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MUNI MED Department of Pathological Physiology

Molecular analysis of oral pathogens and saliva, dental caries

Lecture topics

- Factors involved in development of dental caries:
  - Oral microbiome
    - Dental plaque (bacterial biofilm)
  - Saliva
    - Saliva composition and its functions
  - Behavioral and environmental factors
  - Genetic factors
    - tissue susceptibility to caries
- Molecular analysis of saliva
- Molecular analysis of oral microbiome
- Genetic basis of dental caries
  - Genetic association studies related to dental caries

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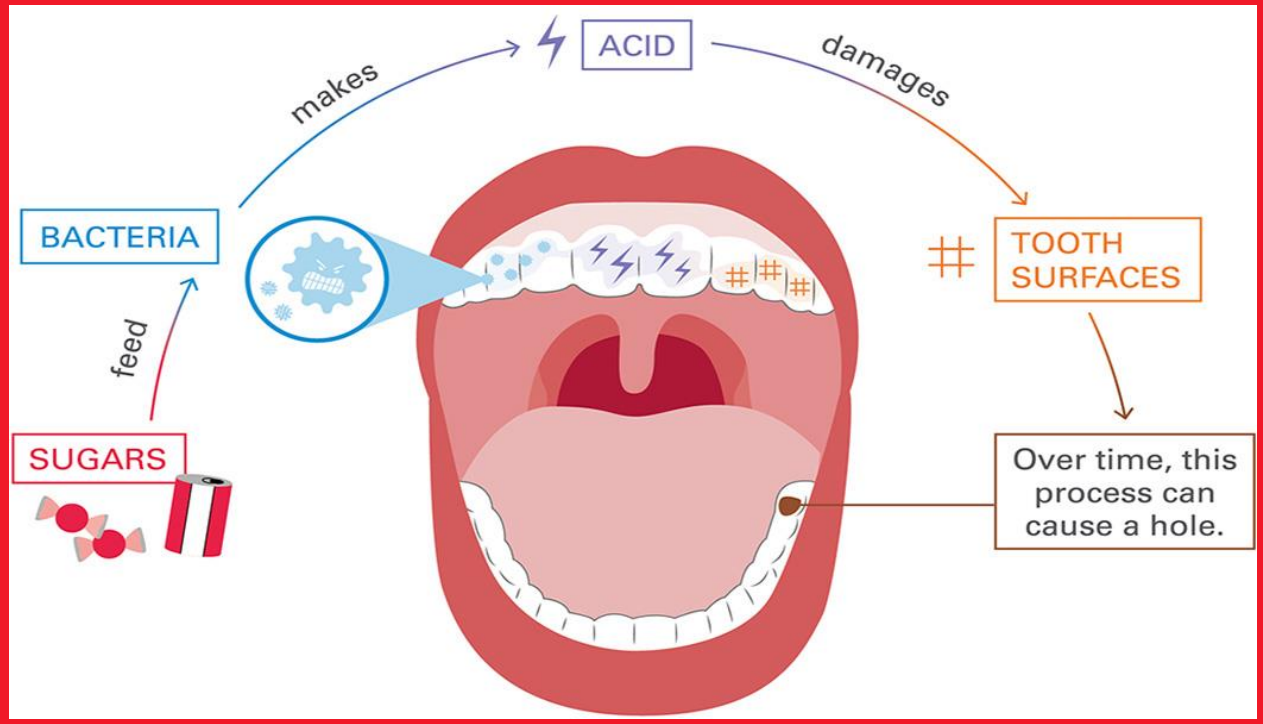
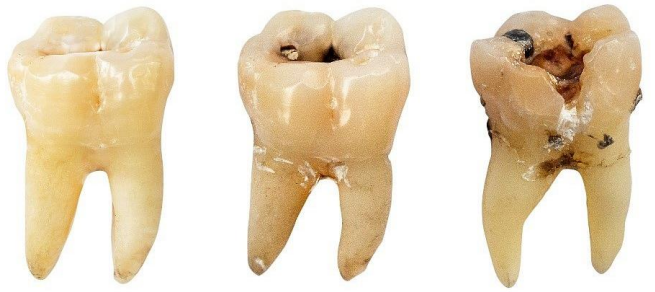
Dental caries

and factors affecting their development



Dental Health Services Victoria
(www.betterhealth.vic.gov.au)

Dental caries



Puwadol Jaturawutthichai
(www.shutterstock.com)

- the most common disease
  - 32 % of the world population
- non-communicable disease (according to FDI World Dental Federation)
  - shares risk factors with many other non-communicable diseases
  - however → *Streptococcus mutans* transmission from parents to infants (Early childhood caries)
- complex disease
  - multifactorial (endogenous and exogenous factors), multiple genes are involved in
- interaction of several factors contributes to formation of caries
  - genetic predispositions
  - composition of oral microflora
  - composition and physical action of saliva
  - overall health condition (immune system disorders, systemic diseases affecting immune system)
  - behavioral and environmental factors
  - time for which the factors act / interact

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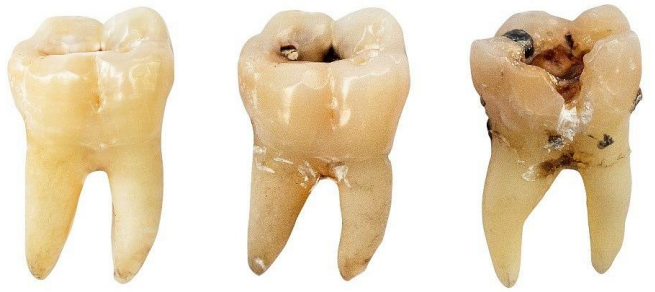
Dental caries



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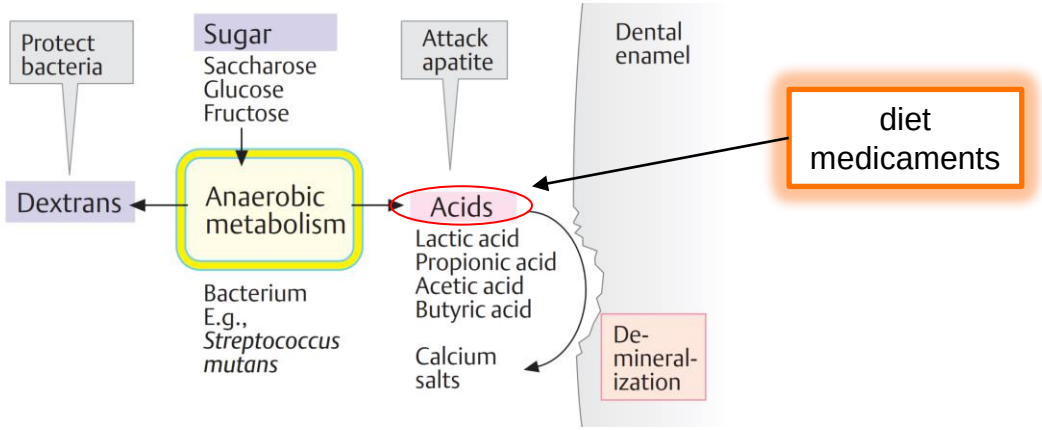
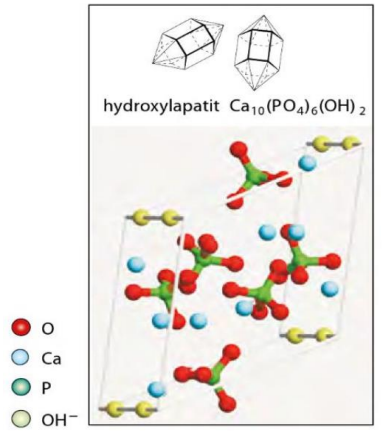
(www.shutterstock.com)

- caries dentium
  - enamel → 95 – 98 % of inorganic matter (apatites - the most important mineral component)
    apatite – cationic complexes = ligands Ca^{2+} and $(PO_4)^{2-}$
    – counter-ions → $Ca_{10}(PO_4)_6CO_3$ (*carbonate apatite*), $Ca_{10}(PO_4)_6(OH)_2$ (*hydroxyapatite*), $Ca_{10}(PO_4)_6F_2$ (*fluoroapatite*)
    dynamic process characterized by alternating periods of demineralization and remineralization of the enamel
    → ↑ demineralization →→ carious lesions progression
  - caries formation → organic acids (bacteria, diet) dissolve the mineral part of the enamel by neutralizing apatite counter-ions (crystal structure disintegration)
    → proteolytic enzymes→ degradation of organic matrix (collagens and proteoglycans)
  - carious lesion → dentin → dentinal tubules → pulp → pulpitis, periodontitis





Color Atlas of Biochemistry
(3rd edition, 2013)

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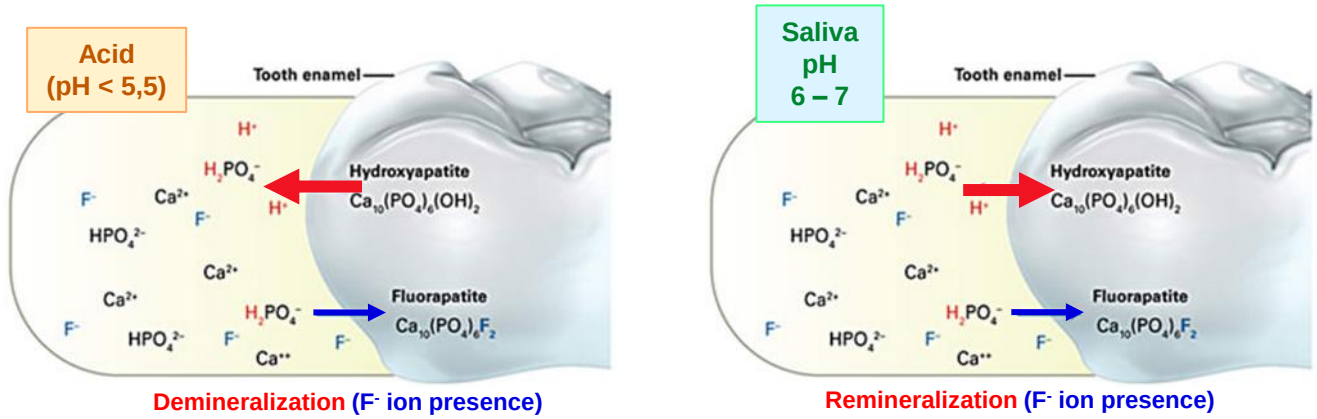
Factors involved in development of caries

- Saliva:
  - complex carioprotective factor
  - maintaining homeostasis
  - physical effect

    saliva flow (washing and lubrication of tissues of oral cavity),
    oral cavity clearance (washing of harmful substances, not adhered microorganisms)
  - components

    gustin (Carbonic anhydrase VI→ buffering capacity), calcium, phosphate, fluoride ions, proteins of specific (IgA, IgG) and non-specific immunity (lysozyme, defensins), other components of immune system
  - ↓ food remnants, ↓ microorganisms, ↓ acidity (dilution, buffer systems - bicarbonate, hydrogen phosphate, proteins), ↑ substances with antibacterial, antifungal and antiviral properties, ↑ balance between re- and demineralization
  - reduced saliva flow ← dehydration, obstruction / hypofunction of salivary glands (complex diseases), medicaments (beta blockers, antidepressants, antihistamines), drugs (methamphetamine, THC)

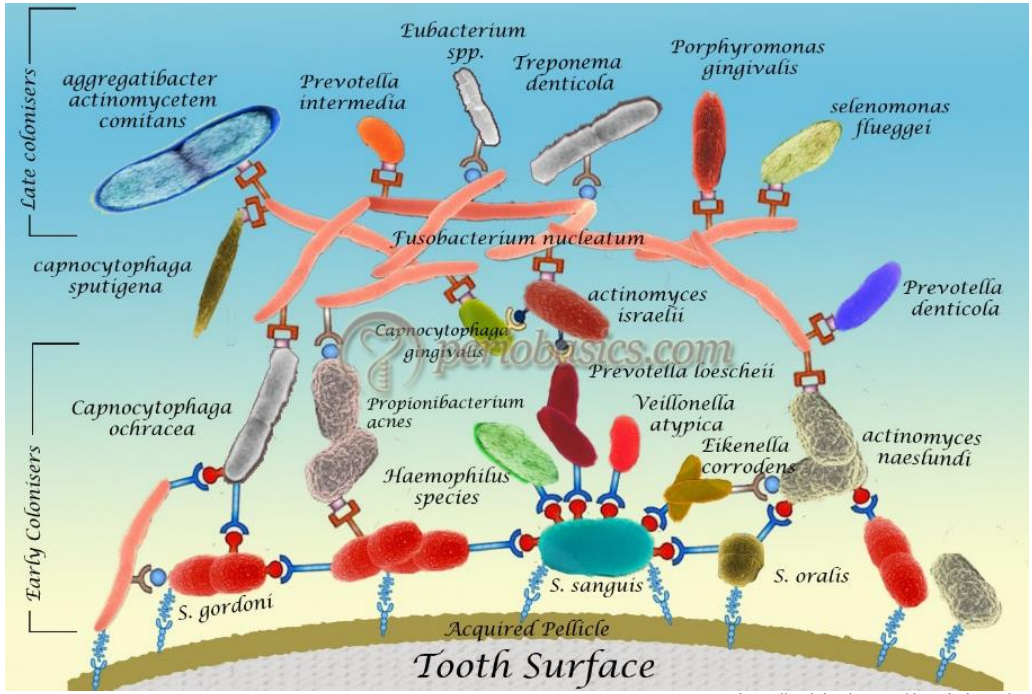
    → promotion of carious lesions formation



www.dentalcare.com/en-us/professional-education/ce-courses/ce410/fluoride-s-mechanism-of-action

Factors involved in development of caries

- Oral microbiome:
  - second largest and diverse (over 700 species of bacteria)
    homeostasis maintenance (competition and displacement of exogenous pathogens → maintaining ecosystem stability), immunomodulation
  - dental plaque = biofilm =
    matrix of extracellular polymeric substances (EPS)
    + aerobic bacteria (*Streptococcus sanguinis*), facultative anaerobes (*S. mutans, S. sobrinus, Lactobacillus* sp.), anaerobes (*Actinomyces* sp., *Veillonella* sp.), fungi (*Candida* sp.)

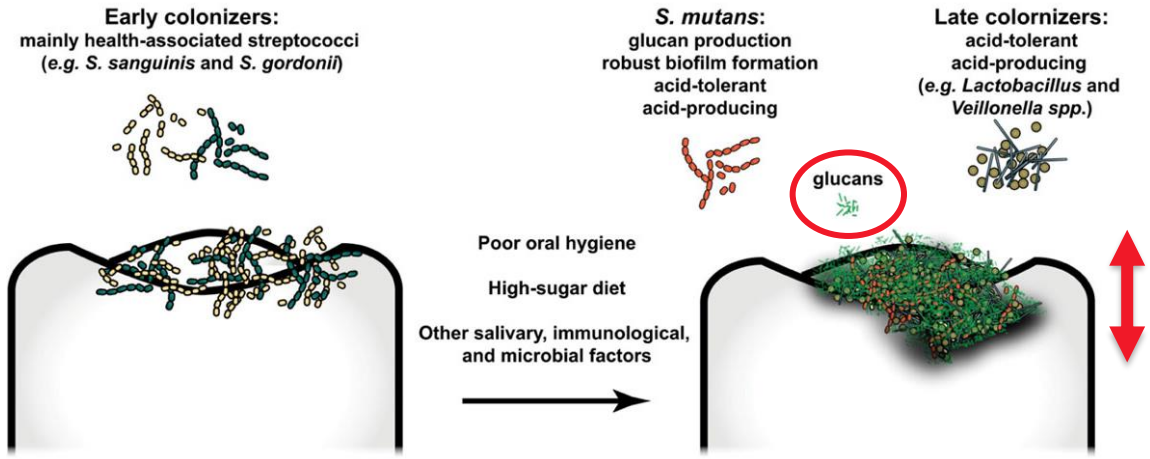


https://periobasics.com/dental-plaque/

  - saliva → proteins with charged surfaces (acidic PRPs, statherin, histatins) → electrostatic interaction with phosphate and calcium ions of apatite
  - → (acquired) pellicle formation (mucins, cystatins, albumin, IgA, IgG, lysozyme, alpha-amylase, carbohydrates, neutral lipids, phospho- and glycolipids, glucosyltransferase)
    → protection against demineralization, partial reduction of microbial adhesion
    → substrate for bacteria→ biofilm formation → **dental plaque**

Factors involved in development of caries

- Dental plaque:
  - problem: dysbiosis of oral microbiome
    disruption of homeostasis



https://doi.org/10.3389/fmicb.2018.03323

  - → ↑ cariogenic species (ferment carbohydrates to organic acids + tolerate low pH environment)
    → prevailing *Streptococcus mutans* a *Streptococcus sobrinus, Lactobacillus* sp., *Candida* sp.

  - factors promoting cariogenic species
    ↓ saliva, ↓ oral hygiene → ↑ plaque thickness; ↑ intake of sugars / acids → acidification; ↓ immunity, inflammation,…

    ↑ dental plaque → lack of oxygen → ↑ anaerobic metabolism→ metabolism of fermentable carbohydrates → organic acids → ↓ pH → demineralization → →→ caries

    S. mutans → dextran (α-1,6-D-glucan) → extracellular insoluble polysaccharide → ↑ protection of bacteria against adverse environment (low pH, antimicrobial factors), ↑ co-adhesion of other species, ↑ plaque adhesion

Factors involved in development of caries

- External factors:

  poor oral hygiene

  poor eating habits (excessive intake of fermentable carbohydrates)

  smoking

  alcohol consumption

  medicaments (salivary glands function impairment, acidification of oral cavity, antibiotics)

  poor access to quality food, drinking water, hygiene supplies, medical care

- Time

Factors involved in development of caries

- Genetic predisposition:
  - complex disease (genetic, epigenetic and exogenous factors)
    - multiple genes
    - genetic heterogeneity – locus heterogeneity (mutations in genes at different loci), allelic heterogeneity (different mutations at the same locus)
    - incomplete penetrance – pathological phenotype is not manifested in all individuals carrying disease-causing gene (positive effects of other alleles or exogenous factors)
    - phenocopy – pathological phenotype is manifested by individuals who are not carrying disease-causing gene
    - high frequency of risk alleles in population
    - ethnic variability (disease-causing genes can vary among populations, variant alleles can have different impact on phenotype in different populations)

    → it is possible to determine only genes (alleles) which act as risk factors → predisposition (predisposing genotype can increase probability of disease development, but does not determine the disease)

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Molecular analysis of saliva

Saliva as a Diagnostic Fluid

Molecular analysis of saliva

- Saliva as a diagnostic fluid
  - saliva collection: non-invasive, easy, painless, repeatable (available material), can be used for all age categories
  - markers of diseases of the oral cavity, systemic diseases (disease diagnostics, monitoring of disease development depending on therapy)
  - **salivaomics** – uses high-throughput technologies (genomics, transcriptomics, proteomics, metabolomics, lipidomics and microbiomics) to study saliva components and identify biomarkers

Table describing examples of commonly analyzed biomarkers in whole mouth saliva; CRP – C-reactive protein; HPLC – high performance liquid chromatography; IC – ion chromatography; LC-MS – liquid chromatography mass spectrometry; MALDI-TOF MS - matrix assisted laser desorption ionization-time of flight mass spectrometry; RT-LAMP – reverse transcriptase loop-mediated isothermal amplification; AOPP – Advanced Oxidation Protein Products; TBARS – Thiobarbituric Acid Reactive Substances; TAC – Total Antioxidant Capacity; FRAS – Free Radical Analytical Systém.

Group of molecules	Biomarkers	Method
Cytokines	Interleukins Tumor-necrotising factor Interferons, Chemokines	Multiplex array Luminex fluoresce technique
Acute phase proteins	CRP	ELISA
Inflammatory proteins	Myeloperoxidase Neutrophil elastase	ELISA
Antibodies	Anti-HIV, Anti-RO Anti-La	RT-LAMP ELISA
Hormones	Testosterone Estradiol Cortisol	ELISA HPLC
Enzymes	Amylase Lysozyme	ELISA MALDI-TOF MS
Proteins-polypeptides	Immunoglobulin A Lactoferrin	MALDI-TOF MS ELISA
Nucleic acids	DNA methylation DNA mutations microbiome	Microarray Sequencing
Vitamins	25(OH)D(3) vitamins A, C, E	LP-MC ELISA
Ions	Na^+, K^+, Mg^{2+}, Cl^-, Ca^{2+}, NH_3^-	IC
Oxidative stress	AOPP TBARS	Spectrophoto- Spectrofluorometric methods ELISA
Antioxidant status	TAC FRAS	Spectrophoto- Spectrofluorometric methods ELISA

Janšáková et at.,
Klin. Biochem. Metab., 26 (47), 2018, No. 1, p. 21–26

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Molecular analysis of saliva

- Saliva as a diagnostic fluid
  - worse reproducibility of results ← high variability
    - technical (sampling, processing, method used)
    - interindividual
    - biological (influence of the condition of the oral cavity, systemic diseases (Sjögren's syndrome), xerostomia (medicaments),
    - ↓↓↓ analyte concentration in comparison with serum → ↑ saliva sample volume, detection limit, depletion of abundant proteins

→ standardization
- → correlation of protein markers to saliva total protein concentration
- → standardization of used methods

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Antibodies	Anti-HIV, Anti-RO Anti-La	RT-LAMP ELISA
Hormones	Testosterone Estradiol Cortisol	ELISA HPLC
Enzymes	Amylase Lysozyme	ELISA MALDI-TOF MS
Proteins-polypeptides	Immunoglobulin A Lactoferrin	MALDI-TOF MS ELISA
Nucleic acids	DNA methylation DNA mutations microbiome	Microarray Sequencing
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Molecular analysis of saliva

- Saliva as a diagnostic fluid – dental caries susceptibility

  - <u>point-of-care testing (chair-side diagnostic kits):</u>

  - physical properties: volume, flow rate, viscosity, consistency
  - pH and buffering capacity of saliva

    commercial kits (visual or colorimetric detection)

  - lactate

    commercial kits (colorimetric detection)

  - determination of cariogenic bacteria *S. mutans* and *Lactobacillus* sp.

    commercial kits (immunochromatographic detection of antigen, cultivation kit)

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Molecular analysis of saliva

- Saliva as a diagnostic fluid – dental caries susceptibility
  - saliva protein biomarkers associated with caries susceptibility:

    ↑ saliva total protein, total antioxidant capacity (TAC)
    ↑ alpha-amylase, mucins (MUC1 a MUC5B)

    ↓ arginine deiminase system, albumin, proteinase 3, PRP1/3, statherin, histatin 1
    ↓ concentrations of calcium and bicarbonate ions
    ↓ urease activity

  - saliva protein biomarkers associated with susceptibility to early childhood caries:

    ↑ PRPs, histatins, IgA, IgG

    ↓ statherin

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Molecular analysis of saliva

- Saliva as a diagnostic fluid – examples of disease biomarkers

  - autoimmune diseases
    Sjögren's syndrome - α-amylase, carbonic anhydrase VI, lactoferrin, β2-microglobulin
  - neurodegenerative diseases
    Alzheimer's disease - total tau protein, phosphorylated tau protein, amyloid-β and alpha-synuclein
  - genetic diseases
    cystic fibrosis – Ca, PO_4^{2-}, Na, K, Cl, ↓ saliva volume, urea, uric acid, prostaglandin E_2
  - cancer
    squamous cell carcinoma – IL-8, IL-6, IL-1*β*, IL-4, IL-1, VEGF, HER2, tissue polypeptide antigen (TPA) and EGFR, LDH, N-α-acetyltransferase 10 protein (Naa10p), carcinoembryonic antigen (CEA) protein, serum basic fibroblast growth factor (bFGF), transferrin, cyclin D, Maspin, specific mRNAs …….
    breast cancer - HER2/neu (C-erbB-2), VEGF, EGF, specific mRNAs, autoantibodies against HER2 and MUC-1
    pankreas cancer – transcriptomic markers of mRNAs (*KRAS, MBD3L2, ACRV1* a *DPM1*), specific miRNA, lactoperoxidase, Cyclophilin B, Cytokeratins (14, 16 a 17)
  - endocrine diseases
    Cushing's syndrome and Addison's disease - cortisol
    sex hormones - polycystic ovary syndrome, menopause / andropause, anovulation, hypogonadism, hyperestrogenism

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Molecular analysis of saliva

- Saliva as a diagnostic fluid – examples of disease biomarkers

  - cardiovascular diseases
    CK-MB, myoglobin, troponin I, myeloperoxidase, inflammation markers (CRP, TNF-α, MMP-9), cellular adhesion molecules (soluble CD40 and ICAM-1)
  - metabolism
    diabetes mellitus type 2 - 1,5-anhydroglucitol, CRP, leptin, IL-6, TNF-α
  - infectious diseases
    HIV - antibodies against HIV
    viruses - IgM / IgA antibodies, viral RNA
    Candidiasis, amebiasis - *Candida* sp., *Entamoeba histolytica* (antibodies)
    Hepatitis - DNA of HBV virus
    Peptic ulcer disease, gastritis - *Helicobacter pylori* (IgG antibodies, *H. pylori* DNA)
  - allergy
    food allergies - IgE and IgG_1
  - periodontitis
    bacteria of ‚red complex' (*Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola*), *Aggregatibacter actinomycetemcomitans*
    biomarkers - MMP-8, MMP-9, osteoprotegerin, ALT, α-amylase

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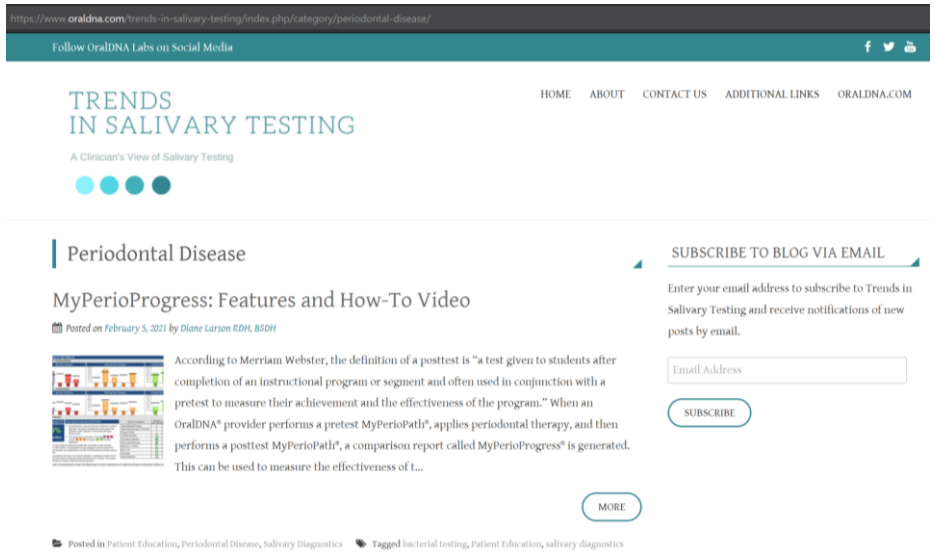
Molecular analysis of saliva

– Saliva as a diagnostic fluid
  – diseases biomarkers



https://www.oraldna.com/trends-in-salivary-testing/

Table 2. Examples of Commercially Marketed Oral Fluid Tests[18, 22-25]

Test (Manufacturer)	Intended Use
23andMe® Health + Ancestry	Detect genetic health risks (e.g., BRCA1/BCRA2 status), carrier status, physical traits, and wellness features
Alert 2™ (OralDNA Labs)	Combine MyPerioPath® and MyPerio ID® IL-6
Celsus One™ (OralDNA Labs)	Evaluate genetic markers related to inflammatory response
DNA DrugMap™ (OralDNA Labs)	Detect drug metabolizer status
Intercept® i2™, Intercept® i2he™, and Intercept® Oral Fluid Drug Test (OraSure Technologies, Inc.)	Detect drugs of abuse (e.g., marijuana, cocaine and opiates)
MyPerio ID® IL-6 or IL-1 (OralDNA Labs)	Detect genetic polymorphisms associated with increased genetic risk for severe periodontal disease
MyPerioPath® (OralDNA Labs)	Evaluate the number and concentration of bacteria implicated in periodontitis
OraMark™ Test (Vigilant Biosciences)	Detect CD44 and total protein associated with oral cancer
OraQuick® In-Home HIV Test, OraQuick® HIV Self Test, and OraQuick ADVANCE® Rapid HIV-1/2 Antibody Test (OraSure Technologies, Inc.)	Detect HIV-1 and/or HIV-2 antibodies in oral fluid
OraRisk HPV® (OralDNA Labs)	Screening tool to identify the type(s) of oral HPV present
OraRisk HSV® (OralDNA Labs)	Detect HSV-1 or HSV-2 present in the oral cavity
OraRisk® Candida (OralDNA Labs)	Detect and identifies all common species of *Candida* present in the oral cavity
OraRisk® CT/NG (OralDNA Labs)	Detect the presence of *Chlamydia trachomatis* and/or *Neisseria gonorrhea* in the oropharynx
OraSure® HIV-1 (OraSure Technologies, Inc.)	Detect HIV-1 antibodies in oral fluid
Q.E.D. Saliva Alcohol Test (OraSure Technologies, Inc.)	Detect alcohol in oral fluid
SaliMark OSCC® (PeriRx, LLC)	Detect increased levels of certain mRNAs associated with increased risk of oral cancer

https://www.ada.org/en/member-center/oral-health-topics/salivary-diagnostics

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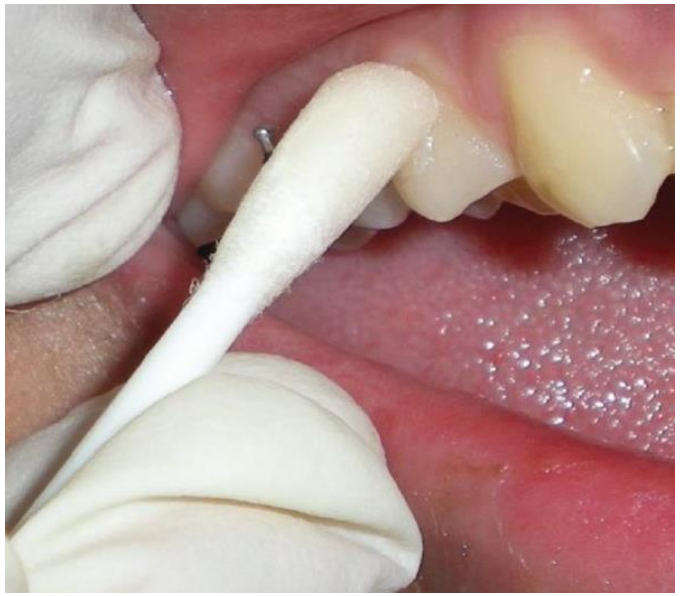
Molecular analysis of oral microbiome



https://www.jorthodsci.org/viewimage.asp?img=JOrthodontSci_2014_3_4_125_143233_f6.jpg

Molecular analysis of OM

- Oral microbiome
  - biomarker of dental caries and periodontitis
  - polymicrobial infection → microbiological profile of patients

→ disease susceptibiity

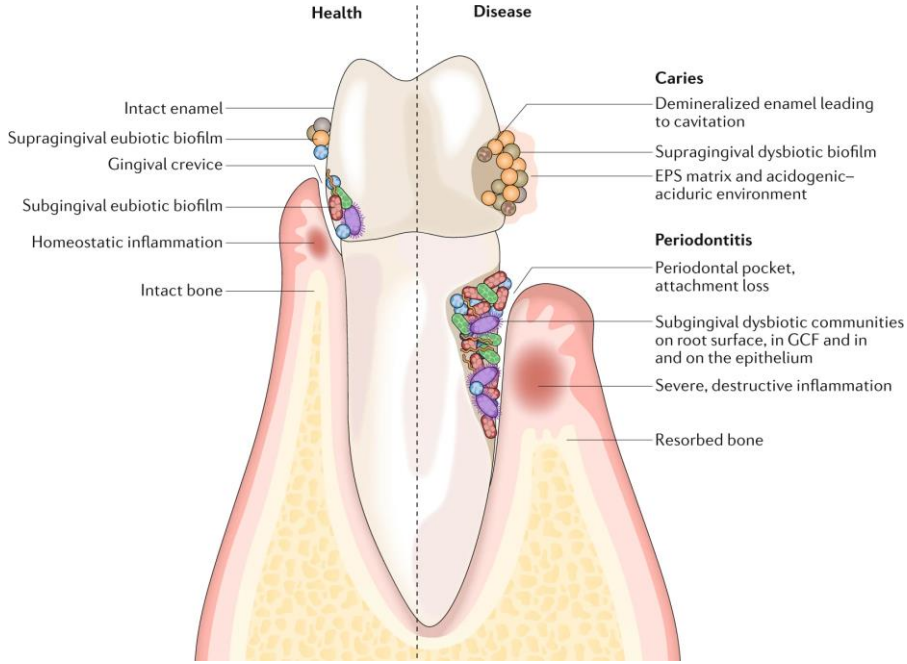
→ early diagnosis of disease (before onset of symptoms)

→ monitoring the course of disease and effectiveness of treatment (shift of microbiota from dysbiosis to eubiosis), targeted treatment

→ an effective tool for disease prevention (evidence of patient dental care)

→ development of new therapeutic approaches, personalized dental treatment

→ research - an effort to fully characterize a "healthy" microbiome (Which components of the microbiome should be monitored to evaluate the return of the microbiome from dysbiosis to a state compatible with health? Is it sufficient to monitor only selected key species or is it necessary to use multispecies assays?)



Microbial colonization occurs on all available surfaces, and microorganisms can also penetrate epithelial tissues and cells. The microbiota assembles into biofilm communities on the abiotic and biotic surfaces. In health (left), eubiotic biofilms maintain a homeostatic balance with the host. In disease (right), caries and periodontitis ensue when biofilms become dysbiotic, resulting in increased levels and duration of low pH challenge and the induction of destructive inflammatory responses, respectively. EPS, extracellular polymeric substance; GCF, gingival crevicular fluid.
https://doi.org/10.1038/s41579-018-0089-x

Molecular analysis of OM

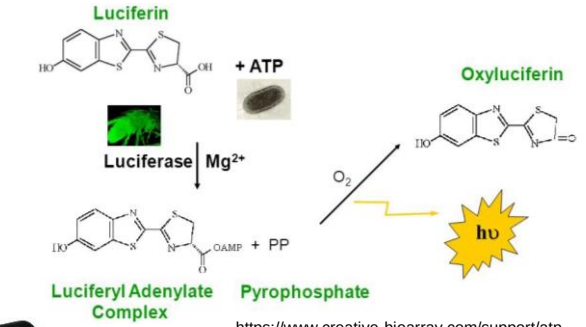
- Oral microbiome – methods of analysis
  - point-of-care testing (chair-side diagnostics) :

    dental caries risk – device **CariScreen Susceptibility Testing Meter** (Oral BioTech LLC) – after wiping the plaque off the tooth surface by special brush a reaction of ATP occurs nad bioluminescence is measured by the device → bacterial activity *S. mutans*

  - analysis in laboratory

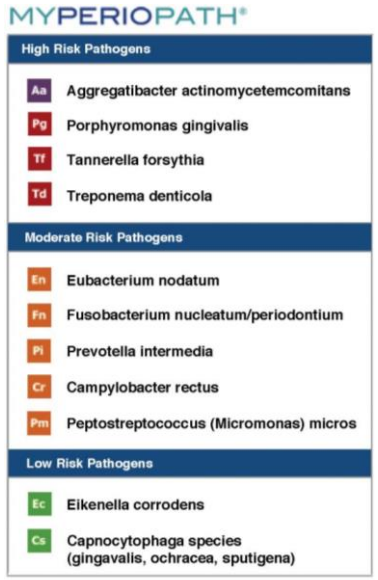
    commercial kits – an example

    periodontitis – kit MyPerioPath® (OralDNA Lab), saliva test, testing presence and amount of 11 bacteria species that promote periodontitis onset and development (quantitative real-time PCR analysis)



https://www.creative-bioarray.com/support/atp-cell-viability-assay.htm

https://carifree.com/product/pro-cariscreen-testing-meter/



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Molecular analysis of OM

- Oral microbiome – methods of analysis
  - sampling

    → microbial communities present on different parts of the oral cavity (saliva, tongue, palate, buccal mucosa, tooth surfaces, gums, supra- / subgingival plaque, tonsils, throat) show an overall similarity, but with small differences

    → sample collecting from a specific site / rinsing of the whole mouth

Molecular analysis of OM

- Oral microbiome – methods of analysis
  - microbiological cultivations
    → OM is one of the most complex microbial communities in the human body → some species have not yet been cultivated
  - 16S rRNA sequencing
    → sequencing of the conserved gene for 16S rRNA, the most common method, taxonomic data only
  - whole genome shotgun sequencing (WGS)
    → DNA is randomly cut and then subjected to Sanger sequencing or NGS; a tool for metagenomic analysis; not only taxonomic data but also biological functional profiles of the microbial community
  - qPCR
    → not only the gene for 16S rRNA, but also other genes; also allows quantification
  - ELISA test
    → antigens, *P. gingivalis*

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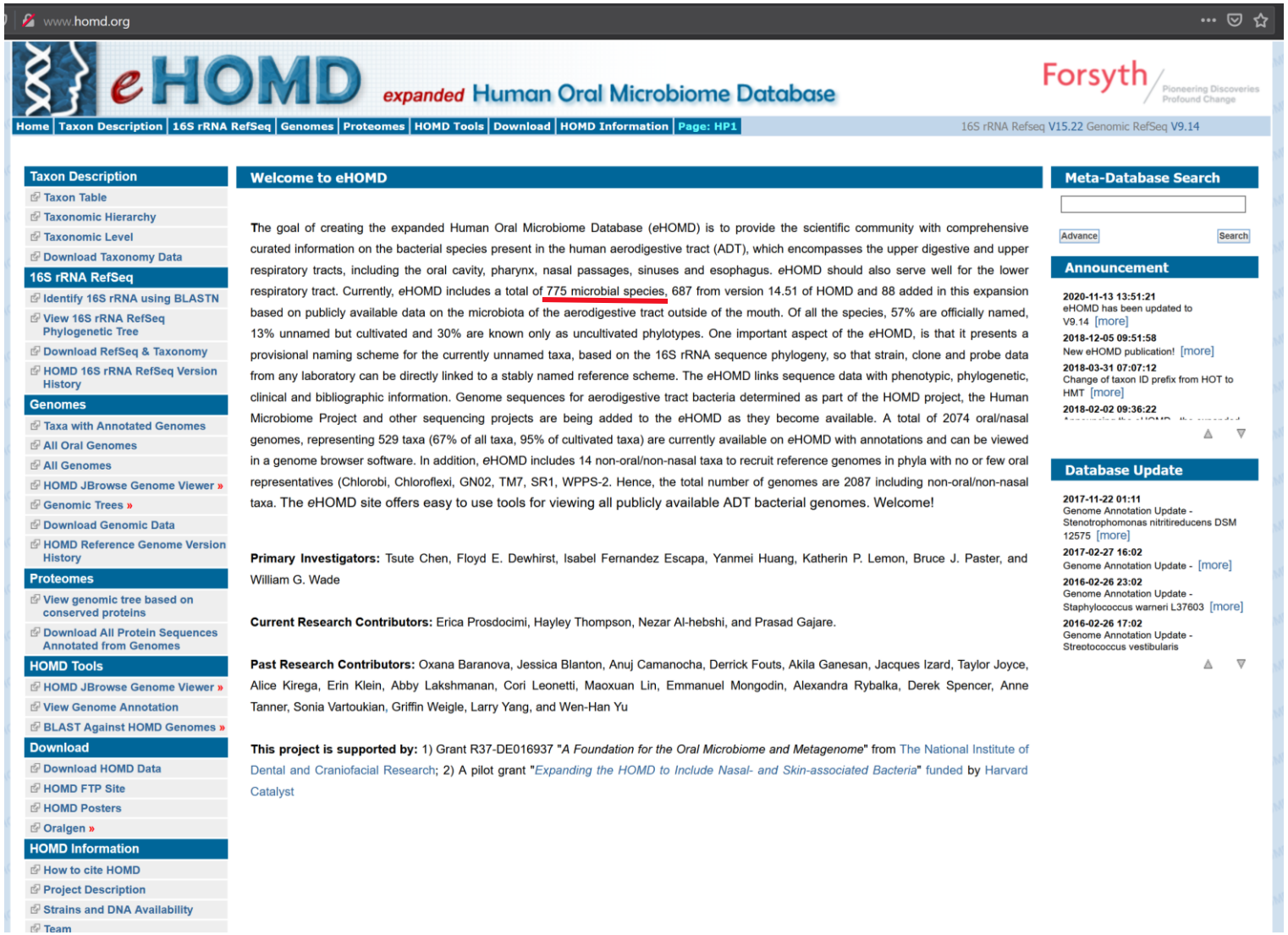
Molecular analysis of OM

- Oral microbiome
  - database



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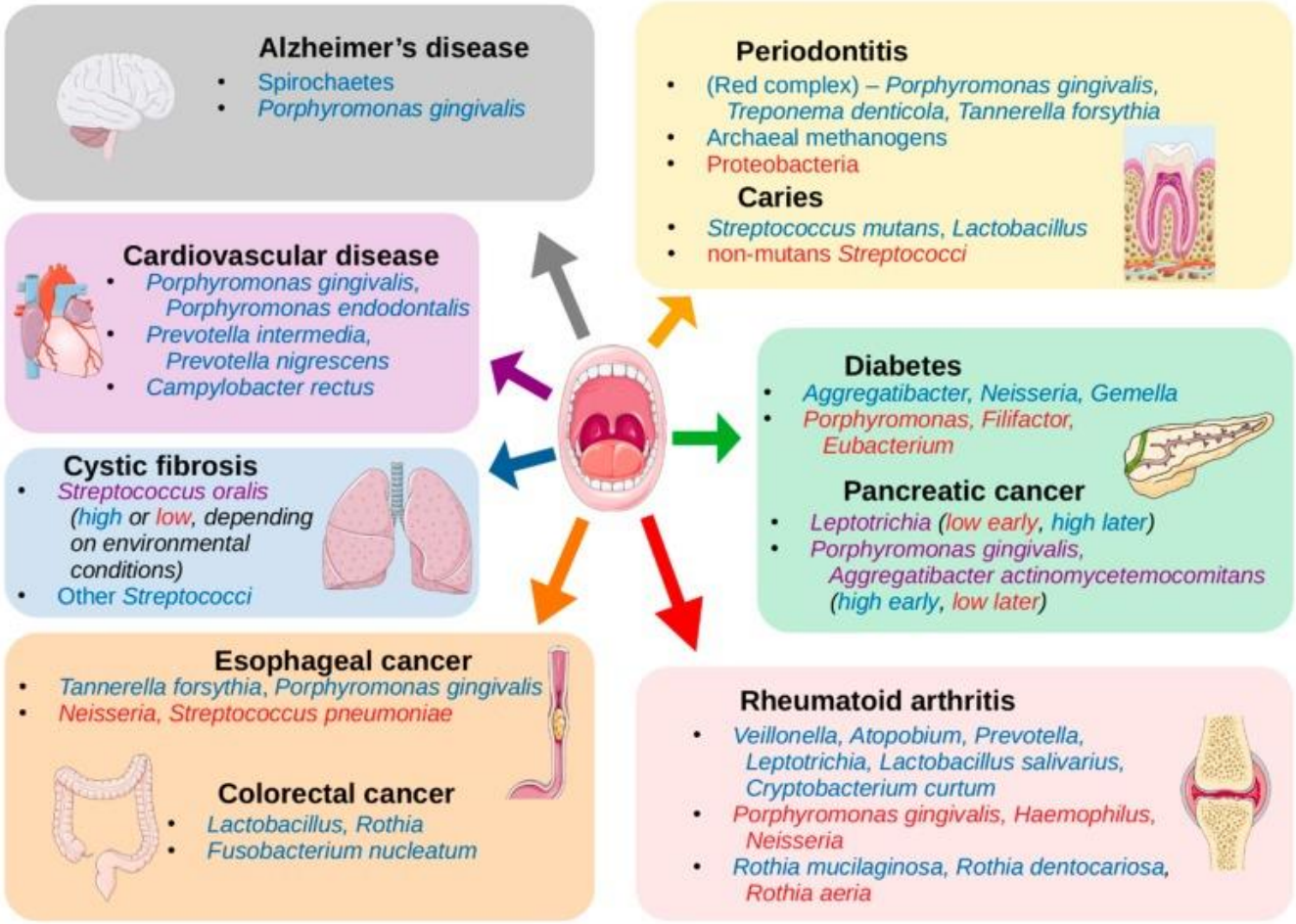
Molecular analysis of OM

dysbiosis as a non-invasive biomarker

- Oral microbiome
  - a potential biomarker of systemic diseases



Oral and systemic diseases associated with the oral microbiome. A representation of the associations found between diseases with increases or decreases of the abundances of organisms in the oral cavity. Organisms listed in blue have been shown to be increased in abundance in the oral cavity in individuals presenting with the noted disease, and organisms listed in red have been shown to be decreased. Those in purple may be either increased or decreased depending on the conditions or progression of the disease.

doi:10.3390/microorganisms8020308

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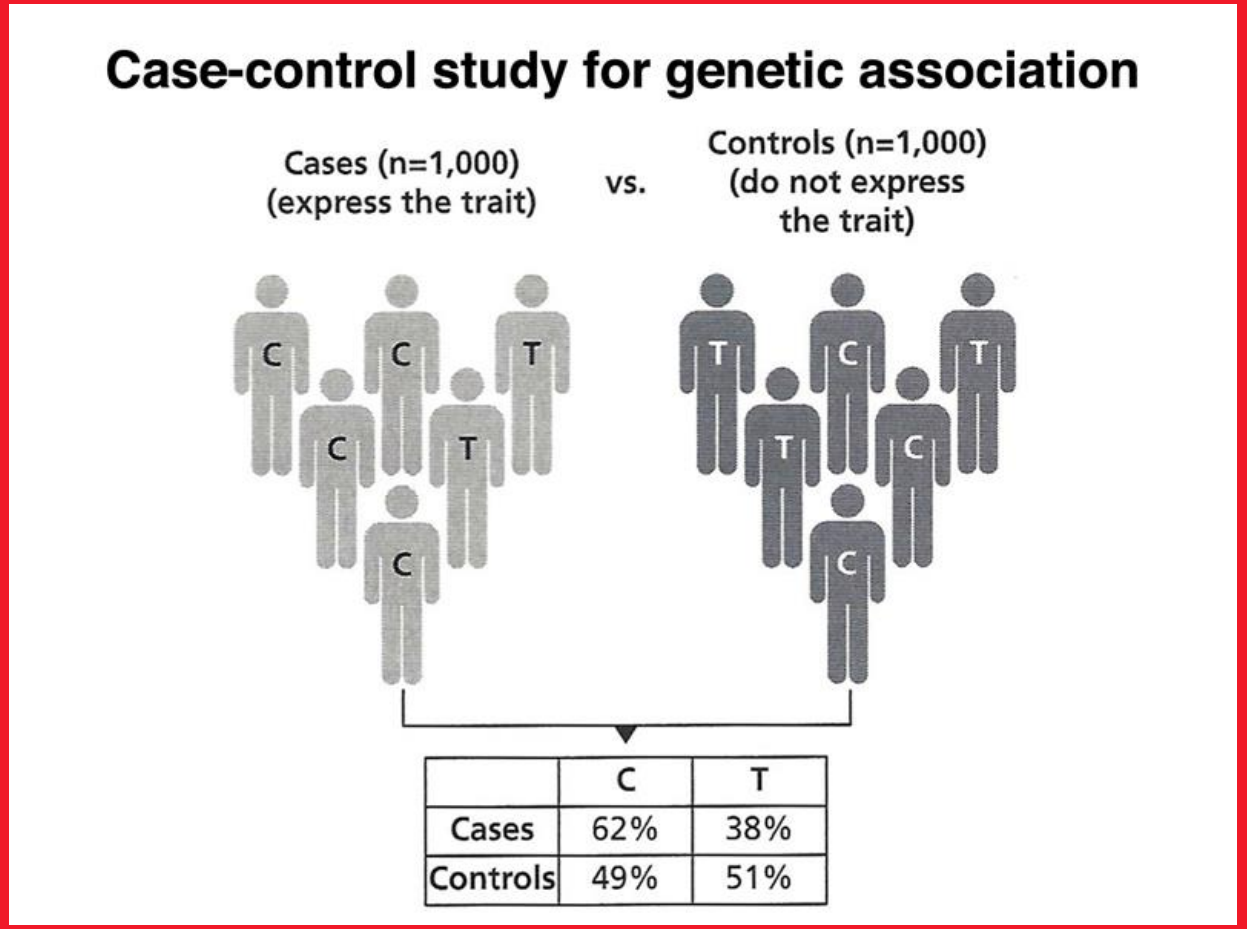
Genetic association studies - candidate gene approach (case-control studies)



	C	T
Cases	62%	38%
Controls	49%	51%

http://www.discoveryandinnovation.com/BIOL202/notes/lecture25.html

Genetic association studies

- Candidate genes
  - Selection of suitable candidate genes

    in general, based of known biological, physiological and/or functional relevance to the disease

    search for new potential genes (alleles) in the whole genome (GWAS, QTL - quantitative trait locus)

  - Suitable candidate genes for caries association studies

    genes participating in tooth development and affecting its morphology
    genes related to immune response
    genes related to production and composition of saliva
    genes related to taste preferences

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Genetic association studies

- Candidate genes
  - Selection of alleles (polymorphisms)

    SNP, CNV, VNTR

    based on studies already performed in other populations, GWAS, QTL

    minor allele frequency is sufficient in a given population
    (↓ frequency of allele in the population ← ↑ number of cases / controls)

    linkage disequilibrium among SNPs → tagSNP

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Genetic association studies

- Methods of genotyping
  - selection of an appropriate methodical approach

    number of polymorphisms to be determined

    total number of samples to be genotyped

    quality of DNA sample (genomic DNA - blood, saliva, buccal swab)

    costs - equipment, chemicals, consumables

    availability of commercial genotyping services

Genetic association studies

Comparison of methods used for mannose-binding lectin gene (*MBL2*) genotyping.

	Allele -Specific PCR (AS-PCR)	ARMS [3]/Double ARMS [3] (+ Multiplex Allele -Specific PCR)	PCR and Restriction-Fragment Length Polymorphism (PCR-RFLP)	Commercial TaqMan Assay [5]	High-Resolution Melt Analysis (HRMA)	Commercial INNO-LiPA MBL2 kit (Reverse PCR-SSOP)	Pyro-Sequencing	Sanger Sequencing	*MBL2* SNaPshot Assay
principle of allele discrimination/detection	PCR with a primer specific for one allele	PCR with primers specific for both alleles	allele-specific enzymatic cleavage of PCR amplicon	allele-specific hybridization of fluorescently labelled probe	temperature-dependent allele-specific hybridization of fluorescently labelled probe	hybridization of biotinylated PCR product with membrane immobilized sequence-specific oligonucleotide probes	chemiluminiscence-based detection of nucleotides during sequencing-by-synthesis reaction	detection of the sequence of an oligonucleotide amplified in PCR with fluorescently labelled dideoxyribonucleotides	allele-specific SBE by a single fluorescently labelled dideoxyribonucleotide (minisequencing)
post-PCR analysis	yes	yes	yes	no	no, when real-time PCR thermocycler is used	yes	no	yes	yes
analysis time	2 h [2]	2–3 h [2]	2 h + 1–3 h [4]	1–2 h [6]	1–1.5 h + 2–8 min. [8]	3–4 h	2–3 h	6–7 h	5–6 h
number of work steps	2 (PCR, gel analysis)	2 (PCR, gel analysis)	4 (PCR, gel analysis, RFLP, gel analysis)	1 (real-time PCR)	1 (when real-time PCR thermocycler is used for PCR and subsequent melting temperature analysis)	9 (PCR, gel analysis, denaturation, hybridization, 2 washing steps, 3-step color development)	4 (PCR, gel analysis, purification, pyrosequencing)	5 (PCR, enzymatic cleaning, sequencing reaction, purification, analysis on sequencer)	5 (PCR, enzymatic cleaning, SBE reaction, enzymatic cleaning, analysis on sequencer)
automatic analysis	no	no	no	yes	yes	no	yes	yes	yes
number of analyses for complete *MBL2* haplogenotype [1]	12	6	6	6	5	1	4 [9]	2	1
number of oligonucleotide primers + labelled primers/probes for complete *MBL2* haplogenotype [1]	24 primers	15 primers	6 primers	6 TaqMan assays (12 primers + 12 TaqMan probes)	10 primers + 5 TaqMan probes	4 primers	8 primers + 4 biotinylated primers	2 primers	8 primers
estimated cost of analysis of whole haplogenotype [1]	1 USD	1 USD	2 USD	2 USD	1 USD	product was discontinued	2 USD	5 USD	1.50 USD
input amount of template DNA	20–200 ng	20–200 ng	50–500 ng	1–20 ng	10–20 ng	200–500 ng	10–100 ng	10–250 ng	10–100 ng
assay robustness	low	low	low–medium	medium–high	high	low–medium	medium	medium–high	medium–high
special equipment requirement	-	-	-	real-time PCR thermocycler	real-time PCR thermocycler or fluorescence scanning/detection system	water bath with shaking platform, aspiration apparatus	vacuum prep workstation, pyrosequencing machine	automated DNA sequencer	automated DNA sequencer
SNP genotyping throughput	low	low	low	high	high	medium	high	high	high
software for automatic allele calling	no	no	no	yes (SDS software, SNPman program) [7]	yes real-time PCR instruments with HRMA compatible software with genotype auto-calling function	no	no	yes (Mutation Surveyor, GeneMarker, Minor Variant Finder Software, SeqScape™ Software, Variant Reporter™ Software) [10]	yes (GeneMapper, GeneMarker) [11]
Ref.	[41]	[30,42]	[43,44]	[27]	[28]	[29]	[45]	[37]	-

[1] rs11003125, rs7096206, rs7095891, rs5030737, rs1800450 and rs1800451; [2] Analysis time depends on polymerase chain reaction (PCR) length and gel concentration; [3] ARMS–amplification refractory mutation system; [4] Separation time depends on gel concentration and the length of cleaved fragments; [5] TaqMan® SNP Genotyping Assays (Applied Biosystems), probes: C__11876879_10 (rs11003125), C__27858274_10 (rs7096206), C__26813436_10 (rs7095891), C___2336610_10 (rs5030737), C___2336609_20 (rs1800450) and C___2336608_20 (rs1800451); [6] depends on number of cycles; [7] Sequence Detection Software (SDS) by Applied Biosystems™ (www.thermofisher.com (accessed on 2 November 2020)), SNPman program by Konopac et al. [46]; [8] Time of temperature melt analysis depends on a temperature range and thermal ramp rate of the instrument; [9] Due to the proximity of the three single nucleotide polymorphisms (SNPs) in exon 1 only one analysis is needed to determine these SNPs; [10] Mutation Surveyor® and GeneMarker® by SoftGenetics® (https://softgenetics.com (accessed on 2 November 2020)). Minor Variant Finder Software, SeqScape™ Software and Variant Reporter™ Software by Applied Biosystems™ (www.thermofisher.com (accessed on 2 November 2020)); [11] GeneMapper™ by Applied Biosystems™ (www.thermofisher.com (accessed on 2 November 2020)), GeneMarker® by SoftGenetics® (https://softgenetics.com (accessed on 2 November 2020)). Single-base extension (SBE). Mannose-binding lectin gene (*MBL2*). Reverse hybridization with membrane-immobilized sequence-specific oligonucleotide probes (reverse PCR-SSOP).

Genetic association studies

- Genotyping methods
  - **PCR+RFLP** (restriction fragment lenght polymorphism)
    - → PCR amplification followed by specific restriction digestion

polymorphism is part of a palindromic sequence

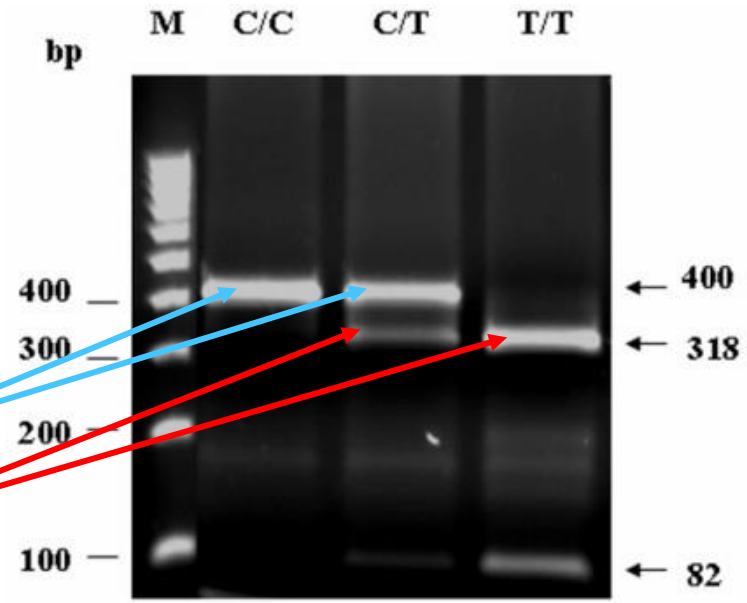
***Hinf* I**

5´… G A N T C … 3´

3´… C T N A G … 5´

I. alela → 5´… G A G C C … 3´

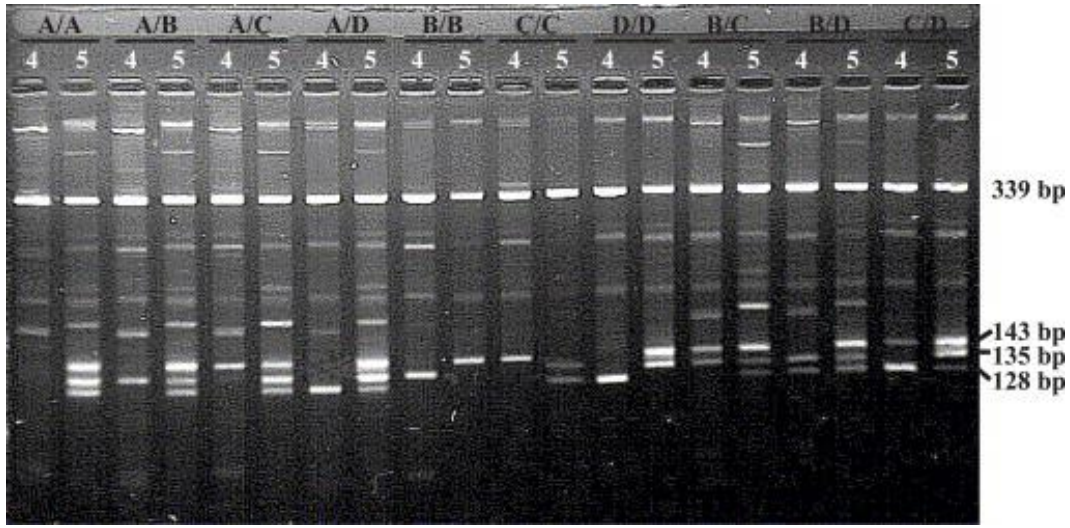
II. alela → 5´… G A G T C … 3´



Huang, BCM Cancer (2008)

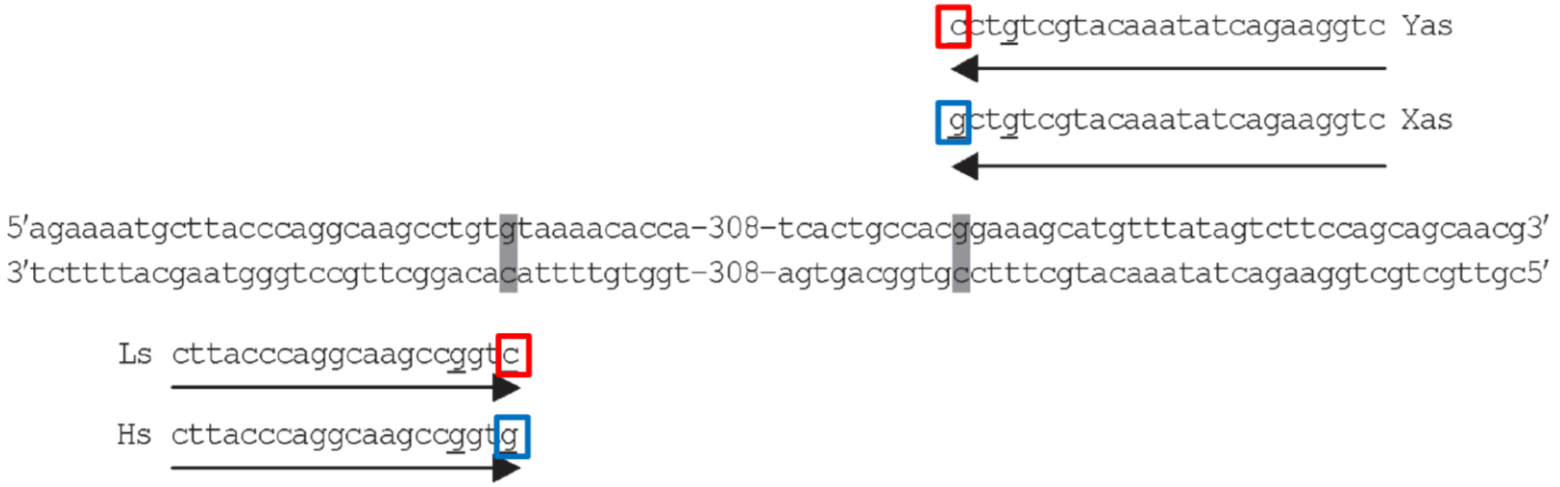
Genetic association studies

- Genotyping methods
  - **allele-specific PCR**



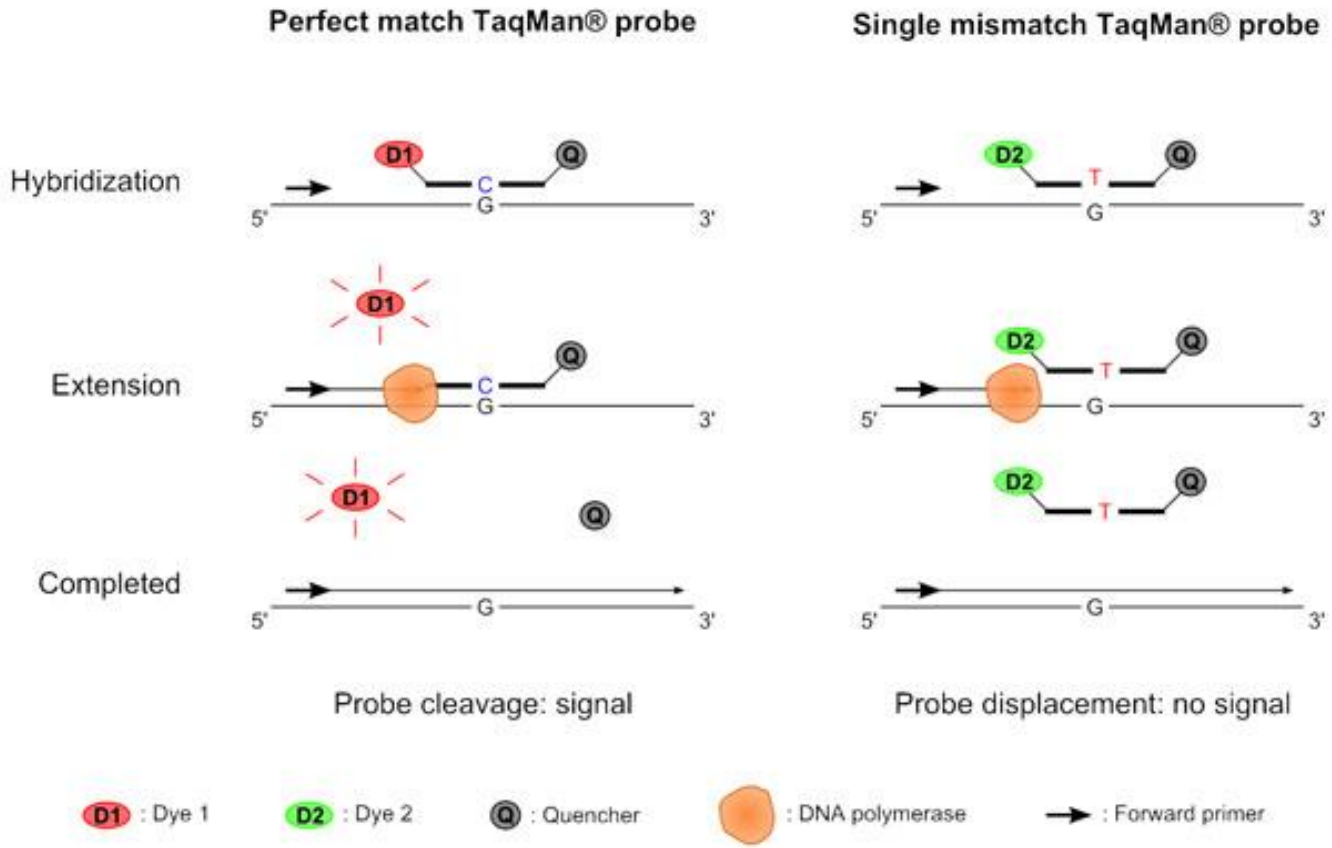
https://doi.org/10.1016/j.jim.2004.10.007

→ primers that are specific for particular allele
→ if the allele is present → amplification product is generated → detection

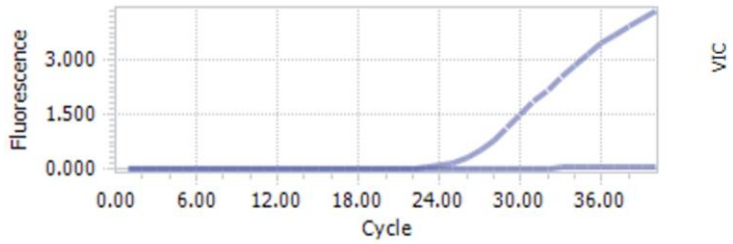


Genetic association studies

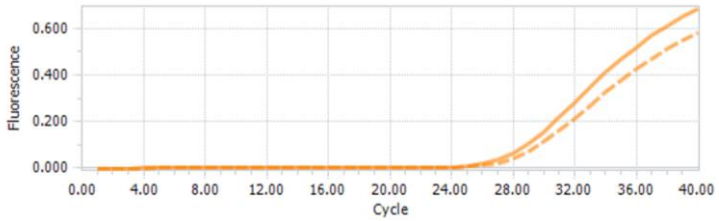
- Genotyping methods
  - **real-time PCR** → fluorescently labeled hybridization probes
    →commercial TaqMan probes

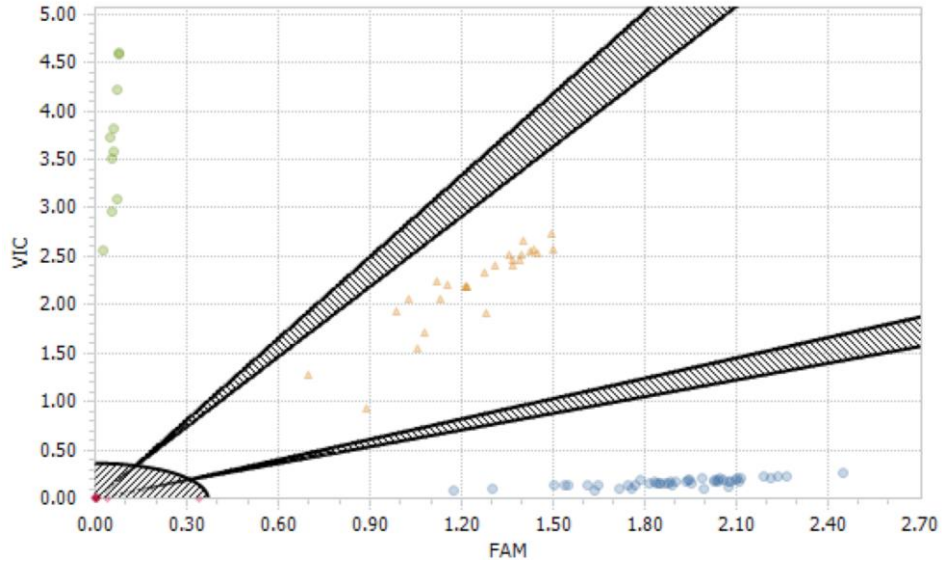


homozygote



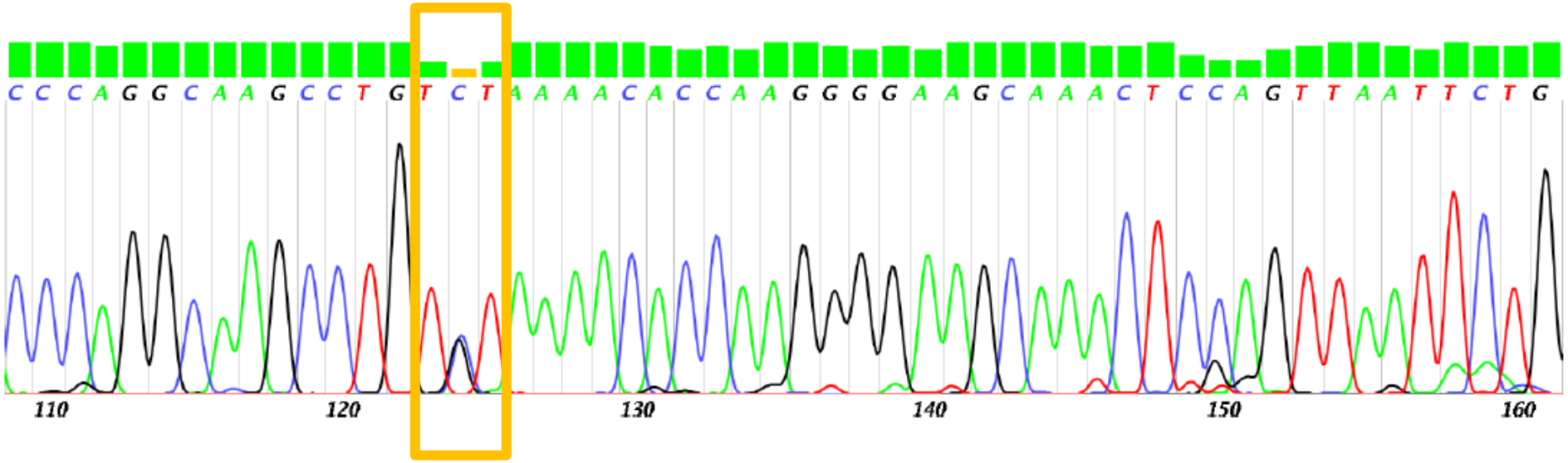
heterozygote





Genetic association studies

- Genotyping methods
  - **Sanger sequencing** → sequence of a part of DNA with polymorphism



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Genetic association studies

- Genotyping methods
  - Single Nucleotide Polymorphism Detection with the iPLEX® Assay and the MassARRAY® System

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Genetic association studies

- Genes that have been associated with increased risk of dental carries
  - Proteins involved in development of enamel

AMELX – Amelogenin gene

ENAM – Enamelin gene

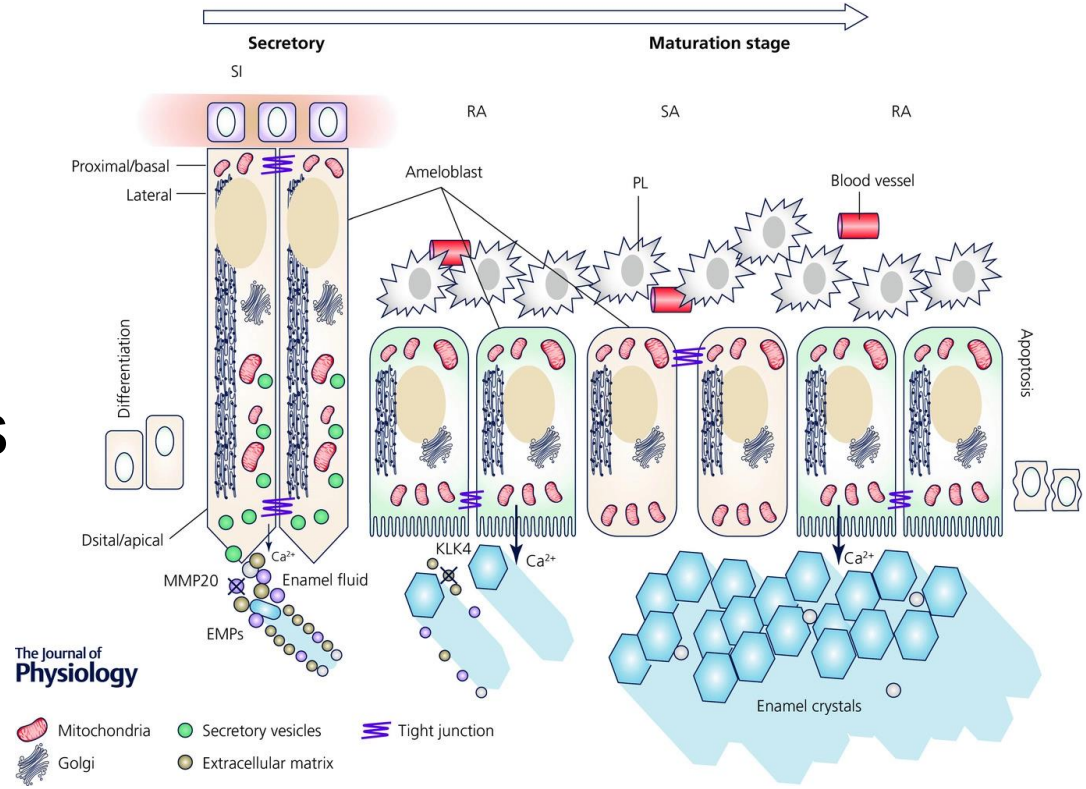
TUFT1 – Tuftelin gene

KLK4 – gene encoding Kallikrein-related peptidase 4

AMBN – Ameloblastin gene

TFIP11 – gene encoding Tuftelin-interacting protein 11

MMPs (*MMP20*) – genes encoding Matrix Metalloproteinases



Schematic diagram of histological changes in amelogenesis. The histological development of enamel crystals goes hand in hand with changes in ameloblast morphology. Undifferentiated epithelial cells receive signals to transform into secretory ameloblast cells of some 75 μm tall and ~5 μm in diameter with a specialized distal cell process (Tomes' process) which plays an important role in matrix exocytosis. These same cells will retransform into shorter cells (~35 μm tall) during maturation devoid of the Tomes' process. In maturation stage, ameloblasts undergo cyclical changes from a cell with a distal ruffled border, the ruffled-ameloblast (RA), to a cell with a smooth distal border, the smooth-ameloblast (SA). Tight junctions are found at the basal and apical pole of secretory ameloblasts. The apical or distal pole is closest to the enamel crystals. In RA cells, tight junctions are found only at the apical pole but in SA cells they are located at the basal pole. Organellar distribution differs in cells at each stage (see text for details). SI = stratum intermedium, PL = papillary layer, EMPs = enamel matrix proteins. MMP20 and KLK4 are the main proteases in AMEL processing. See also organellar distribution at each stage.

https://doi.org/10.1113/JP272775

https://doi.org/10.1016/j.sjbs.2020.11.071

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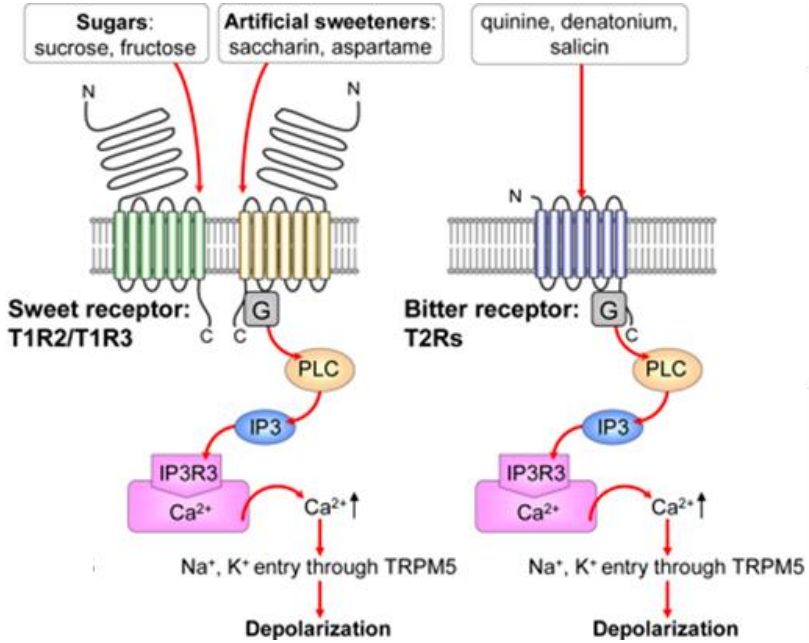
Genetic association studies

- Genes that have been associated with increased risk of dental carries



https://doi.org/10.3390/s100403411

- Taste receptors → associated with heightened preference for sweet taste → ↑ sugar intake

TAS2R38 – gene encoding Taste receptor 2 member 38 → G protein-coupled receptor, responsible for sensitivity to bitter taste

TAS1R2 / TAS1R3 – genes encoding Taste receptor 1 member 2 and 3 → G protein-coupled heterodimeric receptor, responsible for sensitivity to sweet taste

https://doi.org/10.1080/00016357.2020.1832253

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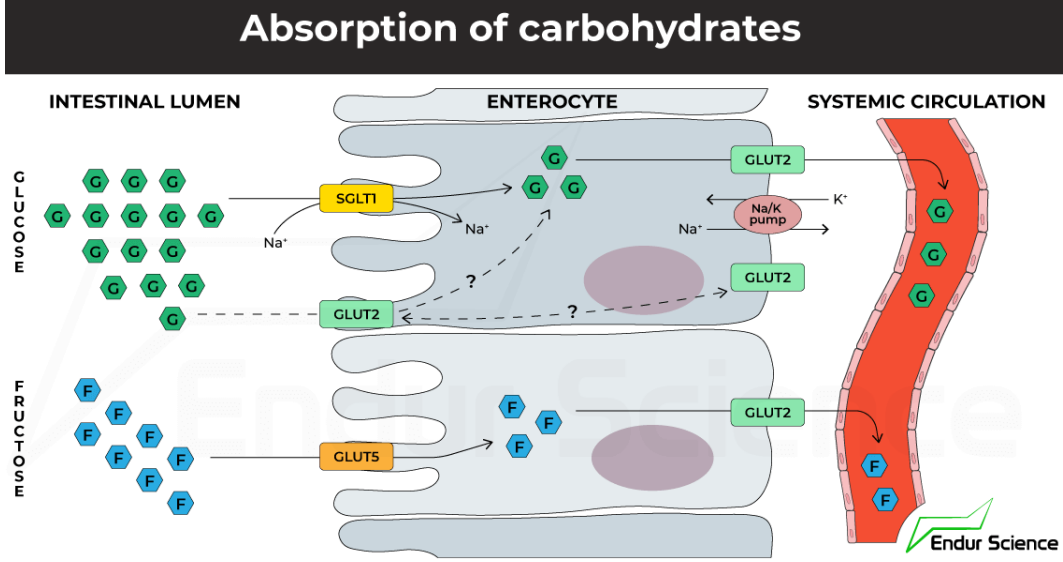
Genetic association studies

- Genes that have been associated with increased risk of dental carries



https://doi.org/10.3390/s100403411

- Glucose transporter → associated with heightened preference for sweet taste → ↑ sugar intake

GLUT2 – gene encoding Glucose transporter 2 – required for glucose-stimulated insulin secretion (pancreatic β-cells), controls perception of glucose (nervous system) → control of food intake

- expression is required for the physiological control of glucose-sensitive genes, its inactivation in the liver leads to impaired glucose-stimulated insulin secretion

https://doi.org/10.1080/00016357.2020.1832253

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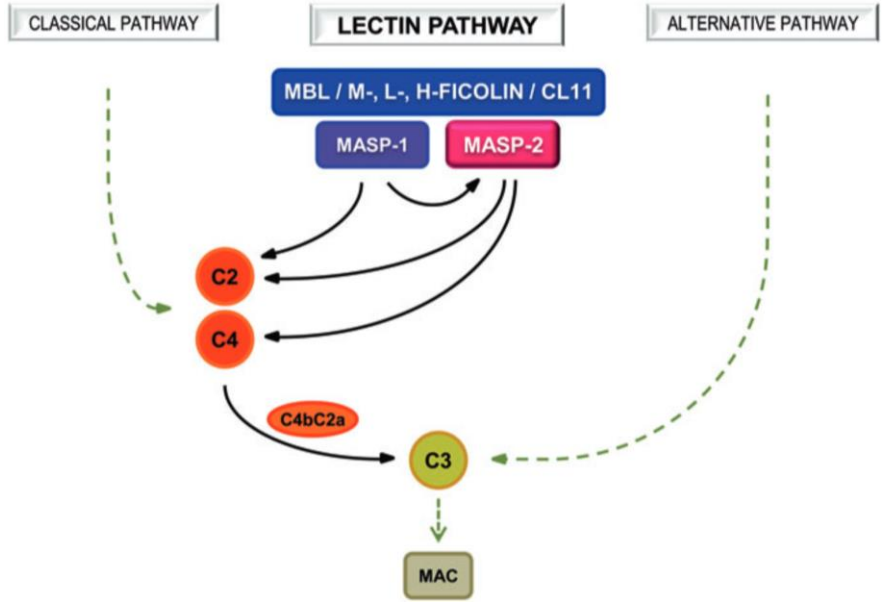
Genetic association studies

- Genes that have been associated with increased risk of dental carries
  - Proteins of immune system



Schematic representation of the lectin pathway of the complement system. The lectin pathway (LP) is triggered by five pattern recognition receptors (PRR): mannose-binding lectin (MBL), ficolin-1, -2, and -3, and collectin 11 (CL11 or CL-K1). The LP is initiated when these PRRs bind to pathogen-associated molecular patterns (PAMPs) on the surface of pathogens or to apoptotic or necrotic cells (damage-associates molecular patterns, DMAPs). Circulating MBL, CL11, and ficolins form complexes with MASP-1 and MASP-2. After the binding of MBL, ficolins, and CL-11 to their targets, MASP-1 auto-activates and triggers MASP-2. Activated MASP-2 cleaves C4 and C2 allowing the assembly of the C3 (C4bC2a) and C5 ($(C4bC2a(C3)_n$)convertases and the subsequent activation of the terminal pathway. Activated MASP-1 also cleaves C2. MAC = membrane attack complex.

https://doi.org/10.1007/978-1-4614-9209-2_7-1

MBL2 – gene encoding Mannose-binding lectin (AKA Mannose-binding protein, Mannan-binding protein/lectin, Collectin 1, MBP1, or Mannose-binding protein C)

– soluble serum lectin recognizing specific carbohydrates on bacterial surfaces → ↓ complement activation

https://doi.org/10.1080/08927014.2020.1856821

Genetic association studies

- Genes that have been associated with increased risk of dental carries
  - Proteins in saliva

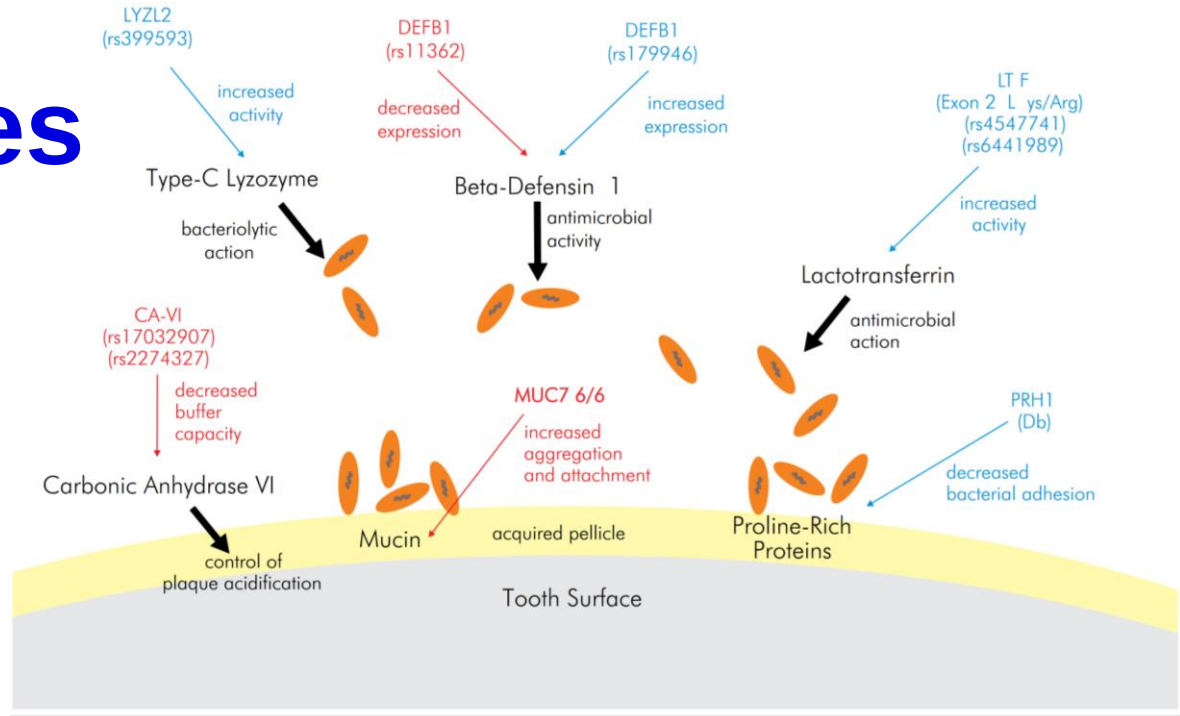


Figure 4. Salivary proteins and functions (black) that present polymorphisms associated positively (red) or negatively (blue) with dental caries experience.

https://doi.org/10.1590/1807-3107bor-2017.vol31.0041

DEFB1 – gene encoding β-Defensin 1 – an antimicrobial peptide from family of Defensins (alpha, beta), which includes cysteine-rich cyclic cationic peptides. They are part of innate immunity, create channels in the cytoplasmic membrane of bacteria, stimulate the immune system incl. complement (classical pathway), act as chemoattractants.

LTF – Lactoferrin gene – transport globular glycoprotein, binds free iron. Part of innate immunity, antibacterial (peroxides are formed when interacting with bacterial membranes), antiviral (competition of adhesion of viral particles to host cells, binding to particles of certain types of viruses), antifungal (against *C. albicans*) activity, stimulation of phagocytosis.

LYZL2 – gene encoding Lysozyme-like protein 2 – part of C-type Lysozyme family. Hydrolyzes glycosidic bonds in peptidoglycans (breaking down the cell wall of G^+ bacteria).

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Genetic association studies

- Genes that have been associated with increased risk of dental carries
  - Proteins in saliva

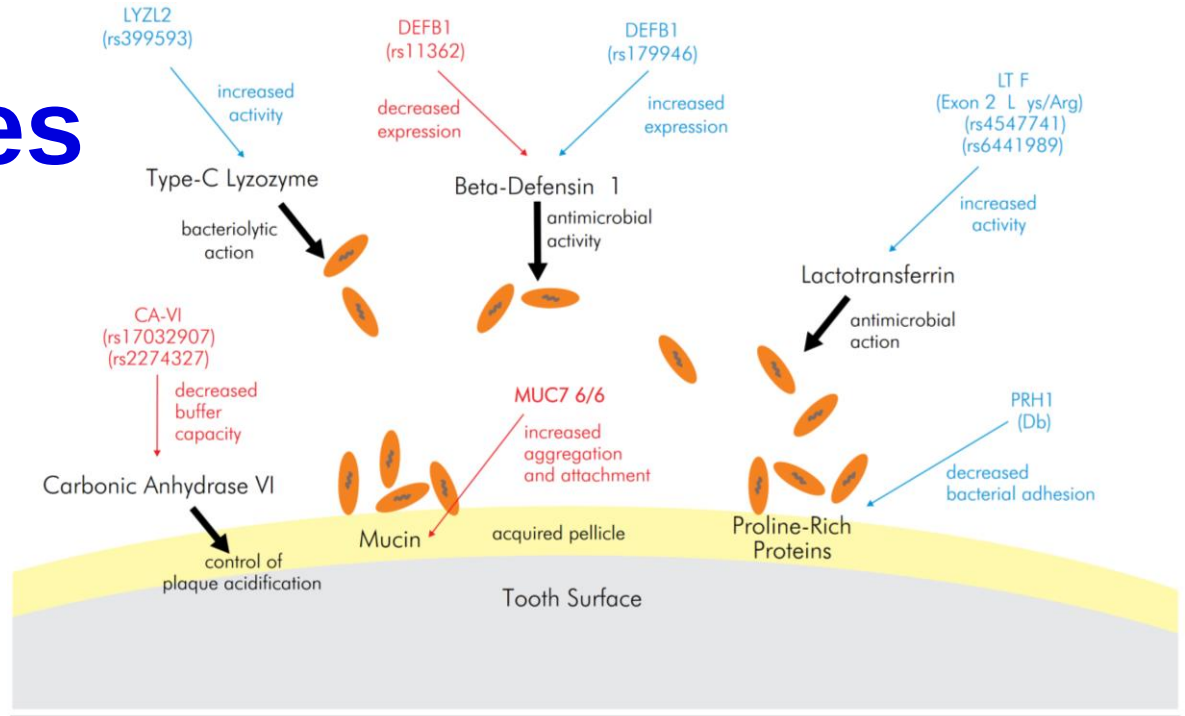


Figure 4. Salivary proteins and functions (black) that present polymorphisms associated positively (red) or negatively (blue) with dental caries experience.

https://doi.org/10.1590/1807-3107bor-2017.vol31.0041

CA6 – gene encoding Carbonic anhydrase VI – enzyme also called ‚gustin‘, catalyzes the hydratation of carbon dioxide to form bicarbonate ions and protons. Saliva pH maintenance (bicarbonate buffer system)

MUC7 – gene encoding Mucin 7 – low molecular weight glycoprotein (MG2), participates in the formation of acquired pellicle and thus in bacterial adhesion and biofilm formation

MUC5B – gene encoding Mucin 5B – glycoprotein (MG2), participates in the formation of acquired pellicle and thus in bacterial adhesion and biofilm formation

PRH1 – gene encoding salivary acidic proline-rich phosphoprotein 1, participates in the formation of acquired pellicle and thus in bacterial adhesion and biofilm formation

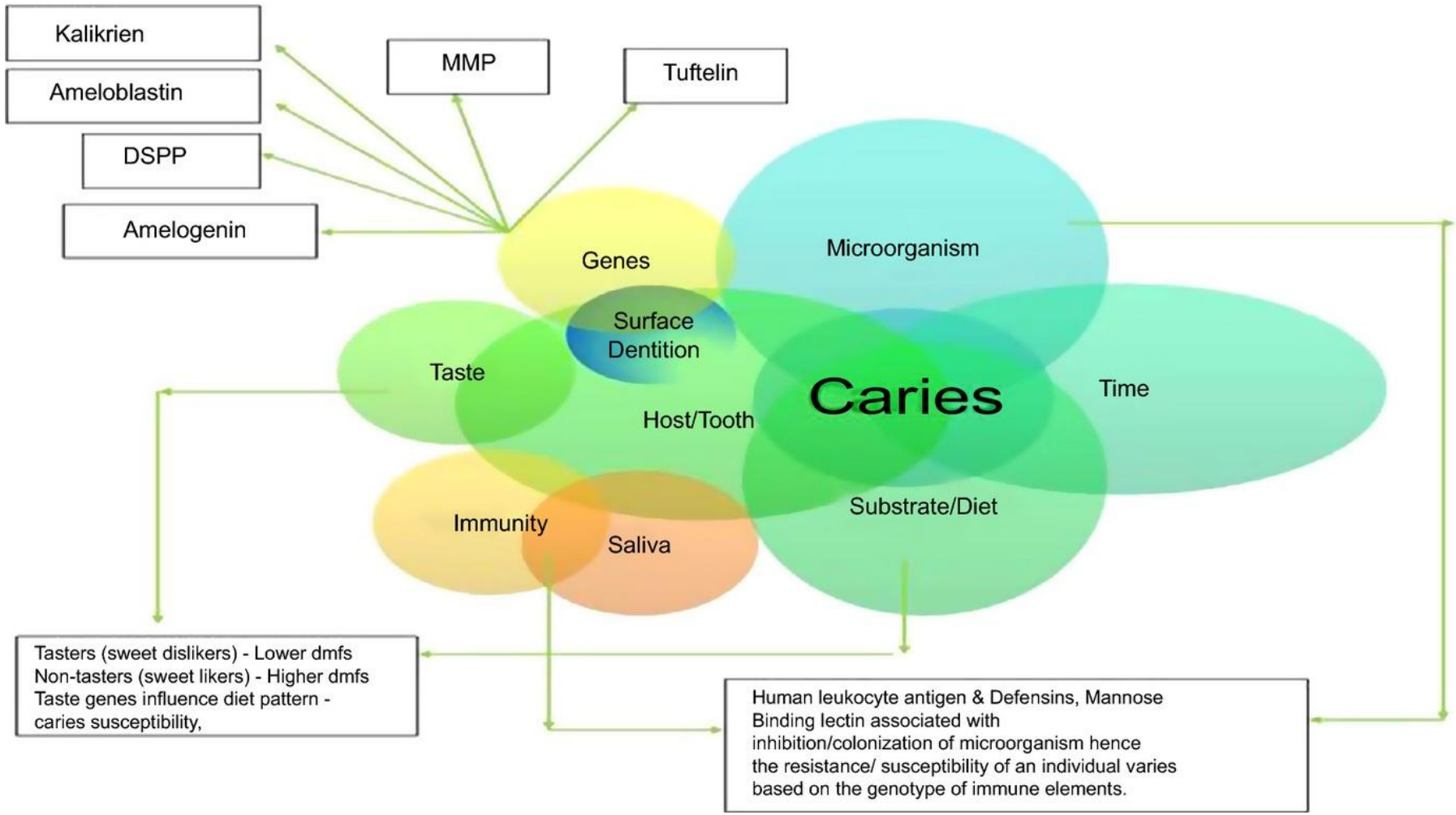
Genetic association studies

- Pitfalls of genetic association studies of dental caries
  - too many factors play a role in etiopathogenesis → set of patients (cases) can never be perfectly categorized → perfectly defined set of cases cannot be created → maximally defined set of cases as far as possible
  - most studies do not confirm the association, only suggests (some studies even give conflicting results)

  → further association studies (more samples) - studies of individual polymorphisms (but their effect may be small), but also genes and loci (gene-based and gene-cluster analysis) → further strengthening of results

  → meta-analysis – a combination of data obtained by an exhaustive search of published and unpublished data worldwide → increasing the consistency of the results (by increasing the strength of the result). Many primary studies are too small to demonstrate an important clinical effect (not strong enough). A combination of all studies that answer the same clinical question → increase in statistical power or significance level

  - detailed questionnaires for evaluating the influence of psychological, sociological, economic and behavioral factors → fragmentation of set of cases to too many groups of too few patients



https://doi.org/10.1111/adj.12262

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