Fusobacterium nucleatum: universal colonizer

Nedeljković, Ema

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DEPARTMENT OF BIOTECHNOLOGY

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Sažetak

Fusobacterium nucleatum je gram-negativna obligatna anaerobna bakterija koja obitava u usnoj šupljini. Igra ulogu u nekoliko oralnih bolesti, uključujući parodontitis i gingivitis, ali je također povezana sa širokim spektrom ljudskih bolesti koje obuhvaćaju cijelo tijelo. Adhezin FadA konzerviran u *F. nucleatum*-u ključni je faktor virulencije i potencijalni dijagnostički marker za bolesti povezane s *F. nucleatum*-om. Ovaj rad razmatra implikacije *F. nucleatum*-a u parodontitisu, kolorektalnom karcinomu i nepovoljnim ishodima trudnoće. U radu se opisuju patogeni mehanizmi uključeni u ove bolesti, s posebnim naglaskom na invaziju, kolonizaciju i indukciju upalnih i tumorigenih odgovora domaćina. Biomarkeri su također istaknuti kako bi se poboljšala dijagnoza i liječenje navedenih bolesti.

Ključne riječi: *F. nucleatum*, parodontitis, kolorektalni karcinom, nepovoljni ishodi trudnoće, FadA

Summary

Fusobacterium nucleatum is a Gram-negative obligate anaerobic bacterium in the oral cavity. It plays a role in several oral diseases, including periodontitis and gingivitis but is also associated with a wide spectrum of human diseases throughout the body. The FadA adhesin conserved in *F. nucleatum* is a key virulence factor and a potential diagnostic marker for *F. nucleatum*-associated diseases. This thesis reviews *F. nucleatum's* implication in periodontitis, colorectal cancer and adverse pregnancy outcomes. The pathogenic mechanisms involved in these diseases are discussed, with a particular emphasis on invasion, colonization, and induction of host inflammatory and tumorigenic responses. Biomarkers are also highlighted in order to improve diagnosis and subsequent treatment of these diseases.

Key words: *F. nucleatum*, periodontitis, colorectal cancer, adverse pregnancy outcomes, FadA

Abbreviations

LPSs – lipopolysaccharides

PAGE – polyacrylamide gel electrophoresis

TLR – toll-like receptor

Il – interleukin

TNF-α – tumor necrosis factor-α

FadA – *Fusobacterium* adhesin A

VE-cadherin – vascular endothelial cadherin

MAP – mitogen activated protein

MMPs – metalloproteases

PBMCs – peripheral blood mononuclear cells

PMNs – polymorphonuclear cells

CRC – colorectal cancer

FISH – fluorescence in situ hybridization

FQ-PCR – fluorescent quantitative polymerase chain reaction

qPCR – quantitative real-time polymerase chain reaction

ddPCR – droplet digital polymerase chain reaction

FFPE – formalin-fixed paraffin-embedded

CDH5 – VE-cadherin 5 (vascular endothelial cadherin 5)

LEF-1 – lymphoid enhancer factor

miR21 – microRNA-21

MYD88 – myeloid differentiation factor 88

FIP – *F. nucleatum's* immunomodulatory protein

MDSCs – myeloid-derived suppressor cells

TAMs – tumor-associated macrophages

DCs – dendritic cells

iNOS – inducible nitric oxide synthase

TIGIT – T cell immunoreceptor with Ig and ITIM domains

NK – natural killer

CTL – cytotoxic T lymphocytes

IFN-γ – interferon-γ

Fn – *Fusobacterium nucleatum*

lncRNA – long non-coding RNA

RASA1 – RAS P21 Protein Activator 1

PANDAR – promoter of CDKN1A antisense DNA damage activated RNA

APO – adverse pregnancy outcome

PROM – premature rupture of membranes

SIDS – Sudden Infant Death Syndrome

EOS – early-onset sepsis

LOS – late-onset sepsis

IAI – intra-amniotic infection

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1. Introduction

Fusobacterium nucleatum is a species of bacteria belonging to the genus *Fusobacterium* of the *Bacteroidaceae* family. The name of the genus is based on the shape of its species, with *fusus* meaning spindle and *bacterion* meaning a small rod, therefore a small spindle-shaped rod. Due to the intracellular granules of the nucleus, visible with both light and electron microscopy, it was decided the name of the species would be *nucleatum*. It is Gram-negative, nonsporeforming and nonmotile, with a genomic size about 2.4×10^6 bp and a GC content of 27-28 mol%. Its cells are 5-10 μm long with sharply pointed ends. It has outer and inner membranes which are separated by a periplasmic space. The inner membrane contains a 50/50 split of phospholipids and proteins, while the outer membrane consists of phospholipids, lipopolysaccharides (LPSs), lipoproteins, and proteins (Figure 1). It does not have any fimbriae, pili, or flagellae, thus being nonmotile, but it does, on occasion, have a mucopolysaccharide capsule of variable thickness, which helps in its pathogenicity [1].

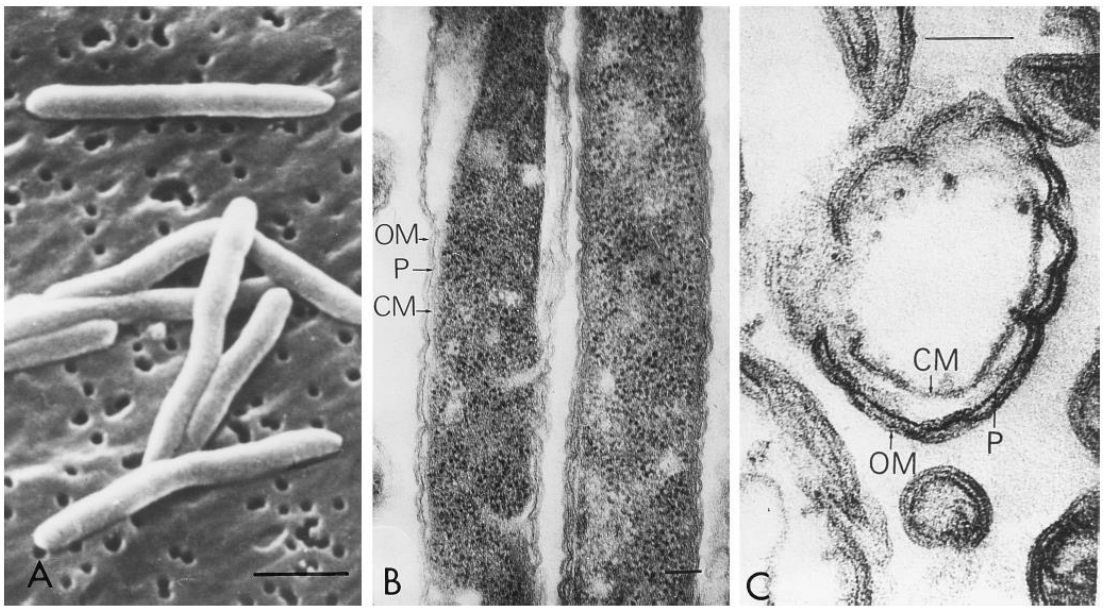

Figure 1. Electron microscopy (EM) of F. nucleatum (A) Scanning EM. Bar, 1 μm. (B and C) Transmission EM showing sections through intact cells. Bars, 100 nm. OM, outer membrane; P, periplasmic space; CM, cell membrane [1].

F. nucleatum as a species is rather heterogenous. Based on the PAGE of the whole-cell proteins and DNA homology, it was, at first, divided into three subspecies: *nucleatum, polymorphum, and vincentii* [2]; but after analyzing the patterns of DNA-DNA hybridization [3], new subspecies were discovered, for 5 subspecies in total (*nucleatum, polymorphum, fusiforme, animalis and vincentii*).

F. nucleatum is an opportunistic pathogen, implicated in all sorts of diseases in various sites of the body. As such, aside from the oral cavity, it has also been isolated from lung, liver, spleen, blood, chest, abdominal, joint, and obstetrical and gynecological abscesses and infections, either as the sole infectious agent or in mixed-species infections. These infections then go on to damage these tissues, causing multiple diseases [4].

F. nucleatum, over the years, has become known to be a type of "facilitator" for other bacterial species. As such, it interacts with a number of other organisms, namely *Porphyromonas gingivalis, Streptococcus cristatus,* and *Pseudomonas aeruginosa*, and facilitates their adherence to or invasion of host cells, increasing their invasion all the way up to 20-fold in certain species. *S. cristatus*, in particular, is noninvasive and can enter the epithelial cells only through ''piggybacking'' with *F. nucleatum*. These observations may explain the mixed-species infections associated with *F. nucleatum*. There are other examples like this, and they all prove a sort of bridging role that *F. nucleatum* also possesses, amongst many other roles [4].

Using expression microarrays, it was discovered that *F. nucleatum* affects many pathways in oral epithelial cells, including the mitogen-activated protein kinase-signaling pathway and toll-like receptor (TLR)-signaling pathway, regulation of actin cytoskeleton, cell cycle, cytokine-cytokine receptor interactions, and focal adhesion, among others. *F. nucleatum* is not known to harbor significant virulence factors but it can modulate an array of host responses upon attachment to and invasion of host cells. It

is capable of interfering with both innate and adaptive immune responses. In general, *F. nucleatum* induces interleukin 1 (IL-1) and tumor necrosis factor alpha (TNF-α), IL-8, collagenase 3, antimicrobial-peptide β-defensins and other proinflammatory cytokines. *F. nucleatum* also suppresses T-cell responses. When it comes to the host's innate immune responses specifically, it can activate TLR-2 and TLR-4 [4]. It is worth noting that *F. nucleatum* does not always induce all of the above-mentioned immune responses, but rather it induces differing ones depending on where in the body it is located.

F. nucleatum has the ability to co-adhese, coaggregate and hemagglutinate [5], as well as attach to and invade epithelial [6] and endothelial cells [7,8], polymorphonuclear leukocytes, monocytes, erythrocytes, fibroblasts, and HeLa cells, as well as salivary macromolecules, extracellular matrix proteins, human IgG [4] and even placental tissue [9]. It has many proteins in its arsenal, including multiple adhesins, such as *Fusobacterium* adhesin A (FadA) [6], RadD [10] and Fap2 [10], lectins [11], LPSs [4] and other protein complexes [12]. With *F. nucleatum* being an opportunistic pathogen, it partakes in a number of infections encompassing the whole of the human body.

FadA is, by far, the best-characterized virulence component from *F. nucleatum*. It exists in two main forms, the intact pre-FadA consisting of 129 amino acids that is anchored to the membrane, and the secreted mature FadA (mFadA) consisting of 111 amino acids that is secreted outside of *F. nucleatum*. The protein is alanine and leucine rich, with a predominantly alpha-helical structure. When mFadA combines with pre-FadA, the pre-FadA-mFadA is internalized, and FadAc is activated. This ensures that *F. nucleatum* binds to and invades host epithelial and endothelial cells, as well as placental tissue. The FadA receptor binding site is located in its loop region, which is only fully exposed at the tip of the filament. However, researchers hypothesize that it is possible that multiple filaments may bundle together to form a cluster of loops, which

would provide additional mechanical support. This ‘‘bundling’’ hypothesis is supported by the observation of FadA filaments of various widths by electron microscopy, which may represent different degrees of bundling [6]. The roles of FadA are seemingly limitless as it has a hand in virtually all of the diseases described earlier by way of both direct invasions into the host cells and pericellular invasion via loosened cell-cell junctions [13].

Fap2 is an autotransporter protein located on the outer membrane of several species of *Fusobacteria*. It has a molecular mass of 389.8 kDa, contains over 3,000 amino acids and has no cysteine residues in order to prevent disulfide bond formation of the protein while it is in the periplasm and facilitate its translocation. It has multiple functions that may facilitate fusobacterial adaptation to different body habitats, such as co-adherence, galactose-sensitive coaggregation and hemagglutination. In the oral cavity, where *F. nucleatum* is ubiquitous, Fap2 is mainly involved in co-adherence to neighboring bacteria in a manner that bridges different species and increases the diversity and the stability of the developing dental plaque. It has also proven to be involved in induction of cell death, presumably due to its ability to enable fusobacterial adherence to host cells [5]. In this way, it is similar to another protein of *F. nucleatum* called RadD, as it is also implicated in cell death, particularly that of human lymphocytes. Other than that, this arginine-inhibitable adhesin has the ability to mediate coaggregation, specifically with the *Streptococcus* genus [10].

LPS extracts from *F. nucleatum* adhere to saliva-coated hydroxyapatite and serum-coated hydroxy beads. This indicates that they play a role in adhering not only to the epithelium but also to tooth surfaces, including root cement. This may become important later as it opens the door for infection of the oral microbiota [1].

2. Aim of thesis

With the advancement of microbial detection technologies in the last decade, an increasing number of previously overlooked microorganisms have been discovered to play important roles in human diseases. *Fusobacterium nucleatum* is one such emerging pathogen and is quickly attracting the attention of the medical and research communities. The purpose of this thesis is to collect and relay information about some of the more prominent infections and diseases in which *F. nucleatum* is implicated. Another purpose is to review the mechanisms of pathogenicity it uses, as well as potential biomarkers that could better identify it in order to improve diagnosis and treatment of these ailments, and in general raise awareness of this colonizing pathogen.

3. Periodontitis

Periodontal disease is one of the two biggest threats to dental health. It is usually the result of infections (mostly bacterial) and subsequent inflammation of the gums and bone that surround the teeth. It has two forms which are characterized depending on the state of gums: gingivitis, the earlier stage, and periodontitis, the later stage [14].

3.1. Pathophysiology

Periodontitis is a chronic inflammatory oral disease, in which there is a progressive decay of gums and bone that support the teeth. Common symptoms include bad breath, bleeding of the gums, receding gums, loosening of teeth, and in extreme cases, tooth loss [15]. The reason why periodontitis is more devastating than its earlier counterpart is due to the formation of deep pockets in the periodontal tissue, which harbor anaerobic organisms that will do more damage over time. These include *Porphyromonas gingivalis*, one of, if not the, biggest contributor in the development of chronic periodontitis [16], *Aggregatibacter actinomycetemcomitans, Eikenella corrodens*, *Actinobacillus actinomycetemcomitans, Bacteroides forsythus, Fusobacterium nucleatum, Campylobacter spp., Prevotella intermedia* and many other gram-negative bacilli [15]. Among them, *F. nucleatum* is one of the most studied, and seems to be crucial in the progression of the disease [17].

3.2. *F. nucleatum* in periodontitis

Fusobacterium nucleatum is numerically dominant in dental plaque biofilms and important in biofilm ecology. It is a prominent component quantitatively and is one of the first Gram-negative species to become established in plaque biofilms. As such, it can be found in various stages of periodontal disease, from gingivitis to chronic periodontitis, but is

generally more prominent in the latter, as it is more abundant in deep periodontal pockets and in places with high inflammation. It is a central "bridging" species in physical interactions between Gram-positive and Gram-negative species, which are likely to be important in biofilm colonization, and it contributes to the reducing conditions necessary for the emergence of oxygen-intolerant anaerobes. Therefore, it is considered as an intermediate colonizer bridging the attachment of commensals that colonize the tooth and epithelial surface with true pathogens. However, it is not responsible for destructive periodontal disease, which is a major cause of tooth loss [17].

3.2.1. Environmental factors

It has been proven that there is a distinct correlation between the abundance of *F. nucleatum* and environmental factors. Namely, smoking drastically increases the numbers of *F. nucleatum,* as it creates a highly diverse, pathogen-rich, commensal-poor, anaerobic microbiome, compared to healthy individuals [18]. The same was observed in type-2 diabetes patients, which had a similar subgingival biodiversity compared with nondiabetic subjects, meaning that the patients had a larger chance of developing periodontitis [19].

3.2.2. The orange complex: the colonizers

In a work by Socransky et al., subgivingal plaque samples from 185 subjects were taken in order to try and distinct certain complexes that a variety of bacterial species were forming. Ultimately, 5 complexes were identified and assigned a specific color: red, orange, yellow, green and purple (Figure 2). Among the five, *F. nucleatum*, along with *Fusobacterium periodonticum, Prevotella intermedia, Prevotella nigrescens* and *Peptostreptococcus micros*, were identified as the core species of the orange complex. This complex is characterized as a sort of bridge, providing structural support or serving as a metabolic cornerstone, and is

a very strong colonizer. Without it, the aggressive bacteria of the red complex (namely *Porphyromonas gingivalis*) would not survive in the human oral microbiota [20].

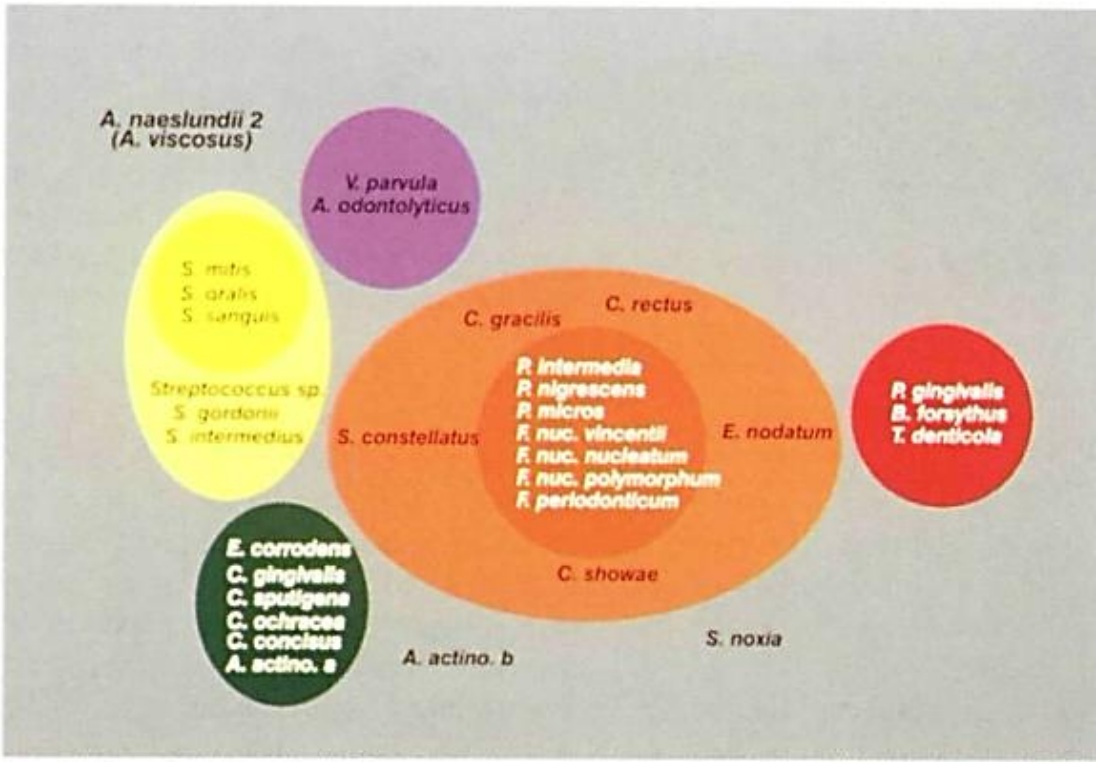

Figure 2. Diagrammatic representation of the relationships of species within microbial complexes and between the microbial complexes [20].

Being a part of the colonizer complex implies that *F. nucleatum* has a way of opening up tissue, which makes it easier for other species to invade. To achieve this, it uses one of its adhesins, FadA. FadA binds to endothelial cells (among others) via the vascular endothelial cadherin (VE-cadherin), a cell–cell junction molecule (Figure 3). This makes the cell-cell junctions loosen and easier to permeate, allowing other bacteria to cross the endothelium through loosened junctions [7]. This might make it sound like *F. nucleatum*, on its own, does not cause much damage to the microbiota, but that could not be further from the truth.

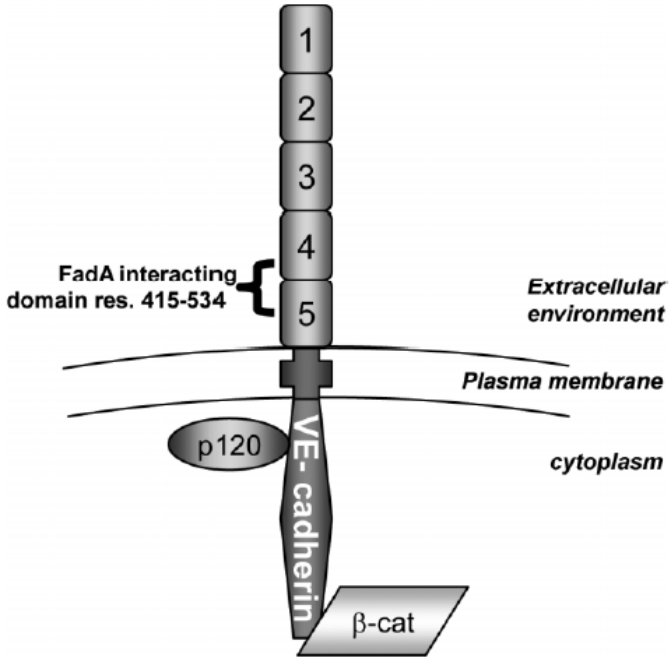

Figure 3. Diagram of VE-cadherin structure and main interacting proteins. VE-cadherin is a cell surface exposed protein composed of five extracellular domains (EC), a unique transmembrane domain, and an intracellular domain interacting with catenins. The region bound by FadA is a sequence of 119 residues composed of the 2nd half of EC4 and the 1st half of EC5 (the parenthesis highlights these halves) [7].

3.2.3. Mechanisms of pathogenicity in periodontitis

There are a few ways through which *F. nucleatum* inflicts damage. For example, to increase local inflammation, *F. nucleatum* stimulates the production of IL-8. However, opinions differ when it comes to discussing the way it achieves this. Some scientists have found that *F. nucleatum* increases IL-8 secretion by epithelial cells, and stimulates IL-8 liberation via LL-37, a cathelicidin. Others have found that it does this through mitogen activated protein (MAP) signaling. Aside from IL-8, *F. nucleatum* also upregulates pro-inflammatory cytokines and matrix metalloproteases (MMPs), which modify inflammatory reactions and cause infected cells to migrate, ensuring their survival. Examples of these MMPs are collagenase 3 (MMP-13) and gelatinase A (MMP-2), as well as MMP-9 [17].

Another way *F. nucleatum* damages the oral tissue is through the secretion of serine proteases, virulence factors employed also by species of the red complex, such as *P. gingivalis* and *T. denticola*. They degrade some elements of the periodontal connective tissue, like the extracellular matrix proteins fibrinogen and fibronectin, as well as collagen I and collagen IV in the case of *F. nucleatum*. The 65 kDa protease is also able to degrade the host defense systems, namely the α-chains of IgA, thus helping the evasion of the host's immune system. The specific activity of this protease is very low, however. Given that *F. nucleatum* is most often found together with other highly proteolytic microorganisms, it makes sense why it does not need to possess a strong proteolytic protease [17].

Lastly, *F. nucleatum* has an immunosuppressive role, yet another property it shares with the species of the red complex. Namely, it induces apoptotic cell death in peripheral blood mononuclear cells (PBMCs) and in polymorphonuclear cells (PMNs). This does, however, require the activation of caspases. *F. nucleatum* is also capable of inhibiting B- and T-cell functions, thus showcasing its ability to induce a generalized paralysis of the immune system [17].

4. Colorectal cancer

Colorectal cancer (CRC) is a type of cancer that occurs in the colon or rectum, usually from polyps that manifest on the lining of the gut. The most common type are adenomatous polyps, which make up 95% of all cases and overall more than 50% occur in the rectum and sigmoid [21]. It is one of the most common malignant tumors of the digestive tract, ranking fourth for incidence (9.2% of total cancer cases) and second in terms of mortality (9.2% of total cancer deaths) as of 2019 [22]. Although the mechanisms are not completely known, it is well established that CRC is a multi-factor and multi-step process caused by the synergy of environment, diet, and lifestyle along with genetic factors [23].

4.1. Symptoms

Colorectal adenocarcinomas grow slowly, and a long interval elapses before they are large enough to cause symptoms, which depend on lesion location, type, extent, and complications. A carcinoma in the ascending (right) colon is by far the hardest to diagnose, as the only symptoms are fatigue and weakness caused by severe anemia that may or may not be accompanied with bleeding. As for the descending (left) colon, an early sign is usually partial obstruction with colicky abdominal pain or complete obstruction. Other than that, the stool may be streaked or mixed with blood and some patients present with symptoms of perforation, usually walled off (focal pain and tenderness), or rarely with diffuse peritonitis. In rectal cancer, the most common initial symptom is bleeding with defecation. Tenesmus or a sensation of incomplete evacuation may be present, along with pain [21].

4.2. F. nucleatum & CRC

The connection between *F. nucleatum* and CRC was discovered in 2012 by two individual groups and since then, further analysis using 16S rRNA gene sequence and application of metagenomic sequencing technology was used to confirm the close relation between the bacteria and the disease [23].

4.2.1. Detection

To detect *F. nucleatum* in CRC, researchers have used several different methods, including fluorescence in situ hybridization (FISH) (Figure 4), fluorescent quantitative polymerase chain reaction (FQ-PCR), quantitative real-time polymerase chain reaction (qPCR), and droplet digital polymerase chain reaction (ddPCR). Sample collection methods also vary among studies, but the usual ones are derived from formalin-fixed paraffin-embedded (FFPE) CRC tissues, CRC frozen tissues, genomic DNA, and feces collected from CRC patients [23].

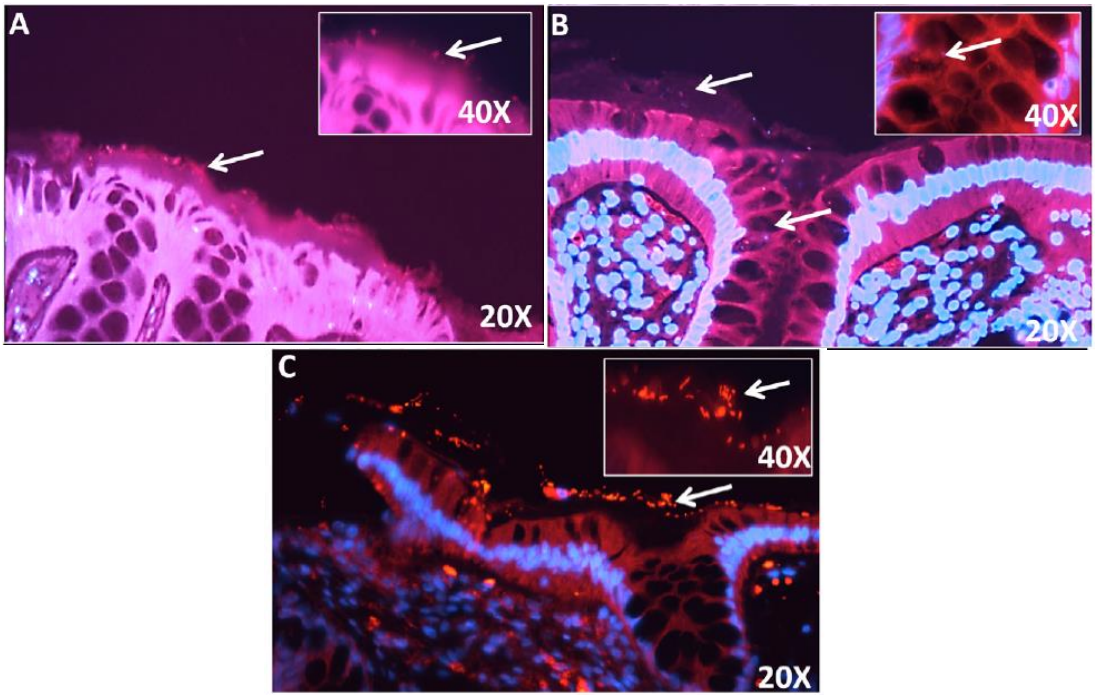

Figure 4. Representative fluorescence in situ hybridization targeting Fusobacterium sp. in colorectal mucosal biopsy sections using bacterial 16S rRNA probes. Fig. 2A–B are composite images of Cy3 and DAPI views of sections hybridized with a Fusobacterium-specific probe. Fig. 2C (20X and 40X) is a positive control and shows sections stained with general bacteria probe (Eub 388). White arrows point to bacteria either in mucus layer above the colonic epithelium or within the crypt [24].

4.2.2. The 5 pathways of pathogenesis in CRC

There are multiple ways in which *F. nucleatum* promotes the progression of tumors, with new ones being discovered every so often. As for CRC, there seem to be 5 main pathways that make up the mechanism of pathogenesis (Figure 7) [23].

The first and second pathway, although quite different, share a common protein that initiates them both, the FadA adhesion protein. From here, the pathways diverge, depending on which receptor the FadA binds. In the first pathway, it binds to the VE-cadherin 5 (CDH5), the host's endothelial receptor for FadA. This allows *F. nucleatum* to attach itself onto endothelial cells and invade them, causing the production of multiple cytokines such as IL-6 [8], IL-8 [8], IL-10 [24] and IL-18 [8], as well as TNF-α [24] and the inflammatory protein NF-κB [8]. The end result is the creation of a proinflammatory microenvironment that will only serve in propagating further cancerogenic progression.

The second pathway sees FadA binding to a different receptor, specifically the cell-adhesion molecule E-cadherin, thus inhibiting its tumor suppressor activity (Figure 5). In a study by Rubinstein et al., it was discovered that FadA binds to E-cadherin on both CRC and non-CRC cells, mediating *F. nucleatum* attachment of, and invasion into, the cells whilst simultaneously stimulating the expression of inflammatory proteins including NF-κB and cytokines IL-6, 8, and 18. FadA modulates (phosphorylates) E-cadherin and activates β-catenin signaling by decreasing its phosphorylation, which leads to β-catenin accumulation in the cytoplasma and translocation into the nucleus, resulting in activation of β-catenin-regulated transcription. This leads to increased expression of transcription factors (such as lymphoid enhancer factor (LEF-1)), *myc* oncogenes, *wnt* genes (7a, 7b and 9a specifically), and inflammatory genes, as well as growth stimulation of CRC cells. Furthermore, while FadA binding to CRC cells is sufficient to turn on *wnt* and oncogenes, its

internalization, mediated by clathrin, is needed to activate the inflammatory genes [8]. On the other hand, *F. nucleatum* infected cells increase the expression of microRNA-21 (miR21) by activating TLR4 signaling to myeloid differentiation factor 88 (MYD88). This leads to the activation of NF-κB, and hyperactive NF-κB subsequently binds to the promoter of miR21 and initiates an oncogenic cascade in CRC [25].

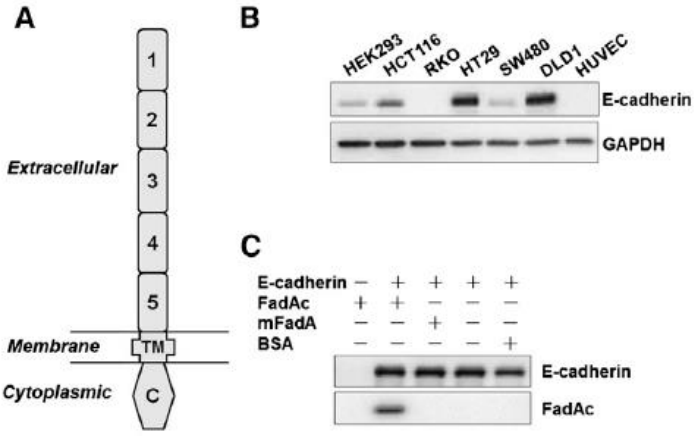

Figure 5. (A) Schematic representation of the E-cadherin (CDH1) structure. E-cadherin has five extracellular cadherin (EC) repeats, numbered EC1–EC5 starting from the N terminus. TM, transmembrane domain; C, cytoplasmic domain. (B) E-cadherin is expressed in epithelial HEK 293 cells and most CRC cells. (C) E-cadherin coimmunoprecipitates with FadAc. HEK 293 cell lysate expressing E-cadherin was mixed with E. coli lysates expressing FadAc or mFadA, or BSA, followed by incubation with mouse anti-CDH1 monoclonal antibodies (mAb), and captured with protein A/G agarose beads [8].

Pathways 3, 4 and 5 all have the same end goal, that being to inhibit the activity of immune cells, which will produce a tumor immunosuppressive microenvironment. However, each pathway has a different way of achieving this. In pathway 3, FIP (immunomodulatory protein extracted from *F. nucleatum*), which was previously shown to be capable of inhibiting human T-lymphocyte activation by both mitogens and antigens, is used as a means of inhibiting mitogen-induced human T-cell proliferation in a dose-dependent fashion. Specifically, in the thymidine incorporation assay, FIP-treated cells fail to incorporate [^{3}H]thymidine and

thus these cells remain in the G_0/G_1 phase of the cell cycle (Figure 6). This disturbance in turn adversely affects the development of normal immunologic defense mechanisms [12].

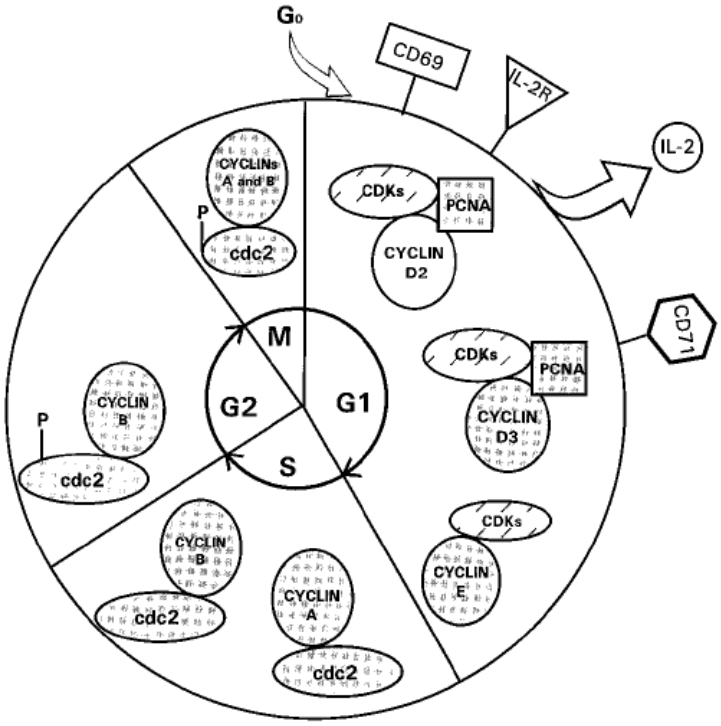

Figure 6. Schematic diagram of the cell cycle in T cells. Shaded symbols indicate factors known to be inhibited in FIP-treated cells; open symbols represent events not altered in these cells; hatched symbols represent events not yet analyzed [12].

Pathway 4 is characterized by products of amino acid metabolism generated by *F. nucleatum*, including formyl-methionyl-leucyl-phenylalanine and short chain fatty acids. These are myeloid cell chemoattractants, which may explain the intratumoral myeloid cell expansion that was observed and interconnect tumor metabolism, bacterial metabolism, and immune cell function within the tumor microenvironment. These chemoattractants specifically attract myeloid-derived suppressor cells (MDSCs), tumor-permissive myeloid cells with potent immune suppressive activity, tumor-associated macrophages (TAMs) and M2-like TAMs, which promote tumor progression and metastasis, and dendritic cells (DCs) that can either dampen or promote

anti-tumor immunity. MDSCs suppress CD4 T cells predominantly via expression of arginase-1 and inducible nitric oxide synthase (iNOS). Additionally, MDSCs showed significant T cell suppressive activity. TAMs inhibit T cell responsiveness via expression of arginase-1, while M2-like TAMs had two-fold higher arginase-1 levels compared to TAMs and exhibited significant suppressive activity on CD4 T cells [26]. The intestine possesses a specific subset of DCs expressing CD103 integrin. These cells play a role in regulation of immune responses by promoting the expansion of Foxp3+ regulatory T cells, a CD4+ T cell subset that suppresses cytotoxic and effector T cells and thus dampen anti-tumor immunity [27]. Collectively, *F. nucleatum* modulates the tumor immune microenvironment which promotes tumor progression.

Lastly, there is pathway 5, in which the spotlight is on Fap2, a virulence factor protein located on the outer membrane. One of its receptors in humans is TIGIT (T cell immunoreceptor with Ig and ITIM domains), a receptor expressed on all NK cells and various T cells. Once Fap2 binds to TIGIT, it sends out an inhibitory signal, effectively preventing immune cells from killing tumor cells. Specifically, it inhibits NK cytotoxicity as well as cytotoxic T lymphocytes (CTL) and T helper cell activities but has no effect on NK cell interferon-γ (IFN-γ) and TNF-α secretion [28]. Along with RadD, Fap2 also has the ability to induce cell death in immortalized human lymphocytes [10]. In addition, Fap2 mediates *F. nucleatum* enrichment by interacting with Gal-GalNAc overexpressed in colorectal tumors [5].

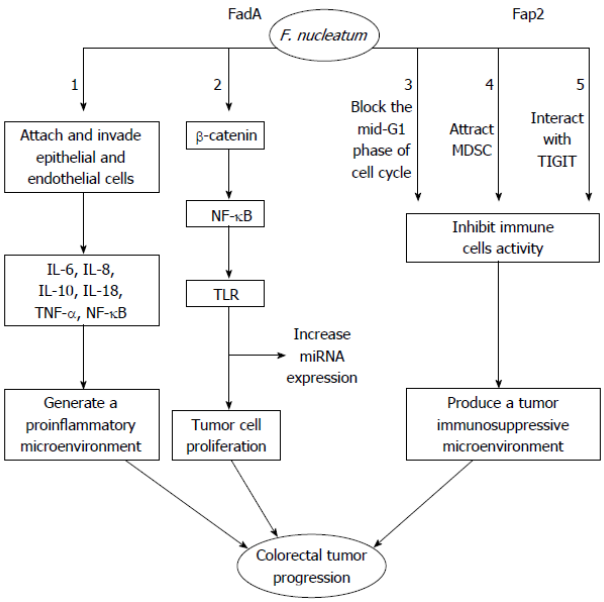

Figure 7. Underlying mechanism of Fusobacterium nucleatum pathogenesis in colorectal cancer. MDSC: Myeloid-derived suppressor cell; TLR: Toll-like receptor. [23]

4.2.3. Biomarkers

With the abundance of research made in order to understand the inner workings of *F. nucleatum*'s invasion, a variety of biomarkers were also discovered. These range from immune antibodies to miRNA or TAMs, and all play a role in *F. nucleatum*-associated CRC.

In infection-associated cancers, a promising approach for the early detection of cancer is the assessment of immune response to antigens of tumor-associated microbes. Such is the case with *F. nucleatum*, and therefore the host's immune system produces anti-Fn antibodies. Specifically, the circulating levels of anti-Fn-IgA in patients with CRC group and its stage I-II groups were significantly higher than those of healthy controls. In addition, the circulating levels of anti-Fn-IgG in patients with CRC group were significantly higher than those of two control groups, but there was no significant difference in stage I-II and control group. Although the highest levels were observed in later stage

CRC (stage III-IV), the serum levels of anti-Fn-IgA and anti-Fn-IgG of CRC patients in stage I-II were not significantly different than stage III-IV. These results indicated that anti-Fn level is similar in both CRC patients in the advanced stage of the disease and in patients in the early stage. The findings validate the performance of anti-Fn-IgA as a plasma marker to increase the diagnostic sensitivity for early detection of CRC, and indicate that the serum levels of anti-Fn-IgA, along with CEA and CA19-9, which are currently used tumor markers for CRC, have a better diagnosis values for screening early stage of CRC than CEA and CA19-9 combined diagnosis [29]. Along with these, lncRNA can also be considered as a potential diagnostic biomarker during the early stage of *F. nucleatum*-positive CRC [23].

Earlier in the review, TAMs were described as cells that promote tumor progression and metastasis. One study made its objective to assess the intensity of the infiltration of TAMs and Tregs in CRC patients as prognostic factors with respect to disease-free survival (DFS) and overall survival (OS). It was discovered that the presence of intense infiltration of TAMs and Tregs in the tumor stroma was related to shorter DFS and OS. Furthermore, the relative risks of recurrence and cancer-related death more than doubled in the group of patients with intense infiltration of TAMs and were more than 12 times higher in patients with intense infiltration of Tregs [30]. This makes TAMs a sort of general poor prognostic markers for *F. nucleatum*-associated CRC.

Another poor prognostic marker is miRNA. As previously mentioned, miR21 is highly expressed by activating TLR4 binding to MYD88 during *F. nucleatum* invasion in pathway 2. Then, miR21 activates the NF-κB pathway and decreases the RAS GTPase Ras p21 protein activator 1 (RASA1) level, leading to an increase in several inflammatory factors that significantly promote the CRC cell proliferation [25]. Alongside these two, LncRNA PANDAR is another poor diagnostic biomarker for clinical prognosis of *F. nucleatum*-positive CRC [23].

Although it may seem that only poor diagnostic CRC biomarkers exist, that is not the case. In fact, there are some markers that are associated with a more positive prognosis. Such is the case with tumor-infiltrating T-cells, specifically $CD45RO^+$-cells, a type of memory T-cells, which are associated with improved prognosis in CRCs, independent of clinical, pathologic and molecular features, including densities of the other T-cell subsets (Figure 8) [31]. And although the $FOXP3^+$ transcription factor is usually associated with a poor prognosis in CRC [31], a specific subpopulation named $FOXP3^{\pm}$ (lo) T cells exhibit a more favorable prognosis [23].

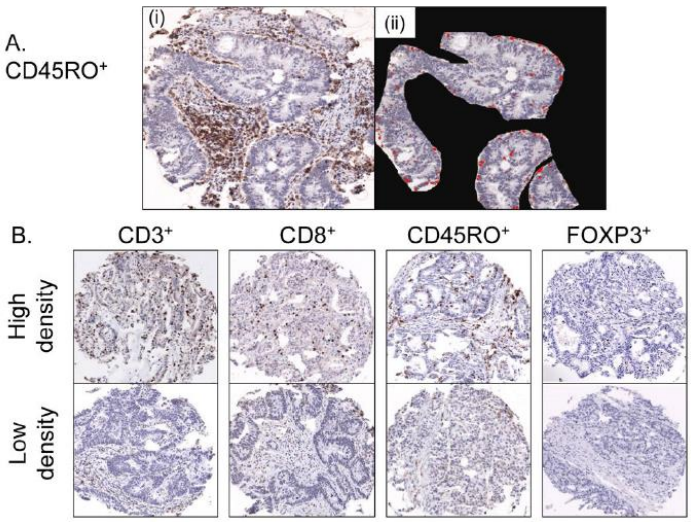

Figure 8. Image analysis of $CD3^+$, $CD8^+$, $CD45RO^+$ and $FOXP3^+$-cells in colorectal cancer [31].

5. Adverse pregnancy outcomes

Adverse pregnancy outcome (APO) is a broad term for any and all complications that can arise during the gestational period. These include preterm labor, intra-amniotic infection (chorioamnionitis), preterm premature rupture of membranes (PROM), preeclampsia, miscarriage, intrauterine growth retardation, low birth weight, stillbirth, and neonatal sepsis. *F. nucleatum* is by far the most prevalent oral species implicated in APO [13], which makes it a prime specimen to study in order to try and minimize its implication in these complications and by extension the occurrence of these complications.

5.1. Preterm birth

Labor, and subsequently birth, that begins before 37 weeks of gestation is considered to be preterm. Premature babies often have complicated medical problems and the earlier the baby is born, the higher the risk of complications. There are 4 classifications of preterm birth: extremely preterm (< 28 weeks), very preterm (28 to 31 6/7 weeks, or day 197 to 224), moderately preterm (32 to 33 6/7 weeks, or day 225 to 238), late preterm (34 to < 36 6/7 weeks, or day 239 to < 259). Symptoms vary depending on which classification the baby falls into, but some of the general ones are small size with a disproportionately large head, fine hair (lanugo) covering much of the body, low body temperature due to a lack of stored body fat or a lack of reflexes for sucking and swallowing [32].

Globally, an estimated 15 million babies are born too early every year, or more than 1 in 10. Out of the 15 million, 1 million children die each year while many survivors face a lifetime of disability, including learning disabilities as well as visual and hearing problems. In almost all countries with reliable data, preterm birth rates are increasing [33].

There are a lot of risk factors when it comes to preterm birth, such as PROM, prior premature births, intrauterine infection, chorioamnionitis, multifetal pregnancy, fetal or placental abnormalities, another ascending uterine infection (commonly due to group B streptococci), uterine abnormalities, pyelonephritis, preeclampsia, poor nutrition during gestation, cigarette smoking, younger or older maternal age (eg, < 16 years, > 35 years), being underweight or overweight before pregnancy, stressful life events, multiple miscarriages or abortions and even some sexually transmitted diseases [32]. Still, a lot of preterm births occur spontaneously [33].

5.1.1. Bacterial infection

Bacterial infection is something a few of these risk factors have in common. Within the uterus, bacteria can infect the area between the maternal tissues and the fetal membranes, within the fetal membranes (chorioamnionitis), within the placenta, within the amniotic fluid (amnionitis), or within the umbilical cord (funisitis) or the fetus (Figure 9). In doing so, it chooses one of the two pathways of achieving infection, depending on its abilities. The first pathway is more direct, in which the uterus is invaded via migration from the abdominal cavity through the fallopian tubes, inadvertent needle contamination at the time of sampling, hematogenous spread through the placenta or passage through the cervix from the vagina. Vaginal organisms first ascend into the choriodecidual space, then cross the intact chorioamniotic membranes into the amniotic fluid, ultimately infecting the fetuses. The second pathway is much more roundabout, and includes organisms, such as oral bacteria, that may reach the uterus hematogenously [34].

It has been well established that pregnancy brings about many changes in various systems of the body, not just the reproductive system. Such is the case with the oral microenvironment, where pregnancy gingivitis, an acute form of gingivitis characterized by erythema, edema, hyperplasia, and

increased bleeding of the gingival tissue, occurs in approximately 30-75% of all pregnant women. But just as pregnancy has an effect on gingival and subsequently periodontal tissues, the opposite is also true, with periodontal disease maybe able to affect pregnancy. It has been observed that pregnant women with severe periodontal disease more often deliver low birth weight and preterm babies, making periodontal disease a statistically significant risk factor. Moreover, periodontal therapy has been shown to reduce the rates of preterm birth in a population of women with periodontal disease [35].

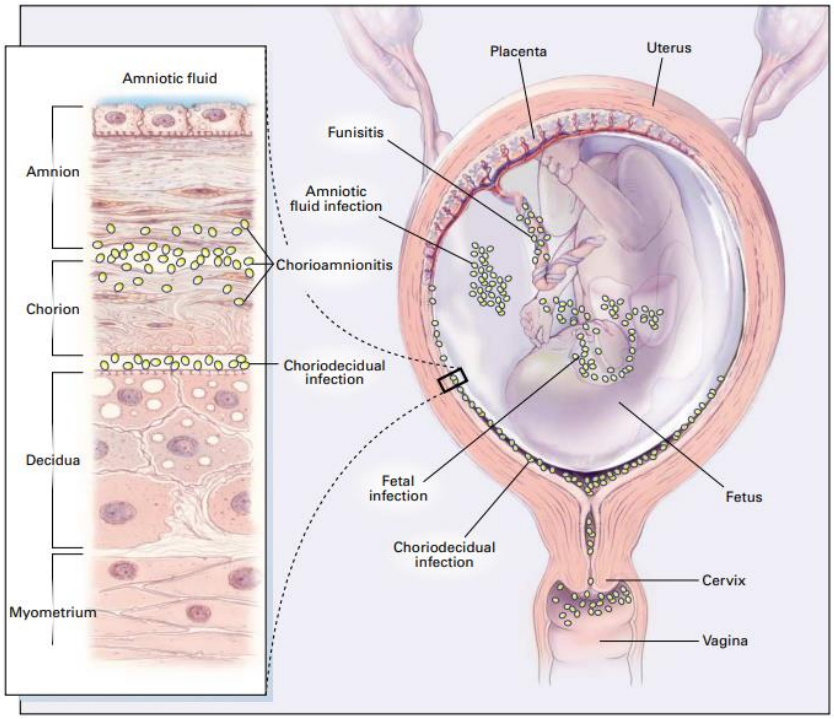

Figure 9. Potential sites of bacterial infection within the uterus [34].

5.1.2. *F. nucleatum* in preterm birth

As mentioned in previous chapters, *F. nucleatum* plays a major role in promoting periodontal disease and has also proven to have multiple ways to adhere to and infect a variety of cells. So, it comes as no surprise that

it is a prime example of a bacteria that utilizes (at least) the second pathway described earlier and subsequently infects various parts of the reproductive system. In fact, *F. nucleatum* is associated with both preterm labor with intact membranes and with preterm PROM. In an article by Chaim et al., the presence of Fusobacterium in amniotic fluid cultures of patients with premature labor and intact membranes was in fact significantly higher than in patients with preterm PROM [36], further propagating the latter finding that *F. nucleatum* is the most frequently isolated species from amniotic fluid cultures among women with preterm labor and intact membranes [37]. Since then, *F. nucleatum*, using 16S rRNA-based culture independent methods, has been detected in placental and fetal tissues, including fetal membranes [38], cord blood [39] and neonatal gastric aspirates [40].

In a study by Gonzales-Marin et al., it was observed that out of the 4 subspecies of *F. nucleatum* found in neonates, three of them matched better with the strains amplified from the maternal oral cavity (*F. nucleatum* subspp. *polymorphum, vincentii* and *nucleatum*) in contrast with *F. nucleatum* subsp. *animalis*, which was more commonly observed in vaginal samples [40]. Other studies also support these findings, even going as far as proving that *F. nucleatum* may have originated from the partner's oral microflora [37]. Interestingly enough, *F. nucleatum* subsp. *polymorphum* is more commonly observed in periodontally healthy sites, whilst *F. nucleatum* subsp. *nucleatum* is observed mainly in disease, meaning that destructive periodontal disease may not be strictly necessary for bacterial translocation to occur. It is more likely that the sole presentation of gingival inflammation, as observed frequently in pregnancy gingivitis, may suffice to allow opportunistic pathogens such as *F. nucleatum* to translocate and invade the amniotic cavity [40].

5.2. Stillbirth

Stillbirth is characterized as delivery of a dead fetus at or after 20 weeks of gestation [41,42], though stillbirth occurring during delivery is rare in modern countries [42]. It can be further classified, depending on which week it occurs as early stillbirth (between 20 and 27 completed weeks of pregnancy), late stillbirth (between 28 and 36 completed pregnancy weeks) or term stillbirth (between 37 or more completed pregnancy weeks). In the US, each year about 24,000 babies, or approximately 1 in 160, are stillborn. That number corresponds to the number of babies that die during the first year of life and it is more than 10 times as many deaths as the number that occur from Sudden Infant Death Syndrome (SIDS). Because of advances in medical technology, the number of late and term stillbirth has reduced significantly, but the rate of early stillbirth has remained about the same over time [41].

Although it can happen spontaneously, which is then pronounced as "unexplained stillbirth" [41], there are certain risk factors, such as being of African-American or African descent, being 35 years of age or older, lower socioeconomic status (these are tied to maternal health, income, access to quality health care, stress, social and emotional support resources and cultural factors), smoking, drinking, drug use, intrahepatic cholestasis of pregnancy, certain medical conditions (high blood pressure, diabetes, obesity), chorioamnionitis, multiple pregnancies such as triplets or quadruplets and previous pregnancy loss or complications [41–43]. Sometimes, the fetus may die if it has certain conditions like anemia, chromosomal or genetic abnormality, birth defect or infection. Other times, there may be problems with the placenta, for instance placental abruption, prolapsed umbilical cord, conditions that reduce blood flow to the fetus, bleeding, vasa previa or other problems with the umbilical cord (such as a knot) [43].

5.2.1. *F. nucleatum* in stillbirth

Unlike with preterm birth, not many experiments have been done specifically with stillbirth in mind. In a case study by Han et al., *F. nucleatum* was found in the mother's oral cavity, but not in her vaginal or rectal flora, suggesting a hematogenous pathway from the oral microbiota to the uterus, not unlike the one described earlier. It was proposed that the mother might have had pregnancy-associated gingivitis, and that the upper respiratory tract infection that followed suit might have weakened the mother's immune system enough to provide a window of escape for the bacteria to colonize in the uterus, causing the stillbirth [44]. Aside from that, a work by Ebersole et al. found that women with stillbirths had significantly higher serum antibody levels to *F. nucleatum* when compared to those with live births [45].

5.3. Neonatal sepsis

Neonatal sepsis is a type of blood infection that occurs in an infant younger than 90 days old [46]. It is categorized as either early onset (0-3 days of life) or late onset (4 or more days of life) [47]. In early-onset, 85% infants are diagnosed within 24 hours (median age of onset being 6 hours), 5% within 24-48 hours, and a smaller percentage within 48-72 hours. It is also more rapid in premature neonates [47] but is more preventable these days due to screening during pregnancy [46].

Early-onset sepsis (EOS) is caused by organisms prevalent in the maternal genital tract or in the delivery area, with bacterial infections being the most prevalent, specifically group B streptococci and *E. coli*. However, it can also be caused by fungi, parasites or viruses [48]. The infection happens most commonly via ascending infection from the cervix but can also be hematogenous or transplacental [47]. Risk factors include prematurity, low birth weight, premature and prolonged rupture of

membranes, maternal fever, urinary infection and chorioamnionitis [48]. On the other hand, late-onset sepsis (LOS) is caused by the organisms in the external environment, whether that be hospital or home with the infection being transmitted through the hands. A big risk factor is preterm delivery, as well as invasive procedures such as resuscitation in delivery room, intubation, mechanical ventilation, central venous catheters, exposure to antibiotics, surgical procedures and staying in intensive care for a prolonged period [46,48].

5.3.1. *F. nucleatum* in neonatal sepsis

In a study by Wang et al., 16S rRNA-based genomic analyses were conducted in order to connect intra-amniotic infection (IAI) and EOS. The reasoning behind it was that now many previously unrecognized species could be identified as to being the cause of EOS and other APO cases, which would prior be labeled as "idiopathic". A total of 44 pregnant women participated and samples of their amniotic fluid and cord blood were taken. It was then discovered, for the first time, that *F. nucleatum* has a much bigger role in causing EOS. Specifically, that it was the most prevalent species detected in the cord blood in those presumably suffering from EOS. This puts it on par with *E.coli*, a bacteria previously acknowledged to play a major role in causing EOS [39].

5.4. Mechanisms of *F. nucleatum* pathogenicity in APOs

Previously, two pathways that bacteria can take in infecting the uterus were described. One of them was the hematogenous pathway, with the bacteria originating in the oral microbiota. As there is not much data that describes the mechanisms of this pathway, aside from in murine studies [9], one can only hypothesize as to how *F. nucleatum* utilizes this pathway in humans. However, the fact that *F. nucleatum* can enter the bloodstream from mouth ulcers in immunosuppressed patients, such as

pregnant women, and that the second gestational trimester is associated with pregnancy gingivitis, and a general increase of *F. nucleatum* in the oral microbiota, points to a higher probability of the hypotheses being true [37].

Once it has entered the bloodstream, *F. nucleatum* presumably makes it to the placenta. This is where its adhesin FadA comes into play and binds to the VE-cadherin. It is naturally expressed in the placenta and therefore makes an ideal entrance point into it. The binding causes a relocation of VE-cadherin away from the cell–cell junctions, increasing the endothelial permeability, which allows the bacteria to cross the endothelium through loosened junctions [7]. Once in, *F. nucleatum* can colonize the placenta and reach the amniotic fluid and fetus, cause inflammation and ultimately harming the fetus, just like with the murine models [9]. The loosening of tight junctions also makes it possible for other species to disseminate, such as *E. coli*. This makes *F. nucleatum* a sort of "facilitator" (as was described in an earlier chapter) and helps explain why it is frequently detected concurrently with other oral species in intrauterine infections in humans [7].

A study by Coppenhagen-Glazer et al. identified Fap2 as a galactose-sensitive adhesin involved in hemagglutination, coaggregation, and adherence to mammalian cells in *F. nucleatum* subsp. *nucleatum*. They demonstrated this by comparing a wild-type *F. nucleatum* with one that had a Fap2 mutant with his ability to invade the placenta impaired. The wild-type was two-fold more successful in colonization, suggesting that, aside from FadA, Fap2 also plays an important role in the invasion of the placenta (Figure 10). Similarly to CRC, the placenta has also been shown to overexpress Gal-GalNAc, which with Fap2 can interact, creating a more immunosuppressive microenvironment [5].

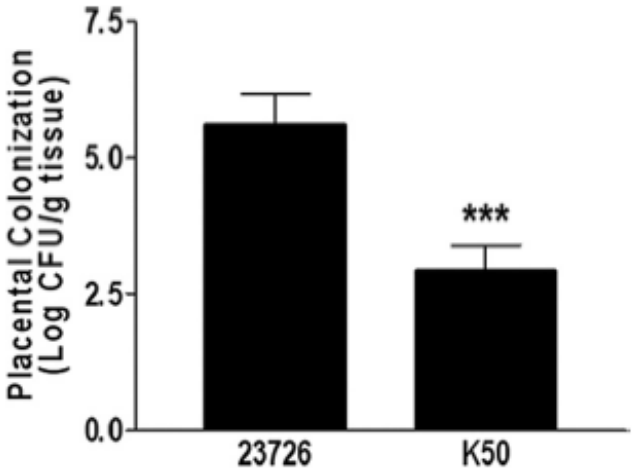

Figure 10. The Fap2 adhesin is involved in placental colonization. Wild-type F. nucleatum ATCC 23726 or the hemagglutination-deficient mutant K50 was injected into the tail veins of pregnant mice. After 24 h, the placentas were harvested and homogenized, and bacterial CFU were determined [5].

6. Discussion

F. nucleatum as a species was discovered around the 1980s, and quickly drew attention to itself for its implications in periodontal diseases. As such, researchers began analyzing its various characteristics as well as its genome. Soon, it was discovered that *F. nucleatum* has several subspecies, each with its own unique attributes. However, some studies would omit spp. *vincentii*, while others would omit spp. *fusiforme*. The reasoning behind this is seemingly unknown, as there were not any recent research papers on this topic. Still, there is a noteworthy study by Kook et al., in which it was proposed that the 4 subspecies of *F. nucleatum* (*nucleatum, polymorphum, vincentii* and *animalis*) should be classified as *F. nucleatum, F. polymorphum, F. vincentii,* and *F. animalis*, respectively [49]. It would seem as though further analysis is needed in order to better identify subspecies and subsequently provide better treatment in the future.

Although *F. nucleatum* is not the only bacterial species associated with CRC, its abundance has been found to be significantly higher in CRCs compared to healthy human control groups [23]. Still, a substantial amount of research that describes the pathways *F. nucleatum* utilizes in its pathogenicity in CRC was done with murine models or in vitro [24–27]. This means that the pathways may be different in humans, making further research mandatory if we are to better the diagnosis and treatment of *F. nucleatum*-associated CRC.

Further usage of the FadA has been discovered in its potential to be a CRC biomarker. This is due to the discovery that the FadA protein is highly conserved among *F. nucleatum*. Moreover, it was revealed that FadA gene copies gradually increase from the baseline of normal individuals to adenomas and normal tissues adjacent to adenomas and adenocarcinomas, and to adenocarcinomas. This makes FadA an ideal potential diagnostic marker to identify individuals at risk for developing

adenomas and/or adenocarcinomas as it would be possible to define healthy, precancerous, and cancerous states according to FadA gene copy levels [8].

A few years after the initial discovery of *F. nucleatum*, its implications in APOs were discovered [36]. However, there is very little data that explains the pathogenesis of *F. nucleatum* in APOs in humans. Therefore, most of it is speculation based on murine models [9]. For example, in a work by Goldenberg et al., they hypothesized two ways in which *F. nucleatum* could cause PROM. The first one is through its high phospholipase A2 content, which enhances prostaglandin synthesis and may therefore precipitate labor. The second one is by the body's own production of IL-1 and TNF in response to bacterial infection, which again can stimulate prostaglandin production by amnion and decidua [36]. Aside from preterm birth, speculation is also made in regards to the pathogenic pathways of *F. nucleatum* in stillbirth and neonatal sepsis as described in an earlier chapter, and further research is definitely required.

Two pathways that oral bacteria can take in infecting the uterus were also described. Unfortunately, there is not much research about the pathway in which *F. nucleatum* ascends from the abdominal cavity to the uterus. There is some data linking *F. nucleatum* ssp. *animalis*, which is often found more in the vagina [37], with intra-uterine infections [50] however there is no data as of yet that describes in what way the bacteria crosses from the abdominal cavity into the placenta. In summary, *F. nucleatum's* involvement with APOs in humans as a whole is still relatively unknown. This needs to be corrected in the coming years as understanding the mechanisms involved in transformation of this oral commensal organism into systemic pathogens lays the foundation for developing prevention and treatment strategies to circumvent bacterial-induced pregnancy complications.

7. Conclusion

Ever since the discovery of *F. nucleatum*, scientists around the world have been baffled by the sheer number of infections and diseases it has been implicated with. From periodontitis to CRC and even APOs, there seems to be no tissue that *F. nucleatum* cannot invade and colonize. It starts off in the oral microbiota and, not long after, travels to various parts of the body via several hematogenous pathways. Once there, it uses its vast array of proteins to bind and enter different body systems and cause damage to them. This work touched upon some of the more researched diseases it is associated with, however, it is imperative that the research is continued and done more thoroughly as time passes and modern medicine advances. Another important action to take is to spread awareness of this pathogen and how to prevent it from spreading by actively practicing proper dental hygiene. By doing this, I hope *F. nucleatum's* activity in infections subsides over time, thus providing a brighter and healthier future for everyone.

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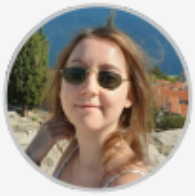
9. CV



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ABOUT ME

Dedicated and passionate. Looking to broaden my horizons and conquer new challenges!

WORK EXPERIENCE

01/07/2019 – 01/10/2019 – Rijeka, Croatia

Fry cook
McDonalds

I was responsible for making fries and various types of hamburgers as well as keeping the pantry stock in check. I also have experience in making beverages and deserts.

EDUCATION AND TRAINING

01/10/2018 – CURRENT – Radmile Matejčić 2, Rijeka, Croatia

Undergraduate course: Biotechnology and drug research
Department of biotechnology Rijeka
https://www.biotech.uniri.hr/hr/

01/09/2014 – 22/05/2018 – Vukovarska 58, Rijeka, Croatia

Secondary education
Natural Science and Graphics school of Rijeka
https://www.biotech.uniri.hr/hr/

LANGUAGE SKILLS

MOTHER TONGUE(S): Croatian

OTHER LANGUAGE(S):

English

Listening	Reading	Spoken production	Spoken interaction	Writing
C2	C2	C1	C1	C1

DIGITAL SKILLS

Microsoft Word / Microsoft Excel / Microsoft Office / Outlook / Microsoft Powerpoint / Social Media / Google Drive / Facebook / Instagram / VMD – Visual Molecular Dynamics / AVOGADRO for drawing molecule structure / MarvinSketch / PyMOL + Molecular Visualization Program / UCSF Chimera / SciDAVis / wxMacMolPlt / General Atomic and Molecular Electronic Structure System (GAMESS)