UNIVERSITÀ DEGLI STUDI DI SALERNO
Dipartimento di Medicina, Chirurgia e Odontoiatria
"Scuola Medica Salernitana"

CORSO DI DOTTORATO DI RICERCA IN MEDICINA TRASLAZIONALE
DELLO SVILUPPO E DELL'INVECCHIAMENTO ATTIVO
XXXIII CICLO

COORDINATORE: Prof. P. Monteleone
Curriculum: tecnologie innovative

TESI DI DOTTORATO
IN

"*Parodontite e carcinoma del colon-retto: la possibile associazione genetica e patogenetica*"

Tutor:
Chiar.mo Prof. Ludovico Sbordone
Co-Tutor:
Chiar.mo Prof. Vincenzo Pilone

Dottorando:
Dr. Federica Di Spirito
Matricola:
8800900023

Anno Accademico 2020/2021

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1. Background

Periodontitis is a multifactorial microbially-associated inflammatory disease affecting tooth-supporting structures and, finally, causing tooth loss (Di Spirito et al., 2020; Lang & Bartold, 2018; Sbordone et al., 2009; Tonetti, et al., 2018)

New evidences associates periodontitis to systemic inflammatory conditions and pathologies, such as atherosclerosis, rheumatoid arthritis, obesity (Di Spirito et al., 2019; Soory, 2010) and to solid cancers, including prostate, breast, lung, pancreas and kidney malignant neoplasms (Fitzpatrick & Katz, 2010; Lee et al., 2018; Michaud et al., 2008;). In particular, periodontitis seems to be related to IBD (Soory et al., 2010), to an augmented risk of gastrointestinal cancer, especially colorectal adenoma and CRC (Hussan, et al., 2017; Lee et al., 2018; Momen-Heravi et al., 2017), and to, an increased mortality from CRC (Lee et al., 2018; Ren et al., 2016).

Colorectal cancer (CRC) accounts for approximately 10% of new cancer cases wordwilde in males and 9.2% in females (Ferlay et al., 2015). Considering the high mortality rate (8% and 9% of the cases corresponding to 700000 estimated deaths/year), together with CRC morbidity, an insight into etiopathogenic mechanisms of the CRC may be crucial to advancement in both early diagnosis and treatment customization (Guo et al., 2017).

Only in 10% of cases, CRC recognizes genetic susceptibilities and/or family history positivity (Krzystek-Korpacka et al., 2013). Identified risk factors for CRC are Inflammatory Bowel Disease (IBD) (Triantafillidis et al., 2009), comprising Crohn's disease and ulcerative colitis (Abraham & Cho, 2009). Other putative carcinogenic factors in CRC are unhealthy behaviors (i.e. red meat and alcohol consumption, smoking habit, reduced physical activity) and diseases and conditions (i.e. type 2 diabetes, obesity) related to systemic inflammation (Flood et al., 2008; Li & Martin, 2016). Indeed, it has been hypothesized that chronic inflammation may play a central role in CRC development (Krzystek-Korpacka et al., 2013; Li & Martin, 2016), and may be the key link between CRC, on the one hand, and obesity, IBD (Petersen & Ogawa, 2012) and periodontitis (Fitzpatrick & Katz, 2010; Michaud et al., 2008), on the other hand.

However, mechanisms underlying the association between the disorders are still not completely understood and may be attributable to direct and indirect effects of the virulence factors belonging to periodontal pathogens and to the continuously increasing inflammatory mediators and carcinogenic factors (i.e. nitrosamines) (Han, 2013; Ren et al., 2016). In addition, CRC carcinogenesis is related to multiple environmental and cellular factors, as well as to genetic factors (Guo et al., 2017). As for the last, a bioinformatics method, called 'leader gene approach' (Covani et al., 2008), was carried out to identify the

restricted set of genes potentially involved in and shared in both CRC and periodontitis pathogenesis.

2. Aims

The primary aim of the present in silico study was to assess the genetic linkages between periodontitis and human colorectal cancer, through the identification of all the genes involved in the etiopathogenesis, their ranking into cluster in descending order of predominance, and, the final recognition of those genes presumed to be “leader” in the association between the disorders and, consequently, possible molecular targets for further investigations and focused therapies.

Secondarily, the study aimed to the evidence-based characterization of the main function of leader gene products, of their involvement in biological processes and role in the onset and progression of CRC and periodontitis, in order to determine the putative pathogenic mechanisms linking periodontitis and CRC and, accordingly, point out the possible clinical implications of such genetic linkages.

3. **Methods**

The present study, being performed on computer, did not require informed consent nor ethical approval and was concluded on the 3 April, 2019.

3.1 Gene clustering analysis

Gene clustering analysis of the genes involved in colorectal cancer and periodontitis onset and development included several steps, and for each of them a different software program was used.

First of all, an introductory set of genes related to the above-mentioned phenomenon was established using several integrated cross-search databases (PubMed, Medgen, Genedx,GenBank, GeneCards, OMIM, GenAtlas) via the search engine Entrez (http://www. ncbi.nlm.nih.gov/).

The search strategy included the following pertinent key words, obtained from several papers dealing with either colorectal cancer or periodontitis or both of them (Covani et al., 2009; Hai Ping et al., 2016; Han et al., 2018; Hu et al., 2018; Jiang et al., 2018; Momen-Heravi et al., 2017; Ren et al., 2016; Shamoun et al., 2018; Song et al., 2019; Yu et al., 2004), which were combined using the

three boolean operators AND, OR, NOT, for every search in the above-mentioned databases:

(1) gene AND human

(2) cancer

(3) carcinoma

(4) (2) OR (3)

(5) colon

(6) colonic

(7) rectal

(8) CRC (acronym of ColoRectal Cancer)

(9) 5 OR 6 OR 7 OR (8)

(10) periodontitis

(11) periodontal disease

(12) periodontal inflammation

(13) gingivitis

(14) periodontal disruption

(15) 10 OR 11 OR 12 OR 13 OR 14

(13) 1 AND 4AND 9 AND15.

The iterative search consisted of a consecutive expansion-filtering loop: gene expansion was performed with the Web available software STRING version 11.0 (https://string-db.org/) (Szklarczyk et al., 2019). The new genes in the

expanded set were filtered by a further search with PubMed to eliminate any false positive.

Once a new gene was obtained, its name was verified by means of the official Human Genome Organization (HUGO) Gene Nomenclature Committee, or HGNC (available at http:// https://www.genecards.org), and the approved gene symbol was applied erasing previous symbols or aliases. The free web-based software STRING (version 11.0) (Szklarczyk et al., 2019) was the source of a collection of combined predicted associations with a higher level of confidence (that is a result with a score ≥ 0.9) between each gene and all the other genes involved in the investigated phenomenon.

The sum of the combined predicted association scores gave a numeric variable called the "Weighted Number of Links" (WNL) for each gene.

A synthesis of the gene clustering analysis procedure is shown in Figure 1.

Figure

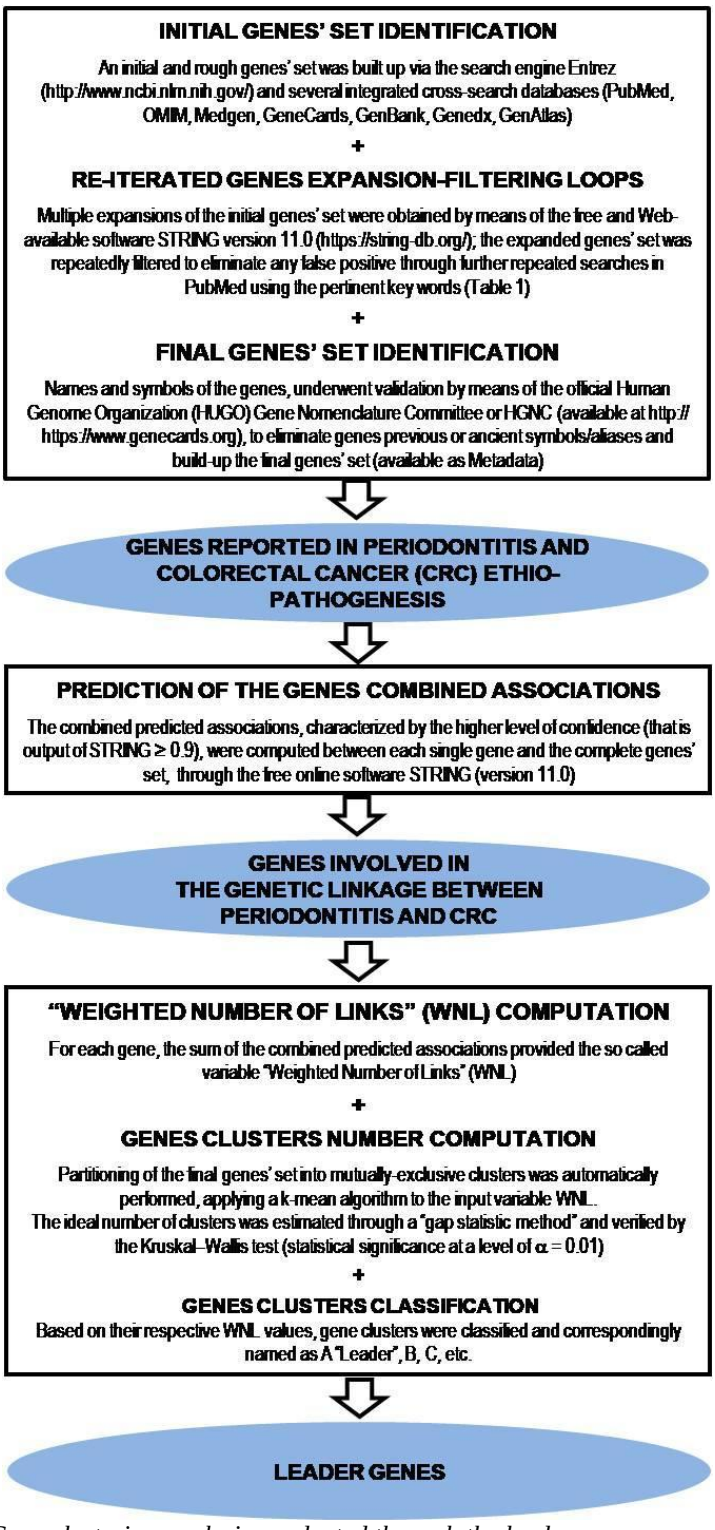

Figure 1: Gene clustering analysis conducted through the leader gene approach (Di Spirito et al., 2020)

3.2 Identification of leader genes

All gene-related data were entered into a matrix laboratory, allowing calculations to be performed automatically. A k-mean algorithm was applied to the input variable WNL, and a partitioning of the overall dataset of genes into mutually-exclusive clusters was automatically performed.

A “gap statistic method” was applied to the set of data for estimating the optimal number of clusters $\hat{k}$ (Barone et al., 2015; Covani et al., 2008; Covani et al., 2009; Sbordone et al., 2009; Toti et al., 2013) for the clusters from 2 to 12. Significant differences among WNLs of cluster groups obtained by the gap statistic method, were found by the Kruskal–Wallis test (statistical significance at a level of $\alpha = 0.01$), verifying the accurate estimate of the number of clusters. Gene clusters were ranked in decreasing order, according to their respective value of WNL centroid, and they were named as A, B, C and so on. The first cluster was named as ”leader” suggesting a leading role in the process, whereas the last class, named as ”orphans”, had genes without identified predicted associations (WNL=0).

3.3 Determination of the putative pathogenic mechanisms associating periodontitis and CRC

The characterization of the main function of leader gene products and their involvement in biological processes of the identified leader genes was conducted via the free online software STRING (Version 11.0) (Szklarczyk et al., 2019).

An additional literature search, using the reported keywords, was performed on ScienceDirect and PubMed/MEDLINE search engines, to disclose the role of leader genes in the genesis/progression of both CRC and periodontitis and to highlight their putative pathogenic mechanisms in the genetic linkage between periodontitis and CRC.

4. Results

4.1 Gene clustering analysis and identification of the leader genes in the genetic linkages between periodontitis and human colorectal cancer

The final data set consisted of 137 genes, described in Table 1.

Table

Gene Acronym	Gene Identification Number	Gene Official Name	Protein main function/biological process(es) involvement	Gene Cluster Assignment
CBL	12	***E3 ubiquitin-protein ligase CBL***	Cell Signalling	**A**
CTNNB1	26	***Catenin beta-1***	Cell Signalling	**A**
FOS	43	***Proto-oncogene c-Fos***	Gene(s) Transcription, Cell Signalling, Cell proliferation and differentiation	**A**
GRB2	46	***Epidermal Growth Factor Receptor-Binding Protein***	Cell Signalling	**A**

		GRB2		
IL1B	52	***Interleukin 1 beta***	Inflammation	**A**
IL4	54	***Interleukin 4***	Immune response	**A**
IL6	56	***Interleukin 6***	Immuno-inflammatory process	**A**
IL10	58	***Interleukin 10***	Inflammation	**A**
JUN	63	***Transcription factor AP-1***	Gene(s) Transcription	**A**
PIK3CA	96	***Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit alpha isoform***	Cell proliferation, Cell survival	**A**
PIK3R1	97	***Phosphatidylinositol 3-kinase regulatory subunit alpha***	Cell Signalling	**A**
RELA	109	***RELA Proto-Oncogene, NF-KB Subunit or Transcription factor p65***	Sub-unit of the Transcription factor NF-kappa-B	**A**
AKT1	2	***RAC-alpha serine/threonine-protein kinase***	Cell proliferation, Cell survival, Angiogenesis	**B**
CD19	16	***B-lymphocyte antigen CD19***	Immune response	**B**
CD79A	19	***B-cell antigen receptor complex-associated protein alpha chain***	Immune response	**B**
CD79B	20	***B-cell antigen receptor complex-associated protein beta chain***	Immune response	**B**
EP300	35	***Histone acetyl transferase p300***	Regulates genes transcription via chromatin remodeling	**B**
IGLL5	48	***Immunoglobulin lambda like polypeptide 5***	Associated with Solitary Osseous	**B**

			Plasmacytoma	
IKBKB	50	***Inhibitor of nuclear factor kappa-B kinase subunit beta***	Cell Signalling (NF- kappa-B pathway)	**B**
IL-1a	51	***Interleukin-1 alpha***	Immuno-inflammatory process	**B**
IL1R1	53	***Interleukin-1 receptortype 1***	Cell Signalling	**B**
SRC	117	***Proto-oncogene tyrosine-proteinkinase Src***	Gene(s) transcription, Immune response, Cell cycle regulation, Cell adhesion and migration	**B**
TP53	134	***Cellular tumor antigen p53***	Cell cycle regulation	**B**
CCND1	13	***G1/S-specific cyclin-D***	Cell cycle regulation	**C**
CRK	25	***Adapter molecule crk***	Phagocytosis of apoptotic cells, Cell motility	**C**
FGFR3	41	***Fibroblast growth factor receptor 3***	Cell proliferation, differentiation and apoptosis, Skeleton development	**C**
IL4R	55	***Interleukin-4 receptor subunit alpha***	Immune response	**C**
IL6R	60	***Interleukin-6 receptor subunit alpha***	Immuno-inflammatory process	**C**
IRF4	62	***Interferon regulatory factor 4***	Immune response, Dendritic cell	**C**

			differentiation	
LTA	73	***Lymphotoxin-alpha***	Immune response	**C**
NFKB1	91	***Nuclear factor NF-kappa-B p105 subunit***	Cell Signalling, Immuno-inflammatory process, Cell cycle regulation and differentiation, Tumorigenesis	**C**
SMAD4	115	***Mothers against decapentaplegic homolog 4***	Muscle physiology	**C**
TLR2	125	***Toll-like receptor 2***	Immune response	**C**
TLR4	126	***Toll-like receptor 4***	Immune response	**C**
TLR6	127	***Toll-like receptor 6***	Immune response	**C**
AURKA	5	***Aurora kinase A***	Cell cycle regulation	**D**
B2M	10	***Beta-2-microglobulin***	Immune response	**D**
CD38	18	***ADP-ribosylcyclase/cyclic ADP-ribosehydrolase 1***	Synthesizes the second messengers cyclic ADP-ribose and nicotinate-adenine dinucleotide phosphate	**D**
IGJ	49	***Immunoglobulin J chain***	Immune response	**D**
IL6ST	61	***Interleukin-6 receptor subunit beta***	Cell Signalling, Immune response, Hematopoiesis, Pain control, Bone metabolism	**D**
MMP9	83	***Matrix metalloproteinase-9***	Extracellular matrix degradation, Leukocyte migration,	**D**

			Bone osteoclastic resorption	
NFATC1	90	***Nuclear factor of activated T-cells, cytoplasmic 1***	Immuno-inflammatory process, Osteoclastogenesis	**D**
PMS1	99	***PMS1 protein homolog 1***	DNA repair	**D**
PMS2	100	***Mismatch repair endonuclease PMS2***	DNA repair	**D**
POU2AF1	103	***POU domain class 2-associating factor***	Immune response	**D**
PTGS2	104	***Prostaglandin G/H synthase 2***	Inflammation	**D**
TNFRSF1A	131	***Tumor necrosis factor receptor superfamily member 1A***	(Pro)Apoptosis	**D**
APC	4	***Adenomatous polyposis coli protein***	Tumor suppressor (Wnt pathway)	**E**
AXIN2	6	***Axin-2***	Cell Signalling(Wnt pathway)	**E**
BAX	7	***Apoptosis regulator BAX***	(Pro)Apoptosis	**E**
BMPR1A	8	***Bone morphogenetic protein receptor type-1A***	Chondrocyte differentiation, Adipogenesis	**E**
CALR	11	***Calreticulin***	Cell endoplasmic reticulum formation	**E**
CD27	17	***CD27 antigen***	Immune response	**E**
HLA-B	47	***HLA class I histocompatibility antigen, B-7 alpha chain***	Immune response	**E**
LTF	74	***Lactotransferrin***	Immuno-inflammatory process, Protection against cancer development and	**E**

			metastasis	
MLH1	77	***DNA mismatch repair protein Mlh1***	DNA repair	**E**
MME	79	***Neprilysin***	Opioid peptides, angiotensin-2, -1, -9 and atrial natriuretic factor degradation	**E**
MMP2	81	***Matrix metalloproteinase-2***	Inflammation, Tissue repair, Angiogenesis, Tumor invasion	**E**
MSH2	86	***DNA mismatch repair protein Msh2***	DNA repair	**E**
MSH6	87	***DNA mismatch repair protein Msh6***	DNA repair	**E**
NRAS	96	***GTPase NRas***	Binds GDP/GTP and possesses intrinsic GTPase activity	**E**
PDGFRB	94	***Platelet-derived growth factor receptor beta***	Tyrosine-protein kinase acting as cell-surface receptor, playing an essential role in blood vessel development	**E**
POLD1	102	***DNA Polymerase Delta 1 Catalytic Subunit***	Plays a crucial role in high fidelity genome replication, requiring the presence of accessory proteins POLD2, POLD3 and POLD4 for full	**E**

			activity	
SMAD7	116	***Mothers against decapentaplegic homolog 7***	TGF-beta inhibition	**E**
TGFBR2	123	***TGF-beta receptor type-2***	Cell cycle regulation (epithelial and hematopoietic cells) Cell proliferation and differentiation (mesenchymal cells), Immune response	**E**
TLR1	124	***Toll-like receptor 1***	Immune response	**E**
TLR9	128	***Toll-like receptor 9***	Immune response	**E**
TNFRSF17	132	***Tumor necrosis factor receptor superfamily member 17***	Immune response	**E**
TNFSF11	133	***Tumor necrosis factor ligand superfamily member 11***	Immune response	**E**
TRAF2	135	***TNF receptor-associated factor 2***	NF-kappa-B and JNK activation, Cell survival and apoptosis regulation,Immune response	**E**
XBP1	136	***X-box-binding protein 1***	Cardiac, hepatic and ecretory tissue development	**E**
BUB1B	9	***Mitotic checkpoint serine/threonine-protein kinase BUB1 beta***	Cell cycle regulation	**F**
CCL18	14	***C-C motif chemokine 18***	Immune response	**F**
COL1A1	23	***Collagen alpha-1(I) chain***	Member of group I collagen	**F**
DCC	30	***Netrinreceptor DCC***	Nervous system	**F**

			development	
EPCAM	34	***EPCAM Epithelial cell adhesion molecule***	Immune response	**F**
FCRLA	38	***Fcreceptor-like A***	Immune response	**F**
IL8	57	***Interleukin-8***	Immune response	**F**
IL22	59	***Interleukin-22***	Inflammation	**F**
MMP1	80	***Matrix metalloproteinase-1***	Types I, II, III, VII and X collagens degradation	**F**
MMP7	82	***Matrix metalloproteinase-7***	Casein, type I, III, IV, and V gelatins and fibronectin degradation	**F**
MZB1	85	***Marginal zone B- and B1-cell-specific protein***	Immune response	**F**
NAMPT	89	***Nicotinamide phosphoribosyltransferase***	Immune response, Anti-diabetic function	**F**
ZBP1	137	***Z-DNA-binding protein 1***	Immune response	**F**
AEBP1	1	***Adipocyte enhancer-binding protein 1***	Adipocyte proliferation, Enhanced macrophage inflammatory responsiveness	**Orphan**
AMPD1	3	***AMP deaminase 1***	Energy metabolism	**Orphan**
CD14	15	***Monocyte differentiation antigen CD14***	Immune response	**Orphan**
CEACAM21	21	***Carcinoembryonic Antigen Related Cell Adhesion Molecule 21***	Immune response	**Orphan**
CLDN10	22	***Claudin-10***	Cell adhesion	**Orphan**
CPNE5	24	***Copine-5***	Melanocytes formation	**Orphan**
CTR	27	***Calcitonin receptor***	Receptor for	**Orphan**

			calcitonin	
C12orf63	28	***Cilia- and flagella-associated protein 54***	Cilia and flagella assembly	**Orphan**
C8orf80	29	***Nuclear GTPase, Germinal Center Associated***	Genome stability	**Orphan**
DERL3	31	***Derlin-3***	Endoplasmic reticulum stress-induced pre-emptive quality control	**Orphan**
DLC1	32	***Rho GTPase-activating protein 7***	Cell proliferation and migration	**Orphan**
DPEP1	33	***Dipeptidase 1***	Immuno-inflammatory process	**Orphan**
FAM46C	36	***Nucleotidyltransferase FAM46C***	RNA polymerisation	**Orphan**
FAM92B	37	***Protein FAM92B***	Ciliogenesis	**Orphan**
FCRL2	39	***Fc receptor-like protein 2***	Immune response, B-cells tumorigenesis	**Orphan**
FCRL5	40	***Fc receptor-like protein 5***	Immune response	**Orphan**
FLCN	42	***Folliculin***	Tumor suppression	**Orphan**
GALNT12	44	***Polypeptide N-acetylgalactosaminyltransferase 12***	Oligosaccharide biosynthesis	**Orphan**
GPR114	45	***Adhesion G-protein coupled receptor G5***	Cell Signalling	**Orphan**
KCNA3	64	***Potassium voltage-gated channel subfamily A member 3***	Mediates the voltage-dependent potassium ion permeability of excitable membranes	**Orphan**
KCNN3	65	***Small conductance calcium-***	Forms a voltage-	**Orphan**

		activated potassium channel protein 3	independent potassium channel activated by intracellular calcium	
KLHL6	66	***Kelch-likeprotein 6***	Immune response	**Orphan**
LAX1	67	***Lymphocyte trans membrane adapter 1***	Immune response	**Orphan**
LBP	68	***Lipopolysaccharide-binding protein***	Immune response	**Orphan**
LGALS7	69	***Galectin-7***	Cell growth control	**Orphan**
LILRA3	70	***Leukocyte Immunoglobulin Like Receptor A3***	Immune response	**Orphan**
LY9	71	***T-lymphocyte surface antigen Ly-9***	Immune response	**Orphan**
LRMP	72	***Lymphoid-restricted membrane protein***	Immune response	**Orphan**
MCC	75	***Colorectal mutant cancer protein***	Tumor suppression	**Orphan**
MEI1	76	***Meiosis inhibitor protein 1***	Meiosis	**Orphan**
MLH3	78	***DNA mismatch repair protein Mlh3***	DNA repair	**Orphan**
MMP12	84	***Macrophage metalloelastase***	Tissue remodeling	**Orphan**
MUTYH	88	***Adenine DNA glycosylase***	DNA repair	**Orphan**
ODC1	93	***Ornithine decarboxylase***	DNA replication, Cell proliferation and apoptosis	**Orphan**
PDGFRL	95	***Platelet-derived growth factor receptor-like protein***	Associated with colorectal cancer and other malignancies	**Orphan**
PIM2	98	***Serine/threonine-protein kinase pim-2***	Cell proliferation, Cell survival	**Orphan**
PNOC	107	***Prepronociceptin***	Nociception, Neuronal	**Orphan**

			development	
PTPN12	105	***Tyrosine-protein phosphatase non-receptor type 12***	Cell Signalling	**Orphan**
PTPRJ	106	***Protein Tyrosine Phosphatase Receptor Type J***	Cell proliferation and differentiation, Cell adhesion and migration, Platelet activation and thrombosis	**Orphan**
P2RX1	107	***P2X purinoceptor 1***	Synaptic transmission	**Orphan**
RAD54B	108	***DNA repair and recombination protein RAD54B***	DNA repair	**Orphan**
RPS11	110	***Ribosomal protein S11***	40S sub-unit ribosomal protein	**Orphan**
SAA1	111	***Serumamyloid A-1 protein***	Inflammation	**Orphan**
SIRT2	112	***NAD-dependent protein deacetylase sirtuin-2***	Cell cycle regulation	**Orphan**
SLAMF7	113	***SLAM family member 7***	Immune response	**Orphan**
SLC17A9	114	***Solute carrier family 17 member 9***	ATP storage and exocytosis	**Orphan**
SPAG	118	***RNA polymerase II-associated protein 3***	RNA polymerization	**Orphan**
SSR4	119	***Translocon-associated protein subunit delta***	Retention of ER resident proteins regulation	**Orphan**
STK11	120	***Serine/threonine-protein kinase STK11***	Tumor suppression	**Orphan**
ST6GAL1	121	***Beta-galactoside alpha-2,6-sialyltransferase 1***	Transfers sialic acid from CMP-sialic acid to galactose-containing acceptor substrates	**Orphan**

TAS1R3	122	***Taste receptor type 1 member 3***	Umami taste stimulus response	**Orphan**
TMEM156	129	***Transmembrane protein 156***	Transmembrane protein	**Orphan**
TNFa	130	***Tumor necrosis factor***	Cell proliferation and differentiation, Tumor cells death	**Orphan**

Table 1 reports gene numbers, acronyms, official names, primary functions, and biological process(es) involvement description, as per the free online software STRING (version 11.0) (Szklarczyk et al., 2019) and cluster assignment (Di Spirito et al., 2020).

The optimal number of clusters, defined as the ˆk number of clusters (Figure 2A), was estimated through the use of a clustering algorithm and the gap statistic method. Depending on the weighted number of links (WNL), related to the number of predicted interaction of each gene, shown in Figure 2B, all clusters were hierarchically ranked based on the prominence of the genes in the phenomenon under investigation and were correspondingly designated as A or leader cluster, followed by B, C, D, E, F clusters, and, lastly, by the cluster of the orphan genes. The distance of the WNLs between clusters resulted statistically significant ($p = 0.0034$). The links of predicted interactions for the present phenomenon, as per the above-mentioned STRING program is illustrated in Figure 2C.

Figure

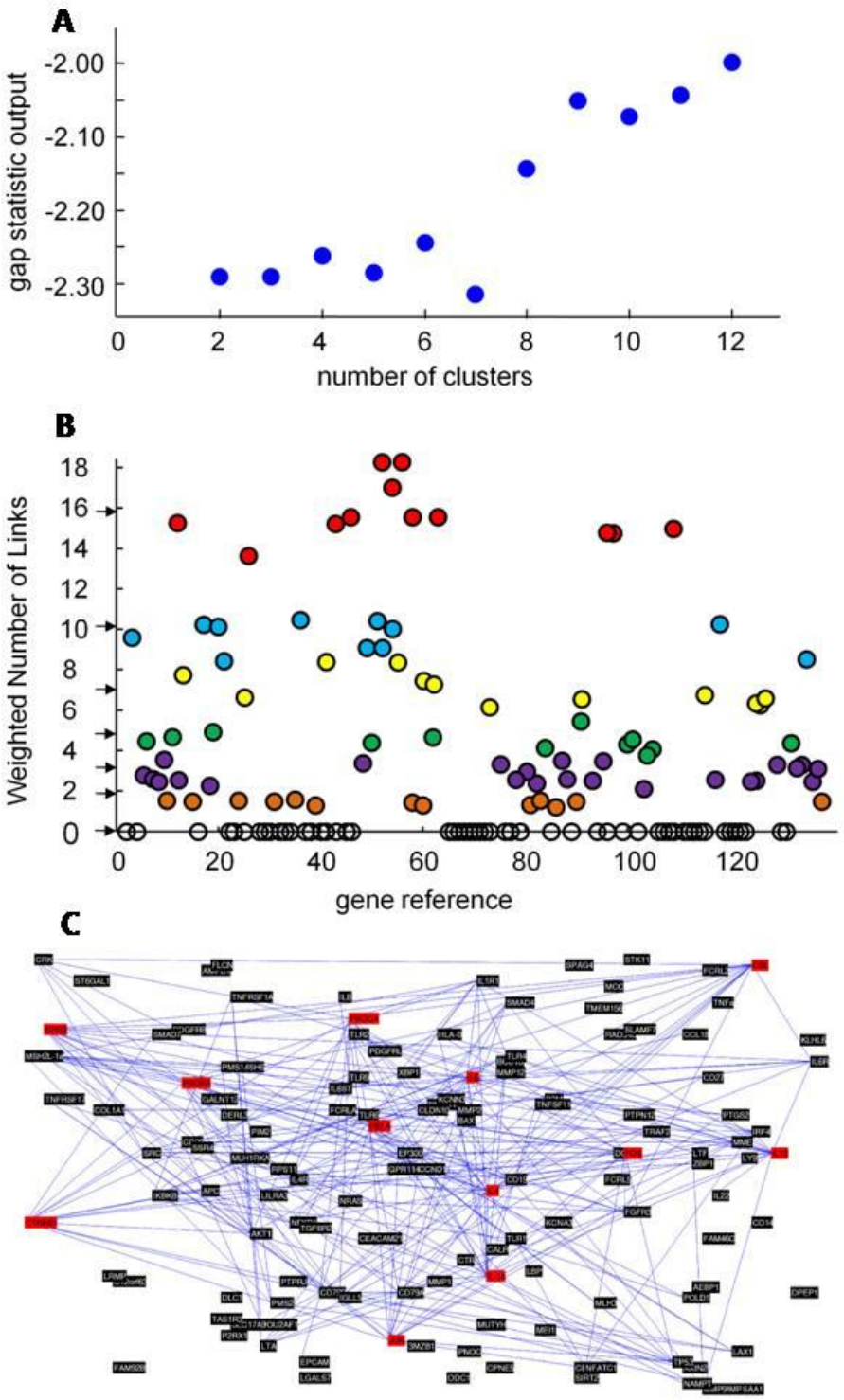

Figure 2 A: gap statistic method for estimating the optimal number of clusters; B: Weighted Number of Links (WNL) for the identified genes, centroids of the cluster groups (black), leader genes (red); cluster B genes (light blue), cluster C genes (yellow), cluster D genes (green),

cluster E genes (purple), cluster F genes (orange), orphan genes (clear); C: map of predicted interactions (lines connecting single genes) of the overall 137 genes involved in the genetic linkage between periodontitis and CRC according to STRING, with the leader genes in red. (Di Spirito et al., 2020)

Totally 7 clusters were identified.

Leader cluster consisted of 12 genes (Figure 3): E3 ubiquitin-protein ligase (CBL), Catenin beta-1 (CTNNB1), Proto-oncogene c-Fos (FOS), Growth factor receptor-bound protein 2 (GRB2), Interleukins1B,4,6,10 (IL1B, IL4, IL6, IL10), Transcription factor AP-1 (JUN), Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit alpha isoform (PIK3CA), Phosphatidylinositol 3-kinase regulatory subunit alpha (PIK3R1) and RELA Proto-Oncogene NFKB Subunit or Transcription factor p65 (RELA).

Eleven genes, RAC-alpha serine/threonine-protein kinase (AKT1), B-lymphocyte antigen CD19 (CD19), B-cell antigen receptor complex-associated protein alpha chain (CD79A), B-cell antigen receptor complex-associated protein beta chain (CD79B), Histone acetyltransferase p300 (EP300), Immunoglobulin lambda like polypeptide 5 (IGLL5), Inhibitor of nuclear factor kappa-B kinase subunit beta (IKBKB), Interleukin-1 alpha (IL1A), Interleukin-1 receptor type 1 (IL1R1), Proto-oncogene tyrosine-protein kinase Src (SRC) and Cellular tumor antigen p53 (TP53) resulted belonging to cluster B (Figure 3).

Cluster C, D, E and F consisted of 12, 12, 24 and 13 genes, respectively (Figure 3).

Finally, the 53 genes found without identified predicted interactions set up the orphan cluster.

Figure

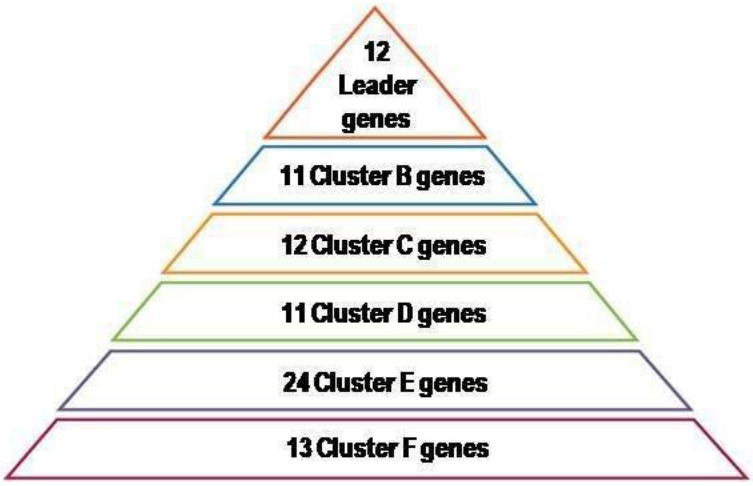

Figure 3: *Gene ranking into the seven hierarchical clusters, excluding the orphan genes (Di Spirito et al., 2020).*

4.2 Characterization of the leader genes in the pathogenic linkage between periodontitis and human colorectal cancer

The 12 leader genes, among the 137 genes identified in both CRC and periodontitis, resulted involved in different signalling (i.e. CTNNB1, CBL, GRB2, PIK3CA, PIK3R1) and transcriptional (i.e. JUN, RELA) pathways, as

well as in cell proliferation/differentiation (i.e. FOS) and in inflammatory processes (i.e. IL1B, IL4, IL6, IL10).

Four out of the 5 leader genes identified in periodontitis (Covani et al., 2008) are also ranked as leader genes in the genomic relationship between CRC and periodontitis: CBL, GRB2, PIK3R1 and RELA; Nuclear factor NF-kappa-B p105 subunit (NFKB1), instead, is considered as leader gene in periodontitis, but addressed to cluster C genes in the currently described phenomenon.

NFKB1 (p50) and RELA (p65), active in inflammatory and immune processes, make-up the NFKB complex, although encoded by distinct genes.

RELA expression is higher in CRC cells compared to normal colonic cells, as well as in breast, liver, pancreatic and gastric cancerous cells, although its role in cancerogenesis is still not fully elucidated (Yu et al., 2004). NFKB regulates the transcription of several genes encoding for pro-inflammatory cytokines. It is constitutively inactivated by the binding of IκB. IκB ubiquination and proteasomal degradation activates NFKB. Indeed, a de-regulation in the ubiquitin–proteasome system, with subsequent NFKB activation, may affect immuno-inflammatory response and has been related to atherogenesis, neurodegenerative and autoimmune diseases, as well as to cancer and IBD (Kumaradevan et al., 2018; Tsuchida et al., 2017). Current knowledge about the role of NFKB activation and ubiquitin–proteasome system de-regulation in periodontitis is still limited but it may explain the presence of the E3 ubiquitin-protein ligase (CBL) gene among both the leader genes in periodontitis and the

currently investigated phenomenon, even though no evidence is available relating CBL to periodontitis (Covaniet al., 2008). NFKB complex activity increases in many inflammatory diseases, in periodontal lesions (Covani et al., 2008) and in oral gingival cells, in the presence of *Porphyromonas gingivalis* and *Fusobacterium nucleatum* (Tsuchida et al., 2017).These periodontal pathogens are recognized by the Toll-like receptor (TLR) (Milward et al., 2007), consistent with the current identification of TLR-2, -4, -6 as cluster C genes.

GRB2 gene encodes for a protein binding the Epidermal Growth Factor (EGF) receptor, activating several signaling pathways. GRB2 products stimulate colonic cells proliferation (Weinberg et al., 2017).In particular, the Grb2 associated binding protein 2 (Gab2) has been found responsible for epithelio-mesenchymal transition and consequent CRC metastasis development (Jiang et al., 2018). In periodontal tissue, EGF signaling, indirectly affected by GRB2 expression, is considered essential in tissue regeneration; thus, its interruption may affect healing and regeneration processes. EGF ligands alterations, secondary to the effect of the Peptidylarginine Deiminase enzyme, released by *P.gingivalis*, interferes with EGF signaling, and, potentially, favoures periodontitis progression (Pyrc et al., 2013).

PIK3CA is the most frequently mutated gene in breast cancer and plays a central role in other cancers as well; it phosphorylates PIK3R1, also involved in

human cancers and downregulated in CRC cells (Zeng et al., 2019). PIK3R1 is indicated as a marker of severe periodontitis (Suzuki et al., 2004).

CTNNB1 encodes for b-catenin, a subunit of the adherens junctions complex, regulating cell growth and adhesion and Wnt responsive genes (i.e. c-Myc) expression, leading to cell cycle progression. CTNNB1is mutated in up to 90% of the colonic tumors, being responsible for initial tissue dysplastic transformation (Pandurangan, 2018). B-catenin is detectable in periodontal ligament cells nuclei in mice, where Wnt stimulus induces osteogenic lineage commitment (Lim et al., 2014). Conversely, Wnt depletion is involved in alveolar bone loss, and, potentially, in periodontal ligament homeostasis (Lim et al., 2014).

FOS is an oncogene encoding for the c-Fos protein, which heterodimerizes with c-Jun, encoded by JUN, to form the Transcription factor AP-1, which is involved in cell proliferation, differentiation, apoptosis and cancerous transformation (Chen et al., 2019; Hu et al., 1994). FOS expression increases in CRC lesions (Kou et al., 2015); rs7101 and rs1063169 FOS Single Nucleotide Polymorphisms are considered at higher risk of CRC onset (Chen et al., 2019). In addition, a different member of the FOS family, named Fra-1, is over-expressed in colonic cancer cells, particularly in those acquiring motility and invasive ability (Kou et al., 2015). Moreover, FOS may participate in the inflammatory microenvironment associated with the CRC (Kou et al., 2015).

FOS may also be implicated in periodontitis development and progression through the interaction with Prostaglandin-Endoperoxide Synthase 2, affecting the T Cell Receptor (TCR) signaling (Song et al., 2015).

Among the leader genes, IL1B, IL4, IL6, IL10 have also been identified. These cytokines play a central role in periodontitis onset and progression and may constitute the pathogenic link between periodontitis and systemic disease. Furthermore, such cytokines may predispose to neoplastic transformation of chronic colitis, favouring colorectal carcinogenesis (Guo et al., 2017), and are produced by CRC cells themselves (Krzystek-Korpacka et al., 2013).

IL6 induces CRC cell growth and invasion and higher levels of serum IL6 have been detected in CRC patients compared to controls (Balkwill, 2006). IL6 stimulates osteoclastogenesis (da Silva et al., 2017), has been found associated with aggressive and chronic periodontitis and, together with IL6ST, IL1R1, IL6R, and IL4R may link periodontitis to other diseases (Covani et al., 2008).

IL1b acts in immune response against microbial agents (da Silva et al., 2017). IL1-889 C/T gene polymorphism has been associated with severe periodontitis (da Silva et al., 2017) and its role in periodontitis pathogenesis has long been advocated (Wilkins et al., 2017). In addition, IL1b has been involved in tumorigenesis, since it is also produced in higher concentrations in CRC cells when compared to healthy surrounding tissues, possibly activating the NFKB signaling pathway (Hai Ping et al., 2016).

IL4, produced by activated T helper 2 lymphocytes, may reduce cancer-directed response operated by the immune system, encouraging cancer invasion and metastasis. Through its binding to Type II IL-4 receptor α (IL-4Rα) and JAK/STAT signaling activation, promotes cancer cells survival and immunosuppression, so that a dysregulation in IL-4 signaling or IL-4Rα gene polymorphisms may be associated with cancer, including CRC (Shamoun et al., 2018). Conversely, IL4 plays a protective role in periodontitis progression, reducing alveolar bone loss; consequently, IL4 gingivo-crevicular fluid levels results higher in periodontally healthy subjects and after non-surgical periodontal treatment and the IL4-590 C/T polymorphism has been related to an increased risk of periodontitis development (Yan et al., 2014).

IL-10 is an anti-inflammatory cytokine, down-regulating monocyte-macrophage response. Its gene polymorphism has been associated with periodontitis development in Caucasians (da Silva et al., 2017). IL10 deficiency favours IBD malignant transformation to CRC (Mantovani et al., 2008; Triantafillidis et al., 2009), through the so called "inflammation-dysplasia-carcinoma sequence", which is alternative to the well known "adenoma-carcinoma sequence" (Guo et al., 2017).

Table

Leader Genes	Main Function	Role in CRC	Role in Periodontitis	Putative Pathogenic Mechanisms
CTNNB1	*Cell signaling*	Mutated in up to 90% of colonic tumors; responsible for initial tissue dysplastic transformation; encodes for β-catenin, a subunit of the	Its product, β-catenin, is detectable in periodontal ligament cell nuclei in mice, potentially influencing periodontal	**Cell cycle dysregulation**

		adherens junctions complex, regulating cell growth and adhesion and Wnt responsive genes (i.e., c-Myc) expression, leading to cell cycle progression.	ligament homeostasis ; regulates Wnt responsive genes. Wnt stimulus induces osteogenic lineage commitment, while Wnt depletion is involved in alveolar bone loss.	
FOS	*Gene(s) transcription, cell signaling, cell proliferation and differentiation*	rs7101 and rs1063169 FOS single nucleotide polymorphisms are considered at higher risk of CRC onset and its expression increases in CRC lesions. In addition, a different member of the FOS family, named Fra-1, is over-expressed in colonic cancer cells, particularly in those acquiring motility and invasive ability. Moreover, FOS may participate in the inflammatory microenvironment associated with CRC.	May be implicated in periodontitis development and progression through the interaction with prostaglandin-endoperoxide synthase 2, affecting the T-cell receptor (TCR) signaling.	**Cell cycle dysregulation**
JUN	*Gene(s) transcription, cell signaling, cell proliferation, and differentiation, inflammation*	Its product, c-Jun, heterodimerizes with c-Fos protein, encoded by FOS, to form the transcription factor AP-1 (see above). Involved in cell proliferation, differentiation, apoptosis, and malignant transformation.	Its product, c-Jun, heterodimerizes with c-Fos protein, encoded by FOS, to form the transcription factor AP-1 (see above). Involved in cell proliferation, differentiation, apoptosis, and malignant transformation.	**Cell cycle dysregulation**
GRB2	*Cell signaling*	Its products stimulate colonic cell proliferation; in particular, the Grb2-associated binding protein 2 (Gab2) has been found responsible for epithelial mesenchymal transition and consequent CRC metastasis development.	Its products bind to the epidermal growth factor (EGF) receptor. EGF signaling in the periodontal tissue, indirectly affected by GRB2 expression, is considered essential in tissue regeneration; thus, its interruption may affect healing and regeneration processes. Indeed, EGF ligand alterations, secondary to the effect of the peptidylarginine deiminase enzyme, released by porphyromonasgingivalis, interfere with EGF signaling, and, potentially, favor periodontitis progression.	**Cell cycle dysregulation**
PIK3CA	*Cell*	The most frequently mutated	n.a.	**Cell cycle**

	proliferation, cell survival	gene in breast cancer and is centrally involved in other malignancies.		**dysregulation**
PIK3R1	*Cell signaling*	Phosphorylated by PIK3CA, it is downregulated in CRC cells.	It is considered as a marker of severe periodontitis.	**Cell cycle dysregulation**
IL6	*Inflammation*	Induces CRC cell growth and invasion; and higher levels of IL6 have been detected in the serum from CRC patients compared to controls.	Stimulates osteoclastogenesis, has been found associated with chronic as well as aggressive periodontitis and, together with IL6R, IL6ST, IL4R, and IL1R1 may link periodontitis to other diseases.	**Immuno-inflammatory response**
IL1B	*Immune response*	In CRC cells it is produced in higher concentrations compared to healthy surrounding tissues, possibly activating the NFKB signaling pathway.	IL1-889 C/T gene polymorphism has been associated with severe periodontitis [34] and its role in periodontitis pathogenesis has long been advocated.	**Immuno-inflammatory response**
IL4	*Immuno-inflammatory process*	Produced by activated T helper 2 lymphocytes, may reduce cancer-directed response operated by the immune system, encouraging cancer invasion and metastasis. Through its binding to Type II IL-4 receptor α (IL-4Rα) and JAK/STAT signaling activation, it favors survival of cancer cells and immunosuppression, so that a dysregulation in IL-4 signaling or IL-4Rα gene polymorphisms may be associated with cancer, including CRC.	Plays a protective role in periodontitis progression, reducing alveolar bone loss. Consequently, IL4 gingivo-crevicular fluid levels are higher in periodontally healthy subjects and after non-surgical periodontal treatment. In addition, the IL4-590 C/T polymorphism has been reported as potentially associated with an increased risk of periodontitis development.	**Immuno-inflammatory response**
IL10	*Gene(s) transcription*	Its deficiency favors IBD malignant transformation to CRC, through the so called "inflammation-dysplasia-carcinoma sequence", an alternative to the well-known "adenoma-carcinoma sequence".	Anti-inflammatory cytokine, down-regulating monocyte-macrophage response. Its gene polymorphism has been associated with periodontitis development in Caucasians.	**Immuno-inflammatory response**
RELA	*Cell signaling*	Its expression is higher in malignant compared to healthy colonic cells, as well as in breast, liver, pancreatic, and gastric cancers, although its role in cancerogenesis, as well as in periodontitis, is still not fully elucidated.	It is also classified as leader gene in periodontitis probably because it is functionally related to NFKB pro-inflammatory activity.	**Immuno-inflammatory response**
CBL	*Cell signaling*	It may be related to inflammatory bowel disease (IBD) and CRC, as well as to atherogenesis, and neurodegenerative and	No evidence is available relating CBL to periodontitis.	**Immuno-inflammatory response**

		autoimmune diseases, by a de-regulation in the ubiquitin–proteasome system, with subsequent NFKB activation and immuno-inflammatory response enhancement.		

Table 2: Description of the leader genes identified in the genetic linkage between periodontitis and human colorectal cancer (Di Spirito et al., 2020).

5. Discussion

Periodontitis and human colorectal cancer are multi-factorial disorders, linked to several genes, interconnected by various networks, and whose products participate in a multitude of biological pathways (Covani et al., 2008). Highlighting the genetic traits of such complex disorders may pave the way for primary prevention strategies, in order to reduce both biological impact and the healthcare costs, especially of CRC.

The improved understanding of the putative pathogenic mechanisms associating periodontitis and CRC may promote a multidisciplinary more comprehensive approach to CRC, which is strongly advocated for such a complex multifactorial disorder.

5.1 The genetic linkage between periodontitis and human colorectal cancer: leader genes

Among the 137 genes identified in periodontitis and CRC ethio-pathogenesis, 83 were involved in the genetic linkage between both disorders and 12 were

considered to play a predominant role in such an association, being, therefore, defined as leader genes

Notheworthy, four of the leader genes, namely, CBL, GRB2, PIK3R1, and RELA, were also identified among the five leader genes in periodontitis (Covani et al., 2008), and, although NFKB1 gene, which is considered as a leader gene in periodontitis, was currently assigned to cluster C, the presented results may support the existence of a possible genetic linkage between periodontitis and CRC.

The identified leader genes were characterized (see Table 2), revealing their involvement in several biological processes, such as cell proliferation/differentiation (i.e., FOS), cell signaling (i.e., CTNNB1, CBL, GRB2, PIK3CA, PIK3R1), immuno-inflammatory processes (i.e., IL1B, IL4, IL6, IL10; see Table 2) and transcriptional pathways (i.e., JUN, RELA) (Szklarczyk et al., 2019). Moreover, the roles of the leader genes in CRC and periodontitis pathogenesis, shown in Table 2, suggested that the pathogenic mechanisms underlying the linkage between periodontitis and CRC may rely on the effect of the products of the leader genes on cell cycle dysregulation and immuno-inflammatory response alteration, as illustrated in Figure 4.

Figure

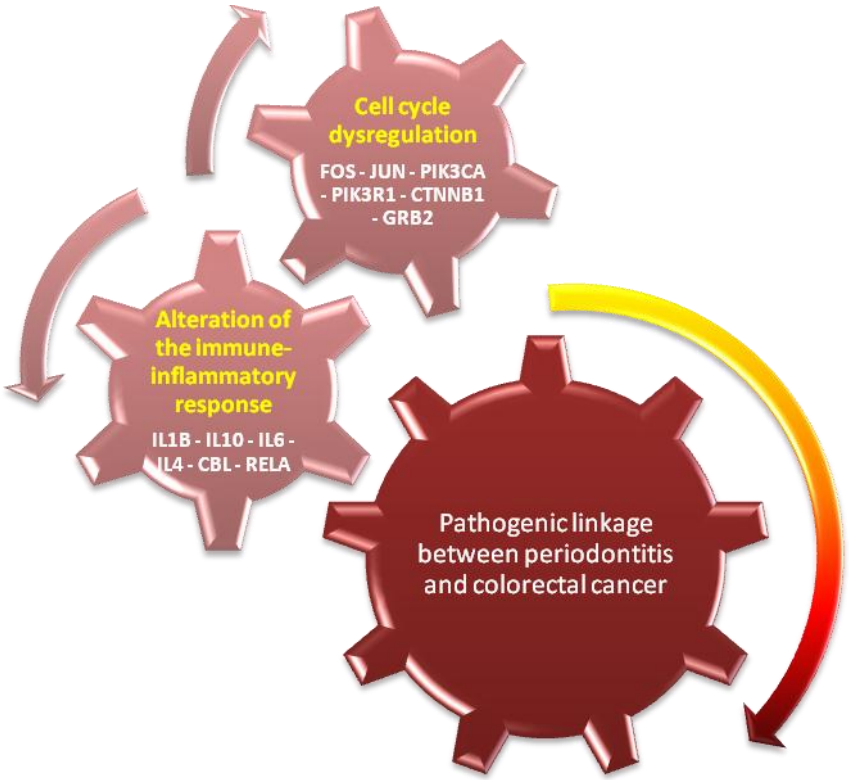

Figure 4: The putative pathogenic mechanisms linking periodontitis and CRC may rely on the effect of the leader genes products on cell cycle dysregulation and immuno-inflammatory response alteration (Di Spirito et al., 2020).

FOS, JUN, PIK3CA, PIK3R1, CTNNB1 and GRB2, acting in cell cycle regulation, may, if dys-regulated, alter cell homeostasis in both colonic and periodontal tissues, promoting colonic cell proliferation and malignant transformation and periodontitis onset and progression (Table 2).

IL1B, IL10, IL6, IL4, CBL and RELA, involved in the immune-inflammatory response (Table 2), are supposed to support a bi-directional relationship between the disorders, as described later. In addition, NFKB, which is functionally associated to RELA and involved in inflammation, has been

currently addressed to cluster C genes; NFKB may be also related to CBL and affected by cellular ubiquitin–proteasome system dysregulation. In more detail, a dysregulation in the ubiquitin–proteasome system, reported in several neurodegenerative processes, autoimmune disorders and atherogenesis, may play a role in IBD and CRC (Jepsen et al., 2018; Tsuchida et al., 2017) as well as in periodontitis pathogenesis, although no evidence is available (Covani et al., 2008).

5.2 The pathogenetic linkage between periodontitis and human colorectal cancer: cytokines and systemic inflammation

Periodontitis is microbially initiated and later sustained by the ongoing local increase of pro-inflammatory cytokines, secondary to the dysregulation of the immune-inflammatory processes (Chapple et al., 2013). As a consequence, cytokines released in inflamed periodontal tissues, along with oral and periodontal microorganisms and toxins may access the systemic blood stream and determine systemic inflammation (Chapple et al., 2013; Yoon et al., 2012) and, finally, affect the clinical course of systemic inflammatory diseases. In this perspective, periodontal cytokines may constitute the pathogenic link between

periodontitis and several systemic diseases, including IBD and CRC (Chapple et al., 2013; Lee et al., 2018; Michaud et al., 2008; Soory et al., 2010; Trius et al., 2019; Yan et al., 2014).

Indeed, it has been recognized that IBD has common oral mucosal manifestations, such as pyostomatitis vegetans and aphthous stomatitis, and subjects with Crohn's disease have an increased risk of periodontitis onset and severity when compared to non-IBD patients (Chi et al. 2018; Momen-Heravi et al., 2017; Yu, 2018). Moreover, a growing body of evidence support the hypothesis that oral pathogens, especially *Fusobacterium nucleatum* and *Campylobacter concisus*, may be related to IBD (Lee et al., 2018). Such inter-relationships may rely on the fact that IBD and periodontitis share a multifactorial etiology and those pathogenic mechanisms leading to the de-regulation of the local immuno-inflammatory response and to the genesis of a systemic inflammation (Chi et al. 2018; Yu, 2018; Kojima et al., 2004). Furthermore, periodontal *F. nucleatum* may also be related to colorectal adenomas (Lauritano et al., 2017) and CRC, since it has been found able to bind, through Fusobacterium adhesin A (FadA), which is unique to oral Fusobacteria, to the (E)-cadherins on epithelial cells, inducing the transcription of the oncogenes regulated by the b-catenin, encoded by the leader gene CTNNB1, stimulating the growth of neoplastic cells; in addition, the transcription IL6 gene, currently classified as leader gene, and of other genes

involved into the immune-inflammatory response, such as NFKB, ranked into cluster C genes, is also stimulated, (Lauritano et al., 2017).

In addition, it has been reported that periodontal cytokines may affect the neoplastic transformation of inflamed colonic cells in IBD, promoting CRC (Guo et al., 2017; Hu et al., 2018; Lauritano et al., 2017).

From the reported evidence, compliant with the presented results, periodontitis may be considered as a possible risk factor for CRC development in IBD subjects (Taylor et al., 2008) and as a risk factor for cancer progression in CRC subjects.

On the other hand, after CRC onset, cytokines may be induced by CRC itself, enhancing neoplastic cells growth and interactions with the surrounding stroma and immune cells, with subsequent colorectal progression and invasion (Chi et al., 2018;; Krzystek-Korpacka et al., 2013; Szklarczyk, 2019). Such CRC cytokines have been identified as leader genes and may, as formerly suggested for cytokines released in diabetes (Yoon et al., 2012), be supposed to negatively affect periodontitis genesis and progression, altering the immune-inflammatory processes in periodontal tissues.

5.3 Linkages between periodontitis and human colorectal cancer: clinical implications

The presented results, although requiring validation by larger studies, provide preliminary data highlighting the etio-pathogenic mechanisms associating periodontitis and CRC, suitable for future clinical researches and applications. Indeed, the findings discussed propose a central role of both periodontal and colonic cytokines, with the related systemic inflammation, in the genetic linkage between the disorders, and, if validated, may recommend the inclusion of periodontitis management in CRC prevention and treatment strategies.

Periodontitis diagnosis in IBD subjects, already considered at higher risk for CRC development, may drive even more attention on them, so that periodontal exam may favour the identification of the IBD subjects at enhanced risk of colonic malignant transformation and periodontal treatment may be also proposed as CRC primary prevention strategies, decreasing the systemic inflammation and, consequently, the inflammatory pro-carcinogenic environment. In this perspective, since the threshold of periodontal cytokines related to systemic inflammation is still not known, and their qualitative assessment and even more the quantitative one may be biased by orally administered drugs and by other the accidental detection of oral microorganisms (Barros et al., 2016), periodontal treatment should be provided to all IBD subjects.

In addition, periodontal treatment may be proposed as a CRC secondary and/or tertiary prevention strategy in subjects affected by CRC, reducing the

inflammation in the tumor-associated environment and the related stimulus to neoplastic cells growth, and, noteworthy, reducing the systemic spread of both periodontal pathogens, especially *F. nucleatum*, which has been found able to promote colonic cells malignant transformation and CRC cells growth, and toxins from periodontal microorganisms.

6. Conclusions

Four of the five leader genes identified for periodontitis (CBL, GRB2, PIK3R1, and RELA) were also listed as leader genes in the presented study, suggesting the existence of genetic linkages between periodontitis and CRC, and recommending a more comprehensive multi-disciplinary approach to CRC subjects, integrating periodontitis diagnosis and periodontal treatment into CRC management.

Ranking IL1B, IL4, IL6, IL10 among leader genes proposed a central role for systemic inflammation in the genomic relationship between periodontitis and CRC. In more detail, periodontitis may be associated to IBD, and, in turn, to CRC, affecting both the "inflammation-dysplasia" carcinogenic sequence and the inflammatory pro-carcinogenic colonic environment. For such reasons, periodontitis management may be proposed as a CRC primary prevention strategy, especially in IBD subjects, considered at high risk of CRC development, aiming to decrease the periodontal microbial charge and cytokines level and, as a result, reduce the systemic dissemination of periodontal cytokines, microorganisms and toxins. Furthermore, periodontitis management, achieving healthy periodontal conditions, may decrease the

systemic inflammation and the inflammatory pro-carcinogenic environment, and be, therefore, included in CRC treatment strategies.

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