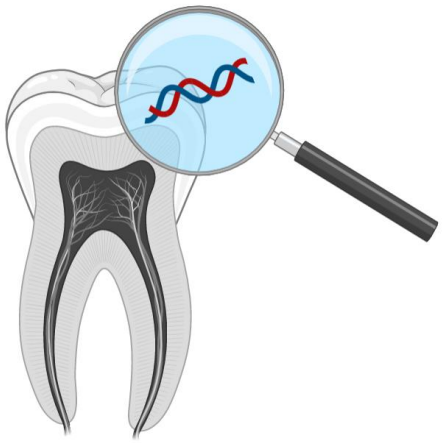
Research Activities in Dentistry – Practical Course

Mgr. Tereza Deissová
Assoc. Prof. RNDr. Petra Bořilová Linhartová, Ph.D., MBA



Masaryk university

Žerotínovo nám. 617/9, 601 77 Brno, Czech Republic
T: +420 549 49 1111, E: info@muni.cz, W: www.muni.cz

Research Activities in Dentistry – Practical Course

Specializations: Dentistry (Faculty of Medicine, M-ZL)

Aims: The students will acquire practical experience in molecular-biological analyses that are routinely used in the field of dentistry, both in research and in the diagnosis of patients with diseases of (not only) the oral cavity.

The tasks: Students will learn how to take samples from the oral mucosa with the subsequent DNA isolation and real-time PCR. Students will learn how to determine two single-nucleotide polymorphisms in the gene for methylenetetrahydrofolate reductase (MTHFR), an enzyme playing an important role in the metabolism of the folic acid. The students will learn to predict the phenotype (i.e., the ability to metabolize the folic acid) after determining the *MTHFR* haplogenotype. If they analyse their own samples of buccal swabs, this information can be useful for them in preventing some diseases. Students preferring to analyze other than their own samples will be given the opportunity to perform the isolation on anonymous samples that are intended for research purposes.

Authors:

Mgr. Tereza Deissová[1,2]

Assoc. Prof. RNDr. Petra Bořilová Linhartová, Ph.D., MBA[1,2,3,4]

[1] Clinic of Stomatology, Faculty of Medicine, Masaryk University Brno

[2] Department of Patophysiology, Faculty of Medicine, Masaryk University Brno

[3] Institute of Medical Genetics, Faculty of Medicine, Masaryk University Brno

[4] Clinic of Maxillofacial Surgery, University Hospital Brno

Opponents:

RNDr. Mária Hovořáková, Ph.D.

Institute of Histology and Embryology, First Faculty of Medicine, Charles University

Assoc. Prof. MUDr. Jakub Suchánek, Ph.D.

Department of Dentistry, Faculty of Medicine in Hradec Kralove, Charles University

Acknowledgements: This course is supported by the project MUNI/FR/1406/2019 Research Activities in Dentistry. The authors would like to thank Prof. MUDr. Lydie Izakovičová Hollá, Ph.D. (Clinic of Stomatology, Faculty of Medicine, Masaryk University Brno) for her critical review of this text.

1. Theoretical background

Folates and methylenetetrahydrofolate reductase

Folate is a general term used to describe various chemical forms of water-soluble vitamin B9 and its derivatives. In some foods, such as citruses, legumes, liver, yeasts, or leaf vegetables, folates naturally occur in the form of so-called pteroylpolyglutamates. These are hydrolyzed in the intestine to pteroylmonoglutamates that cross the intestinal wall into the bloodstream and subsequently converted to tetrahydrofolate. The food supplements and foods artificially supplemented with folates usually contain the pteoryl-monoglutamic acid, widely known as folic acid. Folic acid must be first metabolized to dihydrofolate using the hepatic enzyme dihydrofolate reductase and subsequently to tetrahydrofolate. Tetrahydrofolate methylation then leads to the production of 5,10-methylenetetrahydrofolate, which is involved in the methionine cycle.[1,2] Reduction of 5,10-methylenetetrahydrofolate by the key enzyme. Methylenetetrahydrofolate reductase (MTHFR) produces the active form of folate, 5-methyltetrahydrofolate, serving as a donor of the methyl group in the subsequent reaction resulting in homocysteine remethylation and formation of an essential amino acid methionine,[3] see Fig. 1.

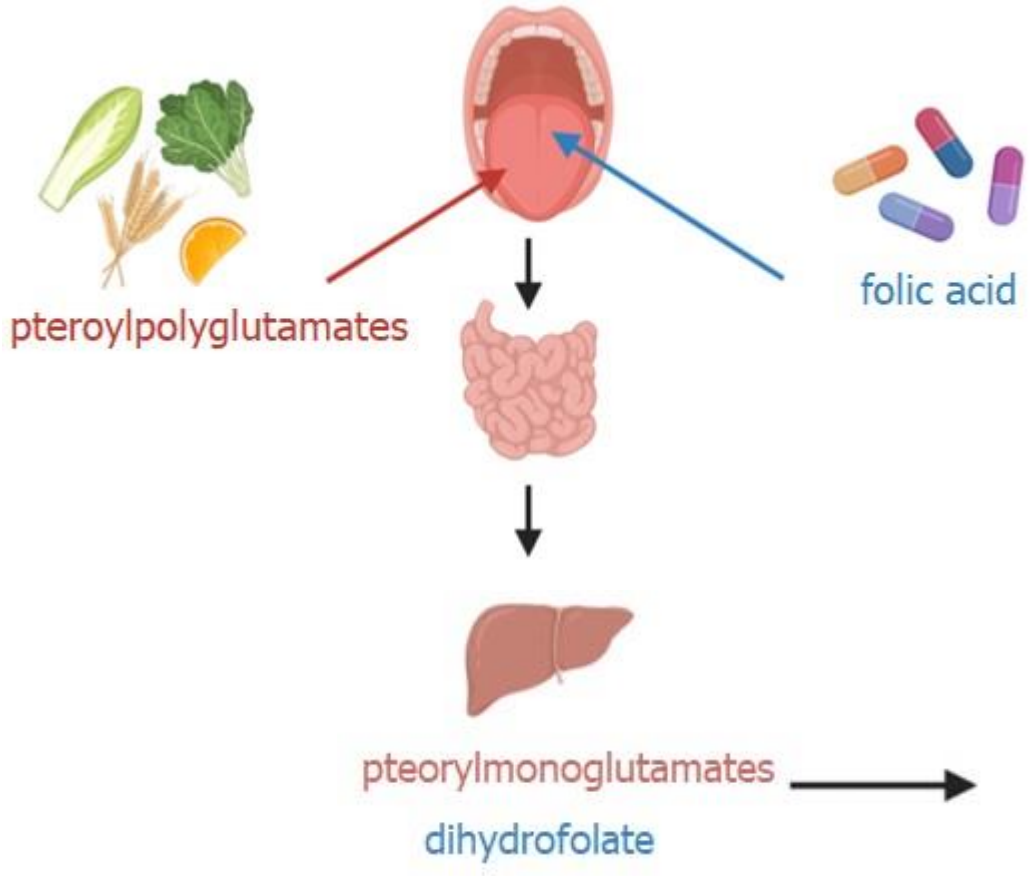


Fig. 1: A simplified scheme of metabolization of natural and synthetic folates (created using the software BioRender.com)- the picture continues on the next page

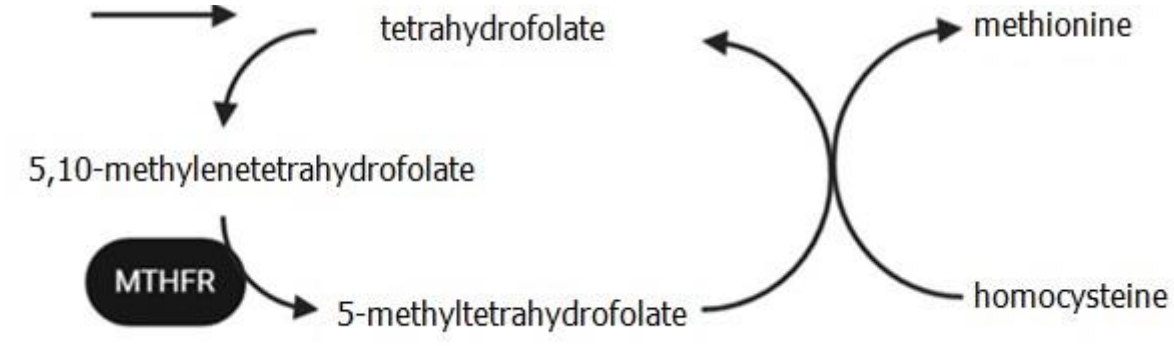


Fig. 1: A simplified scheme of metabolization of natural and synthetic folates (created using the software BioRender.com)

Unlike plants and bacteria, humans do not synthesize vitamin B9 in their body. So they get vitamin B9 as folate from natural foods or as folic acid. For adults, the recommended amount is 400 µg of folate equivalents per day; this recommendation is increased for pregnant women (600 µg/day). The typical diet in the most European countries is, however, deficient in folates. Moreover, their absorption is influenced by many factors. First of all, the folate form is of utmost importance (synthetic folates are taken in more easily than natural ones), other factors include long-term use of certain drugs and alcohol consumption. Folates are also heat-sensitive and susceptible to photodegradation. Besides, some individuals have problems with folate metabolization.[4,5]

Variability in the methylenetetrahydrofolate reductase gene

Two single-nucleotide polymorphisms C677T and A1289C can be found in the gene encoding the MTHFR enzyme. Both reduce the enzymatic activity of MTHFR, thus negatively affecting homocysteine metabolization.[6] The term single-nucleotide polymorphism (SNP) describes the substitution of one nucleotide in the DNA molecule. In these particular cases, cytosine (original allele) is replaced with thymine (mutant allele) at the 677 position of the *MTHFR* gene (C677T polymorphism) and substitution of adenine (original allele) for cytosine (mutant allele) at the 1298 position of the *MTHFR* gene (A1298C). The mutant allele can be either formed „de novo“ or can be inherited from the parents. As both these SNPs are present in the so-called “coding region” of the DNA, the mutations can affect the resulting product of gene transcription, i.e., the MTHFR enzyme.

In a diploid cell, every gene is expressed by two alleles constituting a genotype. Carriers of identical alleles are called homozygotes, carriers of different alleles heterozygotes. In CC homozygotes (in the case of C677T polymorphisms) and AA homozygotes (in the case of A1298C), the enzymatic activity of MTHFR is normal. In the case of heterozygotes (i.e., those who have one mutant allele, the enzymatic activity of MTHFR is reduced. In homozygotes with both alleles mutant, i.e., the TT (in C677T) or CC (A1298C) genotype, respectively, the enzymatic activity of MTHFR is reduced by up to 70 %, see Fig. 2. Moreover, the effect of both polymorphisms on the resulting phenotype is additive; this means that in individuals with the combination of TT (C677T) and CC (A1298C) genotypes, i.e., TTCC, haplogenotype, the MTHFR enzymatic activity is the lowest, see Table 1.

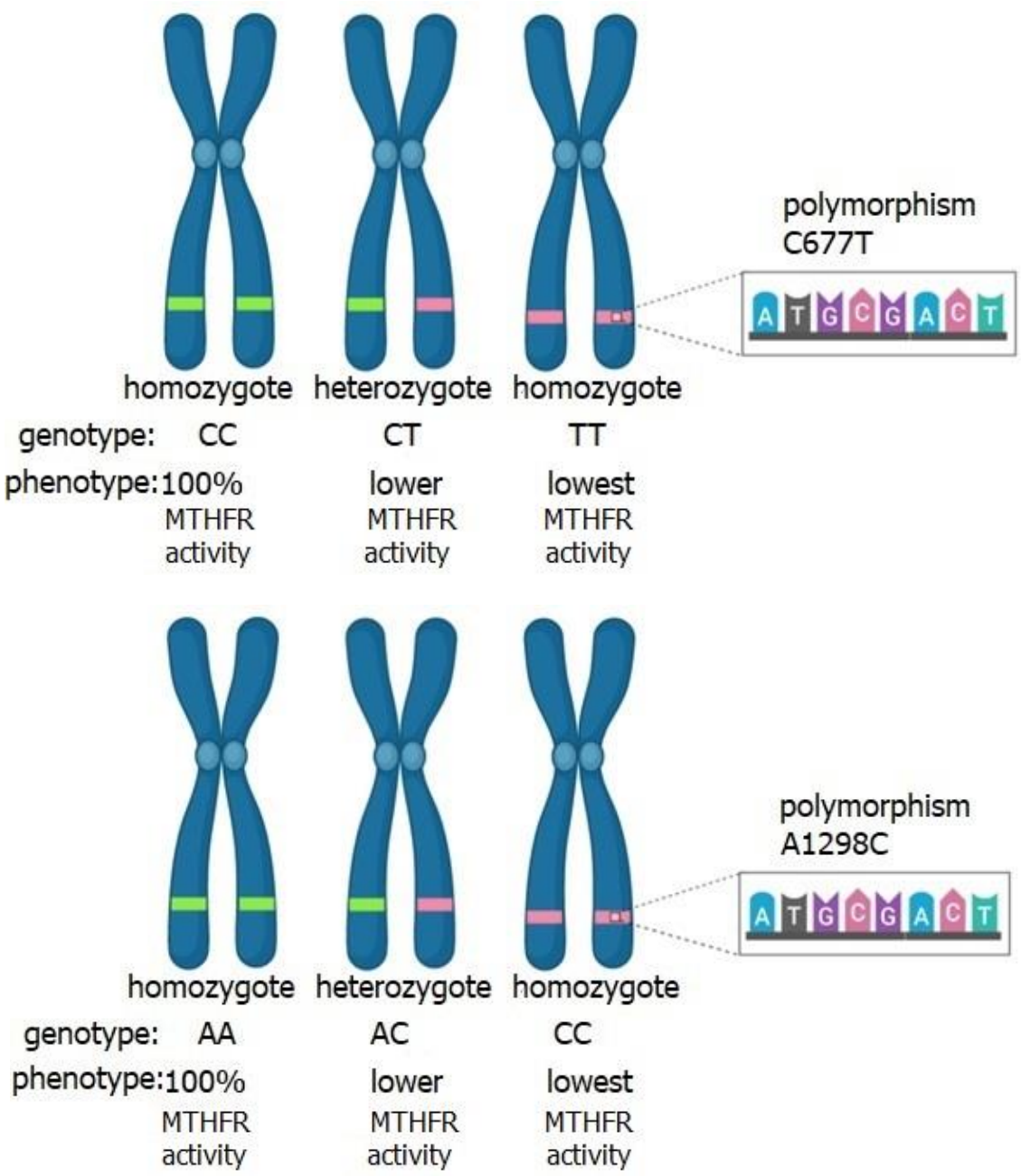


Fig. 2: Polymorphisms in the gene for methylenetetrahydrofolate reductase (*MTHFR* C677T; A1298C) and their effect on the MTHFR enzymatic activity (created using the software BioRender.com)

Table 1: Individual haplogenotypes arising from the combinations of both SNPs

SNP	A1298T genotype AA	A1298T genotype AC	A1298T genotype CC
C677T genotype CC	CCAA 100% MTHFR activity	CCAC 80%	CCCC 60%
C677T genotype CT	CTAA 65%	CTAC 50%	CTCC 30%
C677T genotype TT	TTAA <30%	TTAC <30%	TTCC <10% MTHFR activity

Diseases associated with the lack of vitamin B9 and MTHFR enzymopathy

The insufficient folate intake, potentially combined with the reduced MTHFR enzymatic activity, manifests predominantly at the level of rapidly proliferating cells (lymphocytes, erythrocytes), most commonly as megaloblastic anemia, but it can also be associated with the development of hyperhomocysteinemia and DNA synthesis disorders. All these symptoms are risk factors for cardio- and cerebrovascular diseases and for neural tube defects during prenatal development.[5,7]

Congenital developmental disorders and hormonal contraceptives

In folate deficiency, the risk of congenital developmental disorders (among others cleft defects), pregnancy abortion, or placental abruption is increased. The lack of folic acid can also reduce fertility and it is, therefore, recommended to increase the intake of folates before planned conception. This is particularly important in women with one-sided diet, smokers, and women using oral hormonal contraceptives, whose folic acid levels are significantly reduced. When discontinuing hormonal contraceptives, it takes up to approx. three months to replenish the folate levels to the original concentrations.[8,9]

Cardiovascular diseases

The increased homocysteine concentration in blood is associated with oxidative stress, endothelial damage, an increase thrombogenicity and the risk of atherosclerosis development. A recent meta-analysis confirmed that the supplementation of folic acid reduced the homocysteine concentration and, in turn, a significant reduction of stroke[10] and coronary artery disease was observed.[11]

Recurrent aphthous stomatitis

Recurrent aphthous stomatitis (RAS) is a chronic disease of the oral mucosa manifesting as painful erosions or ulcerations. It is a multifactorial disease, the etiopathogenesis of which may include local trauma, food allergies, microbial dysbiosis, infectious agents, stress, hormonal changes, patient's immunological profile, genetic predispositions, and nutrition

(deficiency of folic acid, Fe ions, or vitamin B12). So far, the treatment of RAS is only symptomatic and the response to treatment is insufficient in some patients.[12]

The analysis of dietary history questionnaires revealed that RAS patients had significantly lower daily intakes of vitamin B12 and folates.[13] Moreover, they have significantly higher homocysteine blood levels and lower concentrations of hemoglobin, Fe ions, vitamin B12, and folic acid.[14] Lower vitamin B12, folate, and ferritin levels were detected in the saliva of RAS patients as well.[15] Kalkan *et al*. (2014) demonstrated the association of the mutant allele T and the TT genotype of the C677T with a higher number/frequency of oral ulcerations in RAS patients.[16] Individuals suffering from RAS, who show a reduced capacity for folate metabolization, can benefit from modified Škach's vitamin treatment (active folate, vitamin B6 and vitamin D3).[17]

Gingivitis and caries

Gingivitis (inflammation of the gingiva) is a relatively widespread disease. Early diagnosis and treatment can prevent further tissue damage and the development of periodontitis. Poor oral hygiene is the principal risk factor for the development of this disease as it facilitates the accumulation of food remains and biofilm formation. Bacteria from that plaque then attack the cells of the mucosa and damage them, which triggers the immune response and a further progression of the inflammation. Other risk factors include smoking and hormonal changes.[18] The use of a mouthwash containing folate at a concentration of 1 mg/1 mL twice a day for 4 weeks led in patients with gingivitis to a reduction in the number of spots with the mucosal discoloration and gingivitis-associated bleeding compared to a placebo control group.[19] The increased dietary intake of folic acid was also shown to have a protective effect in gingival bleeding, which can potentially represent a novel approach in the clinical treatment of gingivitis and in supporting gingival health.[20]

Caries is a widespread disease affecting the hard dental tissue. It is caused by bacteria covering the surface of the teeth that metabolize the fermentable carbohydrates from the food to organic acids. These demineralize the enamel and reduce its hardness. Both environmental and genetic factors contribute to the development of this disease.[21] A high amount of homocysteine in saliva as an oxidative marker caused by reduced folate intake affects the carious activity.[22,23] Based on the research in children of the Czech population, it appears that the original (wild type) alleles of C677T and A1298C polymorphisms in the *MTHFR* gene (that do not affect folate metabolization) are protective factors preventing the development of both these diseases – gingivitis and caries.[24]

Folate deficiency can be countered with food supplements or with foods artificially supplemented with folates. In patients with metabolic defects caused by the MTHFR polymorphisms, however, it is much preferable to supplement the folates directly as 5-methyltetrahydrofolate (active folate) and, therefore, to prevent the accumulation of unmetabolized folic acid in the peripheral circulation, which can be associated with adverse effects.[25]

References

(1) Suitor, C. W.; Bailey, L. B. Dietary Folate Equivalents: Interpretation and Application. *J. Am. Diet. Assoc.* **2000**, *100* (1), 88–94. https://doi.org/10.1016/S0002-8223(00)00027-4.

(2) Lucock, M. Folic Acid: Nutritional Biochemistry, Molecular Biology, and Role in Disease Processes. *Mol. Genet. Metab.* **2000**, *71* (1), 121–138. https://doi.org/10.1006/mgme.2000.3027.

(3) Födinger, M.; Hörl, W. H.; Sunder-Plassmann, G. Molecular Biology of 5,10-Methylenetetrahydrofolate Reductase. *J. Nephrol.* **2000**, *13* (1), 20–33.

(4) McNulty, H.; Pentieva, K. Folate Bioavailability. *Proc. Nutr. Soc.* **2004**, *63* (4), 529–536. https://doi.org/10.1079/PNS2004383.
(5) Stránský, M. Preventivní účinky kyseliny listové. *Interní Medicína* **2011**, *13* (4), 159–162.
(6) Weisberg, I.; Tran, P.; Christensen, B.; Sibani, S.; Rozen, R. A Second Genetic Polymorphism in Methylenetetrahydrofolate Reductase (MTHFR) Associated with Decreased Enzyme Activity. *Mol. Genet. Metab.* **1998**, *64* (3), 169–172. https://doi.org/10.1006/mgme.1998.2714.
(7) CGB laboratoř a.s. Molekulárně genetické vyšetření vybraných sekvenčních variant genů asociovaných s rozvojem kardiovaskulárních onemocnění http://www.pathology.cz/molekularne-geneticke-vysetreni-vybranych-sekvencnich-variant-genu-asociovanych-s-rozvojem-kardiovas--2455.html (accessed Oct 6, 2020).
(8) Doležálková, E.; Unzeitig, V. Kyselina listová a prevence rozštěpových vad centrálního nervového systému. *Čes Gynek 2014* (2), 134–139.
(9) Shere, M.; Bapat, P.; Nickel, C.; Kapur, B.; Koren, G. Association Between Use of Oral Contraceptives and Folate Status: A Systematic Review and Meta-Analysis. *J. Obstet. Gynaecol. Can.* **2015**, *37* (5), 430–438. https://doi.org/10.1016/S1701-2163(15)30258-9.
(10) Wang, Y.; Jin, Y.; Wang, Y.; Li, L.; Liao, Y.; Zhang, Y.; Yu, D. The Effect of Folic Acid in Patients with Cardiovascular Disease. *Medicine (Baltimore)* **2019**, *98* (37). https://doi.org/10.1097/MD.0000000000017095.
(11) Homocysteine Studies Collaboration. Homocysteine and Risk of Ischemic Heart Disease and Stroke: A Meta-Analysis. *JAMA* **2002**, *288* (16), 2015–2022. https://doi.org/10.1001/jama.288.16.2015.
(12) Borilova Linhartova, P.; Valová; Izakovicova Holla, L. Vrozená Náchylnost k Recidivující Aftózní Stomatitidě (Přehledový Článek) Genetic Predisposition to Recurrent Aphthous Stomatitis (Review). *Ceskoslovenská Stomatol.* **2017**, *117*, 27–34.
(13) Kozlak, S. T.; Walsh, S. J.; Lalla, R. V. Reduced Dietary Intake of Vitamin B12 and Folate in Patients with Recurrent Aphthous Stomatitis. *J. Oral Pathol. Med.* **2010**, *39* (5), 420–423. https://doi.org/10.1111/j.1600-0714.2009.00867.x.
(14) Sun, A.; Chen, H.-M.; Cheng, S.-J.; Wang, Y.-P.; Chang, J. Y.-F.; Wu, Y.-C.; Chiang, C.-P. Significant Association of Deficiencies of Hemoglobin, Iron, Vitamin B12, and Folic Acid and High Homocysteine Level with Recurrent Aphthous Stomatitis. *J. Oral Pathol. Med.* **2015**, *44* (4), 300–305. https://doi.org/10.1111/jop.12241.
(15) Pourzare mehrbani, S.; Motahari, P.; Zaheri, F. Evaluation of Salivary Folate, Ferritin, and Vitamin B12 in Patients with Recurrent Aphthous Ulcers. *J. Mashhad Dent. Sch.* **2020**, *44* (3), 209–215.
(16) Kalkan, G.; Karakus, N.; Yigit, S. Association of MTHFR Gene C677T Mutation with Recurrent Aphthous Stomatitis and Number of Oral Ulcers. *Clin. Oral Investig.* **2014**, *18* (2), 437–441. https://doi.org/10.1007/s00784-013-0997-0.
(17) Bořilová Linhartová, P.; Fassmann, A.; Linhartová, J.; Izakovičová Hollá, L. Farmakoterapie Recidivující Aftózní Stomatitidy u Pacientů s Geneticky Podmíněnou Sníženou Schopností Metabolizovat Kyselinu Listovou - Pilotní Studie. *Pharmacother. Recurr. Aphthous Stomatitis Patients Genet. Impair. Abil. Metab. Folic Acid - Pilot Study* **2019**, *119* (1), 4–11.
(18) Cope, G.; Cope, A. Gingivitis: Symptoms, Causes and Treatment. *Dent. Nurs.* **2011**, *7* (8), 436–439. https://doi.org/10.12968/denn.2011.7.8.436.
(19) Pack, A. R. C. Folate Mouthwash: Effects on Established Gingivitis in Periodontal Patients. *J. Clin. Periodontol.* **1984**, *11* (9), 619–628. https://doi.org/10.1111/j.1600-051X.1984.tb00914.x.
(20) Esaki, M.; Morita, M.; Akhter, R.; Akino, K.; Honda, O. Relationship between Folic Acid Intake and Gingival Health in Non-Smoking Adults in Japan. *Oral Dis.* **2010**, *16* (1), 96–101. https://doi.org/10.1111/j.1601-0825.2009.01619.x.
(21) Opal, S.; Garg, S.; Jain, J.; Walia, I. Genetic Factors Affecting Dental Caries Risk. *Aust. Dent. J.* **2015**, *60* (1), 2–11. https://doi.org/10.1111/adj.12262.
(22) LeBlanc, J. G.; Giori, G. S. D. *B Group Vitamins: Current Uses and Perspectives*; BoD – Books on Demand, 2018.
(23) Kumar, D.; Pandey, R. K.; Agrawal, D.; Agrawal, D. An Estimation and Evaluation of Total Antioxidant Capacity of Saliva in Children with Severe Early Childhood Caries. *Int. J. Paediatr. Dent.* **2011**, *21* (6), 459–464. https://doi.org/10.1111/j.1365-263X.2011.01154.x.
(24) Bořilová Linhartová, P.; Mrázková, J.; Novák, D.; Kukletová, M.; Kukla, L.; Izakovičová Hollá, L. Association of methylenetetrahydrofolate reductase (MTHFR) gene variants with dental caries and gingivitis in Czech children. **2018**.
(25) Scaglione, F.; Panzavolta, G. Folate, Folic Acid and 5-Methyltetrahydrofolate Are Not the Same Thing. *Xenobiotica* **2014**, *44* (5), 480–488. https://doi.org/10.3109/00498254.2013.845705.

2. Experimental part

In the experimental part, students will perform a swab from the patient's (classmate's) buccal mucosa. This simple, non-invasive method of sample collection brushes off epithelial cells of the buccal mucosa that are suitable for extraction of DNA in the sufficient quality and amount for the subsequent molecular-biological analyses. Students will also isolate the DNA themselves, evaluate its quality and quantity, prepare qPCR, and assess and interpret the results.

2.1. Collection of the biological material

For 1 hour prior to the sample collection, the "patient" should not eat, drink (except for water) and perform oral hygiene. The epithelial cells are collected using a collection brush.

Method:

- Put protective gloves on
- Take the brush from the package without touching anything; use only the end for holding and manipulating the brush, see Fig. 3

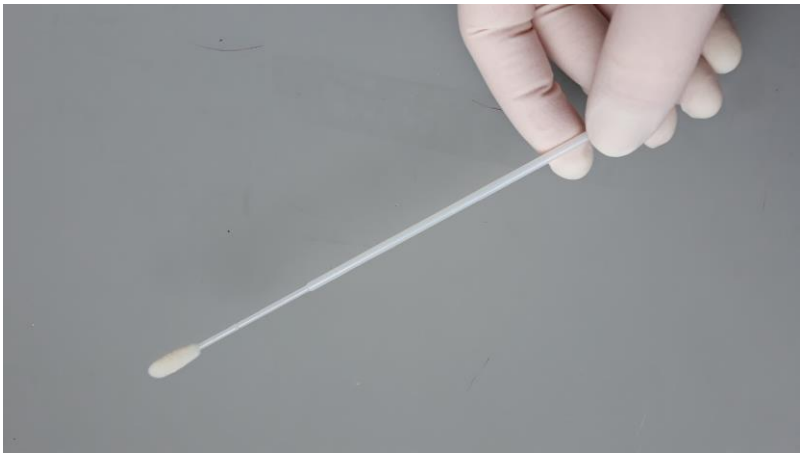


Fig. 3: Manipulation with the collection brush

- Rub the brush across the buccal mucosa for approx. 1-2 minutes, rotating it (see Fig. 4)

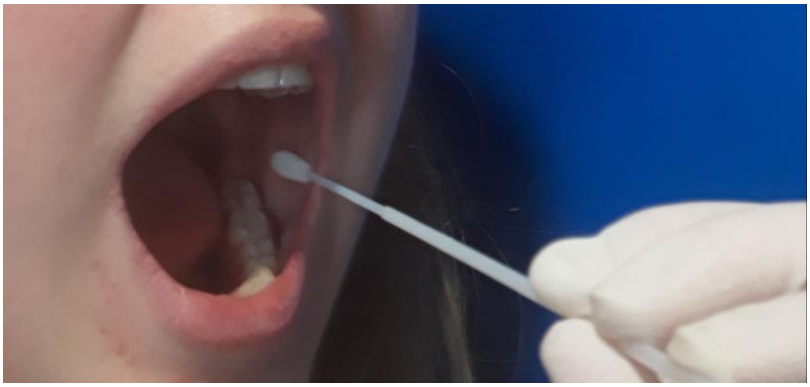


Fig. 4: Cell collection from the buccal mucosa

- Insert the brush into the test tube with the lytic solution and proteinkinase K and break the brush rod off at the designated break-off point

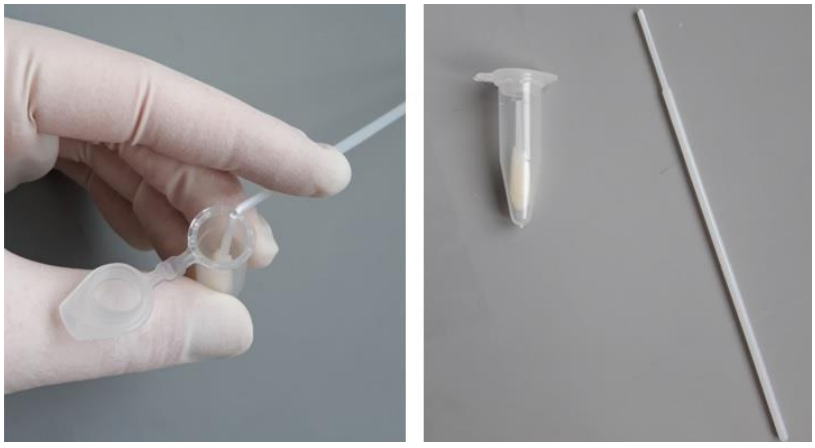


Fig. 5: Breaking off the brush rod into the lytic solution with proteinkinase K

2.2. DNA isolation

There are many DNA extraction techniques, one of which is DNA isolation using commercially available silicate columns. First, the cellular and nuclear membranes of the epithelial cells must be disrupted to release the DNA. This is the purpose of the lytic solution, the principal components of which are the detergent and chaotropic agent. The entire process is assisted by the enzyme proteinkinase K, degrading besides proteins also RNAses and DNAses (i.e., enzymes breaking down nucleic acids). After lysis, DNA is purified through precipitation with 96 % ethanol and subsequently transferred to the silicate column. There, assisted by the high concentration of chaotropic salts, DNA binds to the silicate column via hydrogen bonds. Centrifugation leads to washing out all components but DNA from the column. DNA is then eluted from the column using water or a buffer with low content of salts and second centrifugation.

Method:

- Vortex the tube with 400 µL of the lytic solution T1 and 25 µL of proteinkinase K twice for 5 s, see Fig. 6

Fig. 6: Isolation kit (left) and vortexing (right)

- Briefly **centrifuge** the tube, place it into a thermomixer and incubate and mix for 10 minutes at **56 °C**, see Fig. 7

Fig. 7: Centrifuge (left), thermomixer (right)

- **Remove the brush** from the tube using sterile tweezers
- Add **400 µL** of the **B3** solution and incubate for **5 minutes at 70 °C**
- Remove the tube from the thermomixer and let it **temperate at the room temperature**
- Add **400 µL of 96%** biologically clean **ethanol**
- **Vortex 2 x 5 s** and briefly **centrifuge**
- Transfer **600 µL** of the solution **on the column** and centrifuge for **1 minute at 11 000 g (rcf)**
- **Discard the supernatant** and **return the column** into the tube
- Transfer **another 600 µL** of the solution on the column and **repeat the steps**
- Transfer the **remaining amount** of the solution on the column and centrifuge for **1 minute at 11 000 g (rcf)**
- **Insert the column into a new tube**
- Add **50 µL** of the **B5** solution and centrifuge a for **1 minute at 11 000 g (rcf)**
- Add another **50 µL** of the **B5** solution and centrifuge for **2 minutes at 11 000 g (rcf)**
- Transfer the column into a new tube with a lid
- Add **20 µL** of the **BE** solution right in the middle of the column and centrifuge for **1 minute at 11 000 x g (rcf)**
- Remove the column and leave the tube with an open lid for **30 minutes at room temperature** to allow the remaining ethanol evaporate

2.3. Analysis of the quantity and quality of the isolated DNA

The amount and quality of the isolated DNA are most commonly established through spectrophotometry. Nucleic acids have an absorption maximum at 260 nm. The NanoDrop machine also allows establishing the purity of the nucleic acids through ratios of absorbance values at 260/280 nm and 260/230 nm. For further biological analyses, the concentration of isolated DNA should be approx. 50 ng/µL. The 260/280 absorbance ratio should be ≥ 1.8; values below 1.8 indicate protein contamination because tryptophan, as well as other amino acids, have the maximum light absorption capacity at 280 nm. The 230/260 absorbance ratio should be approx. 2. A lower value indicates contamination with organic agents used during extraction (guanidine, etc.). Contaminated DNA can be purified through re-precipitation with ethanol it is, however, always at the expense of the loss of a certain amount of DNA.

Method:

- Apply **2 µL of the elution solution** (BE) into the beam window, see Fig. 8

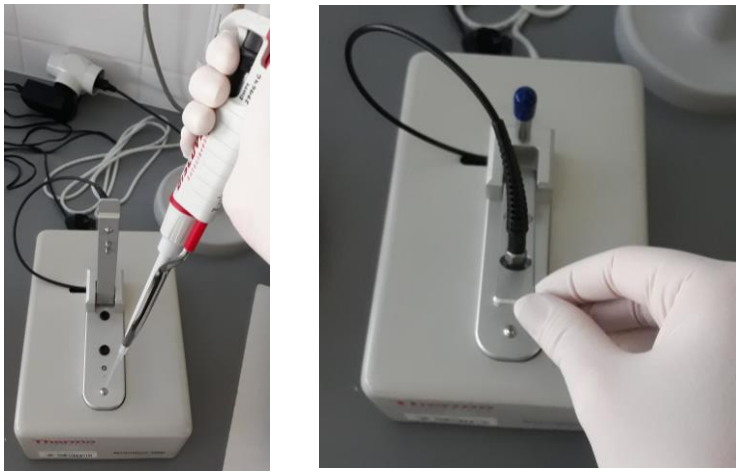


Fig. 8: Sample transfer into the NanoDrop machine

- Push the button **BLANK** and wait for the machine to calibrate
- **Wipe the drop away** using gauze
- Apply **2 µL of the DNA sample** to the same spot
- Push the button **MEASURE** and wait for the result
- Wipe the drop away using gauze
- Record and evaluate the result

2.4. qPCR

Polymerase chain reaction (PCR) is the principal molecular biological method enabling amplification (multiplication) of a certain DNA segment. This method has many modifications; here, we will work with real-time PCR (qPCR) with fluorescently labeled TaqMan probes. Let's start with the preparation of reaction mix, containing besides water and buffer solution also nucleotides (adenine, thymine, guanine, and cytosine – the structural components of which DNA strands are built), DNA polymerase (the enzyme incorporating these components into a newly formed DNA strand) and Mg^{2+} ions as catalyzers of the reaction. In addition, a TaqMan probe is added into the mixture – a solution containing primers that are complementary to a certain sequence of the DNA (in this case, a sequence of the *MTHFR* gene), on which the enzyme DNA polymerase begins to synthesize a new DNA strand, and the probe itself (two types of oligonucleotides, each one with a different fluorophore), which is complementary to the site in the *MTHFR* gene where the polymorphism occurs. For example, in the presence of the nucleotide A in the position of a polymorphism, an oligonucleotide with the VIC fluorophore (and in the case of the G nucleotide, the FAM fluorophore) binds to the DNA at the site of the polymorphism. Mixing the reagents with a DNA sample forms a reaction mix that is placed into a PCR cycler.

The PCR itself comprises several stages. The first stage is denaturation when the strands of two-strand DNA are separated, resulting in single-strand DNA. This stage is usually operated at a temperature of 95 °C. Subsequently, the temperature is reduced to an annealing temperature, at which the primers bind to the complementary DNA regions. The temperature may differ at this step; nevertheless, in the TaqMan probes, it is usually 60 °C. At this step, polymerase also gets to the primer and begins to synthesize a new DNA strand. However, DNA polymerase is capable not only of the synthesis of the new chain but also of the removal of nucleotides that are "in the way" during the synthesis of the new strand (exonuclease activity). Hence, when the DNA polymerase comes upon a nucleotide with a fluorophore, it is removed. The fluorophore is bound to the oligonucleotide together with a fluorescence quencher; therefore, as long as it is bound to the oligonucleotide, no fluorescence can be detected. However, as soon as DNA polymerase removes the individual nucleotides from the oligonucleotide, the fluorophore is released from the quencher and the machine can detect fluorescence (see Fig. 9). Thus, we can, in real time, observe the increase in fluorescence, which is proportional to the amount of the amplification product.

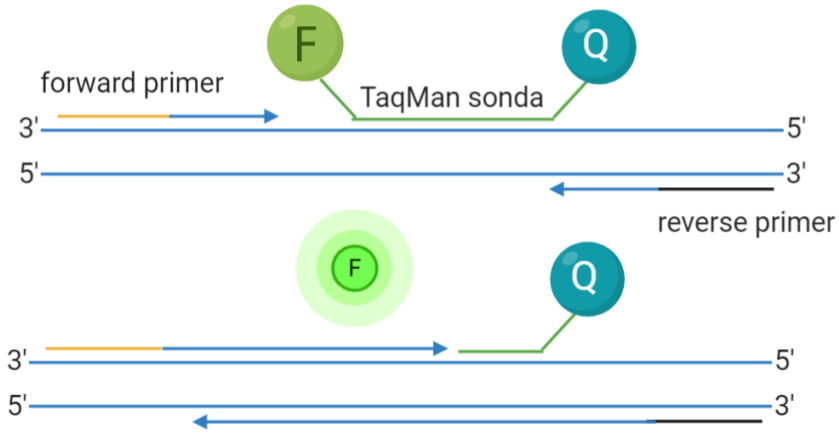


Fig. 9: The principle of PCR using TaqMan probes (created in the software BioRender.com)

Method:

- Pipette the reagents into a 1.5 mL tube according to the table and **prepare the reaction mix**

Reagent	Concentration of the stock solution	Concentration in the reaction mix	Volume
Master Mix	2x	1x	10 µL
Primer-probe Mix	4x	1x	5 µL

- Vortex the reaction mix **2 x 5 seconds**
- Transfer **15 µL of the reaction mix** into the reaction plate/strip
- Add **5 µL DNA**/negative control (water)/positive control
- **Seal the plate** with a film
- **Centrifuge** briefly, see Fig. 10

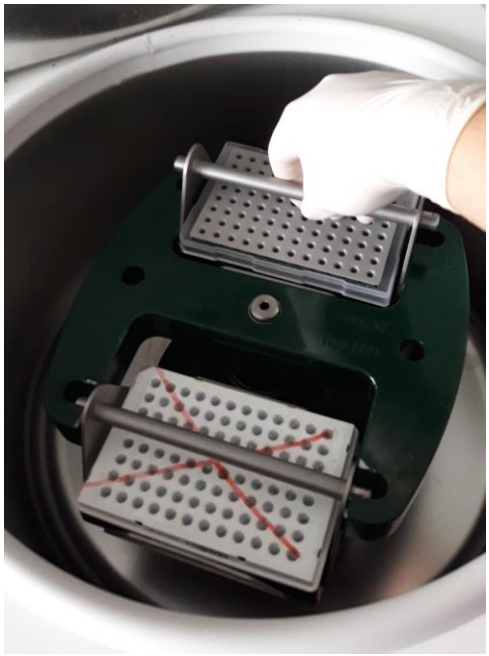


Fig. 10: Centrifugation of a PCR plate

- Set the program in LightCycler 96 Roche to

program	Temperature	Duration	Number of Cycles
denaturation	95 °C	600 seconds	1x
two-step amplification	95 °C 60 °C	10 seconds 30 seconds	40x

- Place the plate into the LightCycler 96 Roche and run the program, Fig. 11

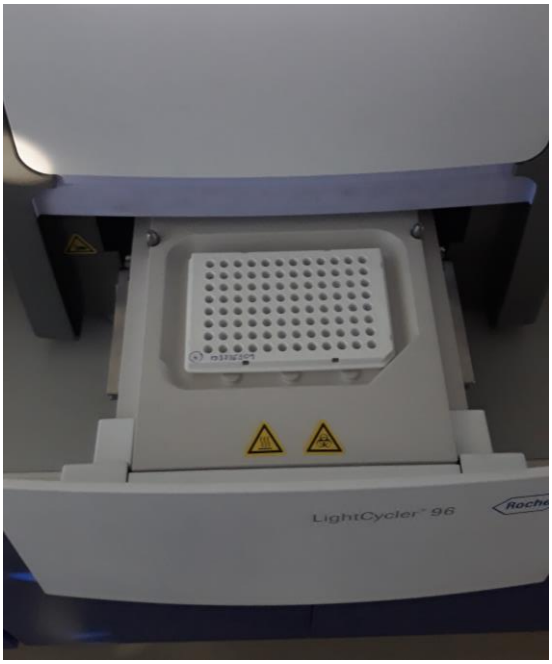
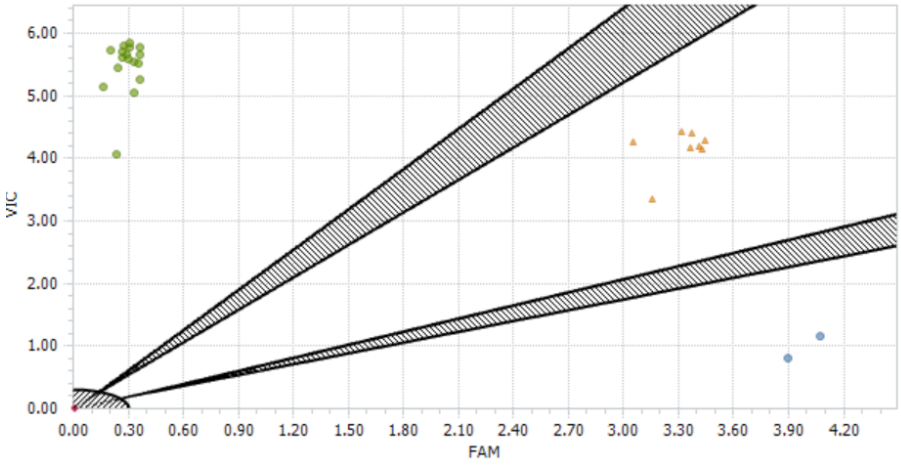
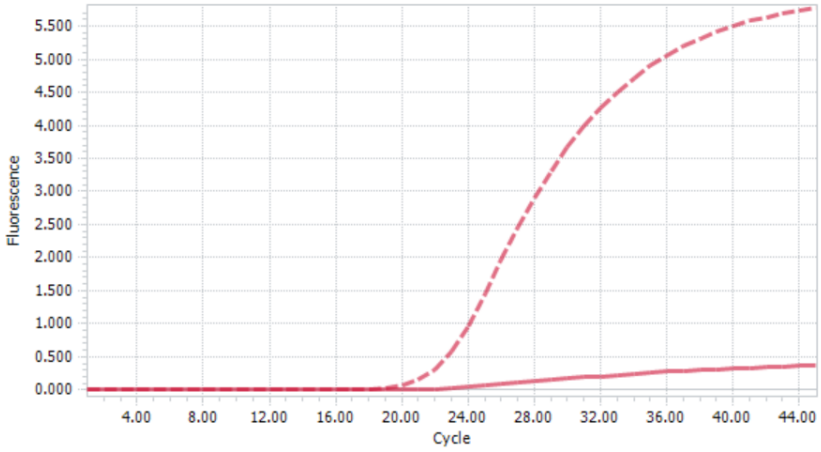


Fig. 11: Placement of the plate in the LightCycler 96 Roche

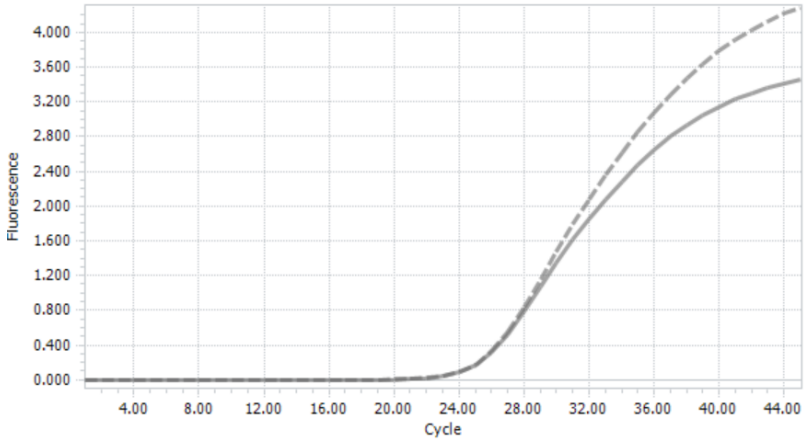
- Evaluate the results, see Figures 12-15



- green circle = VIC homozygote



- yellow triangle = heterozygote



- blue circle = FAM homozygote

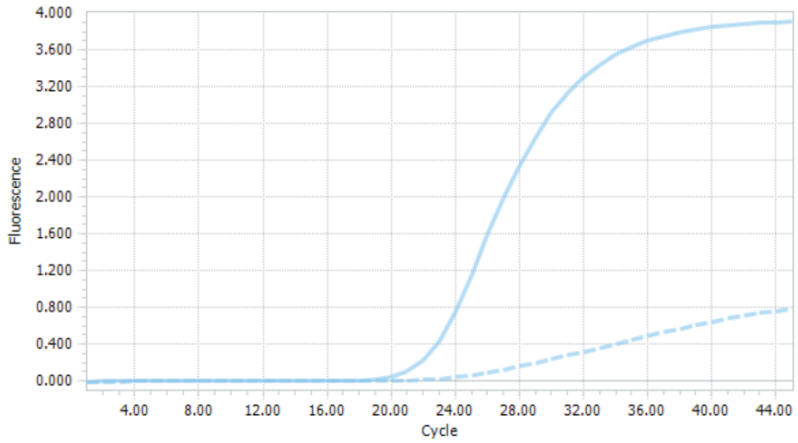


Fig. 12-15: Graphs representing individual genotypes

2.5. Evaluation

Fill in the report (see below) where you will record the concentration and quality of the isolated DNA, the genotype of both polymorphisms in the *MTHFR* gene and, based on the determined haplotype, predict the expected phenotype of the patient (his/her ability to metabolize folic acid). Besides, state what you would, as a dentist, recommend to the patient with this finding, in the report.

<table>
<tr><td colspan="4">Examination for the presence of polymorphisms C677T and A1289C in the MTHFR gene</td></tr>
<tr><td colspan="4">Method: TaqMan PCR</td></tr>
<tr><td>Sample code:</td><td colspan="3"></td></tr>
<tr><td colspan="4">Input material:</td></tr>
<tr><td>DNA concentration:</td><td></td><td>DNA purity (260/280):</td><td></td></tr>
<tr><td colspan="2"></td><td>DNA purity (260/230):</td><td></td></tr>
<tr><td colspan="4">Genotype:</td></tr>
<tr><td>C677T:</td><td></td><td>A1289C:</td><td></td></tr>
<tr><td colspan="2">Haplogenotype (C677T/A1289C):</td><td colspan="2"></td></tr>
<tr><td colspan="4">Phenotype:</td></tr>
<tr><td colspan="4"></td></tr>
<tr><td colspan="4">Recommendation:</td></tr>
<tr><td colspan="4"></td></tr>
</table>