Molecular analysis of oral pathogens and saliva, dental caries
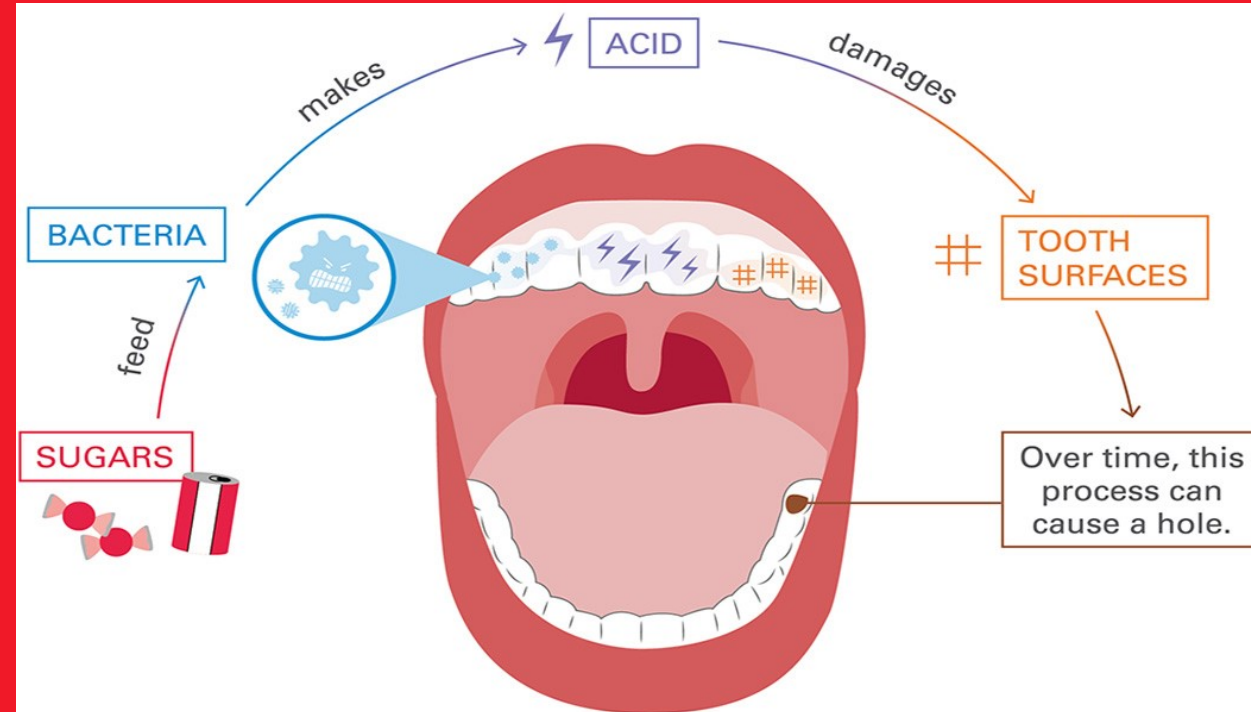
Jana Mrázková, PhD

Department of Pathophysiology MED MUNI

Topics

— Factors involved in development of dental caries
— Molecular analysis of saliva
— Molecular analysis of oral microbiome
— Genetic basis of dental caries
   — Genetic association studies related to dental caries

MUNI
MED

Dental caries
and factors affecting their development

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

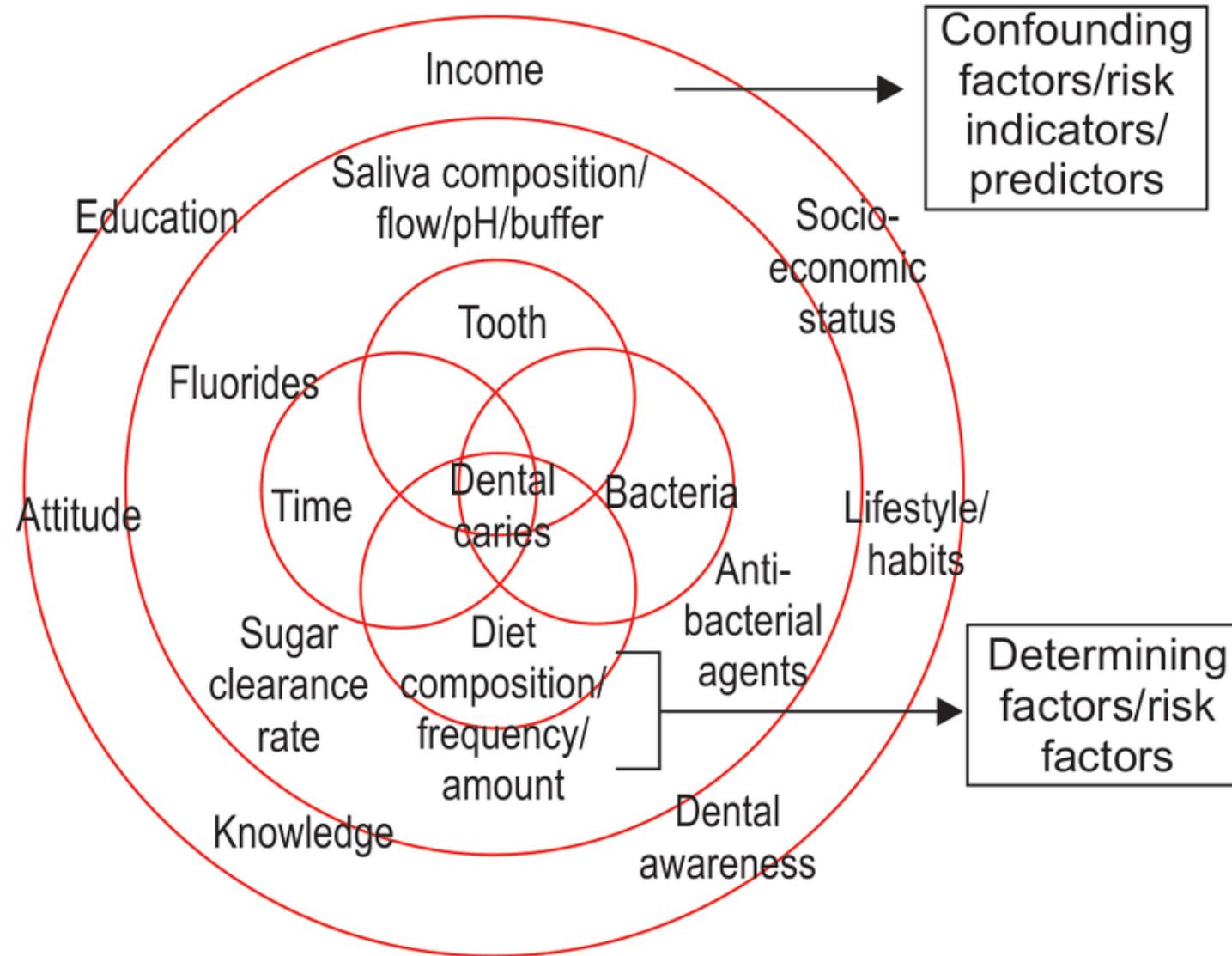# Dental caries

Puwadol Jaturawutthichai
(www.shutterstock.com)

— most common chronic disease
  — 3.5 billion people worldwide (530 million children, according to WHO)
— non-communicable disease (according to FDI World Dental Federation and WHO)
  — shares similar risk factors with other chronic/systemic diseases
— Infectious/transmissible
  — → *Streptococcus mutans* transmission from parents to infants (Early childhood caries), and from one person to another (kissing couples)
— complex disease
  — multifactorial (endogenous and exogenous factors), multiple genes are involved in
— interaction of several factors contributes to formation of caries
  — oral microflora composition (main factor)
  — enamel and dentin properties (tooth surface quality)
  — saliva composition and physical effects
  — genetic predisposition
  — overall health condition (immune system disorders, systemic disease affecting IS)
  — behavioral and environmental factors
  — time for which the factors act / interact

MUNI
MED

https://www.ncbi.nlm.nih.gov/books/NBK551699/

Dental caries

Diagrammatic representation of the determining (risk factors) and confounding factors (risk indicators/predictors) in dental caries disease.

MUNI
MED

Dental caries


Puwadol Jaturawutthichai
(www.shutterstock.com)

– caries dentium

disruption of dynamic process of cyclic alteration between demineralization and remineralization of enamel → ↑ demineralization →→ caries formation begins

- enamel → cca 97 % of inorganic matter (apatites – cationic complexes = **ligands** Ca^{2+} a $(PO_4)^{2-}$ + **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*)

- organic acids (bacteria, diet) → apatite counter-ions neutralization → disintegration of crystal structure units → dissolution of mineral part of the enamel → caries formation
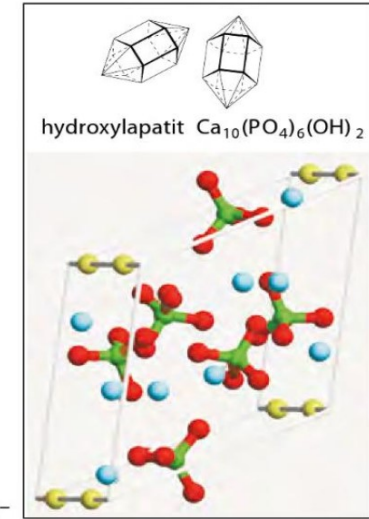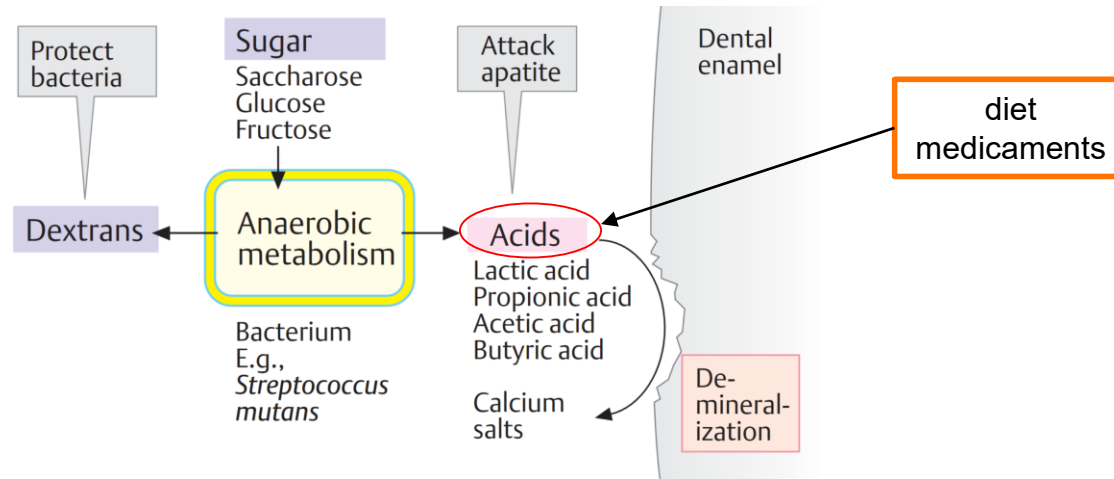
Color Atlas of Biochemistry
(3rd edition, 2013)

- bacterial proteolytic enzymes→ degradation of organic matrix (collagens and proteoglycans)
- carious lesion → dentin → dentinal tubules → pulp → pulpitis, periodontitis

M U N I
M E D

Factors involved in caries development
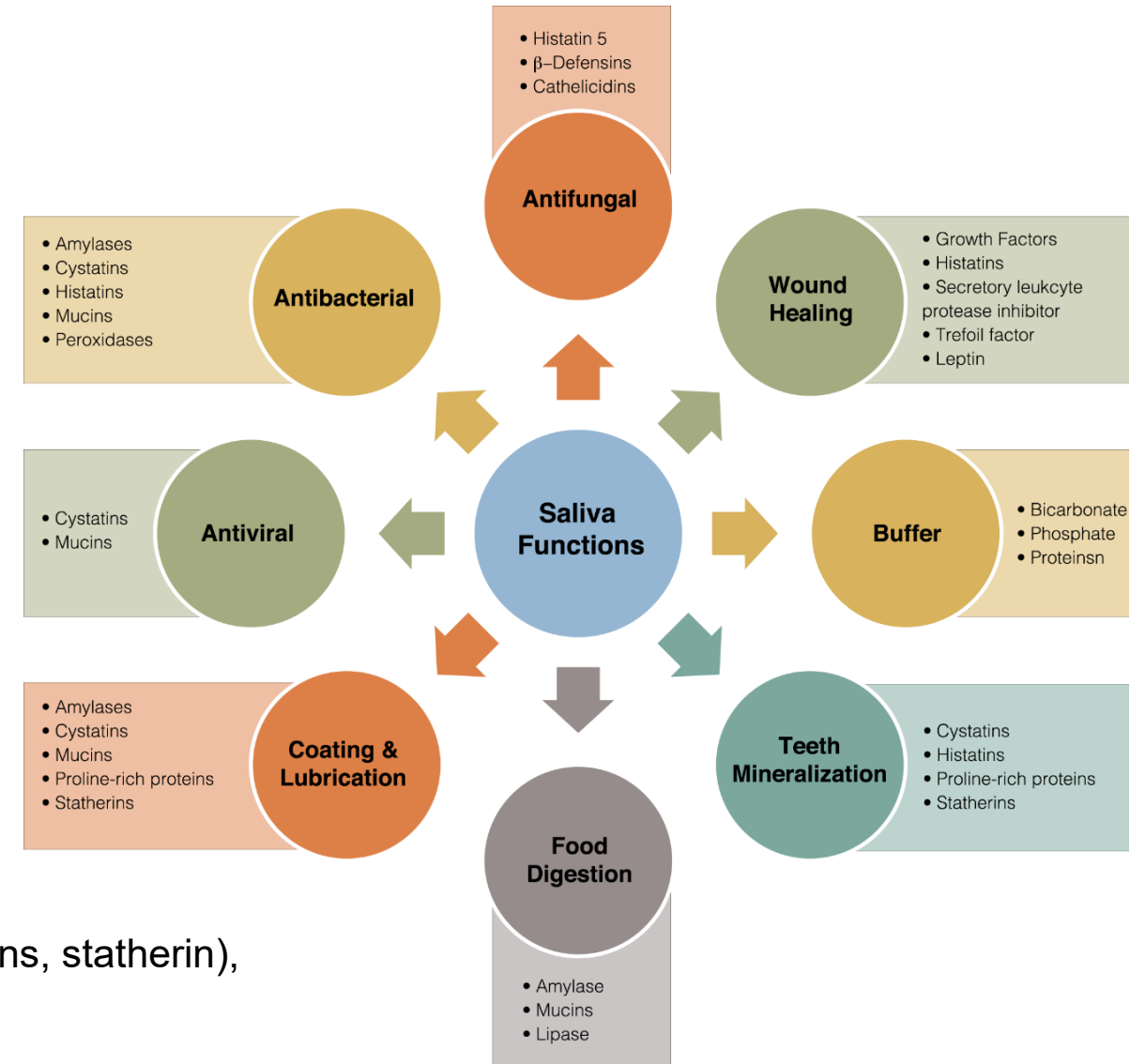
— Saliva:

— complex carioprotective factor

— homeostasis maintenance

— physical factor
  saliva flow (washing and lubrication of oral cavity tissues)
  oral cavity clearance (washing of harmful substances,
  unadhered microorganisms)

— „chemical" factor
  gustin (Carbonic anhydrase VI→ buffering capacity),
  calcium, phosphate, fluoride ions
  lysozyme, lactoferrin,
  proteins of specific (IgA, IgG)
  and non-specific immunity (defensins, cathelicidins, histatins, statherin),
  proline rich proteins (PRPs),
  mucins

• Histatin 5
• β–Defensins
• Cathelicidins

Antifungal

• Amylases
• Cystatins
• Histatins
• Mucins
• Peroxidases

Antibacterial

Wound Healing
• Growth Factors
• Histatins
• Secretory leukcyte protease inhibitor
• Trefoil factor
• Leptin

• Cystatins
• Mucins

Antiviral

Saliva Functions

Buffer
• Bicarbonate
• Phosphate
• Proteinsn

• Amylases
• Cystatins
• Mucins
• Proline-rich proteins
• Statherins

Coating & Lubrication

Teeth Mineralization
• Cystatins
• Histatins
• Proline-rich proteins
• Statherins

Food Digestion
• Amylase
• Mucins
• Lipase

MUNI
MED

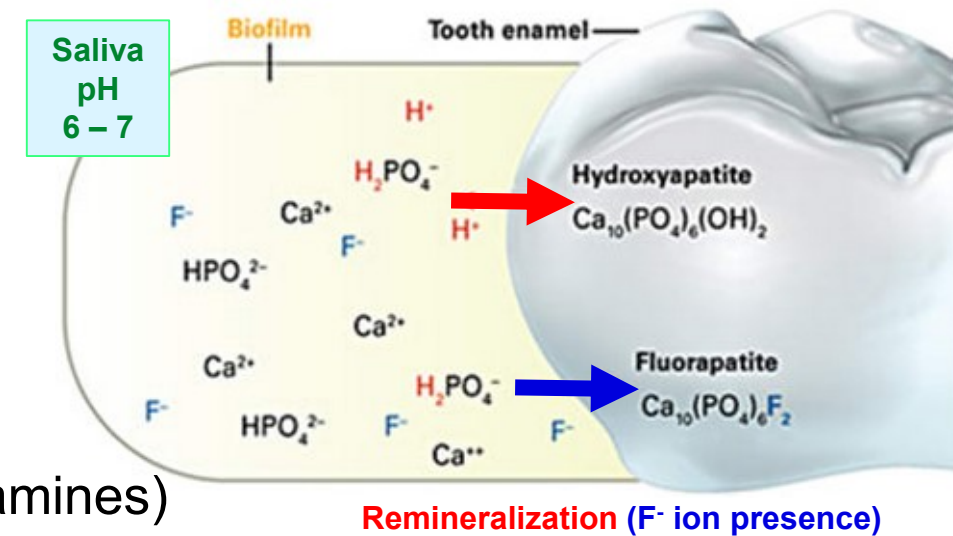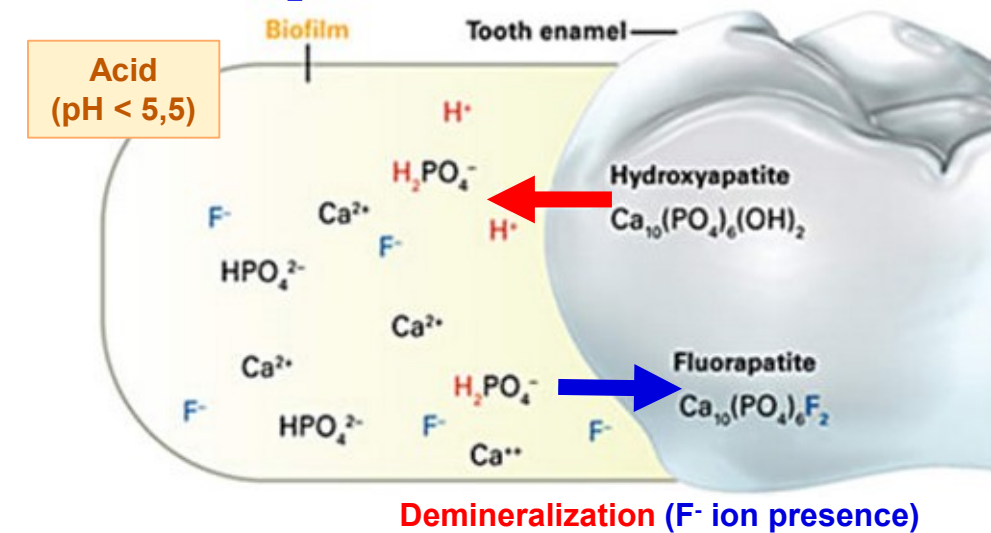# Factors involved in caries development

— Saliva:

— effects of saliva:
  - → ↑ balance between re- and demineralization
  - → ↓ food remnants, ↓ microorganisms,
       ↓ environment acidity (dilution, buffer systems
           - bicarbonate, hydrogen phosphate, proteins)
  - → ↑ substances with antibacterial, antifungal and
       antiviral properties

— problem → reduced production of saliva

  - ← dehydratation, anxiety, obstruction / hypofunction of
      salivary glands (DM, Sjögren's syndrome, AIDS,
      tumors and radioteraphy, acute infection)
  - ← medicaments (beta blockers, antidepressants, antihistamines)
  - ← drugs (methamphetamine, THC)
          → promotion of carious lesions formation

Acid (pH < 5,5)

Demineralization (F⁻ ion presence)

Saliva pH 6 – 7

Remineralization (F⁻ ion presence)

MUNI
MED

Factors involved in caries development

– Oral microbiome:

  – oral cavity → unique microbiological habitat → separate ecological niches (non/desquamating surfaces, saliva) → colonized by specific species of microorganisms

  – second most diverse (up to 1000 species of microorganisms)
    → homeostasis maintenance (competition and displacement of exogenous pathogens → maintaining ecosystem stability)
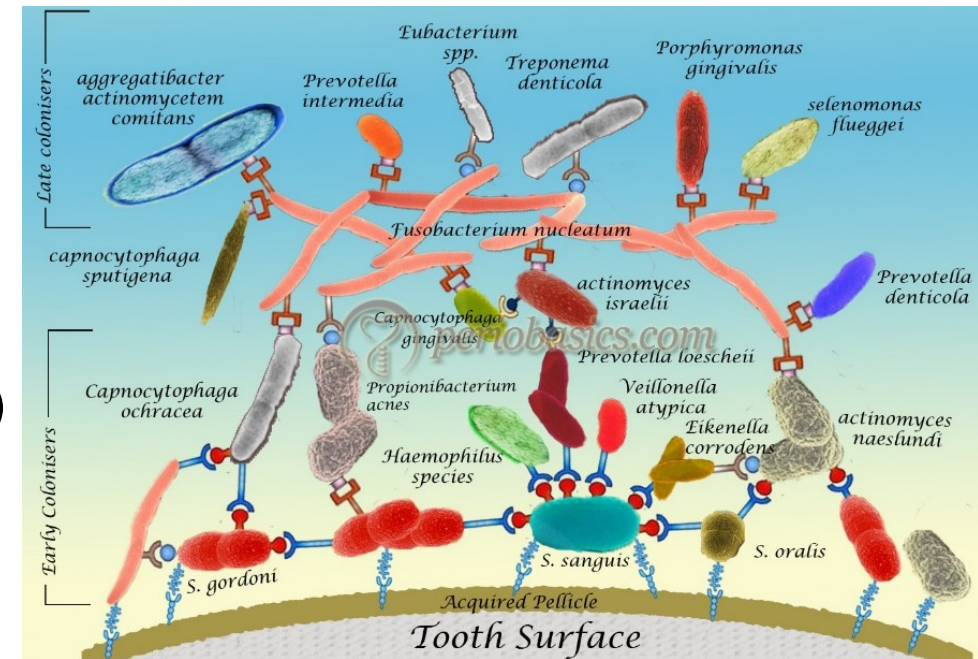   → immunomodulation

  – dental plaque = microbial 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.)

  → saliva → proteins with charged surfaces (acidic PRPs, statherin, histatins) → electrostatic interaction with phosphate and calcium ions of apatite → acelular pellicle formation (mucins, cystatins, albumin, IgA, IgG, lysozyme, alpha-amylase, carbohydrates, neutral lipids, phospho- and glycolipids, glucosyltransferase) → protection against demineralization, <u>partial reduction of microbial adhesion</u> (proteins on the surface are also present in saliva → competition of bacterial binding receptors)
   → <u>substrate for bacteria</u> → → biofilm formation

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

MUNI
MED

Factors involved in caries development

Early colonizers: mainly health-associated streptococci (e.g. *S. sanguinis* and *S. gordonii*)

S. mutans: glucan production robust biofilm formation acid-tolerant acid-producing

Late colornizers: acid-tolerant acid-producing (e.g. *Lactobacillus* and *Veillonella spp.*)

glucans

Poor oral hygiene

High-sugar diet

Other salivary, immunological, and microbial factors

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

— Dental plaque

- problem: oral microbiome <u>dysbiosis</u>
  - → homeostasis disruption → eubiotic balance shift → from mutualism/commensalism to unbalanced parasitic/pathogenic state → disease onset and development
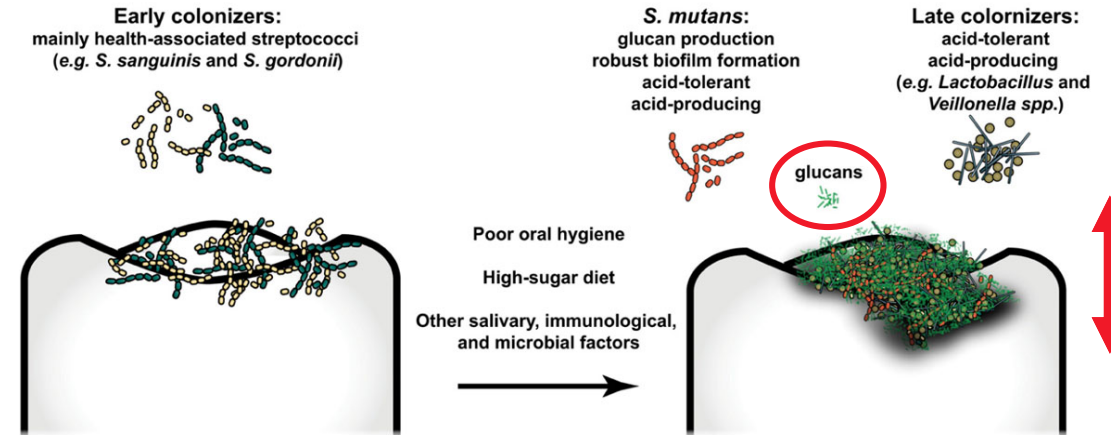
- dental plaque → ↑ cariogenic species (ferment carbohydrates to organic acids + tolerate low pH environment) → predominate *Streptococcus mutans* a *Streptococcus sobrinus, Lactobacillus* sp., *Candida* sp.

- factors supporting cariogenic species predominance
  - → ↑ intake of sugars / acids → acidification; ↓ immunity, inflammation,…
  - → ↓ saliva, ↓ oral hygiene → ↑ plaque thickness
  - → ↑ dental plaque → lack of oxygen → ↑ anaerobic metabolism→ metabolism of fermentable carbohydrates → organic acids → ↓ pH → demineralization
  - → ↑ dental plaque → protects cariogenic bacteria from host defense mechanisms

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

M U N I
M E D

Factors involved in caries development

— External factors:

→ poor oral hygiene

→ poor eating habits (excessive intake of fermentable carbohydrates)

→ smoking (e-cigarettes – filling has a high sugar content)

→ alcohol consumption

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

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

— Time

MUNI
MED

Factors involved in caries development

– 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 in one gene)
- 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) that act as risk factors → predisposition (predisposing genotype can increase probability of disease development, but does not determine the disease)

MUNI
MED

Molecular analysis of saliva

(Salivaomics)

Saliva molecular analysis

— Saliva as a diagnostic fluid

— rich reservoir of peptides and proteins

— saliva components demonstrably change in response to certain diseases and conditions

— more than 100 molecules detected in saliva samples are evaluated as potential diagnostic or prognostic biomarkers for various diseases (eg. tooth decay, periodontitis, cancer, diabetes)
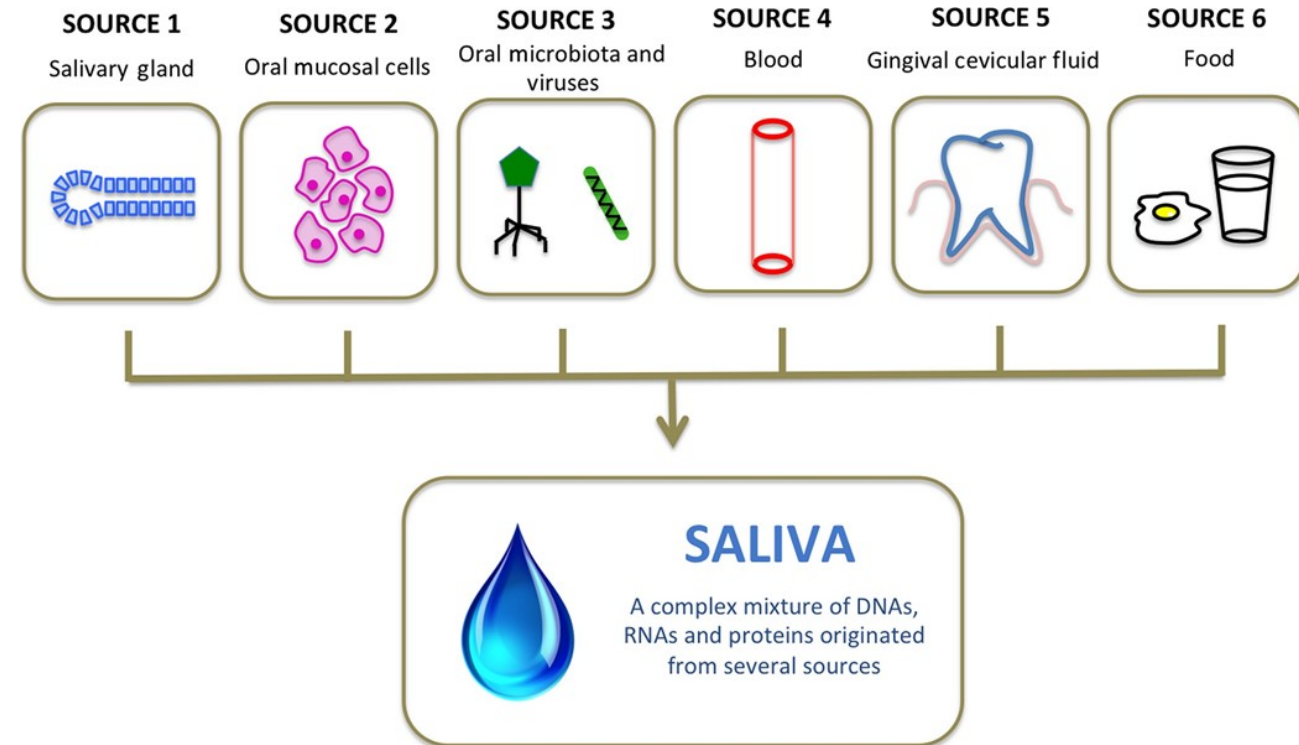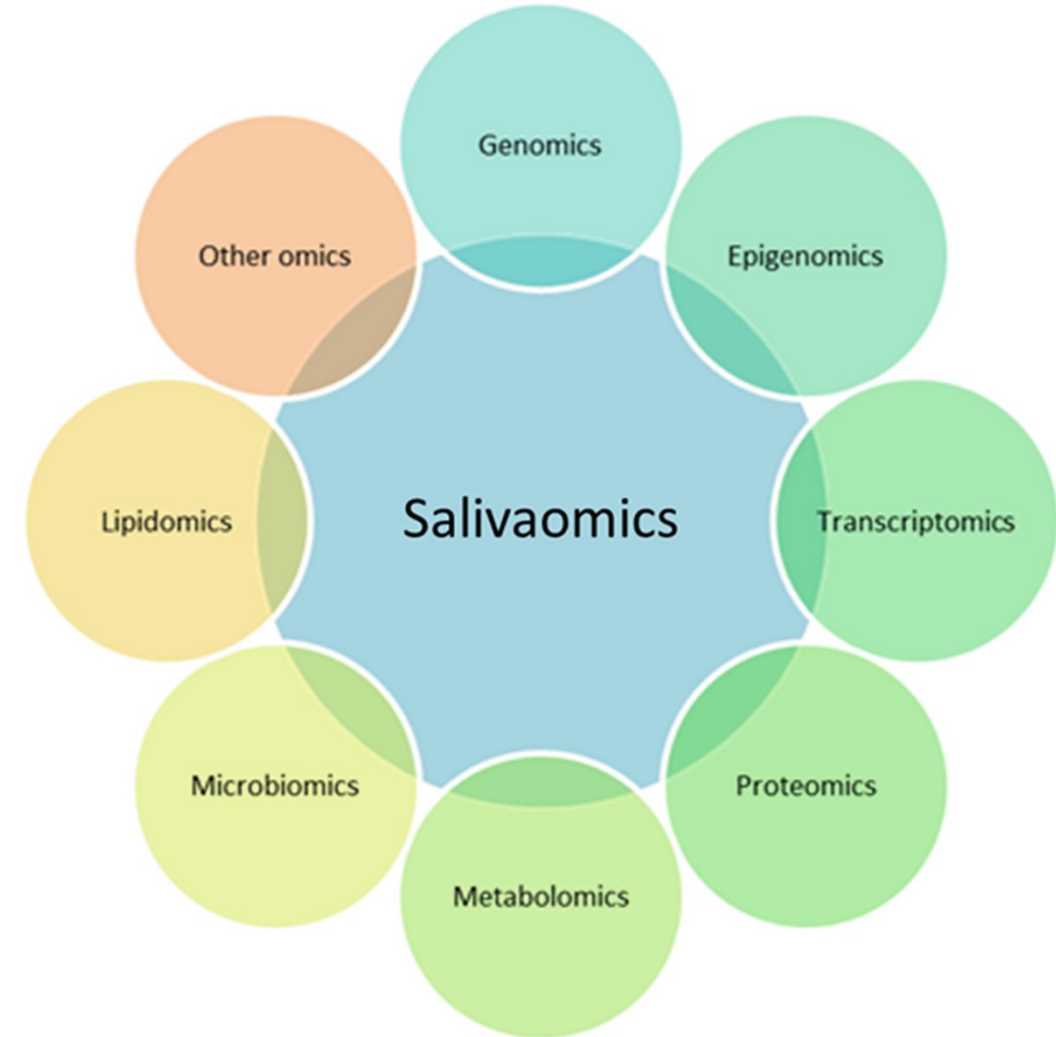

SOURCE 1	SOURCE 2	SOURCE 3	SOURCE 4	SOURCE 5	SOURCE 6
Salivary gland	Oral mucosal cells	Oral microbiota and viruses	Blood	Gingival cevicular fluid	Food

SALIVA
A complex mixture of DNAs, RNAs and proteins originated from several sources

https://doi.org/10.1111/prd.12099

https://doi.org/10.1371/journal.ppat.1008058

MUNI
MED

Saliva molecular analysis

— Saliva as a diagnostic fluid

— **Salivaomics**

→ the term introduced in 2008

→ combines knowledge of various "omic" components
   of saliva
   (proteome, transcriptome, metabolome, microbiome,…)

→ utilizes high-throughput technologies (genomics,
   transcriptomics, proteomics, metabolomics, lipidomics and
   microbiomics,…)

→ saliva analysis → identification of biomarkers

https://doi.org/10.1007/978-3-030-37681-9_4

MUNI
MED

Saliva molecular analysis

— Saliva as a diagnostic fluid

Table 1. Description of Human Saliva Collection Methods.

Type of Whole Mouth Fluid	Method of Collection and Type of Collection Device
Whole Saliva (WS)	Patients should refrain from eating, drinking, and oral hygiene procedures for at least 1 h before saliva collection. (Optimum collection time is 8–10 a.m.). Before collection perform a 1 min oral rinse with distilled water and then after 5 min collect ~5 mL of saliva. Collected sample must be processed in the laboratory within 1 h.
Unstimulated Whole Saliva (USWS)	Passive drooling: In this method restrict oral movement and drain saliva from the lower lip into a plastic vial.
	Spitting method: Instruct subject to spit into a collection vial. In this method 14 times more bacterial contamination is introduced into the sample.
Stimulated Whole Saliva (SWS)	For the stimulation of glands, chewing different things like natural gum, a piece of paraffin wax, citric acids, and powdered drink crystals have been used.
Parotid Gland	Method introduced by Carlson and Crittenden (1910). In this method a double chambered metallic cup with two outlet tubes is used. One end holds the cup in place using vacuum suction. The second half acts as a collection vehicle for saliva. Specimen collection can be enhanced by smearing citric acid (10%; 1 mL) on the dorsum of tongue every 30 s. Discard the first 1.5 mL of saliva prior to sample collection.
Submandibular/Sublingual Gland	Truelove, Bixler, and Merrit (1967) used a "V"-shaped collector. This method is similar to that for parotid gland collection, but in this case the initial 2 mL is discarded.
Minor Glands	Kutscher et al. (1967) used capillary tubes for collecting saliva from minor glands located at the everted surface of the lower lips.

Advantages:

— saliva collection is non-invasive, easy, painless, repeatable (permanent availability of the material), usable for all age categories, untrained staff can do the collection

— large sample volume, stable in time, processing is fast, cheap

— promising potential to replace blood in screening, diagnosis and prognosis of disease

MUNI
MED

Saliva molecular analysis

→ the issue of biomarkers validation for clinical applications

Saliva as a diagnostic fluid

- verification → determination of the biomarker by various techniques → achieving similar results

- validation → preclinical → definitive academic (prospective sampling, retrospective evaluation → multicenter studies

Limitations:

— serum / saliva biomarker levels correlation

  ↓↓↓ analyte concentration when compared to serum → ↑ saliva sample volume, detection limit of the method, depletion of abundant proteins (PRPs, α-amylase, albumin, mucins and secretory IgA can form up to 80%), osmolality

— high variability → worse reproducibility of results
  - technical (sampling, processing, used method)
  - inter- (age, sex, physiological status) and intraindividual (circadian, circulatory)
  - biological (influence of oral condition, circadian rhythm, systemic diseases (Sjögren's syndrome), drugs, chemo / radiotherapy) → saliva volume and composition
  - saliva flow rate and its stimulation → concentration of salivary biomarkers
  - proteolytic enzymes (microbiome / host) → stability of certain biomarkers

— the issue of standardization
  → correlation of protein markers to total saliva protein concentration (same person as sample and control)
  → standardization of used methods, validation of protocols
  → considering all variables (saliva composition variability, sampling, saliva flow rate, sample volume, stimulation, blood contamination, collection kits, analyte integrity)

MUNI
MED

Saliva molecular analysis

— Saliva as a diagnostic fluid

Spectrophotometric methods

— UV/Vis spectrophotometry
  (enzymes, metabolites, proteins, anti / oxidants)

— Atomic absorption / emission spectrometry
  (atoms and ions – Ca, Mg, Cr, Mn, Ni, Pb / Na, K)

— NIR (near infrared) spectroscopy
  (transition metal ions and rare earth metal ions, molecules containing bonds C-H, N-H, S-H, O-H – thyocyanate, IgA, cortisol, salivary α-amylase, urea, phosphates, total protein)

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

MUNI
MED

Saliva molecular analysis

— Saliva as a diagnostic fluid

Immunoassays

- Enzyme-linked immunosorbent assay (ELISA)
  (direct, indirect or sandwich-type – adiponectin, cortisone, cortisol, C-reactive protein, D-dimer, lactoferrin, IgA, IgM, IgG, IgE, myoglobin)

- Chemiluminescence immunoassay (cortisol, testosterone, lactate)

- Fluoroimmunoassay (salivary α-amylase, Haptoglobin, C-reactive protei

- Radioimmunoassay (cortisol, estradiol, oxytocin)

- Unlabeled immunoassays (nephelometry, turbidimetry, immunochromatography, biosensors / chips)

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

MUNI
MED

Saliva molecular analysis

— Saliva as a diagnostic fluid

Liquid biopsy (fluid biopsy)

— cancer, tumors

— simple, non-invasive

— efforts to replace tissue biopsy

— tests that detect circulating tumor cells, exomas, tumor DNA, tumor RNA, and proteins that were released to bloodstream or saliva from the primary lesion

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

MUNI
MED

Saliva molecular analysis

SALIVA SAMPLING

SALIVARY METABOLOME — SALIVARY PROTEOME — SALIVARY TRANSCRIPTOME — SALIVARY GENOME — SALIVARY MICROBIOME

https://doi.org/10.3390/bios11100396

— Saliva as a diagnostic fluid

„Omics" methods

- DNA (genomics and epigenomics - cancer)
- RNA (transcriptomics - biomarkers for chronic periodontitis, Sjögren's syndrome, lung, ovarian, breast and pancreatic cancer)
- proteins (proteomes for Sjögren's syndrome, Down's syndrome, schizophrenia)
- metabolites metabolomics - oral cancer, hepatocellular and colorectal cancers, periodontitis, chronic kidney disease)
- lipids and microbiome (lipidomics, microbiomics) and others

- simultaneous analysis of hundreds of analytes → precise detection of small changes

- high sensitivity, quantitative results

- analysis of a set of biomarkers for particular disease

- none in clinical application yet → „point-of-care" testing

MUNI
MED

https://doi.org/10.1007/978-3-030-37681-9
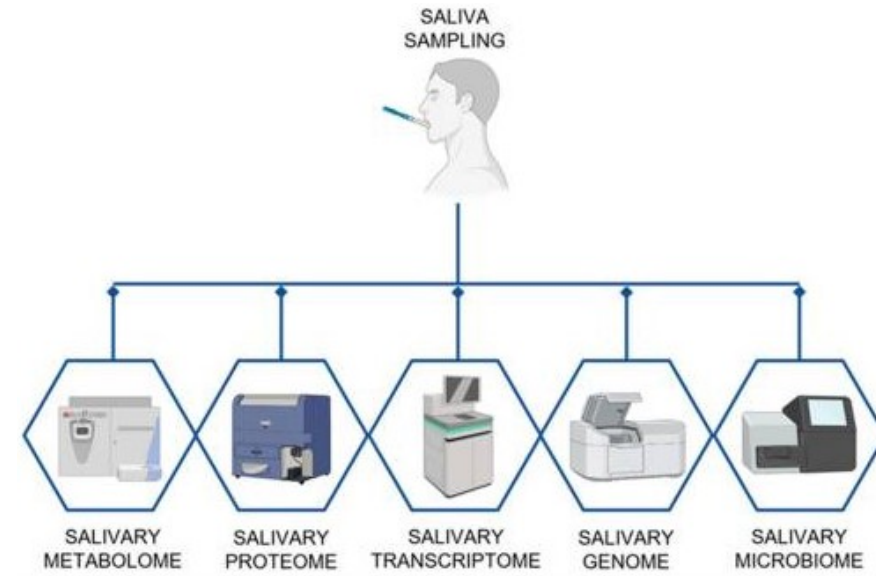
Saliva molecular analysis

– Saliva as a diagnostic fluid – dental caries

  – no diagnostic test

  – caries susceptibility tests → microbiological laboratory

    → determination of presence of cariogenic microflora

    → quantitative determination of the presence of fermenting microorganisms (acidifying environment) in saliva

    → determination of saliva buffering capacity and saliva secretion rate

    → colony quantification of fungus *Candida albicans*

  – efforts to relate the prevalence of tooth decay to the saliva phenotype → ambiguous results

MUNI
MED

Saliva molecular analysis

– Saliva as a diagnostic fluid – dental caries susceptibility

   – salivary protein biomarkers associated with dental caries susceptibility:

       ↑ total protein, total antioxidant activity
       ↑ 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

   – salivary protein biomarkers associated with susceptibility to ECC:

       ↑ PRPs, histatins, IgA, IgG

       ↓ statherin

MUNI
MED

Saliva molecular analysis

– Saliva as a diagnostic fluid – periodontitis, oral cancer

– periodontitis

→ ↑ salivary biomarkers – IL-1β, MMP-8, MMP-9, TNF-α, AST, ligand for receptor activator of nuclear factor κB (RANKL), osteoprotegerin, prostaglandin E_2
– they can be used to detect beginning stages of periodontitis, to distinguish between periodontitis and gingivitis, to predict the progression of periodontitis and monitor the prognosis

→ ↑ red complex bacteria (*Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola*)
→ ↑ *Aggregatibacter actinomycetemcomitans, Prevotella intermedia* (G⁻ anaerobic bacteria)

→ genetic predisposition to susceptibility → IL-1 gene polymorphism

– cancer (oral squamous cell carcinoma – OSCC)

→↑ salivary biomarkers – IL-6, IL-8, IL-1α, IL-1β, CD59, MRP14 (myeloid related protein), profilin 1 protein, catalase, Mac-2 binding protein (M2BP)
→ other potential salivary markers → telomerase, Cytokeratin 19 fragment (Cyfra21-1), tissue polypeptide antigen (TPA), cancer antigen CA 125, CD44, glutathione, transferrin, mRNA (IL8, IL-1β, phosphatase DUSP1, hemagglutinin HA3, enzyme OAZ1, S100 calcium-binding protein P, acetyltransferase SAT), levels of some amino acids, lactate, extracellular DNA, microRNA, carcinoma cells

MUNI
MED

Saliva molecular analysis

— ## Saliva as a diagnostic fluid

- — <u>point-of-care testing, chair-side kits:</u>

- — improving individualized care
  - caries risk determination
  - periodontitis onset risk and progression assesment
  - oral cancer screening

- — caries
  - saliva physical parameters: volume, flow rate, viscosity, consistency
    pH and buffering capacity of saliva

  - lactate

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

commercial kits (visual or colorimetric detection)

commercial kits (colorimetric detection)

commercial kits (immunochromatographic detection of antigen, cultivation kit)

MUNI
MED

Saliva molecular analysis

— ## Saliva as a diagnostic fluid

— ### point-of-care testing, chair-side kits:

— periodontitis
  - detection in saliva – active MMP-8 → PerioSafe® PRO DRS (immunochromatography) a ORALyzer® (analyzer)
  - detection in gingival crevicular fluid – aMMP-8 (ImplantSafe DR®), AST (PerioGard, PocketWatch)

— cancer (OSCC) screening

Table S2: Commercially available POC adjuncts for oral cancer examination

POC Device	Company	Principle	Sample	Sensitivity	Approximate cost of analysis	References
Salivary adjuncts						
Oramark/ OncAlert RAPID Test	Vigilant Biosciences, Fort Lauderdale, Florida	Salivary Biomarkers - CD44 and total protein	Saliva	Qualitative	NA	("OncAlert Oral Cancer LAB Test | RAPID Test | Vigilant Biosciences | Vigilant Biosciences," n.d.)
OncoE6™ Oral Test	Arbor Vita Corporation, Fremont, California	HPV viral E6 oncoprotein	Saliva	Qualitative	NA	("Products-CoVisa | Arbor Vita Corporation," n.d.)
SaliMark OSCC salivary DNA test	PeriRx LLC, Broomall, Pennsylvania	DNA biomarkers	Saliva	Quantitative	200$	(SaliMark ™ OSCC The Most Clinically-Advanced and Scientifically-Validated Molecular DNA Test for Oral Squamous Cell Carcinoma, n.d.)
The OraRisk® HPV Complete Genotype	(OralDNA Labs, Inc., Eden Prairie, Minnesota	Oral HPV biomarkers	Saliva	Quantitative	200$	("OralDNA | Test Menu | OraRisk HPV Complete Genotyping," n.d.)

MUNI
MED

Saliva molecular analysis

– Saliva as a diagnostic fluid

  – periodontitis

Table 2. Examples of biomarker assay kits in the market.

Biomarker Classification	Sampling From	Product Name	Detecting Target	Detecting Principle	Analyzing in
Biochemical assay	GCF	Periocheck	Neutral proteases	Enzymatic digestion reaction (Colorimetric assays)	Chairside
	GCF	PocketWatch	AST	Enzymatic catalysis reaction (Colorimetric assays)	
	GCF	PerioGard	AST	Enzymatic catalysis reaction (Colorimetric assays)	
	Oral rinse	PerioSafe	aMMP-8	Lateral flow test with digital reader (OraLyzer®)	
	GCF	ImplantSafe			
	Oral rinse	SillHa ST-4910	Blood, leukocytes, and protein	Lateral flow test with dual-wavelength reflectometry	

MUNI
MED

Saliva molecular analysis

— Saliva as a diagnostic fluid

   – diseases and examples of 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 α-synuclein

— genetic diseases

   cystic fibrosis – Ca, PO_4^{2-}, Na, K, Cl, ↓ saliva volume, urea, uric acid, prostaglandin E2

— cancer

   squamous cell carcinoma – IL-8, IL-6, IL-1β, 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
   pancreas cancer – transcriptomic markers of mRNAs (*KRAS, MBD3L2, ACRV1* and *DPM1*), specific miRNA, lactoperoxidase, Cyclophilin B, Cytokeratins (14, 16 a 17)

— allergy

   food allergies - IgE and IgG_1

https://doi.org/10.1016/j.medntd.2022.100115

doi:10.7759/cureus.7708

MUNI
MED

Saliva molecular analysis

– Saliva as a diagnostic fluid

   – diseases and examples of 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 – presence of *Candida* sp., *Entamoeba histolytica* (antibodies)
   Hepatitis – presence of DNA of HBV virus
   Peptic ulcer disease, gastritis – presence of *Helicobacter pylori (IgG antibodies, H. pylori DNA)*

– endocrine diseases

   Cushing's syndrome and Addison's disease - cortisol
   sex hormones - polycystic ovary syndrome, menopause / andropause, anovulation, hypogonadism,
   hyperestrogenism

https://doi.org/10.1016/j.medntd.2022.100115

doi:10.7759/cureus.7708

Saliva molecular analysis

– Saliva as a diagnostic fluid – biomarkers analyzed in labs

Saliva molecular analysis

– Saliva as a diagnostic fluid
    – biomarkers analyzed in labs

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

M U N I
M E D

Molecular analysis of oral microbiome

(„Oralome")

Molecular analysis of OM

– Oral microbiome

— community of up to 1000 different microbial species → bacteria, fungi, viruses, archaea, protozoa
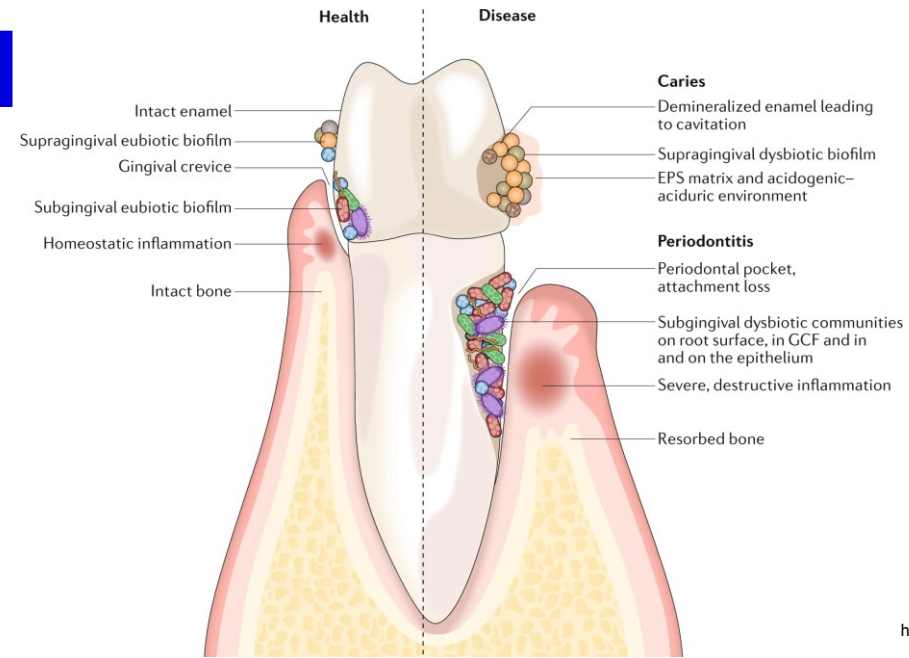→ bacterial species predominate

— „oralome"
→ the summary of the dynamic interactions orchestrated between the ecological community of oral microorganism that live in the oral cavity and the host
(For example, oral microflora is important for the maturation and development of an appropriate oral immune response → the host's immune system must defend itself against pathogenic microbes, but at the same time it must harmonize and protect commensal oral microbes)

MUNI
MED

Molecular analysis of OM

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

– Oral microbiome

  – dysbiosis → oral diseases
    ← host diet
    ← inflammatory reactions
    ← systemic diseases (DM 2, hyperglycemia)
    ← host habits (smoking, alcohol, chronic stress)

  – <u>caries</u> → ↑ dietary carbohydrate intake → ↑ acid production → ↓ saliva pH and buffering capacity → ↑ production of ECM of biofilm → ↑ acid concentration on the enamel surface → ↑ growth support of aciduric and acidogenic species → dysbiosis

  – <u>periodontitis</u> → dysbiosis of subgingival microbial communities (biofilm) → formation and maintaining of gingival and periodontal inflammation → adverse effect on the host IS → blocking of IS subversion and tissue regeneration
    → some types of OM biofilm → etiological agents (red complex bacteria)
    → *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola* → show the ability to manage processes involved in the pathogenesis of periodontal disease by controlling microbiota restructuring and promoting inflammation
    → oral virome may be as important in the pathogenesis of the disease as oral bacteriome

MUNI
MED

https://doi.org/10.1111/prd.12393

Molecular analysis of OM

Caries
- Demineralized enamel leading to cavitation
- Supragingival dysbiotic biofilm
- EPS matrix and acidogenic– aciduric environment

Periodontitis
- Periodontal pocket, attachment loss
- Subgingival dysbiotic communities on root surface, in GCF and in and on the epithelium
- Severe, destructive inflammation
- Resorbed bone

Intact enamel
Supragingival eubiotic biofilm
Gingival crevice
Subgingival eubiotic biofilm
Homeostatic inflammation
Intact bone

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

— Oral microbiome

- dysbiosis → oral microbes can affect the immune system response and pathogenesis of diseases in the system (reservoir of pathobionts)
  → oral cavity → high degree of vascularization, entry into the respiratory and digestive systems
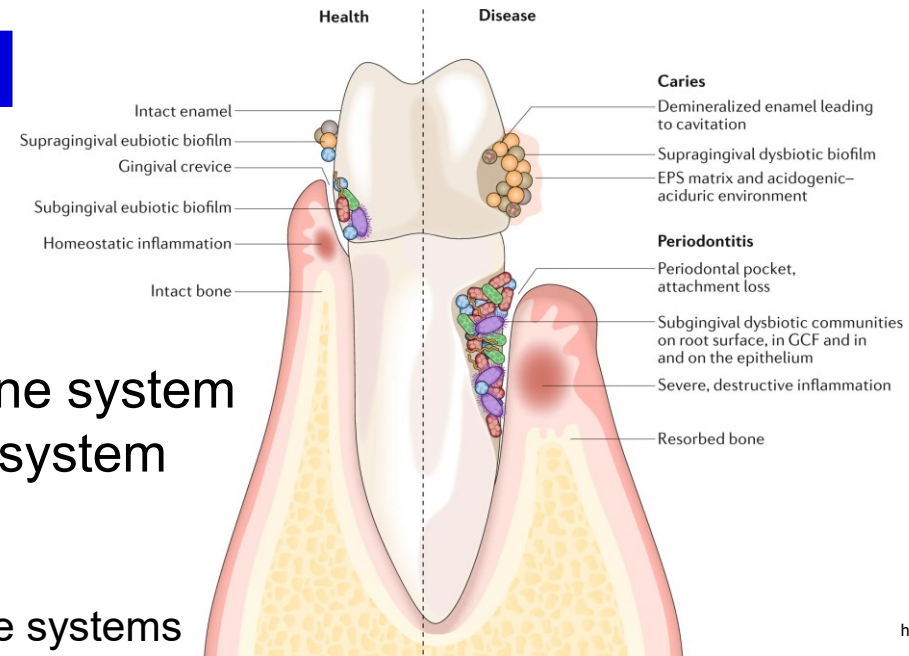
- systemic diseases → primary mechanisms linking oral infection to systemic pathologies
  → spread of infection from the oral cavity due to transient bacteremia
  → circulation of microbial toxins
  → systemic inflammation caused by adverse immunological reactions to oral microbes

- microbes associated with the oral cavity detected in many distant organs (small intestine, lungs, heart, brain, placenta) → colonization depends on the health status of the tissue

- proven association between microbes involved in periodontitis and chronic conditions (cardiovascular diseases, hypertension, inflammatory diseases)
  → subgingival biofilm → a source of bacteria and pro-inflammatory mediators → blood circulation

https://doi.org/10.1111/prd.12393

MUNI
MED

Molecular analysis of OM

– Oral microbiome – a potential biomarker of systemic diseases
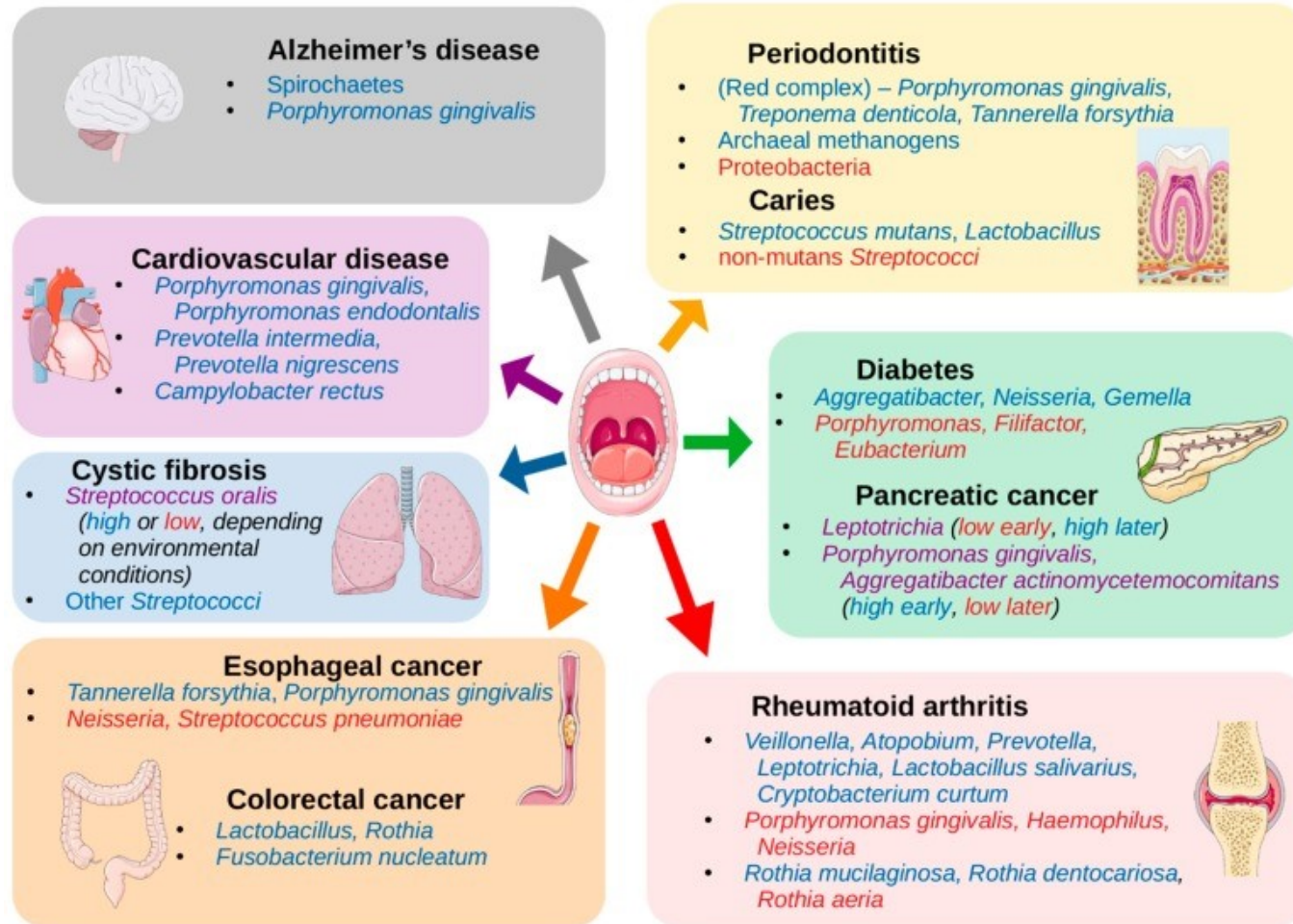
oral microbiome as a non-invasive biomarker

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.

MUNI
MED

Molecular analysis of OM

– Oral microbiome

Table 1. Host Factors to Modulate the Oral Microbiome.

Factor	Reference
Genetics	- Genetic polymorphism in miRNA202 is involved in hBD1 salivary level as well as caries experience [64] - Genes expressed in dental enamel development are associated with molar–incisor hypomineralization [65] - GLUT2 and TAS1R2 genotypes individually and in combination are associated with caries risk [66] - Host genetic control of the oral microbiome in health and disease [67] - Microbial abundance and some aspects of the microbial population structure are influenced by heritable traits in saliva [68]
Immunity	- Immune cell network mediating immune surveillance at oral mucosa and gingiva [69,70] - The innate host response in caries and periodontitis [71] - Secretory immunity with special reference to the oral cavity [72]
Attachment surface	- Surface properties influence oral biofilm formation [73] - Differences in relation to the microbial diversity of modified resins during the initial phase of biofilm maturation [74] - Biomaterial-associated infection of implants and devices [46]
Diet	- Vegan diet influences on the human salivary microbiota [75] - Short- and medium-chain fatty acids exhibit antimicrobial activity for oral microorganisms [76]
Cigarette smoking	- Smoking decreases structural and functional resilience in the subgingival ecosystem [77] - Firmicutes were statistically elevated in smokers at the expense of Proteobacteria and Fusobacteria in non-smokers [78] - Tobacco smoking affects the salivary gram-positive bacterial population [79]
Alcohol	- Alcohol affects to the oral microbiome composition [80–82]
Oral hygiene	- Toothbrushing frequency is related to the incidence and increment of dental caries [83]
Socioeconomic status	- Socioeconomic factors, such as education and income, are associated with disparities in the prevalence and severity of periodontal disease [84] - A strong association between cariogenic bacteria and socioeconomic status was found [85] - Differences in socioeconomic status were reflected in the bacterial profile of saliva [86]

MUNI
MED

Molecular analysis of OM

– Oral microbiome – methods of analysis

    – <u>sampling place – sample location</u>

        → OM at various sites of the oral cavity (saliva, tongue, palate, buccal mucosa, tooth surfaces, gums, supra- / subgingival plaque, tonsils, throat) shows an overall similarity, but with some differences → ecological niches

        → general microbial screening for diagnosis is performed from saliva or site-specifically from gingival crevicular fluid or dental biofilm

MUNI
MED

Molecular analysis of OM

qPCR
→ not only 16S rRNA gene, but also other genes, also allows quantification

— Oral microbiome – methods of analysis

— <u>microbiological cultivations</u>
  → 250 species isolated and characterized
  → OM is complex → some species have not been cultivated

— <u>single-cell sequencing NGS</u>
  → originally developed for immune profiling
  → modified to allow evaluation of individual microbial cells
  → ↑ detection rate of non-cultivable organisms

<u>genomics</u>

— <u>16S rRNA sequencing</u>
  → sequencing of the conserved gene for 16S rRNA (bacteria) or ITS (internal transcribed spacer) DNA region (fungi)
  → most common sequencing method, cheap
  → taxonomic data only (limited differentiation of phylogenetically related species / strains)

— <u>whole genome shotgun sequencing (WGS)</u>
  → DNA is randomly fragmented and then subjected to Sanger sequencing or NGS
  → tool for metagenomic analysis, parallel evaluation of all kingdoms (bacteria, fungi, viruses) in one sample
  → not only taxonomic data but also biological functional profiles of the microbial community
  → evolutionary analysis of specific organisms associated with a particular disease or environment

MUNI
MED

Molecular analysis of OM

— Oral microbiome – methods of analysis

- **transcriptomics** → defining the expression of microbial and host genes in the context of oral health and pathology

- <u>mRNA sequencing (metatranscriptomics)</u>
  - → utilizing random hexamer primers
  - → summary of viable and transcriptionally active microbes
  - → parallel evaluation of microbial and host transcriptomes → examined to assess the interactome

- **metabolomics** → metabolic and functional activities of the host and its microbiome → view of complex interspecies interactions
  - → low molecular weight metabolites, proteins
  - → liquid / gas chromatography with mass spectrometry, NMR

  - → periodontitis → a characteristic shift in the composition of oral bacteria, which is partly mediated by bacterial metabolites → metabolomics → how and why this shift occurs
  - → it can offer guidance for critical time points at which therapeutic interventions could be beneficial

MUNI
MED

Molecular analysis of OM

— Oral microbiome – methods of analysis

— **proteomics** → profile of all proteins in an organism, tissue, cell or biological fluid, or sub-component of any of them → view on health / illness
    → proteins in a sample → yes/no, amount, posttranslational modifications, isoforms, molecular interactions
    → 1-D/2-D gel electrophoresis with mass spectrometry, liquid chromatography with mass spectrometry
    → to characterize changes in gingivitis, or mild, moderate and chronic periodontitis

— high resolution techniques + clinical data + longitudinal studies → understanding the interactions of microbes and hosts from the species level to the molecular level and their implications for oral health

MUNI
MED

Molecular analysis of OM
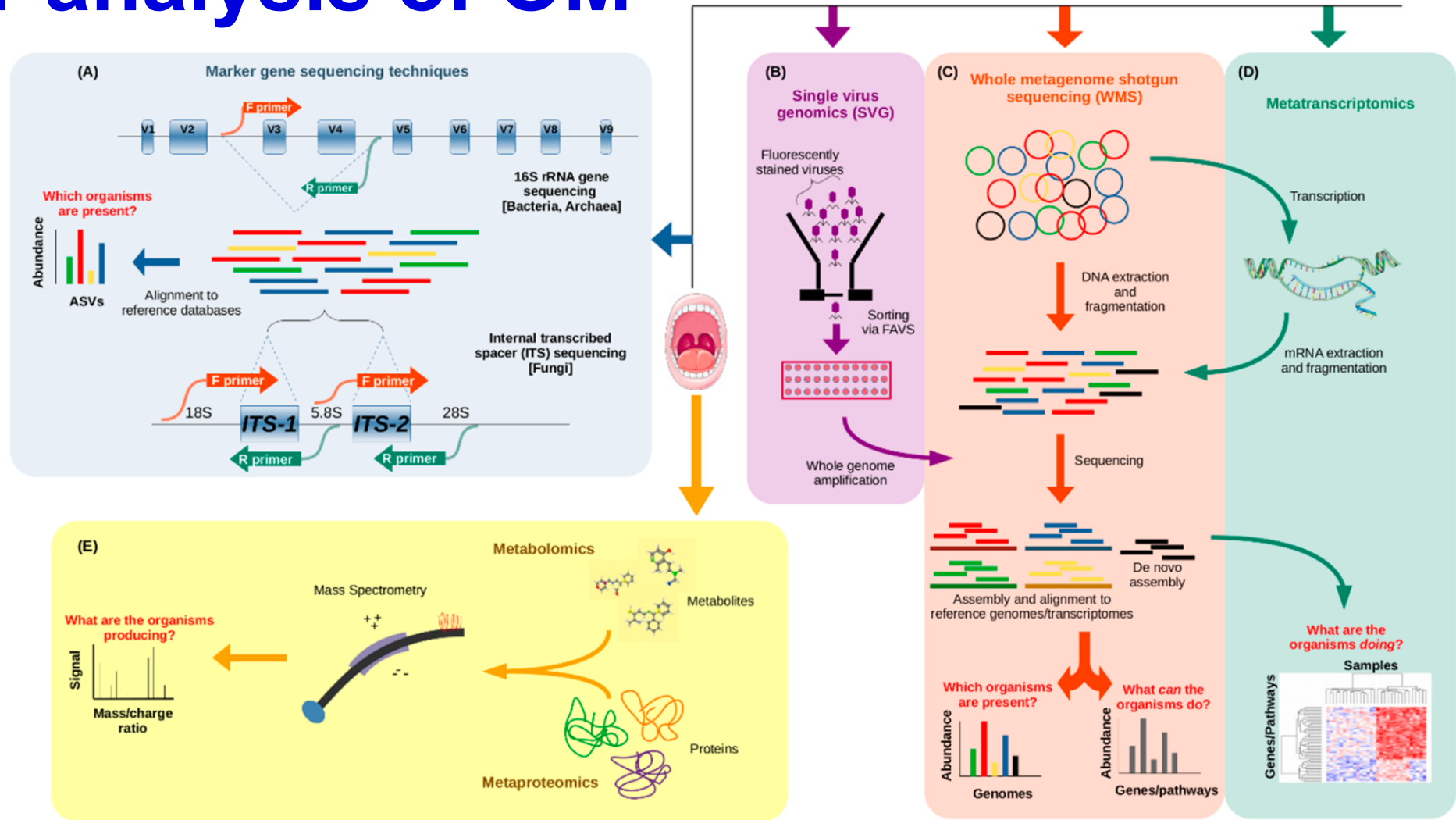
Schematics of standard techniques used in microbiome studies. (A) Marker gene sequencing techniques can use primers to target certain conserved regions of a genome to capture intermittent variable regions, which can then be used to identify organisms in a sample rapidly and inexpensively. The 16S rRNA gene is the most commonly used marker gene in bacteria and archaea, and in the figure, primers are used to capture the V3 and V4 variable regions together, a common approach for 16S sequencing. The internal transcribed spacer (ITS) region of the nuclear rRNA cistron in fungi is made of two segments, which can be captured with primers targeting the 18S, 5.8S, and 28S rRNA sections that surround them. (B–D) Instead of targeting one small segment of the genome, these techniques capture the entirety of the genetic material from an organism. (B) Single virus genomics (SVG) uses a fluorescent stain to isolate individual virus particles in a sample by fluorescence-activated virus sorting (FAVS), wherein they are embedded in an agarose bead before undergoing whole genome amplification and sequencing. (C) Whole metagenome shotgun sequencing (WMS) involves the fragmentation of all DNA in a sample, sequencing of the fragments, and assembly of the sequences, which can then be mapped to reference genomes, or de novo assembly can be performed. (D) Metatranscriptomics also involves a shotgun sequencing approach, but it is performed after mRNA extraction. The outputs then allow for differential gene expression analysis. (E) Metabolomics and metaproteomics allow for quantification of the metabolites and proteins produced by the microbiome in a sample, respectively. Mass spectrometry is a common approach to quantification.

MUNI
MED

Molecular analysis of OM

– Oral microbiome

→ clinical applications

→ prediction of susceptibility to oral diseases

→ microbial screening for systemic diseases

→ early diagnosis of a disease (before onset of symptoms)

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

→ effective tool for disease prevention (evidence of a patient's 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?)

Disease

Microbial factor	Host factor
16S rRNA seq.	Genomics
Metagenome seq.	Epigenomics
Transcriptome	Transcriptomics
Proteome	Proteomics
Metabolome	Metabolomics

Combined omics data analysis

Personalized medicine

MUNI
MED

Molecular analysis of OM

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

https://www.creative-bioarray.com/support/atp-cell-viability-assay.htm
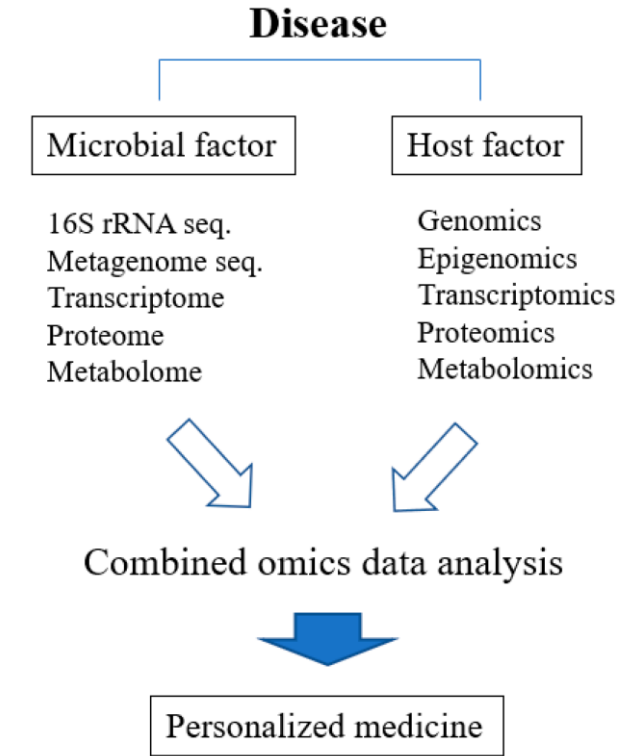
— Oral microbiome – methods of analysis

- — point-of-care testing (chair-side diagnostics):

  caries susceptibility – device **CariScreen Susceptibility Testing Meter** (Oral BioTech LLC)
  - → bacterial aktivity of *S. mutans*
  - → after wiping the plaque off the tooth surface, a bioluminescent reaction ATP occurs in a special brush, which is measured by the device

  periodontitis – BANA-Enzymatic test™ kit, Evalusite kit (immunoassay)

- — laboratory analysis

  commercial tests - example

  periodontitis – kit MyPerioPath® (OralDNA Lab)
  - → salivary test for the presence and amount of 11 bacteria species that contribute to periodontitis (quantitative real-time PCR analysis)

  ELISA test → antigens of *P. gingivalis*

Molecular analysis of OM

Table 2. Examples of biomarker assay kits in the market.

	Subgingival plaque	Evalusite	Aa, Pg, Pi	Sandwich enzyme immunoassay (Colorimetric assays)	Chairside
	Subgingival plaque	BANA-Enzymatic test kit	Pg, Td, Tf	BANA hydrolysis reaction (Colorimetric assays)	
	Gums and plaque	OMNIgene ORAL/ OMR-110	Characterization of virus species of all genome type including Aa, Pg, Pt, Fn, Td, Ec	DNA hybridization	
	Saliva	OMNIgene ORAL/ OM-501, 505			
Microbiological assay	Subgingival plaque	Carpegen® Perio Diagnostik	Aa, Pg, Tf, Td, Fn, Pi	Real-time qPCR	Company or research laboratory
	Oral rinse	MyPerioPath®	Aa, Pg, Td, Tf, En, Fn, Pi, Cr, Pm, Ec, Cs	DNA hybridization	
	Microbiological samples/subgingival plaque	iai Pado Test	Aa, Pg, Pi, Td, Tf, Fa	DNA hybridization	
	Subgingival plaque	micro-IDent®plus11	Aa, Pg, Pi, Tf, Td, Pm, Fn, Cr, En, Ec, Cs	DNA hybridization	
Genetic assay	Cheek swab	PerioPredict™	genes for IL-1	DNA hybridization	Company laboratory
	Oral rinse	MyPerioID® IL-6 or IL-1	genes for IL-6 or IL-1	Genetic polymorphisms detection	

GCF: Gingival crevicular fluid, AST: Aspartate aminotransferase, aMMP: active Matrix metalloproteinase, Aa: Aggregatibacter actino-mycetemcomitans, Pg: Porphyromonas gingivalis, Pi: Prevotella intermedia, Td: Treponema denticola, Tf: Tannerella forsythia, Fn: Fusobacterium nucleatum, Ec: Eikenella corrodens, En: Eubacterium nodatum, Fn: Fusobacterium nucleatum/periodonticum, Cr: Campy-lobacter rectus, Pm: Peptostreptococcus (Micromonas) micros, Cs: Capnocytophaga species (gingivalis, ochracea, sputigena), Fa: Filifactor alocis, IL: Interleukin, qPCR: quantitative polymerase chain reaction.

MUNI
MED

Molecular analysis of OM

— Oral microbiome

— databasis

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

Case-control study for genetic association

Cases (n=1,000) (express the trait) vs. Controls (n=1,000) (do not express the trait)

	C	T
Cases	62%	38%
Controls	49%	51%
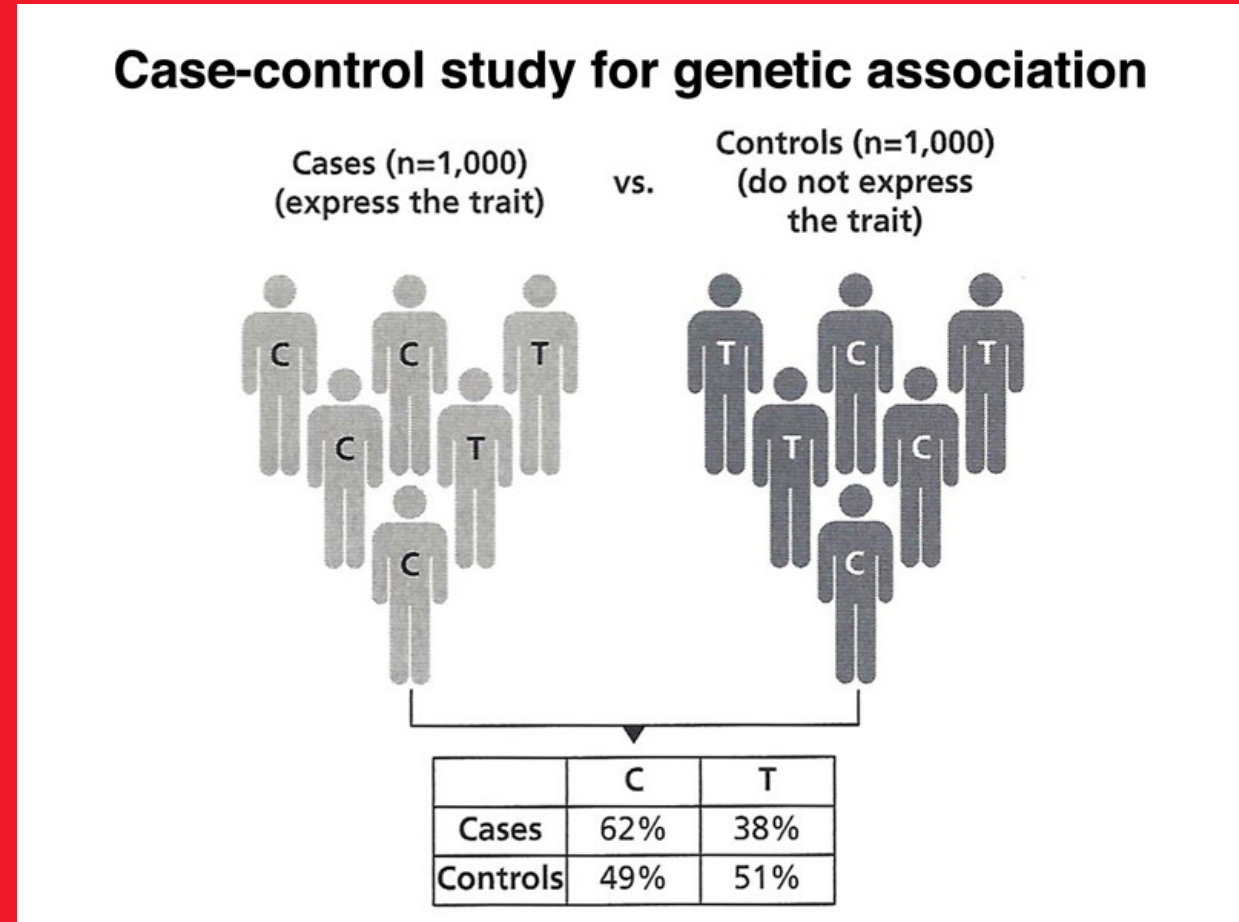
Genetic association studies

– Candidate genes

– <u>Selection of suitable candidate genes</u>

→ based on known biological, physiological or functional significance in relation to the disease

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

– <u>Suitable candidate genes for caries risk association studies</u>

→ 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

MUNI
MED

Genetic association studies

— Candidate genes

  — <u>Selection of alleles (polymorphisms)</u>

    → SNP (Single Nucleotide Polymorhism), CNV (Copy Number Variation), VNTR (Variable Number of Tandem Repeat)

    → based on studies that have been performed in other populations, GWAS, QTL

    → minor allele frequency is sufficient in chosen population
(↓ frequency of allele in the population → ↑ number of cases / controls)

    → linkage disequilibrium among SNPs → tagSNP

MUNI
MED

Genetic association studies

— Genotyping methods

— selection of an appropriate methodical approach

→ number of polymorphisms to be determined

→ total number of samples to be genotyped

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

→ costs of instruments, equipment, chemicals and consumables

→ availability of commercial genotyping services

MUNI
MED

Genetic association studies

— Genotyping methods
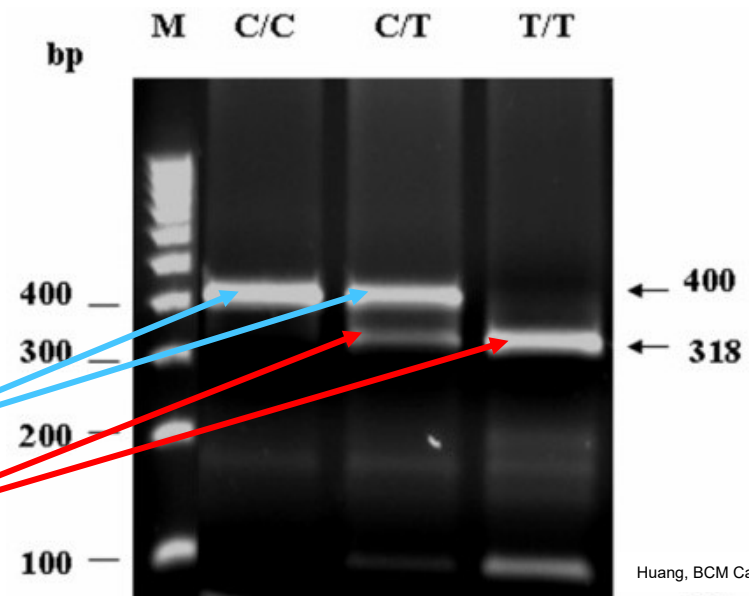
— **PCR+RFLP** (restriction fragment lenght polymorphism)

→ 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

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

— **allele-specific PCR**

→ primers that are specific for particular allele
→ if the allele is present → amplification product is generated → detection
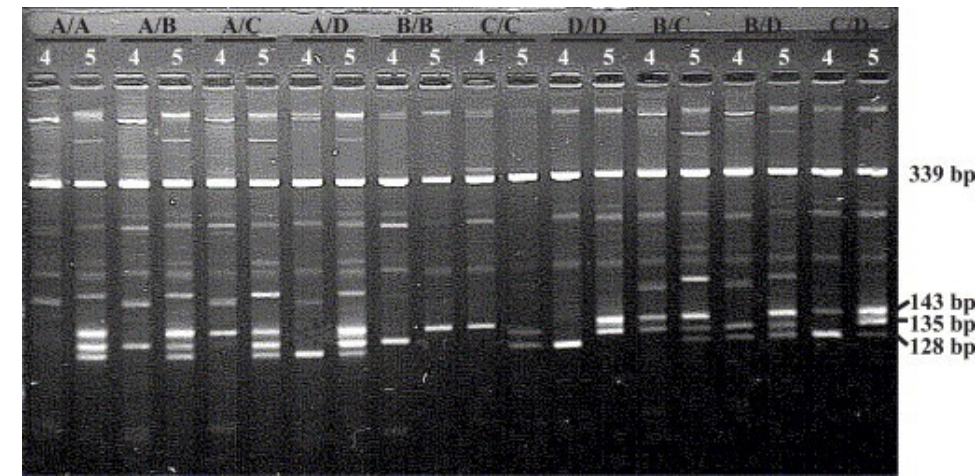
M U N I
M E D

Genetic association studies

— Genotyping methods

 — **real-time PCR** → fluorescently labeled hybridization probes
 →commercial TaqMan probes
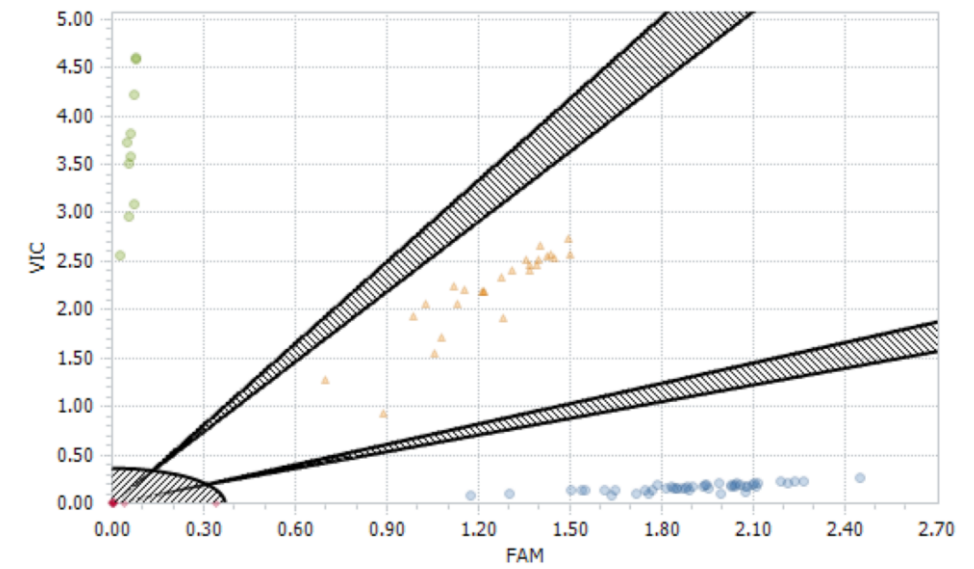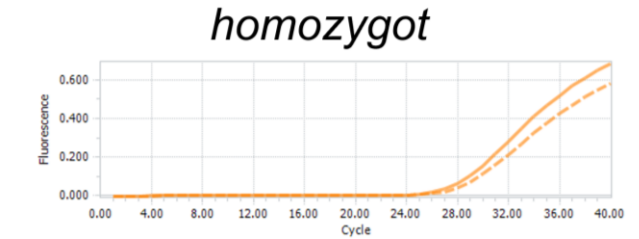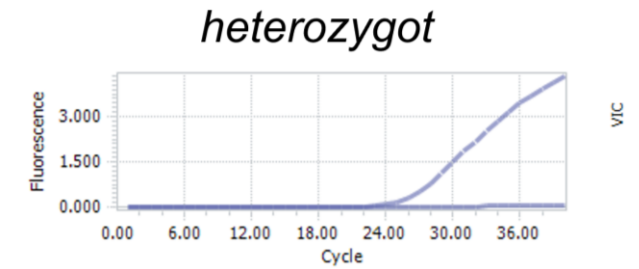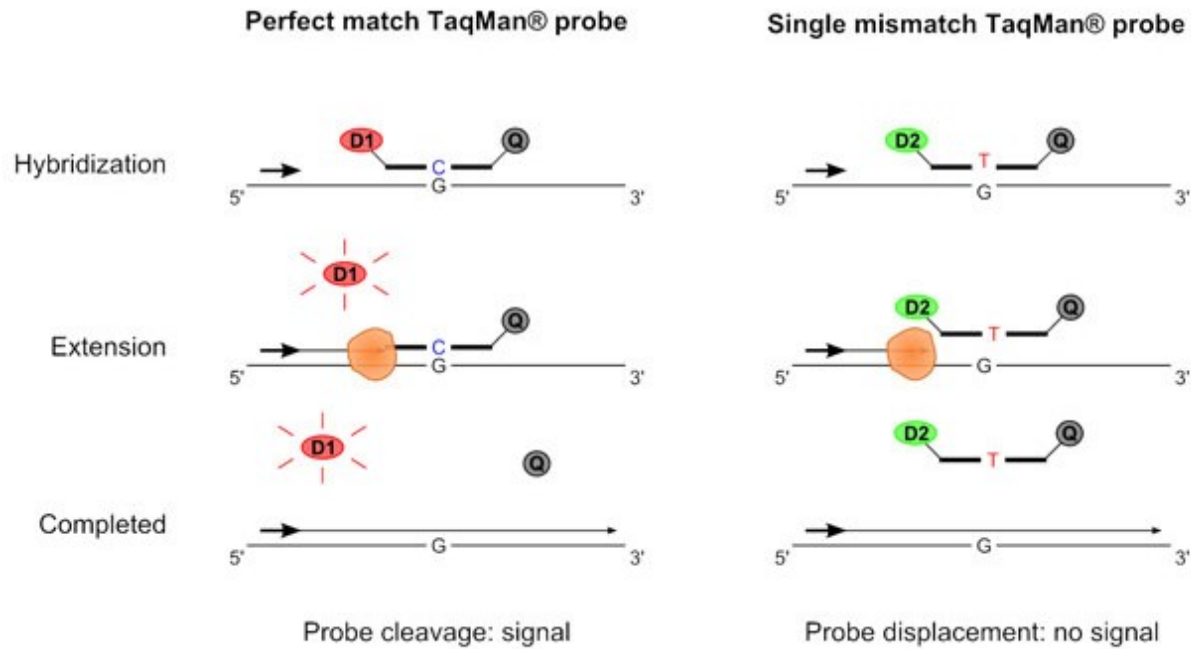
Genetic association studies

— Genotyping methods

— **Sanger sequencing** → sequence of a part of DNA with polymorphism

heterozygote

MUNI
MED

Genetic association studies

– Genotyping methods

  – Single Nucleotide Polymorphism
    Detection with the iPLEX® Assay
    and the MassARRAY® System

MUNI
MED

Genetic association studies

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

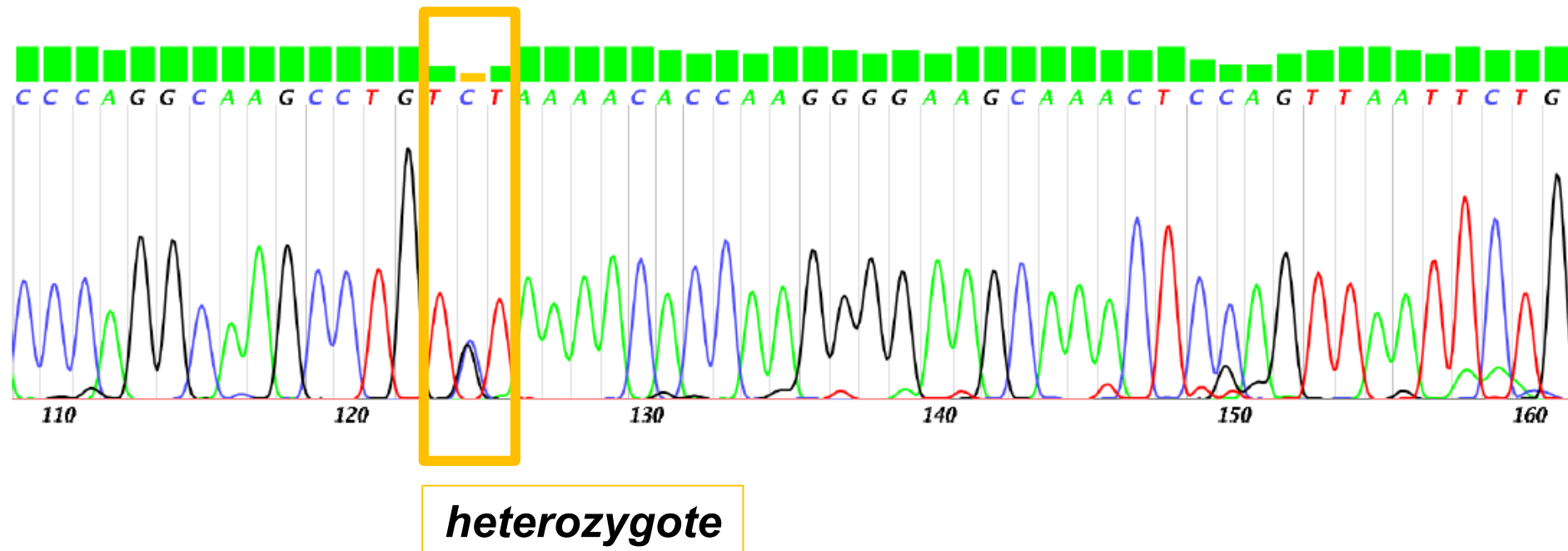|	Allele-Specific PCR (AS-PCR)	ARMS[3]/Double ARMS[3] (+ Multiplex Allele-Specific PCR)	PCR and Restriction-Fragment Length Polymorphism (PCR-RFLP)	Commercial TaqMan Assay[5]	High-Resolution Melt Analysis (HRMA)	Commercial INNO-LiPA MBL2 kit (Reverse PCR-SSOP)	Pyro-Sequencing	Sanger Sequencing	*MBL2* 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; 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

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.

— Candidate genes that have been associated with increased risk of dental carries

   — <u>Proteins involved in development of enamel</u>

     AMELX – Amelogenin gene

     ENAM – Enamelin gene
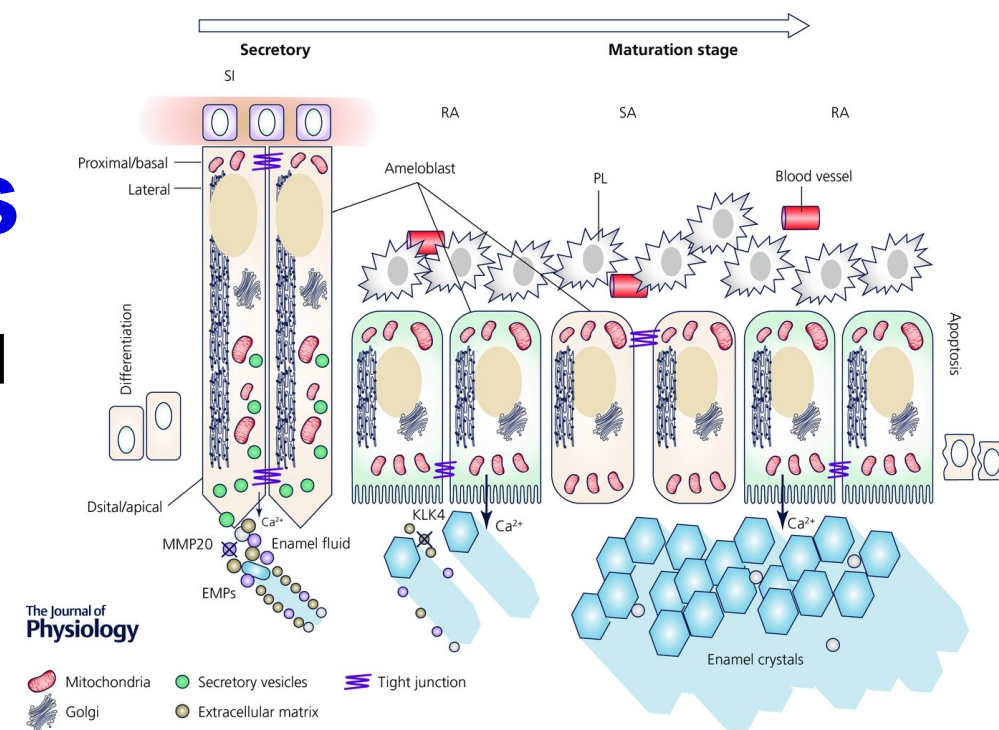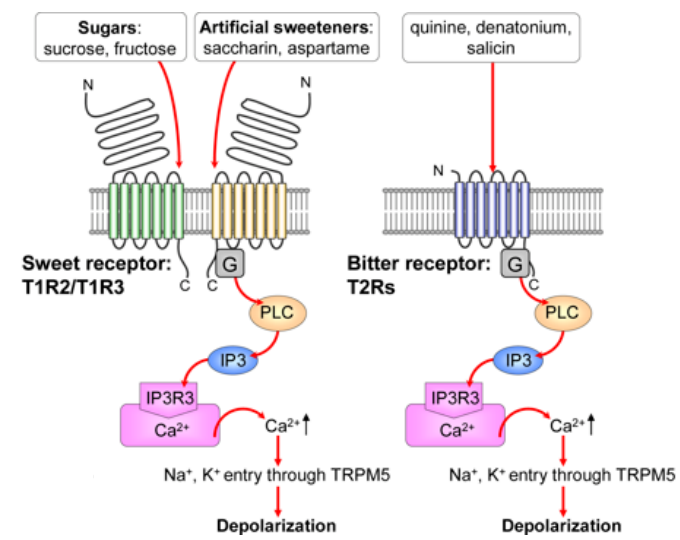
     TUFT1 – Tuftelin gene

     KLK4 – gene encoding Kallikrein-related peptidase 4

     AMBN – Ameloblastin gene

     TFIP11 – gene encoding Tuftelin-interacting protein 11

     MMPs (*MMP20*) – genes encoding Matrix Metalloproteinases

Genetic association studies

– Candidate genes that have been associated
   with increased risk of dental carries

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

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

TAS1R1/TAS1R3 – genes encoding Taste receptor 1 member 2 and 3 → G protein-coupled heterodimeric receptor, responsible for sensitivity to sweet taste

MUNI
MED

Genetic association studies

Absorption of carbohydrates

https://doi.org/10.3390/s100403411

— Candidate genes that have been associated
with increased risk of dental carries
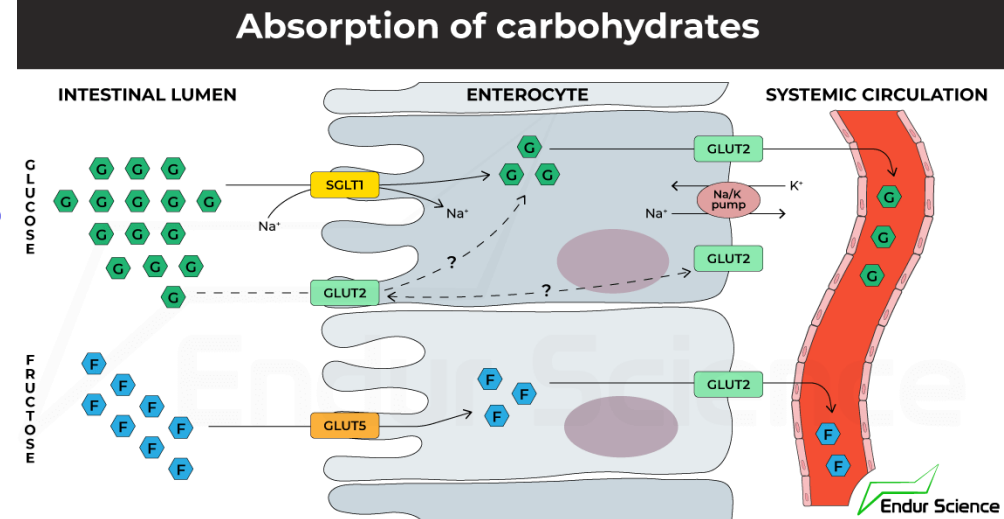
— <u>Glucose transporter → associated with ↑ preference for sweet taste → ↑ sugar intake</u>

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
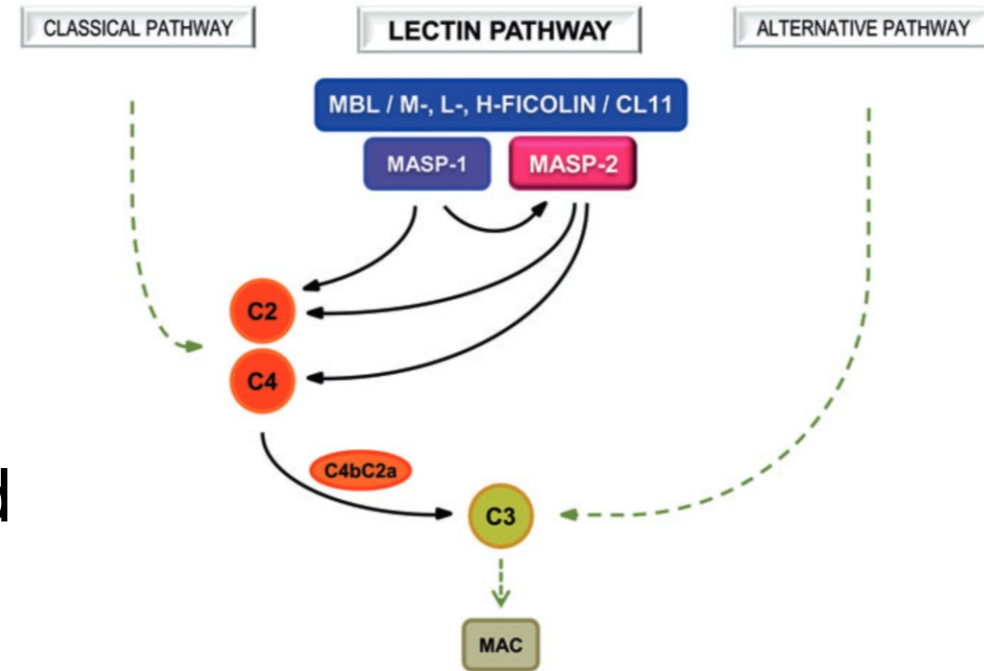
https://doi.org/10.1080/00016357.2020.1832253

MUNI
MED

Genetic association studies

— Candidate genes that have been associated with increased risk of dental carries

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.
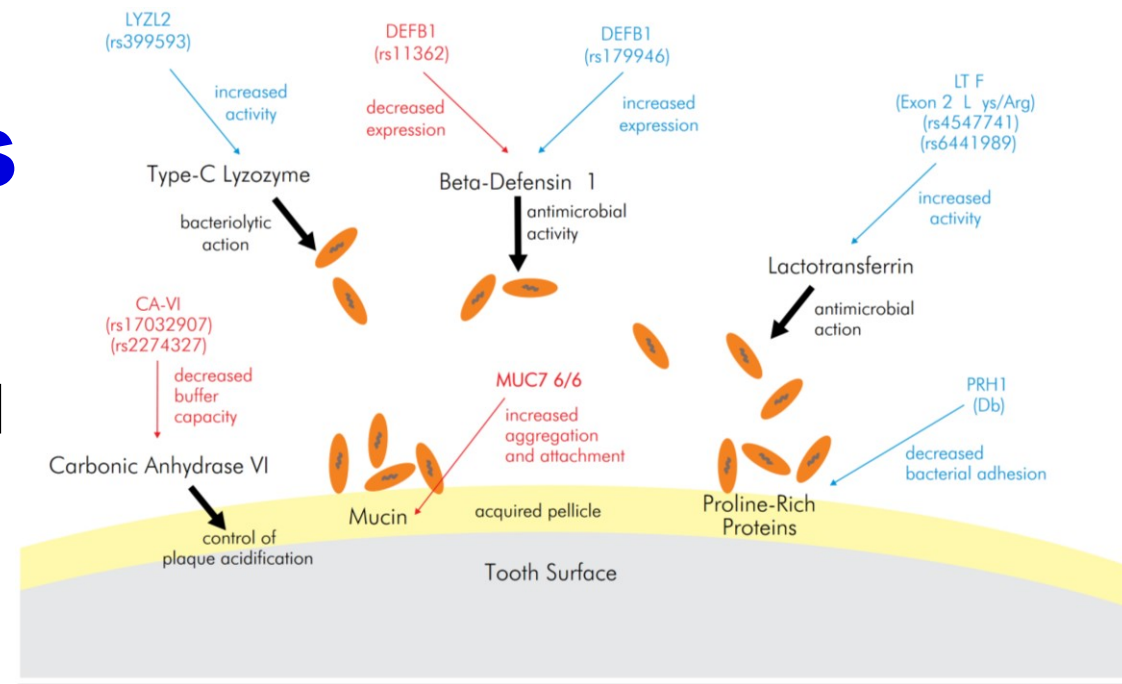
— <u>Proteins of immune system</u>

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

MUNI
MED

Genetic association studies

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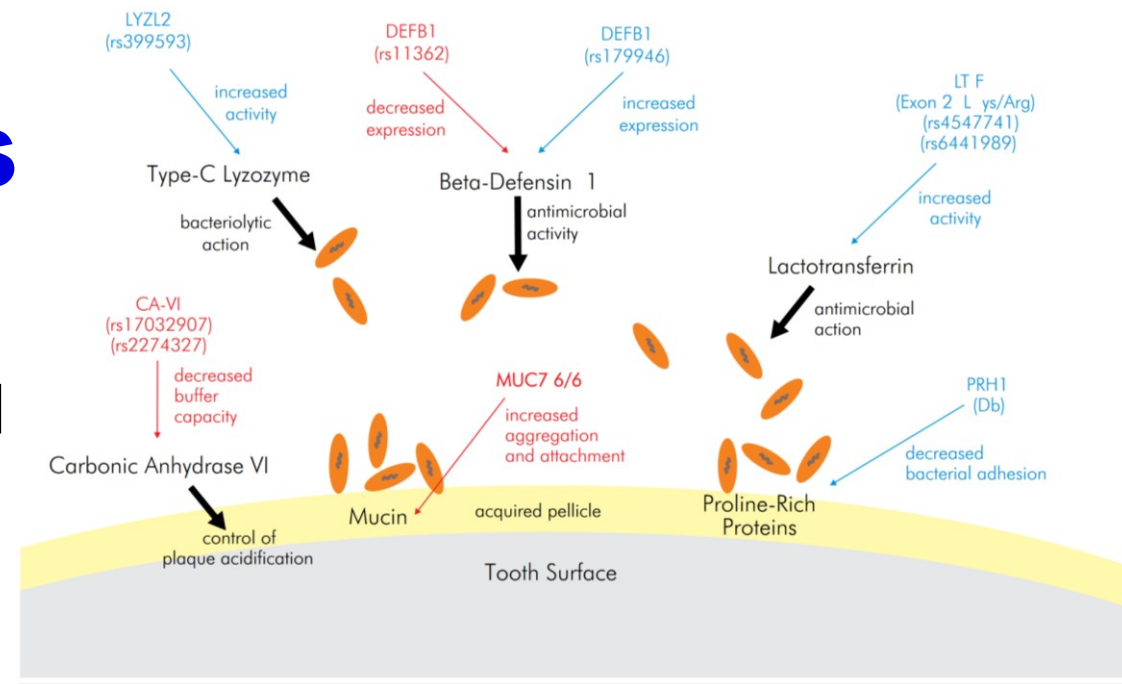
https://doi.org/10.1590/1807-3107bor-2017.vol31.0041

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).

MUNI
MED

Genetic association studies

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

— Candidate genes that have been associated with increased risk of dental carries

  — Proteins in saliva

CA6 – gene encoding Carbonic anhydrase VI – enzyme also called ‚gustin', catalyzes the hydration 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
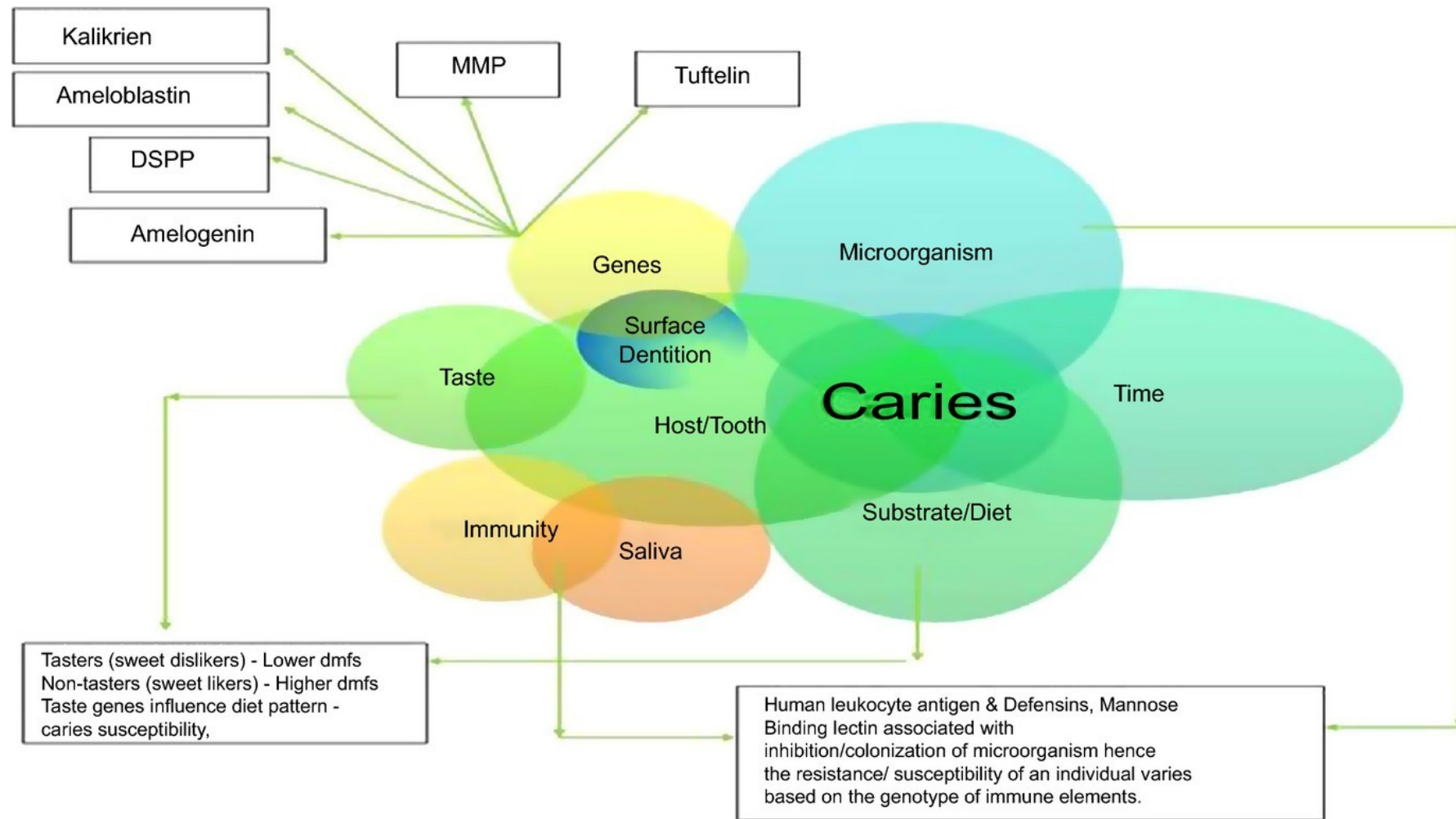
PRH1 – gene encoding salivary acidic proline-rich phosphoprotein 1, participates in the formation of acquired pellicle and thus in bacterial adhesion and biofilm formation

MUNI
MED

Genetic association studies
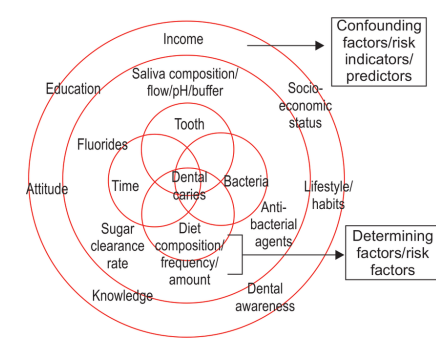
– Pitfalls of genetic association studies of dental caries

- too many factors playing role in etiopathogenesis → set of patients (cases) can never be perfectly categorized → 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 studies are needed to understand the correlations found

- 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) → strengthening of the results

- meta-analysis – a combination of data obtained by an exhaustive search of published and unpublished worldwide data → 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

MUNI
MED

MUNI
MED