing. Seminal cytokine levels were similar among all groups. Interleukin-1β and tumor necrosis factor-α levels were significantly negatively related to sperm total number and concentration, whereas interleukin-4 was correlated with sperm motility.

Discussion and conclusion: Negative correlations between interleukin-1β and tumor necrosis factor-α and sperm number and the overall high levels of semen cytokines indicate a potential detrimental role of severe acute respiratory syndrome coronavirus 2 driven inflammation on spermatogenesis. Overall, our results indicate that male patients recovering from coronavirus disease 2019 deserve accurate follow-up for their fertility status.

KEYWORDS
COVID-19, cytokines, inflammation, men, SARS-CoV-2

1 | INTRODUCTION

The pandemic coronavirus disease 2019 (COVID-19), due to a novel virus called severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), caused one-hundred-millions of infections all over the word in the very first year of the outbreak.[1] The SARS-CoV-2 virus resulted more contagious and infected a higher percentage of young men during the second and third pandemic wave, as compared with the first one.[2] Angiotensin-converting enzyme 2 (ACE2) receptor is the primary receptor mediating the entry of SARS-CoV-2 into human cells. ACE2 receptor is present in spermatogonia, seminiferous tubules, Sertoli, and Leydig cells and is highly expressed in cell lines derived from male genitourinary epithelium of fertile young men.[3–5] After SARS-CoV-2 infection, changes in ACE2 signaling pathways can induce severe oxidative stress and inflammation that may cause significant damage to the male reproductive tract.[6] Therefore, clinical evidence of real COVID-19 impact on male reproductive function, even in the long term, is greatly advocated.[7]

In the last decade, new biomarkers of male genital tract inflammation have been reported as the most promising putative markers of infection.[8] We previously reported that pathological levels of interleukin (IL)-8 were present in semen from 33/43 of men after recovery from COVID-19.[9] Moreover, semen levels of IL-8 were related to the severity of illness (hospitalization and need for oxygen therapy or invasive ventilation)[9] suggesting an impairment of genital apparatus during the viraemic spread.

The high levels of semen IL-8 in the seminal fluid of COVID-19 patients suggest a direct involvement of genito-urinary epithelium in the inflammatory process. In fact, IL-8 production precedes the infiltration of inflammatory cells[9,10] and promotes natural killer (NK) cell recruitment and function.[11,13]

Increasing evidence on the impact of SARS-CoV-2 infection on semen parameters is reported.[9] However, a longitudinal analysis of semen collected by patients after COVID-19 recovery is still lacking.

To improve the knowledge of COVID-19 impact on male reproduction, we analyzed a panel of semen cytokines in COVID-19 patients after an average of 35 days of recovery from the infection.

2 | METHODS

2.1 | Study design and population

A prospective cohort study was designed following the Strengthening the Reporting of Observational Studies in Epidemiology guidelines. Due to the sudden and unexpected spread of the COVID-19 pandemic, no previous knowledge was present on the topic at the initial study design. Therefore, no a priori power analysis and sample dimension calculation were possible.

Male patients with previous SARS-CoV-2 infection and subsequent proven recovery from COVID-19 (two consecutive negative NP swabs for SARS-CoV-2 ribonucleic acid) were screened for the present study. Besides male gender, inclusion criteria were: age between 18 and 65 years, active sexual life, and capability to express informed consent to participate in the current study. Exclusion criteria were ejaculatory disorders or refusal to participate.

The current study was approved by the Institutional Review Board of AOUC – Careggi Hospital, Florence Italy in June 2020, under code 17104. Subsequently, it was registered in clinicaltrials.gov with the identifier NCT04446169. It was designed and conducted according to the Declaration of Helsinki. All enrolled participants signed informed consent.

2.2 | Data recording and specimen collection

Clinical data and semen specimen collection were recorded according to the previously reported scheme.[9] Values of serum inflammatory markers (white blood cell count, procalcitonin, and C-reactive protein)

were retrospectively recorded for hospitalized patients at the time of admission, peak, and discharge.

Semen obtained with masturbation was collected in sterile jars. After liquefaction of semen and assessment of volume, semen samples were analyzed.

2.3 | Immunoplex assay and semen analysis

Detection and quantification of cytokines in semen were performed using the Milliplex Map kit Human Cytokine/Chemokine/Growth Factor Panel A Magnetic Bead Panel (Merck Kgaa, Darmstadt, Germany) following the manufacturer's protocols. The plate was analyzed with the Luminex 200 MAGPIX. The limit of sensitivity (MinDc = minimum detectable concentration) and the linear range of detection for the analyzed cytokines are reported in Table S1. Data were analyzed using a 5-parameter logistic curve-fitting method for calculating analyte concentrations in samples.

Semen collection was done after a period of abstinence 2–7 days prior to the collection was requested to all the enrolled patients, according to World Health Organization recommendations. Semen analysis was performed as reported.[9] Quantification of semen leukocytes was done by counting the number of round cells/ml with an improved Neubauer hemocytometer and evaluating the percentage of leukocytes and immature germ cells after May-Grunwald staining of the examined specimen. All analyses were performed by the same team of our academic center.

2.4 | Statistics

Patients' features were reported using medians and interquartile ranges (IQRs) for continuous variables and numbers and percentages for categorical variables. Patients were subsequently divided into different groups for comparisons according to their semen parameters or hospitalization. All analyses were age-adjusted. Differences were tested with the Kruskal-Wallis H test and Wilcoxon test for continuous variables whenever appropriate and with chi-square and Fisher's Exact tests for categorical variables according to sample dimension. Spearman test was applied to explore correlations between investigated variables. All statistical analyses were performed using IBM SPSS version 20.0 (SPSS Inc, Chicago, IL, USA). Statistical significance was set with a p-value < 0.05 and all statistical tests were two-tailed.

3 | RESULTS

3.1 | Patients' characteristics and clinical features

Of 160 male patients with a double-negative nasopharyngeal swab after SARS-CoV-2 infection invited to participate in the study, 43 men accepted and were enrolled (Figure S1).

Patients' clinical characteristics, including COVID-19 clinically relevant data, uro-andrological questionnaires, and seminal cytokines levels are summarized in Table 1. As expected, age was the main determinant of clinical management of COVID-19, with the youngest men (mean age: 44 years) not hospitalized as compared to the middle-aged men (mean age: 52 years) hospitalized, and elderly ones (mean age: 59 years) needing intensive care.

In our previous study, we found that, at semen analysis carried out on average 35 (IQR: 24–43) days after healing, 32 men were normozoospermic, three were oligozoospermic, and eight were crypto-azoospermic.[9] In addition, semen impairment was directly related to hospitalization requirement and severity of disease ($p < 0.01$).[9] Fifty percent of the recruited patients, admitted to the intensive care unit (ICU) due to COVID-19 complications, were crypto-azoospermic.

3.2 | Serum inflammatory markers at hospitalization

Data on serum inflammatory markers (white blood cells count, procalcitonin, and C-reactive protein), were retrospectively collected from 31 (72.1%) hospitalized patients, but data on procalcitonin were available only in 11 of them (25.6%). The values at admission, peak, and discharge, plus their decreasing time are shown in Table 2. Procalcitonin at admission and C-reactive protein at admission and at the peak were statistically significantly higher in men resulting in crypto-azoospermic after healing compared to normozoospermic ($p = 0.05$; $p = 0.03$ and $p = 0.02$, respectively).

Other values, including white blood cell count at peak, white blood cell count decreasing time, and C-reactive protein decreasing time were slightly above statistical significance and showed higher values in crypto-azoospermic men compared to normozoospermic. A statistically significant negative correlation was found between procalcitonin values at admission and sperm concentration (Rho −0.738, $p < 0.01$) and between sperm total number and procalcitonin at admission (Rho −0.672, $p < 0.01$), and C-reactive protein at admission (Rho −0.360, $p = 0.04$) and at peak (Rho −0.411, $p = 0.03$) (Figure 1).

3.3 | Semen cytokines

The levels of IL-8, IL-4, IL-6, IL-17, tumor necrosis factor α (TNF-α), and IL-1β were measured in the seminal fluid of all patients. All cytokines were remarkably above the cut-off levels[13] in all semen samples (Figure 2). Significant differences among the groups were revealed only for IL-1β which was significantly higher in the group of crypto-azoospermic patients ($p = 0.01$) (Table 3). IL-8 values were statistically significantly higher in patients with severe COVID-19 infections requiring ICU recovery ($p = 0.01$). Both IL-17 and TNF-α showed a similar trend, without reaching statistical significance (Figure 3).

IL-1β and TNF-α values were negatively related to sperm total number (Rho −0.443, $p < 0.01$ and Rho −0.362, $p = 0.03$), respectively)

TABLE 1 Patients' clinical characteristics, globally and comparison according to semen analysis at enrollment

		Total ($n = 43$, 100%)	Normozoospermic ($n = 32$, 74.4%)	Oligozoospermic ($n = 3$, 7%)	Crypto-azoospermic ($n = 8$, 18.6%)	p
Age (years)	51 (45–58)	50 (44–57)	58 (43–60)	59 (53–63)	0.10	
BMI (kg/m^2)	26.3 (23.6–29.2)	26.2 (23.8–30.0)	23 (21.9–23.3)	27.5 (27.0–29.4)	0.06	
Smoking	Never	35 (81.4%)	27 (84.4%)	3 (100%)	5 (62.5%)	0.59
	Current	3 (7.0%)	2 (6.3%)	0	1 (12.5%)	
	Former	5 (11.6%)	3 (9.4%)	0	2 (25.0%)	
Period between first positive-oroparhyngeal swab and healing from COVID-19 (days)		31 (22–40)	32 (22–40)	31 (25–45)	33 (21–43)	0.99
Hospitalization for COVID-19	No	12 (27.9%)	11 (34.4%)	0	1 (12.5%)	**<0.01**
	Clinical ward	26 (60.5%)	20 (62.5%)	3 (100%)	3 (37.5%)	
	ICU	5 (11.6%)	1 (3.1%)	0	4 (50.0%)	
Uro-andrologic questionnaires	IPSS	4 (2–8)	3 (1–7)	5 (2–9)	7 (5–9)	0.20
	NIH-CPSI	0 (0–2)	0 (0–2)	0 (0–2)	1 (0–4)	0.78
	MSHQ-EJD	24 (22–25)	25 (22-25)	23 (15–24)	22 (22–24)	0.70

Abbreviations: BMI, body mass index; ICU, intensive care unit; IPSS, international prostate symptom score; NIH-CPSI, National Institute of Health-chronic prostatitis symptom index; MSHQ:EjD, male sexual health questionnaire - ejaculatory dysfunction.
All values are expressed as median (interquartile range) or n (%). Statistical analyses are Kruskal-Wallis H test and Fisher's Exact test accordingly.

TABLE 2 Patients' inflammatory markers during admission for coronavirus disease 2019 (COVID-19) infection, globally and according to semen analysis

	Total ($n = 31$)	Normozoospermic ($n = 21$, 67.7%)	Oligozoospermic ($n = 3$, 9.7%)	Crypto-azoospermic ($n = 7$, 22.6%)	p
WBC at admission (1000/ml)	5.5 (5.1–7.6)	5.5 (5.1–7.4)	5.4 (5.4–10.1)	7.3 (3.5–8.4)	0.84
WBC at peak (1000/ml)	7.4 (6.0–12.6)	6.7 (6.0–11.2)	5.4 (5.1–10.1)	12.8 (7.7–18.3)	0.08
WBC at discharge (1000/ml)	5.7 (4.4–6.5)	6.0 (4.9–6.6)	4.6 (4.1–5.6)	4.7 (3.0–6.7)	0.12
WBC decreasing time (days)	16 (8–21)	17 (11–21)	4 (2–4)	16 (8–22)	0.07
Procalcitonin at admission (mg/ml)	0.16 (0.10–0.35)	0.12 (0.07–0.18)	NA	0.33 (0.26–0.36)	**0.05**
Procalcitonin at peak (mg/ml)	0.15 (0.10–0.42)	0.14 (0.10–0.17)	0.10 (0.08–0.12)	0.42 (0.36–0.56)	0.16
Procalcitonin at discharge (mg/ml)	0.08 (0.04–0.12)	0.08 (0.04–0.12)	0.18 (0.18–0.18)	0.07 (0.03–0.10)	0.47
Procalcitonin decreasing time (days)	19 (11–24)	19 (11–23)	NA	22 (14–31)	0.48
C-reactive protein at admission (mg/ml)	79 (28–120)	56 (13–93)	58 (31–120)	142 (86–158)	**0.03**
C-reactive protein at peak (mg/ml)	79 (44–142)	56 (18–106)	58 (38–120)	206 (134–291)	**0.02**
C-reactive protein at discharge (mg/ml)	12 (4–39)	9 (4–39)	17 (12–38)	0 (0–43)	0.46
C-reactive protein decreasing time (days)	17 (8–24)	17 (12–24)	4 (4–4)	22 (8–27)	0.07

WBC = white blood cells.
All values are expressed as median (interquartile range) or n (%). Statistical analyses are Kruskal-Wallis H test and Fisher's Exact test accordingly.

and with sperm concentration (Rho −0.414, $p = 0.01$ and Rho −0.372, $p = 0.02$, respectively) (Figure S2). In contrast, IL-4 levels were significantly positively correlated with sperm motility (Rho +0.346, $p = 0.05$).

A trend of positive correlation which however did not reach the statistical significance was also present between IL-4 and sperm total number.

4 | DISCUSSION

Infection of male genital reproductive apparatus has been reported in the course of numerous bacterial and viral infections including COVID-19.[14] We report here that high levels of inflammatory cytokines are present in the semen of men recently (median time, 35 days) recovered

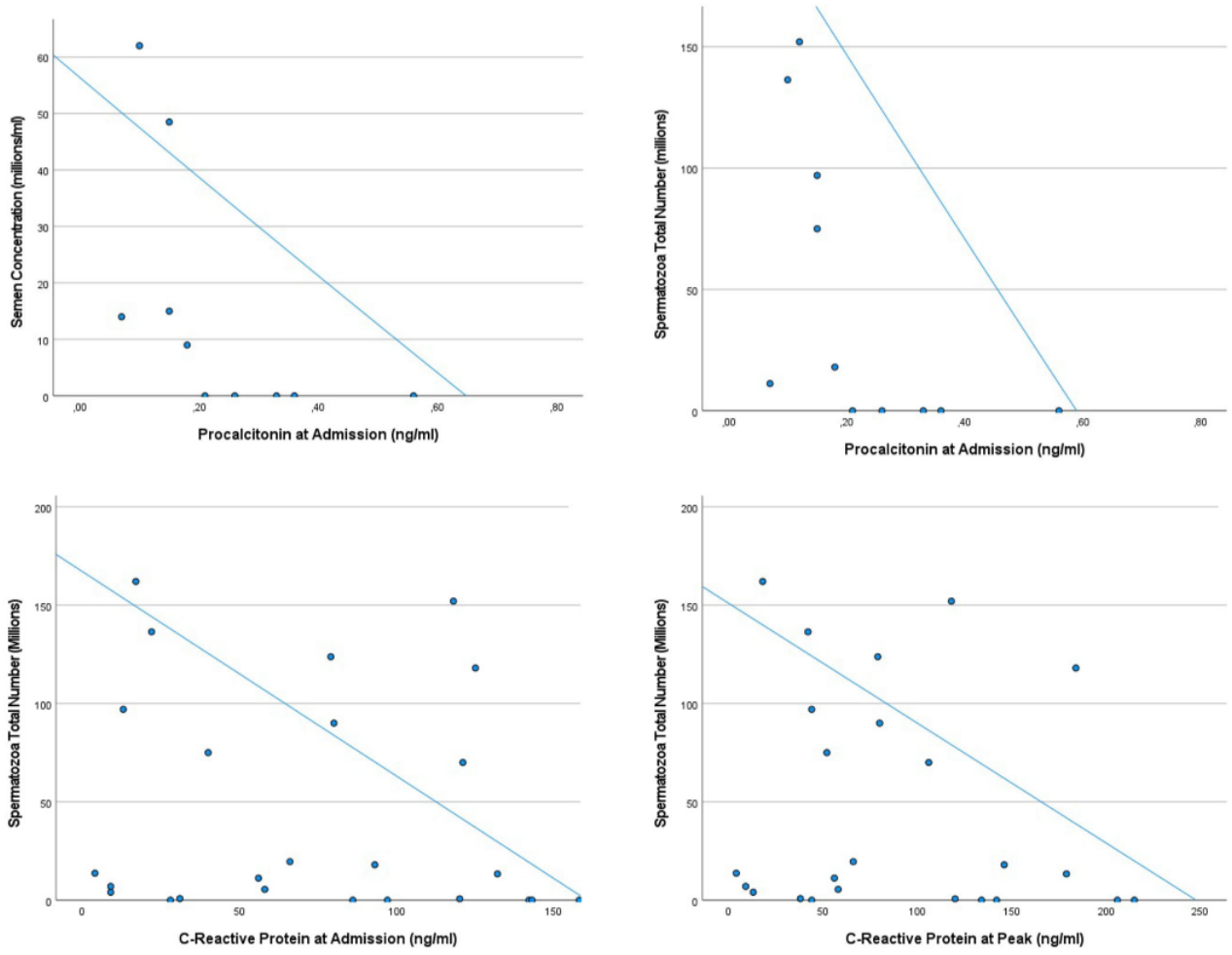

FIGURE 1 Correlation of procalcitonin and C-reactive protein values at admission with sperm concentration and sperm total number

from COVID-19, indicating the presence of an inflammatory condition in the male genital tract.

Viruses typically reach the male reproductive organs through hematogenous spread. The blood-testis barrier protects germ cells in most infections. However, some viruses acquire the property to cross the barrier and, in some cases, can infect testicular cells and/or induce an immune-inflammatory response within the testis.[7,15] Fifteen percent of mumps-infected males undergo bilateral orchitis with severe impairment of testicular function.[16] HIV and Zika viruses have been detected in the semen and can lead to orchitis.[17] Immune-inflammatory response against virally infected cells is largely mediated by macrophages, T cells, NK cells, and to a lesser extent, by B cells. These cells release pro-inflammatory cytokines (e.g., IL-17, IL-15, IL-8, and interferon-γ) which in turn increase the expression of several growth factors including fibroblast growth factor (FGF) and FGF-2, resulting in abnormal proliferation of either epithelial and stromal cells. The increased oxygen due to cell proliferation often leads to local hypoxia producing low levels of reactive oxygen species promoting angiogenesis and the production of additional growth factors (i.e., vascular endothelial growth factor, IL-8, FGF-2, FGF-7, and transforming growth factor ß).[14]

Spermatogenesis impairment was observed in different clinical conditions, including autoimmune orchitis, and it has been repeatedly associated with the increased concentration of inflammatory cytokines in seminal plasma.

IL-6 and TNF-α, in particular, were involved in the damage of the blood-testis barrier.[18–20] IL-8 was found significantly increased in patients with prostatitis.[13]

Patients with severe COVID-19 frequently undergo sepsis[21] and the presence of high viral load in the blood is supposed to facilitate dissemination in different organs and apparatuses including the male reproductive tract and, rarely, semen.[22,23] Several molecular mechanisms may be involved in testicular damage in severe COVID-19 patients. A high concentration of IL-6 is likely involved in the damage of the blood-testis barrier. The viral load may directly induce the testicular damage[24] or indirectly through the immune response, either innate

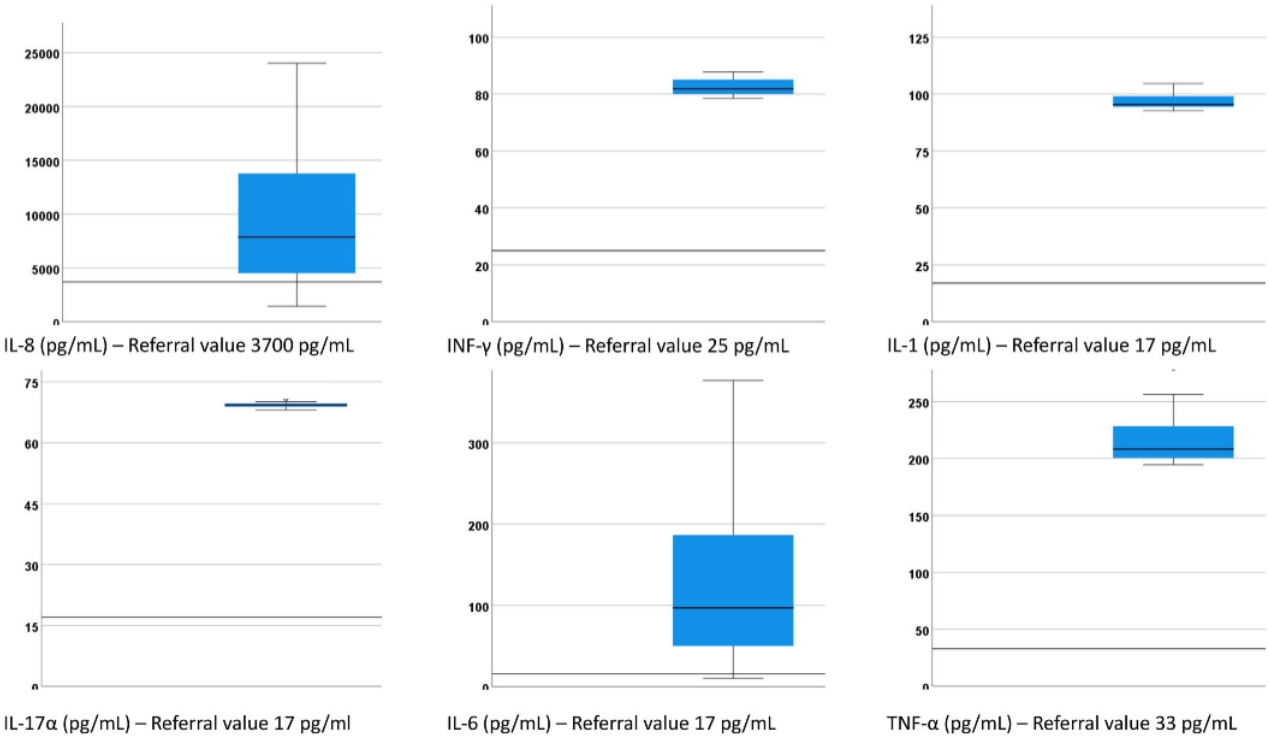

FIGURE 2 The level of interleukin (IL)-8, IL-4, IL-6, IL-17, tumor necrosis factor α (TNF-α), and IL-1β in the seminal fluid of all patients

TABLE 3 Seminal chemokines globally and comparison according to semen analysis at enrollment

	Total ($n = 31$)	Normozoospermic ($n = 21$, 67.7%)	Oligozoospermic ($n = 3$, 9.7%)	Crypto-azoospermic ($n = 7$, 22.6%)	p
IL-8 (pg/ml)	7860 (4412–14,400)	7670 (3757–10,942)	6720 (4680–19,190)	13123 (7046–32,806)	0.21
IL-1 (pg/ml)	95 (94–99)	95 (94–97)	96 (96–96)	116 (103–160)	0.01
IL-4 (pg/ml)	18 (17–18)	18 (17–18)	18 (17–18)	18 (18–19)	0.76
IL-6 (pg/ml)	97 (50–186)	88 (45–186)	111 (90–132)	164 (141–204)	0.49
IL-17 (pg/ml)	69 (69–70)	69 (69–70)	69 (69–69)	70 (70–70)	0.35
TNF-α (pg/ml)	208 (201–228)	206 (200–219)	245 (211–279)	246 (208–329)	0.09
INF-γ (pg/ml)	82 (80–85)	82 (80–86)	82 (81–83)	82 (79–84)	0.86

Abbreviations: IL, interleukin; INF-γ, interferon-γ; TNF-α, tumor necrosis factor-α.
All values are expressed as median (interquartile range) or n (%). Statistical analyses are Kruskal-Wallis H test and Fisher's Exact test accordingly.

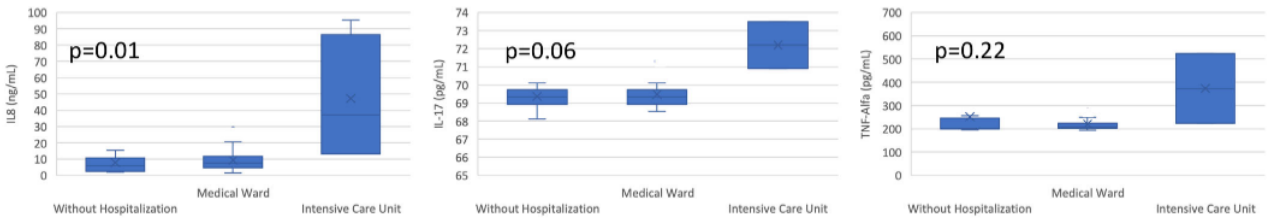

FIGURE 3 Interleukin (IL)-8, IL-17, and TNF-α values in patients divided according to the severity of coronavirus disease 2019 (COVID-19) infections. The lines and "X" represent the median and the average of the sample, respectively

or adaptive, activated to fight against the virus.[25] In addition, the testicular damage can be due to the high local temperatures reached during viral dissemination.[26,27]

C-reactive protein levels were found high in all patients during the disease and were still high after the recovery. Oligo-zoospermia and crypto-azoospermia, found in three and eight patients, respectively, after COVID-19 recovery, were significantly associated with the concentration of plasmatic C-reactive protein measured at admission time and during the peak of the disease.

Systemic inflammatory markers are usually poorly related to semen quality, as suggested by men with metabolic syndrome, which yields higher C-reactive protein blood values compared to healthy subjects without altering substantially semen quality.[28] Similarly, procalcitonin appears to be present both in seminal plasma and in blood serum in case of male urogenital tract infections, but with its seminal levels comparable to healthy men. In our cohort of COVID-19 recovered men, procalcitonin and C-reactive protein, both at admission and the peak, were statistically significantly higher in crypto-azoospermic patients compared to normozoospermic patients ($p = 0.05$; $p = 0.03$ and $p = 0.02$, respectively) suggesting a possible involvement of these markers in testicular impairment.

Although the occurrence of the SARS-CoV-2 genome in seminal fluid appears to be a rare event,[9] high levels of pro-inflammatory cytokines were detected in all semen samples from post-COVID-19 patients. Cytokine concentrations were more than 2–3-fold higher than those found in control subjects (age >18 years) in pre-COVID age (2014) from our group, using the same detection technology.[13]

The levels of seminal IL-8 and, to a lesser extent, of IL-17, were associated with disease severity and were significantly higher in ICU requiring patients compared to other groups suggesting that an inflammatory process is present in the genital tract. Fifty percent of the patients admitted to ICU due to COVID-19 complications were also crypto-azoospermic[9] and testicular damage was significantly correlated with signs of systemic inflammation. Thus, our data may suggest that the impairment of testicular function could be a consequence of systemic inflammation following viral dissemination. Whether such inflammatory status persists after healing is presently unknown and further studies are required to address such eventuality.

High inflammatory infiltration was found in the testis of patients who succumbed to COVID-19, suggesting alterations of testicular cell function, including Leydig cells leading to reduced testosterone production.[29] SARS-CoV genome is 79% homologous to that of SARS-CoV2 and the two viruses utilize the same ACE2-receptor to entry in the cells. Thus, it is conceivable to hypothesize that inflammation-mediated disruptions of testicular cells, including the Leydig cells, also occur during SARS-CoV infection.

The high concentrations of TNF-α, IL-17, and IFN-γ detected in the seminal fluid of patients recovered from COVID-19 indicate that, in addition to innate immunity, Th-1 and Th-17 responses are activated in the upper genital tract as a response to SARS-CoV-2 infection. Activated immune cells infiltrated in the male genital tract may also trigger autoimmune responses following the exposure of hidden antigens, and develop antibodies against the germ cells as reported for patients who died for SARS-CoV infection.[29]

In addition, our data confirm that IL-4 could have a protective effect on the testicular function of COVID-19 patients, as previously reported.[30]

Although novel, our results should be evaluated considering the study design and limitations. First, this is a prospective study including a small cohort of COVID-19 recovered patients with no control group. Besides, in light of the small study sample and the requested active participation, our results could be influenced by selection bias due to several issues, such as education, employment, the desire to have children, or andrological diseases. In addition, we have no data about the semen quality and semen inflammatory markers of enrolled patients before SARS-CoV-2 infection. Moreover, we analyzed data from a median follow-up time of 35 after recovery that this is a rather short time, and further studies to evaluate the long-term impact of covid-19 infection on semen quality are needed. Another limitation of the study might be found in the low number of patients with available serum procalcitonin data, which may affect the statistical power of this part of the study, and might represent a selection bias. In addition, even if TNF-α was statistically significant, the results are affected by levels out of scale (above 280 pg/ml) found in three patients. According to the previous limitations, some of the data presented should be interpreted cautiously. Finally, hormone levels were not assessed in our study. Nevertheless, although several limitations, our study represents the largest population analyzed in current literature with a full panel of seminal inflammatory markers.

5 | CONCLUSION

According to our initial results, patients with coronavirus disease 2019 infections might have high levels of pro-inflammatory cytokines in seminal plasma, in particular in patients with severe impairment of semen parameters, thus suggesting an impact of inflammatory processes on testicular function. Further studies are warranted to evaluate whether such a condition persists after a longer period from recovery as well as to clarify severe acute respiratory syndrome coronavirus 2 mechanisms of action on the male genital tract.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest related to this article.

AUTHOR CONTRIBUTIONS

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SUPPORTING INFORMATION

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Case Report

Persistent Male Genital Tract Inflammation and Semen Impairment: A Long-Term Effect of SARS-Cov-2 Infection

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Abstract

Background: Recent reports evidenced an impairment of semen parameters in men affected by coronavirus disease 2019 (COVID-19). In particular, our group previously reported that 1 over 4 COVID-19 healed men were found to be crypto- / azoo-spermic. Moreover, most patients had elevated IL-8 semen levels at sperm analysis. The aim of our study was to assess semen parameters and inflammation by evaluating a panel of sperm cytokine levels (IL-1, IL-4, IL-6, IL-8, IL-17, INF gamma, TNF-alpha) on average 1 month after the second SARS-CoV-2 negative nasopharyngeal swab and 3 months later. **Methods:** Ten men who showed normozoospermia (n=3), oligozoospermia (n=3) or crypto/azoospermia (n=4) 1 month after healing from COVID-19 in our previous study, were re-called and re-evaluated 3 months after the first semen analysis. Semen parameters were evaluated according to WHO manual and seminal plasma cytokine levels by an ELISA method. **Results:** At 3-months follow-up, 8 men showed an overall increase of semen parameters compared to levels assessed after 1 month. In particular, of

the 4 crypto-/azoo-spermic men 1 month after healing, 2 resulted oligozoospermic, 1 normozoospermic and only 1 remained azoospermic. Two of the 3 oligozoospermic men turned normozoozpermic. Semen cytokine levels were remarkably high one month after healing and remained elevated after 3 months, with the exception of IL-6. **Conclusions:** This is the first longitudinal, prospective study comparing semen parameters and semen inflammatory markers one and three months after recovering from COVID-19. Our data indicate an overall tendency to an improvement of semen parameters although a genital tract inflammatory condition appears to persist at least 3 months after COVID-19 recovery. This condition could have an impact on male fertility requiring a careful follow up of these patients.

Keywords: Covid-19; SARS-Cov-2; Sperm; Semen, Inflammation, Fertility

Introduction

Male reproductive system is vulnerable to several viral infections, including those from ZIKA, Mumps, hepatitis B and C virus or human immunodeficiency virus, which exert different impacts on male fertility [1]. The main types of male gonadal cells (spermatogonia, Leydig and Sertoli cells) express Angiotensin-Converting Enzyme 2 (ACE2) receptor and transmembrane serine protease 2 (TMPRSS2) on their membrane [2]. Several reports suggest a role for ACE2 and TMPRSS2 as the cellular receptor for SARS-CoV-2, allowing the access of the virus to the male reproductive organs, with a consequent risk of testicular impairment in men with Covid-19 [3]. In a recent series of 34 Chinese men (median age: 37) recovering from COVID-19, SARS-CoV-2 was not detected in the semen 1 month after COVID-19 diagnosis [4]. However, subjects with a moderate infection from Covid-19 showed an impairment of sperm quality, even if SARS-CoV-2 RNA is not detected in semen [5]. Gonadal impairment could be due either to a direct effect of SARS-CoV-2 on testicular cells and/or to the high inflammatory response consequent to the disease [3]. In particular, the COVID-19 cytokine storm may produce a widespread tissue damage, resulting in multi-organ failure with the potential involvement of the reproductive system as well [3,6]. We previously demonstrated that at least one-fourth of men recently recovered from COVID-19, presented oligo/crypto/azoospermia and such condition was related to the severity of the illness [7]. In addition, most of these men, showed elevated semen levels of IL-8, a surrogate marker of male genital tract inflammation [7]. Alterations of semen quality in patients healed from COVID-19 have been demonstrated in other studies [8,9]. Even if some evidence indicates the loss of testicular architecture at post-mortem examination of COVID-19 severe cases, data regarding the long-term impact of COVID-19 on male reproductive function and semen quality after recovery are lacking [3,10]. Aim of the present study was the evaluation of semen parameters (Sperm Concentration, Total Spermatozoa Number, Progressive Motility) and semen inflammatory cytokine levels (IL-1, IL-4, IL-6, IL-8, IL-17, INF gamma, TNF-alpha), in 10 sexually active men one and 3 months after recovery from COVID-19.

Materials and Methods

Study Design

A prospective cohort study was designed following the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines. Current study was approved by Ethics Commettee Area Vasta Centro - based at AOU Careggi in June 2020, under the code 17104. Subsequently It was registered in clinicaltrials.gov with the identifier NCT04446169. It was designed and conducted according to the Declaration of Helsinki. All enrolled participants signed an informed consent. The study inclusion criteria are: male patients, between 18 and 65 years of age recovered from covid 19 and confirmed by molecular swab of sars-cov 2 infection. We re-contacted 10 subjects, crypto / azoospermic (n = 4), normozoospermic (n = 3) and oligozoospermic (n = 3) evaluated by spermiogram performed on average 1 months after recovery from COVID-19 according to our previous study7 and offered them a reassessment of sperm parameters 3 months after the first one. Everyone agreed to participate in the study. Data recording, specimen collection and SARS-CoV-2 RNA detection. Clinical data were collected according to the previously reported scheme [7]. Clinical data included: patient demographics, comorbidities, medications, hospitalization time and features (including intensive care need), laboratory tests and treatments (including oxygen therapy) [11].

Semen Analysis

Semen was collected after a period of 2-7 days of abstinence. WHO guidelines [12] were strictly followed for semen analysis. Sperm concentration was determined using an improved Neubauer hemocytometer. The percentages of progressive, non-progressive and immotile spermatozoa were evaluated on 200 spermatozoa/sample by phase contrast microscopy. Sperm morphology was assessed after Diff-Quick staining on 200 spermatozoa at 1000x bright field microscopy. Semen leukocytes were quantified by counting the number of round cells/ml and evaluating the percentage of leukocytes after May-Grunwald staining of the sample.

Immunoplex Assay Semen Plasma

Frozen - thawed samples of seminal plasma were

centrifuged at 800 g for 10 minutes to remove particulates. Detection and quantification of cytokines was performed using the Milliplex® Map kit Human Cytokine/Chemokine/Growth Factor Panel A Magnetic Bead Panel (Merck Kgaa, Darnstadt, Germany) following the manufacturer's protocols. The plate was analyzed with the Luminex 200 MAGPIX® and data were then analyzed using a 5-parameter logistic curve-fitting method for calculating analyte concentrations in samples. All samples were processed on the same day of collection or stored at -80°C until further analysis. Nucleic acids from samples were extracted with Microlab Nimbus IVD system (Seegene Inc, Seoul, South Korea) using the Starmag Universal Cartridge and amplified with the multiplex RT-PCR Allplex™ 2019-nCoV assay (Seegene Inc), targeting RdrP, E and N genes, according to Manufacturers' instructions.

Statistical Analysis

Continuous and categorical variables were reported as median (interquartile range) and number (percentage), accordingly. Patients were then divided according to their semen analysis results, thus normo-, oligo- and azoo-spermic. Statistical comparisons between groups were conducted with Mann-Whitney u-test and univariate ANOVA for continuous variables, and with $\chi 2$ and Fisher's Exact test for categorical variables, as appropriate according to sample size. Intragroup comparison from baseline to follow-up were done with Wilcoxon test for continuous variables. Statistical significance was set with a p-value<0.05. All statistical analyses were performed using IBM SPSS version 20.0 (SPSS Inc, Chicago, IL, USA).

Results

Patient characteristics and the received treatments during COVID-19 infection are reported in Table 1 and 2, respectively. Table 3 reports semen parameters and seminal plasma IL-8 levels 1 month and 3 months after recovery. Levels of the other evaluated cytokines, 1 and 3 months after recovery, are reported in Table 4. In the latter table, semen levels of cytokines in healthy controls and in patients with chronic prostatitis according to a previous study where cytokine levels were measured in the same laboratory13 are shown for comparison. Eight out of the 10 patients showed an increase in sperm count (Figure 1, panels A-B and Table 3) after three months. Interestingly, 1 normozoospermic patient at the time of recovery (#2) tested oligozoospermic 3 months later. He is a young (32-years-old), healthy man, who was treated with remdesivir, hydroxychloroquine and haeparin during hospitalization. One oligozoospermic patient (#6), who was treated with remdesivir and hydroxychloroquine during hospitalization, showed a small increase in sperm concentration (from 1.1 to 7.6 x106/mL). Progressive motility (Figure 1, panel C) showed an improvement in 5 out of 10 patients, although only 4 patients (2 normozoospermic [#2, #3], 1 oligozoospermic [#4] and 1 crypto/azoospermic [#10]) reached values above the 5th percentile of WHO reference [12], at 3 months follow up. None of the enrolled patients required new medications or hospitalization during the follow-up period. IL-8 levels were above the cut-off value of 3.8 ng/ml [13] in all enrolled patients at the time of recovery (Table 3). Three months later, the levels of IL-8 were still high, with the exception of 1 patient (#1) (Figure 2). Overall, we demonstrate a halving of IL-8 levels between first (median: 95) and second (median: 9,) semen analysis, although more than 3 months after healing from COVID-19, IL-8 levels remained more than double of cut-off values (8) in the majority of patients. Semen cytokines were all elevated 1 month after recovery and were persistently high after 3 months, with the exception of IL-6 (Figure 3). Mean cytokines concentrations in post COVID-patients were all higher when compared with healthy population [13] both 1 month after recovery and after further 3 months (Table 4). Interestingly, the mean levels of INFγ and TNFα were significantly higher as compared to those reported in men with proven prostatitis13 (Table 4).

Figure 1

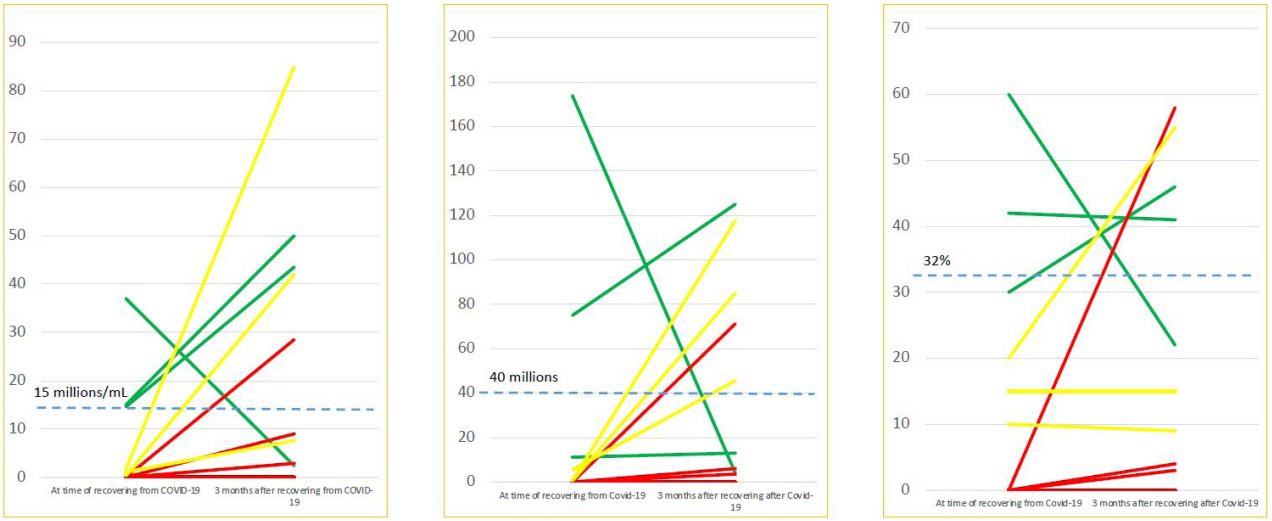
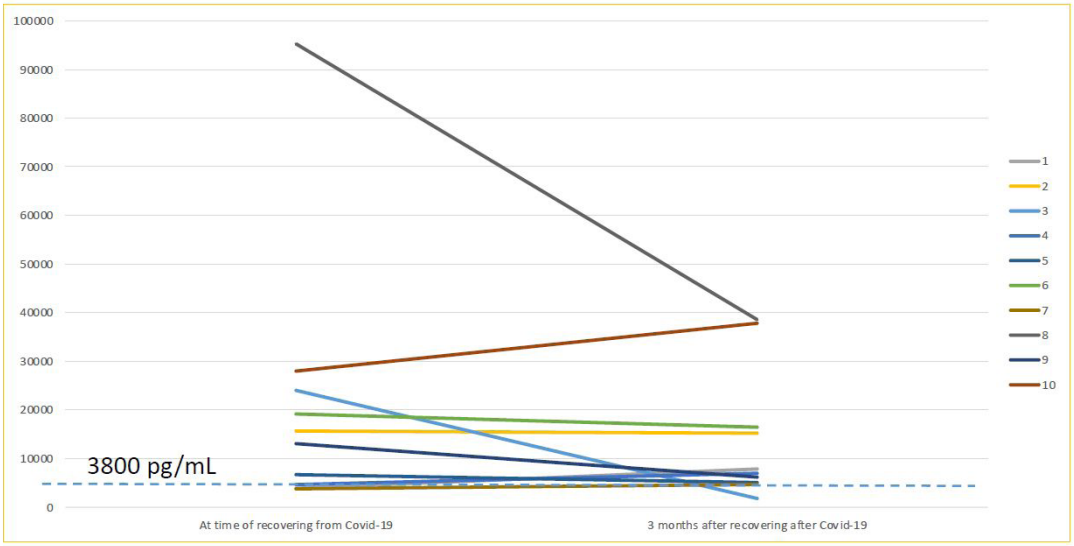


Figure 2



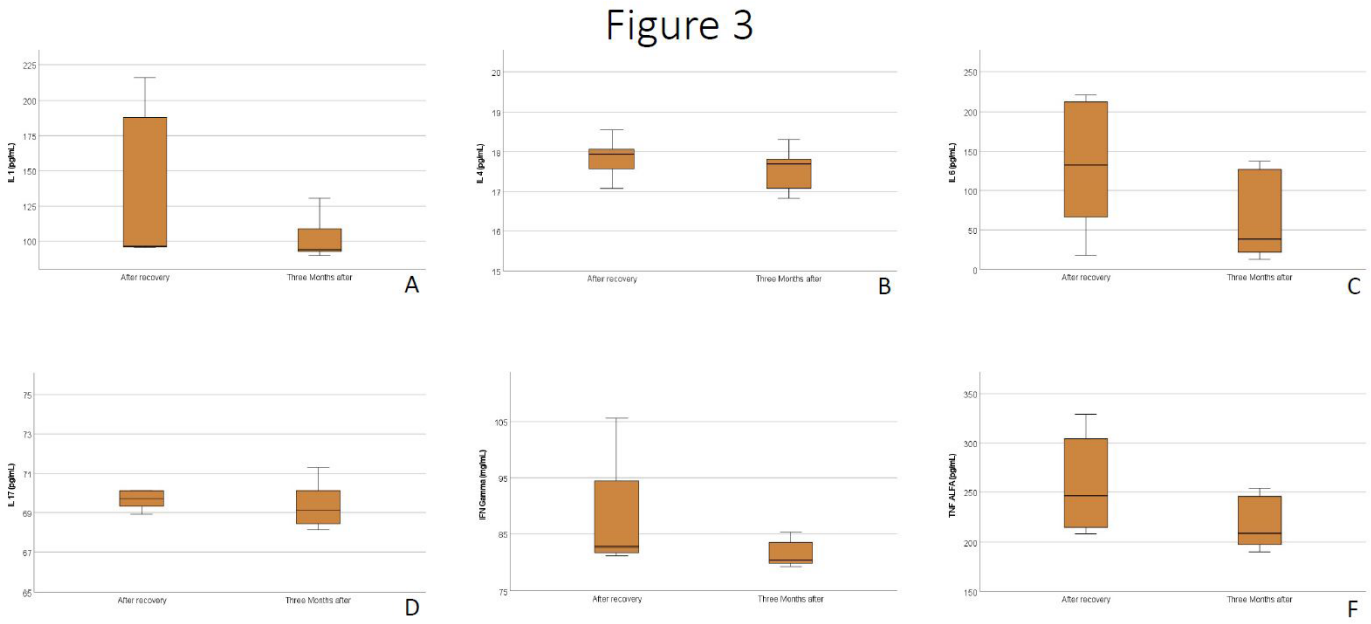


Figure 1: Semen changes from one month a3er COVID-19 recovering to a3er further three months a3er recovering, Dashed lines represent referral values, Green = normospermic patients, Yellow= oligospermic patients, Red = azoospermic patients. 1 A: Sperm Concentra;on (Millions/mL), 1 B: Total Spermatozoa Number (Millions), 1 C: Progressive Mo;lity (a+b) (%).

Figure 2: Interleukin 8 changes from one month after COVID-19 recovering to after further three months after recovering, Dashed line is the referral cut-off value for Interleukin 8 levels in semen, 3800 pg/mL.

Figure 3: Box plot comparison of Cytokines and Chemokines values "One Month AJer Recovery" and "AJer further three months". Box represents interquartile range, while lines maximum and minimum values. All values are expressed as pg/mL. A: Interleukin 1β; B: Interleukin 4; C: Interleukin 6; D: Interleukin 17; E: Interferon γ; F: Tumor Necrosis Factor α.

	Normozoospermic			**Oligozoospermic**			**Crypto-azoospermic**			
	1	2	3	4	5	6	7	8	9	10
Age, years	35	32	47	44	60	58	62	64	49	40
BMI, Kg/m2	29,2	23,6	23,1	21,8	22,9	23,2	27,4	30,1	27,4	28,7
Smoke	No	No	No	No	No	No	No	No	Former	No
IPSS	6	6	2	5	9	2	10	12	6	3
IPSS QoL	2	1	2	0	2	0	1	3	2	0
IIEF-5	25	24	25	24	15	23	15	22	25	22

Paternity	0	0	1	0	2	2	1	1	2	1

BMI: Body Mass Index (calculated as weight in kilograms divided by height in meters squared); IQR: Interquartile Range. [a]International Prostatic Symptoms Score; [b]International Prostatic Symptoms Score Quality of Life; [c]International Index of Erectile Function Questionnaire.

Table 1: Patients' Characteristics.

	Normozoospermic	Oligozoospermic	Crypto-azoospermic
	(n=3)	(n=3)	(n=4)
Non-Hospitalized (n, %)	1 (33.3)	0 (0)	1 (25.0)
Hospitalized (n, %)	2 (66.7)	3 (100)	1 (25.0)
Intensive Care (n, %)	0 (0)	0 (0)	2 (50.0)
Antibioticsa (n, %)	0 (0)	0 (0)	3 (75)
Antivirals (n, %)	2 (66.7)	3 (100)	3 (75)
Steroids (n, %)	0 (0)	0 (0)	1 (25)
Heparin (n, %)	2 (66.7)	2 (66.7)	3 (75)
Hydroxychloroquine (n, %)	3 (100)	3 (100)	3 (75)
Oxygen therapyb	2 (66.7)	1 (33.3)	1 (25)
Low Flow O2 (n, %)	1 (33.3)	2 (66.7)	1 (25)
Invasive Vent. (n, %)	0 (0)	0 (0)	2 (50)

Abbreviations: COVID-19: coronavirus disease 2019.
aAntibiotics include amoxicillin and clavulanic acid, piperacillin and tazobactam, azythromycin.
bLow Flow Oxygen Therapy includes nasal cannula, simple face mask and partial rebreather mask while High Flow Oxygen includes Trans Tracheal Catheters, Venturi Mask, Aerosol Mask, Tracheostomy collars, non-rebreathing mask with reservoir and one way valve and high humidity face tents.

Table 2: Patients' treatments during COVID-19 infection, according to semen analysis.

	Normozoo spermic (n=3)		Oligozoospermic (n=3)		Crypto-azoospermic (n=4)	
	1 month after recovering	3 Months later	1 month afterrecovering	3 Months later	1 month after recovering	3 Months later
Volume (ml) (median)	4.7	1.7	2.2	3	0.5	1.4
Number (millions) (median)	75	13.1	0.8	85	NA	6
Concentration (millions/ml) (median)	15	43.5	1	42	NA	6

pH (median)	7.4	7.2	8	8	6.9	7
Prog. Motility (a+b) (%) (median)	42	41	15	15	NA	58
Morphology (NF) (%) (median)	4	5	4	5	NA	1
Leucocytes (103/ml) (median)	0.2	0	0	0	NA	0
IL-8 (ng/ml) (median)	15.7	7.9	6.7	7	20.6	22
Abbreviations: IQR: interquartile range. aTime needed to carry the samples to laboratory.						

Table 3: Patients' semen characteristics, according to time of analysis.

Cytokines (pg/ml) Median (Interquartile Range)	Healthy controls [13]	Covid Patients – One month after recovery (n=10)	CP IIIA13	Covid Patients – After further three months (n=10)
IL8	1984 (1164-2444)	14387 (4488-25113)	15240 (10630-19501)	7045 (4328-16160)
IL-1β	17 (10-41)	96 (95-215)	61 (22-108)	94 (93-110)
IL-4	NA	18 (17-18)	NA	18 (17-18)
IL6	16 (10-26)	132 (43-221)	99 (31-130)	38 (20-132)
IL-17α	NA	70 (69-70)	NA	69 (68-70)
INFγ	25 (9-55)	83 (82-104)	44 (17-63)	80 (80-84)
TNFα	33 (22-65)	246 (211-329)	58 (37-95)	209 (197-250)
13 Penna et al, Eur Urol, 2007 (https://doi.org/10.1016/j.eururo.2006.07.016) Legend: CP IIIA= Chronic Prostatitis patients stage IIIa				

Table 4: Semen Cytokines levels in Covid-19 recovered patients at recovery and three months after, together with healthy controls and Chronic Prostatitis patients stage IIIa.

Discussion

Our study indicates that alterations of semen parameters observed shortly after recovering from COVID-19 may be transient as most patients show an improvement of semen quality after three months although an inflammatory status of the male genital tract seems to persist. Several viruses, including HBV, HCV, HPV, HSV, Mumps, may cause an impairment of testicular function and can be detected in semen [14]. Some viruses such as MuV, HIV and SARS-CoV can affect testicular cells, resulting in severe orchitis, which can compromise male fertility [15]. During the very first weeks after recovery from Covid-19, the majority of men show high semen levels of IL-8, and at least one-fourth of these men, show alterations of semen parameters [7]. A recently published systematic review based on data collected from 70 studies confirms these data [16]. In particular, the Authors suggest that COVID-19 may cause an inflammatory condition of the testis which is correlated to the severity of the disease16. SARS-CoV-2 is very contagious and has already infected a higher proportion of young men during the second pandemic wave, as compared with the first one [17]. Since the highest expression of ACE2 in testicular cells occurs at about thirty years of age, the interaction between SARS-CoV-2 and ACE2 receptor and TMPRSS may cause severe damage to the testis in young males, rising concerns about male fertility [18]. Impaired gonadal function can be due to inflammation an autoimmune response [12] as well as high fever and medications during the course of the disease [19]. In particular, a significant increase of semen levels of inflammatory cytokines, such as IL-6 and TNF-α was observed in COVID-19 patients1. Furthermore, evidence suggests that both testicular function and semen quality may be reduced following severe COVID-19, due to several aspects of the disease including high fever and medications, indicating the need for andrological evaluation of recovered men [7,9]. Such a recommendation is also indicated in the official website of the government of Hubei Province which posted a bulletin inviting all men recovered from SARS-CoV-2 to undergo fertility checks, suggesting a big concern for a possible link between this novel disease and the male reproductive system [20].

In the present study, we report a longitudinal prospective case series of sexually active men based on two consecutive sperm analysis performed after healing from COVID-19 and 3 months later, after a complete new cycle of spermatogenesis. Overall, 3 out of 7 (43%) oligo-crypto-azoospermic patients at the time of recovery, showed an improvement of testicular function with an increase of sperm number, 3 months after healing, suggesting that the detrimental effects of COVID-19 could be temporary. However, in few cases, no improvement in sperm number has been achieved. Since semen quality of these men before COVID-19 was not available, whether or not persistence of crypto/azoospermia is due to the illness cannot be ascertained. However, 6 out of 7 patients (86%) reported paternity before COVID-19 [7]. In most patients, an improvement of sperm progressive motility was observed, but values still remained below the 5th percentile of the WHO reference values in 6 of them. As previously reported, IL-8 is a cytokine associated with inflammation of prostate, seminal vesicles and epididymis [13]. Semen IL-8 concentrations 1 month after recovery from COVID-19 were related with severity of the illness, including the need of hospitalization, intensive care, oxygen therapy and invasive ventilation7. Interestingly, semen IL-8 levels remain high (more than double of the cut-off value of 3.8 ng/ml) in most of the enrolled patients also 3 months after COVID-19 healing, suggesting the persistence of an inflammatory condition in the male genital tract that may require further assessment. Similarly, semen levels of 7 cytokines which are part of the SARS-CoV-2 cytokine storm [21] resulted elevated both one and 3 months after COVID-19 recovery in all patients recruited in this study, with the exception of IL-6 which decreased significantly after 3 months. In particular, levels of some cytokines (IL-8, IL-1, IL-6, INF gamma and TNF-alpha), resulted higher respect to those found in patients affected by prostatitis (Table 4) [13], indicating that the inflammatory status of the male genital tract of patients recovering from COVID-19 may be similar or even more compromised. A limitation of our study regards the lack of semen parameters before COVID-19 diagnosis. Another limitation regards the small cohort of evaluated patients. However, our intent was to determine whether those patients that showed alterations of semen quality 1 month after healing from COVID-19 [7], could recover after 3 months, representing an entire spermatogenetic cycle. Our study has also strengths. To our knowledge, this is the first study comparing semen parameters in healed men from COVID-19 at 1 and 3 months after recovery, although in a small number of subjects. In addition, we evaluated a wide panel of seminal plasma cytokines involved in male genital tract inflammation. In conclusion, our study demonstrates that, despite an overall amelioration of semen quality, COVID-19 induced inflammation in the male reproductive tract may persist 3 months after recovery, with a possible detrimental effect on male fertility. Further studies are needed to understand the link between the cytokine storm during COVID-19 and the damage to reproductive system in order to minimize the potential sequelae on male fertility. Our study indicates that a careful follow up is needed for patients in reproductive age recovering from COVID-19. The dataset(s) supporting the conclusions of this article is(are) included within the article.

Declarations

Ethics approval and consent to participate: Current study was approved by our Institutional Review Board in June 2020, under the code 17104. Subsequently It was registered

in clinicaltrials.gov with the identifier NCT04446169. It was designed and conducted according to the Declaration of Helsinki. All enrolled participants signed an informed consent.

Availability of data and materials: The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no competing interests. The authors report no involvement in the research by the sponsor that could have influenced the outcome of this work.

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