Univerzita Karlova v Praze

Přírodovědecká fakulta

Studijní program: Analytická chemie

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Analýza proteinů a jejich změn v biologických tkáních
Analysis of proteins and their changes in biological tissues

Dizertační práce

Vedoucí disertační práce: prof. Ing. Ivan Mikšík, DrSc.
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Praha, 2020

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V Praze, 17. dubna 2020 Podpis

Tato dizertační práce vznikla v letech 2014 až 2020 během mého Ph.D. studia na Katedře analytické chemie Přírodovědecké fakulty Univerzity Karlovy v Praze, Hlavova 2030, 128 43, Praha 2. Experimentální část práce byla prováděna na oddělení Translačního metabolismu na Fyziologickém ústavu Akademie věd České republiky, v. v. i..

Dizertační práce vznikla za finanční podpory Grantové agentury Karlovy Univerzity v Praze, projekt GA UK č. 322216, Ministerstva zdravotnictví České republiky Program na podporu zdravotnického aplikovaného výzkumu (č. 17-31564A) a Grantové agentury České republiky (č. 16-12420Y).

Školitel: prof. Ing. Ivan Mikšík, DrSc.
Oddělení Translační metabolismus
Fyziologický ústav Akademie věd České republiky, v. v. i.

Školitel-konzultant: prof. RNDr. Věra Pacáková, CSc.
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Přírodovědecká fakulta, Univerzita Karlova v Praze

Dizertační práce je založena na následujících pěti vědeckých pracích, které byly publikovány v mezinárodních impaktovaných časopisech:

1. **Kulhavá, L.**; Eckhardt, A.; Pataridis, S.; Bartoš, M.; Foltán, R.; Mikšík, I.: Differences of Saliva Composition in Relation to Tooth Decay and Gender; Folia Biologica (Praha) 64, 195-203 (2018)
2. **Kulhavá, L.**; Eckhardt, A.; Pataridis, S.; Foltán, R.; Mikšík, I.: Proteomic analysis of whole saliva in relationship to dental caries resistance; v tisku v časopise *Folia Biologica* (Reference number: 2019-1359)
3. Eckhardt, A.; **Kulhavá, L.**; Mikšík, I.; Pataridis, S; Hlaváčková, M.; Vašinová, J.; Kolář, F.; Sedmera, D.; Ošťádal, B.: Proteomic Analysis of cardiac ventricles: baso-apical differences; Molecular and Cellular Biochemicstry, 445:211-219 (2018).
4. Sechovcová, H.; **Kulhavá, L.**; Fliegerová, K.; Trundová, M.; Morais, D.; Mrázek, J.; Kopečný, J.: Comparison of enzymatic activities and proteomic profiles of Butyrivibrio fibrisolvens grown on different carbon sources; Proteome Science 17:2 (2019).
5. Eckhardt, A.; Novotný, T.; Doubková, M.; Hronkova, L.; Vajner, L.; Pataridis, S.; Hadraba, D.; **Kulhavá, L.**; Plencner, M.; Knitlová, J.; Lišková, J.; Uhlík, J.; Žaloudíková, M.; Vondráček, D.; Mikšík, I.; Ošťádal, M.: Novel Contribution to Clubfoot Pathogenesis: The Possible Role of Extracellular Matrix Proteins; Journal of Orthopaedic Research, 37:769-778 (2019).

Prohlášení o spoluautorství

Jako zástupce spoluautorů prohlašuji, že se Mgr. Lucie Kulhavá podílela na níže uvedených publikovaných pracích měrou uvedenou v závorkách:

1. **Kulhavá, L.**; Eckhardt, A.; Pataridis, S.; Bartoš, M.; Foltán, R.; Mikšík, I.: *Differences of Saliva Composition in Relation to Tooth Decay and Gender*; Folia Biologica (Praha) 64, 195-203 (2018).

   (podíl 70%)

2. **Kulhavá, L.**; Eckhardt, A.; Pataridis, S.; Foltán, R.; Mikšík, I.: *Proteomic analysis of whole saliva in relationship to dental caries resistance*; v tisku

   (podíl 80%)

3. Eckhardt, A.; **Kulhavá, L.**; Mikšík, I.; Pataridis, S; Hlaváčková, M.; Vašinová, J.; Kolář, F.; Sedmera, D.; Ošťádal, B.: *Proteomic Analysis of cardiac ventricles: baso-apical differences*; Molecular and Cellular Biochemicstry, 445:211-219 (2018).

   (podíl 30%)

4. Sechovcová, H.; **Kulhavá, L.**; Fliegerová, K.; Trundová, M.; Morais, D.; Mrázek, J.; Kopečný, J.: *Comparison of enzymatic activities and proteomic profiles of Butyrivibrio fibrisolvens grown on different carbon sources*; Proteome Science 17:2 (2019).

   (podíl 30%)

5. Eckhardt, A.; Novotný, T.; Doubková, M.; Hronkova, L.; Vajner, L.; Pataridis, S.; Hadraba, D.; **Kulhavá, L.**; Plencner, M.; Knitlová, J.; Lišková, J.; Uhlík, J.; Žaloudíková, M.; Vondráček, D.; Mikšík, I.; Ošťádal, M.: *Novel Contribution to Clubfoot Pathogenesis: The Possible Role of Extracellular Matrix Proteins*; Journal of Orthopaedic Research, 37:769-778 (2019).

   (podíl 10%)

…….……

prof. Ing. Ivan Mikšík, DrSc.

Předmětová hesla

Analytická chemie, separační metody

Klíčová slova

Analýzy proteinů, gelová elektroforéza, hmotnostní spektrometrie, biologické materiály

Subject words

Analytical chemistry, separation methods

Key words

Analysis of proteins, gel electrophoresis, mass spektrometry, biological materials

Poděkování

Na tomto místě bych ráda poděkovala svému školiteli prof. Ing. Ivanu Mikšíkovi, DrSc. za vedení mé dizertační práce a školitelce-konzultantce prof. RNDr. Věře Pacákové, CSc. za věcné připomínky, rady, diskusi a podporu během celého doktorandského studia. Dále také kolegům oddělení Translačního metabolismu za ochotu předání zkušeností v experimentálních přístupech a kolegyním RNDr. Pavle Sedlákové, Ph.D a Ing. Janě Svobodové, Ph.D. za příjemné pracovní a přátelské prostředí na oddělení a podporu vedoucího oddělení doc. Ing. Tomášovi Čajkovi, Ph.D.

V neposlední řadě bych ráda poděkovala své rodině, rodičům, bez kterých bych nikdy doktorandské studium nemohla studovat, za podporu a trpělivost, kterou mi věnovali během mého studia.

Abstrakt (CZ)

Proteiny jsou důležité biologicky aktivní látky organismu, jejich kvalitativní i kvantitativní složení rozhoduje o funkcích živého organismu. Analýzy proteinů v biologickém materiálu jsou významnou součástí biologického a lékařského výzkumu.

Tato dizertace je věnována optimalizaci přípravy vzorků - biologického materiálu, a to zpracování vzorků v preanalytické fázi a jejich přípravě pro vlastní analýzy a zavádění nových postupů v jejich přípravě pro příslušné analýzy. U jednotlivých biologických materiálů byly vždy optimalizovány podmínky použitých analytických metod jednorozměrné a dvourozměrné gelové elektroforézy a hmotnostní spektrometrie. Získané výsledky byly statisticky zpracovány a navrženy další možné přístupy k výzkumu zmíněných problematik.

Hlavním studovaným biologickým materiálem byla lidská slina a to s ohledem na orální zdraví. Byla provedena srovnávací studie vzorků lidské sliny, která byla získána od lidí se sanovanou (ošetřenou) denticí a lidí se zdravou denticí. Bylo studováno složení sedimentu (část vzorku celkové sliny získán centrifugací) ve vztahu k zubnímu kazu. Byly nalezeny odlišné exprese identifikovaných proteinů ve sledovaných skupinách lidí se sanovanou a zdravou denticí. Pomocí přístupu label-free kvantifikace byly prokázány rozdíly v proteinovém zastoupení ve vzorcích lidské sliny s ohledem na pohlaví a na orální zdraví. V supernatantu bylo vyhodnoceno 14 proteinů se signifikantně vyšší expresí ve skupině lidí se zdravou denticí v porovnání se sanovanou denticí, z toho polovina proteinů patřila do kategorie proteinů imunitního systému a antibakteriální proteiny (např. lysozym c, protein S100-A8, polymerní immunoglobulinový receptor, konstantní řetězec immunoglobulinu kappa). Nalezené proteiny mohou hrát důležitou roli v prevenci orálního zdraví. V sedimentové části vzorku lidských slin byly nalezeny 3 signifikantně rozdílné exprese proteinů (cornulin, protein 14-3-3, annexin A1) s vyšší expresí ve skupině lidí s ošetřenou denticí ve srovnání se skupinou se zdravou denticí. Jedná se o vápník vázající proteiny, které mohou přispívat k dekalcifikaci zubu, k jeho oslabení, a může tak docházet k vzniku zubního kazu.

Dalším zkoumaným biologickým materiálem byla tkáň – části srdce potkana, kde byly popsány rozdíly v proteinovém profilu levé a pravé komory srdeční a bazo-

apikální rozdíly na základě analýz pomocí dvourozměrné gelové elektroforézy. Popsané rozdíly v proteinovém profilu byly výraznější v levé komoře srdeční potkana, které je vystaveno vyššímu pracovnímu zatížení. Byly identifikovány mitochondriální proteiny, patřící do metabolických a energetických drah (laktátdehydrogenasa B, kreatinkinasa M) a kontraktilní protein (myosinový lehký řetězec 3).

Dalším studovaným materiálem byla kontrahovaná tkáň z části talonaviculárního skloubení – část tkáně postižené onemocněním idiopatická *pes equinovarus* (clubfoot). Výsledky analýz label-free kvantifikace s hmotnostním spektrometrem přispěly k novému poznání o patogenezi této deformity. Většina z 11 nalezených proteinů s rozdílnou expresí je součástí extracelulární hmoty. Právě tyto proteiny mohou hrát specifickou roli v patogenezi clubfoot.

Metody jednorozměrné a dvourozměrné elektroforézy byly rovněž aplikovány při analýze extracelulárních proteinů z bakterie *Butyrivibrio fibrisolvens* Bf3071 kultivované na čtyřech substrátech s následným porovnáním účinku změny substrátu (glukóza, xylan, směs xylanu a glukózy a xylóza). Rozdílné exprese proteinů byly pozorovány u extracelulárních proteinů účastnících se glykolýzy. Výsledky tohoto srovnání poskytují informace o substrátové preferenci.

Abstract (EN)

Proteins are the important biologically active substances for the organism, their qualitative and quantitative composition determines thein function in the organism. Protein analysis in biological material is an important part of biological and medical research.

This dissertation is focused on the optimalization of sample preparation – the biological materials – namely the pre-analytical phase of sample processing and their preparation for their own analases and also introducing new procedures in theie praparation. The conditions of individual analysis approaches were always optimized by means of one-dimensional and two-dimensional gel electrophoresis and mass spectrometry. The obtained results were statistically evaluated and other possible approaches to futher research were outlined.

The main biological material studied was human saliva with regard to oral health. Comparative study of human saliva was performed (human saliva obtained from caries-free and caries-susceptible people). The saliva composition of individual fractions was investigated with regard to oral health. The study was carried out to compare differences in the abundances of proteins in the saliva of caries-free and caries-susceptible persons, based on label-free mass spectrometry quantification, and to perform a gender comparison. A total of 14 proteins were found with higher expression in the caries-free group in comparison with the caries-susceptible group, and half of the proteins are immune and antimicrobial ones (for example lysozyme C, protein S100-A8, polymeric immunoglobulin receptor, immunoglobulin κ constant). The specificity of these proteins could play an important role in oral protection. Three proteins (cornulin, protein 14-3-3, annexin A1) were found in pellet fractions with higher signifiant expression in caries-susceptible group in comparison with caries-free group. These proteins are calcium binding proteins that could decalcify enamel of tooth, tooth decay may occur.

Another examined biological material was tissue - parts of the heart of the rat, where the left and right ventricular differences and baso-apical differences were described on the basis of two-dimensional gel electrophoretic analysis. The described changes were more pronounces in the left ventricular, which is subjected to higher workload. Mitochondrial proteins belonging to the “metabolism and energy pathways”

(l-lactate dehydrogenade, creatine kinase M-type) and contractile protein like myosin light chain 3 were found.

Another material studied was the contracted tissue from part of the talonavicular articulation - part of the tissue affected by the disease idiopathic *pes equinovarus* (clubfoot). Label-free quantification was used and most of the 11 differently expressed proteins found are components the extracellular matrix. These proteins could play specific roles in the pathogenesis of this deformity.

One-dimensional and two-dimensional electrophoresis methods were also applied in the analysis of extracellular proteins from *Butyrivibrio fibrisolvens* grown on four substrates (glucose, xylan, xylan / glucose mixture and xylose) and the effect of substrate on protein expression was studied. The different expression found was observed in the extracellular proteins involved in glycolysis. The results of this comparison provide information about substrate preference.

Seznam zkratek a použitých symbolů

AK	aminokyseliny
AQUA	přístup absolutní kvantifikace (Absolute Quantification Approach)
CE	kapilární elektroforéza (Capillary Electrophoresis)
DGGE	elektroforéza v gradientovém denaturačním gelu (Denaturing Gradient Gel Electrophoresis)
DNA	deoxyrinobukleová kyselina (Deoxyribonucleic Acid)
DMFT	index rozpadlých, chybějících a plněných zubů (Decayed, Missing, Filled Teeth)
ECC	zubní kaz v raném dětství (Early Childhood Caries)
ESI	ionizace elektrosprejem (ElectroSpray Ionization)
ICAT	izotopické značení proteinů (Isotope-Coded Affinity Tag)
IEF	izoelektrická fokusace (Isoelectric Focusing)
IPG	imobilizovaný pH gradient (Immobilized pH gradient)
i-TRAQ	izobarické značení pro relativní a absolutní kvantifikaci (Isobaric Tag for Relative and Absolute Quantitation)
LC	kapalinová chromatografie (Liquid Chromatography)
MALDI	laserová desorpce/ ionizace pomocí matrice (Matrix-Assisted Laser Desorption/Ionization)
NGS	sekvenování nové generace (Next Generation Sequencing)
nLC	nano-kapalinová chromatografie (nano-liquid chromatography)
PCR	polymerázová řetězová reakce (Polymerase Chain Reaction)
PEC	onemocnění pohybového aparátu *pes equinovarus congenitus* (clubfoot)
pI	izoelektrický bod (isoelectric Point)
SDS	dodecylsíranu sodný (Sodium Dodecldodecyl Sulfate)
SDS-PAGE	elektroforéza v polyakrylamidovém gelu v přítomnosti dodecylsíranu sodného (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis)
SILAC	značení stabilními izotopy aminokyseliny v buněčné kultuře (Stable Isotope Labeling with Aminoacids in Culture)
TOF	hmotnostní analyzátor doby letu (Time of Flight)
1-DE	jednorozměrná gelová elektroforéza (One Dimensional Electrophoresis)

2-DE	dvourozměrná gelová elektroforéza (Two-Dimensional Gel Electrophoresis)
2-DE-DIGE	dvourozměrná diferenční gelová elektroforéza (Two-Dimensional Difference Gel Electrophoresis)

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1 Úvod

1.1 Proteiny a jejich úloha v živém organismu

Proteiny patří mezi vysokomolekulární přírodní látky. Jejich stavebními jednotkami jsou aminokyseliny (AK), které jsou navzájem vázány peptidovou vazbou. Podle počtu aminokyselin, které jsou navázány v molekule, rozlišujeme oligopeptidy (obsahují 2-10 AK), polypeptidy (11-100 AK) a vlastní proteiny (více jak 100 AK).

Struktura proteinů se rozlišuje na primární, sekundární, terciální a kvarterní. Primární struktura je dána pořadím aminokyselin v polypeptidovém řetězci. Sekundární struktura je geometrické uspořádání polypeptidového řetězce mezi několika po sobě jdoucími aminokyselinami a je podmíněna vznikem vodíkových můstků mezi NH- a C=O skupinami peptidové vazby. Mezi nejčastější sekundární struktury patří α-helix a β-skládaný list. Terciální strukturou se označuje trojrozměrné uspořádání celého peptidového řetězce. Kvarterní struktura dává informace o uspořádání proteinů, které jsou tvořeny z dvou nebo více polypeptidových řetězců.

Struktury vyššího řádu jsou vytvářeny jen slabými nekovalentními interakcemi (především vodíkovými vazbami a hydrofobními interakcemi). K rozrušení nekovalentní vazby se používají činidla jako močovina nebo SDS (dodecylsulfát sodný), které rozruší i sekundární, terciální i kvarterní strukturu a dojde i ke ztrátě biologické aktivity (denaturaci).

Vztah mezi strukturou proteinu a jeho biologickou funkcí je velmi blízky. Proteiny jsou jedny z nejdůležitějších látek pro správné fungování organismů. Proteiny mají funkci katalytickou (enzymy, např. trypsin, DNA polymeráza), regulační (hormony, např. insulin), obrannou (protilátky, interferony), zásobní (např. ferritin, kasein), transportní (např. hemoglobin, myoglobin), strukturní (např. kolagen), kontraktilní (myosin, aktin), genetické (histony), ale i některé toxiny patří mezi proteiny.

Rozdělení proteinů může být provedeno na základě chemického složení, jednoduché (proteiny složené pouze z aminokyselinových jednotek) a složené (obsahující i neproteinovou složku; lipoproteiny, glykoproteiny, fosfoproteiny, metaloproteiny, hemoproteiny, nukleoproteiny).

Analýzy lidského proteomu mají velkou škálu praktického využití v biologii a lékařství. Biologické materiály obsahují důležité látky z diagnostického a fyziologického hlediska, a jejich analýzy jsou velmi přínosné i v době, kdy je vyřešena sekvence lidského genomu (Collins a kol., 2003).

1.2 Proteiny a jejich analýza

Proteomika (analýza proteinů) se zabývá studiem biologických procesů na úrovni proteinů. Výstupy analýz proteinů poskytují informace o jejich identifikaci, funkci a kvantitě, což přináší důležité informace o proteinových sekvencích, proteinových strukturách, míře exprese, posttranslačních modifikacích nebo protein-proteinových interakcích. K analýze proteinů se využívá celá řada metod, jedná se velice často o kombinaci elektroforetických a chromatografických metod s hmotnostní spektrometrií.

V současnosti v hmotnostní spektrometrii jsou přístupy – „Bottom-up“, „Shotgun“ a „Top-down“ analýzy proteinů. Schéma (Obr. 1) znázorňuje obecné postupy v proteomických analýzách (Herrero a kol., 2012).

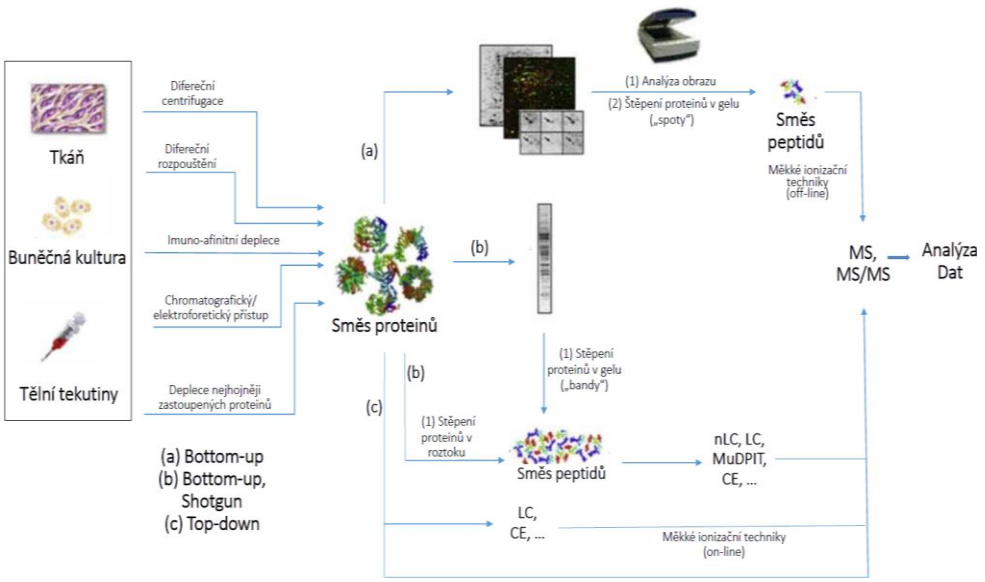
Přístupy „Bottom-up“ a „Top-down“ jsou dvěma základními strategiemi používanými pro identifikaci a charakterizaci proteinů (peptidů). „Bottom-up“ proteomika je klasický přístup identifikace proteinů - tzv. gelový přístup založený na rozdělení směsi proteinů gelovou elektroforézou.

Předběžná separace proteinů se provádí pomocí jednorozměrné, dvourozměrné gelové elektroforézy nebo diferenční gelové elektroforézy (DIGE). Jednorozměrná elektroforéza (1-DE) je elektroforéza v polyakrylamidovém gelu v přítomnosti dodecylsíranu sodného, ve kterém dochází k rozdělení směsi proteinů podle molekulové hmotnosti (SDS-PAGE). Dvourozměrná gelová elektroforéza (2-DE) slouží k separaci směsí proteinů. V prvním rozměru probíhá izoelektrická fokusace (IEF), dochází k separaci proteinů podle jejich izoelektrických bodů (pI), a ve druhém rozměru dochází k rozdělení proteinů podle molekulové hmotnosti.

Následně se vyřezávají spoty (skvrna, proužek), které jsou podrobeny enzymatickému štěpení proteinů na peptidy - „in gel digestion“. Zpravidla se

k enzymatickému štěpení používá trypsin. A dále jsou získané peptidy analyzovány pomocí hmotnostního spektrometru. Obvykle se využívá měkké ionizace jako laserová desorpce/ ionizace pomocí matrice MALDI (MALDI = Matrix Assisted Laser Desorption Ionization) nebo ionizace elekrosprejem ESI (ESI = ElectroSpray Ionization) (Obr. 1a). Přístup bez předběžné separace proteinů pomocí 1-DE a 2-DE je tzv. štěpení směsi proteinů v roztoku - „In-solution digestion“ (Obr. 1b).

V případě „Shotgun“ proteomiky je identifikace proteinů založena na enzymatickém štěpení neseparované směsi bílkoviny na peptidy, které jsou následně rozděleny vhodnou separační metodou (např. kapalinovou chromatografii) a potom sekvenovány tandemovou hmotností spektrometrií (Obr. 1b). Naproti tomu přístup „Top-down“ funguje bez předchozího štěpení proteinů a intaktní proteiny jsou přímo analyzovány hmotnostním spektrometrem (Obr. 1c).



Obr. 1 Schéma proteinových přístupů pomocí MS (modifikováno dle Herrero, 2012)

Hmotnostní spektrometrie se stala nedílnou součástí v diagnostice a léčbě nemocí a spolehlivým a nezbytným nástrojem pro objasnění biologických procesů na úrovni proteinů (Li a kol., 2017). Současný rozvoj hmotnostní spektrometrie směřuje k miniaturizaci a využití přenosných nástrojů v oblastech forenzních analýz, potravinářství, průzkumu vesmíru, environmentálních analýz atd. (Mielczarek a kol.,

2019). Přenosné hmotnostní spektrometry se rychle vyvíjejí v biomedicínských oblastech - s výhodou specifické a spolehlivé informace v daném místě a bez časově náročné předúpravy vzorků a bez zasílání vzorků do příslušné laboratoře (Pu a kol., 2019).

Biochemické metody se tradičně využívají k analýze proteinů, jedná se o techniky separace a kvantifikace. Laemmli již v roce 1970 představil elektroforézu v polyakrylamidovém gelu za přítomnosti dodecylsulfátu sodného, SDS-PAGE), ve které byl použit detergent dodecylsulfát sodný k rozvolnění trojrozměrné struktury proteinů, a byl jim tak udělen jednotný záporný náboj. Po následném vložení napětí proteiny migrují od anody ke katodě póry polyakrylamidového gel rychlostí nepřímo úměrnou jejich velikosti (Laemmli, 1970). SDS-PAGE má i dnes velké využití v různých odvětví proteomického výzkumu. Další široce využívanou metodou analýzy proteinů je kombinace SDS-PAGE a izoelektrické fokusace, dvoudimenzionální (dvourozměrná) elektroforéza (2-DE). V tomto přístupu jsou v prvním rozměru proteiny rozděleny podle jejich izoelektrického bodu a následně v druhém rozměru podle velikosti (Bjellqvist a kol., 1982). Ke kvantifikaci proteinů byla používána pouze metoda 2-DE a její modifikace diferenční gelová elektroforéza (DIGE), využívající ke značení vzorků fluorescenční barviva (Unlü a kol., 1997).

Dříve bylo pro sekvenci proteinů používáno pouze Edmanovo odbourávání (Edman, 1949), ale rozvoj hmotnostní spektrometrie tento způsob identifikace proteinů do značné míry nahradil. Průlom v analýze proteinů přinesla ionizace elektrosprejem a laserová desorpce a ionizace za účasti matrice, které se využívají jako ionizační techniky v hmotnostní spektrometrii. Poslední roky je zaznamenáván rozvoj ambientních ionizačních technik, které umožňují přímou analýzu objektu s minimální předúpravou vzorku (Feider a kol., 2019; Lemr a Borovcová, 2020). Spojení hmotnostní spektrometrie s kapalinovou chromatografií umožňuje separaci i identifikaci proteinů.

Pro kvantifikaci proteinů pomocí gelových technik se využívají přístupy 2-DE a DIGE. A to na základě porovnání proteinových profilů jednotlivých vzorků porovnávaných skupin mezi jednotlivými polyakrylamidovými gely. Diferecní gelová elektroforéza je modifikace 2-DE, umožňující separovat dva vzorky směsi proteinů na jednom gelu. Výhodou této metody je minimalizace problémů spojených s nízkou

reprodukovatelností metody. Tato metoda využívá ke značení vzorků fluorescenční barviva CyDyes^{TM} (GE Healthcare Little Chalfont, Velká Británie).

Po separaci proteinů je nezbytné proteinové spoty na gelu vizualizovat (provedení barvení). Barvení je možné pomocí Coomassie Briliant Blue (citlivost 10-30 ng/skvrnu; kompatibilní s MS analýzou), stříbrem (redukce stříbrného iontu na kovové stříbro formaldehydem v alkalickém prostředí uhličitanu sodného, citlivost 0,5 ng/skvrnu; obtížněji kompatibilní s MS analýzou), a také fluorescenční barvení (DIGE flourescenční barviva Cy3, Cy5 citlivost 0,025 ng/skvrnu; barvivo Cy2 citlivost 0,075 ng/skvrnu; barviva SYBRO Orange, SYBRO Red citlivost 4-8 ng/skvrnu; barvivo SYBRO Ruby 1-2 ng/skvrnu; nevýhoda vyšší cena, nezbytnost 2-3 laserového scanneru). A dále také Western blotting, kdy jsou proteiny přenáseny z gelu na membrány (nitrocelulóza, polyvinylidenfluorid), kde jsou detekovány specifickými protilátkami, a pak jsou proteiny vizualizovány barvivem např. Coosmassie blue, Ponceau S, amidočerň. Přístup detekce pomocí Western blotting má vysokou citlivost i specifičnost, ale vyžaduje další pracovní kroky.

Gely po vizualizaci jsou skenovány a příslušné skeny pomocí softwaru analyzovány, vyhodnocují se na základě optické denzity shodných spotů. Provádí se obrazová analýza skenů, kterou získáme relativní poměr intenzit shodných proteinových spotů mezi jednotlivými gely. Z vybraných spotů s odlišnou denzitou se extrahují peptidy, a identifikují pomocí hmotnostní spektrometrie (MS/MS).

Další možností kvantifikace proteinů je pomocí hmotnostní spektrometrie, a to na základě stabilního izotopového značení, nebo pomocí tzv. label-free kvantifikace. Hmotnostní spektrometrie umožňuje rychlé měření, jednoduchou interpretaci výsledků a díky vysoké citlivosti a specifičnosti je vhodnou metodou pro kvantifikaci (Bantschell a kol., 2007).

Absolutní kvantifikace umožňuje stanovení přesného množství nebo koncentrace proteinů ve směsi pomocí izotopicky značeného peptidového standardu o známém množství ze sledovaného proteinu, kdežto relativní kvantifikace určuje „up-“ nebo „down- “ regulaci proteinu. Metody relativní kvantifikace umožňují určit snížení či zvýšení exprese proteinů ve dvou a více sledovaných stavech buňky (Elliott a kol.,

2009). V dnešní době existuje mnoho rozmanitých metod pro relativní i absolutní kvantifikaci.

Metody absolutní kvantifikace umožňují určení přesného množství nebo koncentrace proteinu ve vzorku. Nutnou podmínkou je znát kvantifikovaný protein (sekvenci jeho peptidu/ peptidů), který lze analyzovat hmotnostní spektrometrii. Podle příslušné sekvence peptidu je vytvořena izotopově značená kopie (např. ^{13}C, ^{15}N) lišící se od původního peptidu hmotností. K absolutní kvantifikaci se využívá metoda AQUA (AQUA = Absolute Quantification Approach), která stanovuje koncentraci peptidu z poměru ploch nebo výšek píků peptidu a interního standardu (Gerber a kol., 2003).

Metody relativní kvantifikace umožňují určit změnu exprese proteinů v porovnávaných vzorcích bez určení přesné koncentrace kvantifikovaných složek. Lze mezi sebou porovnávat dva a více vzorků (což je limitováno počtem dostupných izotopických značek pro danou metodu). Izotopické značky se do struktury molekuly začleňují metabolickou, enzymatickou nebo chemickou cestou (Dayon a Affolter, 2020). Změna exprese jednotlivých proteinů ve srovnávaných vzorcích je určena z poměru ploch nebo výšek píků odpovídajících izotopicky značeným variantám peptidů.

Relativní kvantifikace mohou být prováděny například pomocí metod izotopově značených aminokyselin, izotopově značených proteinů, izotopově značených volných primárních aminoskupin proteinů, enzymatickou reakcí začlenění atomů ^{18}O výměnou za ^{16}O ($^{16}O/^{18}O$ labeling - $^{16}O/^{18}O$ značení), nebo chemickým značením peptidových fragmentů pomocí izobarických značek.

Značení stabilními izotopy SILAC (SILAC = Stable Isotope Labeling with amino ACids in cell culture) je metoda užívaná k relativní kvantifikaci proteinů ve dvou paralelně kultivovaných buněčných kulturách v médiu s lehkými nebo těžkými (izotopicky modifikovanými) aminokyselinami (např. Arg, Lys ^{13}C, ^{15}N). Nutnost je dosáhnout vysoké úrovně inkorporace těžkých aminokyselin do buněk (Ong a kol., 2002). Izotopově značených proteinů využívá ke kvantifikaci metoda ICAT (ICAT = Isotope-Coded Affinity Tag). Molekuly izotopové značky mají biotinovou afinitní část, reaktivní skupinu se specifickou thiolovou skupinou a linker. Kontrolní vzorky se označují lehkou formou značky a sledovaný vzorek těžkou formou, vzorky se

kombinují. Pomocí afinitní chromatografie se kombinovaná směs štěpených peptidů rozdělí na peptidy obsahující aminokyselinu cystein a na ty, které ji neobsahují, a následně jsou peptidy obsahující aminokyselinu cystein analyzovány pomocí hmotnostní spektrometrie. Lze pozorovat dvojice píků peptidů se stejnou sekvencí aminokyselin, lišící se pouze ve formě izotopické značky (rozdíl mezi pozorovanými peptidovými páry je 8 Da (Gygi a kol., 1999).

Metoda iTRAQ (iTRAQ = isobaric Tags for Relative and Absolute Quantification) využívá chemického značení peptidových fragmentů pomocí izobarických značek, lze tak označit až 8 vzorků. Izobarická značka je molekula složená z reaktivní aminoskupiny, reportérové a vyrovnávací skupiny. Po izolaci proteinů ze studovaného vzorku, jsou proteiny štěpeny. iTRAQ značky jsou navázany na vazebné aminové skupiny peptidu a smíchány. Kvantifikace proteinů je prováděna na základě módu MS/MS (odlišnost od kvantifikovaných značek používaných v metodách SILAC, ICAT). V MS^1 spektru se zobrazí značky jako jeden jediný pík. V MS/MS se izobarická značka s navázaným peptidem rozštěpí na část reportérovou s nábojem a balanční (bez náboje, neutrální fragment). Dochází k zobrazení uvolněných reportérových iontů s odpovídající hmotností značky. Z intenzit jednotlivých píků reportérových iontů jsou vypočítána relativní množství proteinů ve srovnávaných vzorcích (Weise a kol., 2007).

Existují též metody hmotnostní spektrometrie využívající statistické zpracování naměřených MS, resp. MS/MS dat (tzv. label-free metody). Label-free kvantifikace je založená na porovnání intenzity signálu jednotlivých peptidů bez předchozího značení. Využívá se k relativní kvantifikaci proteinů. Při dodržení identických podmínek separace existuje vztah mezi intenzitou MS signálu peptidu a jeho abundanci, plochy píků identifikovaných peptidů rostou v závislosti na množství peptidů ve vzorku a tak i s množstvím proteinů v původním vzorku (i ve složité směsi) (Zhu a kol., 2010). Tento přístup využívá přesná hmotnostní spektra s vysokým rozlišením získaných peptidových signálů na úrovni MS^1, a tím je oddělena kvantifikace od procesu identifikace MS^2. Celkový iontový proud peptidového signálu je integrován a použit ke kvantifikaci původní koncentrace peptidu. Jedná se o porovnání iontových intenzit peptidů. Přístup label-free kvantifikace je hodně používán v klinické proteomice (Megger a kol., 2013). Label-free kvantifikace našla uplatnění například v oblasti stomatologie - ve výzkumu proteinových profilů zubní pelikuly s ohledem na gastroezofageální reflux a eroze zubů

(Martini a kol., 2019), dále byla využitá při odhalování změn proteinového profilu vzorků lidské sliny s ohledem na parodontální onemocnění (Bostanci a kol., 2018); v urologii – v identifikování proteinového biomarkeru rakoviny močového měchýře v moči (protein nukleární matrix 22) (Chakraborty a kol., 2019); v pneumologii – identifikování změn exprese proteinů v plicní extracelulární hmotě v souvislosti s onemocněním plic jako např. chronické obstrukční plicní choroby a astmatu (Burstaller a kol., 2017) a v dalších lékařských oborech a jejich výzkumu.

Label-free kvantifikaci lze provádět také na základě počtu MS/MS spekter peptidů pocházejících z jednoho proteinu mezi dvěma nebo více vzorky. Peptidy pocházející z jednoho proteinu poskytují MS/MS spektra, a jejich množství je úměrné množství proteinu ve vzorku (pokud je ve vzorku proteinu více, zvýší se tak počet detekovaných peptidů, vyšší počet MS/MS spekter). Metodu založenou na počítání MS/MS spekter využívající ke kvantifikaci nazýváme „spectral counting“.

Label-free metody jsou alternativou využití značení stabilními izotopy při kvantifikaci. Přístup label-free nevyžaduje využití drahých chemikálií ke značení a odpadají další náročné kroky v přípravě vzorků při značení a z toho plynoucí možnosti chyby při stanovení. Nevýhodou však je náročné statistické zpracování dat a nutnost reprodukovatelné LC separace.

Spojení kapalinové chromatografie s hmotnostní spektrometrií představuje v dnešní době nejpoužívanější analytickou metodu, a postupně nahrazuje metody založené na separaci pomocí gelové elektroforézy (2-DE) a v případě kvantifikace diferenční gelové elektroforézy (DIGE).

Významný pokrok v oblasti hmotnostní spektrometrie, která umožňuje identifikaci proteinů a jejich kvantifikaci v biologických matricích (biologických tkáních, tělních tekutinách), směřuje k nalezení nových biomarkerů související se sledovaným onemocněním. Nejběžněji je vyšetřovaným materiálem v klinickém výzkumu krev (Tessitore a kol. 2013). V dalších tělních tekutinách (cervikální hlen, moč, mozkomíšní mok, sliny, slzy, sputum, sperma, pankreatická šťáva, plodová voda), které jsou produkovány jednotlivými orgány, mohou být identifikovány specifické biomarkery pro sledované onemocnění (Václavková a kol., 2020). Tzv. omické přístupy (jako jsou genomika, metabolomika, proteomika) poskytují výsledky genů, metabolitů,

proteinů lišící se ve skupinách zdravých a nemocných jedinců (Teclemariam a kol., 2020; Chung a Kang, 2019; Cajka a kol., 2017; Zuo a kol., 2016). Nejčastější návrh proteomické studie na identifikaci proteinů jako biomarkerů pro sledované onemocnění je zaměřen na identifikaci proteinů, jejichž obsah je statisticky rozdílný mezi zdravou skupinou a skupinou s příznaky sledovaného onemocnění. Při statisticky dostatečném počtu vzorků pro jednotlivé sledované skupiny lze identifikovat a kvantifikovat proteiny, které jsou charakteristicky zastoupeny pro konktrétně sledované onemocnění, a nejsou zapříčiněny intraindividuálními rozdíly (rozdíly v rámci jedince) (Rapson a kol., 2020; Kovalčíková a kol., 2019; Džunková a kol., 2018).

Nejčastěji vyšetřovaným biologickým materiálem pro klinický výzkum i praxi je lidská krev. Jsou rutinně známé možnosti odběrů a její zpracování. Poskytuje komplexní proteomický profil, protože protéká celým tělem a mísí se, a zastává systémové funkce (Tessitore a kol., 2013). Většina orgánů v lidském těle sekretuje specifickou tělní tekutinu, v nichž konktrétně mohou být biomarkery ještě ve vyšších koncentracích oproti systémové krvi. Jako příklad je možné uvést biomarkery pro - onemocnění zubním kazem identifikované ve vzorcích lidské sliny (Gao a kol., 2016), dále pomocí iTRAQ přístupu hmotnostní spektrometrie biomarkery onemocnění zubním kazem a věkové rozdíly v proteinovém profilu vzorků lidské sliny (Wang a kol., 2018a), biomarkery pro onemocnění ústní sliznice (*lichen planus*), systémového onemocnění lupénky, nádorů v dutině ústní identifikované ve vzorcích lidských slin (Martine a kol., 2020). Dále také možné identifikovat biomarkery pro Alzheimerovu chorobu ve vzorcích lidských slin (Gleerup a kol., 2019), provádět analýzy plodové vody v souvislosti s předčasným porodem a aneuplodie - genová mutace, dochází k chybění nebo nadbytku chromozómů ve všech buňkách určitého organismu (Kamath-Rayne a kol., 2014), nebo analýzy mozkomíšního moku v souvislosti s onemocněním roztroušenou sklérozou (přítomnost oliklonální IgG) (Wright a kol., 2012).

Předložená dizertační práce odráží pokroky v proteomické analýze, lze v ní vysledovat postupy zmíněného pokroku v trendu proteomických analytických metod – od metod využívající separaci v polyakrylamidovém gelu s přídavkem dodecylsulfátu sodného, dvoudimenzionální elektroforézu přes diferenční gelovou elektroforézu k analýzám vzorků pomocí kapalinové chromatografie ve spojení s hmotnostní spektrometrií a label-free kvantifikace.

2 Cíle práce

- Provést optimalizaci přípravy vzorků biologického materiálu a jejich následnou analýzu pomocí separačních metod (jednorozměrná gelová elektroforéza, dvourozměrná gelová elektroforéza, dvourozměrná diferenční gelová elektroforéza a kvantifikace pomocí hmotnostní spektrometrie přístupem label-free kvantifikace).

- Provést porovnání proteinového profilu vzorků lidských slin získaných od žen a mužů, a provést tak genderové srovnání identifikovaných proteinů ve slinách.

- Identifikovat proteiny obsažené v jednotlivých frakcích vzorků lidských slin (supernatant a sediment, získané frakce centrifugací vzorku celkové sliny), a následné vyhodnotit unikátní proteiny pro dané frakce.

- Využít gelovou diferenční elektroforézu pro získání proteinových profilů vzorků lidských slin v souvislosti s onemocněním dutiny ústní.

- Porovnat proteomické a mikrobiologické profily vzorků lidských slin (odebrány před a po vyčištění dutiny ústní po noci).

- Optimalizovat podmínky separace biologického materiálu v konkrétních problematikách fyziologického výzkumu (bazo-apikálních rozdílu srdce potkana, analýzy tkáně - kloubního pouzdra u onemocnění *pes equinovarus congenitus*, a srovnávací studie proteinových profilů získaných z bakterie pocházející z bachoru *Butyrivibrio fibrisolvens*, která byla kultivována za různých podmínek).

3 Analýza proteinů v lidské slině s ohledem na orální zdraví a pohlaví

3.1 Teoretický úvod

3.1.1 Lidská slina a onemocnění v dutině ústní

Lidská slina je tělní tekutinou, která je produkována slinnými žlázami. Slinné žlázy (*glandula salivariae*) jsou uloženy v maxilofaciální oblasti. Všechny vývody slinných žláz jsou vedeny do dutiny ústní. U člověka jsou párové velké slinné žlázy: příušní (*grandula parotis*), podčelistní (*grandula submandibularis*), podjazyková (*grandula sublingualis*) a drobné slinné žlázy: *grandula* (*gll.*) *labiales* – z vnitřní strany rtů, *gll. buccales* – na vnitřní straně tváří, *gll. molares* – bukální, na úrovni molárů, *gll. palatinae* – pod sliznicí patra, *gll. linguales* – na jazyku (Kilián, 1999). Normální produkce lidských slin v ústech je 1,5 - 2 litry denně (produkce slin je ovlivněna druhem a četností příjmu potravy, dodáváním tekutin, onemocněním (např. Sjogrenův syndrom (Vivino, 2017)) a vývojovými vadami (Taji a kol., 2011). V noci dochází k poklesu produkce slin.

Slina splňuje několik funkcí: mechanickou (díky ní dochází k odstranění zbytků potravy (orální clearence), v trávení, má antimikrobiální vlastnosti - obsahuje antimikrobiální proteiny (laktoferrin, aglutininy) (Khurshid a kol., 2016), je důležitým zásobníkem vápníku a fosfátových minerálů důležitých pro remineralizační procesy ve sklovině zubů, selektivní adheze a kolonizace bakterií a funguje jako nárazníkový systém - stabilizuje výkyvy pH v ústech, a to díky bikarbonátovému, fosfátovému a proteinovému systému (Dawes a kol., 2015). Lidská slina plní také protektivní funkci tvrdých a měkkých tkání v dutině ústní (Pedersen a kol., 2018). Důležité je množství a složení lidské sliny.

Zubní kaz je nejčastější multifaktorové infekční onemocnění dutiny ústní, postihující velkou část populace (Petersen, 2003). Zuby jako tvrdá tkáň dutiny ústní jsou v nepřetržitém kontaktu se slinou, kde její hlavní složky a vlastnosti hrají zásadní roli při výskytu a progresu zubního kazu (Gao a kol., 2016). Mnoho měřitelných vlastností lidské sliny je potenciálním biomarkerem zubního kazu, které jsou využity pro predikci, diagnostiku, prognózu a léčbu zubního kazu. Existují výsledky srovnávacích studií, které dávají do souvislostí zubní kaz a určité parametry, vlastnosti lidské sliny a vliv přítomnosti, nebo nepřítomnosti biologicky aktivních látek ve slinách (Gao a kol., 2016).

Vlastnosti lidské sliny jako její nízká produktivita, snížená pufrovací kapacita sliny a změna kolonizace bakterií (např. riziko zvýšeného výskytu bakterie *Streptococcus mutans*) je dána do souvislosti s vyšším výskytem zubního kazu (Gao a kol., 2016; Hemadi a kol., 2017). Za biomarkery určující náchylnost k zubnímu kazu jsou považovány - mikroorganismy ve slinách (*Streptococcus mutans*, *Lactobacillus*, *Candida albicans* atd.) (Tanner, 2015; Yang a kol., 2015) a jejich diverzita, elektrolyty obsažené ve slinách, proteiny a peptidy identifikované ve vzorcích lidské sliny (Vitorino a kol., 2006; Preza a kol., 2009; Laputková a kol., 2018) a funkční vlastnosti sliny (tok sliny, pH sliny, pufrovací kapacita, slinná clearence). Byly popsány i metabolity identifikované ve slinách v souvislosti se zubním kazem (Pereira a kol., 2019).

Zubní kaz v raném dětství (ECC) přináší velké riziko do dutiny ústní (ECC = early childhood caries) (Merglová a Ivančáková, 2009; Tinanoff a kol., 2019; Kirthiga a kol., 2019). ECC je označení pro výskyt zubního kazu do 6 let věku dítěte. Rizikové faktory pro ECC jsou sociodemografické, dietní a orální hygiena (Kirthiga a kol., 2019). Nízký socioekonomický status, nízké vzdělání, první dítě, domácnost s jedním rodičem jsou uváděny jako sociodemografické faktory pro výskyt ECC (Östberg a kol., 2017; Evans a kol., 2013). Mezi dietní faktory patří zvýšený příjem cukru v potravě (převážně přijímaní kariogenní stravy – potraviny a tekutiny bohaté na sacharidy) (de Sousa a kol., 2020; Watanabe a kol., 2014), pití slazených nápojů v noci (Lulic-Dukic a kol., 2001), mléčné výrobky (Tanaka a kol., 2012), nedostatek vitamin D – dochází ke kalcifikaci zubů, důsledek hypoplazie skloviny zubu (Schroth a kol., 2014; Seminario, 2016; Singleton a kol., 2019). Absence fluoridu (v zubních pastách) je prezentována jako zásadní nedostatek v orální hygieně (Targino a kol., 2011), a následně také nedostatečná hygiena a u dětí nedostatečný dozor u čištění zubů do 10 let věku dítěte (Sandstrom a kol., 2011). Uvedená rizika pro předčasný zubní kaz jsou uváděny i pro výskyt zubního kazu u dospělé populace (Kutsch, 2013).

3.1.2 Analýza proteinů ve slinách v souvislosti s infekčním onemocněním zubním kazem

Pomocí proteomické analýzy mohou být vyhodnoceny specifické proteiny, a to na základě jejich zvýšené nebo snížené exprese při sledovaném

onemocnění (Jenkins a Pennington, 2001; Mohanty a kol., 2017; Vít a kol., 2019). Srovnávací studie vzorků lidské sliny zabývající se proteinovými profily lidí se zdravým a sanovaným chrupem byly již publikovány, ale tyto studie se ve výsledcích rozcházejí, popisují opačné trendy ve zvýšené/snížené expresi proteinů v jedné ze srovnávaných skupin vzorků.

Například studie Vitorina a kolektivu (Vitorino a kol., 2005) ukazuje na korelaci mezi vysokou hodnotou DMFT indexu (DMFT indey = Decayed, Missing, and Filled Teeth index) a vysokou hladinou proteinu laktoferrinu, kdežto studie Jentsche a kolektivu (Jentsch a kol., 2004) ukazují na zvýšenou kazivost u lidí s nízkou hladinou laktoferrinu. Neshoda dosažených výsledků může být zapříčiněna velkou variabilitou vzorků - interindividuální i intraindividuální variabilitu (Rosa a kol., 2016), i různými přístupy stanovení jednotlivých proteinů (různá příprava vzorku, rozdílné separační metody, použití rozdílných komerčních setů).

Dosud byly prováděny studie vždy pouze na části vzorku lidské sliny, a to na supernatantu získaném centrifugací vzorku celkové sliny (celková slina – sekret velkých i drobných slinných žláz). Například byly analyzovány vzorky slin produkující příušní žlázy (vzorek lidské sliny odebrán přímo z výtoku z příušní žlázy do dutiny ústní) s ohledem na výskyt zubů s ošetřenými kořenovými kanálky (Preza a kol., 2009) nebo analýza peptidů identifikovaných ve vzorcích lidské sliny s ohledem na orální zdraví (Vitorino a kol., 2005). Byla rovněž provedena komplexní studie srovnání proteinových profilů u dětí s ohledem na sanovaný a zdravý chrup (Wang a kol., 2018b).

Srovnávací studie pomocí dvourozměrné gelové elektroforézy ukázala rozdílné exprese u proteinů (proteiny β-2 mikroglobulin a transferrin se zvýšenou hladinou ve slinách u žen ve srovnání se slinou od muže) s ohledem na pohlaví (Fleissig a kol., 2010). Byly prováděny srovnávací studie vzorků lidských slin s ohledem na pohlaví a jejich vlastnosti jako pH a produkce slin (Li-Hui a kol., 2016).

Významné genderové rozdíly v biochemii vzorků lidských slin - vyšší enzymová aktivita enzymu lysozymu stanovená ve vzorcích lidské sliny získaných od žen ve srovnání se vzorky lidské sliny získaných od mužů a oproti tomu vyšší hodnoty pH vzorků lidské sliny a vyšší aktivita enzymu chitinasy ve vzorcích lidské sliny získaných od mužů ve srovnání se vzorky získaných od žen je popsána v práci Prodan a kol.

(Prodan a kol., 2015). Taktéž byla provedena srovnávací studie interindividuálních variací u peptidových profilů pomocí MALDI-TOF hmotnostní spektrometrie (Prodan a kol., 2016). Ve studii Prodana a kolektivu bylo provedeno srovnání 268 vzorků lidských slin od zdravých dospělých, které byly charakterizovány s ohledem na aktivity enzymů lysozymu (součást obranného systému (Fábián a kol., 2012)) a chitinasy (přispívá k ochraně dutiny ústní před patogenními kvasinkami (např. *Candida albicans*) (van Steijn a kol., 2002)) (Prodan a kol., 2016).

3.2 Výsledky a diskuze – komentář k publikaci I

Odběr vzorku slin byl přesně předem nadefinován (preanalytická část) (Příloha 9; 10). Vzorky byly sbírány od dospělých lidí různé věkové kategorie (s ohledem na celkové zdraví, celkové orální zdraví, zkušenost se zubním kazem, pohlaví, dietní návyk, kouření). Vlastní odběr slin si skupina lidí zařazených do studie prováděla sama do sterilních zkumavek (přibližně 1 ml tekutiny), nalačno, před vyčištěním zubů (pro analýzu vzorků slin z mikrobiologického hlediska) nebo po vyčištění zubů (nejdříve však za 0,5 h), vzorky byly skladovány při teplotě -20 °C, a následně zpracovány. Byla odebírána nestimulovaná slina (odběr vzorku celkové sliny bez podnětu, bez chuťového a čichového stimulu, bez žvýkání hmoty - vosku, žvýkačky) (Ghezzi a kol., 2000).

Cílem sdělení v této publikaci je prezentovat rozdílné proteinové profily slin získaných od mužů (n=30) a žen (n=30) a provést srovnání získaných profilů pomocí label-free kvantifikace s ohledem na orální zdraví dárce vzorku slin a na jejich pohlaví.

Všechna měření byla prováděna na nano-kapalinovém chromatografu (n-LC; Proxeon Easy-nLC; Proxeon, Odense, Dánsko) spojeném s hmotnostním spektrometrem s kombinací dvou analyzátorů, kvadrupólu s průletovým analyzátorem (MaXis quadrupole time-of-flight (Q-TOF); Bruker Daltonics, Bremen, Německo). V experimentech byly použité předkolony a kolony (NS-MO-10 Biosphere C 18, NS-AC-C18 Biosphere C18; Nieuwkoop, Holansko). Separace směsi peptidů (získaných štěpením trypsinem jednotlivých vzorků lidských slin; části vzorku celkové sliny - supernatant, která byla získána centrifugací vzorku celkové sliny) byla provedena pomocí lineárního gradientu s mobilními fázemi A (voda) a B (acetonitril), přičemž obě obsahovaly 0,1% (v/v) kyseliny mravenčí. Nastavení hmotnostního spektrometru pro

analýzy MS a MS/MS bylo použito dle Mikšíka a kol. (Mikšík a kol., 2018). Data MS/MS byla zpracovávána pomocí softwaru ProteinScape (verze 3.0., Brucker Daltonics, Bremen, Německo). Proteiny byly identifikovány korelací tandemového hmotnostního spektra vzorků lidských slin s mezinárodní databází IPI databáze (následně i pomocí UniProt databáze). Prohledávání databáze bylo prováděno s taxonomií omezenou na *Homo sapiens* a nastavena kritéria pro validní protein a peptid (Mascot scóre ≥ 80 pro proteiny, Mascot scóre ≥ 20 pro peptidy; http://www.matrixscience.com). Výsledky byly statisticky zpracovávány pomocí Studentova t-testu ($p < 0,05$).

Bylo zjištěno 9 proteinů (α-amylasa 1, annexin A1, protein S100-A9, aktin, protein glutamingamaglutamyltransferasa E, protein S100-A8, annexin A2, serpin B3 a peptidyl-prolylcis-transisomerasa A) s vyšší expresí ve skupině vzorků lidských slin mužů se sanovaným chrupem ve srovnání se vzorky lidských slin od mužů se zdravou denticí a 7 proteinů (androgenem regulovaný protein 3B podčelistní žlázy, protein C6orf58, polymerní immunoglobulinový receptor, cystatin-SN, mucin-5B, lysozym c, lipokalin-1) s vyšší expresí ve skupině vzorků lidských slin získaných od žen se zdravou denticí v porovnání se skupinou vzorků od žen se sanovanou denticí.

Bylo provedeno srovnání proteinových profilů vzorků lidských slin získaných od lidí se sanovaným chrupem a zdravou denticí bez ohledu na pohlaví, a 5 proteinů bylo nalezeno s různou hladinou v porovnaných skupinách (α-amylasa 1, annexin A1, protein S100-A8, glyceraldehyd-3-fosfátdehydrogenasa, triose-fosfátisomerasa) (Příloha 1).

Poprvé bylo provedeno srovnání proteinových profilů ve vzorcích lidské sliny s ohledem na pohlaví. Byly vyhodnoceny statisticky významné rozdíly mezi expresí jednotlivých proteinů s ohledem na pohlaví. Ve srovnání profilů proteinů získaných ve vzorcích lidských slin od žen a mužů se sanovanou denticí bylo nalezeno 18 proteinů se statisticky rozdílnou expresí v porovnávaných skupinách. Identifikovali jsme 14 proteinů se statisticky významnou změněnou exprese ve srovnání žen a mužů bez ohledu na orální zdraví (Příloha 1).

Nalezené proteiny jako α-amylasa, annexin A1 a protein S100A9 jsou nejhojnější proteiny v lidské slině (Glassl a kol., 2016; Esteves a kol., 2019). Zjistili jsme 6 proteinů (α-amylasa, protein glutamingamaglutamyltransferasa E, protein S100-

A8, protein S100-A9, annexin A1, annexin A2) s vazebnými vlastnostmi vápníku ve skupině vzorků lidských slin od lidí se sanovanou denticí.

Proteiny S100-A9 a S100-A8 jsou vápník vázající proteiny a podílejí se na regulaci zánětlivého procesu (Ryckman a kol., 2003) a proteiny imunitního systému. Protizánětlivý mediátor annexin A1 může ovlivnit migraci a buněčné reakce vrozeného imunitního systému (Weyd, 2016). Lze předpokládat, že důvodem vyšší koncentrace imunitních proteinů ve vzorcích lidských slin od lidí se sanovanou denticí může být předchozí zkušenost s onemocněním v dutině ústní (zubní kaz). Studie Shina a kol. ukázala proteiny S100-A8 a S100-A9 jako biomarkery periodontitidy ve slinách (periodontitida – zánětlivé postižení periodontia (ozubice, závěsní parodontální vazy, spojení zubu a čelisti), které může být akutní a chronické; *periodontitis chronica*, *periodontitis acuta*) (Shin a kol., 2019). Slizniční muciny (mucin 5B) byly popsány v souvislosti s ochranou zdraví ústní dutiny (Frenkel a Ribbeck, 2015). Snížená koncentrace mucinů MUC5B a MUC7 je spojena s rozvojem zubního kazu, a stanovení hladiny mucinu MUC7 může být vhodné při vyhodnocení rizika vzniku zubního kazu (Szkaradkiewicz-Karpinska a kol., 2019).

Předpokládá se, že proteiny jako mucin-5B, laktoferrin, lysozym c mají specifickou ochrannou roli v ústní dutině, a mohly by být slibnými biomarkery zubního kazu. Preza a kol popsal změny proteomu lidské sliny získané odběrem vzorku lidské sliny přímo z příušní žlázy s ohledem na orální zdraví (konktrétně ošetření kořenových kanálku zubů) (Preza a kol., 2009). V naší práci jsme nenašli změny v těchto proteinech, které uvádí studie Preza a kolektiv. Studie Vitorina a kol byla zaměřena na proteinové složení lidských slin s ohledem na orální zdraví. Námi nalezené proteiny lipokalin 1 a cystatin-SN se zvýšenou expresí ve skupině vzorků lidských slin od lidí se zdravou denticí ve srovnání se vzorky lidských slin od lidí se sanovanou denticí jsou ve shodě s výsledky Vitorina a kolektivu (Vitorino a kol., 2006).

Srovnání proteomu lidských slin s ohledem na pohlaví dárce slin a s ohledem na orální zdraví (výskyt zubního kazu) bylo provedeno Lukacsem (Lukacs, 2011). V práci došli k závěru, že ženy mají vyšší míru dispozice zubního kazu než muži. Jako důvody uvádějí dřívější erupce zubů (prořezání zubů – průnik zubů z čelisti a dásní na povrch a růstový postup zubu až do zaujetí pracovní polohy) u dívek a v těhotenství. Vyšší

výskyt zubního kazu v souvislosti s těhotenstvím je znám, do tohoto procesu je zapojeno mnoho faktorů, jako jsou vyšší množství a frekvence spotřeby kariogenní stravy, snížení pH způsobené častým zvracením a snížení pozornosti na udržení orální hygieny (Cristensen a kol., 1998; Laine, 2002). Sociodemografický status může mít vliv na neznalost prevence ochrany orálního zdraví, a vede k vyššímu výskytu zubního kazu (ElKarmi a kol., 2019).

Práce Fleissinga a kol. uvádí šest proteinů s výrazně vyšší expresí ve vzorcích lidských slin získaných od žen v porovnání se vzorky lidských slin od mužů (Fleissing a kol., 2010). V našich výsledcích je inhibitor leukocytové elastázy (SERPINB1) vyhodnocen s výrazně vyšší expresí ve skupině vzorků lidských slin od mužů se sanovanou denticí ve srovnání se vzorky lidských slin od žen se sanovanou denticí. Protein calgranulin A (S100-A8) byl zjištěn ve všech našich genderových srovnání. Rozdílné výsledky mohou být způsobeny různými experimentálními metodami i věkem dárců vzorků slin. (Fleissig a kol využil ke srovnávací studii metodu dvourozměrnou elektroforézu, zatímco my jsme prováděli analýzy pomocí label-free kvantifikace hmotnostní spektrometrií.).

Rozdílné exprese proteinů získaných ze vzorků lidských slin byly pozorovány před a po fyzické aktivitě, a dále také rozdíly exprese proteinů mezi vzorky lidských slin získaných od žen a mužů. Jednalo se o 3 proteiny (lysozym c, antileukoproteinasa) se zvýšenou expresí ve skupině vzorků lidských slin od mužů ve srovnání se vzorky lidské sliny od žen, a 3 proteiny (α-amylasa 2B, pankreatická α-amylasa, α-amylasa 1), se zvýšenou expresí ve skupině vzorků lidských slin od žen ve srovnání se vzorky lidských slin od mužů (Franco-Martínez a kol., 2020). Nalezené změny v proteomu lidských slin nebyly shodné s námi nalezenými výsledky.

Průřezová srovnávací studie interindividuálních biochemických variací v nestimulované slině (dárci vzorků 18-30 let) popisuje snížené hladiny pH a nižší expresi proteinu MUC5B ve slinách u žen než ve slinách mužů (Prodan a kol., 2015), což je ve shodě s našimi výsledky. Stanovit jednoznačnou korelaci mezi biochemickými parametry, včetně složení minerálů, proteinů, aktivit enzymů ve slinách a krevní plazmě je obtížné. Ale byly nalezeny některé korelace mezi stanovením ve vzorcích lidské sliny a plazmě, a to v případě močoviny, u které je možné sledovat i její nárůst s věkem

dárce, kyseliny močové, stanovení pH, ALT (alaninaminotransferasa) a LDH (laktátdehydrogenasa) (Bel´skaya a kol., 2020). Ovlivnění složení vzorků lidské sliny může být způsobeno také časem odběru. Byly popsány změny ve složení vzorků lidské sliny s ohledem na biorytmus. Dynamika studovaných parametrů (koncentrace vápníkových a draselných iontů, koncentrace minerálů) během 24 hodin je charakterizována významnými změnami, které vyplývají ze změny sekrece slin během 24 hodin a z působení hormonů (Bel´skaya, 2017).

Pozorovali jsme rozdíly v proteinovém složení ve vzorcích lidské sliny s ohledem na pohlaví. Složení proteinů ve vzorcích lidské sliny bylo sledováno pouze v jednom časovém intervalu, a výsledky by mohly být proto ovlivněny přítomností falešně pozitivní identifikací rozdílů. Je třeba zdůraznit, že podmínky sběru byly přísně dodržovány, aby bylo zabráněno předanalytickým chybám.

V naší budoucí práci bychom rádi rozšířili počet jednotlivců v jednotlivých sledovaných skupinách a předložili dárcům vzorků lidských slin rozšířený dotazník (interindividuální a intraindividuální variabilita (Příloha 6: Obr. V); rozšíření otázek ohledně zdraví dárce – interní vyšetření), s několika časovými intervaly odběru vzorků lidských slin s možností zmapovat možné efekty (ovlivnění v důsledku menstruačního cyklu u žen, cirkadiálního rytmu, stravovacího návyku stejně jako stravy v předcházejících dnech před odběrem vzorku, atd.).

3.3 Výsledky a diskuze – komentář k publikaci II

V druhé části práce byly vzorky lidské sliny zpracovány pouze od mužů (n=27). Vzorky byly rozděleny do dvou skupin na základě orálního zdraví: skupina mužů se zdravou denticí (n=12) a skupina mužů se sanovanou denticí (n=15). Dárci vzorků vyplnili dotazník (Příloha 9).

Vzorky lidkých slin byly zpracovány podle požadavků pro příslušné vybrané metody. Pro jednorozměrnou, dvourozměrnou a dvourozměrnou diferenční gelové metody (Příloha 6: Obr. III, IV, VI, VII) a pro přípravu vzorků lidských slin pro analýzy pomocí hmotnostních spektrometrií byly vzorky lidkých slin rozděleny pomocí centrifugace na supernatanty a sedimenty (podmínky 13 000, 30 minut, při 4 °C).

Primární zkoušky byly prováděny na dodaných vzorcích na vatových válečcích, z tohoto typu odběru slin bylo ustoupeno, docházelo k velkým ztrátám proteinů. Všechna publikovaná měření byla prováděna na vzorcích slin odebraných neivazivním odběrem, samovolným nasliněním do sterilních zkumavek (Příloha 10).

Všechny vzorky lidské sliny (supernatanty a sedimenty, získané centrifugací vzorků celkové sliny) byly zpracovávány nejprve pro analýzy pomocí jednorozměrné, dvourozměrné gelové elektroforézy, diferenční dvourozměrné gelové elektroforézy. Jednotlivé optimalizační kroky v přípravě vzorků nebyly publikovány, jednotlivé komentáře k optimalizačním krokům pro příslušné metody naleznete v příloze (Příloha 6: Obr. I-VII).

Vzorky lidské sliny (získané části tohoto vzorku centrifugací – supernatanty a sedimenty) byly analyzovány pomocí hmotnostní spektrometrie a kvantifikovány pomocí přístupu label-free kvantifikace (Příloha 2). Poprvé bylo provedeno srovnání proteinových profilů částí vzorků celkové sliny (supernatanty, sedimenty) s ohledem na orální zdraví.

Při porovnání supernatanových frakcí získaných od skupin se zdravou a ošetřenou (sanovanou) denticí bylo nalezeno 14 proteinů se statisticky rozdílnou expresí ve sledovaných skupinách s ohledem na orální zdraví. Porovnáním sedimentů získaných od skupin se zdravou a ošetřenou denticí byly nalezeny 3 proteiny s rozdílnou expresí ve srovnání sledovaných skupin s ohledem na orální zdraví (Příloha 2).

Proteiny zjištěné pomocí label-free kvantifikace hmotnostním spektrometrem ve frakci supernatant (14 proteinů) byly nalezeny s vyšší expresí ve skupině získaných od mužů se zdravou denticí ve srovnávání se skupinou mužů se sanovanou denticí. Polovina těchto proteinů jsou proteiny imunitního systému a antibakteriální proteiny (např. lysozym c, protein S100-A8, konstantní řetězec imunoglobulinu kappa, polymerní imunoglobulinový receptor). Tyto výrazně zvýšené proteiny mohou hrát určitou roli v prevenci zubního kazu.

U tří proteinů ve frakci sediment byla statisticky zjištěna s vyšší exprese ve skupině vzorků sedimentů získaných od mužů se sanovanou denticí ve srovnání se skupinou od mužů se zdravou denticí. Popravé bylo provedeno srovnání části vzorků lidské sliny – sedimentů a jejich proteinových profilů s ohledem na zubní kaz v dutině ústní. Dosud nebyla publikována práce, která by se na tuto problematiku zaměřila.

Proteinové profily frakcí získaných centrifugací vzorků celkové sliny (supernatanty a sedimenty) byly porovnávány s ohledem na orální zdraví pomocí dvourozměrné gelové elektroforézy a label-free kvantifikace hmotnostním spektrometrem. Oběma přístupy byl nalezen protein annexin A1, který byl nalezen shodně s vyšší expresí ve skupině se zdravou denticí pomocí dvourozměrné gelové elektroforézy a hmotnostní spektrometrie přístupem label-free kvantifikace v části vzorku lidské sliny - sedimentu (Příloha 2; Příloha 6: Obr. VII C).

Byla provedena srovnávací studie proteinových profilů frakcí získaných centrifugací vzorků celkové sliny (supernatant a sediment), byly tak nalezeny unikátní proteiny pro dané frakce (Příloha 2 – Fig. 1). Srovnání bylo provedeno ze tří pohledů: 1) Srovnání všech supernatantů (n= 27) a všech sedimentů (n=27) bez ohledu na orální zdraví dárce (v obrázku označeno A), bylo identifikováno 207 proteinů, z nichž 25 proteinů splnilo předdefinované kritérium jedinečnosti proteinové frakce. Kritérium pro identifikaci jedinečnosti proteinové frakce (supernatant nebo sedimentu) byla přítomnost proteinu v alespoň v 50% vzorcích jedné frakce (ve skupinách se zdravou nebo sanovanou denticí), maximální tolerance proteinové nečistoty byla stanovena na přítomnost ve 2 vzorcích v druhé frakci.). 2) Srovnání supernatanty vs. sedimenty získaných od skupiny mužů se sanovanou denticí (identifikováno bylo 145 proteinů, z toho 25 splnilo kritérium pro jedinečnost v proteinové frakce, kruh B). 3) Srovnání

supernatanty vs. sedimenty získaných od skupiny mužů se zdravou denticí (bylo identifikováno 185 proteinů, z toho 3 byly klasifikovány jako jedinečné pro frakci, kruh C).

Výsledky byly pozoruhodné z hlediska distribuce proteinu pozorované mezi sedimenty a supernatanty. Je z nich zřejmé, že proteinové složení vzorků celkové sliny ve skupinách se sanovanou a zdravou denticí, je velmi specifické, což ukazuje, že vzorky celkové sliny skupiny se sanovanou denticí obsahují hlavně proteiny nalezené jedinečné pro sediment a vzorky celkové sliny skupiny se zdravou denticí obsahují hlavně jedinečné proteiny pro supernatant.

Identifikované proteiny (protein S100-A14, cornulin, proteinglutamingamaglutamyltrasferasa E, spojovací plakoglobin, protein deletovaný v maligních mozkových nádorech 1) patří do skupiny proteinů, které jsou vápník vázající a jsou na fungování Ca^{2+} závislé. Tyto identifikované proteiny byly nalezeny jedinečně v sedimentu ve skupině vzorků lidské sliny se sanovanou denticí a mohou naznačovat určité spojení s onemocněním zubním kazem (odvápnění skloviny).

Proteiny laktotransferrin, protein S100-A8, člen 1 a 2 rodiny proteinů B obsahující skupinu BPI a lysozym c patří do skupiny antimikrobiálních proteinů. Tyto proteiny byly identifikovány jedinečně v supernatantu ve skupině mužů se zdravou denticí. Proteiny laktotransferrin, imunoglobulin lambda variabilní 1-44 se podílejí na imunitní odpovědi. Role těchto proteinů (antimikrobiální vlastnosti a účast na imunitní dopovědi) mohou hrát hlavní roli v prevenci zubního kazu.

Ve frakci sediment byly zjištěny proteiny patřící do skupiny plakinů (periplakin, desmoplakin, plakophilin 1, envoplakin, fillaggrin), keratinů a histonů, patřící do cytoskeletových proteinů. Výsledky ukazují, že nalezené proteiny ve frakci sediment jsou důležité pro etiologii zubního kazu. Nalezené výsledky naznačují, že proteiny podporující obranu proti zubnímu kazu jsou přítomny hlavně v supernatantu vzorku lidské sliny.

Naše výsledky jsou částečné v souladu s prací Wang a kol (Wang a kol., 2018a). Wang a kolektiv analyzoval supernatant slin pomocí LC-MS/MS spojeného s iTRAQ, ve své práci prezentují 14 věkově specifických proteinů s ohledem na orální zdraví.

V případě námi identifikovaného proteinu cornulin, který jsme statisticky vyhodnotili ve frakci sediment s vyšší expresí ve skupině vzorků poskytnutých od mužů se sanovanou denticí oproti se zdravou denticí, a proteinu S100A9, vyhodnoceného ve frakci supernatant s vyšší expresí ve skupině vzorků poskytnuté od skupiny se zdravou denticí ve srovnání se sanovanou denticí, dochází se shodě s prací Wang a kol..

Kvalitativní rozdíly mezi proteomy získaných ze vzorků supernatantů od lidí s ohledem na DMFT index dárce vzorku celkové sliny byly popsány v práci Laputková a kol. (Laputková a kol., 2017). Experiment byl prováděn na vzorcích lidské sliny od 19 letých dárců, rozdělen do skupin s ohledem na DMFT index (skupina s DMFT = 0, a DMFT > 0 (tj. 4 – 9)). Ve vzorcích supernatantů pomocí nana-kapilonové chromatografie a MALDI-TOF /TOF hmotnostní spektrometrie bylo identifikováno 554 (695) proteinů ve skupině s DMFT > 0 (DMFT = 0). Vyhodnocení MS dat pomocí „Gene Ontology (GO) term enrichment Analysis“ odhalilo kvalitativní rozdíly ve skupinách vzorků lidských slin získaných od lidé s DMFT=0 a DMFT > 0. Proteiny, které se podílejí na imunitní odpovědi, byly nalezeny převážně ve skupině s nenulovým DMFT indexem, což v naší srovnávací studii byly tyto proteiny ve skupině vzorků lidských slin se zdravou denticí s vyšší expresí ve srovnání se skupinou se vzorky získaných od mužů se sanovanou denticí. Vápník vázající proteiny byly identifikovány ve skupině s DMFT > 0, což se shoduje s našimi výsledky.

Analýzy biologického materiálu, vzorky lidské sliny, jsou dobrým zdrojem informací pro stanovení diagnózy, sledování progrese onemocnění, a zvláště pro předvídání rizikových skupin pro studovaná onemocnění. Velkou výhodou je neinvazivní odběr biologického materiálu (Czöcz a kol., 2017). Kvantitativní proteomika je současný moderní metodický přístup (Katsani, 2019).

Od našich primárních výsledků, které studovaly problematiku orálního zdraví (konkrétně onemocnění zubní kaz) pouze z pohledu proteinového profilu vzorků lidské sliny, by se dále měly provést komplexnější studie, zaměřené primárně na proteinové profily vzorků lidské sliny (vzorků lidských slin, sliznic, odběry z jednotlivých částí dutiny ústní (odběry vzorků celkové sliny, vzorků z jednotlivých slinných žláz, odběr pelikuly (Odanaka a kol., 2020)) a také odběry pro mikrobiologické analýzy (primární výsledky - Příloha 6 – Obr. VIII A, B; IX A, B). Bude třeba kompletovat výsledky

z proteomických a mikrobiologických analýz (primárná výsledky – Příloha 7) a dále identifikovat metabolity ve vzorcích lidských slin.

V primární srovnávací studie proteinových profilů (odběr vzorků celkové sliny po noci před vyčištěním zubů) byl nalezen cystatin-S se zvýšenou expresí ve skupině lidí, kteří uvedli pozitivní odpověď na kouření. Cystatin-S byl také popsán se zvýšenou expresí u lidí se závislostí na kouření (Batista a kol., 2019). Ve srovnání proteinových profilů vzorků celkové sliny získaných od žen a mužů (Příloha 8 – srovvnání I, III a IV) byl nalezen protein calmodulin 3 se zvýšenou expresí u skupiny vzorků celkové sliny od mužů (bez ohledu na věk dárce vzorků celkové sliny), jedná se o vápník vázající protein.

Primární výsledky mikrobiologického přístupu elektroforézy v gradientovém denaturačním gelu byl získán bakteriální profil ústního mikrobiomu. Touto metodu nebyly pozorovány významné rozdíly v zastoupení bakterií u jednotlivých vzorků celkové sliny (Příloha 6: Obr. VIII A - A-C). Ani za pomocí statistického vyhodnocení softwarem BioNumerics 7.6 nebyly zjištěny významné rozdíly (Příloha 6: Obr. VIII B - E a F). Výrazné spoty byly vyříznuty a podrobeny sekvenovaní pro jejich identifikaci (Příloha 6: Obr. VIII B - D). Z výsledků sekvenování jsme získali pouze 2 bakterie (*Ureibacillus suwonensis*, *Rothia mucilaginosa*), které byly identifkovány s pravděpodobností 97% a více. Tyto výsledky mohly být způsobeny nízkým výskytem bakterií ve slině, vzhledem k tomu, že slina obsahuje 98% vody. Důvodem malé mikrobiální diverzity může být také to, že se jednalo o stejnou sociální vrstvu a úroveň ústní hygieny a jednalo se z velké části o dárce v rodinném příbuzenském vztahu. Mikrobiologický přístup sekvenování nové generace vzorků slin (odběr vzorků lidské sliny odebrané nalačno, před čištěním zubů) a zpracování získaných dat pomocí softwaru Quime neposkytl také žádné významné odchylky v zastoupení jednotlivých bakterií ve srovnání s ohledem na věk, pohlaví, kouření a orální zdraví (Příloha 6: Obr. IX A; B).

Z našich výsledků vyplývá, že sediment obsahuje velkou část unikátních proteinů, avšak často není neanalyzována z důvodu použití pouze supernatanové části vzorku lidské sliny. V dalších srovnávacích studií by se měl proteinový profil vzorků sedimentů dát do souvislosti se studovaným onemocněním (např. plakiny ve vzorcích

lidské sliny). Pro další studie by bylo nutné vypracovat pracovní protokol pro přípravu vzorku sedimentu, který by vedl k vyšší výtěžnosti proteinů (peptidů) z příslušného vzorku (extrakce, rozpouštění).

4 Bazo-apikální rozdíly - proteomické složení levé a pravé komory srdeční

4.1 Teoretický úvod

Srdeční komory vykazují řadu morfologických, biochemických i funkčních rozdílů, kterou jsou dány charakterem zátěže srdce. Zatímco levá komora čerpá krev do vysokotlakého systémového řečiště, pravá komora do nízkotlakého plicního řečiště. Levá komora má proto větší hmotnost, silnější stěnu, myocyty o větším průměru – funguje primárně jako tlakové čerpadlo. Pravá komora funguje jako objemové čerpadlo. Z toho vyplývají odlišné energetické nároky a proteinové složení. Kromě pravolevých rozdílů existují i rozdíly mezi bazální a apikalní části komor a transmurální rozdíly (subepikard versus subendokard).

Byly porovnány hmotnosti levé a pravé komory u člověka pomocí magnetické rezonance (Lorenz a kol., 1999). A také byly porovnávány hmotnosti levé a pravé komory srdeční u mužských atletů a vytrvalostních sportovců spojené s hypertrofií srdce (srdeční zátěž u sportovce vede prokazatelně k zvětšení srdce) (Scharhag a kol., 2002). Výsledky Waskova-Arnostové a kolektivu uvádí, že pravá komora má vyšší aktivitu aerobního glykolytického metabolismu, a může být tak schopna reagovat rychleji na stresující podněty než levá komora (Wakova-Arnostova, a kol., 2013). Byla popsaná změna remodelace (přestavba) levé srdeční komory s ohledem na pohlaví atletů (nižší objem v levé komoře u atletek ve srovnání s atlety získaný z 3D echokardiografie) (Lakatos a kol., 2020).

U psů byla zjištěna menší spotřeba kyslíku v pravé komoře oproti levé komoře (Kusachi a kol., 1982). Bylo provedeno srovnání spotřeby kyslíku srdce jednotlivých srdečních komor u mužů pomocí pozitronové emisní tomografie v klidu a během cvičení (jízdě na kole). Byla zjištěna dvojnásobná až trojnásobná spotřeba kyslíku v obou komorách při cvičení oproti klidovému režimu. A také provedené srovnání frakce extrahovaného kyslíku byla v klidovém režimu stejná v obou komorách, ale při cvičení byla vyšší v pravé komoře (Kudomi a kol, 2019).

Bylo prokázáno, že vzhledem ke značným rozdílům ve velikosti srdce a srdeční frekvenci je srdeční anatomie u myši a člověka pozoruhodně podobná (vývoj srdce, vytvoření čtyřkomorového srdce), a v důsledku toho může být myš vhodným modelem pro studium srdce (Wessels, 2013).

Byla provedena srovnávací studie proteomu levé a pravé komory srdce u myší (Comunian a kol., 2011). Dále bylo provedeno srovnání proteomu pravé komory lidského srdce při srdečním selhání a její srovnání s proteomem levé komory pomocí dvourozměrné gelové elektroforézy (Su a kol., 2015). Gregorich a kol. sledoval posttranslační modifikace proteinů myofilamentu při srdeční kontraktilitě (stažlivostí) pomocí „Top-down" hmotnostní spektrometrie a popsal transmurální rozdíly v proteinech (srdeční troponin T, tropomyosin) obsažených v myofilamentu (Gregorich a kol., 2015).

Rozdíly v proteomu v levé a pravé komoře za aerobních podmínek a odezva na ischemii byly pozorovány u potkana (Cadete a kol., 2012). Rozdíly v proteomu srdečních komor u hypertenzního potkana (18 myokardiálních proteinů s vyšší expresí v pravé komoře ve srovnání s levou komorou srdeční, 8 myokardiálních proteinů s vyšší expresí v levé komoře srdeční ve srovnání s pravou komorou srdeční) byly pozorovány též v práci Manakov a kol. (Manakov a kol., 2016). Proteinová analýza srdce člověka odhalila odlišné proteomy srdečních komor se závislostí na onemocnění (ischemická choroba srdeční a skleróza aortální chlopně) (Littlejohns a kol., 2014).

Doll a kolektiv popsal podrobný proteom zdravého lidského srdce (vzorky získané od zdravých mužů (*post mortem*). Analyzovali vzorky srdce, které rozdělili na 16 částí, a kvantifikovali skoro 11 tisíc proteinů. Byly popsány proteomy srdečních siní a komor, které jsou odlišné v důsledku fyziologické úlohy jednotlivých částí srdce. Nebyl však popsán kvantitativní rozdíl mezi levou a pravou komorou (Doll a kol., 2017).

4.2 Výsledky a diskuze – komentář k publikaci III

V této publikaci bylo popsáno proteinového složení levé a pravé komory srdeční potkana se zaměřením na baso-apikální rozdíly.

V tomto experimentu byli použiti dospělí samci potkana kmene Wistar (n=5; stáří 3 měsíce; 484±26 g hmotnost). Pravá komora a volná stěna levé komory byly odděleny a rozdělena na 3 části – bazální, střední a apikální. Lyofilizované vzorky byly homogenizovány a následně analyzovány pomocí dvourozměrné gelové elektroforézy.

Proteomické analýzy jednotlivých částí levé a pravé komory byly provedeny pomocí dvourozměrné gelové elektroforézy. Před provedením dvourozměrné gelové elektroforézy byla realizována jednorozměrná gelová elektroforéza vzorků (z příslušných částí obou komor). Bylo provedeno 30 dvourozměrných gelových elektroforéz (15 jich bylo opakováno). Jednotlivá skenování gelů byla provedena pomocí programu QuantityOne a skeneru GS-800 Calibrated Imaging Densitometer (Bio-Rad, USA). Kvantitativní vyhodnocení jednotlivých spotů bylo provedeno pomocí softwaru PDQuest^{TM} (Bio-Rad, USA).

Bazoapikální rozdíly v proteinovém složeny nebyly doposud popsány. Naše práce poprvé odhaluje významné bazoapikální rozdíly v expresi několika proteinů. Bylo nalezeno pět proteinů s vyšší expresí v apikální oblasti levé komory srdeční ve srovnání s bazální části levé komory srdeční (myosinový lehký řetězec 3, laktátdehydrogenasa B, kreatinkinasa M, dihydrolipoyldehydrogenasa, protein tepelného šoku HSP60). Z toho tři proteiny jsou mitochondriální a patří do metabolických a energetických drah. V pravé komoře byla pozorována vyšší exprese u dvou proteinů (mitochondriální proteiny) (Příloha 3).

Výraznější změny byly popsány v levé komoře, která je vystavena větší tlakové zatížení. Signifikantně vyšší exprese dvou proteinů byla nalezena v levé komoře ve srovnání s pravou komorou (myosinový lehký řetězec 3, myosinový protein C, srdeční typ).

Srovnání proteinového složení levé a pravé komory odhalilo překvapivě pouze dva rozdílné proteiny. Oba tyto proteiny patří do skupiny kontraktilních proteinů. Existují studie popisující proteomy levé a pravé komory srdce myší (Comunian a kol., 2011), které identifikovaly tisíce proteinů, z toho 16 proteinů ve vyšší koncentraci v levé komoře ve srovnání s pravou komorou a 47 proteinů s vyšší koncentrací v pravé komoře ve srovnání s levou komorou.

V obou komorách byla stanovena výrazně vyšší koncentrace proteinů v apikální oblasti ve srovnání s bazální oblasti, což odpovídá vyšším energetickým nárokům těchto části obou komor ve srovnání s bázemi. Výsledky ukazují, že heterogenita proteinového složení komorového myokardu zahrnuje kromě popsaných proteinových rozdílů levé a

pravé komory a transmurálních rozdílů také významné kvantitativní rozdíly mezi apikální a bazální částí obou komor (Příloha 3).

Práce zabývající se profilováním proteinů levé a pravé komory srdce jsou závislé na analytickém postupu, druhu, věku a pohlaví zkoumaného experimentálního modelu a na charakteru případných patologických projevů.

Výsledky naší studie zabývající se bazoapikálními rozdíly srdečních komor u potkana zdůrazňují důležitost volby vhodného místa pro odběr vzorků pro biochemické analýzy myokardu. Identifikace proteinů v jednotlivých částech srdečních komor může pomoci k objasnění mechanismů vedoucích k srdečním chorobám a k nalezení vhodných biomarkerů.

Výstup ze srovnávacích studií (včetně našeho porovnání proteinového profilu bazální a apikální části srdeční komory) může pomoci k pochopení mechanismu srdce s ohledem na pohlaví a například s onemocněním infarktu myokardu (Ošťádal a kol., 2009). Dalším možným směrem studia by mělo být porovnání jednotlivých části srdečních komor s ohledem na pohlaví potkana s možností využít modernější přístupy k analýze proteinů jako například analýzy pomocí hmotnostní spektrometrie (přístup label-free kvantifikace), což by umožnilo kvantifikovat více identifikovaných proteinů pomocí hmotnostní spektrometrie než dvourozměrnou gelovou elektroforézou.

5 Rozdílné proteinové profily při kultivaci bakterie Butyrivibrio fibrisolvens Bf3071

5.1 Teoretický úvod

Bachor je primárním trávicím orgánem přežvýkavců, a představuje vrcholný symbiotický vztah mezi mikrobiotou a hostitelem. Jedná se o unikátní systém pro zpracování rostlinné biomasy.

Butyrivibrio fibrisolvens Bf 3071 je anaerobní bakterie izolována z bachoru patřící do rodiny *Lachnospiraceae*, řád *Clostridiales*, třída *Clostridia*, rod *Firmicutes*. Patří do rodiny dědičných bachorových druhů, které představují téměř polovinu populace bachoru (López-García a kol., 2018).

Bakterie *Butyrivibrio fibrisolvens* Bf3071 patří mezi xylanolytické bakterie, účastní se degradace xylanu na jednoduché cukry, které jsou přeměňovány na těkavé mastné kyseliny (acetát, butyrát, atd.), které slouží jako zdroj energie pro hostitelské zvíře.

5.2 Výsledky a diskuze – komentář k publikaci IV

Bakterie *Butyrivibrio fibrisolvens* Bf 3071 byla kultivována na čtyřech různých substrátech. Při výběru substrátu pro kultivaci bakterií se vždy přihlíží k preferenci sledované bakterie. Bachorové bakterie (v našem případě *Buryrivibrio fibrisolvens Bf3071*) se obecně snadno kultivují na tzv. lehkých substrátech, kterými jsou např. celobióza nebo glukóza. Tyto „lehké“ substráty jsou pro bakterie snadno dostupné a tím pádem i lépe rostou. Tzv. těžké substráty, ke kterým patří třeba xylan, jsou vhodné pro xylanolytické bakterie, tedy v našem experimentu pro *Butyrivibrio fibrisolvens* Bf3071. Bakteriální populace je snadno kultivovatelná, protože bakterie štěpí svůj preferovaný substrát a získávají z něho energii.

Jako zdroj uhlíku byly použity glukóza (univerzální zdroj uhlíku), xylan (sledovaná bakterie je xylanolytická), směs xylanu a glukózy (přídavek glukózy pro zlepšení podmínek kultivace) a xylóza (pentóza; jednotky xylanu se skládají především z xylózy).

Jednotlivé kultivace byly provedeny ve dvojím provedení, tedy kultivace s glukózou (n=2), s xylanem (n=2), se směsí xylanu a glukózy (n=2) a s xylózou (n=2).

Z jednotlivých kultivací byla izolována jejich extracelulární matrix. Před provedením experimentů dvourozměrné gelové elektroforézy byla provedena jednorozměrná gelová elektroforéza se vzorky jednotlivých izolovaných extracelulárních matrix pro příslušené podmínky kultivace. Byla také provedena detekce xylanolytických enzymů pomocí zymogramů.

Bylo provedené porovnání proteinových profilů bakterie *Butyrivibrio fibrisolvens* Bf 3071 kultivované na čtyřech různých substrátech. Ke kvantifikaci dvourozměrné gelové elektroforézy byl použit software PDQuest^{TM} (Bio-Rad, USA), výsledky byly statisticky zpracovány (Studentův t-test, $p < 0,05$). Spoty splňující statistickou významnost byly vyříznuty a analyzovány pomocí hmotnostní spektrometrie. Identifikované proteiny poskytují informace o substrátové preferenci a způsob k získávání živin a energie (Příloha 4).

Vyhodnocení srovnávací studie jednotlivých substrátů použitých při kultivaci bakterie *Butyrivibrio fibrisolvens* Bf 3071 odhalily omezený počet významně rozdílně exprimovaných proteinů. Námi identifikované proteiny se účastní glykolýzy, syntézy proteinů a syntézu butyrátu. Vyšší hladiny detekovaných enzymů centrální uhlíkové katabolické dráhy byly také získány u bakterií *Ruminiclostridium cellulolyticum* (Badalato a kol., 2017), stejně jako v celém bachorovém mikrobiomu (Snelling a Wallace, 2017). Pozorované změny v proteinové expresi (glyceraldehyd-3-fosfátdehydrogenasa a fosfoglycerátkinasa) se shodují s prací Snelling a Wallace (Snelling a Wallace, 2017).

Proteomická studie *Butyrivibria fibrisolvens* Bf 3071 ukazuje, že tento kmen může hrát důležitou roli pro centrální metabolismus bachoru. Vzhledem k obrovskému potenciálu využití rostlinné biomasy, jako obnovitelnému zdroji energie, by bylo přínosné pochopit mechanismy štěpení biomasy enzymaticky za přispění enzymů bakterií. Enzymatické štěpení je především ekologičtější než štěpení chemické, a dalo by se také využít i v různých průmyslových odvětvích – papírenský průmysl, potravinářství, v bioplynových stanicích.

V experimentu byla použitá k analýze proteinů dvourozměrná gelová elektroforéza. V dalších krocích by mohla být použita kvantifikace pomocí hmotnostní spektrometrie, a to label-free kvantifikace. Výsledky by nebyly omezeny použitím pI

rozsahu při prvním rozměru dvourozměnrné gelové elektroforézy, a poskytly by cenné informace o jedinečné cestě štěpení biomasy.

6 Nový příspěvek ke vzniku a vývoji vrozeného onemocnění pohyblivého aparátu – pes equinovarus

6.1 Teoretický úvod

Idiopatická *pes equinovarus congenitus* (PEC; clubfoot) je vrozená deformita chodidel dolních končetin. Clubfoot patří do skupiny fibroproliferativních poruch, a dodnes nebyla popsána příčina tohoto onemocnění. Incidence PEC evropské populaci je přibližně 1-2 na 1000 živě narozených dětí (Pavone a kol., 2018; Smythe a kol., 2017). Léčbou PEC je fyzioterapie a Ponsetiho metoda (sádrování, tenotomie (chirurgické přetětí šlachy) Achillovy šlachy, transpozice (přemístění) *m. tibialis ant.* (*musculus tibialis anterior*) (Ošťádal a kol., 2017; Kadhum a kol., 2019).

Existuje hypotéza o zapojení extracelulárních proteinů, zejména z fibroblastů, růstových proteinů do patogenetických mechanismů zodpovědných za vývoj clubfootu (Li a kol., 2001; Cai, a kol., 2020). Již dříve byla provedena proteomická analýza vazivové tkáně u pacientů s *pes equinovarus congenitus*. Byly zjištěny kolageny typu I a III a TGFβ (transformační růstový faktor β), které byly již dříve popisovány v této tkáni, ale i další typy kolagenů V, VI a XII. Srovnání mediální s laterální části kloubního pouzdra přineslo rozdílné exprese proteinů. Byla pozorována vyšší exprese proteinů kolagenu VI, asporinu, mimecanu, prolaminu a TGFβ ve vzorcích z mediální strany kloubního pouzdra ve srovnání s laterální stranou. Bylo identifikováno 18 extracelulárních proteinů ve vzorcích v mediální straně skloubení (Ošťádal a kol., 2015).

6.2 Výsledky a diskuze – publikace V

Komplexní proteinová srovnání PEC pomocí label-free kvantifikace byla provedena na vzorcích kloubního pouzdra mediální strany, n=16 a laterální strany, n=13, a to v návaznosti na předchozí práce Ošťádala (Ošťádal a kol., 2015).

Sedm proteinů bylo signifikantně zvýšeno ve vzorcích z mediální strany kloubního pouzdra (asporin, kolagen typu III, I a VI, versikan, tenascin-C, TGFβ) a čtyři proteiny s vyšší expresí ve vzorcích z laterální strany (kolagen typu XII a XIV, fibromodulin a CILP-2 (protein 2 mezivrstvy chrupavky). Většina nalezených proteinů s různou expresí v porovnávaných vzorcích souvisí se stavbou extracelulární matrix, a

předpokládá se, že může hrát roli v patogenezi této vrozené deformity chodidel a dolních končetin.

Tato studie odhalila 11 kvantitativně významných rozdílů v proteinovém složení vazivové tkáně při onemocnění clubfoot. Všechny tyto proteiny mohou sloužit jako potencionální biomarkery tohoto onemocnění, jejichž další analýzy by mohla přinést nové poznatky morfogenetického vývoje této závažné vrozené vady nohy.

Přístup label-free kvantifikace umožnil porovnat zastoupení 70 proteinů (do dnešního dne nejvyšší počet kvantifikovaných proteinů) (Příloha 5). Výběr a získání správného kontrolního vzorku přineslo etické dilema (nezbytný souhlas rodičů s účastí dítěte na výzkumu, kontrolní vzorky od zdravých dětí (téměř nemožné), zatížení dlouhodobou léčbou).

Nabízí se myšlenka chemického ošetření tohoto onemocnění jako v případě další fibroproliferativní choroby např. Dupuytrenova konktaktura, kdy se injekčně používá enzym kolagenasy *Clostrudium histolyticum* (látka přednostně štěpící kolagen typu I a III) (Burlacu, 2010). Doposud ale neexistuje chemické ošetření idiopatické vrozené vady clubfoot.

Jako největší problém lékaři dětské ortopedie vidí v recidivách onemocnění PEC. K recidivám dochází bohužel často (Ošťádal a kol., 2013). Pro lékaře je nutné rozhodnutí vhodné volby léčby – a to od konzervativního způsobu léčby (sádrování) (Islam a kol., 2020, Yu-Bin a kol., 2020) k chirurgickému zákroku (Stouten a kol., 2018). Otázkou zůstává, jak recidivující onemocnění pes equinovarus předpovídat, a co jsou pro recidivu tohoto onemocnění rizikové faktory.

7 Závěr

- Byly provedeny optimalizace preanalytické fáze zpracování vzorků biologického materiálu a provedeny vlastní analýzy pomocí separačních metod (jednorozměrnou a dvourozměrnou gelovou elektroforézou, dvourozměrnou diferenční elektroforézou a analázy pomocí hmotnostní spektrometrie)
- Vzorky celkové sliny byly odděleny centrifugací – získány frakce supernatant a sediment – a byly provedeny srovnání proteinových profilů vzorků lidské sliny s ohledem na orální zdraví a na pohlaví.
- Bylo poprvé popsáno specifické proteinové složení získaných frakcí vzorku lidské sliny (supernatnant, sediment). (Kulhavá a kol., 2020)
- Přístupem label-free kvantifikace byly poprvné srovnány proteinové profily vzorků lidských slin s ohledem na orální zdraví a na pohlaví. Byly nalezeny specifické genderové rozdíly v proteinovém složení vzorků lidské sliny. (Kulhavá a kol., 2018)
- Poprvé byla frakce sediment vzorku lidské sliny a její proteinové složení dána do souvislosti s orálním zdravím dárce vzorku celé sliny. U lidí se zdravou denticí byly nalezeny se zvýšenou expresí proteiny imunitní odpovědi, které by mohly hrát důležitou roli v prevenci zubního kazu. Ve skupině vzorků slin od lidí se sanovanou denticí byly pozorovány ve zvýšené koncentraci vápník vázající proteiny, které mohou odvápňovat sklovinu zubu, a zvyšovat tím riziko zubního kazu. (Kulhavá a kol., 2020)
- Získané vzorky celé lidské sliny byly podrobeny proteinové a mikrobiologické analýze, tím bylo získáno srovnání proteinových a mikrobiologických profilů a rozšíření pohledu na příčiny onemocnění zubním kazem, které je jedno z nejrozšířenějších infekčních onemocnění. Bohužel v případě mikrobiologických analýz nebyly nalezeny výrazné rozdíly mezi porovnávanými skupinami.
- Poprvé byly popsány bazo-apikálních rozdíly pravé a levé komory srdeční u potkana. (Eckhardt, 2018)
- Byla provedena analýza kontrahované tkáně z části talonaviculárního skloubení – část tkáně postižené onemocněním idiopatická *pes equinovarus*. (Eckhardt, 2019)

- Byla provedena srovnávací studie proteinových profilů získaných z bakterie pocházející z bachoru *Butyrivibrio fibrisolvens* Bf 3071, která byla kultivována za různých podmínek. (Sechovcová, 2019)

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Příloha 1

PUBLIKACE I

Differences of Saliva Composition in Relation to Tooth Decay and Gender

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Folia Biologica (Praha) 64, 195-203 (2018)

Original Article

Differences of Saliva Composition in Relation to Tooth Decay and Gender

(dental caries / DMFT / gender / saliva / proteins)

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Abstract. Most people worldwide suffer from dental caries. Only a small part of the population is caries-resistant and the reason for this resistance in unknown. Only a few studies compared the saliva protein composition of persons with carious teeth and persons with no caries. Our study is the first to relate proteomic analysis of the caries aetiology with gender. In this study, we compared the differences in the abundances of proteins in the saliva between caries-resistant and caries-susceptible females and males by nano-liquid chromatography-tandem mass spectrometry (Label-Free Quantitative Proteomics). Our results demonstrate that the observed differences in the protein levels might have an influence on anti-caries resistance. A total of 19 potential markers of tooth caries were found, for example proteins S100A8 and annexin A1 with higher expression in the caries-susceptible group in comparison with the caries-free group and mucin-5B, lactoferrin, lysozyme C with higher expression in the caries-free group in comparison with the caries-susceptible group. The presented study is the first complex proteomic and gender project where the saliva protein content of caries-free and caries-susceptible persons were compared by label-free MS. The newly detected potential protein markers of dental caries can be a good basis for further research and for possible future therapeutic use.

Introduction

Human saliva is a major body fluid and is very important for oral health (saliva production equals approx. 0.75–1.5 l per day). The physiology of the whole saliva and salivary secretion was reviewed in Proctor (2016).

Saliva includes many markers that can foretell the potential risk of some diseases, for example periodontal diseases, cardiovascular diseases, diabetes mellitus, oncological, psychiatric, viral, gynecological and endocrinological diseases (Podzimek et al., 2016).

As World Health Organization states, "Dental caries is still a major oral health problem in most industrialized countries, affecting 60–90 % of schoolchildren and the vast majority of adults" (http://www.who.int/oral_health/disease_burden/global/en; 26.5.2018). Only a small part of the world's population (ca 10 % with DMFT = 0 (DMFT – decayed, missing, filled tooth) is well protected against the prevalence of dental caries. The social-economic status plays an important role in dental caries, as processed in a cross-sectinal study by Wang et al. (2017). The study shows that people aged 65–74 with a low social-economic status have poor oral health.

Possible saliva biomarkers and their association with dental caries (microorganisms in the saliva, salivary electrolytes, salivary proteins and peptides in reaction to dental caries, immune studies focused on particular individual markers) were reviewed in Gao et al. (2016). Only a few studies compared the protein saliva composition of people with carious teeth and people with no caries, and they were reviewed with different results in

Received November 28, 2018. Accepted December 21, 2018.

The study was supported by Charles University, project GA UK No. 322216. This research was carried out within the framework of Specific University Research (SVV260440). The study has received funding from the Ministry of Health of the Czech Republic Department Programme for Research and Development (No. 17-31564A).

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Abbreviations: DMFT – decayed, missing, filled tooth, MS – mass spectrometry, PRPs – proline-rich proteins, Q-TOF – quadrupole time-of-flight, TCA – trichloroacetic acid, Tgase E – transglutaminase E.

Al-Tarawneh et al. (2011). Comparative proteomic analysis of oral fluids found differences in protein expression based on the gender and age (Fleissig et al., 2010).

The present study was focused on human proteins in the saliva (not on microbiome profiles of saliva samples) and is a continuation of our previous research on protein differences in dental pulp in relation to tooth decay (Jágr et al., 2016). This study, based on label-free mass spectrometry (MS) quantification, was performed to compare differences in the abundances of proteins in the saliva between caries-resistant and caries-susceptible persons and also to perform a gender comparison. No such complex proteomic comparison of the saliva composition has been performed to date.

Material and Methods

Preparation of samples for label-free quantitative analysis

Saliva samples of the whole saliva (100 µl) were collected from healthy female volunteers aged between 20 and 35 years, caries-resistant (N = 14, DMFT ranging from 0 to 1) and caries-susceptible (N = 16, DMFT ranging from 5 to 14) females, and from healthy male volunteers aged between 23 and 47 years, caries-resistant (N = 12, DMFT ranging from 0 to 1) and caries-susceptible (N = 18, DMFT ranging from 5 to 12) males. All procedures performed in the studies involving human participants were in accordance with the Ethical Standards and with the World Medical Association Declaration of Helsinki (version 2008). All of the volunteers were requested not to eat, drink, or brush and wash their teeth for 1–2.5 h prior to the trial. Samples were kept on ice and protease inhibitors (cOmplete Protease Inhibitor Cocktail Tablets (Roche Diagnostics, Indianapolis, IN)) were added to inhibit protease activity. Unstimulated whole-saliva samples were frozen at –80 °C until futher analysis. Saliva collection was performed once in sterile bottles of each volunteer over a period of three months. The criteria for group inclusion were overall health of the volunteeer, age, sex, and number of teeth treated. Seventy % of participants had completed high school education. DMFT was assesesed based on clinical examination by one experienced dentist. Furthermore, panoramic X-ray was evaluated in each patient.

Samples were centrifuged at 13,000 *g* for 30 min at 4 °C. The supernatant (A) of each sample was collected. Proteins were precipitated using trichloroacetic acid (TCA) (Sigma-Aldrich, St. Louis, MO) at a final concentration of 10 % (w/v) and dithiotreitol (0.12 % w/v) (Sigma-Aldrich). After vortexing and incubation at 25 °C for 15 min, the precipitated proteins were centrifuged (13,000 *g*, 15 min, 4 °C). Protein pellets (AI) from the collected supernatants (A) were washed three times with ice-cold 100% acetone and lyophilized. All the samples were then digested in a solution containing NH_4HCO_3 (0.05 mol/l) and trypsin (0.2 mg/ml) (1/50 w/w trypsin/sample) at 37 °C for 16 h. Peptides were extracted using Stage Tips-aided (Rappsilber, 2007) purification of samples for nano liquid chromatography (nLC) MS/MS. The extracted solutions were lyophilized and dissolved in 20 µl of 2% formic acid (v/v).

The protein concentration was determined using a Nanodrop ND-1000 spectrometer (ThermoFisher, Wilmington, DE) (average sample concentration was 0.33 mg/ml). Finally, 0.8 µg of the peptide mixture was loaded to the column.

Analysis of tryptic digests with LC-MS/MS

A nano liquid chromatography (nLC) apparatus Proxeon Easy-nLC (Proxeon, Odense, Denmark) was used for analysing the protein digests, similar to our previous work, coupled to a MaXis quadrupole time-of-flight (Q-TOF) mass spectrometer (Bruker Daltonics, Bremen, Germany) (Ošťádal et al., 2015). Auto MS/MS with active exclusion (after one spectrum and release after 0.3 min) was used for MS/MS analyses.

Database searches were performed as described (Eckhardt et al., 2014; Ošťádal et al., 2015) with the taxonomy restricted to *Homo sapiens* to remove protein identification redundancy. Only significant hits (MASCOT score $\geq$ 80 for proteins; $\geq$ 20 for peptides, http://www.matrixscience.com) were accepted.

Label-free quantification

Label-free quantification is a method for determination of the relative amount of proteins in two (or more) biological samples, i.e., without using stable isotopes. In our case it was based on the comparison of signal intensities (at MS) of individual particular peptides at different sets of samples. Profile Analysis software (version 2.1, Bruker Daltonics GmbH) was used to evaluate differences in the protein composition of the caries-susceptible and caries-resistant persons (females and males) by means of label-free quantification (Student's *t*-test; $P < 0.05$). The peptides under consideration had to be found in at least 50 % of all the samples, regardless of the group, and they had to be found in at least one of the two groups (group of susceptible persons and/or group of resistant persons (females and/or males)) as well as in at least 50 % of the group. For correct evaluation of ions with similar m/z values and similar retention times, the Time Alignment option was enabled.

Results

Comparison of caries-susceptible and caries-free saliva samples

In our complex proteomic study, we found protein differences between caries-susceptible and caries-free groups. We detected nine proteins with higher expression in the caries-susceptible male group and seven proteins with higher expression in the caries-free male group (Table 1). Our comparison of female saliva samples showed four statistically significantly higher values in caries-susceptible females (Table 1). We observed

Table 1. List of over-expressed proteins in the saliva of caries-susceptible and caries-free persons

Accession Number	Protein	Total number of peptides	Molecular function	P	Fold change (caries-susceptible : caries-free)
	Up-regulated in caries-susceptible males				
P04745	AMY1C; AMY1A; AMY1B; AMY2A α-Amylase 1	35	glycosidase/hydrolase	3.12E-03	1.8
P04083	ANXA1 Annexin A1	17	phosholipase A2 inhibitor	5.19E-05	1.8
P06702	S100A9 Protein S100-A9	11	antimicrobial	3.77E-03	2.4
P60709	ACTB Actin	9	ATB-binging	4.56E-03	1.6
Q08188	TGM3 Protein-glutamine γ-glutamyltransferase E	10	acyltransferase/transferase	1.27E-02	1.8
P05109	S100A8 Protein S100-A8	7	antimicrobial	6.46E-03	2.7
P07355	ANXA2 Annexin A2	7	RNA-binding	3.64E-03	1.9
P29508	SERPINB3 Serpin B3	6	protease inhibitor	3.03E-02	1.3
P62937	PPIA Peptidyl-prolyl *cis-trans* isomerase A	3	isomerase	1.46E-02	1.3
	Up-regulated in caries-free males				
P02814	SMR3B Submaxillary gland androgen-regulated protein 3B	16		5.84E-04	0.6
Q6P5S2	C6orf58 C6orf58	11	developmetal protein	6.51E-04	0.6
P01833	PIGR Polymeric immunoglobulin receptor	8	polymeric immunoglobulin receptor activity	4.03E-02	0.8
P01037	CST1 Cystatin-SN	6	protease inhibitor	3.09E-02	0.8
Q9HC84	MUC5B Mucin-5B	4		2.84E-02	0.6
P61626	LYZ Lysozyme C	3	antimicrobial	1.34E-02	0.5
P31025	LCN1 Lipocalin 1	3	chloride and zinc binding	5.29E-03	0.5
	Up-regulated in caries-susceptible females				
P04083	ANXA1 Annexin A1	9	phosholipase A2 inhibitor	1.08E-02	1.7
P01876	IGHA1 Immunoglobulin heavy constant α 1	8	antigen binding	4.02E-02	1.2
P06702	S100A9 Protein S100-A9	9	antimicrobial	4.95E-03	3.5
P25311	AZGP1 Zinc-α-2-glycoprotein	9	transmembrane activity	1.20E-02	1.8
	Up-regulated in caries-susceptible persons				
P04745	AMY1C; AMY1A; AMY1B; AMY2A α-Amylase 1	25	glycosidase/hydrolase	2.38E-02	1.6
P04083	ANXA1 Annexin A1	15	phosholipase A2 inhibitor	4.00E-04	1.7
P05109	S100A8 Protein S100-A8	7	antimicrobial	9.99E-03	2.5
P04406	GAPDH Glyceraldehyde-3-phosphate dehydrogenase	5	oxidoreductase/transferase	2.69E-02	1.4
P60174	TPI1 Triosephosphate isomerase	4	isomerase	4.42E-03	1.4

Accession Number (Uniprot); P – significance; the molecular functions were categorized according to the classification system used in the public database available at http://www.uniprot.org.

five differences in the entire comparison (without gender specification) (Table 1). Most of these protein differences were observed for the first time. We observed 111 proteins in total. The list of identified proteins is attached in Table 3.

The Venn diagram describes the relationships amongst proteins that were found to be differently produced in DMFT comparisons (Fig. 1). The distribution of the biological functions of proteins that were found with different expression in human saliva is shown in Fig. 2.

Gender differences in the saliva

We compared saliva proteins on a gender basis. Most of these gender protein differences were observed for the first time. We identified 18 up-regulated proteins by MS label-free quantification in the group of caries-susceptible males. One protein was up-regulated in the saliva of females (caries-susceptible females) (Table 2). We found six proteins over-expressed in males when we compared samples obtained from caries-free persons (Table 2). We also compared saliva samples obtained from all males and all females (both combined caries-susceptible and caries-free). We identified 14 proteins up-regulated in the group of males compared to females (Table 2). The Venn diagram describes the relationships of proteins that were found to be differently produced in gender comparisons (Fig. 3).

Discussion

The present study provides the most complex proteomic comparison of the saliva to date (in both fields: caries protection and gender differences). It was observed that the quality of the protein composition did not differ in all the compared groups. However, significant differences were observed in the quantitative contents of some proteins (most of them for the first time). In the present project, we found 21 differences in protein expression between caries-susceptible and caries-free persons (Table 1) (Fig. 2) and 23 gender-related differences (Table 2) (Fig. 3). Proteins AMY1A, ANXA1 and S100A9 represent the most abundant proteins in human saliva (based on the study by Grassl et al., 2016).

The incidence of lipoprotein PPIA limits *Streptococcus mutans* phagocytosis, as described in the report by Mukouhara et al. (2011). We found this protein with significantly higher expression in the caries-susceptible male group (Table 1), and it could therefore be a poten-

Table 2. List of over-expressed proteins in the saliva of males and females

Accession Number	Protein	Total number of peptides	P	Fold change (female : male)
	Caries-susceptible persons			
	Up-regulated in saliva of males			
P04745	AMY1C; AMY1A; AMY1B; AMY2A α-Amylase 1	39	2.95E-03	0.9
P04083	ANXA1 Annexin A1	17	4.61E-05	0.8
P60709	ACTB Actin	13	1.32E-02	0.7
Q08188	TGM3 Protein-glutamine γ-glutamyltransferase E	7	1.56E-04	0.6
P05109	S100A8 Protein S100-A8	8	3.48E-03	0.7
Q8N4F0	BPIFB2 Bactericidal/permeability-increasing protein-like 1	5	9.89E-04	0.8
P25311	AZGP1 Zinc-α-2-glycoprotein	7	2.20E-03	0.7
P01037	CST1 Cystatin-SN	7	2.00E-03	0.7
A8K2U0	A2ML1 α-2-Macroglobulin-like protein 1	3	3.46E-02	0.8
P07355	ANXA2 Annexin A2	5	2.61E-02	0.8
P01834	IGKC Immunoglobulin κ constant	8	3.60E-02	0.8
P01704	IGLV2-14 Immunoglobulin λ variable 2-14	6	8.41E-03	0.7
P29508	SERPINB3 Serpin B3	5	1.02E-02	0.7
P30740	SERPINB1 Leukocyte elastase inhibitor	4	1.97E-02	0.8
Q9HC84	MUC5B Mucin-5B	4	5.73E-03	0.7
P04080	CSTB Cystatin-B	3	4.02E-02	0.6
P01591	IGJ Immunoglobulin J chain	3	1.65E-02	0.7
P31025	LCN1 Lipocalin 1	3	2.80E-02	0.7
	Up-regulated in saliva of females			
P02814	SMR3B Submaxillary gland androgen-regulated protein 3B	13	1.32E-02	1.3
	Caries-free persons			
	Up-regulated in saliva of males			
P01876	IGHA1 Immunoglobulin heavy constant α 1	11	1.37E-02	0.8
P02788	LTF Lactotransferrin	4	1.85E-02	0.6
P05109	S100A8 Protein S100-A8	6	1.33E-04	0.7
P25311	AZGP1 Zinc-α-2-glycoprotein	9	2.06E-04	0.6
P01704	IGLV2-14 Immunoglobulin λ variable 2-14	6	1.12E-02	0.8
P80188	LCN2 Neutrophil gelatinase-associated lipocalin	5	7.51E-03	0.7
	Up-regulated in saliva of females			
P04745	AMY1C; AMY1A; AMY1B; AMY2A α-Amylase 1	32	7.71E-04	1.4
	Caries-susceptible and caries-free persons			
	Up-regulated in saliva of males			
P04083	ANXA1 Annexin A1	17	5.77E-07	0.7
P01876	IGHA1 Immunoglobulin heavy constant α 1	12	2.07E-02	0.8
P01833	PIGR Polymeric immunoglobulin receptor	7	1.18E-02	0.9
Q08188	TGM3 Protein-glutamine γ-glutamyltransferase E	6	1.19E-03	0.6
P05109	S100A8 Protein S100-A8	8	7.92E-03	0.7
Q8N4F0	BPIFB2 Bactericidal/permeability-increasing protein-like 1	5	1.19E-02	0.8
P25311	AZGP1 Zinc-α-2-glycoprotein	7	9.38E-05	0.5
P69905	HBA2; HBA1 Haemoglobin subunit α	3	4.25E-02	0.4
P01834	IGKC Immunoglobulin κ constant	5	4.54E-02	0.8
P29508	SERPINB3 Serpin B3	4	2.74E-02	0.7
P30740	SERPINB1 Leukocyte elastase inhibitor	5	1.39E-02	0.7
P04080	CSTB Cystatin-B	4	3.15E-02	0.6
P80188	LCN2 Neutrophil gelatinase-associated lipocalin	4	3.00E-02	0.6
P31025	LCN1 Lipocalin-1	3	4.47E-02	0.7

Accession Number (Uniprot); P – significance; the molecular functions were categorized according to the classification system used in the public database available at http://www.uniprot.org.

tial risk biomarker for dental caries in the saliva. In an earlier study, Vitorino's group also detected a high number of PPIA peptide fragments in the caries-susceptible group, which suggests high proteolytic activity (Vitorino et al., 2005).

We detected six proteins (α-amylase, transglutaminase E, S100A9, S 100A8, annexin A1, annexin A2) with calcium-binding properties at higher concentrations in the caries-susceptible groups (Table 1). As concerns α-amylase and transglutaminase E (Tgase E), the key reason for their occurrence in the dental caries-susceptible group cound be binding of α-amylase to bacteria (Scannapieco et al., 1993). Tgase E is a calcium-dependent acyl-transfer enzyme catalysing cross-links between proteins or peptides (Ahvazi et al., 2004). Calcium- and zinc-binding proteins such as S100A9 and S100-A8 play a role in the regulation of inflammatory processes and immune response. S100A8 a S100A9 are highly expressed in neutrophils and monocytes. These proteins were detected at elevated levels at extracellular

Table 3. List of identified proteins in saliva samples

Row	Accession Number	Protein	MW [kDa]	pI	Mascot Scores
1	IPI00300786	AMY1C; AMY1A; AMY1B; AMY2A α-Amylase 1	57.7	6.5	5857.3 (M:5857.3)
2	IPI00218918	ANXA1 Annexin A1	38.7	6.7	2138.7 (M:2138.7)
3	IPI00386879	IGHA1 Immunoglobulin heavy constant α 1	53.1	6.5	2057.3 (M:2057.3)
4	IPI00784950	IGHA2 Immunoglobulin heavy constant α 2	51.6	5.4	1957.0 (M:1957.0)
5	IPI00023011	SMR3B Submaxillary gland androgen-regulated protein 3B	8.2	10.2	1910.8 (M:1910.8)
6	IPI00374315	C6orf58 UPF0762 protein C6orf58	37.9	5.7	1866.2 (M:1866.2)
7	IPI00426060	IGHA1 Putative uncharacterized protein DKFZp686J11235 (Fragment)	54.4	6.3	1806.1 (M:1806.1)
8	IPI00785067	IGHA2 IGH@ protein	52.0	5.9	1744.8 (M:1744.8)
9	IPI00647704	IGHA1 cDNA FLJ41552	53.3	6.1	1722.6 (M:1722.6)
10	IPI00925547	LTF Lactotransferrin	77.9	9.6	1475.7 (M:1475.7)
11	IPI00027462	S100A9 Protein S100-A9	13.2	5.7	1353.1 (M:1353.1)
12	IPI00654755	HBB Haemoglobin subunit β	16.0	6.9	1291.1 (M:1291.1)
13	IPI00021439	ACTB Actin, cytoplasmic 1	41.7	5.2	1287.6 (M:1287.6)
14	IPI00004573	PIGR Polymeric immunoglobulin receptor	83.2	5.5	1267.9 (M:1267.9)
15	IPI00300376	TGM3 Protein-glutamine glutamyltransferase E	76.6	5.5	1222.5 (M:1222.5)
16	IPI00645363	IGHV4-31 Immunoglobulin heavy variable 4-31	51.7	9.0	1157.4 (M:1157.4)
17	IPI00007047	S100A8 Protein S100-A8	10.8	6.6	1031.4 (M:1031.4)
18	IPI00465248	ENO1 α-Enolase	47.1	7.7	978.0 (M:978.0)
19	IPI00473011	HBD Haemoglobin subunit δ	16.0	9.1	928.1 (M:928.1)
20	IPI00922693	ACTB Actin, α skeletal muscle	38.6	5.1	921.1 (M:921.1)
21	IPI00745872	ALB Serum albumin	69.3	5.9	875.6 (M:875.6)
22	IPI00296654	BPIFB2 Bactericidal/permeability-increasing protein-like 1	49.1	9.5	870.1 (M:870.1)
23	IPI00166729	AZGP1 Zinc-α-2-glycoprotein	34.2	5.7	817.5 (M:817.5)
24	IPI00305477	CST1 Cystatin-SN	16.4	7.6	767.7 (M:767.7)
25	IPI00022463	TF Serotransferrin	77.0	7.0	752.3 (M:752.3)
26	IPI01010670	A2ML1 α-2-Macroglobulin-like protein 1	161.0	5.4	749.7 (M:749.7)
27	IPI00455315	ANXA2 Annexin A2	38.6	8.5	712.5 (M:712.5)
28	IPI00410714	HBA2; HBA1 Haemoglobin subunit α	15.2	9.4	711.1 (M:711.1)
29	IPI00969456	IGKC Putative uncharacterized protein	25.8	9.2	699.5 (M:699.5)
30	IPI00154742	IGLV2-14 Immunoglobulin λ variable 2-14	24.8	5.9	692.6 (M:692.6)
31	IPI00887169	IGLV1-44 Immunoglobulin λ variable 1-44	25.0	8.8	692.0 (M:692.0)
32	IPI00979250	IGKC Ig κ chain C region	25.6	8.8	629.6 (M:629.6)
33	IPI00550731	- Putative uncharacterized protein	26.2	9.2	629.0 (M:629.0)
34	IPI00022204	SERPINB3 Serpin B3	44.5	6.4	622.0 (M:622.0)
35	IPI00784865	IGK@ IGK@ protein	25.8	5.9	620.1 (M:620.1)
36	IPI00853045	IGKC Immunoglobulin κ constant	25.7	9.5	581.6 (M:581.6)
37	IPI00219757	GSTP1 Glutathione S-transferase P	23.3	5.3	558.4 (M:558.4)
38	IPI00013382	CST2 Cystatin-SA	16.4	4.7	533.6 (M:533.6)
39	IPI00219018	GAPDH Glyceraldehyde-3-phosphate dehydrogenase	36.0	9.3	527.7 (M:527.7)
40	IPI00032294	CST4 Cystatin-S	16.2	4.8	515.4 (M:515.4)
41	IPI01014238	SERPINB1 Leukocyte elastase inhibitor	38.7	6.2	512.5 (M:512.5)
42	IPI00411765	SFN 14-3-3 protein ς	24.3	4.6	498.8 (M:498.8)
43	IPI00896380	IGHM Ig μ chain C region	51.8	5.8	488.8 (M:488.8)
44	IPI00060800	ZG16B Zymogen granule protein 16 homologue B	22.7	7.6	460.9 (M:460.9)
45	IPI00021263	YWHAZ 14-3-3 protein ζ/δ	27.7	4.6	448.2 (M:448.2)
46	IPI00021841	APOA1 Apolipoprotein A-I	30.8	5.5	441.3 (M:441.3)
47	IPI00295105	CA6 Carbonic anhydrase 6	35.8	6.6	441.2 (M:441.2)
48	IPI00936444	MUC5B Mucin-5B	590.4	6.2	421.9 (M:421.9)
49	IPI00985211	- Similar to VH-3 family (VH26)D/J protein	18.8	7.2	415.6 (M:415.6)
50	IPI00553177	SERPINA1 α-1-Antitrypsin	46.7	5.3	412.6 (M:412.6)
51	IPI00021828	CSTB Cystatin-B	11.1	7.9	391.1 (M:391.1)
52	IPI01012311	DMBT1 Uncharacterized protein	124.4	5.1	373.6 (M:373.6)
53	IPI00304557	BPIFA2 BPI fold-containing family A member 2	27.0	5.2	372.3 (M:372.3)
54	IPI00022974	PIP Prolactin-inducible protein	16.6	9.3	365.8 (M:365.8)
55	IPI01013763	LCN2 Neutrophil gelatinase-associated lipocalin	22.4	9.8	360.2 (M:360.2)
56	IPI00002851	CST5 Cystatin-D	16.1	7.6	357.2 (M:357.2)
57	IPI00916434	- Anti-(ED-B) scFV (Fragment)	25.1	9.2	350.3 (M:350.3)
58	IPI00908881	GPI Glucose-6-phosphate isomerase	60.0	9.4	342.6 (M:342.6)
59	IPI00216691	PFN1 Profilin-1	15.0	9.4	337.4 (M:337.4)
60	IPI00783810	LPO Lactoperoxidase isoform 3 preproprotein	70.9	8.9	325.2 (M:325.2)
61	IPI00940673	TKT Transketolase	58.9	8.6	313.1 (M:313.1)
62	IPI00642247	SPRR3 Small proline-rich protein 3	17.0	9.5	312.2 (M:312.2)
63	IPI00797270	TPI1; TPI1P1 Triosephosphate isomerase	26.7	6.5	299.4 (M:299.4)
64	IPI00218131	S100A12 Protein S100-A12	10.6	5.8	296.7 (M:296.7)
65	IPI00646773	GSN Gelsolin	80.6	5.5	294.7 (M:294.7)
66	IPI01020720	HSPA1B Heat shock 70 kDa protein 1B	67.5	5.2	294.1 (M:294.1)

67	IPI00019038	LYZ Lysozyme C	16.5	10.6	285.1 (M:285.1)
68	IPI00947235	IGJ Uncharacterized protein	8.2	10.0	257.4 (M:257.4)
69	IPI00478003	A2M α-2-Macroglobulin	163.2	6.0	247.3 (M:247.3)
70	IPI00916818	PGK1 Phosphoglycerate kinase	35.0	9.3	222.7 (M:222.7)
71	IPI00032325	CSTA Cystatin-A	11.0	5.3	221.2 (M:221.2)
72	IPI00009650	LCN1 Lipocalin-1	19.2	5.3	219.8 (M:219.8)
73	IPI00377025	PRH1; PRH2 Proline-rich protein HaeIII subfamily 1	17.0	4.6	213.0 (M:213.0)
74	IPI00100630	MLLT1 Protein ENL	62.0	9.5	212.7 (M:212.7)
75	IPI00297056	CRNN Cornulin	53.5	5.7	212.0 (M:212.0)
76	IPI01013441	PRTN3 Myeloblastin	23.6	10.2	191.2 (M:191.2)
77	IPI00640006	GDI2 rab GDP dissociation inhibitor β isoform 2	45.6	5.9	190.2 (M:190.2)
78	IPI00419920	CES2 Cocaine esterase isoform 2	67.0	6.1	187.4 (M:187.4)
79	IPI00007797	FABP5 Fatty acid-binding protein, epidermal	15.2	7.5	182.2 (M:182.2)
80	IPI01022836	ATP5B ATP synthase subunit β, mitochondrial	55.3	5.1	179.7 (M:179.7)
81	IPI00010471	LCP1 Plastin-2	70.2	5.2	170.0 (M:170.0)
82	IPI00387116	- Ig κ chain V-III region NG9 (Fragment)	10.7	7.1	159.5 (M:159.5)
83	IPI01014727	- cDNA FLJ51983, highly similar to Phosphoglycerate mutase 1	27.1	9.2	153.8 (M:153.8)
84	IPI00220494	SERPINB13 Serpin B13	38.4	5.5	150.2 (M:150.2)
85	IPI00925411	PPIA Peptidyl-prolyl cis-trans isomerase AU	13.0	6.4	149.2 (M:149.2)
86	IPI00872684	EZR Ezrin	65.5	5.6	148.3 (M:148.3)
87	IPI00554798	HIST1H2BM Histone H2B type 1-M	14.0	10.8	146.1 (M:146.1)
88	IPI00465439	ALDOA Fructose-bisphosphate aldolase A	39.4	9.2	144.9 (M:144.9)
89	IPI00216298	TXN Thioredoxin	11.7	4.7	143.6 (M:143.6)
90	IPI00974112	CRISP3 22 kDa protein	21.6	9.2	141.8 (M:141.8)
91	IPI00220146	DSC2 Desmocollin-2	93.7	5.2	136.4 (M:136.4)
92	IPI01009918	PRSS1 Uncharacterized protein	25.4	8.8	135.9 (M:135.9)
93	IPI00431645	HPR 31 kDa protein	31.4	9.3	134.6 (M:134.6)
94	IPI00022488	HPX Haemopexin	51.6	6.6	131.0 (M:131.0)
95	IPI00081836	HIST1H2AH Histone H2A type 1-H	13.9	11.3	127.8 (M:127.8)
96	IPI00908762	LGALS3BP Galectin-3-binding protein	46.4	5.0	127.0 (M:127.0)
97	IPI00952922	KRT13 Keratin, type I cytoskeletal 13	38.5	4.8	125.1 (M:125.1)
98	IPI00022432	TTR Transthyretin	15.9	5.4	124.2 (M:124.2)
99	IPI00009792	IGHV1OR15-1 Ig heavy chain V-I region V35	13.0	10.1	123.4 (M:123.4)
100	IPI00013895	S100A11 Protein S100-A11	11.7	7.5	120.5 (M:120.5)
101	IPI00242956	FCGBP IgGFc-binding protein	571.6	5.0	111.9 (M:111.9)
102	IPI00965713	FGB Fibrinogen β chain isoform 2 preproprotein	49.9	9.0	109.2 (M:109.2)
103	IPI01024806	ACTN1 α-Actinin 1	24.4	5.3	107.4 (M:107.4)
104	IPI00023038	PRB1 Basic salivary proline-rich protein 1	38.5	11.7	99.2 (M:99.2)
105	IPI00641244	PRDX1 11 kDa protein	10.7	9.6	96.9 (M:96.9)
106	IPI00982472	TALDO1 Transaldolase	35.3	9.7	95.1 (M:95.1)
107	IPI00978296	CA1 Uncharacterized protein	9.4	10.0	93.3 (M:93.3)
108	IPI01010447	UBC Uncharacterized protein	10.0	10.3	90.3 (M:90.3)
109	IPI00644531	TAGLN2 21 kDa protein	21.1	9.0	88.8 (M:88.8)
110	IPI00964070	ANXA3 Annexin A3	32.1	5.6	87.5 (M:87.5)
111	IPI00386755	ERO1L ERO1-like protein α	54.4	5.4	82.7 (M:82.7)

locations during inflammatory processes (Ryckman et al., 2003).

Anti-inflammatory mediator annexin A1 can affect migration and cellular responses of the innate immune system (Weyd, 2016). Annexin A1 is a membrane-localized and Ca^{2+}-dependent phospholipid-binding protein. Annexin A2 has an important role in the regulation of the coagulation cascade (Iaccarino et al., 2011). We assume that the reason for higher concentrations of these immune proteins in caries-susceptible groups cound be a consequence of the prior experience with dental caries. Salivary mucins are well recognized as an important factor in conservation of the health of the oral cavity (Frenkel and Ribbeck, 2015), which is in agreement with our measurements, showing mucin-5B in significantly higher concentrations in the caries-free saliva (Table 1). An antimicrobial protein such as lysozyme C could take part in protecting teeth against tooth decay, which is also in agreement with our measurements. We assume that all three of the above-mentioned proteins (mucin-5B, lactoferrin, and lysozyme C) could play specific roles in oral protection and could thus be promising "anti-caries" biomarkers.

Preza et al. (2009) compared parotid gland secretion from two groups of elderly persons with and without root caries. Some protein differences unique to the subjects (α-1-acid glycoprotein 1; cathepsin D; collagen α-1 (VI) chain; collagen α-2 (VI) chain; cytokeratin-17; glucose-regulated protein-78 kDa; glutathione S-transferase P; Ig κ chain V–IV region LEN; SPARC-like protein 1) were observed in this comparison. Similar changes were found in patients with Sjogren's syndrome, a condition associated with dental decay (Preza et al., 2009). In our results described here, we did not found any changes in these proteins.

A study by Vitorino et al. (2006) was focused on salivary protein composition in cases of *in vitro* dental pellicle formation and its possible correlation with dental

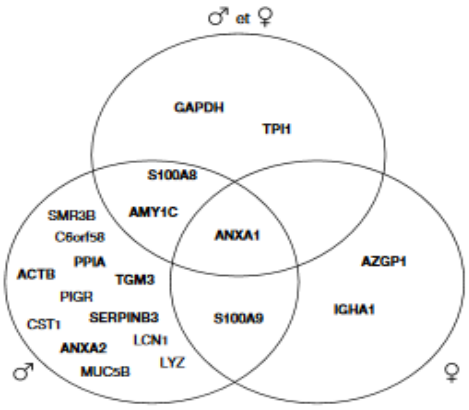

Fig. 1. Comparison of caries-susceptible and caries-free individuals. Proteins found in significantly higher concentrations in the caries-susceptible group (bold) and in the caries-free group (plain) in the individual genders

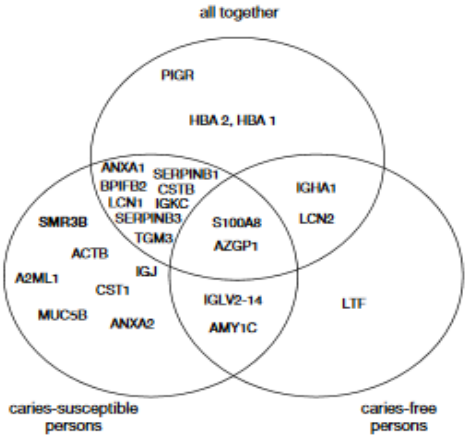

Fig. 3. Gender differences in saliva proteomes. Proteins with significantly higher concentrations in female saliva are shown in bold, and in males in plain fonts

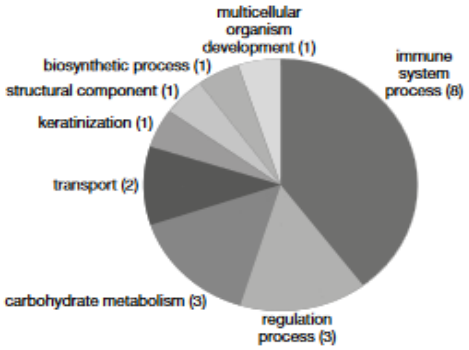

Fig. 2. Distribution of biological processes of proteins found with different expression in human saliva. The protein functions in biological processes categorized according to http://www.uniprot.org.

caries. Analysis of the salivary protein composition showed significantly more abundant concentrations of acidic proline-rich proteins (PRPs), lipocalin, cystatin SN and cystatin S in samples from the caries-free group. In our study we found significantly higher expression of lipocalin 1 and cystatin-SN in the caries-free group and α-amylase in the caries-susceptible group of males, which is in agreement with the results reported by Vitorino et al. (2006), and we assume that these proteins are potential targets for further research of oral health caries.

The present study brings the most detailed gender proteomic comparison of saliva to date. This is the first time that MS quantification was used for this purpose. We discovered 23 gender-related differences (Table 2) (Fig. 3) and assume that these differences could play important roles in the saliva physiology in both genders.

Only a few comparisons of the saliva proteome based on the gender have been performed to date. Lukacs et al. (2011) described sexually-determined proteome differences in the dental caries prevalence. Females were found to exhibit higher prevalence rates than males. The reasons are explained by three factors: earlier eruption of teeth in girls, longer exposure of girls' teeth to a cariogenic oral environment, and pregnancy. The higher incidence of dental caries related to pregnancy is known. There are many factors that are involved in this process, such as higher amount and frequency of consumption of cariogenic diet, reduction of pH of the oral cavity caused by frequent vomiting and decreased attention to maintaining oral hygiene (Christensen et al., 1998; Laine, 2002).

Fleissing et al. (2010) found that gender differences revealed six proteins with significantly higher expression in females. In our results, leukocyte elastase inhibitor (SERPINB1) was observed with significantly higher expression in the caries-susceptible male group and in the group of all males. A second protein, calgranulin A (S100-A8), was found to be up-regulated in the saliva of males in all the gender comparisons. Our results do not agree with those of Fleissing et al. (2010), possibly because of different experimental methods, patient ages, etc.

A cross-sectional study was presented to evaluate inter-individual biochemical variation in unstimulated whole

saliva (18–30 years of age) (Prodan et al., 2015). Females displayed reduced levels of salivary pH, and the protein contents of MUC5B were lower in female subjects compared with male subjects. This is in conformity with our results, showing significantly higher expression of protein MUC5B in the caries-susceptible male group in comparison with the caries-susceptible females (Table 2).

In the study by Li-Hui et al. (2016), where the salivary flow rate was compared, no evident change in the salivary α-amylase was observed. In our work, we found a change in the α-amylase expression, with higher expression in males.

We observed many differences between genders. The saliva protein composition was observed in only one time-point interval, and the results could be influenced by the presence of false-positive identification of differences. The conditions of collection were strictly respected because we wanted to prevent pre-analytical errors. We performed validation of the individual steps of preparation and analyses. In our future work, we would like to extend the number of individuals in the compared groups and present an expanded questionnaire to the volunteers (interindividual and intraindividual variability) with several time-point intervals of sample collection to map the possible effects.

Some correlations were found across the comparisons (DMFT and gender comparisons). We can see, for example, that the protein annexin A1 was found with significantly higher expression in the group of caries-susceptible persons, and this protein was also differently expressed in gender comparisons (annexin A1 was found with significantly higher expression in the male group (comparison of all males vs. all females)).

Our study presents a complex proteomic project, where the saliva protein contents of caries-free and caries-susceptible persons were compared by label-free MS. These results revealed 21 protein differences between caries-susceptible and caries-free persons. These proteins (e.g., with immune and Ca^{2+}-binding functions) could play an important role in oral protection and are promising biomarkers for dental caries and/or for oral health in general. The present study also detected 23 gender-related differences. The specificity of these proteins could play unique roles in the saliva physiology of both genders. Further research including larger groups of the caries-free and caries-susceptible patients is needed.

To date, no similar work has been published describing such a complex proteomic project that included comparisons of the human saliva proteome on the basis of both gender and DMFT by label-free MS.

Acknowledgement

We are grateful to all volunteers for their saliva samples.

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Příloha 2

PUBLIKACE II

Proteomic analysis of whole saliva in relationship to dental caries resistance

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- **Original Article**

Proteomic analysis of whole saliva in relationship to dental caries resistance

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Short title: Pellet in proteomic analysis of saliva

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Abstract

Saliva contains possible biomarkers that are associated with dental caries. The present study aimed to analyse differences in the abundance of proteins in saliva between caries-positive (CP; n=15) and caries-free (CF; n=12) males, and to compare differences in the abundance of proteins between two saliva sample fractions (supernatant and pellet). We found fourteen differently significantly expressed proteins in the CF group when comparing the supernatant fractions of the CP and CF groups, and three proteins in the pellet fractions had significantly higher expression in the CP group. Our results indicate very specific protein compositions of the saliva in relationship to dental caries resistance (the saliva of the CP group contained mainly pellet proteins and the saliva of the CF group contained mainly supernatant proteins). This was the first time that the saliva pellet fraction was analysed in relation to dental caries status. We

detected specific calcium binding proteins that could decalcified enamel in the saliva pellet of CP group. We also observed significantly upregulated immune-proteins in the saliva supernatant of CF group that could play an important role in caries prevention. The particular protein compositions of the saliva pellet and supernatant in groups with different susceptibilities to tooth decay is promising finding for future research.

KEY WORDS: dental caries / fractions of whole human saliva / Label-Free Quantification / mass spectrometry / proteins

Introduction

Oral fluids are very important in oral health. The physiology of human saliva and salivary secretion were reviewed in (Proctor, 2016). Saliva includes many markers that can indicate a risk of certain diseases (Podzimek et al., 2016). Possible biomarkers for dental caries were also previously reviewed, such as salivary electrolytes, microorganisms, proteins and peptides in saliva, and the functional properties of saliva (Gao et al., 2016). Only a small part of the population at the age of 30 is classified as caries-free, and the reason for the resistance is unknown. There are limited studies on the differences in the saliva protein composition between people with carious teeth and people without caries (Al-Tarawneh et al., 2011; Laputkova et al., 2018). Differences in the abundance of salivary proteins between caries-susceptible and caries-free people and a gender comparison were presented in our previous study (Kulhavá et al., 2018). Age-specific variations in the salivary proteome of caries-susceptible adults and elderly people have also been investigated (Wang et al., 2018). The influence of salivary protein composition on *in vitro* dental pellicle and their correlation with dental caries was studied by two-dimensional electrophoresis (Vitorino et al., 2006). Salivary protein roles as predictors of caries risk were described in a recent review (Laputkova et al., 2018). However, studies on the protein composition of saliva in relation to dental caries have varied (different approaches, methods, and the use of gender-based comparisons). A study by Vitorino et al. described an assessment of pre-treatment saliva samples using in-gel and off-gel approaches, and they presented, for the first time, the pellet fraction protein content (but not related to caries) (Vitorino et al., 2012). All comparative studies of caries-positive and caries-free individuals have analysed only the supernatant saliva fraction. The present study, based on analyses by mass spectrometry (MS) (and label-free mass spectrometry quantification), was performed to compare differences in salivary protein abundance between caries-free and caries-positive

males. We compared differences in the abundance of proteins in two saliva sample fractions: the supernatant and pellet. Here, the pellets were analysed in this way for the first time, and a unique comparison between these saliva fractions was carried out.

Material and Methods

Saliva sample collection

Unstimulated whole saliva was collected from 27 healthy male non-smoking volunteers (without diabetes mellitus). The subjects had been characterised according to the DMFT index (decayed, missing, and filled teeth index) into two groups: caries-positive (CP) (n=15, aged 38.4±5.6, DMFT ranging from 7 to 12) and caries-free (CF) (n=12, aged 31.8±7.6, DMFT ranging from 0 (n=10) to 1(n=2)). All procedures performed in studies involving human participants were in accordance with the Ethical standards and with the World Medical Association Declaration of Helsinki (version 2000). All of the volunteers were requested to not eat or drink in the morning and to brush their teeth for 1- 2.5 h prior to the trial. Harvesting a 1 mL sample of saliva was performed between 8 and 10 a.m. The samples were kept on ice, and a protease inhibitor (cOmplete Protease Inhibitor Cocktail Tablets (Roche Diagnostics GmbH, Mannheim, Germany)) was added to inhibit protease activity. The unstimulated whole-saliva samples were frozen at -80 °C until further analysis.

Initial sample preparation

The saliva samples (700 µl) were centrifuged at 13000 g for 30 min at 4 °C. The supernatant and pellet (P) of each sample were retained separately. The obtained supernatants and pellets were then aliquoted for use. Each sample was divided into 7 parts; 4 parts were used for two-dimensional electrophoresis, 2 parts were used for two-dimensional difference gel electrophoresis and 1 part was used for MS analysis and label-free (LF) quantification. The pellets were lyophilised before distribution. Seven samples (supernatant/pelet) of human saliva (caries-positive, n=7; caries-free, n=7) were used by two-dimensional electrophoresis, because the high variability between each saliva samples were identified (verified by the previous one-dimensional electrophoresis), as well as different protein concentrations in individual saliva samples. Four samples (supernatant/pelet) of human saliva (caries-positive, n=4; caries-free, n=4) were analysed by two-dimensional difference gel electrophoresis because of the cost of access method.

Preparation of samples for two-dimensional electrophoresis and two-dimensional difference gel electrophoresis

Supernatant proteins were precipitated using a final concentration of 10% (v/v) trichloroacetic acid and 0.12% (w/v) dithiothreitol (Jehmlich et al., 2013). After vortexing followed by incubation at 25 °C for 15 min, the precipitated protein was concentrated by centrifugation (13000 g, 15 min, 4 °C). Than we used a protocol modified from Gonçalves (Gonçalves et al., 2011), ice-cold acetone was used without dithiothreitol. Protein pellets from collected supernatants (S) were washed three times with ice-cold 100% acetone, lyophilised and stored at -80 °C. Pellet proteins (P) were washed with ice-cold acetone and acetonitrile, lyophilised and stored -80 °C.

MS sample preparation—supernatant (S) and pellet (P) protein

Protein from supernatant and pellet samples was obtained as in the sample preparation for the two-dimensional gel electrophoresis. Pellet proteins were digested in a solution containing 0.1 M NH_4HCO_3 and 0.2 M trypsin (1/100) (w/w; trypsin/sample) for 22 h at 37 °C. All samples were supplemented with 0.1 M NH_4HCO_3 to obtain the same volume, and the samples were lyophilised. Samples were dissolved in 100 µl of 2% formic acid.

LC-MS/MS analysis of tryptic digests

A nano liquid chromatography (n-LC) apparatus (Proxeon Easy-nLC; Proxeon, Odense, Denmark) was used for analysing the protein digests and was coupled to a MaXis quadrupole time-of-flight (Q-TOF) mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany) with a nanoelectrosprayer.

The samples were injected onto an NS-MO-10 Biosphere C18 pre-column with an NS-AC-11-C18 Biosphere C18 column, both manufactured by NanoSeparations (Nieuwkoop, Holland). The injection volume was 5 µl. The peptide separation was achieved via a linear gradient between mobile phase A (water) and B (acetonitrile), both containing 0.1% (v/v) formic acid. The separation was started by running the system with 5% mobile phase B, followed by a gradient elution to 30% B in 70 min. The next step was a gradient elution to 50% B over 10 min and then a gradient to 100% B over 10 min. Finally, the column was eluted with 100% B for 30 min. Equilibration between the runs was achieved by washing the column with 5% mobile phase B for 5 min. The flow rate was 0.25 µL/min, and the column was held at ambient temperature (25 °C). Auto MS/MS with active exclusion (after 2 spectrum and release after 0.8 min) was used for MS/MS analyses. The MS settings for the MS and MS/MS experiments were

as previously published (Mikšík et al., 2018), with the spectra rate set to 1 Hz and with the auto MS/MS option set to "off" for MS measurements.

The MS/MS data were processed using ProteinScape software (version 3.0, Bruker Daltonics GmbH, Bremen, Germany). Proteins were identified by correlating the tandem mass spectra of saliva samples to the International Protein Index (IPI databases) and UniProt databases.

Database searches were performed with the taxonomy restricted to *Homo sapiens* to remove protein identification redundancy. Only significant hits (Mascot score ≥80 for proteins; ≥20 for peptides, http://www.matrixscience.com) were accepted.

Label-free quantification

For the label-free quantitation, an MS chromatogram was measured for each sample, and the collected data were processed with Profile Analysis software (version 2.1, Bruker Daltonics GmbH, Bremen, Germany) to obtain the peptide composition values for the individual samples (caries-positive and caries-free). Each peptide must be found in at least 50% of the samples in each group (caries-positive and caries-free group). The peptides that fulfilled this criterion were evaluated by Student's t-test, and significance was defined as $p<0.05$.

Protein fraction uniqueness by MS/MS

The criterion for identifying protein fraction (pellet (P) or supernatant (S)) uniqueness was the presence of the protein in at least 50% of the samples of one fraction (in CP and/or CF groups). The maximum tolerance of protein impurity was determined as the presence of 2 of its peptides in the other fraction.

Comparison of significant and unique fraction proteins by database for annotation

We used the computational prediction of Enrich (https://amp.pharm.mssm.edu/Enrichr) to provide a protein functional and other interpretation of its predicted potential targets. We clarified the relevant functions linked to the predicted gene list, the proteins (their genes) by Gene Ontology (GO) using Enrichr. For each GO term, the p-value following multiple detection corrections, such as false discovery rate (FDR) corrections, were calculated (Chen EY et al., 2013). We compared pellet versus supernatant proteins in both ways: 1) unique proteins in fractions and 2) significantly changed proteins.

Results

1/ Protein composition of the fractions

We analysed all S and P samples obtained from the caries-positive and caries-free subjects**.** The aim was to find unique proteins that were present in only one fraction (S or P). The results are summarised in Figure 1. A Venn diagram was created and included those protein abbreviations where at least one peptide that met the above criterion was found. In total, we identified 207 proteins across all samples (27 supernatant samples and 27 pellet samples). Approximately half of the proteins were found only in the supernatants, a quarter of the proteins were found only in the pellets and a quarter of the proteins were found in both fractions.

We detected 1520 peptides of which 80 met our criterion for fraction uniqueness (see Materials and Methods). These peptides belonged to 31 proteins. We identified seven proteins (annexin A1; actin; cornulin; 14-3-3 protein sigma; histone H2B type 1-K; lysozyme C; and histone H3.3) that exceeded the impurity criterion (unique proteins must be identified in only one fraction).

2/ Composition differences of supernatants and pellets between caries-positive and caries-free samples (label-free quantification and two-dimensional electrophoresis)

We found 162 valid proteins in supernatant samples by the label-free quantification method. The following fourteen proteins had significant higher expression levels in the supernatant samples of caries-free subjects: alpha-amylase 1; serum albumin; protein S100-A9; immunoglobulin heavy variable 4-31; immunoglobulin heavy constant alpha 1; IGK@ IGK@ protein; apolipoprotein A-I; zymogen granule protein 16 homolog B; immunoglobulin heavy variable 1-44; cystatin-B; lysozyme C; polymeric immunoglobulin receptor; annexin A1; and prolactin-inducible protein) (Table 1A).

We found 73 valid proteins in the pellet samples by the label-free quantification method. We identified three proteins (annexin A1, cornulin and 14-3-3 protein sigma) with higher expression levels in the pellet samples of caries-positive subjects (Table 1B).

We compared the supernatant samples of caries-positive (n=7) and caries-free subjects (n=7) using two-dimensional electrophoresis (2DE). We found a significant difference in the level of polymeric immunoglobulin receptor (t-test: p=0.015; fold change CP/CF = 2.7). Additionally, we compared the pellet samples of caries-positive (n=4) and caries-free subjects (n=4) using two-dimensional difference gel electrophoresis. We found significant differences in the levels of annexin A1 (two spots; t-test: p=0.031 and p=0.015; fold change CP/CF=0.08 and

0.18). The samples were applied to broad-range 7 cm, pH 3-10 NL IPG strips (Bio-Rad). Isoelectric focusing was holded with a Protean IEF Cell system. After the equilibration step, transferred to a 12,5% SDS-polyacrylamide gel (supernatant samples; 10,5% SDS-polyacrylamide gel (pellet samples)). The analysis of 2DE and DIGE gels was done using PDQuest™ software (bio-Rad), version 8.0.1. Local regression model was chosen as a normalization method. Spot was used to select statistically significant differential spots (Student´s t-test; $p \leq 0.05$). Spots with differential expression were excised from the gels. The analyses of the tryptic digests were provided with nano-liquid chromatography tandem mass spectrometry.

3/ Comparison of significant and unique fraction proteins by database for annotation

We used GO analysis to identify significantly enriched GO terms (GO enrichment can directly reflect the distribution of target proteins (their genes) for each enriched GO term (GO Biological Process, and Human Gene Atlas)(https://amp.pharm.mssm.edu/Enrichr/; 15th November 2019) . We observed some significantly enriched biological processes in comparison of significantly changed proteins (and also unique proteins) in the CF group: **defense response to bacterium** (GO:0042742) (adjusted p value: $p<0.010$; including these proteins: immunoglobulin heavy constant aplpha 1, lysozyme C, immunoglobulin heavy variable 4-31, protein S100A9) and receptor-mediated endocytosis (GO:0006898) (adjusted p value: $p<0.046$; including these proteins: serum albumin, immunoglobulin heavy variable 1-44, apolipoprotein A-I, immunoglobulin heavy constant alpha 1) (no significance in CP group). We also observed significantly enriched source of protein origin (by Human Gene Atlas) - "trachea" in the CF group (adjusted p value: $p<0.0011$; including these proteins: polymeric immunoglobulin receptor, prolactin inducible protein, lysozyme C) (no significance in CP group).

Discussion

The present study provides a proteomic comparison of saliva fractions obtained from caries-positive and caries-free males. It was observed that the protein composition differs in the groups (caries-positive vs. caries-free) and also in the saliva fractions (supernatant vs. pellet) which is a unique observation to date.

1/ Comparison of saliva fractions (S vs P) via criterion protein fraction uniqueness

Our comparison of the two fractions (supernatant and pellet) was carried out from three perspectives. First, we compared all supernatants vs. all pellets from both the CP and CF groups

together, identifying 207 different proteins of which 25 proteins fulfilled our criterion for protein fraction uniqueness (circle “A” in Figure 1). Second, a comparison was made of the supernatants vs. pellets obtained from the CP group. A total of 145 proteins were identified in the CP group of which 25 were classified as unique to a fraction (circle “B” in Figure 1). Third, a comparison was made of the supernatants vs. pellets of the CF group. A total of 185 proteins were identified in the CF group of which 30 were classified as unique to a fraction (circle “C” in Figure 1). We found 12 common proteins among the three comparisons (intersection of circles “A”, “B” and “C” in Figure 1) which is surprisingly only 29 % from all unique proteins. One of the study’s aims was to identify proteins that were found only in the pellet fraction, because this fraction has been virtually neglected to date in the literature. In total, 15 unique proteins were found in the pellet fractions. The results of the intragroup comparisons of both the CP (circle “B”) and CF (circle “C” in Figure 1) groups were remarkable in terms of the protein distribution observed between the pellet and supernatant. We found specific protein distributions for the two separate groups of subjects. In the CP group, 15 proteins were uniquely detected only in the pellet, and only 10 were unique to the supernatant (Figure 1). The opposite situation occurred in the CF group, where only threeproteins were uniquely detected in the pellet, but 27 proteins were specifically detected in the supernatant (Figure 1). These results indicate very specific protein compositions of the saliva in the different groups (CP vs CF), showing that the saliva of the CP group contains mainly pellet proteins and the saliva of the CF group contains mainly supernatant proteins. These findings indicate the need for further study in this area to gain a better understanding of the specific mechanisms of anti-caries protection. It appears that CF individuals accumulate their specific proteins in the supernatant fraction (possibly by blocking aggregation) and CP individuals accumulate their specific proteins in the pellet fraction (possibly by enhance aggregation).

We found 12 proteins in the pellet fraction that fulfilled our criterion for protein fraction uniqueness (circle “B” = CP group in Figure 1) and 17 proteins in the supernatant fraction that fulfilled our criterion for protein faction uniqueness (circle “C” = CF group in Figure 1). Proteins such as protein S100-A14 (S100A14), cornulin (CRNN), protein-glutamine gamma-glutamyltransferase E (TGM3), junction plakoglobin (JUP), deleted in malignant brain tumors 1 protein (DMBT1) are calcium ion binding, calcium-dependent protein binding or cadherin binding, which are dependent on calcium Ca^{2+} ions to function. These proteins were found uniquely in the pellet (caries-positive) and calcium binding proteins might be associated with dental caries through the mechanism of calcium dependent aggregation. These proteins might be detected in caries-positive individuals because these proteins are better retained in the saliva. There may be just an association between the disease state rather than mechanistically

contributing to the disease state. Proteins such as lactotransferrin (LTF), protein S100-A8 (S100A8), BPI fold-containing family B member 1 (BPIFB1), BPI fold-containing family B member 2 (BPIFB2) and lysozyme (LYZ) help to provide an antimicrobial humoral response. These proteins were unique to the supernatant of CF group. The proteins lactotransferrin, immunoglobulin lambda variable 1-44 (IGLV1-44), protein S100-A8 and lysozyme support the immune response. Overall, the roles of these proteins (antimicrobial humoral response and immune response) may play a major part in caries prevention. One quarter (~40) of the total identified proteins were found only in the pellet fraction but did not meet our criterion. These are from interesting groups of proteins, such as plakins, keratins and histones. The detected keratins, plakins and tubulins belong to a group of cytoskeleton proteins. We also found periplakin, desmoplakin, plakophilin 1, envoplakin, and fillaggrin, which are intermediate filament-binding proteins (Ruhrberg et al., 1997). These findings indicate that saliva pellets should also be analysed because they probably contain proteins that are important for the aetiology of tooth caries. In contrast, our results suggest that proteins supporting anti-caries defence are mainly present in the saliva supernatant.

2/ Comparison of caries-positive and caries-free groups

The primary intention of this study was to compare saliva samples obtained from caries-positive and caries-free males using three methods (two-dimensional electrophoresis; two-dimensional difference gel electrophoresis; and label-free quantification). However, we found that two-dimensional electrophoresis was not appropriate for these comparisons due to insufficient repeatability of the method, the small number of samples, and their differing concentrations. One other reason was the incomplete dissolution of the samples mainly high abundance protein in the lysis buffer. Only ~ 40 spots were evaluated beside with label-free quantification (comparison of supernatant fraction – 162 valid proteins; comparison of pelet fraction – 73 valid proteins). We observed 14 proteins in the supernatant fraction with a significantly higher expression in the CF group (comparison of samples of CP supernatants vs. CF supernatants by label-free quantification) (Table 1A). Half the number (7) of these proteins are immuno- and/or antibacterial-proteins (lysozyme C, protein S100-A8, immunoglobulin heavy variable 1-34, immunoglobulin heavy constant alpha 1, immunoglobulin kappa constant, immunoglobulin lambda variable 1-44, polymeric immunoglobulin receptor). These significantly upregulated protein in CF group could play an important role in caries prevention. Three proteins in the pellet fractions with significantly higher expression levels were observed in the caries-positive group (comparison of samples of caries-positive pellets vs. caries-free pellets by label-free quantification) (Table 1B). The concentration of annexin A1 was significantly higher in the CF supernatant and also in the CP pellet (by both methods: MS

and 2DE). These results appear to be contrasting; however, they are understandable in the context of the main results of this study, where there is possible specific protein aggregation according to caries susceptibility (different ratio of proteins (pellet/supernatant) in CP and CF groups). In this context, annexin could declared higher protein aggregation in the saliva of the caries-positive group or less protein aggregation in the saliva of the CF group. We have not simple explanation for this possible sample-specific aggregation. Vitorino et al. described a saliva sample preparation method, including the pellet fraction, and demonstrated it using three saliva samples obtained from males (Vitorino et al., 2012). In contrast, our study presents the results of the analysis of both the supernatant and pellet fractions. These results were practically used to investigate differences in protein (peptide) expression according to dental caries status (in pellet for the first time).

Our results are partly consistent with the work in Wang, 2018. They analysed the saliva supernatant using iTRAQ-coupled LC-MS/MS. They found 14 age-specific proteins associated with caries and verified non-age-specific proteins (histatin-1) and BPI fold-containing family B member 1) as important candidate biomarkers for common dental caries (Wang et al., 2018). In the current study, we found that cornulin (in the pellet fraction) was more highly expressed in the caries-positive group. Protein S100A9 was more highly expressed in the caries-free group and was found in both the supernatant and pellet fraction. These results are in agreement with those of Wang (Wang et al., 2018). The observed differences in results may be due to the analysis of salivary samples (only male volunteers vs. female and male volunteers together, age average of sample donations, other mass spectrometry analysis approach). Another i-TRAQ-coupled LC-MS/MS methods was used in Wang´s studies about characterization of the healthy and cariogenic salivary proteome and determine the changes in salivary protein expression of children with varying degrees of the active caries (Wang et al., 2018).

Compared to our previous study (Kulhavá et al., 2018), we found 2 disagreements (alpha-amylase, protein S100A9) and one agreement (lysozyme). The reason for this could be that the samples were prepared by different procedures (purification conditions and sampling). Additionally, the samples were analysed by MS, and the evaluation of the results was also performed by a different approach compared to our previous publication (Kulhavá et al., 2018). Cornulin, 14-3-3 protein sigma and annexin A1 were found with significant higher expression in samples of pellet in the caries-positive group (comparison samples of caries-positive pellets vs samples of caries-free pellets). Cornulin is a calcium ion binding protein (Contzler et al., 2005). Cornulin expression is associated with lesion progression in oral epithelial dysplasia (Santosh et al., 2019) and with the grade of displasia in predicting malignant progression of oral

leukoplakia (Schaaij-Visser et al., 2010). Protein 14-3-3 (SFN) is a catherin binding protein. SFN was found to be decreased in the whole, unstimulated saliva of subjects with generalised aggressive periodontitis compared to healthy volunteers (Wu et al., 2009). Annexin A1 binds to phospholipids in a Ca^{2+}-dependent manner (Mailliard et al., 1996). These results complement our findings that more calcium-binding proteins (calcium-dependent) are found in the pellet fraction of the CP group (Figure 1, Table 1B) which could be a reason for decalcification of enamel.

Annexin A1 is a anti inflammatory and inflammatory resolving protein that is likely involved in a number of chronic conditions such as salivary gland disorders, periodontal disease and diabetes mellitus. The differences in annexin may be also related to periodontal status. This aspect must be included in the experimental design (it is necessary to exclude or clarify the effect on results with respect to other diseases, to be considered in the evaluation in future study; however people with diaberes mellitus were not included in the study).

3/ Comparison of significant and unique fraction proteins by database for annotation

We observed some significantly enriched biological processes in the comparison of significantly changed and also unique proteins of both groups (CP and CF). First of all, there are specific immune processes in the CF group: **defense response to bacterium** (GO:0042742),which confirm our results from label-free quantification (7 significantly upregulated immune- and/or antibacterial proteins in the CF group, Table 1A) and declares their important defence role against caries. Another significantly enriched biological process in the CF group is receptor-mediated endocytosis (GO:0006898). This process by which cells absorb proteins, hormones, metabolites and viruses may play an important role in oral health, however there are not enough data for its better characterization.. We also observed significantly enriched source of protein origin: “trachea” in the CF group. The trachea origin in the CF group may just represent the lack of comprehensive investigation in the oral cavity and not a transfer. It not uncommon for trachea and nasal origins to show up in the oral cavity and may actually not be transient proteins but exist for individuals at all times.

This study shows protein specificity of saliva fractions after centrifugation in relationship to the dental caries experience of the saliva sample donor. We surmise that specific proteins facilitating the formation of tooth caries are found in the saliva supernatant and also pellet fractions. We detected 7 significantly upregulated specific immune-proteins in the saliva supernatant of the CF group that could play an important role in caries prevention. In the pelt of CP group we observed 5 specific calcium binding proteins that could decalcified enamel. The

particular protein compositions of the saliva pellet and supernatant in groups with different susceptibilities to tooth decay is promising finding for future research.

Acknowledgments

This work was supported by the Charles University in Prague, project GA UK No. 322216, within the framework of Specific University Research (SVV 260560), and by the Czech Science Foundation (No. 17-31564A).

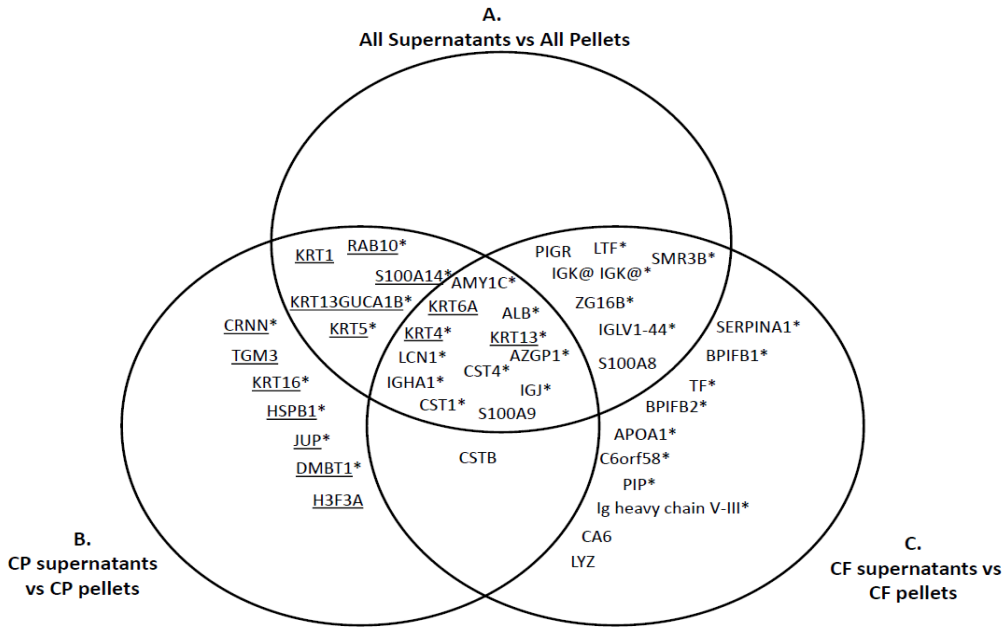

Figure 1 Comparison of protein composition saliva supernatants and pellets.

(Criterion was described Material and Methods- Protein fraction uniqueness by MS/MS). Protein in which at least one peptide (important peptide) was found according to the criterion in the supernatants (normal font) and in the pellets (underlined). Protein abbreviations with "*" indicate that all peptides of the protein were found only in the fraction in which the important peptide was found.

List of abbreviations (alphabetically): **AMY1C** Aplha-amylase 1 (IPI00300786); **ALB** Serum albumin (IPI00745872); **AZGP1** Zinc-aplha-2-glycoprotein (IPI00166729); **BPIFB1** BPI fold-containing family B remenber 1 (IPI00291410); **BPIFB2** BPI fold-containing family B remenber 2 (IPI00296654); **CA6** Carbonic anhydrase 6 (IPI00295105); **CSTB** Cystatin-B (IPI00021828); **CST1** Cystein-SN (IPI00305477); **CST4** Cystein-S (IPI00032294); **CRNN** Cornulin (IPI00297056); **C6orf58** Protein LEG1 homolog (IPI00374315); **HSPB1** Heat shock protein beta-1 (IPI00025512); **H3F3A** Histone H3.3 (IPI00909530); **IGHA1** Immunoglobulin heavy constant aplha1 (IPI00386879); **Ig heavy chain V-III** Immunglobulin heavy chain V-III region (IPI00854644); **IGJ** Immunoglobulin J chain (IPI00947235); **IGK@ IGK@** IGK@ IGK@ protein (IPI00784985); ; **IGLV1-44** Immunoglobulin lambda variable 1-44 (IPI00887169); **JUP** Junction plakoglobin (IPI00554711); **KRT6A** Keratin, type II cytoskeletal 6A (IPI00909059); **KRT1** Keratin, type II cytoskeletal 1 (IPI00220327); **KRT4** Keratin, type II cytoskeletal 4 (IPI01022175); **KRT5** Keratin, type II cytoskeletal 5 (IPI00009867); **KRT13** Keratin, type I cytoskeletal 13 (IPI00009866); **KRT13 GUCA1B** KRT13 GUCA1B protein (IPI00930614); **LCN1** Lipocalin-1 (IPI00009650); **LTF** Lactotransferrin (IPI00925547); **LYZ** Lysozyme C (IPI00019038); **PIGR** Polymeric immunoglobulin receptor (IPI00004573); **PIP** Prolactin-inducible protein (IPI00022974); **RAB10** Ras-related protein Rab-10 (IPI00016513); **TGM3** Protein-glutamine gamma-glutamyltransferase E (IPI00300376); **TF** Serotransferrin (IPI00022463); **S100A8** Protein S100-A8 (IPI00007047); **S100A9** Protein S100-A9 (IPI00027462); **S100A14** Protein S100-A14 (IPI00010214); **TGM3** Protein-glutamine gamma-glutamyltransferase E (IPI0090597); **TF** Serotransferrin (IPI00022463); **ZG16B** Zymogen granule protein (IPI00060800)

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Table 1A Significantly overexpressed proteins in the saliva supernatants of CF group in comparison with CP group

Accession Number	Protein (peptide)	Total number of peptides [a]	Number of peptide [b]	p [c]	Average CS : Average CF [d]	MASCOT Score	n CS : CF [e]
IPI00300786 P04745	**Alpha-amylase 1**	46	10				
	R.TSIVHLFEWR.W			**0.0449**	**0.061**	**72.3**	
	R.ALVFVDNHDNQR.G			**0.0483**	**0.121**	**101.5**	
	K.LGTVIR.K			0.117	0.112	46.4	
	R.NMVNFR.N			0.094	0.125	30,5	
	R.YQPVSYK.L			0.052	0.073	41.8	
	R.LSGLLDLALGK.D			0.101	0.044	88.7	
	K.NWGEGWGFMPSDR.A			0.164	0.167	96.8	
	K.TGSGDIENYNDATQVR.D			0.102	0.076	147.6	
	K.GFGGVQVSPPNENVAIHNPFRPW WER.Y			0.097	0.172	109.2	
	R.YFENGKDVNDWVGPPNDNGVTK .E			0.975	1.008	97.4	
IPI00745872 P02768	**Serum Albumin**	31	3				
	K.KVPQVSTPTLVEVSR.N			**0.0381**	**0.092**	**93.2**	
	K.LVNEVTEFAK.T			0.053	0.234	68.6	
	R.FKDLGEENFK.A			0.052	0.043	78.1	
IPI00027462 P06702	**Protein S100-A9**	17	8				
	K.MHEGDEGPGHHHKPGLGEGTP .-			**0.047**	**0.301**	**70.3**	
	K.MSQLER.N			0.133	0.662	44.0	
	K.DLQNFLK.K			0.174	0.363	25,8	
	R.LTWASHEK.M			0.071	0.178	45.3	
	K.DLQNFLKK.E			0.071	0.424	31,5	
	K.LGHPDTLNQGEFK.E			0.18	0.513	104.8	
	K.VIEHIMEDLDTNADK.Q			0.192	0.307	130.5	
	R.NIETIINTFHQYSVK.L			0.69	1.173		
IPI00645363 P0DP07	**Immunoglobulin heavy variable 4-31**	14	1				
	R.VVSVLTVLHQDWLNGK.E			**0.0477**	**0.113**	**120.9**	

IPI00386879 P01876	**Immunoglobulin heavy constant alpha 1**	13	5			
	R.WLQGSQELPR.E			**0.0183**	**0.025**	**91.8**
	R.DASGVTFTWTPSSGK.S			**0.0424**	**0.063**	**91.7**
	R.VAAEDWK.K			**0.016**	**0.023**	**42.4**
	K.YLTWASR.Q			**0.034**	**0.05**	**39.6**
	K.TPLTATLSK.S			**0.018**	**0.039**	**59.8**
IPI00784865 P01834	**Immunoglobulin kappa constant**	10	2			
	K.DSTYSLSSTLTLSK.A			**0.0226**	**0.1159**	**78.0**
	K.VDNALQSGNSQESVTEQDSK.D			**0.0303**	**0.014**	**181.9**
IPI00021841 P02647	**Apolipoprotein A-I**	12	1			
	R.DYVSQFEGSALGK.Q			**0.0255**	**0.2167**	**65.9**
IPI00060800 O60844	**Zymogen granule protein 16 homolog B**	9	2			
	K.YFSTTEDYDHEITGLR.V			**0.0392**	**0.0387**	**136.4**
	R.VSVGLLLVK.S			**0.0228**	**0.1202**	**33.7**
IPI00887169 P01699	**Immunoglobulin heavy variable 1-44**	8	1			
	K.AGVETTTPSK.Q			**0.0305**	**0.0397**	**64.7**
IPI00021828 P04080	**Cystatin-B**	6	4			
	K.SQVVAGTNYFIK.V			**0.0413**	**0.0542**	**69.6**
	R.VFQSLPHENKPLTLSNYQTNK.A			0.487	0.139	132.0
	K.VHVGDEDFVHLR.V			0.071	0.048	115.3
	K.FPVFK.A			0.15	0.147	32.4
IPI00019038 P61626	**Lysozyme C**	8	2			
	R.STDYGIFQINSR.Y			**0.0255**	**0.2167**	**76.6**
	R.LGMDGYR.G			0.086	0.2395	41.7
IPI00218918 P10107	**Annexin A1**	29	1			
	R.ALYEAGER.R			**0.0413**	**0.129**	**41.2**
IPI00004573 P01833	**Polymeric immunoglobulin receptor**	21	2			
	R.LFAEEK.A			**0.0297**	**0.05**	**43.5**
	K.VYTVDLGR.T			**0.0246**	**0.015**	**54.8**
IPI00022974 P12273	**Prolactin inducible protein**	5	1			
	R.FYTIEILKVE.-			**0.0299**	**0.3216**	**40.0**

Table 1B Significantly overexpressed proteins in the saliva pellets of CO group in comparison with CF group

Accession Number	**Protein (peptide)**	**Total number of peptides [a]**	**Number of peptide [b]**	**p [c]**	**Average CS : Average CF [d]**	**MASCOT Score**

IPI00218918 P04083	**ANXA1 Annexin A1**	44	5			
	R.ALYEAGER.R			**0.0379**	**2.699**	**40.0**
	K.GDRSEDFGVNEDLADSDAR.A			0.301	1.XI	89.9
	K.GVDEATIIDILTKR.N			0.172	I.65	104.3
	R.SEDFGVNEDLADSDAR.A			0.086	I.38	118.3
	K.GVDEATIIDILTK.R			0.218	0.45	92.8
IPI00411765 P31947	**SFN 14-3-3 protein sigma**	8	1			
	K.SNEEGSEEKGPEVR.E			**0.00462**	**2.968**	**117.9**
IPI00297056 Q9UBG3	CRNN Cornulin	16	0			
	M.PQLLQNINGIIEAFRR.Y			0.041	2.369	88.8
	R.WMQVSNPEAGETVPGGQAQTGA STESGRQEWSSTHPR.R			0.048	2.432	113.5
	R.ISPQIQLSGQTEQTQK.A			0.349	1,03	145.2

Accession Number - IPI database and Uniprot

Bold results (these peptides fulfill significance (ratios of their intensities are statistically significant)

a - the number of valid peptides of protein

b - the number of peptides that were found in at least 50% of the samples in the group

c - significance

d - the ratio of the CS and CF averages obtained from the individual intensities data for the given peptide for each sample of the Profile Analysis program

e - the number of samples in the group where the peptide was found

f - t-test of averages all peptides that were found in at least 50% of the samples in the group (Average CS : Average CF)

Příloha 3

PUBLIKACE III

Proteomic Analysis of cardiac ventricles: baso-apical differences

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Molecular and Cellular Biochemicstry, 445:211-219 (2018).

Molecular and Cellular Biochemistry (2018) 445:211–219
https://doi.org/10.1007/s11010-017-3266-8

Proteomic analysis of cardiac ventricles: baso-apical differences

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Received: 7 September 2017 / Accepted: 23 December 2017 / Published online: 4 January 2018

Abstract

The heart is characterized by a remarkable degree of heterogeneity. Since different cardiac pathologies affect different cardiac regions, it is important to understand molecular mechanisms by which these parts respond to pathological stimuli. In addition to already described left ventricular (LV)/right ventricular (RV) and transmural differences, possible baso-apical heterogeneity has to be taken into consideration. The aim of our study has been, therefore, to compare proteomes in the apical and basal parts of the rat RV and LV. Two-dimensional electrophoresis was used for the proteomic analysis. The major result of this study has revealed for the first time significant baso-apical differences in concentration of several proteins, both in the LV and RV. As far as the LV is concerned, five proteins had higher concentration in the apical compared to basal part of the ventricle. Three of them are mitochondrial and belong to the "metabolism and energy pathways" (myofibrillar creatine kinase M-type, L-lactate dehydrogenase, dihydrolipoamide dehydrogenase). Myosin light chain 3 is a contractile protein and HSP60 belongs to heat shock proteins. In the RV, higher concentration in the apical part was observed in two mitochondrial proteins (creatine kinase S-type and proton pumping NADH:ubiquinone oxidoreductase). The described changes were more pronounced in the LV, which is subjected to higher workload. However, in both chambers was the concentration of proteins markedly higher in the apical than that in basal part, which corresponds to the higher energetic demand and contractile activity of these segments of both ventricles.

Keywords Proteomics · Heart · Ventricle · Myocardial heterogeneity · Two-dimensional electrophoresis · Ventricular myocardium

Introduction

The heart is characterized by a remarkable degree of heterogeneity [1], the basis of which is a subject to active investigation. Since different cardiac pathologies affect different cardiac regions, it is important to understand the molecular mechanisms by which these regions respond to pathological stimuli. Important dissimilarities between the two ventricles can already be found in the early phases of ontogenetic development. The right ventricle (RV) and the RV outflow tract are derived from the anterior heart field, whereas the left ventricle (LV) and atria are derived from the primary heart field [2]. Transcription factors such as HAND1 and HAND2 appear to play an important role in chamber-specific heart formation [3]. RV and LV free-wall thickness and force development are equal throughout fetal life. After lung expansion and clamping of the umbilical cord at birth, the peak systolic pressure in the LV and the systemic vascular resistance rise, while RV pressure, pulmonary artery

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11010-017-3266-8) contains supplementary material, which is available to authorized users.

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pressure and pulmonary vascular resistance decrease. The increased workload resulting from these circulatory changes is considered to be the stimulus for a more rapid growth of the LV [4, 5]. The RV and LV of the adult mammalian heart work in series to deliver oxygenated blood to the body. Although both have identical cardiac outputs, their afterload pressures differ significantly (25 vs 120 mmHg); the workload ratio of the left to right heart approaches 5 [6]. The reported ratios of LV to RV mass range between 3.4 [7] and 2.6 [8] and are not proportional to the fivefold difference in work. This discrepancy suggests that in addition to workload there are other underlying differences between the ventricles that determine LV/RV mass, including architecture, contractile properties, and metabolic activities [6]. In contrast to the ellipsoidal shape of the LV, the RV appears triangular when viewed in cross section. The RV wall is mainly composed of superficial circumferential and longitudinally oriented deep muscle layers; the LV contains obliquely oriented myofibers superficially, longitudinally oriented myofibers in the subendocardium, and predominantly circular fibers in between. This arrangement contributes to the more complex movement of the LV, which includes torsion, rotation, translation, and thickening [9]. Oxygen consumption in the resting RV is less than that in LV [10, 11], implying that the metabolic stress (ATP generation rate/maximum ATP generation rate) of the RV is lower, based on a wet weight comparison. Additionally, oxidative capacity in the LV is apparently more sensitive to hypoxia than RV [12]. Furthermore, the RV has a higher concentration of collagenous proteins [13] and higher activity of aerobic glycolytic metabolism than LV [14]. Moreover, several reports have demonstrated significant right/left differences in myocardialsusceptibility to various insults: RV is more resistant to ischemia-induced injury [15] as well as to anthracycline toxicity as compared to LV [16].

To add insight into the underlying mechanisms of differences between the LV and RV, great effort has been put into gene expression analyses of LV and RV [17, 18]. However, such analysis has yet to be performed at the proteomic level: proteomic technology allows us to examine global differences in protein expression and asses the posttranslational modification status of LV and RV; McGregor reviewed over 5000 proteins in both ventricles [19]. So far there are only few reports dealing with the comparison of protein profiling in the RV and LV. Comunian et al. proposed the first comparative characterization of LV and RV using multidimensional protein identification in mice; they have observed significant quantitative differences in the representation of individual proteins [20]. Special attention was paid to the transmural differences in the RV and LV (epi–endocardial). It has been observed that some histochemical changes as well as ultrastructural picture revealed significant transmural differences [21, 22]. The first attempt to assess chamber-specific transmural heterogeneity in myofilament protein phosphorylation by top-down mass spectrophotometry was done by Gregorich et al. [23]. They have observed that phosphorylation of cardiac troponin I and T vary in the two chambers; however, no significant transmural differences were observed in the phosphorylation of the myofilament proteins analyzed.

During development, the differences in gene expression in the ventricles were studied by Krejci et al. in the chick embryo. While the number of differentially expressed genes increased during the septation, the general pattern seemed to be that the right ventricle is lagging about 2 days behind the left ventricle [24].

In addition to the transmural heterogeneity, possible baso-apical differences, observed already during embryonic life, have to be taken into consideration. Differential growth of regions within the ventricular free walls is important for molding of the entire organ [25]. Two proliferative centers were described in the apical parts of RV and LV [26, 27] and later confirmed by de Boer et al. [28]. Furthermore, there is a recognized difference in left ventricular wall thickness between the apex and the base [29], present already at the early developmental stage [30]. It was hypothesized that this difference is due to differential loading stemming from the ventricular activation sequence from apex to base, due to arrangement of the cardiac conduction system; the early-activated thinner apical myocardium is contracting against less resistance than the thicker late-activated basal myocardium [31]. The data about the possible baso-apical differences in the protein composition of individual ventricles are, however, still lacking. The aim of our study was, therefore, to compare proteomes in the apical and basal parts of the RV and LV.

Materials and methods

Sample preparation

Adult male Wistar rats ($n=5$) 3 months old (484 ± 26 g body wt) were used in this experiment. Rats were killed by cervical dislocation, and their hearts were dissected and washed in ice-cold saline. Free LV and RV walls were separated and divided transversally into three parts: basal, middle, and apical. Samples taken from basal and apical parts were frozen and stored in liquid nitrogen until use (Fig. 1; Supplement Table 1).

Extraction of proteins

Lyophilized transmural samples (0.5 mg dry weight (ca 2 mm^3)) were homogenized, and subjected to sonication (water basin) in 0.5-ml tube (15 min, 20 °C) in 135 mL of

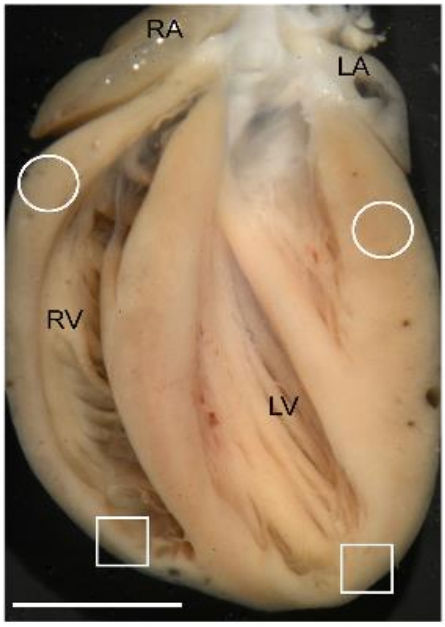

Fig. 1 Four-chamber view of a 3-month-old male rat heart arrested in diastole. The squares show the apical sampling sites while the circles indicated the sampling sites for the basal specimens. *LA* left atrium, *LV* left ventricle, *RA* right atrium, *RV* right ventricle. Scale bar 5 mm

lysis buffer (7 mol/L urea, 2 mol/L thiourea, 2% 3-((3-cholamidopropyl)dimethylammonium)-1-propanesulfonate (CHAPS, w/v), 0.6% Bio-Lyte® ampholytes (3–10 buffer, w/v), 1% dithiothreitol (w/v)), Bio-Rad, Hercules, USA), and centrifugation (1000 g, 20 °C, 3 min). The supernatant was taken for subsequent 2-DE analysis (analyses were performed separately ($n=5$) with replication in case of inconsistent spots, samples were not pooled).

Separation of proteins by two-dimensional electrophoresis (2-DE)

Isoelectric focusing and separation by 2-DE were performed on homogeneous 11% SDS–polyacrylamide gel as described previously [32]. An appropriate amount of the supernatant (125 µL for a 7-cm strip) was transferred to Ready Strip™ IPG Strips (pH range 3–10 NL, Bio-Rad) overnight by active in-gel rehydration (50 V, 15 °C). Isoelectric focusing was carried out at 15 °C with a Protean® IEF cell system (Bio-Rad) under mineral oil. Proteins were focused until 28 kVh per strip was reached. Prior to separation in the second dimension, the strips were equilibrated and transferred to a homogeneous 11% SDS–polyacrylamide gel. Finally, 5 µL of Precision Plus Protein™ Standards (molecular weight range 10–250 kDa, Bio-Rad) was added at the top end of the gel. Mini gels were run in the Mini-Protean Tetra Cell system (5 min for 50 V and then 45 min for 250 V). Gels were stained with colloidal Coomassie brilliant blue stain (Bio-Safe, Bio-Rad) according to the manufacturer's recommendations to enable the visual detection of the various abundances in the spots. Spot normalized volume was used to select statistically significant differential spots ($n=5$, samples were not pooled) by PDQuest Advanced (Bio-Rad). Protein levels showing significant quantitative differences (Student's t test, $p \leq 0.05$) were selected for mass spectrometric analysis.

Identification of proteins—nano-liquid chromatography-tandem mass spectrometry (nLC-MS/MS)

Spots with differential expression were excised from the Coomassie-stained gels and then processed as described previously [33]. The separation of peptides was achieved via a linear gradient between mobile phase A (water) and B (acetonitrile), both containing 0.1% (v/v) formic acid. The nano-LC apparatus used for protein analysis was a Proxeon Easy-nLC (Proxeon, Odense, Denmark) coupled to a maXis Q-TOF (quadrupole—time of flight) mass spectrometer with ultra-high resolution (Bruker Daltonics, Bremen, Germany) by nanoelectrosprayer. The nLC-MS/MS instruments were controlled with the software packages HyStar 3.2 and micrOTOF-control 3.0. The data were collected and analyzed using the software packages ProteinScape 3.0 and DataAnalysis 4.0 (Bruker Daltonics). Peptide mixtures were injected into a NS-AC-11-C18 Biosphere C18 column (particle size: 5 µm, pore size: 12 nm, length: 150 mm, inner diameter: 75 µm), with a NS-MP-10 Biosphere C18 pre-column (particle size: 5 µm, pore size: 12 nm, length: 20 mm, inner diameter: 100 µm), both obtained from NanoSeparations (Nieuwkoop, Netherlands). All nLC-MS/MS analyses were performed in duplicates.

Database search

Database searches were performed as described in [34], and the taxonomy was restricted to *Rattus norvegicus* to remove protein identification redundancy. Proteins were identified by correlating tandem mass spectra with the UniProt/SwissProt database (http://www.uniprot.org), using the MASCOT online search engine for protein identification using

mass spectrometry data (http://www.matrixscience.com). An initial peptide mass tolerance of ± 0.05 Da was used for MS/MS analysis. Only significant hits (MASCOT score ≥ 80 for proteins) were accepted.

Results

Baso-apical differences in the LV and RV

A total of about 150 most abundant proteins were identified in the LV and RV. It has been observed that the quality of protein composition was identical in basal and apical parts of both ventricles. However, significant baso-apical differences were observed in the quantitative representation of some proteins.

As far as the LV is concerned, the concentration of five proteins was significantly higher in the apical part as compared with the basal part: myosin light chain 3, L-lactate dehydrogenase B chain, creatine kinase M-type, dihydrolipoyl dehydrogenase, 60 kDa heat shock protein (Table 1; Fig. 2a). From these five proteins with different baso-apical concentrations, three participate in the metabolism and energy pathways (L-lactate dehydrogenase B chain, creatine kinase M-type, dihydrolipoyl dehydrogenase); three of them are mitochondrial (L-lactate dehydrogenase B chain, dihydrolipoyl dehydrogenase, 60 kDa heat shock protein) (Table 1; Fig. 3) (additional data about differently produced proteins in both ventricles are listed in Supplement Table 2).

The same concentration gradient (i.e., a higher concentration in the apical part) was observed also in two proteins in the RV: creatine kinase S-type (S-CK) and NADH:ubiquinone oxidoreductase core subunit V1 (Fig. 3). On the other hand, the opposite gradient (a higher concentration in the basal part) was detected for aldo–keto reductase family member C1 and succinate semialdehyde dehydrogenase (Table 1; Fig. 2b). All four RV proteins with different baso-apical concentrations participate in metabolism and energy pathways, and three of them are mitochondrial (S-CK, NADH:ubiquinone oxidoreductase, and succinate semialdehyde dehydrogenase) (Table 1).

RV/LV differences

In two proteins, there were detected higher concentrations in the LV: myosin light chain 3 in the apical part and two spots of myosin-binding protein C in the basal part (Table 2; Fig. 4) (additional data about differently produced proteins in the left ventricle in comparison with the right ventricle are listed in Supplement Table 2). Both proteins participate in the contractile function.

Discussion

For the proteomic analysis, 2DE was used, which is the most common method in proteomics despite well-known problems associated with membrane proteins and a rather inaccurate characterization of the molecular mass (membrane and small proteins could be missed out) and isoelectric point of a selected protein. Despite these limitations, 2D gels have a unique ability to separate complete proteins with all

Table 1 List of baso-apical differentially expressed proteins in the left ventricle (part A), the right ventricle (part B)

Part A	UniProt[a]	Ventricle	Protein	Apical/basal (fold)	p[b]	Mw[c]	pI[d]	n. in Fig. 2a[e]
	P16409	Left	Myosin light chain 3	1.758	0.024	22.1	4.9	1
	P42123	Left	L-lactate dehydrogenase B chain	1.624	0.048	36.6	5.6	2
	P00564	Left	Creatine kinase M-type	1.679	0.029	43	6.6	3
	Q6P6R2	Left	Dihydrolipoyl dehydrogenase	1.582	0.029	54	9	4
	P63039	Left	60 kDa heat shock protein	2	0.025	60.9	5.8	5
Part B	UniProt	Ventricle	Protein	Apical/basal (fold)	p	Mw	pI	n. in Fig. 2b[e]
	Q6P8J7	Right	Creatine kinase S-type	1.809	0.045	47.4	9.4	6
	Q91YT0	Right	NADH:ubiquinone oxidoreductase core subunit V1	1.81	0.006	50.7	9.4	7
	Q3MHS3	Right	Aldo–keto reductase family 1, member C1	0.425	0.049	37.2	7.8	8
	P51650	Right	Succinate-semialdehyde dehydrogenase	0.495	0.015	66.1	9.9	9

[a]Protein number in UniProt database (http://www.uniprot.org)

[b]Significance

[c]Theoretical molecular weight

[d]Theoretical isoelectric point

[e]Spot number in the Fig. 2

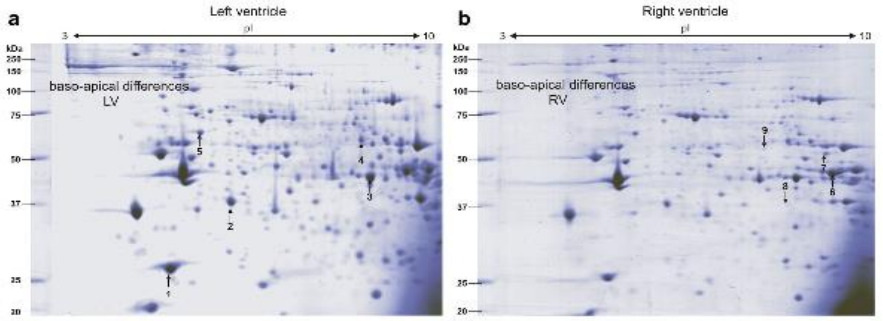

Fig. 2 Representative two-dimensional gel electrophoresis maps of the LV (**a**), and RV (**b**). **a** Protein differences between basal and apical part of the LV. Arrows express up- or down-regulation to apical part of LV (1- myosin light chain 3; 2- L-lactate dehydrogenase B chain; 3- creatine kinase M-type; 4- dihydrolipoyl dehydrogenase; 5–60 kDa heat shock protein). **b** Protein differences between basal and apical part of RV. Arrows indicate up-regulation or down-regulation to apical part of RV (6- creatine kinase S-type; 7- NADH:ubiquinone oxidoreductase; 8- aldo–keto reductase family member C1; 9- succinate semialdehyde dehydrogenase)

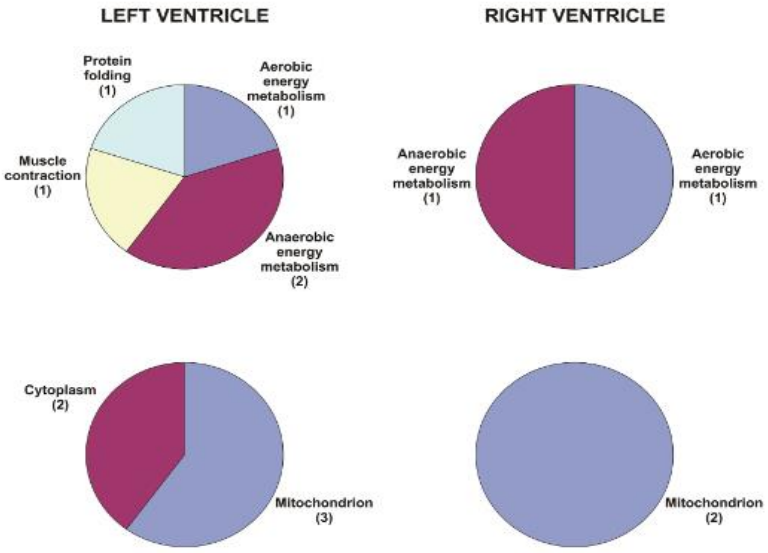

Fig. 3 Functional categorization and subcellular localization of significantly highly expressed proteins in the apical part of the LV and RV in comparison to their basal part. Numbers in brackets indicate numbers of significantly differently expressed proteins

Table 2 List of significantly higher expressed proteins in left ventricle in comparison with the right ventricle

UniProt[a]	Ventricle part	Protein	LV/RV (fold)	p^b	Mw[c]	pI[d]	n. in Fig. 4[e]
P16409	apical	Myosin light chain 3	1.71	0.02934	22.1	4.9	1
P56741	basal	Myosin-binding protein C, cardiac-type	2.05	0.01462	141.2	6.5	2
P56741	basal	Myosin-binding protein C, cardiac-type	3.18	0.03485	141.2	6.5	3

[a]Protein number in UniProt database (http://www.uniprot.org)
[b]Significance
[c]Theoretical molecular weight
[d]Theoretical isoelectric point
[e]Spot number in the Fig. 4

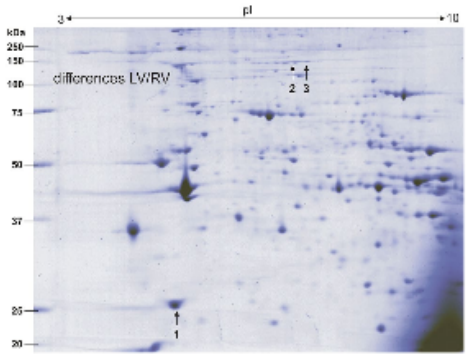

Fig. 4 Representative two-dimensional gel electrophoresis map differences between LV and RV. The arrows express up-regulation of proteins in the apical part of the LV (1-myosin light chain 3) and in the basal part of the LV (2, 3-both spots are isomers of myosin-binding protein C)

their modifications, and 2D gel-based proteomics presents a powerful tool for studying modification landscapes in the heart muscle [35].

Baso-apical gradients in LV

The major result of our proteomic study has revealed for the first time significant baso-apical differences in concentration of some proteins, both in the LV and RV. As far as the LV is concerned, five proteins have a higher concentration in the apical as compared to the basal part of the ventricle (Table 1). They include the following:

Myofibrillar creatine kinase M-type (MM-CK) reversibly catalyzes transfer of phosphate between ATP and various phosphagens (e.g., creatine phosphate). It is necessary to mention that the heart muscle expresses four creatine kinase isoforms; MM–CK represents about 88% of the total CK activity in the human heart [36]. The expression of multiple components of cardiac CK system under pathological conditions was summarized by Zervou et al. [37]. The expression of CK in the cardiac muscle is not homogenous: in addition to our results, transmural endo-epicardial variations of CK were detected in the canine LV wall [38]. These results suggest the possible functional 3D gradient of CK in the LV.

L-lactate dehydrogenase is very important enzyme of energy metabolism. This protein reduces pyruvate to L-lactate when oxygen supply is absent or reduced; it performs the reverse reaction during the Cori cycle. Enzymes involved in the L-lactate metabolism have recently been reviewed [39].

Myosin light chain 3 is contractile protein and its higher apical concentration is in agreement with the increased contractile force in this part of the ventricular wall. This finding supports the observation of Taylor et al. that phosphorylation of myosine binding protein-C contributes to the genesis of ventricular wall geometry, linking myofilament biology with multiscale cardiac mechanisms and myoarchitecture [40].

Dihydrolipoamide dehydrogenase serves as E3 subunit of three mitochondrial enzyme complexes: branched chain α-ketoacid dehydrogenase complex, α-ketoglutarate dehydrogenase complex, and pyruvate dehydrogenase complex [41].

60 kDa heat shock protein (HSP60) increases in response to different pathological conditions, e.g., inflammation, infection, hyperthermia, and presence of toxic metabolites [42].

Three of these proteins are mitochondrial and belong to the "metabolism and energy pathways" group of proteins (myofibrillar creatine kinase M-type, L-lactate dehydrogenase, dihydrolipoamide dehydrogenase); myosin light chain 3 is a contractile protein and HSP60 is the protein involved in folding of other proteins and possibly helping to stabilize them under pathological conditions. The biological function of these proteins corresponds with the higher contraction energetic demand of the apical part of the LV (Table 1; Fig. 3).

Baso-apical gradients in the RV

In the RV, higher concentration in the apical part has been observed in two proteins; both of them belong to the "metabolism and energy pathway" group (Table 1; Fig. 3).

Creatine kinase S-type (sarcomeric)(S-CK) is an important mitochondrial protein [43]; its concentration in the apical part was higher in comparison with the basal part, similarly as in the case of another CK in the LV. Our results, together with the observation of transmural variation of CK observed by Robitaille et al., suggest a possible functional 3D gradient of CK in the whole heart [38].

Proton pumping NADH:ubiquinone oxidoreductase (member of mitochondrial complex 1) is the largest and most complicated enzyme of the respiratory chain; the role of subunits and functional analysis of mitochondrial complex 1 has been recently summarized by Wirth et al. [44].

On the other hand, in two cases, higher protein concentration was observed in the basal part of the RV.

Aldo–keto reductase family member C1 belongs to the aldo–keto reductase superfamily, which catalyzes the NADPH-dependent reduction of various substrates including steroid hormones [45].

Succinate semialdehyde dehydrogenase is one of the two enzymes necessary for gama-aminobutyric acid (GABA) recycling from the synaptic cleft [46].

Our results thus confirmed the hypothesis that cardiac heterogeneity in protein composition also involves significant differences between the apical and basal part of both chambers; the observed changes were more pronounced in the LV, characterized by a higher workload. In this connection, it is necessary to mention that regional mechanical function in the LV varies longitudinally from basal to apical segments. Similarly, RV contraction is sequential, starting with the contraction of the inlet and trabeculated myocardium and ending with the contraction of the infundibulum, which is of longer duration than contraction of the inflow region [47]. The timing of electrical excitation in the LV wall is also known to be heterogeneous, with excitation generally spreading from apex to base [48]. Different protein compositions of the basal and apical parts of both ventricles are likely closely related to the described functional baso-apical gradients. These differences, corresponding to different workloads, are also demonstrated at the morphological level by different thickness of the wall [29].

These differences already appear very early in the embryonic development prior to ventricular septation [30].

Comparison of the LV and RV

The comparison of protein composition between the LV and RV in the present study has surprisingly revealed differences in only two proteins; both of them belong to the group of contractile proteins. Myosin light chain 3 expression was higher in the apical part of the LV; on the other hand two spots of myosin-binding protein C were higher in the basal part of the LV (Table 2).

The data on the comparison of proteomic characteristics of the LV and RV are very rare and controversial. At the first attempt, Comunian et al. in their study on the mouse heart utilized multidimensional protein technology to characterize murine LV and RV proteomes [20]. They identified thousands of distinct proteins: 16 proteins were more abundant in the LV compared to RV, 47 proteins were more abundant in the RV. Differences between proteomes of the LV and RV, both under aerobic conditions and in response to ischemia/reperfusion, were observed by Cadete et al. in rats [49]. They found ten protein spots whose levels were different between the aerobic LV and RV. Manakov et al. investigated proteome differences in the LV and RV in spontaneously hypertensive rats [50]. They identified 26 differently expressed proteins, out of which 18 were upregulated in the RV and 8 in the LV. The first analysis of the human myocardium demonstrated that the LV and RV have distinct proteomes, and that the differences further depend on the type of disease [51]. On the other hand, proteomic analysis of Phillips et al. has surprisingly revealed that expression levels for more than 600 RV and LV proteins detected were similar between the rabbit and pig hearts [6]. They have suggested that the RV–LV differences in overall workload are managed by modifying the amount of cytosol, rather than its composition. It seems, therefore, that the results dealing with the protein profiling of the LV and RV are dependent on the analytic technology, species and the type of pathological conditions. In addition, we are convinced that the protein composition of both ventricles may be influenced by age and sex of investigated experimental model.

Conclusions

Our results for the first time suggest that heterogeneity of protein composition of the ventricular myocardium involves, beside the already described LV/RV and transmural differences also significant quantitative differences between the apical and basal part of both ventricles. The described changes were more pronounced in the LV. In both chambers, the concentration of proteins, mitochondrial and contractile, was markedly higher in the apical than that in the basal part, which corresponds to the higher energetic demand and contractile activity of these segments of both ventricles.

Acknowledgements This work was supported by the Czech Science Foundation (Nos. 16-12420Y, 16-02972S, and 13-12412S), by the Charles University in Prague, project GA UK No. 322216, and within the framework of Specific University Research (SVV 260440).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures have been performed in accordance with the ethical standards and with the approvement of the Ethical Committee of the Institute of Physiology CAS in Prague.

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Příloha 4

PUBLIKACE IV

Comparison of enzymatic activities and proteomic profiles of Butyrivibrio fibrisolvens grown on different carbon sources

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Proteome Science 17:2 (2019).

Sechovcová et al. Proteome Science (2019) 17:2
https://doi.org/10.1186/s12953-019-0150-3

Proteome Science

RESEARCH Open Access

Comparison of enzymatic activities and proteomic profiles of *Butyrivibrio fibrisolvens* grown on different carbon sources

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Abstract

Background: The rumen microbiota is one of the most complex consortia of anaerobes, involving archaea, bacteria, protozoa, fungi and phages. They are very effective at utilizing plant polysaccharides, especially cellulose and hemicelluloses. The most important hemicellulose decomposers are clustered with the genus *Butyrivibrio*. As the related species differ in their range of hydrolytic activities and substrate preferences, *Butyrivibrio fibrisolvens* was selected as one of the most effective isolates and thus suitable for proteomic studies on substrate comparisons in the extracellular fraction. The *B. fibrisolvens* genome is the biggest in the butyrivibria cluster and is focused on "environmental information processing" and "carbohydrate metabolism".

Methods: The study of the effect of carbon source on *B. fibrisolvens* 3071 was based on cultures grown on four substrates: xylose, glucose, xylan, xylan with 25% glucose. The enzymatic activities were studied by spectrophotometric and zymogram methods. Proteomic study was based on genomics, 2D electrophoresis and nLC/MS (Bruker Daltonics) analysis.

Results: Extracellular β-endoxylanase as well as xylan β-xylosidase activities were induced with xylan. The presence of the xylan polymer induced hemicellulolytic enzymes and increased the protein fraction in the interval from 40 to 80 kDa. 2D electrophoresis with nLC/MS analysis of extracellular *B. fibrisolvens* 3071 proteins found 14 diverse proteins with significantly different expression on the tested substrates.

Conclusion: The comparison of four carbon sources resulted in the main significant changes in *B. fibrisolvens* proteome occurring outside the fibrolytic cluster of proteins. The affected proteins mainly belonged to the glycolysis and protein synthesis cluster.

Keywords: *Butyrivibrio fibrisolvens*, Proteomics, Rumen, Carbon sources

Introduction

B. fibrisolvens was isolated from rumen fluid in the mid-fifties by Bryant and Small [1]. It is a strictly anaerobic, non-spore-forming, monoflagellated, butyrate producing bacterium, which is a member of the family Lachnospiraceae (order Clostridiales, class Clostridia, phylum Firmicutes). This genus belongs to the core or heritable rumen species, which represent nearly half of the rumen population [2]. Together with the genera *Ruminococcus*, *Butyrivibrio*, and the Christensenellaceae family, all members of Firmicutes, they form an enriched rumen microbiota population in the prepartal period [3]. *Butyrivibrio* together with *Pseudobutyrivibrio* species also belong to the very first 25% colonizers of feed fibre [4]. The total numbers of both groups can amount to 0.6–2% of total bacterial counts [5]. In the colon, their levels fall to 0.001% [6]. Most strains can ferment various soluble sugars, disaccharides and oligosaccharides, producing butyrate as the major end product [7]. This feature makes *Butyrivibrio* strains an important component of digestive

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tract microbiota influencing the healthy state of colonocytes for which butyrate is the main energy source [8].

In the rumen, butyrivibria represent an important fibre degrader group which are able to utilize various hemicelluloses and xylans, pectin and starch, however exhibiting poor or no growth on cellulose [9]. In grasses, the predominant component of hemicellulose is arabinoxylan, containing β (1 → 4) linked xylose units, with α (1 → 2) and α (1 → 3) linked arabinose units with D-galactose, possibly D-glucose, as well as side chains of L-rhamnose [10]. The enzymes and genes involved in the decomposition of xylan by *Butyrivibrio fibrisolvens* strains have been thoroughly studied [11] and characterized after xylanase cloning and expression in *E. coli* [12] or after its purification from the culture medium [13]. Valuable data coming from the whole genome sequencing and proteome analysis has helped to supply the respective databases (UniProt, Cazy) with relevant information and create an integrated picture of the enzymatic map of this important bacterium for biomass conversion in ruminant animals.

The crucial enzymes of xylan hydrolysis are three types of endo-β-1,4-xylanases (EC 3.2.1.8) from GH family 10, 11, 30 and two types of 1,4-β-xylosidases (EC 3.2.1.37) from GH family 1 and 43 [14]. Endo-xylanase of the GH 10 family requires two unsubstituted xylopyranose units, GH 11 family needs three xylopyranose units in row, and GH 30 family is a xylanohydrolase (glucuronoarabinoxylan endo-β-1,4-xylanase (EC 3.2.1.136)) acting on glucuronoxylan [15]. 1,4-β-xylosidase of family GH 43 is involved in xylooligosaccharide hydrolysis [16] and 1,4-β-xylosidase of family GH 1 splits off xyloside unit from the non-reducing end of xylan chain [17].

The utilization of hydrolytic products from hemicelluloses usually occurs under carbon catabolic repression (CCR), resulting in a preference for hexose over pentoses in bacteria [18]. The mechanism of simultaneous pentose and hexose utilization was observed in thermophilic G+ anaerobes (TGPA). An isolate of *Thermoanaerobacter* sp. utilized both hexose and pentose simultaneously. It was found that its glycobiome is organized into 13 modules (4–138 genes), and these genes are functionally coherent, presumably based on positive co-expression [19]. In Butyrivibrio species, both CCR and simultaneous metabolism were observed. The type strain of *B. fibrisolvens* 3071 is able to utilize xylose and glucose simultaneously [20]. Therefore these substrates were chosen for this proteomic study.

Nowadays, molecular methods for fibre degradation including genomics and proteomics are preferred to obtain a deep understanding of the digestion process [21]. Among ruminal xylanolytic bacteria, advanced proteomic and mass spectrometric methods for exploring xylan degradation have been used exclusively for *B. proteoclasticus*. Its complete genome sequence [22] together with the studies of its extracellular polysaccharide-degrading proteome, its cytosolic oligosaccharide-degrading proteome [23, 24] and its carbohydrate transporting membrane proteins [25] substantially expanded current knowledge about the hydrolytic capability of the *B. proteoclasticus* type strain B3176^T.

The hydrolytic system of *B. fibrisolvens* is however still unexplored and far less investigated than that of *B. proteoclasticus*. Therefore in this study, we proposed to construct a theoretical secretome of *B. fibrisolvens* 3071 and to compare proteomic profiles of *B. fibrisolvens* 3071 grown on four substrates, complex polysaccharide (xylan), simple pentose (xylose), simple hexose (glucose) and carbohydrate mixture (xylan+glucose), which resulted in six pair-wise comparisons.

Material and methods

Bacterial strain, culture conditions and sample preparation

Butyrivibrio fibrisolvens strain DSMZ 3071 (*B. fibrisolvens* 3071) was obtained from the DSMZ culture collection. *B. fibrisolvens* 3071 was cultivated to the late stationary phase under anaerobic conditions at 37 °C in medium M10 [26] without rumen fluid. Various substrates including 4% (w/v) D-xylose (Sigma-Aldrich), 4% (w/v), D-glucose (Sigma-Aldrich), 4% (w/v) beechwood xylan (Fluka), and the combination of 3% (w/v) beechwood xylan (Fluka) with 1% (w/v) D-glucose (Sigma-Aldrich) were used as carbon sources. Bacteria were collected by centrifugation at 10000 g for 20 min at 4 °C. The supernatants containing extracellular enzymes were further processed at 10 °C in a stirred ultrafiltration cell (Millipore) using a Millipore PES membrane with a 10 kDa cut off and stored immediately at −24 °C. The 6-fold concentrated extracellular enzyme extracts were used for monitoring the proteins secreted in response to the different growth substrates.

Isolation of DNA and genome sequencing

Genomic DNA was isolated from the *B. fibrisolvens* 3071 strain cultivated on DSMZ medium 330 (http://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium330.pdf) under anaerobic conditions [27]. DNeasy UltraClean Microbial kit (Qiagen) was used for nucleic acid isolation with a modified lysis step with prolonged incubation at 70 °C. Genomic DNA concentration was determined using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific) and the quality and integrity of DNA were checked by electrophoresis on 0.8% agarose gel. The genome was sequenced, trimmed and assembled with PacBio technology by GATC Biotech (Germany). The contigs of draft genome were processed with Geneious 9.1.8 software (Biomatters Ltd., New Zealand). The processing of output contigs was performed

with KEGG's internal annotation tools [28] and PROKKA software tool [29].

Enzyme assay

Spectrophotometric determination of xylanase enzyme activity was performed according to Lever [30]. The assay mixture contained 100 µl of culture supernatant, 100 mmol/l phosphate buffer (pH 6) and 0.5% (w/v) carboxymethyl (CM) xylan as a substrate [31] and was incubated for 60 min at 40 °C. The reaction was stopped by 0.3 M Ba $(OH)_2$ and 0.3 M $ZnSO_4$, and mixture was centrifuged (6000x g,10 min). The PAHBAH reagent was added (0.9 ml) and the mixture was boiled for 10 min. After cooling the reducing sugars were measured spectrophotometrically at 410 nm (Biomate 5, UK).

The activity of β-xylosidase was determined according to Bidochka [32]. The assay mixture contained 100 µl of culture supernatant, 100 mmol/l phosphate buffer (pH 6) and 0.5% (w/v) p-nitrophenyl-β-D-xylopyranoside (*p*-NPX) (P-LAB) as the substrate and was incubated for 60 min at 40 °C. The reaction was stopped by 0.8 ml 2% (w/v) Na_2CO_3 and mixture was centrifuged (6000x g, 10 min). The reducing sugars were measured spectrophotometrically at 410 nm (Biomate 5, UK).

The protein concentration was determined according to Bradford [33]. Bovine serum albumin (BSA) solution (Sigma-Aldrich) was used for calibration. Two hundred µl of working solution (Coomassie Brilliant Blue G-250) (Bio-Rad) and 4 µl sample (or standard) were pipetted into the 96 well microplate. The absorbance was measured at 595 nm (Sunrise, Tecan, Scholler) and the concentration of proteins in the sample was calculated using the linear regression method.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and zymography

SDS-PAGE of crude extracellular extracts of *B. fibrisolvens* 3071 grown in medium with different substrates as described above was performed according to Laemmli [34] on a slab of 8% polyacrylamide gel in a Mini-Protean Tetra cell system (Bio-Rad) at 110 V for 1.5 h. Protein bands were visualized with Bio-Safe Coomassie R-250 Staining Solution (Bio-Rad) and analysed in a GS-700 Imaging Densitometer (Bio-Rad). Zymograms were prepared according to Flint et al. [35] using 0,1% (w/v) carboxy-methyl (CM) xylan as the substrate [31] on a slab of 8% polyacrylamide gel under the same conditions as for SDS-PAGE. The gel was washed with 1% (v/w) Triton X100 (Fluka) for 30 min and three times with 25 mM phosphate buffer (pH 7,5) to allow the renaturation of enzymes. The gel was then incubated for 20 min at 39 °C in 25 mM phosphate buffer (pH 7,5) and stained in 0.1% (v/w) Congo Red (Sigma-Aldrich) for 30 min. The highlighted spots indicated xylanase activity against a red background after destaining with 1 M NaCl.

Two dimensional gel electrophoresis

The comparison of four different carbon sources was based on pair-wise matching with two replicates for each substrate (8 gels) (Additional file 2: Table S2, Figures S1-S12). Protein samples were precipitated for 1 h using 10% trichloroacetic acid (w/v) and pelleted by centrifugation at 4 °C, 7500 x g for 15 min. Pellets were washed with 1 ml of ice-cold acetonitrile, incubated with acetonitrile at 4 °C for 1 h, and centrifuged at 4 °C, 7500 x g for 15 min. The washing step was repeated with acetone and acetonitrile, and the pellets were dried for 1 h at room temperature. Samples were resuspended in lysis buffer (7 M urea, 4 M thiourea, 4% (w/v) CHAPS, 0.6% (w/v) Biolyt and 1% (w/v) DTT) to achieve a final protein concentration of 1 mg/ml. One hundred and forty µl of sample was applied to a 7-cm IPG strip with a linear gradient of pH 4–7 (ReadyStripTM IPG Strips, Bio-Rad). Isoelectric focusing (IEF) was carried out on IPG strips using a Protean IEF cell system (Bio-Rad). Before the second dimension step, focused strips were equilibrated for 10 min in 1 ml of equilibration solution (0,375 mM Tris/HCl, pH 8.8, 6 M urea, 20% (v/v) glycerol, 2% (w/v) SDS, 0,005% BPB (bromophenol blue) and 2% (w/v) DTT (dithiothreitol), and then in the same buffer solution supplemented with 4% (w/v) IA (iodoacetamide) instead of DTT. The strips were then rinsed in Tris-glycine buffer (pH 8.5) and the second dimension was performed in a homogeneous 10% SDS-PAGE gel at 110 V for 1.5 h using a Mini-Protean Tetra cell system (Bio-Rad). The gels were fixed and stained with Bio-Safe Coomassie R-250 Staining Solution (Bio-Rad) and scanned using a GS-800™ Calibrated Imaging Densitometer (Bio-Rad). The protein spots were detected and their intensities quantified using PDQuest TM software (version 8.0.1. Bio-Rad). For statistical analysis, the two-tailed Student t-test ($p < 0.05$) was used to assess the statistical significance of changes in protein abundance.

Mass spectrometry and bioinformatics

Spots indicating significant differences between the used substrates ($p < 0.05$) were excised from the gels and processed according to Schevchenko et al. [36]. The excised spots were digested in solution containing NH_4HCO_3 (0.05 mol/l) and trypsin (0.02 mg/ml) at 37 °C for 16 h. Extracted peptides were purified using Stage Tips [37]. Extracted solutions were lyophilized and dissolved in 20 µl of 2% formic acid (v/v) for consequent nLC MS/MS analysis. Protein digests were analysed by a nano-liquid chromatography in a Proxeon Easy-nLC (Proxeon, Odense, Denmark) coupled to a MaXis quadrupole time-of-flight (Q-TOF) mass spectrometer (Bruker Daltonics, Bremen, Germany) according to Ošťádal et al. [38]. The samples of the peptide mixture were injected into an NS-AC-11-C18 Biosphere column (particle size 5 µm, length 150 mm, pore size: 12 nm, inner diameter 75 µm) with an NS-MP-10 Biosphere C18

precolumn (particle size 5 µm, length 20 mm, pore size: 12 nm, inner diameter 100 µm) (NanoSeparations, Netherlands).

Data were processed with the software ProteinScape v. 3.0.0.446 (Bruker Daltonics, Bremen, Germany). Proteins were identified by correlating tandem mass spectra to the extracted database for *Butyrivibrio fibrisolvens* from the NCBI database (downloaded on 26th February 2018; 33,312 proteins), using the MASCOT search engine v. 2.3.0 (http://www.matrixscience.com).

Trypsin was selected as the enzyme parameter. One missed cleavage was allowed, an initial peptide mass tolerance of ±10.0 ppm was used for MS and 0.05 Da for MS/MS analysis. Cysteines were assumed to be carbamidomethylated, proline and lysine to be hydroxylated, serine, threonine and tyrosine to be phosphorylated and methionine was allowed to be oxidized. All of these possible modifications were set to be variable. Monoisotopic peptide charge was set to 1+, 2+, and 3+. The Peptide Decoy option was selected during the data search process to remove false-positive results. The hits (MASCOT score ≥ 80, www.matrixscience.com) were accepted as significant. The metabolic pathways involving the observed enzymes were found using the website www.kegg.jp.

The isoelectric point of the proteins was estimated using the EMBOSS iep online tool (http://emboss.bioinformatics.nl/cgi-bin/emboss/iep). The molecular weight of the proteins was calculated using the online tool Protein Molecular Weight (http://www.bioinformatics.org/sms2/protein_mw.html). The carbohydrate metabolism proteins were identified using the Blast Koala online tool [28].

Results

Genome sequencing, theoretical proteome and enzyme assay

The genome of *B. fibrisolvens* 3071 encodes 4079 coding sequences. According to the KEGG annotation, the major group of genes belonged to the Environmental Information Processing group (13.7%), followed by Carbohydrate metabolism genes (12.8%) and genes of Protein families for genetic information processing (12.5%), Protein families for signalling and cellular processes (11.1%) and Genetic Information Processing (11.3%). The Carbohydrate metabolism group includes 99 distinct Glycoside Hydrolase (GHs), 8 Carbohydrate Esterase (CEs) and 48 Glycoside Transferase (GTs) genes (Fig. 1a). Resulting annotated genome files (Additional file 1: Table S1) are in the Supplementary data.

The theoretical 2DE map constructed on the basis of the sequencing data indicates that the proteome of *B. fibrisolvens* 3071 consists of 4035 proteins. There were 2941 proteins (73%) possessing a theoretical p*I* < 7, the majority of them ranging between pI 4 and 7, other 1095 proteins (27%) had theoretical p*I* > 7 and only 172 proteins (4%) had pI > 10. Ninety-three percent of the predicted proteins (3754) had predicted polypeptide masses between 10 and 150 kDa (Fig. 1b).

Seven genes of endo-β-1,4-xylanase (EC 3.2.1.8) and 8 genes of xylan 1,4-β-xylosidase (EC 3.2.1.37) were detected in the genome. To verify the extracellular xylanolytic potential of strain 3071, the activities of xylanase, cleaving β-1,4- xylosidic linkages randomly within the xylan chain, and the activities of xylosidase, which removes successive xylose residues from the non-reducing termini, were investigated in the culture medium of *B. fibrisolvens* 3071 grown on four different substrates. Figure 2 shows that the production of both enzymes is dependent on the nature of the carbon source. Both enzymes were induced by the presence of xylan, especially xylanase, while xylosidase was also induced by monomeric xylose.

SDS PAGE, 2-D electrophoresis and protein characterization

SDS-PAGE analysis of the extracellular protein pattern of *B. fibrisolvens* 3071 grown on four different carbon sources revealed the major protein bands, with a higher intensity of estimated masses from 40 to 80 kDa when grown on xylan and a combination of xylan and glucose. Several protein bands of 58 to 80 kDa were visible for the strain cultivated on xylan and xylan+glucose, while these fragments were absent for cultivation on glucose and xylose. The zymogram shows differences in xylanase isoenzymes, in the range from 32 to 130 kDa, when xylan and xylan+glucose were used as the substrate. Only weak bands at 70 and 58 kDa were detected on the zymogram for enzymes from the strain grown on glucose and xylose (Fig. 3).

Two-dimensional electrophoreses were carried out to identify the protein spot profiles of *B. fibrisolvens* 3071 grown on four different carbon sources. Substrates were compared to each other, amounting to six comparisons: (I) glucose/xylan, (II) glucose/xylan + glucose, (III) glucose/xylose, (IV) xylan/xylan + glucose, (V) xylan/xylose, (VI) xylan + glucose/xylose. Each comparison was performed in duplicate. From these eight 2-D electrophoresis gels, a total of 141 spots were excised and processed. Analysis of the nLC/MS protein peaks revealed that only 14 proteins (28 spots) fulfilled the MASCOT Score Criteria and were found as differentially expressed depending on the used carbon source, as shown in Fig. 4 and summarized in Table 1. These groups of proteins are involved in glycolysis, protein synthesis and butyrate synthesis. The identified proteins were functionally categorized as hydrolases (2), lyases (1), isomerases (1), transferases (3), oxidoreductases (2) and 3 proteins matched with Elongation factor Ts. The functions of these proteins and their involvement in the metabolic pathway are described in Table 2.

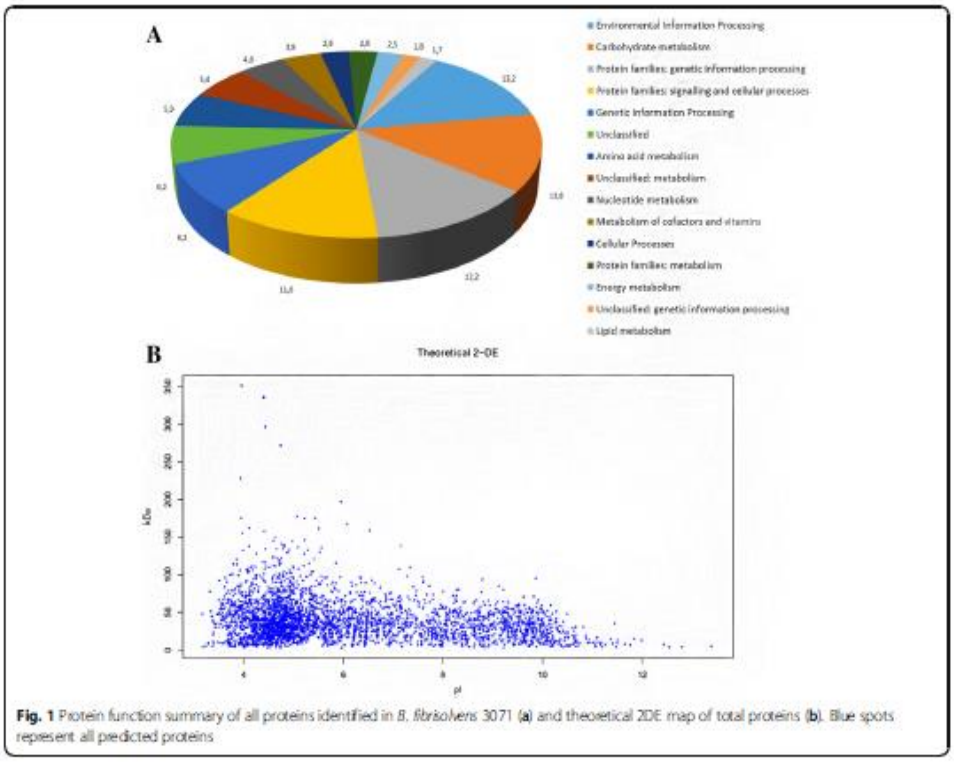

Fig. 1 Protein function summary of all proteins identified in *B. fibrisolvens* 3071 (**a**) and theoretical 2DE map of total proteins (**b**). Blue spots represent all predicted proteins

The enzymes involved in the process of degrading structural polysaccharides in the rumen were mapped using the KEGG database and the position and role of proteins of *B. fibrisolvens* 3071 in metabolic pathways were identified (Fig. 5).

Discussion

Butyrivibria are considered to be an integral part of the bovine rumen bacteriome associated with the host genetic background, thus forming inheritable microbiota [2]. The biggest advantage of these bacteria is in the

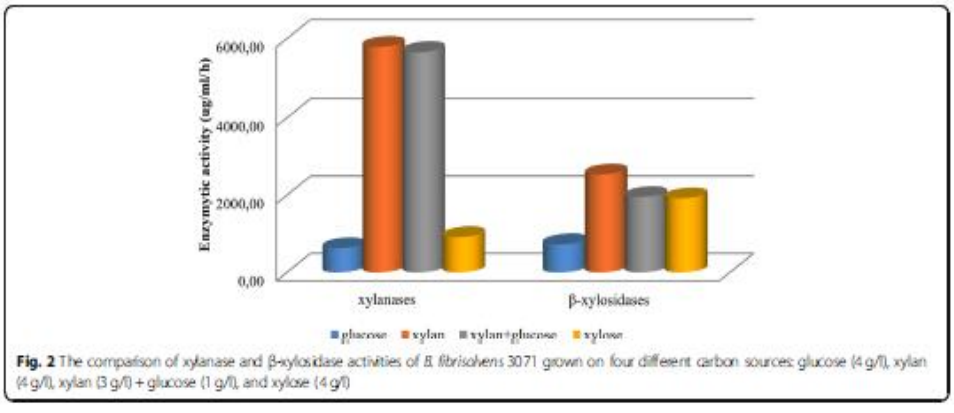

Fig. 2 The comparison of xylanase and β-xylosidase activities of *B. fibrisolvens* 3071 grown on four different carbon sources: glucose (4 g/l), xylan (4 g/l), xylan (3 g/l) + glucose (1 g/l), and xylose (4 g/l)

Table 1 List of *B. fibrisolvens* 3071 proteins identified and confirmed by nLC/MS as significantly different. The spots were excised from the 2-D electrophoresis gels comparing glucose/xylan (I), glucose/xylan + glucose (II), glucose/xylose (III), xylan/xylan + glucose (IV), xylan/xylose (V), xylan + glucose/xylose (VI)

Substrate comparison	Spot number	Accession number	Protein	Total number of peptides	Fold change	MW	pI
I	1a	SHI27642.1	diaminopimelate dehydrogenase	3	3.99 $\uparrow g^1$	36.2	5.5
II	2b	SHH89614.1	glucose-6-phosphate isomerase	18	0.61 $\uparrow x+g^3$	56.9	5.1
	3b	SHI23166.1	N-acetylmuramoyl-L-alanine amidase	6	0.44 $\uparrow x+g^3$	38.9	5.2
	4b	SHH52657.1	1-phosphofructokinase	9	0.61 $\uparrow x+g^3$	32.6	4.7
	5b	SER24636.1	phosphoglycerate kinase	6	2.02 $\uparrow g^1$	43.8	5.2
III	6c	BAD51424.1	beta-hydroxybutyryl-CoA dehydrogenase	7	2.29 $\uparrow g^1$	31.5	5.7
IV	7d	SHI29611.1	fructose-bisphosphate aldolase, class II	19	0.61 $\uparrow x+g^3$	33.9	5.2
V	8e	SHI15246.1	elongation factor Ts	13	0.81 $\uparrow xyl^4$	34.6	4.8
	9e	SHI83999.1	glyceraldehyde 3-phosphate dehydrogenase	8	0.88 $\uparrow xyl^4$	36.9	5.8
	10e	SHH82532.1	3-deoxy-D-arabinoheptulosonate-7-phosphate synthase	2	0.69 $\uparrow x^2$	38.5	6.0
	11e	SHI15246.1	elongation factor Ts	13	0.93 $\uparrow x^2$	34.6	4.8
VI	12f	SER24636.1	phosphoglycerate kinase	2	2.22 $\uparrow x+g^3$	46.8	5.2
	13f	SER75936.1	fructose-bisphosphate aldolase, class II	7	1.04 $\uparrow x+g^3$	33.9	5.2
	14f	SHI15246.1	elongation factor Ts	2	0.62 $\uparrow xyl^4$	34.6	4.8

Superscript 1, 2, 3, and 4 indicate glucose, xylan, xylan and glucose, and xylose, respectively

relatively broad spectrum of utilizable substrates and especially important is their xylanolytic activity [12]. The substrate flexibility of butyrivibria species was clearly documented by a study of the *B. proteoclasticus* glycobiome which covered a wide range of degrading and transporting proteins for different structural and storage polysaccharides [23], as well as a wide spectrum of oligosaccharides [24]. Due to the mobility of *Butyrivibria*, mediated by flagella, these strains also represent the most rapid colonizers of solid substrates in the rumen [4].

The sizes of genomes of *B. fibrisolvens* species sequenced to date range from 4462 up to 5121 kb. This corresponds well with our results, and shows that *B. fibrisolvens* as well as *B. proteoclasticus* [22, 49] have bigger genomes than other *Butyrivibria* and *Pseudobutyrivibria*. Their genomes have been determined in the range from 2100 to 4404 kb. In the *B. fibrisolvens* 3071 genome ten different endo-1,4-β-xylanase genes or precursors, and eight 1,4-β-xylosidase genes have been identified. The enzyme activity measurement and zymogram analysis confirmed that xylanase isoenzymes were in the extracellular fraction and were induced by xylan, even in the presence of a minor amount of glucose. These results are in agreement with the studies [50–53], however Sewell et al. [11] observed that xylosidase activity was mainly cell-associated and cellular xylanase activity rapidly declined in the presence of a small amount of glucose.

This study however mainly aimed to examine changes in the extracellular protein expression of *B. fibrisolvens* 3071 when this xylanolytic organism was grown on a variety of bioenergy-relevant substrates in order to identify the proteins responsible for substrate-specific breakdown and/or utilization. The substrates chosen ranged from simple (monomeric) to complex (polymeric), and varied in their general composition (C5 or C6 sugars). Proteomic analysis resulted in the highest number of spots when xylan was used as a substrate. Nearly all proteins were located in the central pI region. Comparison of proteome derived from simple sugars (glucose, xylose) exhibited a higher fold change in the proteins in the strain cultured on glucose, which indicates a tendency for a preferred sugar, which in the rumen is generally glucose, released by the hydrolysis of polysaccharides [18]. Xylose is not as efficiently utilized as glucose, which is caused by the different metabolic pathways of pentose and hexoses [54]. Pentose sugar represents about one-third of the total carbohydrate monomers in lignocellulosic biomass, which is a mixture of carbohydrate polymers (mainly cellulose and hemicellulose) and lignin [55]. The model of hemicellulose degradation by *Butyrivibria* was suggested by Palevich et al. [56] showing that the unsubstituted xylan chain is mainly degraded to xylose, which is transferred into the bacterial cell via xylose ABC transporters. Substituted xylo-oligosaccharides are transported by other ABC transporters and degraded by specific glycosidases in the cell cytoplasm.

In contrast to the results of Dunne et al. [24], who described debranching arabinofuranosidases, esterases, endo-β-1,4-xylanase and β-xylosidase enzymes in intracellular proteome *Butyrivibrio proteoclasticus* cultures, we did not identify the xylanolytic enzymes as significantly

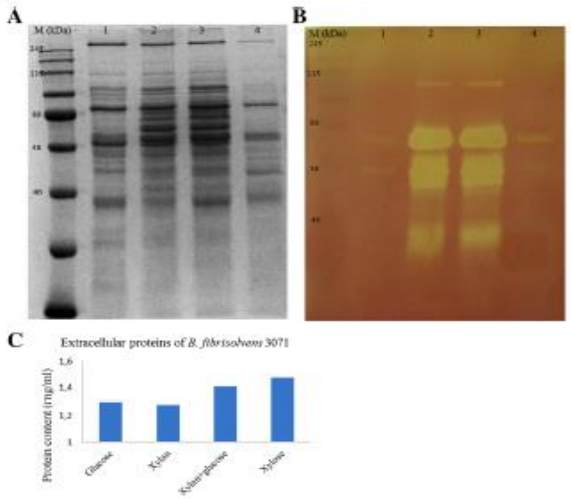

Fig. 3 SDS PAGE (**a**) and zymogram (**b**) of extracellular proteins of *B. fibrisolvens* 3071. Strain was grown on the following carbon sources: 1 - glucose, 2 - xylan, 3– xylan and glucose, 4 – xylose. The MW protein standard was in the range 11–245 kDa. The protein concentrations in samples loaded onto both gels above is illustrated by blue columns (**c**)

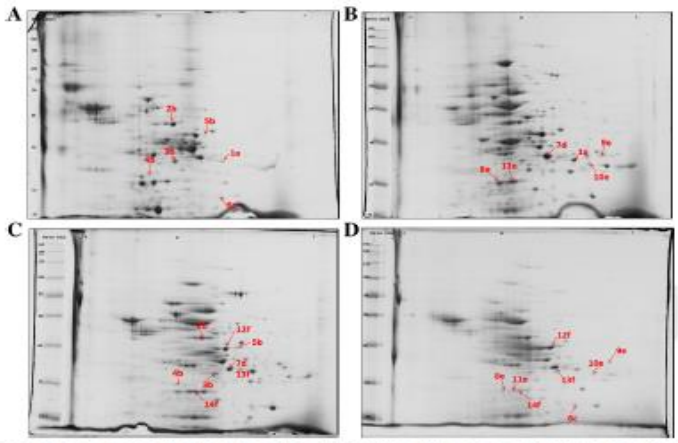

Fig. 4 Two-dimensional gel electrophoresis of extracellular proteins of *B. fibrisolvens* 3071 grown on different substrates. The gels (**a**, **b**, **c**, **d**) show spots from cultivation on glucose, xylan, xylan + glucose, and xylose, respectively. The letters a, b, c, d, e, f indicate the identical spots, however with significantly different intensity, in the comparison of *B. fibrisolvens* 3071 grown on glucose/xylan, glucose/xylan + glucose, glucose/xylan + glucose, xylan/xylan+glucose, xylan/xylose, xylan+glucose/xylose, respectively. Annotations correspond to spot numbers in Table 1. The MW protein standard was in the range 11–245 kDa

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Table 2 Classification and metabolic functions of proteins detected in this study. Proteins in italic are involved in glycolysis

Name	Enzyme Nomenclature	Class	Metabolic pathway (KEGG)	Function	Reference
diaminopimelate dehydrogenase	3.5.1.28	hydrolases	lysine biosynthesis, Metabolic pathway, Biosynthesis of secondary metabolisms, Biosynthesis of amino adds	deaves the amide bond between N-acetylmuramoyl and L-amino adds in bacterial cell walls	Gao et al., 2017 [39]
glucose-6-phosphate isomerase	5.3.1.9	isomerases	glycolysis/Gluconeogenesis, Pentose phosphate pathway, Starch and sucrose metabolisms, Amino sugar and nucleotide sugar metabolisms, Metabolic pathway, Biosynthesis of secondary metabolisms, Microbial metabolism in diverse environments, Biosynthesis antibiotics, Carbon metabolisms	catalyses the conversion of D-glucose 6-phosphate to D-fructose 6-phosphate	Verhees et al., 2001 [40]
N-acetylmuramoyl-L-alanine amidase	3.5.1.28	hydrolases	cationic antimicrobial peptide CAMP (resistance)	deaves the amide bond between N-acetylmuramoyl and L-amino adds in bacterial cell walls (prefeentially D-lactyl-L-Ala)	Loessner et al., 1995 [41]
1-phosphafructokinase	2.7.2.56	transferases	glycolysis/Gluconeogenesis, Metabolic pathway Biosynthesis of secondary metabolites, Microbial metabolism in diverse environments, Biosynthesis of antibiotics, Carbon metabolism, Biosynthesis of amino acids	catalysed the transfer of a phosphoryl group from adenosinetriphosphate to fructuose-6-phosphate to obtain adenosinetriphosphate and fructose-1,6-bisphophate	Kimmel et al., 2000 [42]
1-phosphoglycerate kinase	2.7.1.56	transferases	glycolysis/Gluconeogenesis, Metabolic pathway Biosynthesis of secondary metabolites, Microbial metabolism in diverse environments, Biosynthesis of antibiotics, Carbon metabolism, Biosynthesis of amino acids	conversion of 1,3-bisphosphate glycerate to 3-phosphoglycerate	Yon et al., 1990 [43]
beta-hydroxybutyryl-CoA dehydrogenase	1.1.1.157	oxidoreductases	–	catalyses the third step in the β-oxidation cycle, production of butyrate	Taylor et al., 2010 [44]
fructose-bisphosphate aldolase, class II	4.1.2.13	lyases	glycolysis/Glycogenesis, Pentose phosphate pathway, Fructose and mannose metabolism, Methane metabolism, Metabolic pathway, Biosynthesis of secondary metabolites, Microbial metabolism in diverse environments, Biosynthesis of antibiotics, Carbon metabolism, Biosynthesis of amino acids	catalyses the reversible aldol cleavage or condensation of fructose-1,6-bisphosphate into dihydroxyacetone-phosphate and glyceraldehyde 3-phosphate	Thomson et al., 1998 [45]
elongation factor Ts	–	–	–	catalyses the enzymatic binding of aminoacylt-RNA to ribosomes	Schwartzbach and Spremulli, 1989 [46]
glyceraldehyde-3-phosphate dehydrogenase	1.2.1.12	oxidoreductases	–	responsible for the interconversion of 1,3-diphosphoglycerate and glycealdehyde-3-phosphate, a central step in glycolysis and gluconeogenesis. Forms exist which utilise NAD (EC:1.2.1.12), NADP (EC:1.2.1.13) or either (EC:1.2.1.59)	Fillinger et al., 2000 [47]
3-deoxy-D-arabinoheptulosonate-7-phosphate synthase	2.5.1.54	transferases	phenylalanine, tyrosine and tryptophan biosynthesis, Metabolic pathway, Biosynthesis of secondary metabolites, Biosynthesis of antibiotics, Biosynthesis of amino acids, Quorum sensing	catalyses the metabolic reactions called the shikimate pathway responsible for the biosynthesis of the amino acids phenylalanine, tyrosine, and tryptophan	Hermann, 2001 [48]

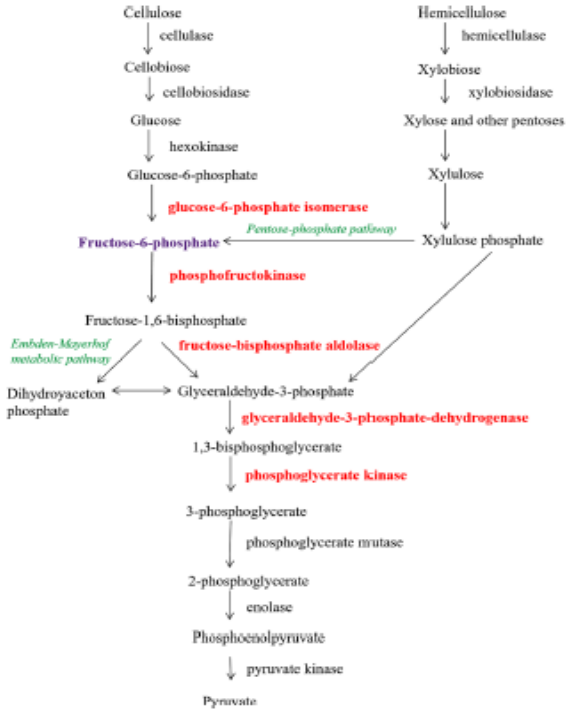

Fig. 5 Scheme of metabolic pathway of decomposition of plant carbohydrates by *B. fibrisolvens* 3071. Red colour indicates enzymes detected in this study as spots with significantly different intensity depending on the carbon source

different with the carbon source used in this study. However, the design of our experiment was differed from study of Dunne et al. [24]. We did not study the whole proteome, but we aimed to compare the proteins which were differentially produced by *B. fibrisolvens* 3071 cultivated on different carbon sources.

In our work a significant regulation was observed in the extracellular fraction of groups of proteins involved in glycolysis and protein synthesis. The proteins affected in our analysis are the same as identified by Snelling and Wallace [21], who explored the ruminal digesta proteome of cattle, sheep and reindeer. These authors identified the glyceraldehyde-3-phosphate dehydrogenase, phosphoenolpyruvate carboxykinase, phosphoglycerate kinase and triosephosphate isomerase as a dominant enzymes of ruminal bacterial proteome. Two of these enzymes were also detected in our work as differentially expressed depending on carbon source. We also detected enzymes fructose-1,6-bisphosphate aldolase, glucose-6-phosphate isomerase involved in glycolysis. Snelling and Wallace [21] detected the beta-hydroxybutyryl-CoA dehydrogenase of *B. fibrisolvens* as the abundant prokaryotic protein in the rumen. These results support the high importance of *B. fibrisolvens* in the rumen ecosystem in spite of its low number [57]. The proteins involved in glycolysis, protein synthesis and carbohydrate metabolism were identified as the predominant pathways also by Hart et al. [58], who moreover also described the protein family containing the elongation factor Tu as being the most highly abundant in the bovine rumen ecosystem. Upregulated proteins of central carbon catabolic pathways have also been described by *Ruminiclostridium cellulolyticum* cultivated on different sources of fibre [59]. We find very interesting that proteomic studies performed on rumen fluid of different animals [21, 58] brought the same results as our work studying one pure bacterial strain. In our opinion this can indicate that the proteins of

glycolysis, which provides sources energy to host animal, play a very crucial role and are essential for the metabolism of ruminants.

Conclusion

This study was focused on the effect of substrates (glucose, xylan, xylan and glucose and xylose) on the expression of extracellular proteins. In *B. fibrisolvens* 3071 cultures, both extracellular β-endoxylanase activity and xylan β-xylosidase were induced by xylan. Xylan induced several hemicellulolytic isoenzymes in the interval from 32 to 130 kDa.

Proteomic analysis comparing complex and simple carbon sources revealed only limited numbers of significantly differently expressed proteins. These proteins are involved in glycolysis, protein synthesis and butyrate synthesis. Such a result was unexpected but not exceptional. Higher levels of the enzymes from the central carbon catabolic pathways were obtained in a bacterial monoculture of *Ruminiclostridium cellulolyticum* [59], as well as in the whole rumen microbial community [21, 58]. This is the first proteomic study of *B. fibrisolvens* 3071 indicating that this strain can play the important role for the central rumen catabolic metabolism.

Additional files

Additional file 1: Table S1. Genome of *Butyrivibrio fibrisolvens* 3071 sequenced by PacBio annotated with Blast Koala (KEGG). (DOCX 15 kb)

Additional file 2: Table S2. Results of statistic evaluation (Student's test) and six pair-wise comparisons of 2D gels separating proteins of B. fibrisolvens 3071 grown on four different substrates (I – glucose, II - xylan, III xylan+glucose, 4 – xylose). **Figure S1.** 2D gel separation of proteins of *B.fibrisolvens* 3071 cultivated on glucose (I). **Figure S2.** 2D gel separation of proteins of *B.fibrisolvens* 3071 cultivated on xylan (II). **Figure S3.** 2D gel separation of proteins of *B.fibrisolvens* 3071 cultivated on glucose (I). **Figure S4.** 2D gel separation of proteins of *B.fibrisolvens* 3071 cultivation on xylan+glucose (III). **Figure S5.** 2D gel separation of proteins of *B.fibrisolvens* 3071 cultivated on glucose (I). **Figure S6.** 2D gel separation of proteins of *B.fibrisolvens* 3071 cultivated on xylose (IV). **Figure S7.** 2D gel separation of proteins of *B.fibrisolvens* 3071 cultivated on xylan (II). **Figure S8.** 2D gel separation of proteins of *B.fibrisolvens* 3071 on xylan+glucose (III). **Figure S9.** 2D gel separation of proteins of *B.fibrisolvens* 3071 cultivated on xylan (I). **Figure S10.** 2D gel separation of proteins of *B.fibrisolvens* 3071 cultivate on xylose (IV). **Figure S11.** 2D gel separation of proteins of *B.fibrisolvens* 3071 cultivated on xylan+glucose (III). **Figure S12.** 2D gel separation of proteins of *B.fibrisolvens* 3071 cultivated on xylose (IV). (DOCX 2222 kb)

Abbreviations

2D: Two Dimensional; 2DE: Two dimensional Electrophoresis; BPB: Bromophenol Blue; BSA: Bovine Serum Albumin; C5 sugar: Five Carbon Sugar; C6 sugar: Six Carbon Sugar; CCR: Carbon Catabolic Repression; CE: Carbohydrate Esterase; CHAPS: 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate; CM: Carboxymethyl; DSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen; DTT: Dithiothreitol; EC Number: Enzyme Commission Number; GH: Glycoside Hydrolase; GT: Glycoside Transferase; IA: Iodoacetamide; IEF: Isoelectric Focusing; IPG: Immobilized pH gradient; MS: Mass Spectrometry; MS/MS: Tandem Mass Spectrometry; MW: Molecular weight; nLC: Nano Liquid Chromatography; PAHBAH: 4-hydroxybenzoic acid hydrazide; PES: Polyethersulfone; *p*-NPX: *p*-Nitrophenyl-β-D-xylopyranoside; SDS-PAGE: Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis; TGPA: Thermophilic Gram Positive Anaerobes; UK: United Kingdom

Acknowledgements

The authors acknowledge Ing. Statis Pataridis and Adam Eckhardt, Ph.D. (Institute of Physiology, CAS, v.v.i.) for their help and assistance with nLC/MS.

Funding

The authors would like to acknowledge funding support from the Czech Science Foundation (16-12431S) and the EU FP7 project RUMINOMICS (No. 289319).
The study was supported by Charles University in Prague, project GA UK No. 322216. This research was carried out within the framework of Specific University Research (SVV260440).
This study was supported by Project Excellence (No. CZ.02.1.01/0.0/0.0/15_003/0000460).
We gratefully acknowledge the support of this work by institutional support RVO 86652036.

Availability of data and materials

We are ready to share our data.

Authors' contributions

HS experiment setup, data interpretation, manuscript writing; JK evaluation of enzyme activities, genome sequencing and manuscript writing; KF major contributor in writing the manuscript; LK participated in 2-D electrophoresis and proteomic techniques; MT proteomic consulting; DM bioinformatics analysis; JM genomics and sequencing. All authors read and approved the final manuscript.

Ethics approval and consent to participate

B. fibrisolvens 3071 was provided by the Collection of Microorganisms of the Institute of Animal Physiology and Genetics CAS, Prague, Czech Republic.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Publisher's Note

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Received: 21 November 2018 Accepted: 15 May 2019
Published online: 01 June 2019

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Příloha 5

PUBLIKACE V

Novel Contribution to Clubfoot Pathogenesis: The Possible Role of Extracellular Matrix Proteins

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Journal of Orthopaedic Research, 37:769-778 (2019).

Novel Contribution to Clubfoot Pathogenesis: The Possible Role of Extracellular Matrix Proteins

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Received 27 September 2018; accepted 11 December 2018
Published online 7 January 2019 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/jor.24211

ABSTRACT: Idiopathic *pes equinovarus* (clubfoot) is a congenital deformity of the feet and lower legs. Clubfoot belongs to a group of fibro-proliferative disorders but its origin remains unknown. Our study aimed to achieve the first complex proteomic comparison of clubfoot contracted tissue of the foot (medial side; $n = 16$), with non-contracted tissue (lateral side; $n = 13$). We used label-free mass spectrometry quantification and immunohistochemistry. Seven proteins were observed to be significantly upregulated in the medial side (asporin, collagen type III, V, and VI, versican, tenascin-C, and transforming growth factor beta induced protein) and four in the lateral side (collagen types XII and XIV, fibromodulin, and cartilage intermediate layer protein 2) of the clubfoot. Comparison of control samples from cadavers brought only two different protein concentrations (collagen types I and VI). We also revealed pathological calcification and intracellular positivity of transforming growth factor beta only in the contracted tissue of clubfoot. Most of the 11 differently expressed proteins are strongly related to the extracellular matrix architecture and we assume that they may play specific roles in the pathogenesis of this deformity. These proteins seem to be promising targets for future investigations and treatment of this disease. © 2019 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 37:769–778, 2019.

Keywords: clubfoot; fibrosis; extracellular matrix; proteomics; collagen

Idiopathic *pes equinovarus* (clubfoot) belongs to a group of fibroproliferative disorders involving the musculoskeletal system and its origin remains unknown. This defect occurs approximately once per 1000 births.[1] The guideline on the diagnosis and treatment of this disease was recently published by Besselaar et al.[2] These methods, although successful, are of a long-term nature and very demanding for the child patients and their parents alike because a number of plasters need to be applied starting at a very young age.

To explain the aetiology of clubfoot, a number of hypotheses have been proposed: Vascular insufficiencies in the talus,[3] environmental factors, abnormal muscle insertions,[4] and genetics.[5] Recently, it was agreed that clubfoot is probably multifactorial in origin and thus the aetiology of the disease is still not clear.

Ostadal et al.[6] reported a significant difference between the evaluation of the short-term and long-term results of the Ponseti method treatment of clubfoot. The number of relapses indicated for surgical intervention significantly increases with the time of follow up. The surgery of relapsed clubfoot has relieved that the structure of the connective tissues macroscopically differs: The medial part of the tarsus (a disc-like mass of fibrotic tissue localized between the *medial malleolus*, *sustentaculum tali*, and navicular bone) is much more rigid than the lateral part.[7] This observation supports the hypothesis that extracellular matrix (ECM) proteins, particularly fibroblasts, and growth factors are involved in the pathogenetic mechanisms responsible for the development of clubfoot,[8] and we started our research with these assumptions. In our previous proteomic study of the medial (contracted) side clubfoot tissue, we identified 18 ECM proteins.[9] The study presented here is the logical continuation of that research. This time we compared the protein composition of the medial (contracted) and lateral sides of clubfoot tissue together with corresponding tissues obtained from healthy adult cadaver controls, by means of label-free mass spectrometry. This method allowed us to compare the most abundant proteins (about 70) in these tissues for the first time. These analyses are necessary for determination of clubfoot biomarkers, better understanding of their roles in the pathological processes, and for their possible future therapeutic use.

[Correction added on March 15, 2019, after first online publication: Affiliations updated].
Conflict of Interest: The authors declare no conflict of interest.
All procedures have been performed in accordance with the ethical standards in the 1964 Declaration of Helsinki.
Institutional approval for the present study was obtained from the ethical committee of the Institute of Physiology of the Czech Academy of Sciences, v.v.i., and the parents of all patients provided written, informed consent to participate.
Grant sponsor: The Ministry of Health Department Program for Research and Development; Grant number: 17-31564A; Grant sponsor: Charles University in Prague, projects GA UK; Grant number: n. 336218; Grant sponsor: Charles University in Prague; Grant numbers: RVO:67985823, n. SVV 260440; Grant sponsor: The KZCR, a.s., Czech Republic, IGP KZ; Grant number: n. 217116002.
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METHODS

This study is analytical, prospective, level of evidence IIB.

Biological Material

A total of 13 patients (9 boys, 4 girls, mean age 58.4 months (SD = 20.2); details in Supplement Table S1) with idiopathic clubfoot were treated by the Ponseti method at the Department of Orthopaedics, Bulovka Hospital in Prague (The Czech Republic). Treatment consisted of gentle manipulation of the foot and the application of 5 to 15 (average 8) long-leg plaster casts (toes to thigh) as described by Ponseti et al.[10] Tissue samples were obtained from patients undergoing surgery for relapsed clubfeet: Contracted tissue was obtained between the ***medial*** malleolus, *sustentaculum tali*, and navicular bone (contracted side, "disc-like tissue"—**M-side**); non-contracted tissue was obtained from the **lateral** surface of the calcaneocuboid joint (non-contracted side—**L-side**) (Figure 1).[8,11] Tissue samples were taken from the feet of the aforementioned 13 patients. In three cases, samples from patients' both feet were taken, but an insufficient amount of tissue was obtained from the L-side, resulting in an uneven number of samples ($n = 16$ for M-side, $n = 13$ for L-side) (Supplement Table S1). The samples (ca 0.3 cm^3) were stored frozen at −80°C.

Control samples for mass spectrometry were taken from 10 human cadavers (5 male, 5 female; age 70 ± 10 years) in the Department of Pathology, the Municipal Hospital Litomerice, (The Czech Republic). The cause of death was not associated with any bone or joint disorder, metabolic disease (with the exception of diabetes) or systemic inflammation. No evidence of such diseases was documented during post-mortem pathological examination and autopsy.[12] The routine autopsies were performed less than 24 h after death. Tissue samples were obtained from the deltoid ligament of the talocalcaneal joint (M-side) and from the lateral surface of the calcaneocuboid joint (L-side). The samples (ca 0.3 cm^3) were stored frozen at −80°C.

Institutional approval for the present study was obtained (ethical committee), and the parents of all patients provided informed consent to participation. All procedures were performed in accordance with the 1964 Helsinki declaration and the laws of the Czech Republic.

Sample Preparation and Mass Spectrometry Quantification

All the obtained samples ($n = 16$ for M-side, $n = 13$ for L-side) were about 10 mg dry weight. Control samples (taken from cadavers) were also about 10 mg dry weight ($n = 10$ for M-side, $n = 10$ for L-side). All samples were processed as described in our previous study.[9] Sample purification was improved by extracting tryptic peptides using Stage Tips.[13] Nano-liquid chromatography procedure, mass spectrometry (MS), and tandem MS (MS/MS) analyses were performed as described in our previous studies,[14] with upgraded software. Our MS device (Maxis quadrupole time-of-flight mass spectrometer (Bruker Daltonics, Bremen, Germany)) allowed us to compare up to 100 most abundant proteins in two data sets using label-free quantification.

Comparisons of M-side to L-side samples were conducted separately in clubfoot patients and in cadaver controls. Samples within cadaver controls were compared to determine if there were any differences between M-side and L-side tissues in healthy feet.

Database searches were performed as described in Eckhardt et al. with the taxonomy restricted to *Homo sapiens*.[15] Only significant hits (MASCOT score ≥80 for proteins; ≥30 for peptides, http://www.matrixscience.com) were accepted.

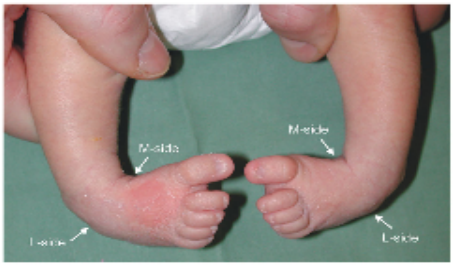

Figure 1. The medial (M-side) and the lateral (L-side) side of foot are marked with arrows.

Histopathological Investigation

Clubfoot samples from ten patients ($n = 10$ for M-side, $n = 10$ for L-side) were fixed with Baker's fluid and embedded in paraffin. Von Kossa staining was performed on longitudinal sections (5 μm) for basic histological assessment and detection of calcified structures. Subsequently, the sections were processed by immunohistochemistry (IHC). All special IHC reagents were purchased from Abcam, Cambridge, UK. Primary antibodies anti-collagen VI (ab6588, 1:200, 30 min at room temperature), anti-transforming growth factor beta (anti-TGFβ) (ab66043, 1:200, overnight at 4°C), and anti-transforming growth factor beta-induced protein (anti-TGFβip) (ab170874, 1:250, 1 h at room temperature) were used. Antigen retrieval was performed with the citrate buffer at 96°C for 15 min. Hydrogen peroxide block, protein block, secondary antibody reaction, and visualization were performed according to the Abcam protocol using the EXPOSE rabbit specific HRP/DAB detection IHC kit. Slides were counterstained with hematoxylin. Extracellular positivity of anti-collagen VI and anti-TGFβip IHC intensity were evaluated using an image analyzer (NIS Elements 3.0 AR, Laboratory Imaging Ltd., Prague, The Czech Republic) signal thresholding. Signal intensity from 10 independent parts of each sample was detected and the percentage of positive area was calculated.

Statistics

The software, Profile Analysis (version 2.1, Bruker Daltonik GmbH), was used to evaluate differences in the protein composition of the L-side and the M-side by means of label-free quantification. The peptides under consideration had to be found in at least 50% of all samples, regardless of the group, and they had to be found in at least one of the two groups (L or M) as well as in at least 50% of the group. The p-value returned by two-sample t-tests was corrected for multiple-testing by false discovery rate (FDR) based on frequency histogram (FDR adjusted p-value threshold 0.05).[16]

The IHC positive percentage area data from the L- and M-sides were checked for normality (Q-Q plot) and compared by two-sample t-test. The significance level to reject the null hypothesis was set to 0.05.[16]

RESULTS

MS Label-Free Quantification

Eleven differently expressed proteins were identified between the M-side and non-contracted L-side clubfoot tissue by MS label-free quantification. Seven proteins were significantly over-expressed in the M-side, and four proteins in the L-side of clubfoot (Table 1).

Apart from the aforementioned proteins, we detected a significantly higher concentration of three blood proteins in the M-side: Apolipoprotein A-I, hemoglobin subunit alpha, and hemoglobin subunit beta. These plasma proteins are not in direct connection with ECM and therefore are not relevant in the context of this study (data not shown).

In contrast, only two differently expressed proteins were identified between the M-side and the L-side tissue of control cadavers by MS label-free quantification. Both proteins were significantly over-expressed in the M-side (Table 2).

IHC Staining

Anti-Collagen VI IHC

The percentage of collagen VI positive area was significantly increased (p-value = 0.0417) in the M-side (Figure 2A) in comparison with the L-side (Figure 2B).

Anti-Transforming Growth Factor Beta (TGFβ) IHC

TGFβ intracellular positivity of fibroblast-like cells was observed in the M-side exclusively (Figure 3A). No intracellular positivity for TGFβ was observed in the L-side (Figure 3B).

Anti-Transforming Growth Factor Beta Induced Protein (TGFβip) IHC

TGFβip positivity was significantly higher (p-value = 0.0001) in the M-side (Figure 4A) as compared to low positivity in the L-side (Figure 4B).

Calcification Analysis

Calcified structures, which diffused along the collagen fibres of the tissue, were detected only in the M-side (Figure 5). No calcifications were detected in the L-side.

DISCUSSION

To date, only a few studies have compared the protein compositions of clubfoot tissue with healthy controls,[17,18] and some authors compared the tissue of the medial (contracted) and lateral (non-contracted) sides of clubfoot.[8,11] However, all these studies were concerned with only one or several proteins. Label-free mass spectrometry allowed us to compare the most abundant proteins (about 70) of both medial and lateral sides of the clubfoot as well as both sides of cadaveric control tissue for the first time in one study.

The Selection of a proper control for the clubfoot samples presented an ethical dilemma, however. Getting a necessary parental consent for children participation in research is extremely hard to accomplish, so obtaining control samples from healthy pediatric donors proved to be nearly impossible. This also applies to pediatric cadavers. Moreover, such samples are frequently affected by a long-term medical treat-

Table 1. List of Proteins With Significantly Different Concentrations Between the Medial (M-Side; Contracted) and Lateral (L-Side; Non-Contracted) of Clubfoot Patients

Accession number	Protein	Total number of peptides	Number of signif. different peptides	$p<$	FDR adjusted p-value	M/L (fold)
	Upregulated in M-side					
P12111	Collagen VI alpha-3 chain	89	30	0.0001	0.0004	1.246
P12109	Collagen VI alpha-1 chain	20	10	0.0001	0.0004	1.468
P12110	CollagenVI alpha-2 chain	23	7	0.0001	0.0004	1.236
P20908	Collagen V alpha-1 chain	6	6	0.0001	0.0004	1.587
P02461	Collagen III alpha-1 chain	83	9	0.0001	0.0004	1.152
P13611	Versican core protein	3	0	0.0030	0.0084	1.522
Q9BXN1	Asporin protein	6	2	0.0030	0.0084	1.401
Q15582	TGFBI Transforming growth factor-beta-induced protein	10	3	0.0040	0.0102	1.418
P24821	Tenascin-C	8	1	0.0180	0.0388	1.240
	Upregulated in L-side					
Q05707	Collagen XIV alpha-1 chain	23	19	0.0001	0.0004	0.417
Q06828	Fibromodulin	14	1	0.0001	0.0004	0.813
Q99715	Collagen XII alpha-1 chain	13	8	0.0005	0.0018	0.669
Q8IUL8	CILP-2 Cartilage intermediate layer protein 2	7	1	0.0080	0.0187	0.630

Proteins with significantly different concentrations between the medial (M-side) and lateral (L-side) are lined up by their "p" (significance). The total number of peptides indicates the number of successfully compared tryptic peptides by label-free MS detected at least in 50% of all samples, specific accession numbers of proteins are used from the UNIPROT database (www.uniprot.org) and the M/L (fold) symbolized value of protein concentration fold change (M-side/L-side).

Table 2. List of Proteins with Significantly Different Concentrations Between the Medial (M-side) and Lateral (L-side) of Controls (Cadavers Without Clubfoot)

Accession number	Protein	Total number of peptides	Number of signif. different peptides	$p<$	FDR adjusted p-value	M/L (fold)
	Upregulated in M-side					
P02452	Collagen I alpha-1 chain	177	10	0.0001	0.0007	1.154
A0A087WTA8	Collagen I alpha-2 chain	124	14	0.0001	0.0007	1.152
P12111	Collagen VI alpha-3 chain	63	12	0.0001	0.0007	1.426
P12110	Collagen VI alpha-2 chain	19	4	0.0005	0.025	1.926
P12109	Collagen VI alpha-1 chain	24	5	0.0083	0.033	1.619
	Upregulated in L-side					
	None					

Proteins with significantly different concentrations between the medial (M-side) and lateral (L-side) are lined up by their "p" (significance). The total number of peptides indicates the number of successfully compared tryptic peptides by label-free MS detected at least in 50% of all samples, specific accession numbers of proteins are used from the UNIPROT database (www.uniprot.org) and the M/L (fold) symbolized value of protein concentration fold change (M-side/L-side).

ment applied to children in intensive care units before their death. Therefore, we had to use more accessible adult cadavers as controls.

The examination of the ECM composition of contracted tissues is important in the context of better understanding the fibrotic processes, in clubfoot as well. The major original findings of our study are (i) the significant quantitative differences of protein compositions between contracted (M-side) and non-contracted (L-side) clubfoot tissues; (ii) pathological calcifications located only in the M-side tissue; and (iii) different intracellular concentrations of TGFβ in fibroblast-like cells from different sides of the clubfoot (M-side, L-side).

We are considering our research as an effort to deepen and confirm the previous findings, as well as a continuation thereof. We have brought new proofs that comparison of clubfoot samples from the M- and L-side are an appropriate model for studying this disease. The calcifications detected in the M-side (but not in the L-side) are clear signs of a pathological condition of that tissue, since there is no soft tissue which would contain such calcifications in physiological conditions.[19] Our other finding shows different intracellular concentrations of TGFβ, which for the first time prove different state and/or behavior of fibroblast-like cells in clubfoot M- and L-side. As in another fibroproliferative pathologies (Dupuytren's disease, Peyronie's disease), TGFβ is regarded to be a key stimulator of fibroblast-like cells activity. This stimulation pathway leads to production of excessive levels of extracellular matrix proteins.[20,21]

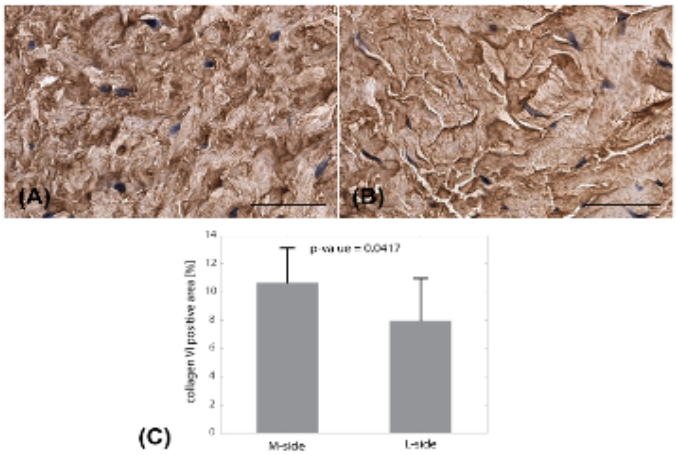

Figure 2. Immunohistochemical staining by anti-collagen-VI antibody detected a positive area in the M-side (A), and the L-side (B) (difference in intensity of brown colour areas, bar = 20 μm). The significant difference in percentages of collagen VI positive area in the M-side and the L-side are described in graph (C); $p < 0.0417$.

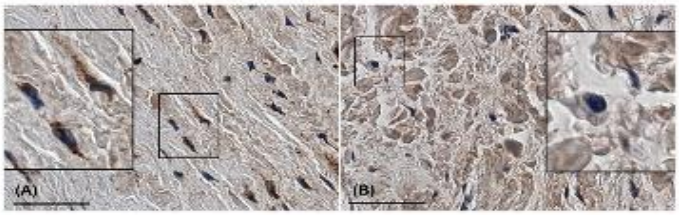

Figure 3. The staining by anti-TGFβ IHC detected (A) significant intracellular positivity in cytoplasm of fibroblast-like cells in the M-side, in comparison with (B) no intracellular positivity in cytoplasm of fibroblast-like cells in the L-side (bar = 20 μm).

Whereas the quality of protein compositions of both clubfoot sides was comparable, the quantity of 11 proteins differs. The concentrations of seven proteins were significantly increased in the M-side and four in the L-side (Table 1). Control samples had only two protein upregulations in the M-side and none protein was detected as upregulated in the L-side (Table 2). The only protein upregulated in both M-side of clubfoot patients and M-side of cadaver controls was collagen VI.

Proteins With Higher Concentration in the M-Side

The presented study has revealed seven ECM related proteins upregulated in the M-side (Table 1) (Figures 2A-C, 4A-C).

Collagen III

Observed over-expression of collagen III confirmed the results of several groups[8,11,17] which showed the protein significantly increased in the M-side (Figure 6). This observation also correlates with the higher ratio of collagen III to/collagen I in wound repair.[22] Poon et al. state that collagen III is more abundant in contracted tissue (also in Dupuytren's disease) due to elevated beta-catenin signaling pathway.[11]

Collagen V and Collagen VI

Collagen V and collagen VI are significantly upregulated in the M-side of clubfoot (Table 1), and moreover upregulated collagen VI was proved by IHC, too (Figure 2A–C). Type V collagen is particularly abundant in vascular tissues and contributes to binding to other connective tissue cells.[23] This collagen type was detected as upregulated in human skin keloid formation.[24] The major function of collagen VI is probably as an anchoring meshwork connecting collagen fibers to the surrounding matrix, but the precise role of collagen VI in cartilage has not been clearly defined.[25] Veidal et al. suggested that collagen VI turnover is a central player in liver fibrogenesis.[26] Moreover, the C-

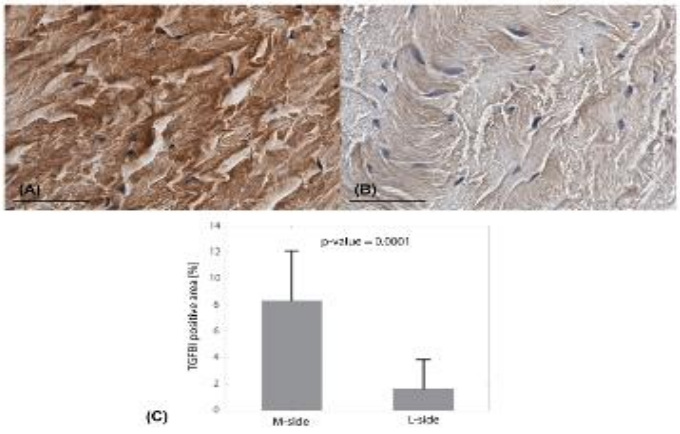

Figure 4. The staining by anti-TGFβip (TGFBI) IHC detected an increase in the percentage of TGFβip positive area in the M-side (A) in comparison with the L-side (B); (bar = 20 μm). The significant difference in percentages of TGFβip positive area in the M-side and the L-side are described in graph (C); $p < 0.0001$.

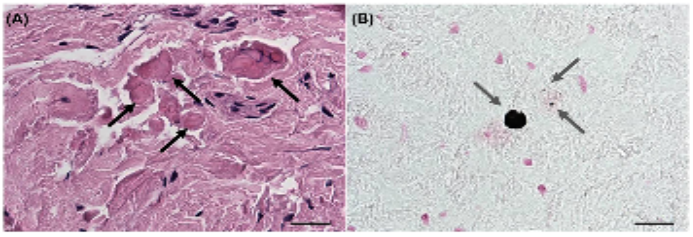

Figure 5. The staining detected calcium deposits in the M-side samples (arrows) (A—hematoxylin-eosin stain, black arrows; B—von Kossa stain, gray arrows; bar = 20 μm). No calcifications were detected in the L-side (picture not displayed).

terminate peptide of collagen VI chain alpha 3 (endotrophin) is known to promote phenotypic modulation and proliferation of cells.[27]

Both these types of collagens could be important molecules in clubfoot pathogenesis (Figure 6). However collagen VI was observed in significantly higher concentrations also in the M-side of controls (Table 2), therefore we do not know if its concentration in clubfoot M-side is higher naturally or due to clubfoot disease.

TGFβ and TGFβip

There is ample evidence that TGFβ signaling is a key regulator of myofibroblast biology in fibrotic tissues (reviewed in Piersma et al.[28]). TGFβ and platelet-derived growth factor (PDGF) were expressed at high levels in the contracted tissues of clubfoot. The blockade of TGFβ and PDGF in cells isolated from clubfoot led to decreased collagen expression and fibroblast proliferation.[8] In the presented study we did not detect TGFβ or PDGF by MS label-free quantification probably due to the low concentration of these growth factors (we were able to compare to ca 100 most abundant proteins due to our MS device).

TGFβip, also known as βig-H3, TGFBI and keratoepithelin, is a protein with the ability to bind collagen. TGFβip plays a role in the early development of cartilage (enhances the growth and adhesion of the pre-chondrogenic cells).[29] In addition, Lorda-Diez et al. documented that TGFβ may also function as a profibrogenic factor for embryonic limb mesoderm. They

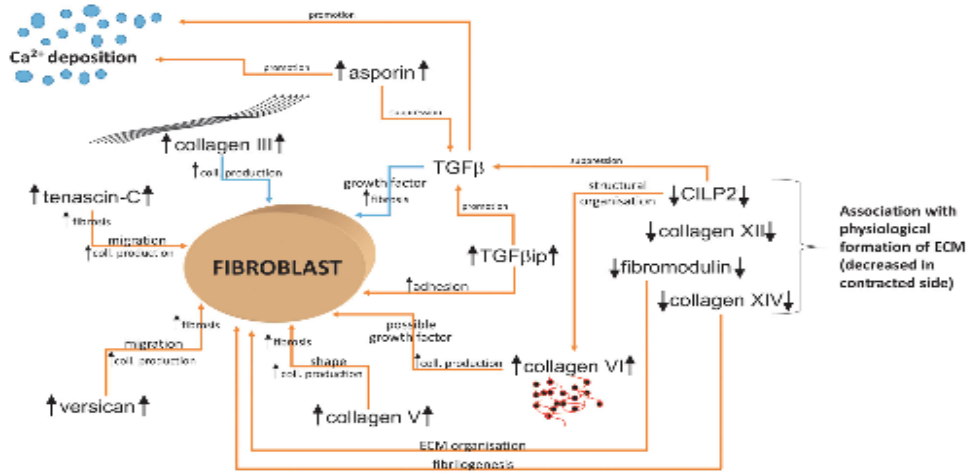

Figure 6. Doubled arrows express upregulation (↑protein↑) or downregulation (↓protein↓) of proteins to the M-side. All upregulated ECM proteins have some relationship to fibroblasts. Some upregulated proteins are implicated in fibrosis (↑fibrosis) and/or take part in migration (migration) and/or enhanced collagen production (↑coll. production) in dermal fibroblasts as reviewed in Tracy et al.[31] Orange arrows designate predicted interactions based on the upregulated proteins in the contracted side. The upregulation of these proteins was detected in this type of fibrotic tissue for the first time. Already described possible pathways are expressed in blue lines. All observed downregulated proteins associate with the physiological formation of ECM.

identified TGFβip as a factor downstream to TGFβ signaling (regulated by Smad 2 and 3), which is highly expressed in joint capsules and the differentiating tendons. Their findings indicated that TGFβip promotes the fibrogenic influence of TGFβ signaling in the pro-fibrogenic pathway.[30]

We observed intracellular presence of TGFβ, detected by IHC in fibroblast-like cells of the M-side (and no intracellular TGFβ in the L-side) (Figure 3A and B). This result suggested that fibroblast-like cells are in a different state in the M-side in comparison with the L-side. We also detected a higher concentration of TGFβip by means of label-free MS (Table 1) and IHC (Figure 4A–C) in the M-side. Our observations suggest that both of these proteins play specific roles in clubfoot pathology (Figure 6). In addition, TGFβ has direct relationships to collagen calcification, and also to another two proteins significantly changed in the presented study—asporin and CILP2 (Figure 6).[31,32]

Versican

The effects of versican (a large fibroblast proteoglycan binding hyaluronic acid) to fibrosis (wound healing) has been reviewed by Tracy et al.[33] Compared to the non-contracted tissues, uterine fibroids and keloid scars contain higher relative amounts of versican.[34] These results correlate with our observation.

Asporin

This protein interacts with TGFβ, binds collagen, and may be one of the key proteins involved in the calcification of blood vessels, tumors, or osteoarthritis. The role of asporin in collagen fibrillogenesis and biomineralization was reviewed by Kalamajski.[35] Possible mechanisms for the involvement of asporin in osteoarthritis pathology are the inhibition of TGFβ function and induction of collagen mineralization.[33,36]

These observations, together with our results (upregulated asporin (Table 1) and the presence of histologically confirmed calcium deposits in the M-side (not in the L-side) (Figure 5)) strongly support the hypothesis that asporin is an important player in clubfoot pathogenesis (Figure 6).

Tenascin-C

This ECM glycoprotein is over-expressed in fibrotic disease, including keloids.[37] The effects of tenascin-C on fibrosis (wound healing) are reviewed in Tracy et al.[33] This protein also plays a central role in maintaining inflammation in arthritic diseases.[38] By in situ detection of protein and mRNA expression, Mikic et al. demonstrated that the morphological abnormalities that result from embryonic immobilization are associated with an altered expression of tenascin-C.[39] Tenascin-C also has an influence on fibroblast migration (Figure 6).[33,40]

Three Plasma Proteins

Apart from changes in concentrations of ECM-related proteins, we detected a significantly increasing expression of three blood proteins in the M-side of clubfoot—apolipoprotein and two globulins. We assume that this may be a consequence of tissue stiffness in the M-side, due to which the blood was enclosed inside the veins in this tissue and could not be completely washed away.

Proteins With Higher Concentration in the Non-Contracted Side (L-side) of Clubfoot

The presented study has revealed that four ECM related proteins were upregulated in the L-side (Table 1).

Collagen XII and Collagen XIV

Both these collagens are members of the FACIT collagens (Fibril Associated Collagens with Interrupted Triple helices). Collagen XII was over-expressed in human corneal scars[41] and could play a role in fibril organization and the stromal architecture.[42] Collagen XII is associated with collagen I fibrils in human tendons, and by analogy, to collagen XIV, in connective tissues enriched in collagen I.[43] Agarwal et al. supposed that anchoring plaques are interconnected to the interstitial network in the papillary dermis by the collagens XII and XIV.[44] Characterization of collagen XIV-deficient mouse tendons showed a shift toward large-diameter fibrils and, thus regulated fibrilogenesis.[45]

These results indicate that both these types of collagen are important for the fully functional (supra-) structure of ECM (Figure 6).

Fibromodulin

This protein regulates the fibrogenic response (ECM organization) to liver injury in mice.[46] An insufficient concentration of fibromodulin in the M-side could be a factor in ECM disorganization.

Cartilage Intermediate Layer Protein 2 (CILP2)

CILP2 mediates interactions between components of the articular cartilage matrix, and may be associated with collagen VI.[47] Seki et al. concluded that CILP suppresses TGFβ signaling and this regulation plays a crucial role in the aetiology of lumbal disc disease.[31,48] This hypothesis could also fit into the complex aetiology of clubfoot. Both observations, mentioned above,[47,48] support our results showing that CILP2 was decreased in the M-side of clubfoot tissue samples.

All four proteins over-expressed in the L-side (and downregulated in the M-side) have strong relationships to the ECM architecture, and we assume that their scarcity in the M-side could play an important role in the development of clubfoot disease (Figure 6).

Possible Novel Pathogenetic Mechanisms in Clubfoot

The presented study revealed significantly different concentrations of 11 proteins (between the M-side and the L-side of clubfoot), which are important for physiological development of the ECM. We assume

that these proteins may play a substantial role in clubfoot pathogenesis. For example, collagens XII and XIV which have been detected in significantly lower concentration in contracted tissue, probably represent a partial absence of the structural components of the ECM physiological structure (Figure 6). These collagens could play a role in fibril organization (type XIV also in fibrilogenesis[45]), and also it is supposed that they interconnect anchoring plaques to the interstitial network.[42–44] Collagens type XII and XIV (downregulated in the M-side) could likely be replaced in prenatal development with collagen V and/or collagen VI (upregulated in the M-side and collagen VI C-terminate peptide, endotropin, is a potential growth factor).[27] The structure of the contracted ECM could thus be completely changed.

Moreover collagen XII together with tenascin-C could play a role in the developing of joint structures.[39] It was demonstrated that the morphological abnormalities (absence of menisci, disorganized cellularity in the fused region of joints) resulting from embryonic immobilization are associated with an altered expression of collagen XII and tenascin-C.[39] Mikic et al. also suggested that tenascin-C expression is sensitive to external changes in the mechanical loading environment within developing joint structures.[39] These observations could bear some relationship to clubfoot aetiology, because its pathology starts in the perinatal period (Figure 6).

Native full-length tenascin-C also arrests fibroblast cell-cycle progression in the G1 phase and promotes fibroblast migration along the fibrin-fibronectin matrices, characteristic of early wounds.[40] Conversely, fragmented tenascin-C was shown to almost completely inhibit fibroblast migration as a part of physiological wound healing. The persistence of the full-length tenascin-C in fibrotic disease may thus contribute to pathological healing.[33] This mechanism could be a part of a new hypothesis for clubfoot aetiology (Figure 6).

Tenascin-C is not the only protein with influence on cell migration. In the M-side of clubfoot we also found upregulated protein versican (highly present in keloid scars[34]), so alteration in tissue concentration of these proteins could be relevant in clubfoot pathology (Figure 6).

There are also other proteins whose concentration in the M-side was significantly higher, and which are moreover involved in the process of collagen mineralization. We observed specific calcified structures in the M-side of clubfoot for the first time (Figure 5). This could bring a direct connection with overexpression of asporin (which plays a role in biomineralization[32,49]), TGFβip (essential for TGFβ signaling[30]), and the intracellular pool of TGFβ (relationships to collagen calcification[32]) in the M-side. Moreover Seki et al. declared that overexpressed CILP suppressed TFGβ signaling in lumbal disc disease.[31] In our study, CILP2 in the M-side has a significantly lower concentration, and therefore TGFβ signaling could be disregulated. That assumes an important role for CILP2 in ECM. The connection of these four proteins (asporin, TGFβ, TGFβip, and CILP2) with pathological calcifications in clubfoot needs future research (Figure 6).

The current state of knowledge of the clubfoot disease is still limited, and we are not able to strictly determine what the causes and effects are in this pathological process. Only with a detailed description of the clubfoot contracted tissue, it may be possible to accelerate treatment of this disease with adjunctive therapeutic agents. The present study has revealed 11 significant quantitative differences in the protein composition of the contracted and non-contracted tissues of clubfoot, and suggests a new hypothesis about clubfoot pathogenesis. All these proteins may serve as potential biomarkers of the clubfoot disease. Their further analysis might bring new insights into the mechanisms of morphogenetic development of this serious congenital malformation. Chemical treatment is already clinically used in cases of some other fibroproliferative diseases, for example, Dupuytren's conctracture, where collagenase clostridium histolyticum is frequently injected.[50] However, there is no alternative or additional chemical treatment for idiopathic clubfoot to date.

AUTHORS' CONTRIBUTION

AE, MO, TN responsible for conception and design of study. MO responsible for acquisition of samples. AE, LK, DV, JK, JL, TN, JU responsible for acquisition of data. SP, TN, MD, MP, LV, IM: data analysis. DH, LH, MZ, TN responsible for statistics. AE, MO, MD, TN, JU, LV, LK interpreted the data analyses and prepared the manuscript. All authors have contributed to this research, revised it critically, and approved the final version of the manuscript.

ACKNOWLEDGMENTS

The study was supported by the Ministry of Health of the Czech Republic, Department Program for Research and Development (n. 17-31564A), the KZCR, n.s., IGP KZ (n. 217116002), and Charles University, project GA UK (n. 336218), as part of the Specific University Research (SVV 260440), and long-term conceptual development of research organization (RVO:67985823).

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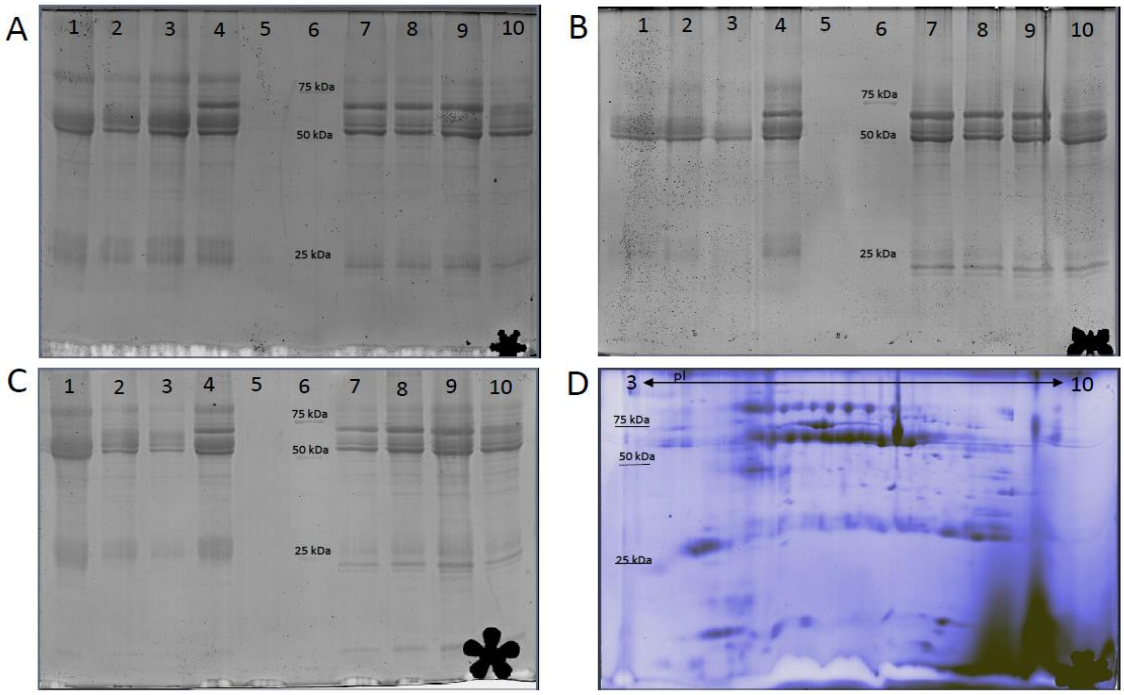
SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's website.

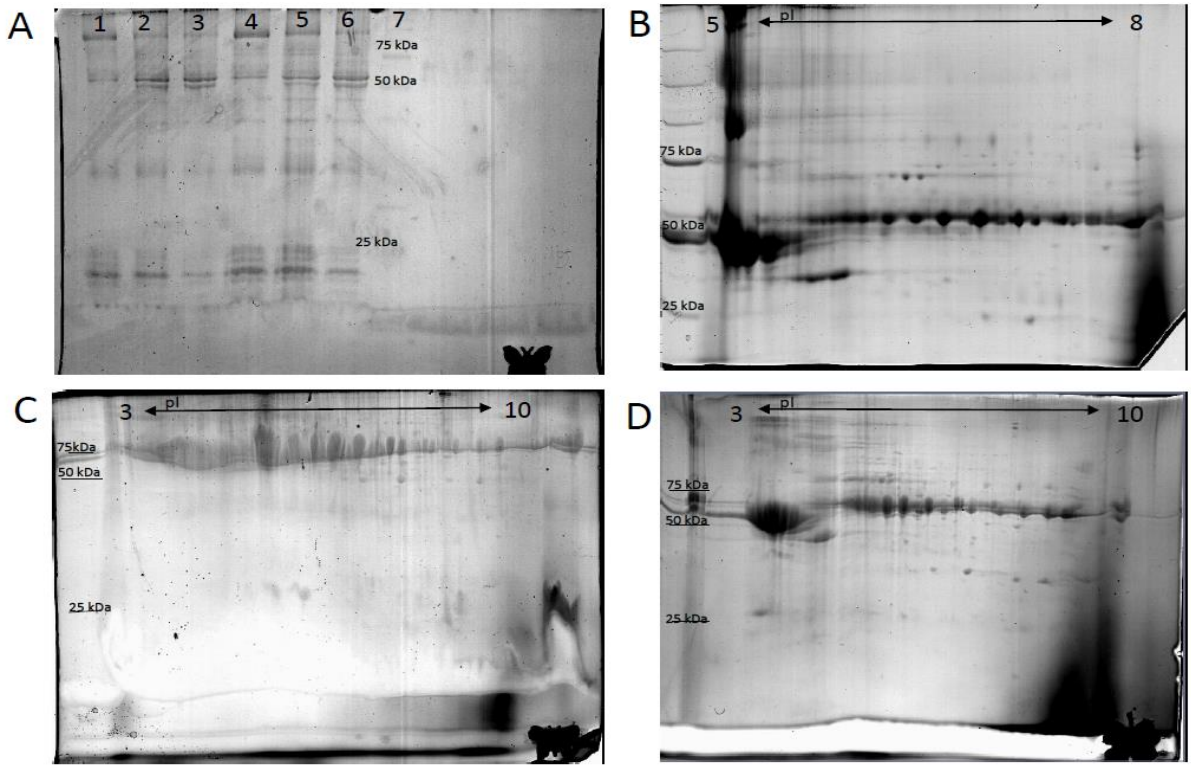
Příloha 6

Nepublikované výsledky jednorozměrné a dvourozměrné elektroforézy a diferenční dvourozměrné gelové elektroforézy a mikrobiologických analýz



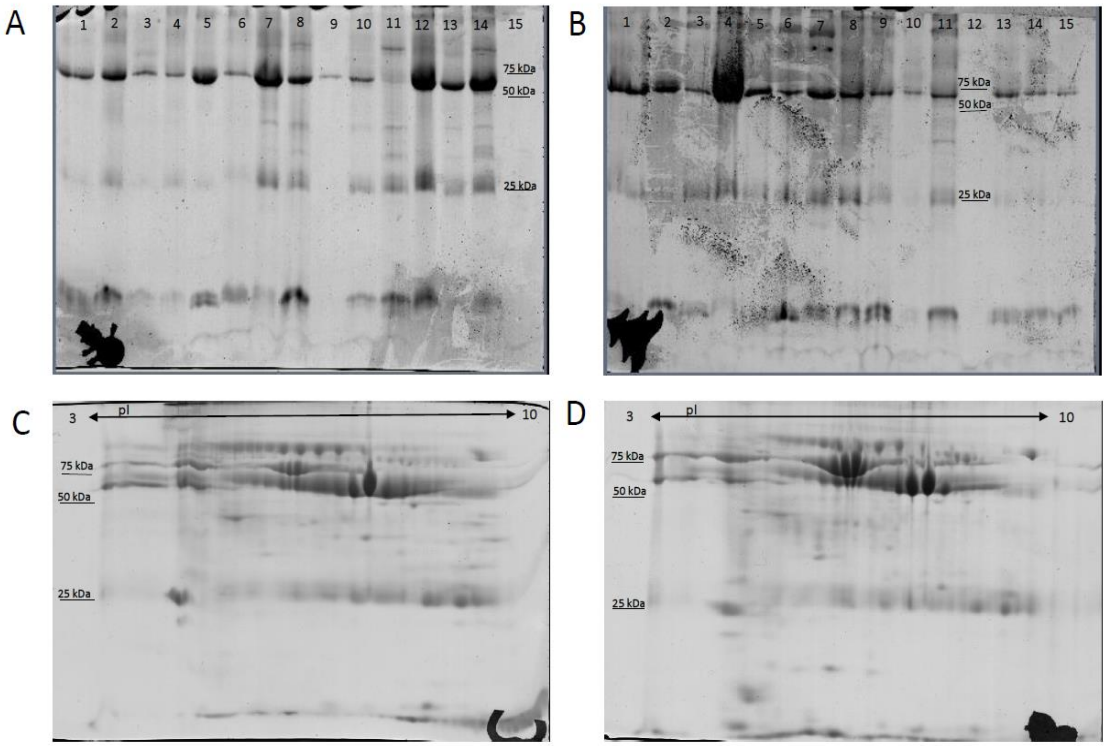
Obrázek I: Optimalizace přípravy sraženého sedimentu proteinů vzorků lidských slin pro jednorozměrnou a dvourozměrnou elektroforézu (nalačno, po vyčištění zubů) - frakce supernatant: A. Jednorozměrná elektroforéza SDS-PAGE vzorků slin (frakce supernatant) s různým oplachem vysrážených proteinů (aceton/acetonitril), 8% separační gel; **B.** Jednorozměrná elektroforéza SDS-PAGE vzorků slin (frakce supernatant) s různým oplachem vysrážených proteinů (aceton/acetonitril), 10,5% separační gel; **C.** Jednorozměrná elektroforéza SDS-PAGE vzorků slin (frakce supernatant) s různým oplachem vysrážených proteinů (aceton/acetonitril), 12,5% separační gel; **D.** Dvourozměrná elektroforéza vzorku slin – frakce supernatant.

Všechny optimalizační kroky byly prováděny minimálně na 3 vzorcích (3 biologické materiály- 3 vzorky lidských slin od různých lidí. Vzorky lidských slin byly nejprve centrifugovány 13 000 g, 30 minut při 4 °C. Byly získány 2 frakce – supernatant a sediment. Ke sražení proteinů ve vzorku ve frakci supernatant byl použit roztok 10% (v/v) kyseliny trichloroctové s přídavkem 0,12% (w/v) dithiothreitolu (Jehmlich, 2013) **po dobu 30 minut při laboratorní teplotě (25 °C). Následně byla provedena centrifugace 13 000 g, 15 minut při 4 °C. K oplachu vysrážených proteinů byl využit roztok aceton a acetonitrilu vychlazený předem v mrazáku, a následně chlazen na ledu. A** – 1- vzorek 1, nanáška 2 µl, oplach sedimentu 3x aceton; 2- vzorek 1, nanáška 4 µl, oplach sedimentu 3x acetonem; 3- vzorek 1, nanáška 4 µl, oplach 5x aceton; 4- vzorek 1, nanáška 4 µl, oplach 5x aceton+ 5x acetonitril, 5- vzorek 1, nanáška 4 µl, oplach 5x acetonitril; 6- standard; 7- vzorek 2, nanáška 4 µl, oplach sedimentu 3x acetonem; 8- vzorek 2, nanáška 4 µl, oplach 5x aceton; 9- vzorek 2, nanáška 4 µl, oplach 5x aceton+ 5x acetonitril, 10- vzorek 2, nanáška 4 µl, oplach 5x acetonitril; 8% separační gel, B- nanášky vzorků do stejných pozic jak v A; 10,5% separační gel, 4% zaostřovací gel; C – nanášky vzorků do stejných pozic jak v A; 12,5% separační gel, 4% zaostřovací gel; D – vzorek 1, frakce supernatant, 12,5% separační gel, rozsah pI 3-10, barvení pomocí Bio-Safe™ Coomassie G-250 Stain (Bio-Rad, USA). Všechny gelové elektroforézy v této práci byly barveny pomocí této barvy od firmy Bio-Rad, focení gelů bylo prováděno pomocí GS-800 Calibrated Imaging Densitometer, Bio-Rad, USA).



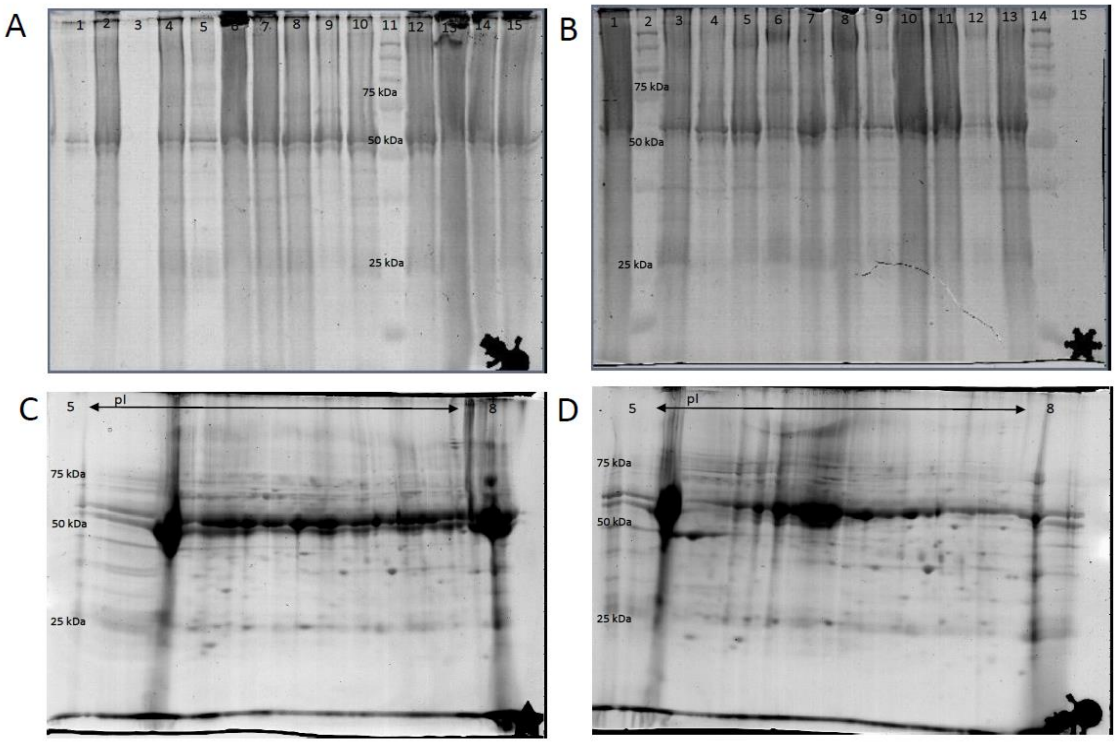
Obrázek II: Optimalizace přípravy sedimentu proteinů vzorků slin pro jednorozměnrnou a dvourozměrnou elektroforézu (nalačno, po vyčištění zubů) – frakce sediment: A. Jednorozměrná elektroforéza SDS-PAGE vzorků slin s různým oplachem sedimentu (aceton/acetonitril), která byly získána po centrifugaci vzorku celkové sliny, 12,5% separační gel; **B** – Dvourozměrná elektroforéza vzorku sedimentu získaného po první centrifugaci vzorku celkové sliny rozsah pI 5-8; **C** – Dvourozměrná elektroforéza vzorku sedimentu (sediment získáný po první centrifugaci vzorku celkové sliny) pouze opláchnut acetonem; **D** – Dvourozměrná elektroforéza vzorku sedimentu (sediment získáný po první centrifugaci vzorku celkové sliny), který byl opláchnut acetonitrilem i acetonem

Všechny optimalizační kroky byly prováděny minimálně na 3 vzorcích (3 biologické materiály- 3 vzorky lidkých slin od různých lidí. Vzorky slin byly nejprve centrifugovány 13 000 g, 30 minut při 4 °C. Centrifugací vzorku celkové sliny byly získány 2 frakce – supernatant a sediment. K oplachu získaného sedimentu po centrifugaci byl využit roztok aceton a acetonitrilu vychlazený předem v mrazáku, a následně chlazen na ledu. A – 1- vzorek 1, nanáška 2 µl, oplach pouze acetonem; 2 – vzorek 1, nanáška 2 µl, oplach acetonem i acetonitrilem; 3 – vzorek 1, nanáška 2 µl, oplach pouze acetonitrilem; 4 – vzorek 1, nanáška 4µl, oplach pouze acetonem; 5 – vzorek 1, nanáška 4 µl, oplach acetonem i acetonitrilem; 6- vzorek 1, nanáška 4 µl, oplach pouze acetonitrile, 7 – standard; **B** – rozsah pI 5-8, 8% separační gel; **C**- rozsah pI 3-10, 12,5% separační gel; **D** – rozsah pI 3-10, 10% separační gel.



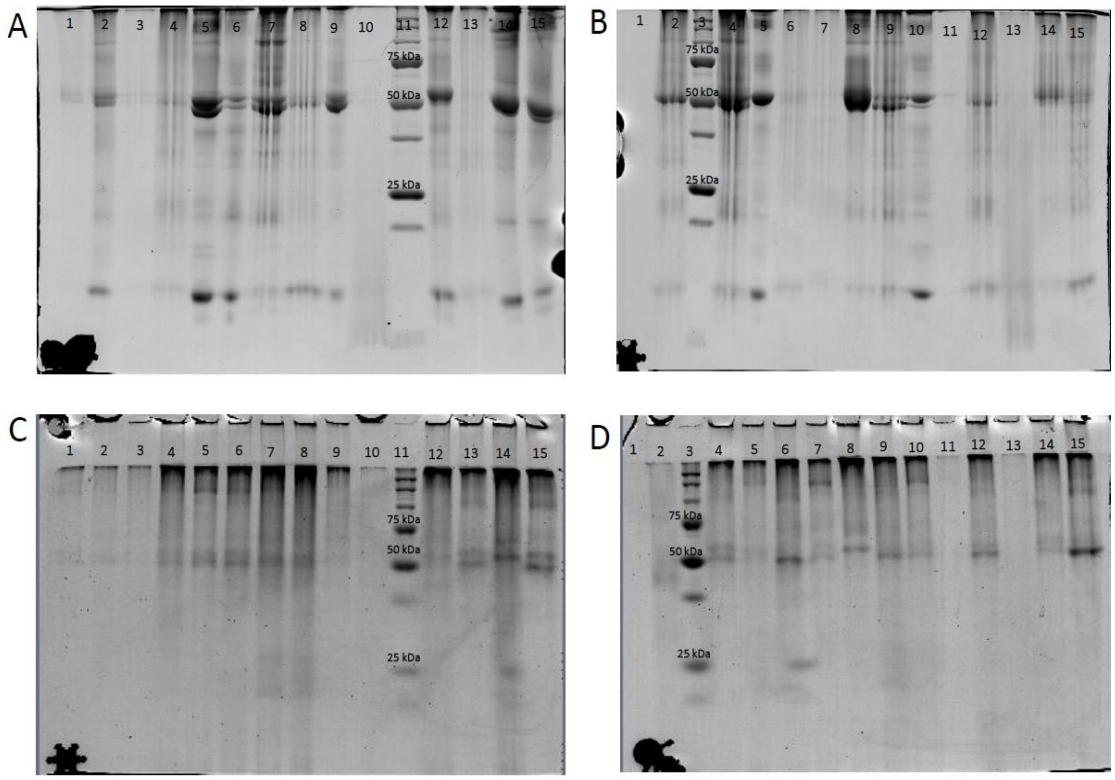
Obrázek III: Jednorozměrná a dvourozměrná elektroforéza vzorků slin mužů (frakce supernatant): Frakce supernatant získána po centrifugaci vzorku celkové sliny (horní část) A. Jednorozměrná elektroforéza vzorků slin (frakce supernatant) od mužů, kteří mají sanovaný chrup (vzorky označeny I S – XV S); B. Jednorozměrná elektroforéza vzorků slin (frakce supernatant) od mužů, kteří mají zdravý chrup (nikdy se ve své dutině ústní nesetkali se zubním kazem) (vzorky označeny XXI S – XXXII S); C. Dvourozměrná elektroforéza vzorku slin (frakce supernatant) (vzorek VII S, získaný od muže se sanovaným chrupem); D. Dvourozměrná elektroforéza vzorku slin (frakce supernatant) (vzorek XXI S, získaný od muže se zdravím chrupem)

A – 12,5% separační gel, 4% zaostřovací gel, vzorky bez povaření v redukujícím pufru, naneseny rozpuštěné v lysis bufferu pro 2-DE; 1- vz I S; 2- vz II S; 3- vz III S; 4- vz IV; 5- vz V S; 6- vz VI S; 7- vz VII S; 8- VIII S; 9- vz IX S; 10- vz XI S; 11- vz XII S; 12- vz XIII S; 13- vz XIV S; 14- vz XV S; 15- standard. **B** – 12,5% separační gel, 4% zaostřovací gel, vzorky bez povaření v redukujícím pufru, naneseny rozpuštěné v lysis bufferu pro 2-DE 1- vz XXI S; 2- vz XXIII S; 3- vz XXIV S; 4- vz XXV S; 5- vz XXVI S; 6- vz XXVII S;, 7- vz XXVIII S; 8- vz XXIX S; 9- vz XXX S; 10- vz XXXI S; 11- vz XXXII S; 12- standard; 13- vz I S; 14- vz II S; 15- vz XS. **C** – 12,5% separační gel, 150µg proteinu vzorku VII S naneseno na strip (rozmezí pI 3-10). **D** - 12,5% separační gel, 150µg proteinu vzorku XXI S naneseno na strip (rozmezí pI 3-10). Všechno vyhodnocení dvourozměrných gelových elektroforéz bylo prováděno pomocí PDQuest™ software (Bio-Rad), version 8.0.1.



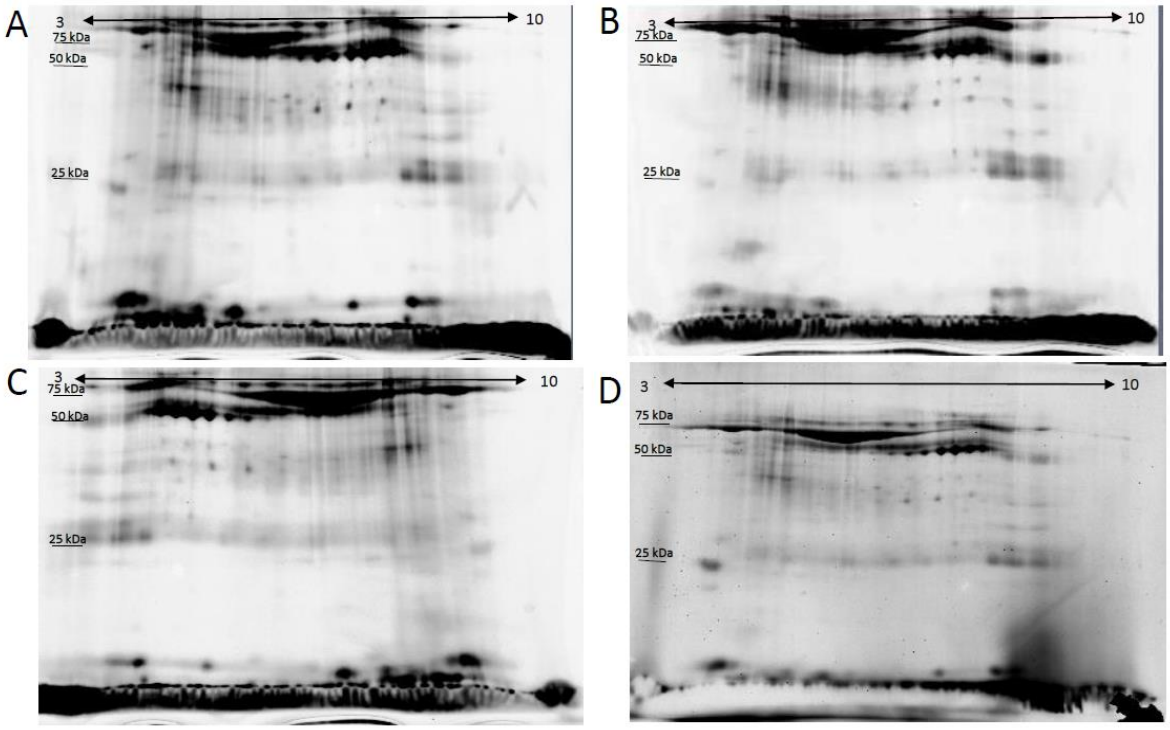
Obrázek IV: Jednorozměrná a dvourozměrná elektroforéza vzorků slin mužů (frakce sediment): A. Jednorozměrná elektroforéza vzorků (sedimentů) slin od mužů, kteří mají sanovanou dentici (vzorky označeny I P – XV P); B. Jednorozměrná elektroforéza vzorků (sedimentů) slin od mužů se zdravou denticí (nikdy se ve své dutině ústní nesetkali se zubním kazem)(vzorky označeny XXI P – XXXII P); C. Dvourozměrná elektroforéza vzorku slin (frakce sediment)(vzorek X P, získaný od muže se sanovanou denticí); D. Dvourozměrná elektroforéza vzorku slin (frakce sediment)(vzorek XXX P, získaný od muže se zdravou denticí).

A – 10% separační gel, 4% zaostřovací gel, vzorky bez povaření v redukujícím pufru, naneseny rozpuštěné v lysis bufferu pro 2-DE; 1- vz I P; 2- vz II P; 3- vz III P; 4- vz IV P; 5- vz V P; 6- vz VI P; 7- vz VII P; 8- VIII P; 9- vz IX P; 10- vz X P; 11- standard; 12- vz XI P; 13- vz XII P; 14- vz XIII P; 15- vz XIV P. **B** – 10% separační gel, 4% zaostřovací gel, vzorky bez povaření v redukujícím pufru, naneseny rozpuštěné v lysis bufferu pro 2-DE 1- vz XV P; 2- standard; 3- vz XXI P; 4- vz XXII P; 5- vz XXIV P; 6- vz XXV P;, 7- vz XXVI P; 8- vz XXVII P; 9- vz XXVIII P; 10- vz XXIX P; 11- vz XXX P; 12- XXXI P; 13- vz XXXII P; 14- standard; 15- x. **C** – 10% separační gel, 150µg proteinu vzorku X P naneseno na strip (rozmezí pI 5-8). **D** - 10% separační gel, 150µg proteinu vzorku XXX P naneseno na strip (rozmezí pI 5-8).



Obrázek V: Jednorozměrná elektroforéza vzorku slin (frakce supernatant a sediment) rozpuštěných v lyzačním pufru pro dvourozměrnou diferenční elektroforézu (DIGE): A. Jednorozměrná elektroforéza vzorků slin (frakce supernatant) I – vzorky rozpuštěny v lyzačním pufru pro DIGE; **B.** Jednorozměrná elektroforéza vzorků slin (frakce supernatant) II – vzorky rozpuštěny v lyzačním pufru pro DIGE; **C.** Jednorozměrná elektroforéza vzorků slin (frakce sediment) I – vzorky rozpuštěny v lyzačním pufru pro DIGE; **D.** Jednorozměrná elektroforéza vzorků slin (sediment) II – vzorky rozpuštěny v lyzačním pufru pro DIGE

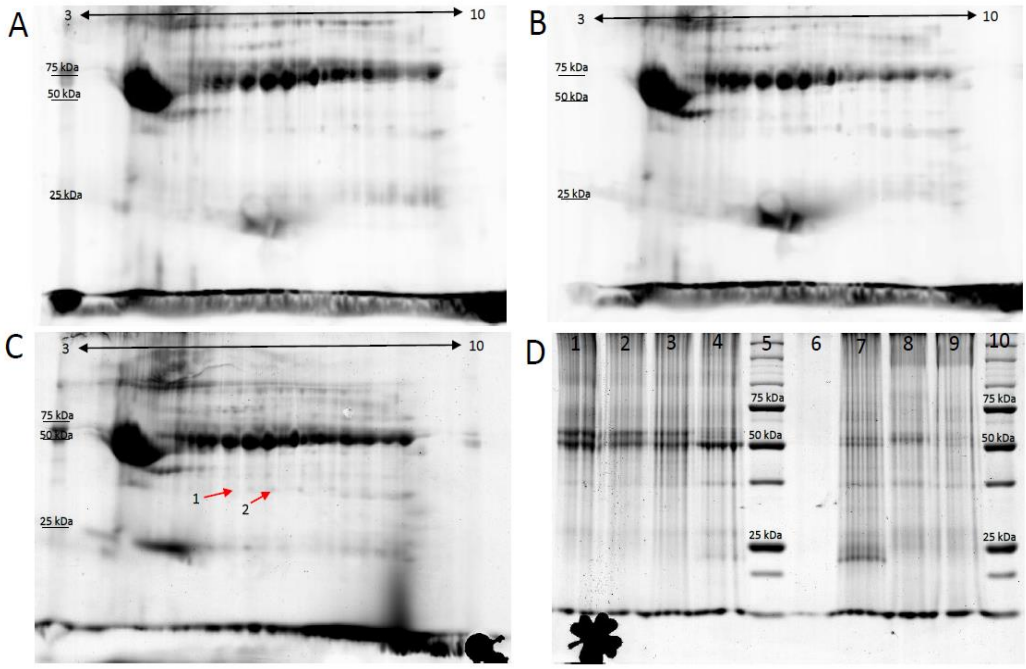
Vzorky bez povaření v redukujícím pufru, naneseny rozpuštěné v lyzačním pufru pro DIGE; 10,5% separační gel, 4% zaostřovací gel. **A.** 1- IS; 2- IIS; 3- IIIS; 4- IVS; 5- VS; 6- VIS; 7- VIIS; 8- VIIIS; 9- IXS; 10- XS; 11- standard; 12- XIS; 13- XIIS; 14- XIIIS; 15- XIVS; **B.** 1- x; 2- XVS; 3- standard; 4- XXIS; 5-XXIIS; 6- XXIIIS; 7- XXIVS; 8- XXVS; 9- XXVIS; 10- XXVIIS; 11- XXVIIIS; 12XXIXS; 13- XXXS; 14- XXXIS; 15- XXXIIS; **C.** 1- IP; 2- IIP; 3- IIIP; 4- IVP; 5- VP; 6- VIP; 7-VIIP; 8- VIIIP; 9- IXP; 10- XP; 11-standard; 12- XIP; 13- XIIP; 14- XIIIP; 15- XIVP; D. 1- x; 2- XVP; 3- standard; 4- XXIP; 5-XXIIP; 6- XXIIIP; 7- XXIVP; 8- XXVP; 9- XXVIP; 10- XXVIIP; 11- XXVIIIP; 12- XXIXP; 13- XXXP; 14- XXXIP; 15- XXXIIP.



Obrázek VI: Dvourozměrná diferenční elektroforéza (DIGE) vzorků slin mužů (frakce supernatant): A. DIGE vzorku vz1 - VIII S (vzorek slin od muže (frakce supernatant) se sanovanou denticí) obarven barvou Cy3; **B.** DIGE vzorku vz2 – XXXI S (vzorek slin od muže (frakce supernatant) se zdravou denticí) obarven barvou Cy5; **C.** DIGE vzorku vz1+vz2 – směsní vzorek (interní standard) obarven barvou Cy2; **D.** Dvourozměrná elektroforéza vzorků VIII S a XXXI S barvených pomocí tzv. minimálního barvení (použitá fluorescenční barviv CyDyes)

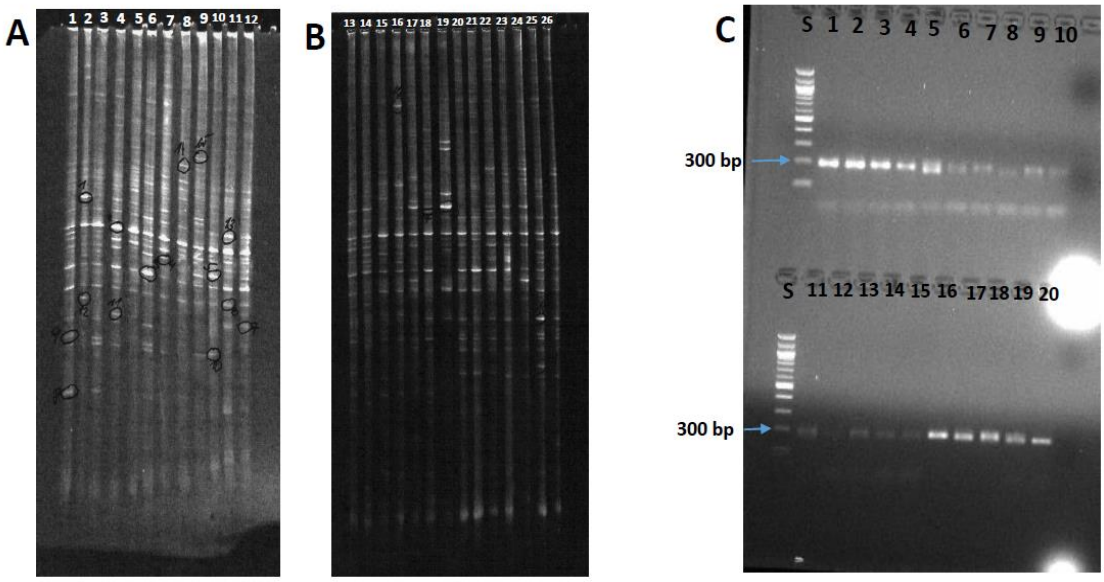
Minimální barvení bylo provedeno přídavkem 400 pmol roztoku barviva CyDyes (rozpuštěného v dimethylformamidu DMF) k 50 µg vzorku (v případě vz1 (vzorek celkové sliny získán od muže se sanovaným chrupem) – použita fluorescenční barvivo Cy3; vz2 (vzorek slin získán od muže se zdravou denticí – použité fluorescenční barvivo Cy5; směsný vzorek v poměru 1:1 (vz1 a vz2) byl obarven fluorescenčním barvivem Cy2). Roztoky byly promíchány a uloženy do tmy na ledu. Po 30 minutách byla reakce ukončena přídavkem 1 µl mM lysinu. Po provedení prvního a druhého rozměru gelové elektroforézy bylo provedeno skenování dle příslušných podmínek pro každé barvivo pomocí fluorescenčního skeneru (Pharos FX^{TM}, Molecular Imager, Bio-Rad, USA) Použité vlnové délky pro jednotlivé barvy CyDyes: Cy2 - absorpční maximum v DMF 491±3 nm, emisní maximum v DMF 506±5 nm; Cy3 - absorpční maximum v DMF 552±3 nm, emisní maximum v DMF 572±5 nm; Cy5 - absorpční maximum v DMF 648±3 nm, emisní maximum v DMF 669±5 nm.

Následně byl SDS-PAGE obarven pomocí Bio-Safe^{TM} Coomassie G-250 Stain (Bio-Rad, USA). K vyhodnocení změn exprese proteinů pro jednotlivé porovnávané skupiny byl využit PDQuest™ software (Bio-Rad, USA), version 8.0., stejně jako u vyhodnocení dvourozměrné gelové elektroforézy. V případě porovnání získaných touto metodou nebyly nalezeny žádné rozdíly v expresi proteinů.



Obrázek VII: Dvourozměrná diferenční elektroforéza (DIGE) vzorků slin mužů (frakce sediment): A. DIGE vzorku vz1 - V P (vzorek slin od muže (frakce sediment) se sanovanou denticí) obarven barvou Cy3; **B.** DIGE vzorku vz2 – XXVII P (vzorek slin od muže (frakce sediment) se zdravou denticí) obarven barvou Cy5; **C.** Dvourozměrná elektroforéza vzorků V P a XXVII P barvených pomocí tzv. minimálního barvení (použitá fluorescenční barviv CyDyes); **D.** Jednorozměrná elektroforéza vzorků sedimentu, které byly následně vybrány k porovnání pomocí softwaru PDQuest

Minimální barvení bylo provedeno stejným způsobem, jak je uvedeno u předchozího obrázku č. 8. K vyhodnocení byly vybrány 4 vzorky sedimentu získaných od mužů se sanovaným chrupem a 4 vzorky sedimentů získaných od mužů se zdravou denticí. Pomocí programu PDQuest, vypočtu T-testu a následnou MS analýzou spotů, byly nalezeny rozdíly v expresi proteinu annexin A1 (2 spoty; t-test: p=0,031 a p=0,015; poměr mezi skupinou muži se sanovaným chrupem a skupinou muži se zdravou denticí byl vypočítán 0,08 a 0,18; spoty jsou zakresleny červenou šipkou s čísly 1 a 2 v obrázku C. D - vzorky sedimentu rozpuštěné v lyzačním pufru pro DIGE naneseny do jamek rozostřovacího gelu, separační gel 10,5%; 1 - XIV P, 2 - XXI P, 3 - XII P, 4 - XXXIIP, 5 - standard, 6 - VIII P (špatné nadávkování), 7 - XXVI P, 8 - V P, 9 - XXVII P, 10 - standard.



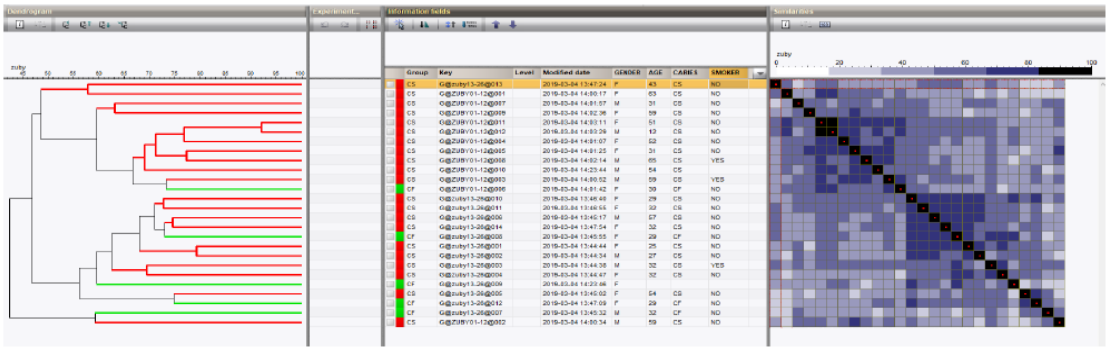
Obrázek VIII A: Výsledky z denaturační gradientové gelové elektroforézy (DGGE) vzorků slin: **A**. DGGE I vzorky číslo 1-12 (odběr vzorku celkové sliny, nalačno, bez vyčištění zubů, po noci) (zakreslené výřezy příprava pro Sangerovo sekvenování (komerčně; SEQme.eu); **B.** DGGE II vzorky číslo 13-26 (odběr vzorku celkové sliny, nalačno, bez vyčištění zubů, po noci); **C.** Izolace DNA z výřezů při DGGE a ověření bandu 300 kDa na agarozové elektroforéze s ethydium bromidem;

A a **B** - Denaturační gradientová gelová elektroforéza umožňující separaci molekul DNA v polyakrylamidovém gelu (lineární gradient koncentrace denaturačního roztoku, zpravidla formamidu a močoviny) na základě odlišné sekvence nukleotidů. Principem metody je putování dvouvláknové DNA gelem rychlostí určenou její molekulové hmotnosti až do doby než vstoupí do části gelu s koncentrací denaturačních látek způsobující denaturaci dvouvláknové DNA na jednovláknovou, změní se tak její pohyblivost. Konečná pozice fragmentu DNA v gelu závisí tedy na denaturačním bodu (Muyzer, 1993). Denaturačního gradientu lze dosáhnout teplotním gradientem. C – Agarozová elektroforéza, při které se při přípravě gelu použije přídavek roztoku ethidium brodmidu se využívá pro detekci produktů PCR (polymerázová řetězová reakce) fragmentů DNA (např. ověření účinnosti izolace DNA pomocí komerčního setu)(Lee PY, 2012). Detekce je založena na focení gelu pomocí UV záření, pod kterým ethidium bromid navázaný na DNA emituje světlo (Voytas S, 2001).

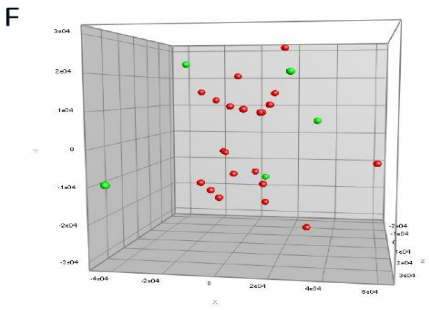
D

	Description	Max score	Total score	Query cover	E value	Ident	Accession
1	nedetekováno						
2	nedetekováno						
3	Streptococcus vestibularis strain ATCC 49124 16S ribosomal RNA, partial sequence	148	148	98%	4,00E-36	90.00%	NR_042777.1
4	nedetekováno						
5	Caecibacterium sporoformans strain 77-5d 16S ribosomal RNA, partial sequence	183	183	78%	2,00E-46	91.13%	NR_159147.1
6	Ureibacillus suwonensis strain 6T19 16S ribosomal RNA gene, partial sequence	58.4	58.4	23%	1,00E-08	97.06%	NR_043232.1
7	Veillonella tobetsuensis strain JCM 17976 16S ribosomal RNA gene, partial sequence	246	246	99%	2,00E-65	94.16%	NR_113570.1
8	Rothia mucilaginosa strain DSM 20746 16S ribosomal RNA gene, partial sequence	220	220	100%	1,00E-57	99.18%	NR_044873.1
9	nedetekováno						
10	Veillonella atypica strain KON 16S ribosomal RNA gene, partial sequence	231	231	100%	5,00E-61	94.41%	NR_041880.1
11	nedetekováno						
12	nedetekováno						
13	Scopulibacillus darangshiensis strain DLS-06 16S ribosomal RNA gene, partial sequence	104	104	54%	1,00E-22	89.33%	NR_115059.1
14	nedetekováno						
15	nedetekováno						
16	nedetekováno						
17	nedetekováno						
18	Veillonella atypica strain KON 16S ribosomal RNA gene, partial sequence	200	200	99%	1,00E-51	91.24%	NR_041880.1
19	Actinomyces graevenitzii strain CCUG 27294 16S ribosomal RNA, partial sequence	219	219	100%	4,00E-57	92.36%	NR_042167.1
20	Haemophilus parainfluenzae strain CIP 102513 16S ribosomal RNA gene, partial sequence	248	248	100%	6,00E-66	93.59%	NR_116168

E



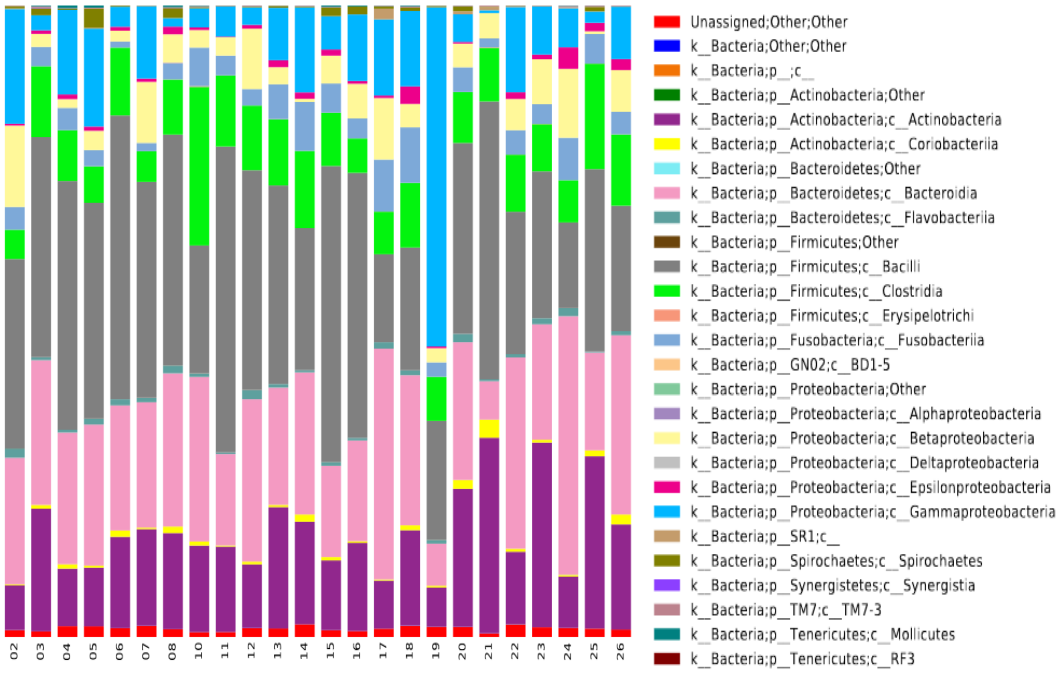
F



Obrázek VIII B: Výsledky z denaturační gradientové gelové elektroforézy (DGGE) vzorků slin: **D.** Tabulka výsledků z Sangerova sekvenování – určení dle databáze příslušné druhy s uvedením identifikační shodnosti (limit pro správné identifikování, limit pro určení daného druhu (97%)); **E.** Dendogram výsledků DGGE – porovnání zastoupení přítomnosti bandů (BioNumerics 7.6); **F.** PCA analýza výsledků porovnání jednotlivých profilů na DGGE

D – Získané výsledky z SEQme.eu byly zobrazeny jako chromatogramy pomocí prohlížeče Chromas prohlížeč (seřazení jednotlivých bází za se sebou v sekvenci, bylo provedeno určení začátku a konce sekvence, exportováno do textu („in plain text“) .fasta. Následné prohledávání v databázi Nucleotide BLAST byly získány příslušné identifikace (limit pro správné identifikování, limit pro určení daného druhu (97 %)).

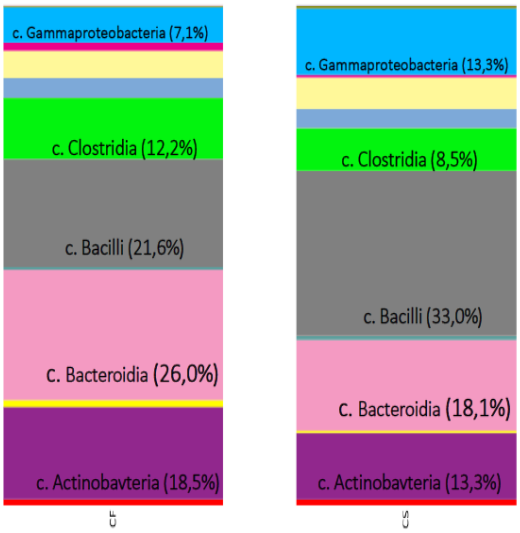
A



Doména (k); kmen (p); třída (c)	Total	vz 2	vz 3	vz 4	vz 5	vz 6	vz7	vz 8	vz 10	vz 11	vz 12	vz13	vz14	vz15	vz16	vz17	vz18	vz19	vz20	vz21	vz22	vz23	vz24	vz25	vz26
Unassigned;Other;Other	1.4%	1.1%	0.9%	1.7%	1.7%	1.4%	1.8%	1.2%	0.8%	0.8%	1.4%	1.4%	2.0%	1.1%	0.9%	1.3%	1.8%	1.6%	1.6%	0.6%	2.0%	1.5%	1.4%	1.3%	1.2%
k__Bacteria;Other;Other	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.1%	0.1%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__;c__	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Actinobacteria;Other	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Actinobacteria;c__Actinobacteria	15.1%	7.1%	19.4%	9.1%	9.3%	14.4%	15.3%	15.2%	13.7%	13.5%	10.1%	19.2%	16.2%	11.0%	14.0%	7.6%	15.1%	6.2%	21.9%	30.9%	11.5%	29.3%	8.1%	27.3%	16.6%
k__Bacteria;p__Actinobacteria;c__Coriobacteriia	0.7%	0.2%	0.6%	0.7%	0.3%	1.0%	0.2%	1.0%	0.6%	0.2%	0.4%	0.3%	1.1%	0.5%	0.2%	0.2%	0.8%	0.3%	1.4%	2.9%	0.5%	0.4%	0.3%	0.9%	1.6%
k__Bacteria;p__Bacteroidetes;Other	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Bacteroidetes;c__Bacteroidia	21.5%	20.0%	22.9%	20.9%	22.3%	19.8%	19.9%	24.3%	26.1%	14.5%	25.7%	18.6%	22.5%	14.5%	16.0%	36.6%	23.8%	6.7%	21.8%	6.1%	30.3%	18.3%	41.0%	15.5%	28.4%
k__Bacteria;p__Bacteroidetes;c__Flavobacteriia	0.7%	1.5%	0.5%	0.4%	0.9%	1.0%	0.7%	1.2%	0.5%	0.2%	1.5%	0.6%	0.3%	0.6%	0.4%	1.0%	0.8%	0.6%	1.3%	0.3%	0.5%	0.9%	1.3%	0.2%	0.7%
k__Bacteria;p__Firmicutes;Other	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Firmicutes;c__Bacilli	30.6%	30.0%	34.9%	39.4%	34.2%	45.0%	34.2%	36.6%	20.2%	48.4%	34.8%	31.4%	22.5%	46.9%	42.0%	13.9%	19.4%	18.8%	30.2%	44.0%	22.6%	23.3%	13.5%	28.9%	19.8%
k__Bacteria;p__Firmicutes;c__Clostridia	9.5%	4.6%	11.2%	8.0%	5.8%	10.7%	4.9%	8.7%	25.1%	11.2%	10.2%	10.5%	12.2%	8.4%	5.4%	6.7%	10.2%	7.0%	8.1%	8.5%	9.0%	7.5%	6.7%	16.7%	11.3%
k__Bacteria;p__Firmicutes;c__Erysipelotrichi	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.1%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.1%	0.0%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Fusobacteria;c__Fusobacteriia	4.0%	3.6%	3.0%	3.5%	2.6%	1.0%	1.3%	2.5%	6.1%	3.1%	2.6%	5.5%	7.7%	4.6%	3.2%	8.2%	8.8%	2.3%	3.9%	1.4%	3.9%	3.1%	6.7%	4.7%	3.6%
k__Bacteria;p__GN02;c__BD1-5	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Proteobacteria;Other	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.1%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria	4.9%	12.9%	2.0%	1.4%	3.0%	1.7%	9.7%	4.5%	2.8%	2.9%	9.6%	2.7%	0.5%	4.4%	5.5%	9.7%	3.7%	2.2%	3.8%	4.0%	4.9%	7.1%	10.9%	0.4%	6.6%
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Proteobacteria;c__Epsilonproteobacteria	0.9%	0.3%	0.6%	0.7%	0.7%	0.6%	0.5%	1.2%	0.4%	0.1%	0.6%	1.1%	1.0%	1.0%	0.4%	0.4%	2.8%	0.3%	0.3%	0.0%	1.1%	0.7%	3.4%	1.3%	1.7%
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria	9.7%	18.2%	2.4%	13.4%	15.5%	3.2%	11.5%	1.3%	3.0%	4.8%	2.8%	8.3%	13.4%	5.3%	10.5%	12.1%	11.9%	53.7%	4.4%	0.4%	13.5%	7.6%	6.2%	1.8%	8.3%
k__Bacteria;p__SR1;c__	0.1%	0.1%	0.0%	0.0%	0.1%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	1.7%	0.0%	0.0%	0.5%	0.5%	0.1%	0.0%	0.1%	0.0%	0.0%
k__Bacteria;p__Spirochaetes;c__Spirochaetes	0.5%	0.4%	1.1%	0.2%	3.1%	0.2%	0.1%	1.6%	0.2%	0.1%	0.1%	0.3%	0.1%	1.4%	1.2%	0.4%	0.5%	0.2%	0.8%	0.1%	0.1%	0.1%	0.0%	0.7%	0.1%
k__Bacteria;p__Synergistetes;c__Synergistia	0.0%	0.0%	0.1%	0.1%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__TM7;c__TM7-3	0.1%	0.1%	0.2%	0.0%	0.1%	0.0%	0.0%	0.1%	0.1%	0.0%	0.1%	0.0%	0.1%	0.0%	0.0%	0.1%	0.2%	0.0%	0.1%	0.2%	0.0%	0.1%	0.2%	0.2%	0.1%
k__Bacteria;p__Tenericutes;c__Mollicutes	0.1%	0.0%	0.1%	0.4%	0.3%	0.0%	0.0%	0.3%	0.1%	0.0%	0.0%	0.0%	0.0%	0.2%	0.2%	0.1%	0.1%	0.0%	0.0%	0.0%	0.0%	0.0%	0.1%	0.0%	0.0%
k__Bacteria;p__Tenericutes;c__RF3	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%

Obrázek IX A: Výsledky ze sekvenování nové genové generace (NGS) pro zjištění zastoupení jednotlivých bakterií v jednotlivých srovnán (Grilli a kol., 2016; Mrázek a kol., 2019)**. Výsledky NGS vyhodnoceny pomocí software Qiime dle jednotlivých vzorků** (vz 6, vz 19-21, vz 24- skupina se zdravou denticí).

B



Doména (k); kmen (p); třída (c)	Zastoupení CF	Zastoupení CS
Unassigned;Other;Other	1,23%	1,19%
k__Bacteria;Other;Other	0,00008%	0,00010%
k__Bacteria;p__;c__	0,00004%	0,00002%
k__Bacteria;p__Actinobacteria;Other	0,000002%	0,00%
k__Bacteria;p__Actinobacteria;c__Actinobacteria	**18,55%**	**13,33%**
k__Bacteria;p__Actinobacteria;c__Coriobacteriia	1,37%	0,41%
k__Bacteria;p__Bacteroidetes;Other	0,000005%	0,000008%
k__Bacteria;p__Bacteroidetes;c__Bacteroidia	**26,04%**	**18,14%**
k__Bacteria;p__Bacteroidetes;c__Flavobacteriia	0,57%	0,81%
k__Bacteria;p__Firmicutes;Other	0,00007%	0,01%
k__Bacteria;p__Firmicutes;c__Bacilli	**21,57%**	**33,00%**
k__Bacteria;p__Firmicutes;c__Clostridia	**12,22%**	**8,52%**
k__Bacteria;p__Firmicutes;c__Erysipelotrichi	0,03%	0,02%
k__Bacteria;p__Fusobacteria;c__Fusobacteriia	3,94%	3,81%
k__Bacteria;p__GN02;c__BD1-5	0,00%	0,000003%
k__Bacteria;p__Proteobacteria;Other	0,000002%	0,000001%
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria	0,000009%	0,00002%
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria	5,47%	6,29%
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria	0,00%	0,00002%
k__Bacteria;p__Proteobacteria;c__Epsilonproteobacteria	1,61%	0,51%
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria	**7,06%**	**13,26%**
k__Bacteria;p__SR1;c__ 0.000527829661828	0,05%	0,05%
k__Bacteria;p__Spirochaetes;c__Spirochaetes	0,21%	0,44%
k__Bacteria;p__Synergistetes;c__Synergistia	0,000004%	0,01%
k__Bacteria;p__TM7;c__TM7-3	0,14%	0,07%
k__Bacteria;p__Tenericutes;c__Mollicutes	0,00006%	0,05%
k__Bacteria;p__Tenericutes;c__RF3	0,00%	0,000002%

Obrázek IX B: Výsledky ze sekvenování nové genové generace (NGS) pro zjištění zastoupení jednotlivých bakterií v jednotlivých srovnán. B. Výsledky NDS vyhodnoceny pomocí software Qiime dle porovnání lidí se sanovaným chrupem a se zdravou denticí (vz 6, vz 19-21, vz 24- skupina se zdravou denticí skupina CF, ostatní vzorky (vz 2-5, vz 8-18, vz 22, vz25, vz26- skupina se sanovaným chrupem CS)**.**

Příloha 7

Výsledky label-free kvatifikace vzorků lidských slin nalačno před čištěním zubů.

Accession Number	Název a zkratka proteinu	Počet validních peptidů	Počet peptidů T-test (p<0,05)		
Proteinové srovnání dle pohlaví a věku					
Srovnání I: starší ženy vs mladší ženy (bez ohledu na orální zdraví), pouze věkové kritérium 91 validních proteinů v kompilaci vzorků; starší ženy n=6; mladší ženy n=7					
				ženy starší vs ženy mladší	
IPI01011344	ACTB Actin	11	2	0,21	p=0,0481
				0,22	p=0,0233
IPI00218918	ANXA1 Annexin A1	10	1	0,28	p=0,0264
IPI00411765	SFN Isoform 2 of 14-3-3 protein sigma	4	1	0,36	p=0,0248
IPI00216984	CALML3 Calmodulin-like protein 3	5	1	0,36	p=0,0288
IPI00455315	ANXA2 Isoform 1 of Annexin A2	5	2	0,22	p=0,116
				0,16	p=0,0081
IPI00976027	HSPA1B;HSPA1AHeat shock 70 kDa protein 1	3	1	0,32	p=0,0468
IPI01012311	DMBT1 Deleted in malignant brain tumors 1 protein	3	1	1,48	p=0,0017
IPI00021263	YWHAZ 14-3-3 protein zeta/delta	3	1	0,3	p=0,0270
IPI00554711	JUP Junction plakoglobin	3	1	0,31	p=0,0142
IPI00847989	PKM2 Pyruvate kinase	3	1	0,27	p=0,0494
Srovnání II: starší muži vs mladší muži (bez ohledu na orální zdraví), pouze věkové kritérium 75 validních proteinů v kompilaci vzorků; starší muži n=3; mladší muži n=5					
				muži starší vs muži mladší	
IPI00300376	TGM3 Protein-glutamine gamma-glutamyltransferase E	10	1	2,59	p=0,0090
IPI00021812	AHNAK Neuroblast differentiation-associated protein AHNAK	9	1	1,2	p=0,0417
IPI00982101	YWHAZ Uncharacterized protein	3	1	1,4	p=0,0354
IPI00025512	HSPB1 Heat shock protein beta-1	3	1	0,92	p=0,0043
IPI00220644	PKM2 Isoform M1 of Pyruvate kinase isozymes M1/M2	3	1	1,14	p=0,0199
IPI00013895	S100A11 Protein S100-A11	1	1	0,9	p=0,0190

Srovnání III: starší ženy vs starší muži (bez ohledu na orální zdraví), věkové a genderové kritérium
78 validních proteinů v kompilaci vzorků; starší ženy n=6; starší muži n=3

				ženy starší vs muži starší	
IPI00216984	CALML3 Calmodulin-like protein 3	6	1	0,25	p=0,0121
IPI00647704	IGHA1	7	1	0,35	p=0,0322
IPI00300376	TGM3 Protein-glutamine gamma-glutamyltransferase E	8	1	0,16	p=0,0107
IPI00411765	SFN Isoform 2 of 14-3-3 protein sigma	4	1	0,2	p=0,0199

Srovnání IV: mladší ženy vs mladší muži (bez ohledu na orální zdraví), věkové a genderové kritérium
90 validních proteinů v kompilaci vzorků; mladší ženy n=7; mladší muži n=5

				ženy mladší vs muži mladší	
IPI00027462	S100A9 Protein S100-A9	8	1	1,7	p=0,0403
IPI00216984	CALML3 Calmodulin-like protein 3	5	1	0,82	p=0,0273
IPI01012311	DMBT1 Deleted in malignant brain tumors 1 protein	2	1	0,15	p=0,0114
IPI00010796	P4HB Protein disulfide-isomerase	3	1	0,17	p=0,0038
IPI00909530	H3F3A Histone H3.3	3	1	1,55	p=0,0498

Srovnání V: všechny ženy vs všichni muži (bez ohledu na orální zdraví), genderové kritérium
103 validních proteinů v kompilaci vzorků; ženy n=14; muži n=11

				ženy vs muži	
IPI00297056	CRNN Cornulin	10	1	0,39	p=0,0123
IPI00013933	DSP Isoform DPI of Desmoplakin	16	1	1,57	p=0,0520
IPI00218918	ANXA1 Annexin A1	12	1	0,39	p=0,0293
IPI00027462	S100A9 Protein S100-A9	8	2	1,72	p=0,0175
				1,96	p=0,0232
IPI00007047	S100A8 Protein S100-A8	12	2	2,34	p=0,0326
				1,57	p=0,0475
IPI00966829	ALB 69 kDa protein	15	2	1,77	p=0,0299
				1,62	p=0,0361
IPI00936444	MUC5B Mucin-5B	9	2	0,29	p=0,0175
				1,11	p=0,0200
IPI00411765	SFN Isoform 2 of 14-3-3 protein sigma	6	1	0,43	p=0,0220
IPI00216984	CALML3 Calmodulin-like protein 3	6	1	0,43	p=0,0062
IPI00386879	IGHA1	7	1	0,52	p=0,0512
IPI00060800	ZG16B Zymogen granule protein 16 homolog B	5	1	0,07	p=0,244
IPI01012311	DMBT1 Deleted in malignant brain tumors 1 protein	3	1	0,29	p=0,0279

IPI00019502	MYH9 Isoform 1 of Myosin-9	4	1	1,14	p=0,0027
IPI00796316	GSN Isoform 2 of Gelsolin	3	1	5,07	p=0,0085
IPI00166729	AZGP1 Zinc-alpha-2-glycoprotein	2	1	1,99	p=0,0046
IPI00032294	CST4 Cystatin-S	3	1	0,38	p=0,0167
IPI00414676	HSP90AB1 Heat shock protein HSP 90-beta	4	1	0,47	p=0,0279
IPI00947235	IGJ	2	1	1,49	p=0,0462
IPI00028064	CTSG Cathepsin G	3	1	2,36	p=0,0225
IPI00017526	S100P Protein S100-P	2	1	3,14	p=0,0414
IPI00909530	H3F3A Histone H3.3	3	2	1,48	p=0,0112
				2,14	p=0,0187

Accession Number	Název a zkratka proteinu	Počet validních peptidů	Počet peptidů T-test (p<0,05)

Proteinové srovnání dle kazivosti

Srovnání VI: slina od lidí se zdravou denticí vs slina od mladších lidí se zkušeností se zubním kazem
90 validních proteinů v kompilaci vzorků; lidé se zdravou denticí n=4; mladší lidé (CS) n=12

				lidé se zdravou denticí vs mladší lidé (CS)	
IPI00386879	IGHA1	7	1	1,88	p=0,0024
IPI00216984	CALML3 Calmodulin-like protein 3	5	1	0,96	p=0,0422
IPI00060800	ZG16B Zymogen granule protein 16 homolog B	5	1	1,25	p=0,0361

Srovnání VII: slina od lidí se zdravou denticí vs slina od starších lidí se zkušeností se zubním kazem
94 validních proteinů v kompilaci vzorků; lidé se zdravou denticí n=4; starší lidé (CS) n=8

				lidé se zdravou denticí vs starší lidé (CS)	
IPI00218918	ANXA1 Annexin A1	11	1	2,27	p=0,0498
IPI00386879	IGHA1	7	1	3,25	p=0,0054
IPI01010670	A2ML1 Alpha-2-macroglobulin-like protein 1	3	1	1,06	p=0,0410
IPI00909530	H3F3AHistone H3.3	3	1	0,27	p=0,0424
IPI00426051	IGHG2	1	1	0,27	p=0,0471

Proteinové srovnání s ohledem kouření

Srovnání VIII: slina od lidí kuřák pozitivní vs slina od lidé kuřák negativní (bez ohledu na orální zdraví, gender, věk)

103 validních proteinů v kompilaci vzorků; kuřáci n=4; nekuřáci n=19

				kuřáci vs nekuřáci	
IPI00013933	DSP Isoform DPI of Desmoplakin	16	2	0,45	p=0,0070
				0,43	p=0,0040

IPI00027462	S100A9 Protein S100-A9	8	3	0,44	p=0,0347
				0,28	p=0,0085
				0,14	p=0,0392
IPI01011344	ACTG1 Actin, cytoplasmic 2	13	1		p=0,0540
IPI00007047	S100A8 Protein S100-A8	12	1	0,36	p=0,0283
IPI00386879	IGHA1	7	2	0,27	p=0,0460
				0,37	p=0,0305
IPI00026256	FLG Filaggrin	7	1	0,41	p=0,0348
IPI01013544	HSPA1B Heat shock 70 kDa protein 1	5	2	0,32	p=0,0268
				0,42	p=0,0116
IPI00032294	CST4 Cystatin-S	4	2	1,67	p=0,0495
				3,45	p=0,0251
IPI00028064	CTSG Cathepsin G	3	1	0,34	p=0,0429
IPI00017526	S100P Protein S100-P	2	1	0,17	p=0,0146

Příloha 8

Identifikované proteiny v jednotlivých frakcích vzorku lidských slin (supernatant, sediment) a vzorky lidských slin nalačno před vyčištění zubů (Srovnání V (Tabulka 1))

A Kompilace identifikovaných proteinů ve vzorcích supernatantů (Kulhavá, 2020)

Pořadí	Accession Number	Název a zkratka proteinu	MW [kDa]	pI	Scores	Počet peptidů	SC [%]
1	IPI00300786	AMY1C;AMY1A;AMY1B;AMY2A Alpha-amylase 1	57.7	6.5	3566.0	46	84.1
2	IPI00218918	ANXA1 Annexin A1	38.7	6.7	2049.7	29	63.9
3	IPI00745872	ALB Serum albumin	69.3	5.9	1645.7	31	38.9
4	IPI01010737	A2M 165 kDa protein	164.8	6.1	1474.6	25	21.8
5	IPI00021439	ACTB Actin, cytoplasmic 1	41.7	5.2	1436.9	22	54.1
6	IPI00300376	TGM3 Protein-glutamine gamma-glutamyltransferase E	76.6	5.5	1405.5	23	41.3
7	IPI01010670	A2ML1 Uncharacterized protein	161.0	5.4	1363.7	25	23.4
8	IPI00465248	ENO1 Isoform alpha-enolase of Alpha-enolase	47.1	7.7	1303.5	22	60.4
9	IPI00553177	SERPINA1 Alpha-1-antitrypsin	46.7	5.3	1289.5	23	51.0
10	IPI00027462	S100A9 Protein S100-A9	13.2	5.7	1230.5	17	96.5
11	IPI00009650	LCN1 Lipocalin-1	19.2	5.3	1214.1	14	65.9
12	IPI00004573	PIGR Polymeric immunoglobulin receptor	83.2	5.5	1203.8	21	24.6
13	IPI00936444	MUC5B Mucin-5B	590.4	6.2	1196.0	20	4.0
14	IPI00291410	BPIFB1 Long palate, lung and nasal epithelium carcinoma-associated protein 1	52.4	6.9	1167.4	17	45.5
15	IPI00374315	C6orf58 UPF0762 protein C6orf58	37.9	5.7	1122.5	18	52.1
16	IPI00925547	LTF Lactotransferrin	77.9	9.6	1089.6	18	30.1
17	IPI01014238	SERPINB1 Leukocyte elastase inhibitor	38.7	6.2	1083.8	17	61.9
18	IPI00922693	ACTB Actin, alpha skeletal muscle	38.6	5.1	1079.3	1	40.5
19	IPI00166729	AZGP1 Zinc-alpha-2-glycoprotein	34.2	5.7	1032.5	16	52.0
20	IPI00783987	C3 Complement C3 (Fragment)	187.0	6.0	1030.3	20	15.7
21	IPI00903112	LTF Lactotransferrin (Fragment)	76.7	9.5	1020.2	1	29.0
22	IPI00022463	TF Serotransferrin	77.0	7.0	999.5	16	27.2
23	IPI00759806	ENO1 Alpha-enolase	36.9	5.9	970.5	1	59.8
24	IPI00645363	IGHV4-31;IGHG1 Putative uncharacterized protein DKFZp686P15220	51.7	9.0	904.5	14	31.6
25	IPI00022204	SERPINB3 Isoform 1 of Serpin B3	44.5	6.4	903.0	16	44.1
26	IPI00384938	IGHV4-31;IGHG1 Putative uncharacterized protein DKFZp686N02209	52.8	9.5	893.3	1	31.1
27	IPI00386879	IGHA1 Ig alpha-1 chain C region	53.1	6.5	867.2	13	31.8
28	IPI00219018	GAPDH Glyceraldehyde-3-phosphate dehydrogenase	36.0	9.3	852.7	12	46.0
29	IPI00784985	IGK@ IGK@ protein	25.5	6.2	852.4	10	49.4
30	IPI00455315	ANXA2 Isoform 1 of Annexin A2	38.6	8.5	834.4	15	53.7
31	IPI00297056	CRNN Cornulin	53.5	5.7	828.7	8	26.5
32	IPI00784950	IGHA2 IGHA2 Ig alpha-2 chain C region	51.6	5.4	813.8	1	30.9

33	IPI00423462	IGHA1 Putative uncharacterized protein DKFZp686K18196 (Fragment)	56.4	6.6	812.9	1	28.1
34	IPI00032294	CST4 Cystatin-S	16.2	4.8	786.0	11	68.1
35	IPI00296654	BPIFB2 Bactericidal/permeability-increasing protein-like 1	49.1	9.5	757.8	10	29.7
36	IPI00784865	IGK@ IGK@ protein	25.8	5.9	751.1	1	46.2
37	IPI00013890	SFN Isoform 1 of 14-3-3 protein sigma	27.8	4.5	749.5	12	42.3
38	IPI00021841	APOA1 Apolipoprotein A-I	30.8	5.5	746.9	12	54.3
39	IPI00550731	- Putative uncharacterized protein	26.2	9.2	732.6	1	44.4
40	IPI00021263	YWHAZ 14-3-3 protein zeta/delta	27.7	4.6	729.4	10	50.6
41	IPI01013544	HSPA1B;HSPA1A Uncharacterized protein	68.0	5.2	728.6	15	28.7
42	IPI00305477	CST1 Cystatin-SN	16.4	7.6	699.0	6	62.4
43	IPI00060800	ZG16B Zymogen granule protein 16 homolog B	22.7	7.6	681.8	9	52.9
44	IPI00295105	CA6 Carbonic anhydrase 6	35.8	6.6	661.6	8	29.8
45	IPI00013382	CST2 Cystatin-SA	16.4	4.7	638.8	4	60.3
46	IPI00654755	HBB Hemoglobin subunit beta	16.0	6.9	624.7	8	60.5
47	IPI00219757	GSTP1 Glutathione S-transferase P	23.3	5.3	620.4	8	54.3
48	IPI00236554	MPO Isoform H14 of Myeloperoxidase	73.8	10.2	613.4	13	23.5
49	IPI00641737	HP;HPR Haptoglobin	45.2	6.1	575.2	11	23.4
50	IPI01012981	P4HB Protein disulfide-isomerase	55.3	4.5	545.2	9	27.8
51	IPI00426051	IGHG2 Putative uncharacterized protein DKFZp686C15213	51.1	8.9	530.1	2	22.4
52	IPI00896380	IGHM Isoform 2 of Ig mu chain C region	51.8	5.8	524.5	10	23.5
53	IPI01012504	PGD 6-phosphogluconate dehydrogenase, decarboxylating	51.8	7.8	517.6	11	24.9
54	IPI00916434	- Anti-(ED-B) scFV (Fragment)	25.1	9.2	511.9	2	29.0
55	IPI00887169	IGLV1-44 Putative uncharacterized protein	25.0	8.8	501.6	8	40.7
56	IPI00465439	ALDOA Fructose-bisphosphate aldolase A	39.4	9.2	492.2	9	28.8
57	IPI01013543	TPI1;TPI1P1 Triosephosphate isomerase	22.9	6.5	490.2	7	53.1
58	IPI01015050	GSN gelsolin isoform c	78.9	5.6	483.0	9	17.6
59	IPI00339269	HSPA6 Heat shock 70 kDa protein 6	71.0	5.8	471.6	1	14.0
60	IPI00607708	LDHA L-lactate dehydrogenase A chain	36.5	9.1	471.3	9	29.5
61	IPI00304557	BPIFA2 Short palate, lung and nasal epithelium carcinoma-associated protein 2	27.0	5.2	463.8	6	34.1
62	IPI00410714	HBA2;HBA1 Hemoglobin subunit alpha	15.2	9.4	461.5	7	69.7
63	IPI00154742	IGLV2-14;IGLC2 IGL@ protein	24.8	5.9	449.9	2	35.5
64	IPI00007047	S100A8 Protein S100-A8	10.8	6.6	444.3	8	54.8
65	IPI00021828	CSTB Cystatin-B	11.1	7.9	440.9	6	61.2
66	IPI00032325	CSTA Cystatin-A	11.0	5.3	440.7	7	84.7
67	IPI01013763	LCN2 Isoform 2 of Neutrophil gelatinase-associated lipocalin	22.4	9.8	433.6	7	40.9
68	IPI00099110	DMBT1 Isoform 1 of Deleted in malignant brain tumors 1 protein	260.6	5.1	431.8	9	2.7
69	IPI00783810	LPO lactoperoxidase isoform 3 preproprotein	70.9	8.9	422.2	9	19.4
70	IPI00981943	HSPA8 Uncharacterized protein	68.8	5.2	412.5	3	14.4

71	IPI00453473	HIST2H4B;HIST1H4C;HIST1H4J;HIST1H4D;HIST1H4A;HIST2H4A;HIST1H4I;HIST1H4K;HIST1H4E;HIST1H4L;HIST1H4F;HIST1H4H;HIST4H4;HIST1H4B Histone H4	11.4	11.8	407.2	7	51.5
72	IPI00175024	IL1RN Isoform 2 of Interleukin-1 receptor antagonist protein	17.9	5.1	395.1	6	47.8
73	IPI01025882	IGKV3-20 Myosin-reactive immunoglobulin light chain variable region (Fragment)	11.6	9.5	393.0	1	45.9
74	IPI00719373	IGLC1 IGL@ protein	25.1	6.2	388.7	2	31.2
75	IPI00642247	SPRR3 Uncharacterized protein	17.0	9.5	388.2	9	55.3
76	IPI00972963	- Lambda light chain of human immunoglobulin surface antigen-related protein (Fragment)	24.7	5.3	384.4	1	32.3
77	IPI00022488	HPX Hemopexin	51.6	6.6	363.4	7	21.2
78	IPI00216691	PFN1 Profilin-1	15.0	9.4	339.2	6	47.1
79	IPI00019038	LYZ Lysozyme C	16.5	10.6	336.0	8	29.7
80	IPI00759832	YWHAB Isoform Short of 14-3-3 protein beta/alpha	27.8	4.6	333.8	2	29.9
81	IPI00796116	ALDH3A1 Uncharacterized protein	41.6	5.1	331.8	4	15.3
82	IPI00216984	CALML3 Calmodulin-like protein 3	16.9	4.1	318.3	5	38.3
83	IPI01011238	BPIFA1 Uncharacterized protein	25.2	5.6	313.2	4	22.7
84	IPI00982472	TALDO1 Transaldolase	35.3	9.7	309.7	6	19.8
85	IPI00022974	PIP Prolactin-inducible protein	16.6	9.3	302.1	5	39.0
86	IPI00783287	- Immunglobulin heavy chain variable region (Fragment)	13.4	9.4	293.6	2	38.0
87	IPI00220327	KRT1 Keratin, type II cytoskeletal 1	66.0	8.8	290.5	6	9.8
88	IPI00977788	- Similar to Hepatitis B virus receptor binding protein	18.4	9.4	287.9	2	25.6
89	IPI00216298	TXN Thioredoxin	11.7	4.7	267.8	4	42.9
90	IPI00910979	PKM2 pyruvate kinase isozymes M1/M2 isoform e	56.2	9.6	264.6	7	20.7
91	IPI00419585	PPIA Peptidyl-prolyl cis-trans isomerase A	18.0	9.0	262.2	5	39.4
92	IPI00032293	CST3 Cystatin-C	15.8	9.9	257.0	4	23.3
93	IPI00024095	ANXA3 Annexin A3	36.4	5.5	254.1	6	25.1
94	IPI00219806	S100A7 Protein S100-A7	11.5	6.4	252.2	4	42.6
95	IPI00910781	GPI glucose-6-phosphate isomerase isoform 1	64.3	9.6	250.2	7	13.0
96	IPI01015306	TTR Uncharacterized protein	15.1	5.4	249.5	3	29.5
97	IPI00854644	IGKV3D-20 Ig kappa chain V-III region Ti	12.5	4.3	248.7	1	27.6
98	IPI00000874	PRDX1 Peroxiredoxin-1	22.1	9.2	246.8	6	27.1
99	IPI00514669	SH3BGRL3 Uncharacterized protein	9.4	10.0	246.0	4	46.6
100	IPI00220301	PRDX6 Peroxiredoxin-6	25.0	6.0	245.8	4	19.6
101	IPI00942979	TKT Transketolase	67.8	8.5	242.4	5	11.1
102	IPI00298497	FGB Fibrinogen beta chain	55.9	9.3	231.9	4	11.6
103	IPI00419833	HIST1H2BK Histone H2B type 1-K	13.9	10.8	230.6	4	40.5
104	IPI00829956	- Rheumatoid factor C6 light chain (Fragment)	12.5	9.5	229.2	4	37.1
105	IPI00242956	FCGBP IgGFc-binding protein	571.6	5.0	229.1	5	1.1
106	IPI00642632	IGLC7 Ig lambda-7 chain C region	11.3	9.5	228.2	1	41.5
107	IPI01026288	KLK1 Uncharacterized protein	17.4	4.2	219.2	3	20.6
108	IPI00013895	S100A11 Protein S100-A11	11.7	7.5	214.6	4	33.3

109	IPI00022990	STATH Statherin	7.3	9.0	214.5	3	59.7
110	IPI00916818	PGK1 Phosphoglycerate kinase	35.0	9.3	210.2	3	12.8
111	IPI00299729	TCN1 Transcobalamin-1	48.2	4.8	206.9	5	16.4
112	IPI00220146	DSC2 Isoform 2B of Desmocollin-2	93.7	5.2	203.6	4	6.1
113	IPI00985211	- Similar to VH-3 family (VH26)D/J protein	18.8	7.2	201.5	1	18.5
114	IPI00947235	IGJ Uncharacterized protein	8.2	10.0	201.4	3	38.6
115	IPI00010471	LCP1 Plastin-2	70.2	5.2	198.2	5	12.8
116	IPI00877703	FGG Uncharacterized protein	52.3	5.3	195.6	3	10.8
117	IPI00956135	ENDOU Isoform 3 of Poly(U)-specific endoribonuclease	39.6	5.5	190.1	3	15.0
118	IPI00974544	YWHAE Isoform SV of 14-3-3 protein epsilon	26.5	4.6	183.9	1	15.9
119	IPI00023011	SMR3B Submaxillary gland androgen-regulated protein 3B	8.2	10.2	170.8	3	65.8
120	IPI00007797	FABP5 Fatty acid-binding protein, epidermal	15.2	7.5	168.4	4	19.3
121	IPI00909530	H3F3A;H3F3B;H3F3AP6;H3F3AP4 cDNA FLJ52843, highly similar to Histone H3.3	12.9	12.4	166.1	4	31.0
122	IPI00644531	TAGLN2 21 kDa protein	21.1	9.0	165.8	3	20.9
123	IPI00002851	CST5 Cystatin-D	16.1	7.6	165.2	2	14.8
124	IPI00981659	- Similar to Cold agglutinin FS-1 H-chain	14.9	10.4	161.2	1	19.1
125	IPI00021536	CALML5 Calmodulin-like protein 5	15.9	4.1	159.6	3	24.0
126	IPI00847635	SERPINA3 Isoform 1 of Alpha-1-antichymotrypsin	47.6	5.2	156.6	3	11.3
127	IPI00219446	PEBP1 Phosphatidylethanolamine-binding protein 1	21.0	7.8	153.2	3	21.9
128	IPI00816799	- Rheumatoid factor D5 light chain (Fragment)	12.8	9.9	152.2	3	36.4
129	IPI00382577	- Kappa 1 light chain variable region (Fragment)	12.7	9.2	147.0	1	23.3
130	IPI00021304	KRT2 Keratin, type II cytoskeletal 2 epidermal	65.8	8.9	143.3	1	5.9
131	IPI00922330	IGKC Putative uncharacterized protein	9.1	4.3	142.0	1	24.1
132	IPI00783625	SERPINB5 Isoform 1 of Serpin B5	42.1	5.7	141.0	3	10.9
133	IPI00384401	- Myosin-reactive immunoglobulin kappa chain variable region (Fragment)	11.8	9.7	140.1	2	31.2
134	IPI00006560	SERPINB13 cDNA FLJ53019, highly similar to Serpin B13	45.3	5.5	139.8	4	11.5
135	IPI00947307	CP cDNA FLJ58075, highly similar to Ceruloplasmin	108.8	5.4	139.1	3	4.3
136	IPI01013441	PRTN3 Uncharacterized protein	23.6	10.2	139.0	3	12.6
137	IPI01015738	ACTN4 Uncharacterized protein	79.9	4.9	136.7	3	5.5
138	IPI00387120	- Ig kappa chain V-IV region Len	12.6	9.0	136.4	2	23.7
139	IPI00025753	DSG1 Desmoglein-1	113.7	4.8	136.1	2	3.3
140	IPI00952922	KRT13 Uncharacterized protein	38.5	4.8	133.8	3	6.5
141	IPI01014727	- cDNA FLJ51983, highly similar to Phosphoglycerate mutase 1	27.1	9.2	133.4	3	18.0
142	IPI00956602	IGLV1-44 Anti-streptococcal/anti-myosin immunoglobulin lambda light chain variable region (Fragment)	11.6	6.9	130.1	1	26.9
143	IPI00152154	MUC7 Mucin-7	39.1	9.9	129.6	4	11.9
144	IPI00386621	CALM3;CALM2;CALM1 CALM3 protein	16.5	4.2	127.2	2	22.4
145	IPI00552432	PRB2 Basic salivary proline-rich protein 2	40.8	12.1	126.5	1	13.7
146	IPI00009865	KRT10 Keratin, type I cytoskeletal 10	58.8	5.0	125.5	2	6.0
147	IPI00924820	- Similar to Kappa light chain variable region	13.1	9.7	121.9	1	15.0

148	IPI00974112	CRISP3 22 kDa protein	21.6	9.2	120.8	2	5.8
149	IPI00027463	S100A6 Protein S100-A6	10.2	5.2	118.3	3	24.4
150	IPI00081836	HIST1H2AH;HIST1H2AG;HIST1H2AK;HIST1H2AL;HIST1H2AM; HIST1H2AJ;HIST1H2AI Histone H2A type 1-H	13.9	11.3	117.0	2	21.9
151	IPI00976039	NUCB2 Uncharacterized protein	40.3	4.9	113.2	2	8.9
152	IPI00032179	SERPINC1 Antithrombin-III	52.6	6.3	107.8	3	6.5
153	IPI00031547	DSG3 Desmoglein-3	107.5	4.7	106.6	3	3.0
154	IPI00004656	B2M Beta-2-microglobulin	13.7	6.1	106.2)	3	23.5
155	IPI00908762	LGALS3BP Galectin-3-binding protein	46.4	5.0	105.2	3	10.7
156	IPI00465436	CAT Catalase	59.7	7.0	101.0	1	3.6
157	IPI00012024	HTN1 Histatin-1	7.0	9.5	93.8	2	43.9
158	IPI00218131	S100A12 Protein S100-A12	10.6	5.8	90.5	3	43.5
159	IPI00872684	EZR cDNA FLJ54141, highly similar to Ezrin	65.5	5.6	89.6	2	3.1
160	IPI00386755	ERO1L ERO1-like protein alpha	54.4	5.4	86.9	2	6.0
161	IPI00965611	SPARCL1SPARC-like protein 1	61.7	4.6	84.1	1	3.2
162	IPI00947285	SBSN suprabasin isoform 1 precursor	60.5	6.5	80.6	2	5.6

B Kompilace identifikovaných proteinů ve vzorcích sedimentů (Kulhavá, 2020)

Pořadí	Accession Number	Název a zkratka proteinu	MW [kDa]	pI	Scores	Počet peptidů	SC [%]
1	IPI00218918	ANXA1 Annexin A1	38.7	6.7	3179.6	44	63.9
2	IPI00300725	KRT6A Keratin, type II cytoskeletal 6A	60.0	8.9	1904.7	28	37.8
3	IPI00299145	KRT6C Keratin, type II cytoskeletal 6C	60.0	8.9	1872.0	2	37.8
4	IPI00930073	KRT6C Keratin 6E (KRT6E)	60.0	8.6	1664.9	1	35.3
5	IPI01022175	KRT4 Keratin, type II cytoskeletal 4	51.7	6.5	1619.9	24	38.2
6	IPI00009866	KRT13 Keratin, type I cytoskeletal 13	49.6	4.8	1617.8	30	39.5
7	IPI00298057	PPL Periplakin	204.6	5.4	1553.5	27	19.8
8	IPI00910738	KRT6B Keratin, type II cytoskeletal 6B	55.7	9.3	1425.4	2	32.1
9	IPI00293665	KRT6B Keratin, type II cytoskeletal 6B	60.0	8.9	1421.4	2	37.6
10	IPI00217182	DSP Isoform DPII of Desmoplakin	260.0	6.6	1292.6	24	15.4
11	IPI01011344	ACTG1 Actin, cytoplasmic 2	37.4	5.3	1231.1	22	42.0
12	IPI00297056	CRNN Cornulin	53.5	5.7	1213.0	16	46.9
13	IPI00009867	KRT5 Keratin, type II cytoskeletal 5	62.3	8.6	1165.5	12	28.5
14	IPI00023711	EVPL Envoplakin	231.5	6.6	1144.2	24	16.1
15	IPI00220327	KRT1 Keratin, type II cytoskeletal 1	66.0	8.8	1120.2	15	31.8
16	IPI00384444	KRT14 Keratin, type I cytoskeletal 14	51.5	4.9	897.7	12	24.6
17	IPI00021812	AHNAK Neuroblast differentiation-associated protein AHNAK	628.7	5.7	890.3	19	4.6
18	IPI00290857	KRT3 Keratin, type II cytoskeletal 3	64.4	6.1	886.3	5	23.9
19	IPI00217963	KRT16 Keratin, type I cytoskeletal 16	51.2	4.8	764.7	7	28.1
20	IPI00789324	JUP Uncharacterized protein	66.3	5.0	662.5	6	19.1
21	IPI00411765	SFN Isoform 2 of 14-3-3 protein sigma	24.3	4.6	627.5	8	46.8
22	IPI00984879	ACTA2 similar to Actin, aortic smooth muscle	37.3	5.3	573.7	1	19.3
23	IPI00021304	KRT2 Keratin, type II cytoskeletal 2 epidermal	65.8	8.9	500.0	1	7.9

24	IPI00008359	KRT76 Keratin, type II cytoskeletal 2 oral	65.8	9.2	496.2	3	13.8
25	IPI00025512	HSPB1 Heat shock protein beta-1	22.8	6.0	483.0	6	44.4
26	IPI00910597	TGM3 Protein-glutamine gamma-glutamyltransferase E	58.8	6.8	454.0	7	19.7
27	IPI00021263	YWHAZ 14-3-3 protein zeta/delta	27.7	4.6	433.7	4	33.5
28	IPI00930614	KRT13 GUCA1B protein (Fragment)	8.6	5.2	423.4	3	73.3
29	IPI00027462	S100A9 Protein S100-A9	13.2	5.7	408.4	8	84.2
30	IPI00009865	KRT10 Keratin, type I cytoskeletal 10	58.8	5.0	407.9	5	17.8
31	IPI00465248	ENO1 Isoform alpha-enolase of Alpha-enolase	47.1	7.7	401.3	7	19.8
32	IPI00329306	KRT74 Keratin, type II cytoskeletal 74	57.8	8.6	400.0	1	8.5
33	IPI00554711	JUP Junction plakoglobin	81.7	5.7	373.3	3	11.0
34	IPI00478672	SCEL Isoform 2 of Sciellin	75.3	10.0	343.9	7	13.9
35	IPI00453473	HIST2H4B;HIST1H4C;HIST1H4J;HIST1H4D;HIST1H4A;HIST2H4A;HIST1H4I;HIST1H4K;HIST1H4E;HIST1H4L;HIST1H4F;HIST1H4H;HIST4H4;HIST1H4B Histone H4	11.4	11.8	324.6	5	51.5
36	IPI00219018	GAPDH Glyceraldehyde-3-phosphate dehydrogenase	36.0	9.3	323.9	6	28.7
37	IPI00020101	HIST1H2BG;HIST1H2BI;HIST1H2BE;HIST1H2BC;HIST1H2BF;NCALD Histone H2B type 1-C/E/F/G/I	13.9	10.8	315.5	7	57.9
38	IPI00004573	PIGR Polymeric immunoglobulin receptor	83.2	5.5	314.7	6	4.2
39	IPI00026256	FLG Filaggrin	434.9	9.6	282.8	7	2.8
40	IPI00418169	ANXA2 Isoform 2 of Annexin A2	40.4	9.2	278.3	5	23.8
41	IPI00216984	CALML3 Calmodulin-like protein 3	16.9	4.1	260.7	5	30.9
42	IPI00477227	KRT78 Keratin, type II cytoskeletal 78	56.8	5.7	255.9	3	11.5
43	IPI00217468	HIST1H1B Histone H1.5	22.6	11.4	234.5	4	23.0
44	IPI00019038	LYZ Lysozyme C	16.5	10.6	234.4	6	28.4
45	IPI00217465	HIST1H1C Histone H1.2	21.4	11.4	215.2	3	22.1
46	IPI00232492	TRIM29 Isoform Beta of Tripartite motif-containing protein 29	63.8	6.6	214.5	3	5.6
47	IPI00007047	S100A8 Protein S100-A8	10.8	6.6	212.7	4	32.3
48	IPI00028064	CTSG Cathepsin G	28.8	12.1	185.1	3	18.0
49	IPI00300052	KRT84 Keratin, type II cuticular Hb4	64.8	9.0	185.1	1	4.3
50	IPI00879248	GBP6 Uncharacterized protein	58.1	6.0	176.8	3	13.3
51	IPI00060800	ZG16B Zymogen granule protein 16 homolog B	22.7	7.6	175.2	3	15.9
52	IPI00305477	CST1 Cystatin-SN	16.4	7.6	171.2	2	23.4
53	IPI01009809	IVL 51 kDa protein	51.3	4.4	166.3	5	15.3
54	IPI00909570	EEF2 Elongation factor 2	62.8	5.7	162.7	3	4.6
55	IPI00479186	PKM2 Pyruvate kinase isozymes M1/M2	57.9	9.0	159.7	2	10.5
56	IPI00419585	PPIA Peptidyl-prolyl cis-trans isomerase A	18.0	9.0	143.4	2	23.0
57	IPI00410714	HBA2;HBA1 Hemoglobin subunit alpha	15.2	9.4	136.9	4	29.6
58	IPI00940393	EEF1A1 Uncharacterized protein	47.8	9.8	132.2	3	9.5
59	IPI00218528	PKP1 Isoform 1 of Plakophilin-1	80.4	10.0	129.4	2	3.3
60	IPI00926685	TUBB4 cDNA FLJ53341, highly similar to Tubulin beta-4 chain	40.5	4.7	126.3	3	15.2
61	IPI00016513	RAB10 Ras-related protein Rab-10	22.5	9.4	124.2	2	11.5
62	IPI00471928	ATP5A1 ATP synthase subunit alpha	54.5	8.9	116.6	3	7.4

63	IPI00940673	TKT Transketolase	58.9	8.6	114.9	4	6.9
64	IPI01014005	AMY1C;AMY1A;AMY1B;AMY2A AMY1A protein (Fragment)	56.2	9.6	113.2	2	6.5
65	IPI00032294	CST4 Cystatin-S	16.2	4.8	109.7	1	23.4
66	IPI00554773	CA6 Uncharacterized protein	35.3	9.1	106.9	2	6.4
67	IPI00465439	ALDOA Fructose-bisphosphate aldolase A	39.4	9.2	103.7	2	9.3
68	IPI00552768	TXN Uncharacterized protein	9.4	5.7	101.4	1	23.5
69	IPI01025005	RPL6 Uncharacterized protein	25.9	11.1	98.1	1	8.8
70	IPI01021600	TUBA1B Uncharacterized protein	5.3	4.5	97.8	3	81.6
71	IPI00908556	ANXA8L2, similar to Annexin A8	13.6	9.1	95.8	1	13.2
72	IPI00005688	ZNF185Zinc finger protein 185	73.5	6.8	94.6	2	2.6
73	IPI00010214	S100A14 Protein S100-A14	11.7	5.0	81.1	2	19.2

C Kompilace identifikovaných proteinů u vzorků slin - nalačno, před čištěním zubů (Srovnání V)

Pořadí	Accession Number	Název a zkratka proteinu	MW [kDa]	pI	Scores	Počet peptidů	SC [%]
1	IPI01022175	KRT4 Keratin, type II cytoskeletal 4	51.7	6.5	2278.7	38	53.6
2	IPI00009866	KRT13 Keratin, type I cytoskeletal 13	49.6	4.8	1862.5	33	65.3
3	IPI00300725	KRT6A Keratin, type II cytoskeletal 6A	60.0	8.9	1677.0	27	44.0
4	IPI00930073	KRT6C Keratin, type II cytoskeletal 6C	60.0	8.6	1661.1	4	44.3
5	IPI00293665	KRT6B Keratin, type II cytoskeletal 6B	60.0	8.9	1607.3	3	44.0
6	IPI00009867	KRT5 Keratin, type II cytoskeletal 5	62.3	8.6	1353.3	15	40.2
7	IPI00217963	KRT16 Keratin, type I cytoskeletal 16	51.2	4.8	1337.9	21	48.2
8	IPI00008359	KRT76 Keratin, type II cytoskeletal 2 oral	65.8	9.2	1125.8	16	29.9
9	IPI00009865	KRT10 Keratin, type I cytoskeletal 10	58.8	5.0	1063.2	12	28.3
10	IPI00290077	KRT15 Keratin, type I cytoskeletal 15	49.2	4.6	990.0	1	29.4
11	IPI00384444	KRT14 Keratin, type I cytoskeletal 14	51.5	4.9	972.7	6	36.4
12	IPI00220327	KRT1 Keratin, type II cytoskeletal 1	66.0	8.8	941.9	14	25.5
13	IPI00290857	KRT3 Keratin, type II cytoskeletal 3	64.4	6.1	888.4	4	18.5
14	IPI00297056	CRNN Cornulin	53.5	5.7	848.4	10	35.4
15	IPI00013933	DSP Desmoplakin	331.6	6.4	731.8	16	6.0
16	IPI00218918	ANXA1 Annexin A1	38.7	6.7	722.3	12	39.0
17	IPI00027462	S100A9 Protein S100-A9	13.2	5.7	699.8	8	75.4
18	IPI01011344	ACTG1 Actin, cytoplasmic 2	37.4	5.3	691.3	13	41.1
19	IPI00007047	S100A8 Protein S100-A8	10.8	6.6	676.7	12	69.9
20	IPI00300786	AMY1C;AMY1A;AMY1B;AMY2A Alpha-amylase 1	57.7	6.5	661.5	11	28.4
21	IPI00789324	JUP Uncharacterized protein	66.3	5.0	609.2	4	18.3
22	IPI00021304	KRT2 Keratin, type II cytoskeletal 2 epidermal	65.8	8.9	574.2	3	15.2
23	IPI00966829	ALB 69 kDa protein	69.2	6.0	570.8	15	20.2
24	IPI00300376	TGM3 Protein-glutamine gamma-glutamyltransferase E	76.6	5.5	564.9	10	14.6
25	IPI00021812	AHNAK Neuroblast differentiation-associated protein AHNAK	628.7	5.7	560.3	10	2.5
26	IPI00004573	PIGR Polymeric immunoglobulin receptor	83.2	5.5	454.9	6	8.8
27	IPI00936444	MUC5B Mucin-5B	590.4	6.2	445.4	9	1.6

28	IPI00477227	KRT78 Keratin, type II cytoskeletal 78	56.8	5.7	417.0	6	17.1
29	IPI00411765	SFN Isoform 2 of 14-3-3 protein sigma	24.3	4.6	413.0	6	38.0
30	IPI00216984	CALML3 Calmodulin-like protein 3	16.9	4.1	394.7	6	43.0
31	IPI00386879	IGHA1 Ig alpha-1 chain C region	53.1	6.5	359.6	7	12.6
32	IPI01021414	KRT8 Keratin, type II cytoskeletal 8	56.6	5.2	358.3	1	9.2
33	IPI00647704	IGHA1 Ig alpha-1 chain C region	53.3	6.1	357.2	1	12.6
34	IPI00329306	KRT74 Keratin, type II cytoskeletal 74	57.8	8.6	352.8	1	8.5
35	IPI00418169	ANXA2 Isoform 2 of Annexin A2	40.4	9.2	341.8	8	24.4
36	IPI00026256	FLG Filaggrin	434.9	9.6	336.4	7	2.9
37	IPI00784950	IGHA2 Ig alpha-2 chain C region	51.6	5.4	312.4	1	11.1
38	IPI00550731	IGKC Ig kappa chain C region	26.2	9.2	292.9	3	21.3
39	IPI00060800	ZG16B Zymogen granule protein 16 homolog B	22.7	7.6	289.0	5	29.8
40	IPI00019038	LYZ Lysozyme C	16.5	10.6	286.3	6	33.1
41	IPI01013544	HSPA1B;HSPA1A Uncharacterized protein	68.0	5.2	275.9	5	10.3
42	IPI00783987	C3 Complement C3 (Fragment)	187.0	6.0	260.0	5	3.1
43	IPI00300052	KRT84 Keratin, type II cuticular Hb4	64.8	9.0	259.5	1	9.0
44	IPI00103481	KRT72 Keratin, type II cytoskeletal 72	55.8	6.6	254.6	1	9.8
45	IPI00975690	VIM Vimentin variant 3	49.6	5.0	252.5	4	12.5
46	IPI00759806	ENO1 Isoform MBP-1 of Alpha-enolase	36.9	5.9	251.6	6	17.6
47	IPI00784985	IGK@ IGK@ protein	25.5	6.2	249.4	1	19.1
48	IPI00021828	CSTB Cystatin-B	11.1	7.9	247.0	4	53.1
49	IPI00930614	KRT13 GUCA1B protein (Fragment)	8.6	5.2	224.5	1	60.0
50	IPI00021263	YWHAZ 14-3-3 protein zeta/delta	27.7	4.6	207.6	3	15.9
51	IPI01012311	DMBT1 Uncharacterized protein	124.4	5.1	200.9	3	2.3
52	IPI00654755	HBB Hemoglobin subunit beta	16.0	6.9	195.9	4	31.3
53	IPI00013808	ACTN4 Alpha-actinin-4	104.8	5.2	190.5	5	5.6
54	IPI00554696	CA6 Uncharacterized protein	28.7	6.5	186.2	3	12.9
55	IPI00552768	TXN Uncharacterized protein	9.4	5.7	180.9	3	38.8
56	IPI00554711	JUP Junction plakoglobin	81.7	5.7	176.0	3	5.8
57	IPI00453473	HIST2H4B;HIST1H4C;HIST1H4J;HIST1H4D;HIST1H4A;HIST2H4A;HIST1H4I;HIST1H4K;HIST1H4E;HIST1H4L;HIST1H4F;HIST1H4H;HIST4H4;HIST1H4B Histone H4	11.4	11.8	173.5	4	38.8
58	IPI00011692	IVL Involucrin	69.6	4.5	162.8	4	7.6
59	IPI00000874	PRDX1 Peroxiredoxin-1	22.1	9.2	161.2	5	22.1
60	IPI00019502	MYH9 Isoform 1 of Myosin-9	226.4	5.4	159.6	4	3.0
61	IPI00793319	GSTP1 Uncharacterized protein	19.5	5.6	158.2	2	17.2
62	IPI00985064	LOC728638 Conserved hypothetical protein	45.7	4.9	157.6	1	9.1
63	IPI00796316	GSN gelsolin isoform c	77.7	5.4	157.6	3	5.5
64	IPI00007797	FABP5 Fatty acid-binding protein, epidermal	15.2	7.5	155.3	3	18.5
65	IPI00166729	AZGP1 Zinc-alpha-2-glycoprotein	34.2	5.7	145.8	2	11.7
66	IPI00032294	CST4 Cystatin-S	16.2	4.8	142.5	3	23.4
67	IPI00410714	HBA2;HBA1 Hemoglobin subunit alpha	15.2	9.4	138.0	2	15.5
68	IPI00909239	ACTN1 Isoform 2 of Alpha-actinin-1	102.6	5.2	137.4	2	3.0
69	IPI00021536	CALML5 Calmodulin-like protein 5	15.9	4.1	133.0	2	16.4

70	IPI00419215	A2ML1 Alpha-2-macroglobulin-like protein 1	161.0	5.4	132.0	3	2.6
71	IPI00216952	LMNA Isoform C of Prelamin-A/C	65.1	6.4	131.2	3	5.2
72	IPI00022488	HPX Hemopexin	51.6	6.6	130.3	3	8.9
73	IPI00010796	P4HB Protein disulfide-isomerase	57.1	4.6	127.2	3	6.9
74	IPI00414676	HSP90AB1 Heat shock protein HSP 90-beta	83.2	4.8	125.6	4	7.2
75	IPI00479708	IGHM Isoform 2 of Ig mu chain C region	41.2	6.4	125.5	3	8.5
76	IPI00298057	PPL Periplakin	204.6	5.4	125.2	4	2.3
77	IPI00025512	HSPB1 Heat shock protein beta-1	22.8	6.0	125.1	3	14.1
78	IPI00027509	MMP9 Matrix metalloproteinase-9	78.4	5.6	124.1	3	5.4
79	IPI01011970	PGD 6-phosphogluconate dehydrogenase, decarboxylating	50.8	9.1	123.2	3	7.8
80	IPI00947235	IGJ Immunoglobulin J chain	8.2	10.0	122.8	2	27.1
81	IPI00028064	CTSG Cathepsin G	28.8	12.1	121.9	3	10.2
82	IPI00815755	HIST1H2BG;HIST1H2BI;HIST1H2BE;HIST1H2BC;HIST1H2BF HIST1H2BC protein	13.8	10.8	121.5	3	21.4
83	IPI00879248	GBP6 Uncharacterized protein	58.1	6.0	121.0	4	7.6
84	IPI00876888	IGHV4-31;IGHG1 cDNA FLJ78387	51.6	9.3	120.3	3	5.7
85	IPI00847989	PKM2 Pyruvate kinase	50.0	8.7	118.6	3	10.0
86	IPI00965713	FGB fibrinogen beta chain isoform 2 preproprotein	49.9	9.0	117.3	2	6.2
87	IPI00017526	S100P Protein S100-P	10.4	4.6	116.4	2	24.2
88	IPI00022974	PIP Prolactin-inducible protein	16.6	9.3	115.8	3	24.7
89	IPI00909530	H3F3A;H3F3B;H3F3AP6;H3F3AP4 cDNA FLJ52843, highly similar to Histone H3.3	12.9	12.4	115.2	3	17.7
90	IPI00386621	CALM3;CALM2;CALM1 CALM3 protein	16.5	4.2	113.6	1	10.9
91	IPI00908521	BASP1 Isoform 2 of Brain acid soluble protein 1	17.7	4.6	113.2	1	12.1
92	IPI00426051	IGHG2 Putative uncharacterized protein DKFZp686C15213	51.1	8.9	110.3	1	4.3
93	IPI00009650	LCN1 Lipocalin-1	19.2	5.3	107.9	2	12.5
94	IPI00431645	HPR 31 kDa protein	31.4	9.3	105.8	3	10.0
95	IPI00005721	DEFA1;DEFA1B Neutrophil defensin 1	10.2	7.6	101.6	2	19.1
96	IPI00010214	S100A14 Protein S100-A14	11.7	5.0	97.6	2	19.2
97	IPI00013895	S100A11 Protein S100-A11	11.7	7.5	97.2	1	17.1
98	IPI00465436	CAT Catalase	59.7	7.0	91.9	2	3.8
99	IPI00236554	MPO Isoform H14 of Myeloperoxidase	73.8	10.2	90.5	2	5.7
100	IPI00982472	TALDO1 Transaldolase	35.3	9.7	89.0	3	10.1
101	IPI00903112	LTF Lactotransferrin (Fragment)	76.7	9.5	88.4	3	3.4
102	IPI00940393	EEF1A1 Elongation factor 1-alpha 1	47.8	9.8	82.2	1	3.9
103	IPI00027463	S100A6 Protein S100-A6	10.2	5.2	81.6	2	16.7

Příloha 9

Souhlas s účastí na výzkumu a se zpracováním zvláštní kategorie osobních údajů

Dotčená osoba:

Jméno _____

Příjmení _____

Datum narození _____

Účastí na výzkumné studii s názvem "Porovnání proteomického složení slin u lidí se zubními kazy a bez kazu" prováděné Mgr. Lucií Kulhavou, spočívající zejména v bakteriologické a proteomické analýze vzorků slin z dutiny ústní a v následujícím vyhodnocení získaných dat (zejména DNA, proteinové zastoupení), tedy vyplněním následujícího dotazníku zaměřeného na předmětnou problematiku a poskytnutím vzorků slin z dutiny ústní, uděluje dotčená osoba výslovný souhlas se svou účastí na daném výzkumu a potvrzuje, že jí byly poskytnuty veškeré informace jasně a srozumitelně, tedy tak, aby tento její souhlas byl svobodný a informovaný.

Cíl výzkumu: Cílem výzkumu je nalezení rozdílů v bakteriologickém a proteinovém složení slin u lidí s různou zkušeností s onemocněním v dutině ústní především zubním kazem.

Metody a způsob provedení výzkumu: Výzkum je prováděn u osob, které v posledních třech měsících neprodělali antibiotickou léčbu. Odběr je prováděn v ranních hodinách nalačno a před ranním čištěním zubů (za předpokladu vyčištění zubů večer před odběrem způsobem odpovídajícím zvyklostem dotčené osoby), a to nasliněním 2 x do 1,5 ml sterilní ependorky bez zásahu jiné osoby než osoby zúčastněné na výzkumu.

Očekávaný přínos výzkumu: Získání nových poznatků původu vzniku zubního kazu (nalezení rozdílného zastoupení biologicky aktivních látek ve slinách).

Rizika a komplikace odběru vzorku slin: Žádná.

Omezení po odběru vzorku slin: Žádná.

Alternativní možnosti odběru vzorku slin: Odběr vzorků slin nemá v daném případě s ohledem na zaměření výzkumu alternativu.

Dotčená osoba zároveň, svou účastí na předmětné výzkumné studii, potvrzuje, že byla informována o možnosti odmítnout svou účast na výzkumu, a to kdykoliv, i v průběhu jeho provádění.

S ohledem na skutečnost, že předmětem výzkumu je mimo jiné taktéž odběr a využití identifikovatelného lidského materiálu obsahujícího DNA, uděluje dotčená osoba účastí na předmětném výzkumu svůj výslovný souhlas s jeho shromážděním a nakládáním s ním ve smyslu shora popsané metody provádění výzkumu. V této souvislosti taktéž dotčená osoba svou účastí na výzkumu uděluje svůj výslovný souhlas se zpracováním a dalším nakládáním se získanými daty v důsledku provedení bakteriologické a proteomické analýzy vzorků slin z dutiny ústní, a to nejen v souvislosti s jejich publikací v odborných pracích (zejména disertační práce výzkumného pracovníka provádějícího výzkum), ale také v souvislosti s jejich využitím v rámci dalších výzkumů, a to způsobem neumožňujícím jejich přirazení k osobě, které se týkají.

Účastí na předmětné výzkumné studii taktéž dotčená osoba vyjadřuje svůj výslovný souhlas se zpracováním svých osobních údajů, které jsou ve smyslu Nařízení Evropského parlamentu a Rady (EU) 2016/679, obecné nařízení o ochraně osobních údajů, považovány za osobní údaje tzv. zvláštní kategorie (zejména údaje o zdravotním stavu, údaje týkající se mnou podstoupených lékařských zákroků v souvislosti s ústní dutinou a údaje získané v souvislosti s provedenými analýzami, na základě kterých je možná identifikace mé osoby - DNA atd.), a to pro účely provedení výzkumu a jeho vyhodnocení, publikace výsledků v odborných pracích (zejména disertační práce výzkumného pracovníka provádějícího výzkum) a periodikách, a pro účely dalšího využití pro výzkumné účely včetně předání třetí osobě provádějící obdobný či související výzkum, to vše způsobem neumožňujícím jejich přirazení k osobě, které se týkají, a pro dobu nezbytně nutnou. Současně dotčená osoba prohlašuje, že ji byly poskytnuty veškeré informace o zpracování osobních údajů a tyto ji budou poskytnuty kdykoliv na vyžádání.

V ……..… dne …….

_____

podpisDotazník

Odběr vzorků slin z dutiny ústní pro bakteriologické a proteomické analýzy:

Věk:

Pohlaví: ženy – muž

Zdravotní stav: diabetes mellitus, onkologické onemocnění, kuřák, těhotenství

Počet ošetřených zubů:

- **počet sanovaných zubů:**
- **počet proteticky ošetřených zubů**
- **ortodontická léčba: ano- ne**
- **onemocnění dásní: ano – ne**

Preventivní prohlídka zubním lékařem (poslední): (měsíc/rok)

Návštěva dentální hygieny (poslední): (měsíc/rok)

Poslední ošetření u zubního lékaře (sanace chrupu, extrakce zubu...):

Chodím pravidelně dvakrát ročně na preventivní prohlídku k zubnímu lékaři?: ano – ne

Které dentální pomůcky používáte (zubní kartáček, mezizubní kartáčky, dentální niť…):

Stravovací návyky (vegetariánství, bezlepková dieta…):

Příloha 10

Podmínky odběru vzorku slin u vybrané skupiny lidí, kteří byli zahrnuti do srovnávacích studií

Definované podmínky odběru:

1/ Výběr vhodného kandidáta. Odběr slin od lidí, kteří neprodělali v posledních 3 měsících antibiotickou léčbu (pokud se jednalo o odběry slin pro bakteriologické analýzy).

2/ Provést souhlas s účastí na výzkumu a se zpracováním zvláštní kategorie osobních údajů (Příloha 3). A provést poučení o odběru (případně kandidáta poučit a vybavit sterilními zkumavkami domů).

2/ Večer před odběrem si kandidát musí vyčistit zuby večer (standardním postupem, jako vždy svými dentálními pomůckami, žádné další přípravky oproti normálnímu čištění).

3/ Ráno odběr nalačno (pokud se jednalo o odběry i pro bakteriologické analýzy), nebo po vyčištění zubů (minimálně však půl hodiny po vyčištění a maximálně do 2 hodiny po vyčištění) a také nalačno. Odběr vzorku celkové sliny (odběr si provádí kandidát sám v místnosti, v klidu, bez mluvení).

4/ Kandidát nasliní do sterilní zkumavky 2x 1ml vzorek své celkové sliny.

5/ Kandidát doplnit informace do dotazníku (součást souhlasu s účastí na výzkumu).

6/ Vzorek pro další zpracování uložen do mrazáku -20 °C.