Faculdade de Medicina da Universidade de São Paulo

Bruno Fernandes Matuck

Análise da presença do SARS-CoV-2 e dos receptores ACE2, TMPRSS2 nas glândulas salivares e tecidos periodontais: uma avaliação biomolecular, imunohistoquímica e de ultra-estrutura em casos fatais da COVID-19

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*Ao meu amado filho **Gabriel**, que demonstra diariamente que os bons momentos da vida estão dentro do seu abraço.*

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*Aos meus pais, **Regina** e **José Eduardo** por todo o esforço e dedicação para que essa caminhada fosse trilhada ao meu modo.*

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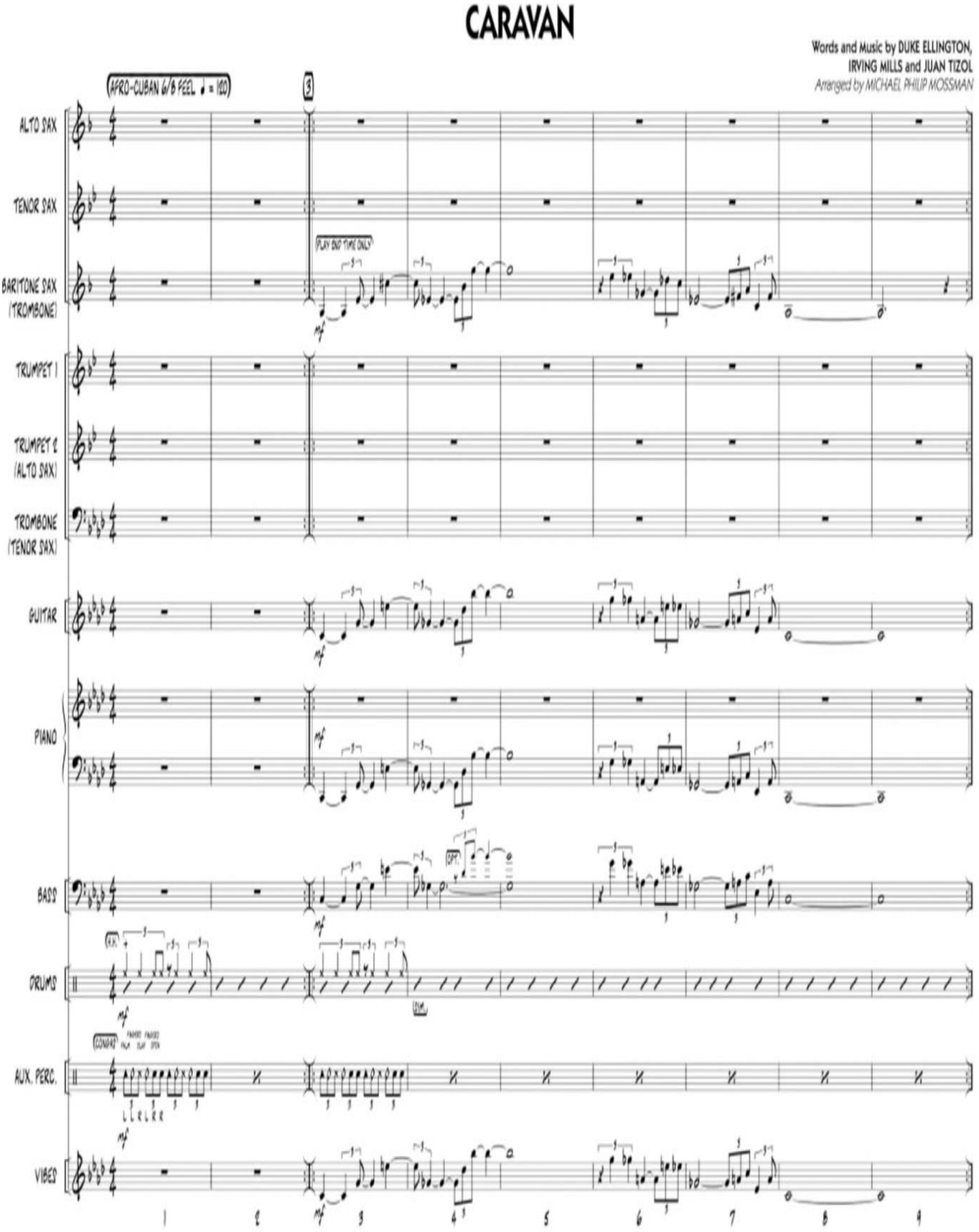
O primeiro ato Concerto para piano nº 5, adagio, Beethoven
Segundo ato Duke Ellington, Caravan



RESUMO

Matuck BF. *Análise da presença do SARS-CoV-2 e dos receptores ACE2, TMPRSS2 nas glândulas salivares e tecidos periodontais:* uma avaliação biomolecular, imunohistoquímica e de ultra-estrutura em casos fatais da COVID-19 [tese]. São Paulo: Faculdade de Medicina, Universidade de São Paulo; 2022.

Resumo: O início de uma nova doença, cujo epicentro se deu em Wuhan - China - com sintomas similares a de uma pneumonia com alto potencial de disseminação, fez com que a Organização Mundial de Saúde decretasse o status de pandemia a está nova doença, chamando a, então, de COVID-19. O agente causador dessa doença foi identificado como um vírus da família *coronoviridae* denominado SARS-CoV-2. Tal patógeno apresenta como seu órgão alvo o pulmão, instalando um dano alveolar difuso e consequentemente a falência respiratória aguda levando milhares de pessoas a óbito através do mundo. Assim como outros vírus da família *coronoviridae*, o SARS-CoV-2 utiliza-se de receptores ACE2 e TMPRSS para ligar-se as células do hospedeiro, apresentando assim intenso tropismo por tecidos epiteliais. Neste racional o objetivo deste trabalho foi aprofundar o conhecimento sobre as interações do SARS-CoV-2 e os nichos epiteliais da cavidade oral a partir de autópsias de pacientes que foram a óbito por complicações da COVID-19, desenvolvendo novas metodologias de coletas e reflexões sobre o papel desta técnica na odontologia com diferentes ensaios. Foram realizadas 40 autopsias minimamente invasivas, coletando múltiplos sítios da cavidade oral com o intuito de identificar e caracterizar a presença do SARS-CoV-2 em tecidos de glândulas salivares e periodonto, assim como, avaliar clinicamente potenciais lesões orais. Por fim, avaliar a interação de agentes descontaminantes do vírus nos tecidos da cavidade oral e demonstrar a importância da utilização das autopsias para a odontologia em diversas abordagens metodológicas. Foram realizadas análises biomoleculares dos tecidos glandulares e periodontais sendo as amostras positivas em 5/7 periodontos e 22/35 das glândulas salivares maiores e 80% das menores. A avaliação IHC anti-SARS-CoV-2 foi positiva 55% dos casos. As reações de imunopostividade para os receptores ACE2 e TMPRSS2 foram francamente positivas em todos os casos de tecido periodontal e glândulas salivares. As avaliações das lesões orais se mostraram majoritariamente como coinfecções decorrentes da deterioração da saúde geral dos pacientes. Visto a cinética viral nos nichos orais, os relatos de casos corresponderam com o racional viral, não tendo capacidade sustentada de erradicar o vírus das amostras avaliadas. A utilização das autópsias para avaliação dos tecidos orais se mostrou uma técnica segura e que permite uma avaliação ampla das amostras, assim como, das interações do mesmo com outros órgãos.

Descritores: COVID-19; SARS-CoV-2; Glândula salivar; Autópsia; Tecido periodontal; Patologia oral.

ABSTRACT

Matuck BF. *Analysis of SARS-CoV-2 and ACE2, TMPRSS2 receptors in salivary glands and periodontal tissues*: a biomolecular, immunohistochemical, and ultrastructural evaluation from COVID-19 fatal cases [thesis]. São Paulo: "Faculdade de Medicina, Universidade de São Paulo"; 2022.

Resume: The beginning of a new disease, in Wuhan – China – that presents pneumonia symptoms and high transmissibility, made the World Health Organization decree a pandemic status, calling this disease COVID-19. The pathogen of this disease was a new virus, named SARS-CoV-2. The virus's main targets are the lung epithelial cells causing diffuse alveolar damage and consequently respiratory distress, leading thousands of people to death. Like other coronaviridae viruses, the SARS-CoV-2 uses ACE2 and TMPRSS2 receptors to bond into membrane host cells, showing intense epithelial tropism. The aim of this research is to understand and create knowledge about the relation between the SARS-CoV-2 and oral epithelial niches based on autopsies from COVID-19 deceased patients, developing new sample methodologies and insights about the autopsy role in dentistry in several methodological approaches. We performed 40 minimally invasive autopsies, sampling multiple oral cavity sites aiming to identify and characterize the SARS-CoV-2 presence in salivary glands and periodontal tissues, as well as, evaluate some clinically evident oral lesions. Finally, evaluate the decontaminate potential from chemical agents and show the value of autopsies in dentistry science. We did analyze tissues from salivary glands and gingiva using biomolecular assays that resulted positive for SARS-CoV-2 in 5/7 periodontal tissues and 22/35 major salivary glands and 80% of minor ones. The immunohistochemistry analysis showed positivity in 55% of the cases. Immunopositivity for ACE2 and TMPRSS2 was frankly positive in all cases involving periodontal and glandular tissue. The evaluation of oral lesions showed in the vast majority as coinfections that appear in recurrence of the poor health status of the patients. The case reports using different methods of decontamination showed transient results, with recontamination during time in the analyzed sample. The use of autopsies to evaluate oral samples seems to be a secure method and allows us an overview of oral and systemic health, the use of new biomolecular techniques must be employed for further validation of new diseases using samples coming from deceased patients

Descriptors: COVID-19; SARS-CoV-2; Salivary gland; Autopsy; Periodontal tissue; Oral Pathology.

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Sumário

Lista de Abreviaturas e Siglas

OMS	Organização Mundial de Saúde
COVID-19	Coronavirus Disease 2019
SARS	Síndrome Respiratória Aguda Grave
MERS	Síndrome Respiratória do Oriente Médio
RT-PCR	Real-Time Reverse Transcriptase Polimerase Chain Reaction
IL	Interleucina
GSCF	Fator de Estimulação de Colônia de Granulócitos
IP	Imunoprecipitação
MCP	Proteína quimioatratante de Monócitos
MIP	Proteína inflamatória de Macrófagos
TNFa	Fator de Necrose Tumoral Alpha
ACE2	Enzima Conversora de Angiotensina 2
TMPRSS2	Protease Transmebranar Serina 2
HCFMUSP	Hospital das Clínicas da Faculdade de Medicina da USP
TCLE	Termo de Consentimento Livre e Esclarecido
EDTA	Ácido Etilenodiamino Tetra-acético
DAB	Diaminobenzina
US-MIA	Autopsia Minimante Invasiva Guiada por Ultrassom
SG	Salivary Gland
ICU	Intensive Care Unit
PISA	Plataforma de Imagens na Sala de Autópsia
HCA	Human Cell Atlas
OCBN	Oral & Craniofacial Bionetwork
SUS	Sistema Único de Saúde
ESB	Equipe de Saúde Bucal
TIC	Tecnologia da Informação e Educação
IHC	Imunohistoquímica

CAPÍTULO 1 – INTRODUÇÃO

1.Introdução

COVID-19

No final de 2019, surgiram relatos de pacientes infectados por um novo patógeno viral, que evoluíam para insuficiência respiratória aguda e apresentavam alta taxa de óbito, o que levou a Organização Mundial de Saúde (OMS) a decretar estado de alerta frente a uma nova doença. Essa infecção respiratória foi chamada de COVID-19 (Coronavirus Disease 2019), sendo seu agente causador um vírus da família *coroviridae* denominado SARS-CoV-2. Desde a identificação da doença até o momento de 2022, o número global de infectados ultrapassou 510 milhões, levando a mais de 6 milhões de óbitos [1].

Em 26 de fevereiro, foi diagnosticado o primeiro caso de COVID-19 no Brasil em um paciente procedente da Itália. Com o objetivo de conter a progressão da doença no Brasil, o Ministério da Saúde (MS) recomendou o isolamento social como medida preventiva. A despeito das medidas instituídas, foi ultrapassada a marca de 22 milhões de casos confirmados no país. [2].

Os primeiros pacientes infectados identificados com a doença apresentaram características de uma síndrome respiratória aguda semelhante a outros dois quadros pandêmicos ocorridos no século XXI, sendo eles o SARS (Síndrome respiratória aguda grave) (2002) e a MERS (Síndrome respiratória do oriente médio) (2011)[3]. Associado aos sintomas respiratórios, os pacientes podiam apresentar febre, mialgia, queixas gastrointestinais, cefaleia dentre os mais diversos sintomas.

Parte desses pacientes, muito rapidamente, necessitavam de suporte ventilatório devido ao grave quadro respiratório e, dessa forma, os primeiros sequenciamentos e a identificação do agente SARS-CoV-2 ocorreram a partir de lavados bronco alveolares de 6 pacientes que trabalhavam onde se imagina ser o epicentro da doença, uma feira livre na qual o contato e o consumo de animais silvestres se dava de forma distinta [4].

A avaliação biomolecular do vírus identificou uma relação de 96,5% de semelhança de nucleotídeos com um coronavirus, e 80% com de semelhança com o SARS presente em morcegos, tratando-se de um betacoronavirus[5].

Durante a progressão da pandemia, o aumento do número casos e a diversidade de transmissão permitiram o aparecimento de novas variantes genéticas do vírus. As mudanças caracterizaram-se por uma alteração no comportamento viral, refletida nos sintomas, na capacidade de transmissão e nos desfechos clínicos[6]. Majoritariamente, temos cinco variantes de preocupação circulantes – B.1.1.7 / Alpha; B.1.351 / Beta; P.1 / Gamma e B.1.617 / Delta / Omicron [7]. Houve constatação da presença da variante B.1.1.7 no Brasil em dezembro de 2020 [8], embora nesta data ainda houvesse predominância das variantes P.1 e delta [9;10]. O sequenciamento de genoma completo do SARS-CoV-2 e o compartilhamento das sequências em bancos de dados, como vem sendo feito em velocidade sem precedentes, é de extrema importância para a descrição de novas variantes. Ademais, a partir do conhecimento da sequência, pode-se adicionar ferramentas complementares como RT-PCR, para acompanhar e monitorar a circulação dessas variantes [11]

Os coronavírus, como o SARS-CoV (síndrome respiratória aguda grave) e MERS-CoV (síndrome respiratória do Oriente Médio), desenvolveram estratégias para diminuir ou retardar a produção de mediadores inflamatórios, desencadeando respostas inflamatórias exuberantes e levando a quadros pulmonares graves [12;13;14]. Acredita-se que a resposta imune desregulada do hospedeiro e a produção de citocinas inflamatórias, conhecida como “tempestade de citocinas”, se correlacionem com a gravidade da doença e com mau prognóstico durante a infecção por SARS-CoV e MERS-CoV [12;15;]. Citocinas e quimiocinas pró-inflamatórias, como CCL-2, CCL-3, RANTES, IL-2 e IL-8, foram altamente expressas durante a infecção por MERS-CoV. Estudos recentes relataram que casos graves de COVID-19 exibem níveis plasmáticos aumentados de IL-2, IL-6, IL-7, IL-10, GSCF, IP-10, MCP1, MIP-1ª e TNFα em comparação com casos leves, indicando que a resposta inflamatória mediada por liberação de citocinas é crítica na progressão da COVID-19 [4;16]. A análise do transcriptoma de amostras de lavado broncoalveolar mostrou um perfil distinto de citocinas inflamatórias para infecção por SARS-CoV-2, mostrando associação entre a patogênese por COVID-19 e a liberação excessiva de citocinas como CCL2 / MCP-1, CXCL10 / IP-10, CCL3 / MIP-1ª e CCL4 / MIP1B [17].

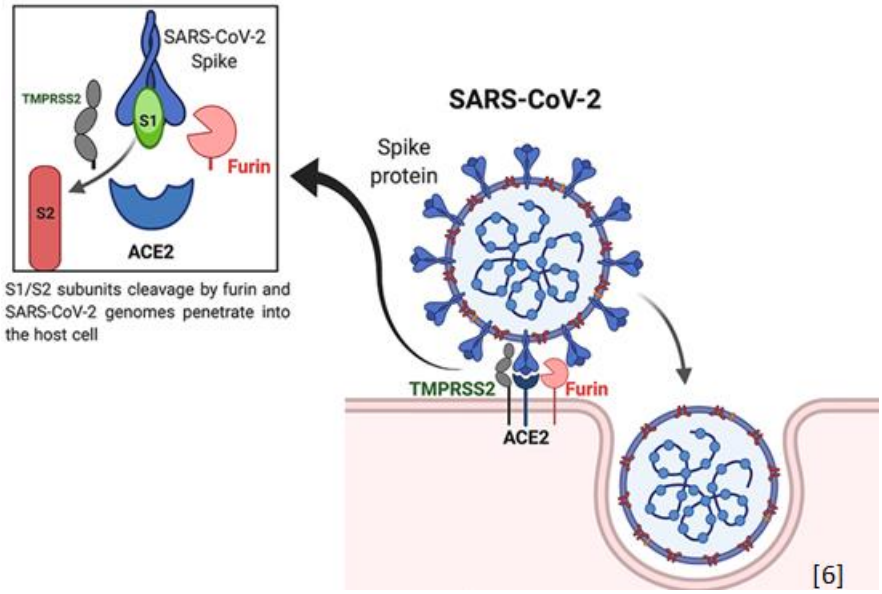
Outras infecções respiratórias causadas por vírus também induzem a produção de ampla variedade de citocinas, que podem estar associadas tanto à recuperação quanto à complicação da infecção. O vírus da influenza e o rinovírus humano, por exemplo, estão associados com uma maior produção de IL-1, IL-6, IL-8, principalmente, além de IFN-1β, IFN-γ e TNF-α [18;19]. Essas citocinas, principalmente a IL-6, quando produzidas em excesso, estão associadas à resposta inflamatória, contribuindo para a severidade da doença respiratória viral. Recentes estudos também mostram envolvimento de citocinas, como IL-17, no processo de agravamento da doença em infecções por Influenza A. Essas também parecem estar envolvidas na infecção do SARS-CoV-2 [19;20;24]. Uma análise combinatória das citocinas e quimiocinas em amostras clínicas pode ser relevante na avaliação da infecção ocasionada por um ou mais vírus respiratórios nesse contexto [20].

Marcadores do processo inflamatório, tais como citocinas e quimiocinas, podem ser medidos na excreta salivar, assim como nos tecidos glandulares, apresentando potencial diagnóstico e prognóstico tanto de doenças da cavidade oral[21;22] como sistêmicas[23;24], abrindo assim a possibilidade do uso deste fluído no para entendimento do perfil inflamatório da COVID-19, diferenciando possivelmente casos leves, moderados e avançados [25;26;27].

CORONAVÍRUS

Os vírus da família *coronaviridae* apresentam, aproximadamente, 30 mil bases em uma fita única de RNA envelopado – não segmentado – sendo assim, a família de vírus de maior código genético corrente no mundo. Seu nome se dá graças a presença de espiculas (Spike) em sua superfície, dando o aspecto de coroa ao patógeno. Tais projeções estão ligadas às proteínas do envelope viral[5], as quais são importantes para o ciclo de replicação. Glicoproteínas ligadas ao envelope viral são capazes de se unir a uma proteína da membrana celular de humanos, a proteína conversora de angiotensina 2 (ACE2)[28]. Para que tal ligação e a invasão celular se inicie é necessário que haja a clivagem da subunidade *spike* por uma enzima, comumente achada nos tecidos pulmonares e do trato respiratório, chamada furina. Após o remodelamento da proteína spike, deixando exposta a subunidade S2 desta proteína, o vírus consegue ser

endocitado pela célula, sendo essa uma via de entrada do agente no corpo humano (como pode ser observada na figura abaixo). A partir de então, o vírus passa a utilizar a sua enzima transcriptase reversa, bem como toda a maquinaria celular para a replicação, montagem e eliminação de novas partículas virais.



A relação entre o vírus e ACE2 inicia uma resposta inflamatória local que pode ser avaliada em todos os sistemas que expressam tais proteínas. Salienta-se que a expressão de ACE2 se dá em capilares e vasos de todos os sistemas, transformando assim, qualquer órgão em um alvo potencial para o vírus[29].

Na cavidade bucal, a expressão de ACE2 vem sendo extensamente estudada como potencial reservatório e porta de entrada do SARS-CoV-2. Alguns estudos já demonstraram a presença do receptor ACE2 nos ductos de glândula salivar menor, assim como, no tecido periodontal e na língua.

Trabalhos realizados em animais demonstraram a presença de outro coronavírus, o SARS, dentro de células epiteliais ductais de glândulas salivares menores[30].

As repercussões que a presença do vírus pode gerar dentro dos tecidos bucais ainda é controversa. A presença do vírus em tecidos da cavidade bucal pode representar, além de uma ameaça às células locais, um reservatório com alto potencial de contaminação, uma vez que o vírus é transmitido entre seres humanos através de gotículas dispersadas durante conversação, espirros e outras formas de disseminação de aerossóis e secreções orotraqueais[31;32;33;34].

A literatura converge no que se refere à capacidade do SARS-CoV-2 em resistir em superfícies contaminadas por estas secreções citadas. Sua dispersão

em aerossóis pode manter o vírus viável por até 4 horas em ambientes fechados e até 72 horas em superfícies plásticas[35], transformando a COVID-19 em uma doença que não se transmite apenas pessoa-pessoa, como também de forma indireta, contaminado superfícies e objetos que serão posteriormente manipulados[36].

O notado sucesso de disseminação da doença se baseia em dois aspectos: o primeiro deles, relacionado ao grande tempo de incubação em seres humanos, que mantém portadores assintomáticos/pré-sintomáticos como vetores de transmissão do vírus por um longo período, sem que os mesmos tomem quaisquer cuidados preventivos[37]. O segundo aspecto relaciona-se à capacidade do vírus de se espalhar através de gotículas, uma habilidade eficaz de transmissão já utilizada por outros vírus respiratórios, que culmina na disseminação da doença em grandes adensamentos populacionais, mesmo em pacientes assintomáticos ou pré sintomáticos[38].

Estima-se que pacientes assintomáticos podem ter sido responsáveis por mais de 80% da transmissão na Alemanha, assim como, mais de 60% na China e 40% em outros locais no sudeste asiático [40].

A capacidade de transmissão está diretamente relacionada com a carga viral no fluido transmissor, que apresenta seu pico de um a dois dias antes do início dos sintomas, e vai diminuindo com o passar do tempo. A literatura relata casos em que a carga viral pode ser encontrada em pacientes com 40 dias após o início dos sintomas[39].

Uma vez que o pulmão é o órgão alvo primário do SARS-CoV-2 e que a contaminação das gotículas, normalmente, se dá por fluidos pulmonares e de vias aéreas superiores (tais quais coriza e restos epiteliais do trato respiratório), que se misturam com excretas salivares, uma possível infecção da excreta das glândulas salivares por si poderia explicar a transmissão em pacientes assintomáticos [41].

GLÂNDULA SALIVAR

A saliva é uma secreção de composição dinâmica que é, majoritariamente, formada por excreta das glândulas salivares maiores e menores. Sendo as glândulas salivares menores responsáveis por 70% dessa secreção[42]. O produto final da saliva se dá a partir da diluição das secreções

salivares, fluido gengival crevicular (secretado pelo periodonto e gengiva inseria) e secreções das vias aéreas superiores carregadas pelo epitélio ciliado respiratório até a cavidade oral.

Devido a esta diversidade na composição da saliva, discute-se intensamente na literatura se a detecção de vírus na saliva se apresenta como uma maneira segura de diagnóstico em massa para doenças infecciosas, particularmente considerando que os indivíduos apresentam proporções diferentes desses componentes na saliva, assim como alguns pacientes assintomáticos podem apresentar menor envolvimento de secreções pulmonares na secreção salivar final. [43]. De qualquer forma, a despeito de limitações específicas, em certas doenças infecciosas a identificação viral a partir da saliva tem se mostrado uma ferramenta útil e segura.

O padrão ouro para detecção do COVID-19 em seres humanos é a coleta de swab naso-orofaríngeo. Tal procedimento apresenta sensibilidade relativa e alguns questionamentos relacionados ao intenso desconforto e, sobretudo, ao modo de operação da coleta, que necessita, para ser efetiva, de um profissional de saúde treinado para a sua realização, expondo o mesmo ao agente infeccioso em questão.

A utilização da saliva como alternativa para detecção de vírus respiratórios pode apresentar-se como uma saída para tais questionamentos, sendo um procedimento menos invasivo e menos desconfortável, de fácil execução, onde os próprios pacientes podem realizar a coleta - diminuindo o risco de transmissão entre os profissionais. Dessa forma, seria possível a coleta em massa na população. [44]

Inúmeros estudos mostram a eficiência do uso de saliva no diagnóstico da COVID-19, com sensibilidade e especificidade variando entre 80% a 100%, com alguns estudos mostrando inclusive maior eficiência da saliva comparada ao swab de naso-orofaringe. [45;46;47]

Como anteriormente descrito, os coronavírus se utilizam dos receptores de angiotensina (ACE2) para seu processo de internalização nas células. Tais receptores estão presentes nos ductos das glândulas salivares. [48]

A relação entre a presença do receptor e o coronavírus já foi demonstrada em modelo animal de macacos Rhesus utilizando imunoflorescência, sugerindo que tais células possam ser a porta de entrada do patógeno nos tecidos. [48]

É importante entender se o vírus em questão está presente nos diversos sítios que secretam a saliva. Até então, não haviam estudos que indicassem a presença do SARS-CoV-2 nas estruturas acinares ou ductais das glândulas salivares.

PERIODONTO

O periodonto é o tecido de sustentação do dente, representado pelo osso alveolar, gengiva inserida, espaço biológico e ligamento periodontal. O receptor ACE2 também está expresso na gengiva e no ligamento periodontal de fibroblastos. Estudos sugerem que o aumento de proteases em pacientes com periodontite crônica pode aumentar o risco da invasão viral nas células desse tecido, uma vez que aumenta a angiogênese local e, consequentemente, a quantidade de receptores de angiotensina.

Outros vírus como Herpes simplex araf (HSV), Esptein-Barr (EBV) e Human Cytomegalovirus (HCMV) [24] foram detectados em tecido periodontais gengivais [49], na placa subgengival [50] e no fluido crevicular – importante sítio que contribui para a composição final da saliva [51]. Pouco se sabe sobre o mecanismo de aparecimento do vírus nesses tecidos, mas acredita-se que possa haver invasão direta a partir dos queratinócitos do epitélio de revestimento da cavidade oral, ou ainda pela migração a partir da corrente sanguínea e liquido intersticial levando o patógeno até os tecidos periodontais. [52]

A literatura levanta a questão se, em pacientes com doenças periodontais, estes tecidos poderiam representar nichos de replicação e armazenamento do SARS-CoV-2, particularmente considerando os níveis elevados de citocinas normalmente observados em pacientes com periodontite. [53]

RECEPTORES

Para entender a fisiopatologia do SARS-CoV-2 se faz necessário entender como seus domínios interagem com receptores de membrana das células eucariontes. O primeiro indicio de que os vírus da família *coronoviridae* utilizavam os receptores ACE2 para sua entrada nas células se dá a partir de um teste realizado em camundongos *knock-out* para os receptores ACE2 infectados

pelo SARS-CoV. Tal estudo mostrou que os camundongos do grupo teste apresentavam menor taxa de infecção celular e consequentemente menor dano alveolar pulmonar do que os animais do grupo controle [54].

Para entender sua relação de infectividade celular, estudos utilizando microscopia eletrônica de transmissão mostraram que a afinidade do SARS-CoV-2 para com o receptor ACE2 é de 10 a 20 vezes maior do que a afinidade do SARS-CoV para com o mesmo [55].

A capacidade dos tecidos em expressar os receptores de angiotensina está diretamente relacionada com o organotropismo celular que o patógeno exerce. Estudos mostraram a expressão, através de imunohistoquímica, dos receptores em 15 diferentes órgãos do corpo humano adquiridos através de biópsias de 93 pacientes, neste estudo nota-se a intensa expressão nos pneumócitos e nos enterócitos [56]. Nas células pulmonares, uma análise de RNA de célula única demonstrou a presença do receptor em franca predileção nos pneumócitos do tipo 2, representando mais de 80% da expressão total do pulmão [57]. Outra análise a partir da expressão de mRNA de múltiplos órgãos mostrou a presença dos receptores ACE2 de forma mais expressiva em pulmões, assim como, sistema renal, cardiovascular e trato gastrointestinal [58].

Esta intensa afinidade do SARS-CoV-2 com os tecidos pulmonares explica, em parte, porque o pulmão é considerado o órgão alvo do vírus, assim como o intenso dano alveolar difuso e atipia celular.

Uma vez dentro da célula, o vírus seguirá seu ciclo de replicação sendo assim, novamente, liberado no parênquima do órgão infectado, causando uma liberação de citocinas inflamatórias, particularmente a IL-6, IL-8 e TNF. Tal liberação atrai por quimiotaxia o recrutamento de neutrófilos e macrófagos para o tecido infectado [59]. Dependendo da intensidade da infecção e do órgão em questão, a resposta inata do sistema imune leva a uma destruição do parênquima. No pulmão, a presença de uma injuria mediada por neutrófilos leva a um edema pulmonar e destruição dos alvéolos. Algo semelhante se dá no sistema renal, ou mesmo nos cardiomiócitos. A gravidade da lesão tecidual tem relação direta com o tropismo do vírus e a quantidade de receptores no órgão alvo [60].

Na cavidade oral, existem poucos estudos sobre a presença do receptor ACE2. Alguns autores inferem que a presença dos receptores pode ser alta, considerando sua distribuição em outras áreas do trato gastrointestinal,

conforme supracitado [61]. Análises de mRNA, com uma pequena amostra de pesquisa, em tecidos normais demonstraram a presença dos mesmos, porém em número menor do que em outros órgãos alvos, como pulmões, rins, coração e esôfago [62].

A proteína TMPRSS2 apresenta-se como passo facilitador e um co-receptor para a entrada do SARS-CoV-2 no arcabouço celular. Faz parte da família das serinas, tendo assim atividade proteolítica intrínseca. A TMRPSS2 desencadeia um processo central na entrada de diversos vírus. Sabe-se que o SARS-CoV-2 não é o único vírus que utiliza da TMPRSS2 para iniciar sua ligação com a membrana celular. O mesmo pode ser observado em outros vírus como Influenza, MERS e SARS-CoV [63].

Conforme dito, TMPRSS2 tem a capacidade de clivar o subdomínio S do envelope viral, expondo assim os resíduos ligáveis ao receptor ACE2. O SARS-CoV-2 pode utilizar outros receptores secundários, tais quais, as catepsinas B e L, porém apenas o TMPRSS2 é essencial para que a entrada do vírus se torne de fato patológica e potencialmente contaminante [64].

Diferentemente do SARV-CoV, o SARS-CoV-2 apresenta certa dependência desse receptor de entrada, pois o vírus sofre a pré-clivagem de outra proteína chamada Furina, presente no epitélio de revestimento da cavidade oral, tornando o envelope viral menos detectável para outras proteases e tornando, desta forma, o reconhecimento viral específico para a TMPRSS2. Já outros vírus respiratórios dispensam esse processo, sendo facilmente ligados a outros co-receptores [65].

Na cavidade oral, a coexpressão dos receptores ACE2 e TMPRSS2 é bastante rara. Em uma análise de células únicas, a presença dos receptores em glândulas salivares maiores é discreta frente a presença nas glândulas salivares menores. O mesmo acontece nos tecidos gengivais quando comparadas as camadas basais às supra basais, sendo estas mais coexpressas do que as basais [66].

1.2 Objetivo

OBJETIVOS INICIAIS

Tendo em vista a convergência da literatura para embasar a hipótese, o objetivo deste trabalho foi identificar e caracterizar a presença do SARS-CoV-2 e dos receptores ACE2 e TMPRSS2 nas glândulas salivares maiores e menores e tecidos periodontais de casos fatais de COVID-19.

Tal caracterização se deu a partir de uma análise histopatológica, comparativa com os padrões de normalidade, de tecidos removidos em autópsias de pacientes que foram a óbito em decorrência de complicações da COVID-19.

Para sedimentar a relação entre as alterações e a presença do patógeno, outras metodologias foram empregadas buscando compor um quadro plural de evidências sobre a presença e as alterações do SARS-CoV-2 nesses tecidos.

Esta compilação de artigos terá como objetivo demonstrar, nos tecidos que compõe a saliva, a presença do SARS-CoV-2 e dos receptores que possibilitam sua entrada.

OBJETIVOS COMPLEMENTARES

Ao longo do desenvolvimento do projeto, buscando atingir os objetivos iniciais, uma série de conexões foram feitas com diferentes pesquisadores em áreas correlatas. A vivência de um grande número de casos, tanto em ambiente clínico como de autópsia, possibilitou o desenvolvimento de outros trabalhos, aqui compilados, que permitiram uma visão mais ampla e multidisciplinar do tema central do trabalho. Assim, os artigos adicionalmente compilados, com a participação do pós-graduando, levaram a objetivos adicionais nesta tese que complementam os iniciais no entendimento do processo de pesquisa e investigação empreendidos durante o desenvolvimento do presente projeto.
Desta forma:

As relações citopáticas e manifestações clínicas observadas durante as autópsias também serão discutidas, afim de entender se as mesmas são provenientes diretamente do vírus ou de complicações da deterioração da saúde geral dos pacientes.

Um capitulo extra será discutido para entender o emprego de novas metodologias que podem colaborar com o entendimento da presença dos vírus nos tecidos da cavidade oral, sendo elucidado por um artigo de um consórcio de autores que estão trabalhando para promover a identificação individual das células da cabeça e pescoço. Tal técnica já foi empregada em outros artigos para avaliar a relação do SARS-CoV-2 e os tecidos da cavidade oral.

Discutiremos a retomada dos atendimentos ambulatoriais odontológicos, frente à pandemia, no âmbito da teleodontologia como alternativa e na possibilidade da descontaminação de superfície, visando um atendimento seguro.

Como reflexões futuras, o ultimo capitulo empregará as possibilidades e a importância da utilização de autópsias para novos estudos dentro da odontologia.

1.3 Metodologia sumarizada

O objetivo do presente capítulo é apresentar, de forma ordenada, consolidada e sumarizada, as metodologias utilizadas nos diferentes artigos posteriormente apresentados, otimizando a avaliação do leitor. Nem toda a metodologia descrita neste tópico foi aplicada em todos os trabalhos publicados. Os métodos usados em cada trabalho bem como suas especificidades são apresentados nos próprios trabalhos

COLETAS DE AMOSTRAS

As amostras biológicas foram coletadas em pacientes com testes RT-PCR positivos para COVID-19 e que foram a óbito no Complexo HCFMUSP. A autópsia foi realizada com autorização dos familiares, manifestada através de assinatura de TCLE aprovado pelo CONEP com parecer 30364720.0.0000.0068. O uso das amostras de tecido periodontal e glândula salivar foi também submetido ao comitê de ética do Hospital das Clinicas da Universidade de São Paulo, aprovado com parecer 45276721.4.0000.0068

As autópsias foram realizadas nas dependências do PISA (Plataforma de Imagem na Sala de Autópsia da FMUSP), por uma equipe composta por dois profissionais previamente treinados pela equipe de autopsia minimamente invasiva do departamento de patologia da FMUSP, sendo um dentista e um técnico de autópsia.

Os corpos foram embalados com plástico protetor e submetidos à Tomografia computadorizada da região de cabeça e pescoço, cujo protocolo já foi previamente testado em projeto da mesma instituição intitulado “Estudos da COVID-19 fatal por meio da autópsia minimamente invasiva guiada por ultrassom”.

A coleta foi realizada em 30 cadáveres do sexo masculino ou feminino com idade entre 6 e 80 anos. A amostragem de tecidos foi realizada por duas técnicas específicas dependendo do tipo de material:

AMOSTRA DOS DIFERENTES ÓRGÃOS E GLÂNDULAS SALIVARES

A biópsia post-mortem foi realizada por punção de órgãos-alvo guiada por ultrassom utilizando equipamento portátil de ultrassonografia (US) SonoSite M-Turbo®(Fujifilm, Bothell, WA, USA) com transdutores banda larga multifrequenciais C60x (5-2 MHz Convexo) e HFL38X (13-6 MHz Linear), imagens padrão DICOM®. Após identificação do órgão alvo, a coleta foi feita por meio de sistema semi-automático com agulhas Tru-Cut® coaxiais de 14G de espessura, com 20 cm de comprimento. Os seguintes órgãos foram biopsiados: parótida, glândula submandibular, além dos diversos outros órgãos envolvidos na autópsia como pulmões, fígado, rins, coração, pulmão, baço e pâncreas.

Na coleta de glândula salivar menor (mucosa labial inferior), a biópsia post-mortem foi realizada por visualização direta. No tecido periodontal (caracterizado pela ameia mesial do primeiro molar superior) a punção foi guiada por sonda nasofibro-optica (ótica Karl-Storz Optical, Tuttlingen –German) acoplada a um smartphone (modelo iphone com aplicativo M-scope (sem contato direto com o paciente). Na ausência do primeiro molar, foi coletado material periodontal do dente adjacente ao espaço protético. A superfície gengival foi degermada por fricção de uma gaze embebida em detergente enzimático na superfície a ser biopsiada.

Foram coletados, no mínimo, 5 fragmentos adequados de cada órgão alvo, conforme protocolo padronizado pela autópsia minimamente invasiva [58]. Um fragmento foi armazenado em nitrogênio líquido e os demais fragmentos colocados em frascos individuais com formol a 10% para análise histopatológica e da ultraestrutura.

PROTOCOLO DE COLORAÇÃO HISTOLÓGICA

As amostras de tecido coletadas foram submetidas a exame histológico de rotina: coradas com hematoxilina-eosina (H&E) e analisadas em microscopia óptica, conforme protocolo já estabelecido pelo laboratório de histoquímica do departamento de patologia da Faculdade de Medicina da Universidade de São Paulo.

Sendo este: inicia-se a etapa de desparafinização e hidratação em sequência de álcoois progressiva, iniciando em álcool 70%, 80%, 95% por um minuto, e dois banhos de álcool absoluto por três minutos

Na sequência, lava-se as lâminas com água destilada por 1 minuto para posterior coloração com hematoxilina de Harris.

As lâminas ficam imersas no corante por 3 minutos em equipamento automatizado, sendo lavadas em água corrente por 10 minutos e contracoradas com eosina por 7 minutos. Por último, as lâminas são desidratadas e clarificadas com sequência de álcoois e xilol em dois banhos de 10 minutos, sendo possível, assim, a montagem das mesmas.

MICROSCOPIA ELETRÔNICA

Para a avaliação ultraestrutural foram identificadas as áreas de interesse nas lâminas coradas por H&E. Os blocos de parafina foram, então, dissecados para remoção destas áreas de interesse, que foram submetidas à desparafinização com xilol, reidratação do tecido em concentrações seriadas de álcool, seguida por re-fixação em glutaraldeido. As amostras foram, então, pos-fixadas em solução de 1% de OsO4 e coradas com solução aquosa de Uranil Acetato a 1% e incluídas em resina araf. Cortes ultra-finos foram contra-corados com uranil acetato e citrato de chumbo. Os cortes finos foram analisados ao microscópio eletrônico de transmissão (Philips Tecnai 10, Hillsboro, OR, EUA, 80 kV) e fotografados utilizando uma câmera (Jeol© JEM 1010 EM 80kV; Tokyo, Japan) acoplada ao microscópio. Os equipamentos estão disponíveis no laboratório de microscopia eletrônica, parte do Laboratório de Investigação Médica 59 – LIM59 – Laboratório de Biologia Celular, coordenado pela Profa. Dra. Elia Caldini.

PROTOCOLO DE IMUNOHISTOQUÍMICA

As reações imunohistoquímicas foram realizadas no laboratório de imunohistoquímica do departamento de patologia da Faculdade de Medicina da Universidade de São Paulo, e no laboratório de imunohistoquímica do Instituto

Adolf Lutz. Para as marcações de tecido glandular e periodontal, foram utilizados os anticorpos primários: ACE2, TMPRSS e Anti-SARS-Cov-2 – nucleocapsid antigen - anticorpo monoclonal [6H3] (http://antibodyregistry.org/AB_2888554), da GeneTex Inc. (Irvine, CA, USA).

Cortes histológicos das amostras, embebidas em parafina, foram feitos numa espessura de 3µm e estendidos sobre lâminas de vidro tratadas com organosilano (3-aminopropyltriethoxysilane) para aumentar a adesividade dos cortes, no laboratório de histopatologia da FMUSP.

Os cortes foram desparafinizados em dois banhos de xilol, sendo o primeiro por 30 minutos e o segundo por 15, ambos em temperatura ambiente e reidratados por meio de banhos consecutivos de 5 minutos cada, em cadeia descendente de etanóis (de absoluto a 85%) e um banho de hidróxido de amônio a 10% em solução de etanol para remoção de pigmento formólico.

As lâminas foram lavadas em água destilada por 5 minutos, e os cortes submetidos ao bloqueio da peroxidase endógena tecidual em solução com peróxido de hidrogênio 20 volumes e metanol na proporção 1:1 em um banho de 45 minutos.

Em seguida, realizado um banho em solução salina de Tris EDTA (Tris 50 mM, NaClmM) por 5 minutos e depois a recuperação antigênica por 20 minutos em banho-maria a 95°C (pH 9.0) seguida por resfriamento em temperatura ambiente.

As secções foram incubadas com o ProteinBlock por 10 minutos, em seguida com o anticorpo primário (ACE2, TMPRSS e Anti SARS-CoV-2) por 16 horas overnight a 4ºC em câmara úmida. Em seguida, incubadas com o polímero livre de biotina (EnVision™) por 30 minutos em temperatura ambiente.

Após as incubações de ACE2 (1;2500) e TMPRSS (1;2500), as secções foram coradas com a solução cromogênica 3,3′-diaminobenzidina tetrahydrochlorida (Revival – HRP DAB polivalente livre de biotina, Spring Bioscience, cod. SPD-125) e cromogênio (Dako Liquid DAB + substrato Cromogen System, Dako, cod. K3460) por 10 minutos. A incubação do Anti SARS-CoV-2 (1:2000) foi corada com fosfatase alcalina por 5 minutos.

Por último, as lâminas foram lavadas em solução de Tris por 5 minutos e os cortes contra-corados com hematoxilina de Mayer (Merck, Darmstadt, Alemanha), reidratados em cadeias crescentes de álcool, diafanizados em xilol e as lamínulas montadas em sistema automatizado.

PROTOCOLO DA RT-PCR

As reações de biologia molecular foram realizadas no Laboratório de Gastroenterologia e Hepatologia tropical – LIM07, do Instituto de Medicina Tropical da Faculdade de Medicina da Universidade de São Paulo, sobre coordenação do Professor Dr. Joao Renato Rebello Pinho.

Extração: As amostras de biópsias coletadas foram submetidas à reação em cadeia da transcriptase reversa polimerase (RT-PCR) para detecção do RNA do 2019-nCOV. As amostras medindo 1 cm3, foram armazenadas a -70 ° C. O tecido foi macerado e a extração de ácido nucleico realizada com o reagente TRIzol (Life Technologies, Carlsbad, CA, EUA), de acordo com as instruções do fabricante. A detecção molecular do 2019-nCOV seguiu protocolos utilizando sondas desenhadas e padronizadas pela CDC e o protocolo Charite. As reações de qRT-PCR consistiram em uma etapa de transcrição reversa a 45 ° C por 10 min, ativação enzimática a 95 ° C por 10 min e 40 ciclos a 95 ° C por 15 se 60 ° C por 45 s para hibridização e extensão com o uso do equipamento ABI7500 (Thermo Fisher Scientific, Waltham, MA, EUA). [59]

ANÁLISE TECIDUAIS

As análises das marcações imunohistoquímicas e alterações morfológicas foram realizadas em microscópio de luz e a partir das fotomicrografias eletrônicas de transmissão.

As lâminas de H&E foram analisadas para avaliação das características teciduais e alterações morfológicas de forma qualitativa.

As marcações imuno-histoquímicas foram realizadas com análise qualitativa para identificar a presença dos vírus e dos diferentes receptores.

As análises das micrografias eletrônicas foram feitas de forma qualitativa para avaliação das alterações da ultraestrutura.

CAPÍTULO 2

Salivary glands are a target for SARS-CoV-2: A source for saliva contamination

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Keywords: COVID-19; Autopsy; Infection Control; Salivary Gland; RT-PCR, SARS-CoV-2; Saliva

Abstract: The ability of the new coronavirus SARS-CoV-2 to spread and contaminate is one of the determinants of the COVID-19 pandemic status. SARS-CoV-2 has been detected in saliva consistently, with similar sensitivity to that observed in nasopharyngeal swabs. We conducted ultrasound-guided postmortem biopsies in COVID-19 fatal cases. Samples of salivary glands (SGs; parotid, submandibular, and minor) were obtained. We analyzed samples using RT-qPCR, immunohistochemistry, electron microscopy, and histopathological analysis to identify SARS-CoV-2 and elucidate qualitative and quantitative viral profiles in salivary glands. The study included 13 female and 11 male patients, with a mean age of 53.12 years (range 8–83 years). RT-qPCR for SARS-CoV-2 was positive in 30 SG samples from 18 patients (60% of total SG samples and 75% of all cases). Ultrastructural analyses showed spherical 70–100 nm viral particles, consistent in size and shape with the *Coronaviridae* family, in the ductal lining cell cytoplasm, acinar cells, and ductal lumen of SGs. There was also degeneration of organelles in infected cells and the presence of a cluster of nucleocapsids, which suggests viral replication in SG cells. Qualitative histopathological analysis showed morphologic alterations in the duct lining epithelium characterized by cytoplasmic and nuclear vacuolization, as well as nuclear pleomorphism. Acinar cells showed degenerative changes of the zymogen granules and enlarged nuclei. Ductal epithelium and serous acinar cells showed intense expression of ACE2 and TMPRSS receptors. An anti-SARS-CoV-2 antibody was positive in 8 (53%) of the 15 tested cases in duct lining epithelial cells and acinar cells of major SGs. Only two minor salivary glands were positive for SARS-CoV-2 by immunohistochemistry. Salivary glands are a reservoir for SARS-CoV-2 and provide a pathophysiological background for studies that indicate the use of saliva as a diagnostic method for COVID-19 and highlight this biological fluid's role in spreading the disease.

Salivary Glands are a target for SARS-CoV-2: A source of saliva contamination

Introduction

Since WHO declared a pandemic status for COVID-19, governments and health care organizations create a series of strategies to mitigate the spread of its etiological agent the SARS-CoV-2. The contagion occurs through infected droplets that are disseminate directly by cough and sneeze[1]. Salivary secretions are the main component of small speech droplets, and thus, play an essential role in the high contamination pattern of COVID-19. The presence of SARS-CoV-2 RNA in saliva droplets have been consistently demonstrated in different stages of the disease and have been used as a reliable COVID-19 diagnostic tool [2,3]. In a series of 70 COVID-19 patients Iwasaki and colleagues detected more copies of SARS-CoV-2 RNA in saliva samples than in the gold standard diagnostic method, the nasopharyngeal smear samples [4].

Saliva is a multi-biological fluid composed of salivary gland secretion, crevicular fluid, respiratory secretion, and exfoliated epithelial cells. The presence of SARS-CoV-2 in saliva may be related to viral proliferation and RNA secretion in any cells and tissues involved in salivary components production such as periodontal tissue, salivary glands and cells of the upper respiratory tract [5]. For instance, we've previously demonstrated the presence of SARS-CoV-2 RNA in periodontal tissue. [6] Determining the how each tissue contribute as a reservoir for SARS-CoV-2 may be a path to better understand the SARS-CoV-2 profile in saliva and develop strategies to improve diagnosis as well as to mitigate the contamination trough salivary droplets.

SARS-CoV-2 infection of the host's cells depends on the cleavage of one of its spike subunits by furin [7], thus allowing the cleaved spike protein to interact with angiotensin-converting enzyme 2 (ACE2) and transmembrane serine protease 2 (TMPRSS) receptors. These interactions initiate cell endocytosis and begin the viral replication cycle [8]. Animal studies have shown the interaction of these receptors and SARS-CoV, suggesting that ACE2, TMPRSS and furin present in salivary gland tissues are early targets of coronavirus infection [9]

Therefore, to better understand the basis of transmission patterns, it is crucial to verify whether SARS-CoV-2 RNA in the saliva is related to viral infection and replication within glandular epithelial cells or is related, instead, only to the respiratory secretion and periodontal component of saliva.

Material and Methods

We conducted post-mortem biopsies of the major (parotid and submandibular) and minor salivary glands (lower lip) during Ultrasound Guided Minimally Invasive Autopsy (US-MIA) from patients who died of COVID-19. Institutional and federal review boards approved this study under protocol number 30364720.0.0000.0068. We performed the US-MIA after obtaining the informed consent of the next-of-kin. The procedure consists of a verbal autopsy questionnaire to gather clinical and medical information followed by ultrasound-guided post-mortem biopsies to obtain samples, following established safety protocols previously described [10].

In each autopsy, we identified the parotid and submandibular glands using a portable SonoSite M-Turbo R (Fujifilm, Bothell, WA, USA) ultrasound system with a HFL38X (13-6 MHz Linear) transducer. We perform post-mortem biopsies using Tru-Cut® semi-automatic percutaneous 14G coaxial needles (20 cm). Punctures were made by percutaneous access to avoid the risk of salivary contamination. In order to access the minor salivary glands, initially we wiped the inner lip mucosa area using a gauze soaked in an enzymatic detergent (Riozyme – Rioquímica, São Jose do Rio Preto, Sao Paulo, Brazil) to clean all superficial contamination and we performed the biopsy using a 0.3 mm punch.

Samples were frozen and stored at −80°C. Tissue samples were macerated, and nucleic acid extracted using the TRIzol® reagent (Invitrogen). Molecular detection of SARS-CoV-2 was performed using the SuperScriptTM III PlatinumTM One-Step qRT-PCR Kit (Invitrogen) and primers/probes sets for *E*, and *N (N1)* genes amplification [11].

The Human *Rnase P* gene was also amplified as nucleic acid extraction control [3]. RT–PCR reactions were performed using the 7500 Fast Real-Time PCR System (Applied Biosystems). They consisted of a step of reverse transcription at 55°C for 10 minutes for reverse transcription, 95°C for 3 minutes

and 45 cycles at 95°C for 15 seconds and 58°C (*E gene*)/ 55°C (*N and RNAse P genes*)/ for 30 seconds.

Additional samples were fixed in buffered 10% formalin solution and were embedded in araffin.We obtainded 3µm thick sections mounted in glass slides for H&E staining and Immunohistochemical reactions for identification of SARS-CoV-2, ACE2 and TMPRSS (protocol details available in the supplementary material).

To perform ultrastructural analyses, we reprocessed the formalin-fixed paraffin-embedded biological tissue. This procedure is especially suitable whenever it is desirable to select a specific area from the sample for transmission electron microscopy.

We identified ductal and acinar areas with pathologic tissue derangement on H&E stained slides of the parotid and submandibular salivary glands and marked with a fiber tip marker on the glass. By macroscopic comparison, we identified corresponding regions on paraffin block surfaces. The target areas were dug out using a razor blade. The resulting small fragments were deparaffinized in xylol, rehydrated in graded alcohol series, and re-fixed glutaraldehyde in 0·15M phosphate buffer, followed by post-fixation in 1% OsO_4, and staining in 1% aqueous uranyl acetate overnight. The specimens were then embedded in an epoxy resin. Ultrathin sections were obtained with a Reichert ultratome and double-stained by uranyl acetate and lead citrate. Micrographs were obtained using a Gatan, Inc. Model 792 BioScan 1K by 1K wide angle CCD camera couplet to a JEOL JEM 1010, 80kV electron microscope.

Results and Discussion

For all patients, the diagnosis was confirmed by real-time reverse-transcriptase polymerase chain reaction (RT-PCR) in nasopharyngeal swab specimens. We evaluated 45 salivary glands (SG) samples (20 parotids, 15 submandibular, and 10 minor SG) from 24 deceased patients. In selected cases, we also performed immunohistochemistry for SARS-CoV-2, ACE2 and TMPRSS receptors (15 cases), and electron microscopy (2 cases). (See methods at supplementary materials for details).

The study included 13 female and 11 male patients, with a mean age of 53.12 years-old (range 8-83). The mean timespan between symptoms onset and

death was 21.12 days (range 4-47). RT-PCR for SARS-CoV-2 was positive in 30 SG samples from 18 patients (60% of total SG samples and 75% of all COVID-19 cases).

Ultrastructural analyses showed spherical 70–100nm viral particles, consistent in size and shape with the *Coronaviridae* family, in the ductal lining cell cytoplasm, acinar cells, and ductal lumen of SG (Fig 1A, 1B). There was also organelles degeneration in viral infected cells and the presence of cluster of nucleopsids, which suggests viral replication in SG cells (Fig 1C). Although cellular degeneration may occur due to the fact that we are using post-mortem samples from paraffin blocks, the high abundance of particles indicates that the damage was probably at least partially due to viral infection explain.

Qualitative histopathological analysis showed morphologic alterations in the duct lining epithelium characterized by cytoplasmic and nuclear vacuolization, as well as nuclear pleomorphism. Acinar cells showed degenerative changes of the zymogen granules and enlarged nuclei (Fig 2A, 2B). Both ductal epithelium and serous acinar cells showed intense expression of ACE2 and TMPRSS receptors (Fig 2C, 2D). The anti-SARS-CoV-2 antibody was positive in 8 (53%) of the 15 tested cases in duct lining epithelial cells and acinar cells of major SG (Fig 2E, 2F). Only 2 (13%) of minor salivary glands were positive for SARS-CoV-2 in immunohistochemistry.

The study of SARS-CoV-2 organotropism is important for understanding the disease's pathogenesis and infection patterns [12]. Salivary glands were reported as a virus reservoir for prevalent diseases such as herpes simplex, EBV, HHV-7 and Cytomegalovirus[5,13]. Viral replication within the SG seems to be an efficient dissemination strategy since the contaminated droplets produced during become the main saliva componention[14]. Even patients from our study who died from non-respiratory causes – including tumors, neurologic events, and vascular causes, presented SARS-CoV-2 infections in salivary gland cells.

For the first time and using different methods, we demonstrate the presence of SARS-CoV-2 infection and its replication in the majors and minor salivary glands. We also present the expression of the cellular viral targets, ACE2 and TMPRSS receptors, in patients with severe COVID-19. Our findings demonstrate that salivary glands are a reservoir for SARS-CoV-2 and provide a pathophysiology background to the recent studies that indicate the use of saliva

as a diagnostic method for COVID-19, and highlight this biological fluid's role in spreading the disease.

Fig.1

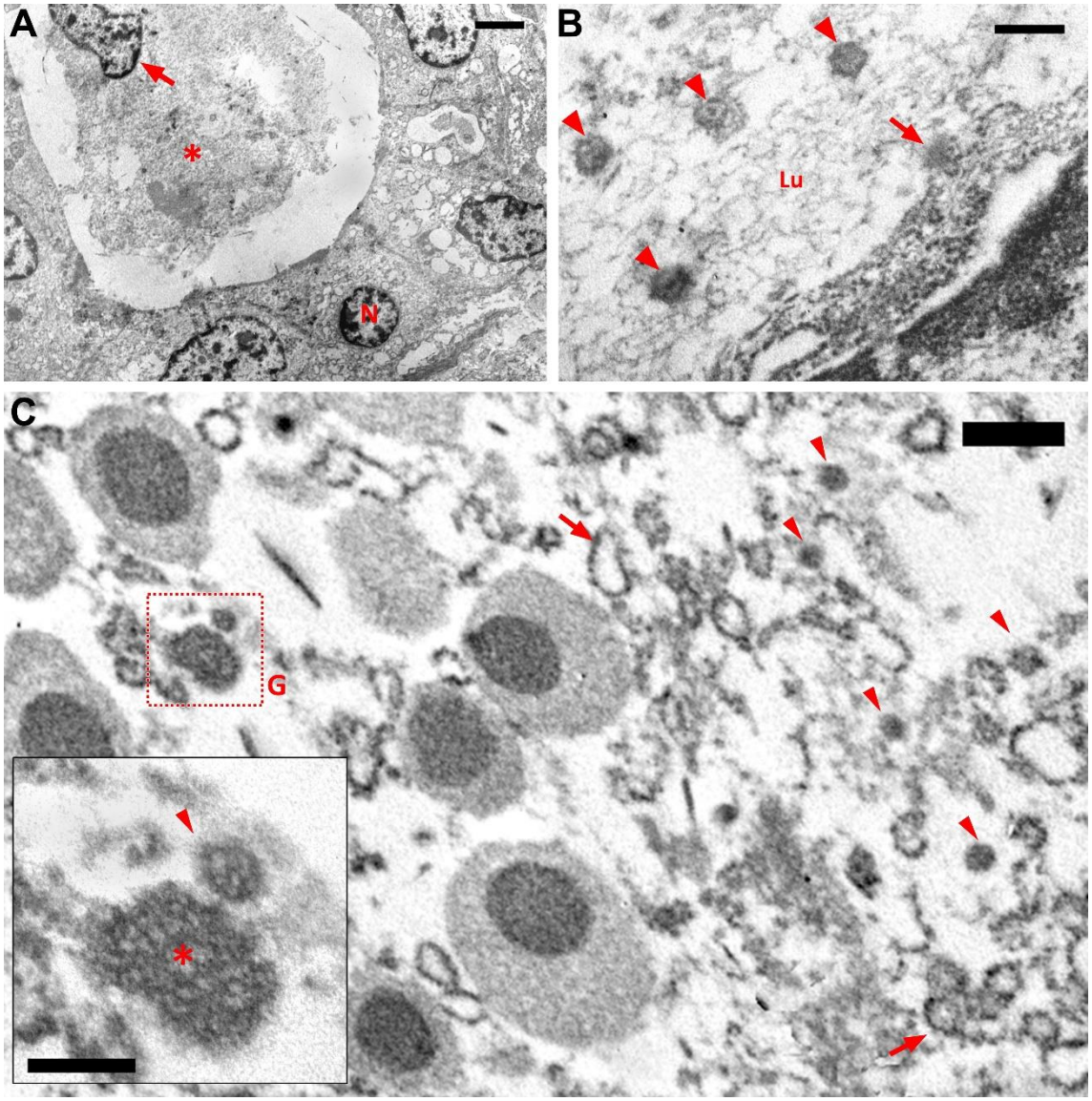

Fig. 2

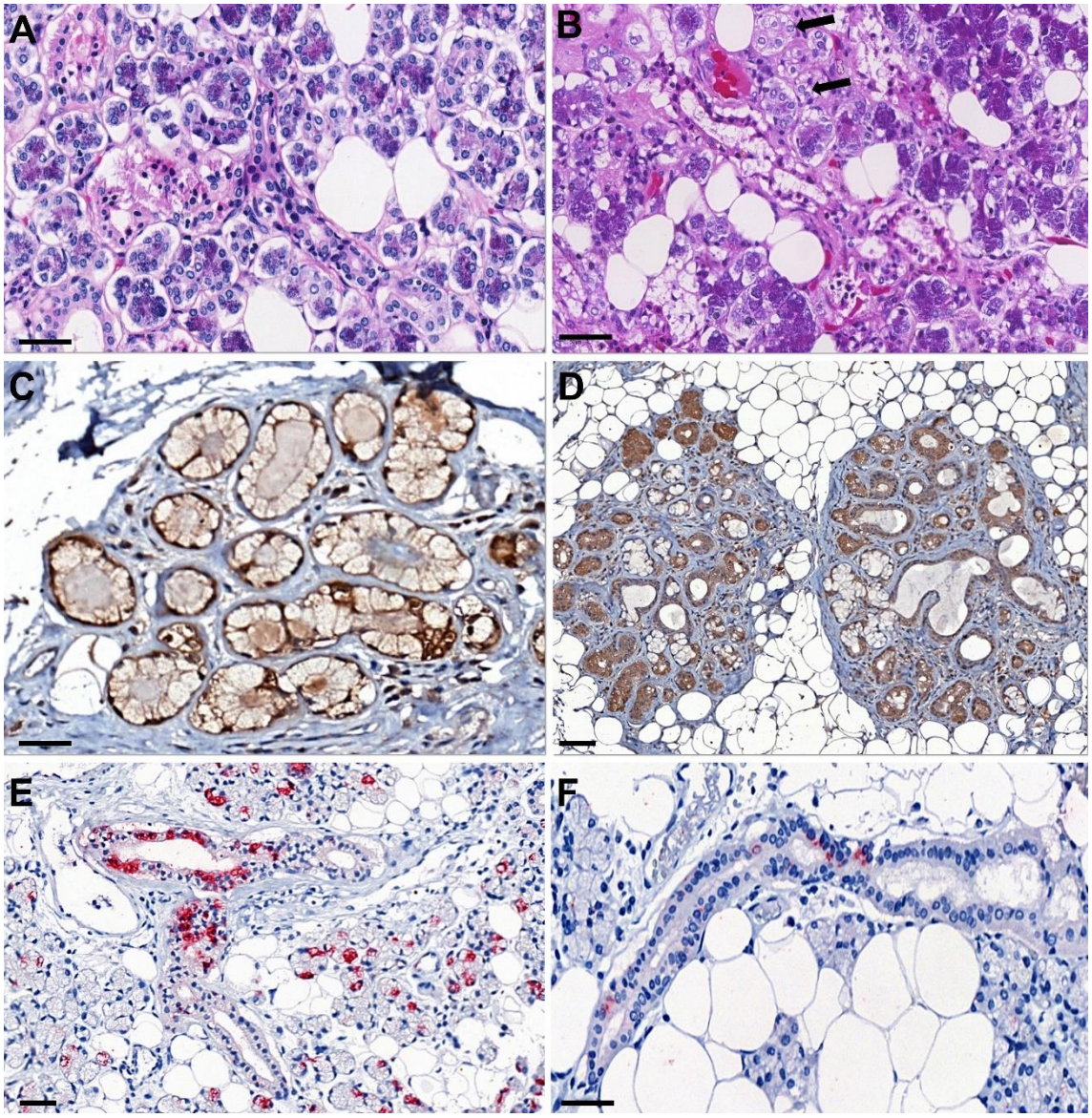

Figure Legends

Figure 1

Post-mortem biopsy histological findings: A: Low magnification electron micrograph of an intralobular duct of the submandibular gland. The ductal epithelium consists of a single layer of cuboidal cells that have a centrally located nucleus (N). The ductal lumen was almost completely obliterated by *debris* accumulation (asterisk), including an isolated cell nucleus (arrow). Bar: 2µm; B: Electron micrograph showing the apical zone of a ductal epithelial cell of the parotid gland. In addition to the viral particles (arrowheads) inside the ductal

lumen (Lu), there is a viral particle leaving the cell by budding through the membrane (arrow). Bar = 200nm; C: The electron micrograph shows part of an acinar cell of the submandibular gland. On the left side of the image, the cytoplasm contains seromucous secretory granules (G) typically formed by strongly stained spherules surrounded by an unstained component. On the right side, the cytoplasm shows degeneration, with viral particles (arrowhead) and microsomal vesicles (arrows). The inset shows a mature viral particle (arrowhead) next to a cluster of nucleocapsids (asterisk). Bar = 500 nm; inset bar = 200 nm.

Figure 2

Post-mortem biopsy histological findings: A: Parotid; B: Submandibular – H&E; duct lining epithelium characterized by nuclear pleomorphism. Acinar cells showing enlarged nuclei (Arrows); condensation of zymogen granules; C: ACE2 receptor – parotid immunohistochemistry targeting the human ACE2 protein (brown) showed staining in acinar cells; D: TMPRSS receptor – submandibular immunohistochemistry targeting the human ACE2 protein (brown) showed staining in acinar and ductal cells. Immunohistochemistry targeting SARS-CoV-2; E: Parotid showing positive staining for SARS-CoV-2 in intercalated duct and striated duct. Acinar cell staining characterized by an apical localization; F: Submandibular SG showing diffuse positive staining for SARS-CoV-2 in a striated duct. Scale bars: 50 µm

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Authors Contribution

Matuck B: contributed to conception, design, acquisition, interpretation, data analysis, drafted and critically revised the manuscript.

Dolhnikoff M: contributed to conception, design, acquisition, interpretation, data analysis and critically revised the manuscript.

Maia G and Gomes S. C: Contributed to design, acquisition, drafted de manuscript.

Sendyk D, Andrade N: Contributed to analysis, interpretation, drafted and critically revised the manuscript.

Zarpellon A: Contributed to acquisition, analysis, interpretation, drafted and critically revised the manuscript.

Nunes-Duarte A, Monteiro R, Kanamura C: Contributed to conception, acquisition, analysis and critically revised the manuscript.

Pinho JR. and Gomes-Gouvêa M: Contributed to design, analysis, interpretation and critically revised the manuscript.

Machado S: Contributed to design, analysis, interpretation, drafted and critically revised the manuscript.

Mauad T: Contributed to conception, design, interpretation and critically revised the manuscript.

Saldiva P: Contributed to conception, design and critically revised the manuscript.

Braz-Silva P:, Caldini E Contributed to conception, analysis, interpretation and critically revised the manuscript.

Silva LF: Contributed to conception, design, acquisition, analysis, interpretation and critically revised the manuscript

All authors gave final approval and agree to be accountable for all aspects of the work.

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Supplementary material

Sample collection and complementary results

All patients were admitted to the Clinics Hospital – University of Sao Paulo School of Medicine and submitted to nasopharyngeal swabs upon hospital admittance. When admitted in poor conditions just before death, the swab was collected immediately after death. Cases with negative RT-PCR in nasopharyngeal swabs were also submitted to post-mortem confirmation by lung RT-PCR for SARS-CoV-2 using primers and probes set for E (envelope) and N genes. All clinical information data during hospitalization was collected.

The minimally invasive autopsy was performed at the “Image Platform at Autopsy Room Research Facility” – Sao Paulo Autopsy Service and University of Sao Paulo School of Medicine.

A total of 24 patients was enrolled for minimally invasive autopsies between May 27th and July 8th, 2020. Exclusion criteria included patients with no positive nasopharyngeal swab for SARS-CoV-2, patients with other viral infectious diseases, and patients whose families did not consent to the minimally invasive autopsy.

Sample collection was performed by the standardized procedure that includes the identification of the organ/site by ultrasound (supplementary Figure 1), followed by the incision in the plastic safety bag that involved the patient, and collection of the sample in a tissue cylinder (2.5 cm long, 0.2 cm in diameter).

Four parotid and nine submandibular samples from 24 patients did not present representative material for PCR analyses or were contaminated with mucosa lining and were excluded. (Supplementary Table 1)

Supplementary Table 1. Clinical information of patients included

Patients						
N	Age	Sex	Cause of death	Time between symptoms and death	Time between admission and death	Smoker
1	63	F	SARS	8	5	no
2	83	F	SARS	13	7	ex
3	41	M	Thrombosis	26	21	no
4	68	M	Cardiac arrest	21	26	no
5	66	M	Cardiac arrest	7	17	ex
6	63	F	SARS	27	4	ex
7	51	M	Cardiac arrest	31	17	no
8	63	M	SARS	21	11	ex
9	71	F	SARS	16	11	no
10	53	F	SARS	25	11	no
11	15	F	Supra-renal tumor	24	9	no
12	55	F	Cardiac arrest	23	21	no
13	46	F	Cardiac arrest	21	14	no
14	67	M	Cardiac arrest	10	34	ex

15	67	F	SARS	34	3	no
16	45	M	Neurologic	20	15	no
17	34	F	SARS	15	8	no
18	38	F	Cardiac arrest	18	9	no
19	22	M	Cardiac arrest	18	11	no
20	8	M	SARS	10	6	no
21	74	M	SARS	29	26	no
22	76	M	SARS	47	40	no
23	55	F	SARS	39	28	no
24	51	F	SARS	4	4	no

SARS: Severe Acute Respiratory Syndrome; Ex: Ex-smoker M: Male F: Female

Biomolecular complementary results

A total of 18 out of 24 patients presented at least one SG with positive RT-PCR, 11 patients showed more than one salivary site with positive RT-PCR for SARS-CoV-2.

Of 20 parotids, 13 were positive with a mean cycle threshold (Ct) of 34.35, indicating a low viral load. Positive submandibular samples were nine out of 15 with a mean Ct of 33.26. The minor SG sampling was positive in 8 out of 10 cases, with a mean Ct of 31.85. (Supplementary Table 2)

Supplementary Table 2. RT-PCR from all samples – cycle threshold values.

	Parotid			Submandibular			Minor		
N	Human Rnase P (Extraction control)	Charité	CDC	Human Rnase P (Extraction control)	Charité	CDC	Human Rnase P (Extraction control)	Charité	CDC
1	-	-	-	23.7	32	31	17.5	38.4	34.8
2	-	-	-	NP	37.8		-	-	-
3	19	Neg	Neg	18.6	Neg	Neg	-	-	-
4	21.2	Neg	Neg	24.5	Neg	Neg	-	-	-
5	18/17.8	34.7	31.5	-	-	-	-	-	-
6	21.4/20	Neg	Neg	30/29.1	38.8	36	-	-	-
7	30	Neg	Neg	31	Neg	Neg	-	-	-
8	22.8	38.2	35	-	-	-	-	-	-
9	22.8	38.2	35	26.9	Neg	Neg	17.6	28.8	26.5
10	23.9	26.9	23.3	20	25.7	22.7	-	-	-
11	30	37	Neg	NP	38.1	34.4	17.4	28	26
12	26.9	Neg	35.7	27.5	35.7/31.7	28.4	-	-	-
13	21.5	29.9	28.4	30.6	Neg	Neg	-	-	-
14	22	36.8	35.3	23.2	33.5	31	19.3	32.7	30.7
15	NP	38.6	34.9	NP	38.8	33	-	-	-

16	21.7	Neg	Neg	25.6	Neg	Neg	-	-	-
17	18.5	Neg	Neg	-	-	-	NP	38.7	32
18	NP	35.8	31.8	-	-	-	NP	35.3	30.9
19	20.7	Neg	Neg	-	-	-	-	-	-
20	19.5	38.2	37	-	-	-	20.2	Neg	Neg
21	-	-	-	-	-	-	16.8	31.6	29
22	-	-	-	-	-	-	26.8	Neg	Neg
23	18.4	38.6	34.5	-	-	-	25	34.4	31.8
24	21	37.7	32	23.5	37	34	-	-	-

Table abbreviations and symbols: NP: Not performed; Neg: Negative; - : Tissue not available; CDC: Central of Disease Control

Immunohistochemistry and Histological Protocols and complementary results

Tissue samples were fixed in buffered 10% formalin, embedded in paraffin and 3 µm sections were stained with hematoxylin and eosin (H&E). Acinar cells showed degenerated zymogen granules and enlarged nuclei, no inflammatory cell infiltration was observed. Patient number 8 showed a periductal lymphomononuclear infiltration.

Immunohistochemistry was performed in 15 salivary glands to detect SARS-COV-2 nucleocapsid antigen using a mouse monoclonal antibody [6H3] (http://antibodyregistry.org/AB_2888554), from GeneTex Inc. (Irvine, CA, USA) at a 1:2000 dilution. Antigen retrieval was performed with citrate at pH 6.0. Amplification was achieved using an alkaline phosphatase conjugated polymer (Polink-2 AP , GBI Labs cat.D24-110, Bothell, WA, USA), and revealed using the permanent Fast Red chromogen (GBI-Permanent Red Substrate, GBI Labs cat. C13-120). The reactions followed standard protocols validated in our laboratories, using positive and negative controls for ACE2 (Abcam, Cambridge, UK, ab15348 at a dilution of 1:2500, 30 minutes incubation time, in citric acid) TMPRSS (Abcam ab109131 – dilution of 1:2500, 30 minutes incubation time in citrate at pH 6.0). Both amplifications were performed with Reveal- Biotin – Free Polyvalent HRP (Spring Bioscience Corp, CA, USA). Positive staining was considered when similar to pulmonary controls. For immunohistochemical negative control, we used a minor SG of a 62-year-old patient deceased from a cardiovascular event, with negative RT-PCR results for SARS-CoV-2 in both pulmonary tissue and nasopharyngeal swab.

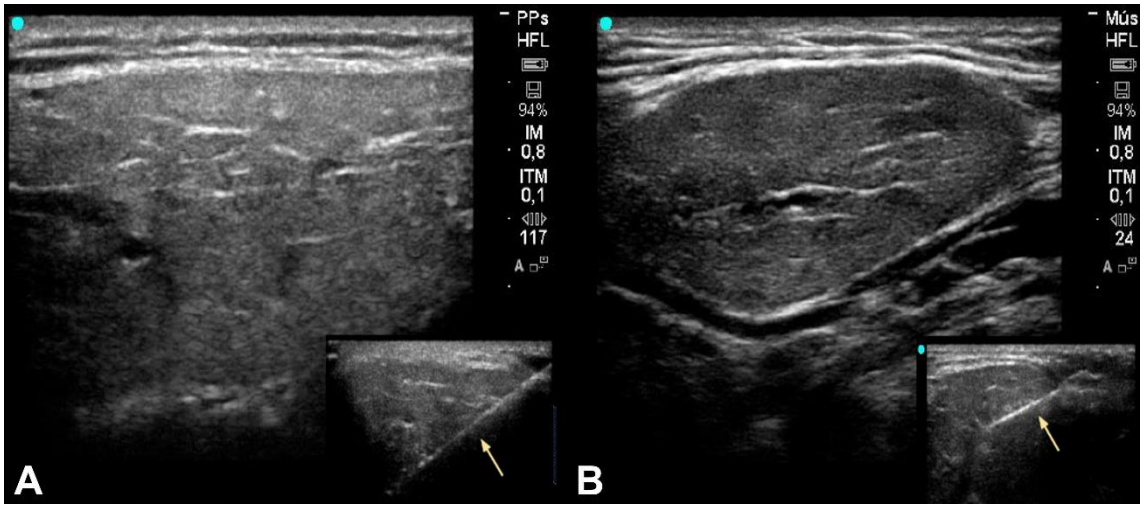
We found positive expression of SARS-CoV-2 in 4/7 parotid, 2/5 submandibular, and 2/3 minor SGs. Positivity was observed in secretory acinar cells and ductal lining structures (Supplementary Table 3, Supplementary Figure 2).

Supplementary Table 3. Immunohistochemistry for SARS- CoV-2

Patient	Parotid	Submandibular	Minor SG
1	-	Positive	-
5	Positive	-	-
9	Positive	-	-
10	Negative	-	-
11	-	Negative	Positive
12	-	Negative	-
14	Negative	Positive	-
15	-	Negative	-
17	-	-	Positive
18	Positive	Negative	-
20	Positive	-	Negative
control	Negative	-	Negative

Table symbol: -: Tissue not available

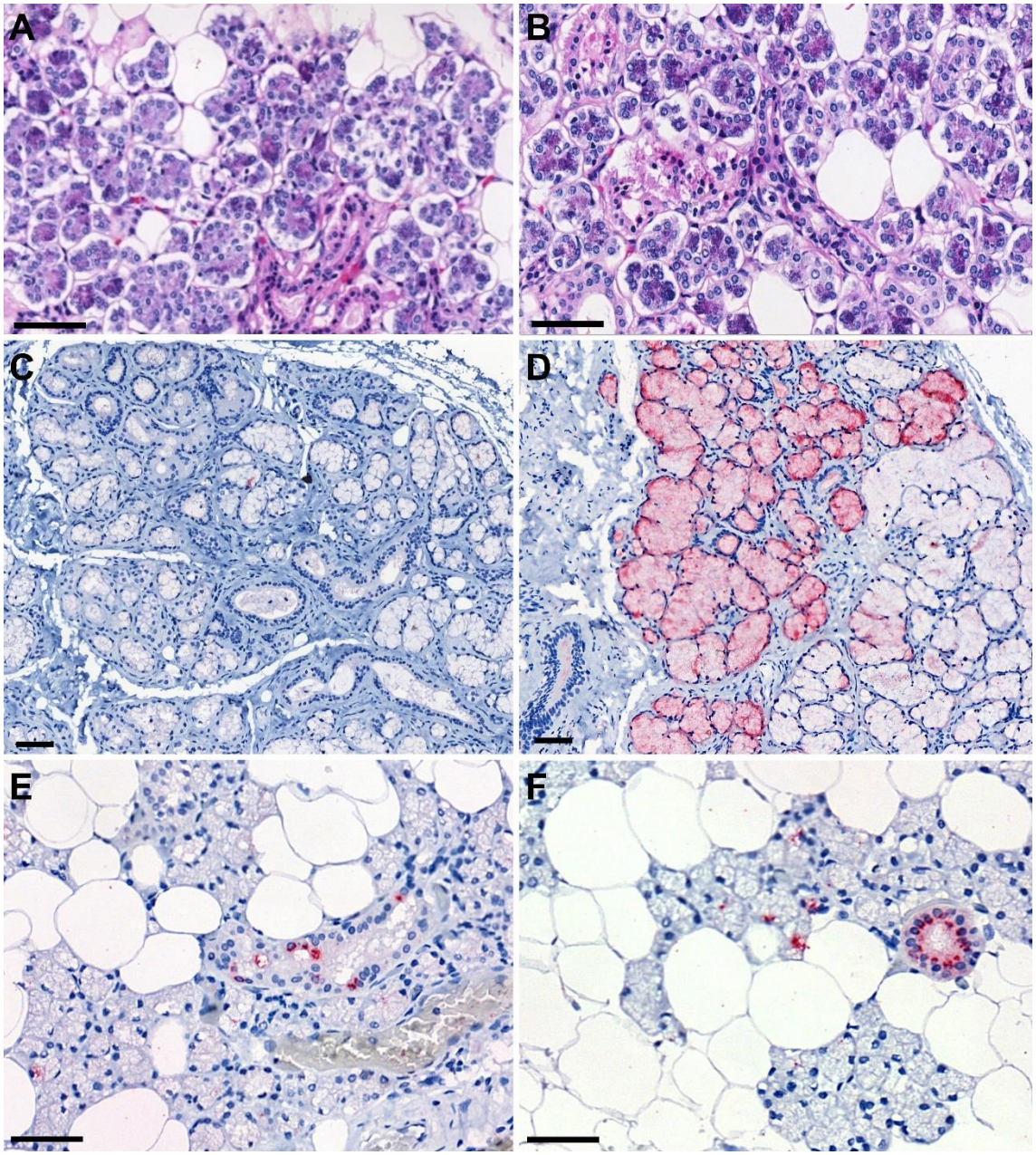
Supp Fig 1.



Supplementary Figure 1.

A: *Post-mortem* parotid ultrasound and biopsy procedure showing the Tru-Cut® needle (arrow); B: *post-mortem* submandibular ultrasound and biopsy procedure showing the Tru-Cut® needle (arrow).

Supp Fig 2



Supplementary Figure 2.

A: Parotid Gland, control patient, H&E; B: Parotid Gland, COVID-19 patient, H&E; C: Minor Salivary gland, Control Patient, immunohistochemistry for SARS-CoV-2 negative; D: Minor salivary gland showing positively stained acinar cells, apical localization. COVID-19 Patient; E: Immunohistochemistry for SARS-CoV-2 in a parotid sample showing positive staining in a striated duct and in acinar cells. COVID-19 Patient; F: Submandibular – Striated duct showing positive staining. COVID-19 Patient. Scale bars: 50 µm.

CAPÍTULO 3

Periodontal tissues are targets for Sars-CoV-2: a *post-mortem* study

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ABSTRACT

Background: The ability of the new coronavirus SARS-CoV-2 to spread is one of the determinants of the COVID-19 Pandemic status. Until June 2020, global COVID-19 cases surpassed 10 million. Asymptomatic patients, with no respiratory impairment, are believed to be responsible for more than 80% of transmission. Other virus has been consistently detected in periodontal tissues. **Objective:** The aim of this study was to investigate the presence of SARS-CoV-2 in periodontal tissue. **Methods:** We conducted video-endoscope minimally invasive post-mortem biopsy in 7 fatal cases of COVID-19. Using a regular endoscope video system associated to a smartphone to locate periodontal tissue. We analyzed the samples using RT-PCR, to identify the SARS-CoV-2 RNA and histopathological analysis. **Results:** The 7 studied autopsies with positive laboratory tests for COVID-19 included 57.14% of female patients and the average age of 47.4 (range 8 to 74). In 5 cases, periodontal tissue was positive for SARS-CoV-2 (RT-PCR). Histopathologic analyses showed morphologic alterations in the keratinocytes of the junctional epithelium, a vacuolization of the cytoplasm and nucleus and nuclear pleomorphism. **Conclusion:** We presented a biomolecular analyses obtained from minimally invasive autopsies. This is the first study to demonstrate the presence SARS-CoV-2 in periodontal tissue in COVID-19 positive patients.

Keywords: COVID-19; Autopsy; Infection Control; Oral manifestation; RT-PCR

Periodontal tissues are targets for SARS-CoV-2: a post-mortem study

INTRODUCTION

An epidemic started in Wuhan (Hubei Province, China) with pneumonia like symptoms, rapidly spread across the world and it was announced by the World Health Organization (WHO) as a pandemic now called COVID-19. Its etiological agent was identified as a new coronavirus (Zhu et al., 2020) responsible for a severe acute respiratory syndrome. The disease leads to a diffuse alveolar damage-causing respiratory distress, and eventually death (Zhou et al., 2020). By June 30rd 2020, the number of confirmed cases adds up to around 8.860.000 with more than 465.000 deaths, affecting 216 countries (https://www.who.int/emergencies/diseases/novel-coronavirus-2019).

SARS-CoV-2 spreads much more rapidly than other respiratory infections and this may be related to long term incubation time and the high ability of the virus to contaminate through coughing or sternutation, during social interaction (Li et al., 2020). Due to the low prevalence of rhinorrhea in COVID-19 patients it is suggested that infected droplets is not only contaminated by nasal sputum and lower respiratory fluids, but also by saliva (Huang C et al., 2020). Some studies suggest that the consistent founds of SARS-CoV-2 in saliva may be used as a point-of-care technology to diagnostic and prognostic, even without understand if the virus is capable to replicate in salivary gland tissues(Hamid et al., 2020).

Saliva is a biological fluid composed by salivary gland excreta, crevicular fluid, lower respiratory secretion and exfoliated epithelial cells, in which SARS-CoV-2 can be found (To et al. 2020a). The presence of the virus in the oral cavity may be related to different sources. SARS-CoV-2 infects cells using angiotensin-converting enzyme 2 (ACE2) receptor as an entrance (Zhou et al. 2020). That receptor may be found in several oral sites such as tongue (Hao X. et al 2020), salivary gland ductal epithelial cells (Liu L. et al 2011) and periodontal tissue

(Oliveira SHP. Et al 2019). The ACE2 receptor was also expressed in gingival and periodontal ligament in human fibroblasts (Santos et al., 2015). Additionally, it was proposed that the increased protease levels in chronic periodontitis, could potentially raise the risk of an oral mucosa mediated coronavirus. (Madapusi Balaji et al., 2020).

Viral genomic of Herpes simplex virus (HSV), Esptein-Barr (EBV) and Human Cytomegalovirus (HCMV) (Cappuyns et al., 2005) have been detected in gingival tissues (Contreras et al., 2000), subgingival plaque (Das et al., 2012) and gingival crevicular fluid (Grenier et al., 2009; Pallos et al.,2020; Kurshid Z et al. 2017). The possible sources of the infection, could be the gingival epithelial cells exposed to the oral cavity and virus migration through the bloodstream (Miller, 2014). It has been hypothesized that the periodontal pocket could be a niche for new coronavirus, due to a favorable environment to replicate and eventually migrate systemically using the capillary periodontal complex (Badran et al., 2020). Moreover predisposing diseases, such as diabetes mellitus, hypertension and cardiovascular diseases and metabolic syndrome, that can contribute to a worst prognostic of COVID-19 are highly associate to periodontal disease, making possible a association between periodontal disease and COVID-19 (Pitones-Rubio et al., 2020)

Due to the risk of contagion, tissues analyses from oral sites have been small, the presence infected saliva and pulmonary fluid mitigated the possibility of biopsies or autopsies in these organs. In this study, we describe a minimally invasive procedure that reduce the direct contact with oral cavity and could be a way to understand COVID-19 mechanisms in oral cavity reducing the chance of contamination.

METHODS

This study was approved by the institutional and federal ethics board, protocol number 30364720.0.0000.0068. The minimally invasive autopsy were performed after informed consent from the next-of-kin.

Deceased patients with SARS-CoV-2 positive test (nasopharyngeal swab), were submitted to minimally invasive autopsy – videoscope guided. These procedures were performed at PISA research center, University of Sao Paulo Autopsy Service and University of Sao Paulo School of Medicine.

Safety protocol

We performed a minimally invasive autopsy with ultrasound-guided post-mortem biopsies to obtain samples, following established safety protocols previously described (Nunes Duarte-Neto. et al. 2020).

The deceased bodies were wrapped in a safety plastic bag, the access to autopsy room was restricted to 3 health care professionals using appropriate personal protective equipment. The professionals were tested for SARS-CoV-2 using PCR and all team members were negative throughout 60 days of procedures.

Sampling protocol

A multidisciplinary team, constituted by an oral and maxillofacial pathologist, an otorhinolaryngologic and an autopsy technician, performed the minimally invasive autopsy. We used a regular endoscope video system (Mscope – Karl-Storz Optical, Tuttlingen -Germany), associated to a smartphone, to achieve oral cavity and localize periodontal tissues (FIGURE 1).

All autopsies were performed in a similar procedure: an incision of 15 cm was made in plastic safety bag that involved the patient; this opening was performed at plastic upper lip region, to allow the access of endoscope to intraoral sites. The optical endoscope was used to locate periodontal tissue - interproximal mesial papilla of first superior molar - in two of the cases the patient had no molars and the tissue collected was from the next mesial tooth available.

Considering the absence of salivary flow, we used gauze soaked in an enzymatic detergent (Riozyme – Rioquímica, Brazil) to clean superficial contamination from gingival biopsy area. The Molt periosteal elevator in a prying motion to elevate interproximal tissue and Takahashi forceps to clamp and collect tissue samples from the site, using endoscope as visual guide.

Once periodontal tissues were removed, two fragments were dissected in longitudinal axis, resulting in two similar parts. One of them was formalin-fixed to histopathological analyses and the other one was frozen (-80ºC) and sent to molecular analyses.

Once tissues were collected the opening in the plastic bag was closed with a transparent adhesive to mitigate the risk of contagion.

Histological and Molecular Diagnosis of SARS-CoV-2

Tissue samples were fixed in buffered 10% formalin, embedded in paraffin and 3 µm sections were stained with hematoxylin and eosin (H&E). Samples measuring 0.5 cm³ were stored at −80°C. Tissue samples were macerated, and nucleic acid extraction was performed using the TRIzol® reagent (Invitrogen). Molecular detection of SARS-CoV-2 was performed with the use of the SuperScriptTM III Platinum™ One-Step qRT-PCR Kit (Invitrogen) and primers/probes sets for E, RdRp and N (N1) genes amplification (Corman *et al*., 2020; CDC 2020). Human RNase P gene was also amplified as nucleic acid extraction control (CDC 2020).

We examined specimens from periodontal tissue - including junctional epithelium, adjacent oral gingival epithelium and connective tissue from fatal cases of COVID-19. Using rRT-PCR we investigated the presence of SARS-CoV-2 RNA of tissues and correlated it with clinical conditions of the patients, since their first symptoms, their ICU time and autopsy findings.

rRT–PCR reactions were performed using the 7500 Fast Real-Time PCR System (Applied Biosystems) and consisted of a step of reverse transcription at 55°C for 10 min for reverse transcription, 95°C for 3 min and 45 cycles at 95°C for 15 s and 58°C (E and RdRp genes)/ 55°C (N and RNAse P genes)/ for 30s.

RESULTS

We included in this study 7 (seven) patients (3 men/4 women). All patients tested positive for SARS-CoV-2 by nasopharyngeal swab. The mean age was 47.43y (8-74y), and the average number of days between the first symptoms and death was 20.14 days (10 - 31 days).

All patients presented severe acute respiratory syndrome and were admitted in the Hospital das Clinicas of Medicine School of Sao Paulo University ICU for mechanical ventilation support. The most frequent symptoms were fever, cough and dyspnea.

Most of the patients presented at least one preexisting comorbidities considering diabetes mellitus, systemic arterial hypertension, malignant neoplasm, cardiovascular disease, asthma or any immunosuppressive condition. There was only one patient free of any comorbidities, and only non-smoker patients were included in this study (TABLE1).

Histopathologic analyses showed morphologic alterations in the keratinocytes of the junctional epithelium, characterized mainly by vacuolization of the cytoplasm and nucleus, and sometimes nuclear pleomorphism (FIGURE 2). Lungs samples presented exudative and proliferative diffuse alveolar damage (DAD), with epithelial atypia which extended throughout the respiratory epithelium as described at Nunes Duarte-Neto et al. 2020.

We detected SARS-CoV-2 by rRT-PCR in 5/7 samples of periodontal tissues with a mean cycle threshold (Ct) value (E primer/probe sets) of 31.38 (27.28 - 36.55).

DISCUSSION

We present the molecular and histopathological features observed in 7 autopsies of COVID-19 patients in Brazil, 5 periodontal tissues from deceased patients were positive. Of the two negative patients, one was a 8-years boy. The pathological aspects of COVID-19 in children still unclear, the disease seems to take mild course. One of the explanations is related to the differences in ACE2

receptor expression in children (Brodin et al. 2020), that can corroborate with the absence of viral genome in periodontal tissue. The presence of vacuolization may be related to complication during hospitalization time. The other patient was a woman with a highest time between the first symptoms and death, the long-term hospitalization time could make possible a clarification the virus presence in the periodontal cells host.

Autopsy is an important tool to understand the pathological mechanisms of viral diseases. We have had a recent experience in Brazil from other two outbreaks of viral infections (yellow fever and zika) and thus it demanded our research group to develop new autopsy procedures to study and contribute with physicians and decision makers (Duarte-Neto et al. 2020). The ultrasound guided minimally invasive autopsy is a reliable alternative to conduct autopsies on COVID-19 cases once it considerably reduces the costs and the production of aerosols (Duate-Neto et al. 2018). This is the first study to associate oral autopsy findings with a minimally invasive procedure - videoscope guided - as a new approach to study viral/pandemic highly contagious diseases and its oral manifestations.

Saliva and gingival crevicular fluid has shown to be a source for human viruses in the oral cavity. A recent study investigating the detection of human herpesviruses in saliva and gingival crevicular fluid in patients with chronic kidney disease found different prevalence between these two sites (Pallos et al. 2020). The same was founded during Zika virus international public health emergency, peptides were identify in saliva of showing a new transmission path of the disease (Zuanazzi et al., 2017). Although our research did not analyze the components of saliva or crevicular fluid, we observed the presence of SARS-CoV-2 RNA copies in the periodontal tissue even many days after the first symptoms. This finding may justify the oral cavity as a source of SARS-CoV-2, as it has been consistently detected in saliva (To et al. 2020; Xu et al. 2020; Kurshid et al 2020; Zou et al. 2020), suggesting that it may be related to the access via cavity-specific crevicular fluid.

In the presented cases, the viral infection seems to be in a longstanding pattern, suggesting that even in patients who had a long course of the disease, viral infection on periodontal tissue persists. The presence of a high viral load of

SARS-CoV-2 in saliva shortly after symptoms of COVID19 is already known (To et al. 2020; Zou et al. 2020). Similar to what we found, To et. Al. have also reported that contagious particles of viral RNA could still be detected in saliva samples from some patients for 20 days or longer after the first symptoms. Our data showed viral RNA of SARS-CoV-2 in periodontal until 24 days after first symptoms in some patients.

Other studies trying to indicate potential targets for SARS-CoV-2, showed that cells from brain, coronoid plexus (Salomon. et al. 2020) and kidney can be a host (Puelles et al 2020) suggesting that organotropism can indicate the course that the disease is going to take. In this study we evaluated a possible organotropism that can influence transmissibility. Saliva is the major component of droplets and responsible for the high contagion pattern of COVID-19. In this context, the presence of SARS-CoV-2 on periodontal tissue can be one of the components that contributes with the saliva viral load.

Detection of SARS-CoV-2 RNA in the periodontal tissues draws our attention to possible implications of periodontal treatment for patients with COVID-19. Supra and subgingival debridement, even without aerosol generation, can be potentially contaminants events. Ultrasonic scalers, air-water syringes and handpieces for maintenance therapy and root planning, are more likely to facilitate transmission due to the spray that can contain particles in droplets of saliva, gingival crevicular fluid, blood and other debris. The Centers for Disease Control and Prevention (CDC, USA) (https://www.cdc.gov/coronavirus/2019-ncov/hcp/dental-settings.html) recommend that dental settings should prioritize urgent visits, carried out following strict biosafety protocols. Clinicians and dental staff should be aware of the importance of using personal protective equipment, disinfection and sterilization.

The results of this investigation should be interpreted with caution due to some limitations. First, our observations are based only in seven cases. Possibly, a greater understanding of the presence of the virus in the gingival crevicular fluid and periodontal tissues will emerge as new findings when larger numbers of cases are reported. Furthermore, the fatal cases described here represent severely ill patients with COVID-19 with prolonged periods of hospitalization, mechanical ventilation and enteral feeding in critical care, requiring oral and nasal

tubes, which could explain the histopathological changes observed. Finally, it is possible that the periodontal tissue response is different in individuals with COVID-19 who are asymptomatic or have only mild symptoms.

We concluded that oral minimally invasive autopsy - videoscope guided - is a safe procedure to obtain samples that can be analyzed by biomolecular and histopathological assays, including autopsies realized during highly contagious pandemic situation. This is the first study do perform oral minimally invasive autopsies. Our findings show that periodontal tissue seems to be a target for SARS-CoV-2, and can contribute in a long term to the presence of the virus in saliva samples. These findings can indicate a new approach to understand the contamination pattern of COVID19.

Authors Contribution

Matuck B: contributed to conception, design, acquisition, interpretation, data analysis, drafted and critically revised the manuscript.

Dolhnikoff M: contributed to conception, design, acquisition, interpretation, data analysis and critically revised the manuscript.

Maia G and Gomes S. C: Contributed to design, acquisition, drafted de manuscript.

Sendyk D: Contributed to analysis, interpretation, drafted and critically revised the manuscript.

Zarpellon A: Contributed to acquisition, analysis, interpretation, drafted and critically revised the manuscript.

Nunes-Duarte A: Contributed to conception, acquisition, analysis and critically revised the manuscript.

Pinho JR. and Gomes-Gouvêa M: Contributed to design, analysis, interpretation and critically revised the manuscript.

Machado S: Contributed to design, analysis, interpretation, drafted and critically revised the manuscript.

Mauad T: Contributed to conception, design, interpretation and critically revised the manuscript.

Saldiva P: Contributed to conception, design and critically revised the manuscript.

Braz-Silva P: Contributed to conception, analysis, interpretation and critically revised the manuscript.

Silva LF: Contributed to conception, design, acquisition, analysis, interpretation and critically revised the manuscript

All authors gave final approval and agree to be accountable for all aspects of the work.

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Figure 1.

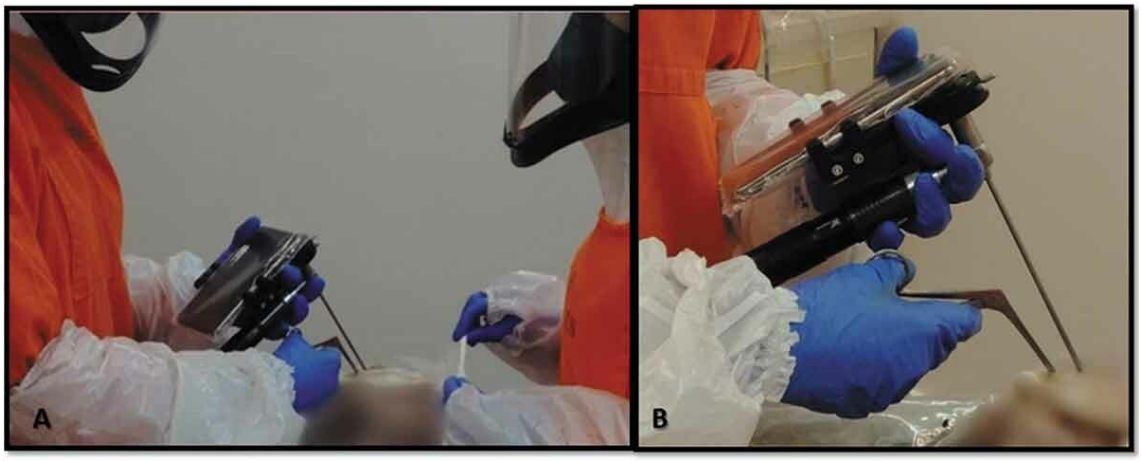

Figure 2.

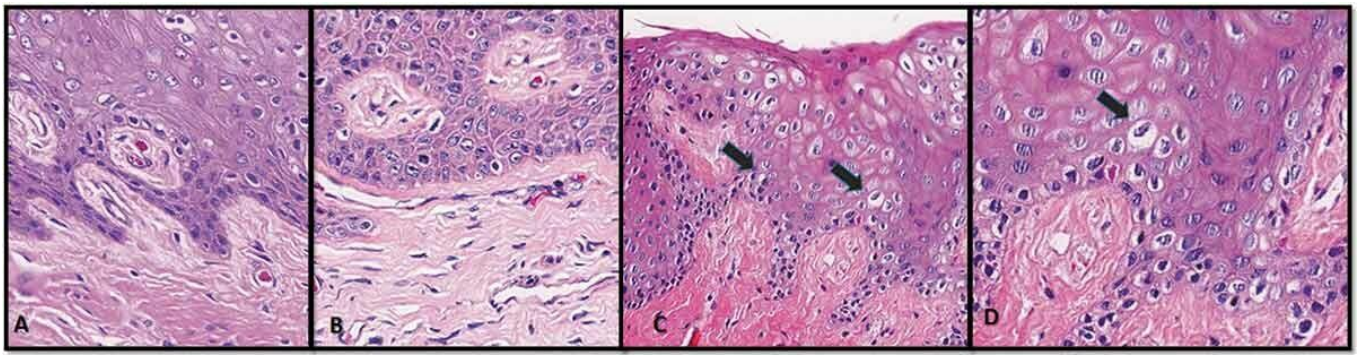

FIGURE LEGENDS

Figure 1. . Minimally invasive autopsy - Videoscope Guided - A. Two Healthcare PPE during autopsy procedure B. Videoscope (MScope, Karl-Storz Optical 4mm, 30º) attached to a smartphone for visual inspection and sampling removal

Figure 2. Histopathological assessment - A. Junctional Epithelium, no infiltration by inflammatory cell is present (20X) B. Cellular and nuclear pleomorphism (20X) C. Cellular vacuolization (20X - Arrow) D. Junctional epithelium with intracellular edema and parabasal vacuolization (40x - Arrow)

Table 1: Characteristic of patients include in this study and results of SARS- CoV-2 detection in periodontal tissues using E-gene primer/probe set

Case number	Gender	Age	DM	SAH	Smoker	Period of hospitalization (days)	Time from symptoms onset to death (days)	CT PCR-RT Periodontal tissue	Nuclear pleomorphism	Vacuolization
1	F	51	No	No	No	14	31	Negative	No	No
2	F	71	Yes	Yes	No	11	16	29.41	No	Yes
3	F	15	No	No	No	9	24	33.23	No	No
4	F	74	No	Yes	No(Ex-smoker)	9	23	27.28	Yes	No
5	M	64	Yes	Yes	No	12	14	30.47	No	No
6	M	49	Yes	No	No	13	23	36.55	Yes	Yes
7	M	8	No	No	No	6	10	Negative	No	Yes

DM: Diabetes mellitus

SAH: Systemic Arterial Hypertension

CT-PCR-RT: Cycle Threshold – Polymerase Chain Reaction – Real time

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CAPÍTULO 4

Oral Lesions and SARS-CoV-2: A Post-Mortem Study

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Oral Lesions and SARS-CoV-2: A Post-Mortem Study

The SARS-CoV-2 entrance into the oral cavity occurs via the angiotensin-converting enzyme 2 receptor (ACE2), which expression has been found in salivary gland ductal epithelium (Liu et al., 2011), tongue (Xu et al., 2020) and periodontal tissue (Fernandes Matuck et al., 2020). Hence, cells with ACE2 receptors may turn into host cells for SARS-CoV-2 and be related to oral manifestations of COVID-19 and inflammatory response in oral sites (Xu et al., 2020). Preliminary studies have reported oral manifestations possibly related to COVID-19 (Amorim Dos Santos et al., 2020; Ansari, Gheitani, & Heidari, 2020; Martín Carreras-Presas, Amaro Sánchez, López-Sánchez, Jané-Salas, & Somacarrera Pérez, 2020). Clinical manifestation showing multiple ulcerative lesions seems to be the most common feature. Some reports related cases suspicious of COVID-19 but had no confirmed diagnosis of SARS-CoV-2 infection and we believe this might have biased the results (Martín Carreras-Presas et al., 2020). The authors do not exclude that the effects of stress may be a trigger for the development of oral lesions, as it is known as a possible causal factor in this kind of conditions (Gallo, Mimura, & Sugaya, 2009).

We present a clinical-pathological report on autopsies of patients who died of COVID-19 in order to elucidate the possibility of oral manifestations of COVID-19 as there have been some divergences in the literatures regarding the oral outcome.

Twenty-six deceased patients (15 females and 11 males) with SARS-CoV-2 were submitted to oral examination during the minimally invasive autopsy procedures (Duarte-Neto et al., 2019). All patients had severe acute respiratory syndrome and were admitted in the ICU of the Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo for mechanical ventilation support. Only SARS-CoV-2 positive cases were included. SARS-CoV-2 tests were performed during hospital admission or ICU stay by using RT-PCR in nasopharyngeal swabs or bronchoalveolar secretion. The mean age was 50 years old (8-76 years), the average number of days of hospitalization was 15.30 days (3-40 days), and the average number of days under mechanical ventilation in the ICU was 12,36 days (0-43 days). The mean time between diagnosis and

death was 10.55 days, and the mean time between death and autopsy was 16.95 hours (5-22 hours). Twenty-four patients were non- or ex-smokers and only two patients had smoking habits (narghile and marijuana). Of the 26 patients, ten (38.46%) were free from comorbidities and the others had at least one disease (i.e., hypertension or diabetes).

A dentist and an otorhinolaryngologist examined the intraoral and perioral sites in the autopsy. Oral evaluation included hard palate, tongue, jugal and gingival mucosa, anterior tonsillar pilar and inner lips. Biopsies were performed with scalpels and punch, resulting in multiple fragments as follows: three fragments of the tongue (i.e., apex, lateral and posterior), two gingival fragments of periodontal tissues, one fragment of the amygdala and one fragment of the inner lip mucosa. Patients who had some oral lesions diagnosed during clinical evaluation, biopsies were performed in the lesion site. Tissue specimens were fixed in buffered 10% formalin and embedded in paraffin, sections were cut and stained with hematoxylin and eosin (H&E). Immunohistochemical staining was performed using the polyclonal Anti-HSV1 Abcam (Ab9533), monoclonal Anti-Cytomegalovirus glycoprotein B (Abcam17073).

Patient 1 and 2 had ulcerative lesions in the lower lip and one in the tongue as well. Both patients were edentulous and stayed in the ICU under mechanical ventilation for 10 and 25 days respectively. Biopsy was performed and histopathological analysis showed diffuse infiltration of inflammatory mononuclear cells, which validates our clinical findings of traumatic injuries resulting from the use of orotracheal tubes for mechanical ventilation support (FIGURE 2).

Patient 3 and 4 showed vesiculo-bullous and ulcerative lesions scattered in the oral mucosa, such as tongue and jugal mucosa. Immunohistochemical staining was performed and indicated the presence of herpes simplex virus (HSV-1) in both cases (FIGURE 2). It is known that critically ill patients have great potential of developing reactivation of some herpesviruses due to ICU and long-term hospitalization (Simonnet et al., 2021). Patient 3 still showed co-infection by fungal forms compatible with *Candida spp* (spores, pseudohyphae and hyphae).

Patient 5 presented ulcerative lesions and the histopathological analysis showed co-infection by typical *Sarcina ventriculi* colonies (FIGURE 2). *S.*

ventriculi is a gram-positive coco that usually appears in gastric biopsies with late gastric emptying and your presence may indicate a gastric pathology (Al Rasheed & Senseng, 2016). Severely ill or immunocompromised patients have a higher probability of suffering from invasive fungal and bacterial co-infections and these findings are consistent with previous reports (Salehi et al., 2020).

Integrity of oral mucosa was preserved in all other cases (FIGURE 1). No other patient examined had atypical oral manifestations, such as vesiculo-bullous lesions or ulcerations in the keratinized epithelium. Histopathological features of the cases with no clinical manifestation in the tongue, gingival papilla, inner lip mucosa and anterior tonsillar pillar showed some morphological alterations in the keratinocytes of the lining and junctional epithelium, characterized mainly by vacuolization of the cytoplasm and nucleus, and sometimes nuclear pleomorphism. Infiltration of inflammatory mononuclear or polymorphonuclear cells was rarely observed, even in cases of severe oral deterioration (FIGURE 2).

The oral manifestations found in our patients and those reported in other studies corroborate the hypothesis of secondary oral lesions induced by co-infection and immune impairment possibly occurring as an adverse reaction from the therapeutic and support measures for COVID-19 (Amorim Dos Santos et al., 2021; Amorim Dos Santos et al., 2020; Martín Carreras-Presas et al., 2020; Putra, Adiarto, Dewayanti, & Juzar, 2020).

The absence of oral lesions related to SARS-CoV-2 in our case series did not exclude the possibility of viral presence in oral tissues. Once the new virus has this distinct organotropism, it is rationale that physicians and dentists try to look for a link between a new disease and oral findings (Puelles et al., 2020). Histopathological, immunohistochemical and biomolecular studies are the gold standard for understanding the cause-effect relations and therefore are essential to a correct link between oral manifestations and SARS-CoV-2 infection.

Patients in ICU are susceptible to lesions resulting from mechanical ventilation, enteral feeding, orotracheal tubes and health deterioration (Dziedzic & Wojtyczka, 2020). Therefore, clinical and physical examinations are highly recommended and indispensable during hospitalization time and sometimes must include biopsies when needed. Biopsies are an important tool to relate

clinical conditions to aetiological agents. Other studies, mainly those on pulmonary manifestations, are trying to find a correlation between histopathological features and clinical conditions (Duarte-Neto et al., 2020). In cutaneous manifestations, histological evaluation showed non-specific inflammatory infiltration, which is consistent with other viral exanthemas (Amatore et al., 2020). In our study, we performed post-mortem biopsy in some oral sites due to the presence of some inflammatory activity, and we found in some cases the presence of herpes simplex virus. *In situ* evaluations should be considered (e.g., hybridization or immunohistochemistry to SARS-CoV-2) to know whether these lesions are related to external conditions or a cytopathic effect.

The oral manifestations of COVID-19 found in our post-mortem case report are secondary lesions related to trauma events, co-infections involving bacterium, *Candida spp*, *Sarcinia ventriculi*, herpes simplex virus, immune impairment due to disease itself and adverse reactions from the therapeutic interventions. Most of the deceased patients evaluated had long-term hospitalization, received mechanical ventilation support and some developed oral injuries during confinement. Different from what the literature suggested in the beginning of the pandemic, oral lesions in severe cases of COVID-19 infected patients seems not to be related specifically to SARS-CoV-2 or due to a cytopathic event that effects oral mucosa.

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Conflict of interest statement

All authors of this work declare no conflict of interest.

Author contribution statement

AZ contributed to conception, acquisition, analysis and interpretation, drafted and critically revised the manuscript. BM contributed to conception, design, acquisition, interpretation, data analysis, and drafted and critically revised the manuscript. MD contributed to conception, design, acquisition, interpretation, data analysis and critically revised the manuscript. GM, SCG and JTF contributed to design, acquisition, and drafted the manuscript. DS contributed to analysis, interpretation and drafted and critically revised the manuscript. AN-D contributed to conception, acquisition and analysis and critically revised the manuscript. SM contributed to design, analysis and interpretation, and drafted and critically revised the manuscript. TM contributed to conception, design and interpretation and critically revised the manuscript. PS contributed to conception and design and critically revised the manuscript. PB-S contributed to conception, analysis, and interpretation and critically revised the manuscript. LFS contributed to conception, design, acquisition, analysis and interpretation and critically revised the manuscript. All authors gave final approval and agreed to be accountable for all aspects of the work

Figure 1.

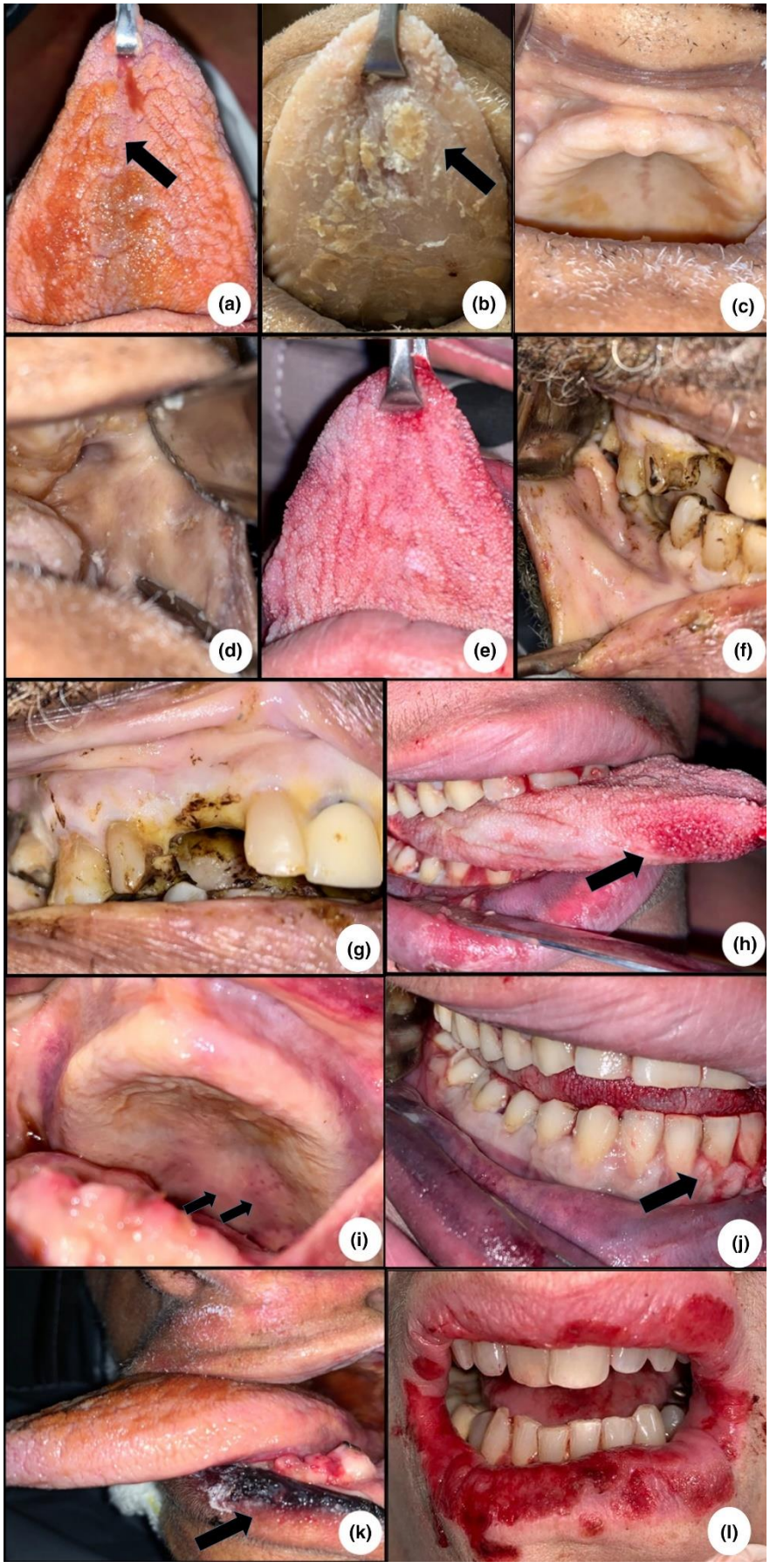
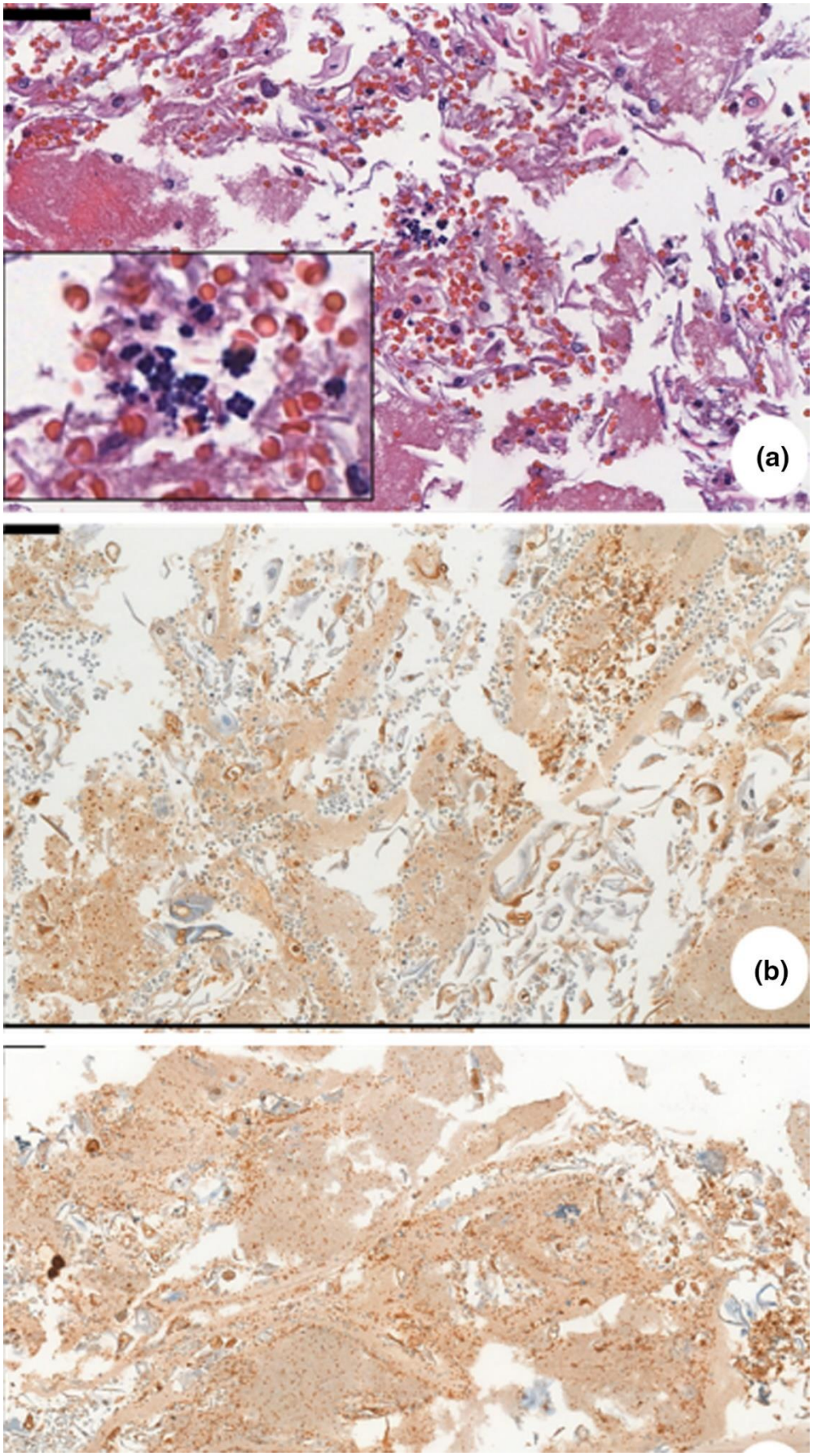


Figure 2.



Captions

Figure 1. Clinical aspects of oral sites on deceased patients of COVID-19. **A, B** Epithelial desquamation due to the shock, masticatory mucosa integrity preserved **C, D E** Patients showing no alterations on mucosa integrity. **F, G** Presence of oral health deterioration with dental caries and missing teeth. **H, I, J** Ulcerative lesion – Inset

showing erythematous lesions in lateral tongue, palate and gingiva. **K.** Large ulcerative lesions on lower lip due to trauma caused by long-term use of orotracheal tube. **H.** Ulcerative lesions in upper and lower lip, extending to gingiva and tongue Scale bars: 50 μm.

Figure 2. 1. Photomicrograph of a cell block of patient pictured in FIG1 H - routine hematoxylin and eosin (HE) stain. Sample of an ulcerative lesion in the lower lip. *Sarcina ventriculi cocci* characterized by multiple gram-positive basophilic colored tetrad arrangement, scattered in epithelium. **2. 2.** Immunohistochemical of a cell block - anti-HSV1 (abcam9533), Sample of an ulcerative lesion on dorsal tongue and buccal mucosa. Immunopositivity seen inside epithelial cell from basal layer.

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CAPÍTULO 5

The Human Oral and Craniofacial Cell Atlas

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Abstract: Among the niches of the human body, the oral and craniofacial tissues are uniquely adapted for continuous function, including the primary functions of breathing, feeding, and communication. To achieve these vital processes, this complex is supported by incredible tissue diversity, variously comprising of epithelia, vessels, cartilage, bone, teeth, ligaments, and muscles as well as mesenchymal, adipose, and peripheral nervous tissue. Recent single cell and spatial multiomics research – i.e., genomics, epigenomics, transcriptomics, proteomics, and metabolomics – has annotated known and new cell types and cell states in these tissues, but the relationships between these subpopulations remain limitedly explored. In this review, we highlight the collaborative and coordinated efforts of the Human Cell Atlas Oral & Craniofacial Bionetwork (OCBN), which aims to leverage approaches in single cell and spatial multiomics to address the hundreds of diseases that affect oral and craniofacial tissues. These powerful assays have already revealed both common and rare cell types that support oral tissues in health and will also help to uncover which cell types and molecular networks are.

The Human Oral and Craniofacial Cell Atlas: A Blueprint for Innovation, Discovery, and Collaboration

The Human Oral & Craniofacial Cell Atlas

Introduction: The Human Cell Atlas Oral and Craniofacial Bionetwork

Oral and craniofacial tissues support the human face and thus, the concept of our individual and collective identity; biologically, they coordinate essential functions to sustain life, including breathing, feeding, and communication. Though often preventable, oral diseases are increasingly burdensome worldwide, affecting over one-third of the globe with a disproportionate effect on socially disadvantaged populations. In addition, chronic oral inflammatory diseases, such as periodontal disease, have been increasingly associated with over 60 systemic diseases such as cardiovascular diseases, diabetes, cancer, pneumonia, inflammatory bowel diseases, obesity, and premature birth (Beck et al. 2019; Byrd and Gulati 2021; Schifter et al. 2010). To achieve the goal of improved and precise *whole-body health* will require the creative and dedicated application of new toolkits to understand the human body in health and disease states.

Recent advances in methods and the resolution of high-throughput single cell and spatial molecular profiling now appear to be the *quantum* leap that precision medicine has required, already revolutionising our ability to study tissue heterogeneity at a remarkable resolution and driving the scientific community to characterise all cells of the human body (Aldridge and Teichmann 2020).

While the origins of the modern single cell revolution can be traced back to quantitative polymerase chain reaction assays using individual neurons nearly 30 years ago, it wasn't until 2016 that The HumanCell Atlas (HCA) initiative was officially launched. Since then, the HCA has established itself as an international and interdisciplinary collaborative effort to create reference cellular maps of the whole body across the lifespan (https://www.humancellatlas.org) (Regev et al. 2017; Regev et al. 2018).

Since 2016, various HCA bionetworks and single cell and spatial biology consortia have made landmark scientific advances, including the discovery of new and rare cell type annotations, referred to as "cell subtypes", as well as disease-associated cellular phenotypes referred to as cell "states".

Additionally, the charting of embryonic, foetal, paediatric, and adult tissues has provided novel insights into cellular differentiation trajectories, pinpointing cells associated with developmental disorders, and highlighting paediatric and adult cancer cells of origin, some of which include previously unknown cell types (Teichmann and Regev 2020; Young et al. 2018).

In 2020, the Oral and Craniofacial Bionetwork was founded within the HCA blueprint to understand the cellular and molecular complexity of oral and craniofacial tissues in health—from embryonic development to older adults. Furthermore, it aims to complement other database resources in the oral and craniofacial development research field, such as FaceBase (Samuels et al. 2020), but with a focus on emergent single-cell technologies applied to human tissues as a primary focus.

Additionally, craniofacial organs and tissues such as the brain, eye, nasal, face skin, adipose, and musculoskeletal system (Baldwin et al. 2021) are described in allied HCA bionetworks. Partnership with these and many other groups is essential to the successful integration of the healthy oral and craniofacial complex data and will facilitate clinical applications.

Organising such a large-scale project is dependent on a multidisciplinary approach relying on the close collaboration between oral surgeons and oral health care providers (to devise quality metrics to obtain and collect clinical samples), together with wet lab scientists and bioinformaticians (for sample processing, data processing and analysis). From a clinician side it is crucial to take into account 1) a careful consideration of sample collection criteria checkpoints, such as medical history screening; 2)the recording of the precise anatomical location of each sample; and 3) review and collect associated donor and sample metadata, including health and disease states. Furthermore, this sort of collaboration will accelerate a shift from description to knowledge and the solving of complex clinical questions.

For the construction of future oral and craniofacial disease atlases in disease, it remains paramount to assess the functions, gene expression, and intercellular interactions of all resident cells in healthy tissue as a reference. This level of annotation will allow for a clearer understanding of how these processes are disrupted in disease states. For instance, small subsets of cells are important in the pathogenesis of a variety of complex diseases (Regev et al. 2017) and studying the breakdown of immune mechanisms and dysregulated pro-

inflammatory pathways on a cell-by-cell basis presents an 111 opportunity to understand how perturbed molecular pathways and processes can lead to disease.

Understanding these processes may identify molecular mechanisms leading to improved targeted therapies, for instance in human craniofacial birth defects such as orofacial clefting or craniosynostosis.

This is foundational knowledge that we intend to be publicly available to advance oral health across the globe.

A Blueprint for The Human Oral and Craniofacial Atlas

Defining oral and craniofacial tissue heterogeneity

The oral and craniofacial complex is supported by tissue niches of incredible diversity, variously comprised of epithelia, blood and lymphatic vessels, cartilage, bone, ligaments, and muscles as well as adipose and peripheral nervous tissue (Nanci and Ten Cate 2017). Broadly, this anatomy has been well-described for decades (Nanci and Ten Cate 2017); however, recent genetic and genomics approaches using mouse models have found intra- and interspecific niche heterogeneity among the periodontium (Nagata et al. 2021; Zhao and Sharpe 2021), tooth (Chiba et al. 2020; Krivanek et al. 2020; Sharir et al. 2019; Takahashi et al. 2019), salivary glands (Hauser et al. 2020; Sekiguchi et al. 2020; Song et al.2018), palate (Byrd et al. 2019; Han et al. 2021; Li et al. 2019), buccal mucosa (Jones et al. 2019), and tongue (Almanzar et al. 2020; Tabula Muris et al. 2018). Some of this data has recently been integrated (Huang et al. 2020), and though primarily focused on healthy murine barrier epithelia, there appears to be both common and unique cell types among these niches left to be discovered and validated.

Traditionally, oral and craniofacial tissues have been broadly defined according to their histological features; distinct keratinised and non-keratinised stratified epithelia and connective tissues composed of fibroblasts, vascular cells, and extracellular matrix (ECM) components (Jones and Klein 2013; Nanci and Ten Cate 2017). In that historical perspective, common oral diseases were largely attributed to environmental factors: smoking, alcohol, and viruses contributing to oral cancer; microbial dysbiosis to dental caries and periodontitis. With increasing advances in the molecular profiling of oral and craniofacial tissues, it is now

established that host susceptibility is equally as important in determining clinical disease outcomes, whether for these commonly studied diseases as well as for the other hundreds of oral and systemic diseases that affect the oral and craniofacial soft tissues (Ariyawardana and Johnson 2019; Stoopler and Sollecito 2014). Therefore, it is crucial to account not only for tissue heterogeneity, but also human ethnic and ancestral diversity in the donor datasets, including microbiome sampling.

Collecting oral and craniofacial atlases as reference datasets

Significant progress has been made by the HCA community with the current total of profiled cells to date numbering about 40 million cells from 15 major organs (Lindeboom et al. 2021) (https://data.humancellatlas.org/). While it is estimated that there are about 40 trillion cells in the human body, we estimate that there are about 2 trillion in the oral and craniofacial tissues and upwards of 200 distinct cell types.

While progress in single-cell profiling human oral tissues has been slow in comparison, researchers have made significant progress as of 2022, publishing atlases of the oral mucosa, including the buccal mucosa, tongue, and gingiva (Consortium and Quake 2021; Huang et al. 2021; Williams et al. 2021), major and minor salivary glands (Consortium and Quake 2021; Costa-da-Silva et al. 2022; Huang et al. 2021), dental pulp (Krivanek et al. 2020; Pagella et al. 2021), periodontal ligament (Pagella et al. 2021), tonsil (King et al. 2021), and even saliva itself(Choudhury et al. 2020) (Figure 1); additional craniofacial niches such as the temporomandibular joint and skeletal muscle are planned and being executed as well.

In sum, these and active studies have already identified regional differences in cellular types, proportions, states, phenotypes, and niche-dependent interactions as well as highlighted the importance of the tissue extracellular microenvironment in shaping resident epithelial, stromal, and immune cells' identity. Within 16 months (between September 2020 and January 2022), the oral and craniofacial tissues made significant strides forward to the modern age of single cell biology (Aldridge and Teichmann 2020), including nearly ~250,000 cells representing the first cell types from 8 distinct niches from healthy adults (Figure 1; Table 1). Within the OCBN, multidisciplinary teams are

already conducting experiments incorporating single cell and spatial multiomics in paediatric and adult human subjects.

While early efforts have primarily focused on the adult oral mucosa, understanding the diverse musculoskeletal development using these toolkits will allow for a more detailed investigation into the molecular mechanisms controlling tendogenesis, chondrogenesis, and osteogenesis which support craniofacial function and a better understanding of craniofacial anomalies and enhance cranial skeletal repair. Studies in cranial skeletogenesis have provided invaluable insights into the cellular and molecular mechanisms that generate and shape cartilage and bone and studies on cranial neural crest cells migration have long-established this process at the centre of multiple craniofacial malformations, such as pharyngeal arch syndromes and neurocristopathies.

Illuminating intercellular communication networks between defined cell types

With this reference dataset, we aspire to accelerate discoveries in the domains of both basic and clinically applied research. Expression profiling of different cell types in adult human tissues has shown how intercellular communication contributes to tissue function by coordinating cell functions in development and homeostasis, thus when there are signalling defects, disease will follow. The study of intercellular communication has significantly accelerated with advances in the single-cell field with several studies discovering novel signalling mediating cellular differentiation and immune responses.

Intercellular crosstalk has been investigated in oral tissues with OCBN studies demonstrating how in periodontal disease there is a shift in the transcriptional signatures of stromal and epithelial oral mucosa cells to an inflammatory profile (Caetano et al. 2021; Williams et al. 2021). In disease, endothelial cells also showed upregulation of pathways related to lymphocyte adhesion and chemokine signaling (Williams et al. 2021).

Given that most cellular crosstalk is spatially restricted with signals working from 0 to 200μm range, spatial transcriptomics data are essential to understand intercellular communication in healthy and diseased tissues.

To investigate how surrounding cells may regulate signalling, several computational methods have been recently developed to integrate spatial information with ligand-receptor analyses (Dries et al. 2021; Efremova et al.

2020). Another key example of intercellular signalling progress includes the functional elucidation of the ECM, which was once thought to be only essential in providing cellular physical support. However, it is now established as a critical component in the form and function of all oral tissues. For example, periodontal ligament and gingiva fibroblasts sense and transmit mechanical stimuli through elements of the extracellular matrix, such as collagens and integrins (Nanci and Ten Cate 2017). However, to translate this to the clinic will require harmonised and consistent cell-type annotation between different human atlases to allow for accurate intercellular communication network modelling as disease networks consistently are sequenced and added to the HCA datasets. Harmonised nomenclature and annotation of cell types/states when integrating different source organ atlases is one of the current efforts of the HCA to achieve a unified reference cell ontology across tissues (Osumi-Sutherland et al. 2021).

While these initial OCBN projects have started to construct high-resolution maps of organs and tissues, there is an unmet need to integrate these atlases to interrogate analogous cellular components across tissues niches and to develop a common coordinate framework for the healthy oral and craniofacial human tissues (Figure 2). So far, most annotations of OCBN single-cell genomics datasets 210 have distinct cell type annotations – even within the same tissue (Table 1).

This discordance makes it difficult to relate findings between studies, highlighting the need for a common ‘language’ for cell annotation. This framework will aim to address clinical and spatial variability within studies and allow for more accurate and precise comparisons between datasets across the human body. Relevant clinical metadata will include (but is not limited to) donor gender/sex/ethnicity, age, tissue type, relevant clinical data, the technology used; spatial data will define the position, tissue plane, site, and size of anatomical structures (Table 1).

Furthermore, future work including these clinical and biological data will highlight the niche-specific cellular diversity that may help to explain why some oral diseases manifest in some oral and craniofacial niches while sparing others. For example, understanding niche-specific cellular heterogeneity in paediatric tissues from neonatal to infancy, juvenile, and adolescence periods will allow the identification of cell states and cell lineages involved in tissue maturation and a better understanding of early disease onset in childhood. However, considering

the vast literature on immune training in early development, these efforts should also reveal the mechanisms of healthy and pathologic aging that may lead to early and accurate prognostics tools allowing for early intervention, similar to what is proposed by the LifeTime Initiative (Rajewsky et al. 2020; 2021).

This additional level of annotation is already relevant. For example, the concept of structural (i.e.niche-specific) immunity has recently been described across the body, suggesting that each tissue's cell-specific composition can instruct their niche-distinct immune response (Krausgruber et al. 2020).

This lens will provide a new framework for interrogating human disease by mapping disease risk genes and also for predicting cell-type specific and co-regulated gene modules, as recently described in periodontitis (Williams et al. 2021).

For the long-term success of the OCBN, high-quality and widely available single cell and spatial multiomic datasets will need to be published along with detailed metadata for each experiment and study.

Integrated analyses within and across multimodal data

A fundamental challenge when constructing biological systems is the correct definition of cell state, which is achieved by applying complementary approaches, such as molecular characterization (transcripts, distribution of chromatin marks and proteins) and functional testing (Figure 2). However, we cannot characterize all types of molecules at the same time, and despite the generation of a growing body of single cell data, gene expression is still the overarching measurement.

To find what the cell is doing, requires proteomics, metabolomics, and functional assays to provide a direct readout of cellular activity. Furthermore, there is a need to interrogate a higher number of molecular dimensions. No single 'omics' technology can fully define the complexity of molecular mechanisms, but taken together, these integrated data have the potential to provide a more comprehensive landscape of basic biological processes and human disease. Multimodal measurements, where distinct molecular parameters can be interrogated in the same cell have been recently developed, and are now allowing us to characterize cells, cell states, and transitions between cell states across multiple levels of regulation.

Multimodal sequencing has the capacity to move the field from descriptive ‘snapshots’ towards a mechanistic understanding of gene regulatory networks, and importantly, to refine sources of cellular heterogeneity as already applied to the immune system (Hao et al. 2021).

The use of multimodal single-cell omics is therefore revolutionizing our understanding of cellular biology; however, relying on the dissociation of cells from their natural tissue environment limits our ability to understand the role of intrinsic and extrinsic factors that underpin cellular communication and organ function. Indeed, today in clinical settings, histopathology is a standard diagnostic tool as many diseases are defined by abnormal cellular organization. Additionally, many scientific discoveries rise from the understanding that cellular organization in tissues is highly connected to biological function. Thus, combining single-cell molecular measurements with histology and microscopy assays will be required to ultimately generate biological insights into human health and disease. Current spatial methods are rapidly developing at the gene expression and chromatin accessibility levels (Thornton et al. 2021), with increasing degrees of resolution. Spatial multimodal measurements are also under rapid development and, so far, they have allowed a high spatial resolution co-mapping of the whole transcriptome and a panel of 22 proteins (Liu et al. 2020).

In any multimodal approach, two emerging concepts of data integration will also be key for this effort. Firstly, spatial multiomic approaches, which include information on the location of cells, will need to be integrated with these single cell multiomic maps. The advantage of these newer assays is for the annotation of regional/cell molecular identities within the tissue architecture, unravelling cell-cell communication with a spatial context, and clarifying tissue microniches, now referenced as “cell neighbourhoods” within tissues (Nitzan et al. 2019; Stahl et al. 2016; Vickovic et al. 2019).

Understanding the cellular context including extracellular components and signalling molecules that contribute to organ homeostasis will help to further identify the functions of specific cell types and interactions as well as provide mechanistic insights into fundamental biological processes in health and disease. Secondly, it will be necessary to integrate human multiomics data with common model organisms as well as patient-derived experimental disease models during the progression from health to disease.

The interrogation of these in parallel will be essential for functional assays for data validation and the development of new testable hypotheses, ultimately accelerating targeted follow-up studies to enter the clinical research space.

Clinically relevant innovation, discovery, and collaboration

The clinical significance of single cell approaches has been successfully demonstrated in various human diseases by allowing the identification of disease-associated cell phenotypes, for example, malignant tumour cells within a tumour's mass (Tirosh et al. 2016) or the identification of immune cells that can predict clinical outcomes and enhance treatment strategies (Sade-Feldman et al. 2018).

In the oral and craniofacial region, there are now glimpses of what is possible. For example, stromal cells promoting neutrophil migration in health that expand in disease have been identified (Williams et al. 2021), and an IgG plasma B cell response was identified as a hallmark of periodontitis(Caetano et al. 2021; Williams et al. 2021). The impact of single cell approaches in understanding human oral disease was further demonstrated during the COVID-19 pandemic; the oral cavity was proved to be an important site for infection with saliva as a potential route of transmission(Huang et al. 2021). This is a minute sampling of the ever-growing clinical advances made possible through single cell approaches.

Many oral conditions will benefit from the molecular characterization of cellular subpopulations.

It will provide valuable insights into factors that affect disease progression of head and neck tumours,such as oral carcinoma; potentially malignant disorders of the oral cavity such as proliferative verrucous leukoplakia (Thompson et al. 2021); oromucosal diseases such as lichen planus and vesiculobullous diseases; salivary gland disorders such as Sjogren's syndrome, and odontogenic and bone pathologies.

Deepening our understanding of molecular characterization of cellular subpopulations will uncover the processes involved in oral manifestations of systemic disease,especially gastroenterology diseases such as Crohn's disease, rheumatological conditions including Sjogren's Disease, and systemic

haematologic diseases including white and red cell dyscrasias, sickle cell anaemia or leukaemia.

Additionally, in partnerships with many foundations and groups like the National Organization for Rare Disorders and the Chan Zuckerberg Initiative Rare as One Network, rare conditions with shared or sole oral and craniofacial manifestations may finally be able to capitalise on their long-standing needs for novel treatments and precise management. Future research may even demonstrate the precise molecular effects of pharmacotherapeutic approaches, to understand the beneficial and adverse effects of therapeutic agents. To succeed in achieving these aims, the network actively seeks to engage all stakeholders including patients, oral physicians, researchers, cell biologists, and data scientists, all of whom share in the collective vision of developing our understanding of oral health and disease—all while adhering to our values of transparency and open science (Regev et al. 2018).

Discussion

Outlining a phased roadmap for the OCBN

While work from the OCBN has just begun to reveal the diverse tissues and fluids of the oral and craniofacial complex (Phase 1), we are already planning to conduct studies that illuminate how these niches harmoniously integrate into the vital functions of communication, defence, breathing, and digestion (Phase 2). The OCBN is committed to establishing an initial version of the Oral & Craniofacial Atlas within two years, with a web-based resource that makes these integrated data easy to access and analyse to encourage a wide-range use from the clinical and research communities. We will fund these initiatives through funding opportunities available via government, charities, industry, and other private partnerships. We will realise this vision by focusing on integrated discovery, utilizing a team science approach that has the potential to advance the development of precise diagnostics, prognostics, and biologics (Figure 3).

Furthermore, by taking an agnostic approach to tissue and organ physical location (e.g., not oral but airway; not skin but stratified squamous epithelia)

should allow for shared discoveries to accelerate human health clinical benefit. For example, while the oral cavity is an important ecosystem, it is also a crossroads that is impacted by the condition of other—even distant—body sites. This is not a surprise; these tissues are intimately connected to the nervous, immune, cardiovascular, and endocrine systems (Tirosh et al. 2016). In a bidirectional manner, the condition of the oral cavity can impact distant sites as well (Beck et al. 2019). Progress towards comprehensive mapping of oral and craniofacial tissues requires not only careful experimental design to robustly capture variation within and across individuals but importantly, a physiological insight to interpret data and curate datasets. To this end, it is essential to increase dialogue between biologists, computational data scientists, clinicians, pathologists, and statisticians to achieve a consensus on data curation and cell annotation and deliver data analyses platforms that are relevant and user-friendly (Figure 3). Moreover, public engagement involving different communities and research participants will be essential to articulate the motivations of the project and to raise awareness of this project's ambitions and research priorities. In addition, public data portals and biorepositories that enable users to easily access and analyse HCA data will be essential to the HCA goal of inclusivity, integrity, and data sharing (https://data.humancellatlas.org; https://oral.cellatlas.io; https://www.covid19cellatlas.org/byrd20/).

Finally, the Human Oral and Craniofacial Cell Atlas encourages and supports the participation of scientists and clinicians from countries around the globe by recognising the need to integrate different ethnicities, environments, and regional diseases, and we invite any interested stakeholder to join the network, participate in our meetings, and contribute their data for the integrated atlas. We are aware of potential challenges and limitations, including available resources, but we are committed to shared protocols (Greenwell-Wild et al. 2021), open and immediate data release, and prioritising international collaborative work. Collective development of research ideas and equitable partnerships guided under the HCA Ethics Working Group is our priority. To enable the advancement of such large-scale projects, we will aim to implement ethically responsible, socially robust, and legally compliant research from the beginning. Continuous monitoring, in the form of yearly consortium meetings, should be tasked with the analysis of emerging ethical and societal aspects from the use of these technologies. These meetings will also attempt to identify weaknesses or areas

where progress was inadequate and take actions to flag areas of expertise that are missing. The task forces formed during the initial meetings should meet regularly to apply concrete changes for patient groups, researchers, or data collectors.

Conclusions

In sum, there is an enormous opportunity for integrated and precise oral health care initiatives that leverage the accessibility of this space to improve oral and systemic health. Profiling of these conditions from a patient-centred perspective will increase our understanding of disease cellular origin, mechanisms, aetiology, and diagnostics—rendering them experimentally tractable to test new hypotheses for better diagnosis and drug discovery. To achieve this grand vision requires the collaboration of a multidisciplinary, international team—spanning the basic and computational sciences to clinical practice. This team science approach will be key to achieving inclusive, ancestrally diverse, open access, multiomic reference atlases of the human oral and craniofacial tissues and fluids across the lifespan. This open data resource will provide quantitative, multi-scale, information sufficient to build integrated prediction models of key oral and craniofacial cell and tissue states to develop breakthroughs in oral health for all.

Figure 1 | A Blueprint for the Human Oral & Craniofacial Cell Atlas

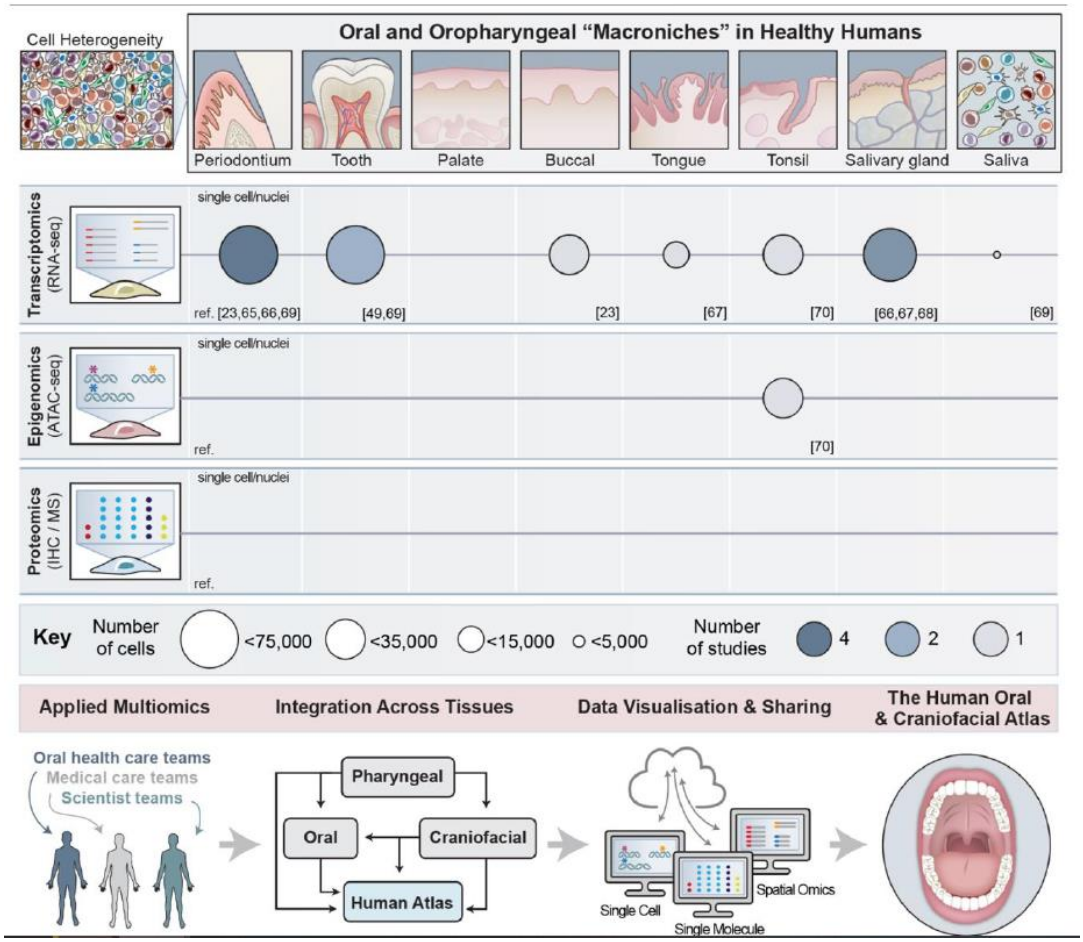
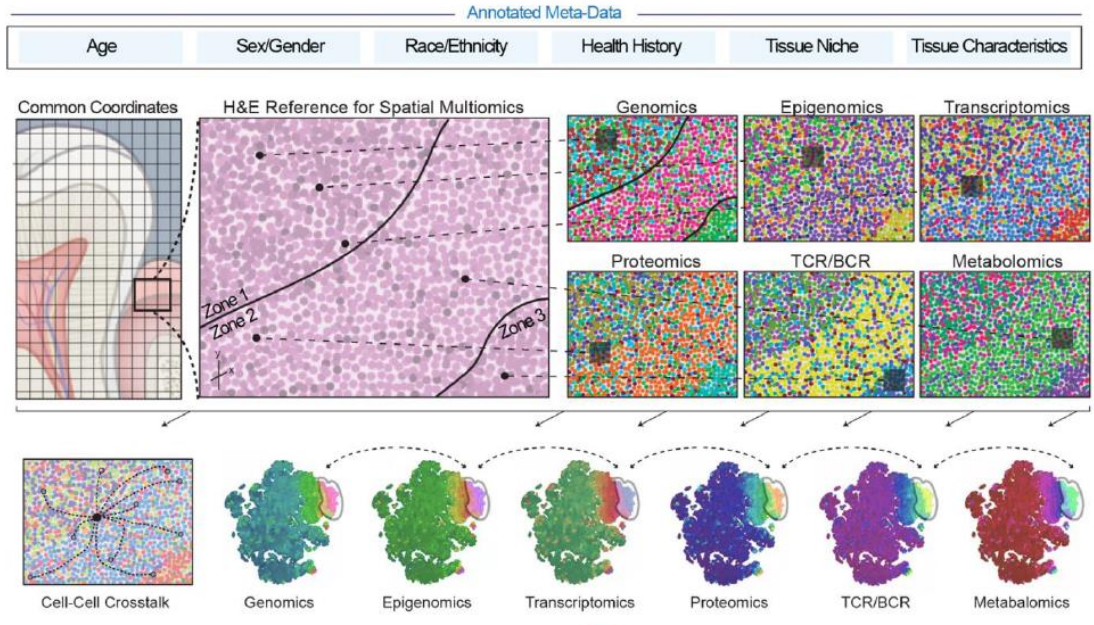


Figure 2 | Oral and Craniofacial Spatial Multiomics for Coordinated and Integrated Analyses



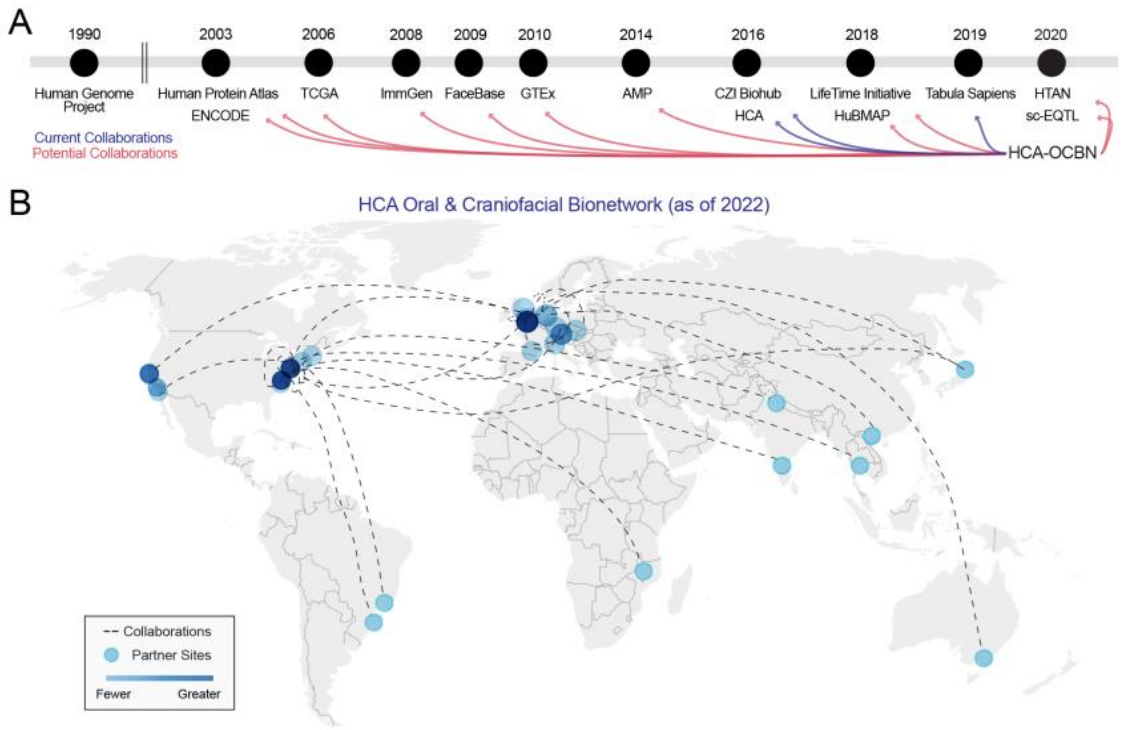


Figure Legends

Figure 1. Oral and craniofacial tissues niches are incredibly diverse, including periodontium, tooth, palate,buccal mucosa, tongue, tonsils, salivary glands, and the fluid from the saliva—these do not account formicroniches that are unaccounted for among these tissue spaces. Each niche is supported by some combination of epithelia, cartilage, bone, ligaments, muscles, adipose tissue, blood and lymphatic vessels, and nerves; and these tissues are harmoniously integrated into the vital functions of communication, feeding, breathing, defence, sensing, and early digestion. The Human Oral & Craniofacial Cell Atlas, supported by the Oral & Craniofacial Bionetwork (OCBN) aims to create comprehensive and integrated cell atlases to understand the common and unique cell types that support these niches in health and uncover which cell types and networks are affected in disease. We will do this using single cell and spatial multiomic (transcriptome, epigenomic, proteomic) approaches and will incorporate additional omics technologies as assays are refined and available. Thus far, most work from the OCBN and others has focused on single cell transcriptomic (scRNAseq) and epigenomic (ATACseq) approaches from healthy adults, including from nearly all major tissue niches and saliva. Work is currently being done with the OCBN for collecting, integrating, visualising, sharing, and

applying the knowledge gained from these early studies. The number of studies developed so far and total number of cells profiled is depicted under each tissue. [Tissue illustrations inspired by Huang et al.(Huang et al. 2021); Credit: Heather McDonald, BioSerendipity, LLC, Elkridge, MD]

Figure 2. Coordinated efforts across the Human Cell Atlas are working toward building a consensus of the necessary metadata (clinical and biological) to generate a common coordinate framework for the human body. Future work including these additional layers of data will highlight the diversity of cell types and states across humans considering age, sex, race, ethnicity, ancestry, oral and systemic health history, as well as the specific niche, the tissue orientation, and the health status of that niche (healthy, inflamed) at the time of sample collection. There is an immense need to interrogate a higher numberof molecular dimensions or human tissues [genome, transcriptome, epigenetic modifications,proteome, T Cell Receptor and B Cell Receptor repertoires (TCR/BCR), and metabolome of the collected sample itself as no single ‘omics’ technology can fully define the complexity of molecular mechanisms, but taken together, these integrated data have the potential to provide a more comprehensive landscape of basic biological processes and human disease. Multimodal sequencing has the capacity to move the field from descriptive ‘snapshots’ towards a mechanistic understanding of gene regulatory networks, and importantly, to refine sources of cellular heterogeneity as already applied to the immune system. The use of multimodal single-cell and spatial multiomics is therefore revolutionising our understanding of cellular biology; however, relying on the dissociation of cells from their natural tissue environment limits our ability to understand the role of intrinsic and extrinsic factors that underpin cellular communication and organ function. Spatial multiomic approaches, which include information on the location of cells, still will need to be integrated with these single cell multiomic maps.

Figure A. Collaboration Opportunity Across Initiatives, Networks, Groups, and Consortia

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CAPÍTULO 6

Teleodontologia e o Sistema Único de Saúde: uma importante ferramenta para a retomada da Atenção Primária à Saúde no contexto da pandemia de COVID-19

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Teleodontologia e o Sistema Único de Saúde: uma importante ferramenta para a retomada da Atenção Primária à Saúde no contexto da pandemia de COVID-19

Introdução

Em 11 de março de 2020, a organização mundial de saúde (OMS) decretou estado de pandemia da COVID-19. Uma nova infecção pulmonar, que se iniciara na cidade de Wuhan (China) e causava sintomas de uma síndrome respiratória aguda grave [1]. O agente biológico causador de tal acometimento é um coronavírus, vírus conhecido por outras duas epidemias do século XXI: a SARS (2002) e a MERS (2011), que tiveram disseminação restrita ao sudeste asiático.

A COVID-19 se difere dessas outras epidemias em sua alta e rápida disseminação pelo mundo, tal eficácia está pautada em três pilares: 1. Longo período de incubação 2. Letalidade relativa 3. Alta transmissibilidade dos assintomáticos [2].

A COVID-19 apresenta especial impacto na odontologia, à medida que estudos indicam sítios da cavidade oral como possíveis focos de entrada do coronavírus nas células do hospedeiro humano. Trabalhos realizados com outros coronavírus em animais, demonstram que receptores de angiotensina presentes nos ductos das glândulas salivares, podem ser o alvo primário de invasão celular do patógeno [3].

O fato deste vírus estar presente dentro de células de tecidos bucais mitiga a chance de que bochechos pré-operatórios eliminem totalmente o coronavírus das secreções salivares e creviculares [4]. Isso faz com que o aerossol gerado durante os procedimentos odontológicos seja potencialmente contaminado.

Portanto, a atividade odontológica é uma das mais críticas, pois grande parte dos procedimentos operatórios gera aerossóis, soma-se a isto, a escassez de equipamentos de proteção individual, a falta de protocolos claros de biossegurança e a necessidade de preservar as equipes de saúde e reduzir os riscos de contaminação dos usuários, diante deste cenário, os sistemas de

saúde suspenderam os procedimentos eletivos em odontologia, com manutenção apenas de urgências e emergências, em diversos países, conforme recomendado por diversas instituições e governos locais [5-9].

Neste contexto, a Teleodontologia tem sido citada por artigos científicos [10-13] e documentos governamentais, como uma alternativa para garantir cuidados em saúde à população. Além disso, no contexto do Sistema único de Saúde (SUS), as Equipes de Saúde Bucal (ESB) podem fazer uso deste tipo de ferramenta para realizar atividades que as integram à equipe multiprofissional no enfrentamento da pandemia, como o rastreamento, Teleorientação e Telemonitoramento dos usuários com suspeita de COVID-19 e seus contactantes. O objetivo deste “short communication” é descrever e analisar, com base nas melhores evidências científicas disponíveis, as possibilidades de atuação e estratégias de implementação da Teleodontologia, como ferramenta estratégica para a oferta do cuidado em saúde bucal no contexto da pandemia de COVID-19, observando as normas brasileiras, as diretrizes do Sistema Único de Saúde e as possibilidades de permanência deste cuidado mediado por tecnologia pós pandemia.

A Teleodontologia: Uma breve revisão da literatura

A Teleodontologia tem se mostrado eficaz tanto no custo quanto na disseminação do acesso, sendo um meio de democratização e equidade, com vantagens relacionadas ao aumento da resolutividade, à redução do tempo de espera e dos custos de tratamento [14-16]. Uma revisão sistemática mostrou a sua relação custo-benefício com pacientes que vivem em áreas rurais, provando ser monetariamente eficaz, com a implementação da Teleodontologia nas práticas odontológicas. Este estudo também mostrou que a Teleodontologia pode ser eficiente na triagem de lesões orais, principalmente em programas escolares, em áreas rurais e com acesso limitado aos cuidados e instalações de cuidados prolongados [17].

O uso das Tecnologias de Informação e Comunicação (TIC) ocorre como parte dos serviços de saúde pública odontológica em países da América Latina, como Brasil, Colômbia e Uruguai, para melhorar a educação continuada e pesquisa colaborativa entre instituições nacionais e estrangeiras [16,18]. No Brasil, através das TIC, foram trocadas informações entre universidades e profissionais

da atenção básica, agregando valores para a relação ensino-serviço e sendo uma forma inovadora de atendimento e qualidade de serviço [19]. Essas tecnologias, que também são úteis para orientação de capacitações e atividades de educação continuada, neste momento de pandemia, têm sido utilizadas para pré-triagem, orientação de profissionais e pacientes, em países como Paraguai e Costa Rica [20,21].

No entanto, há escassez de projetos de Teleodontologia nos países em desenvolvimento, que tem sido atribuída ao conservadorismo dos tomadores de decisão, à falta de recursos, infraestrutura e equipamentos de TIC [22]. Um outro problema é a prestação de serviços de atendimento odontológico ainda se basear em atendimento emergencial e curativo, com falta de protagonismo dos cuidados preventivos [22]. Com a crise da COVID-19, emergiu a necessidade de incorporação da Teleodontologia na rotina do atendimento odontológico, em especial no Sistema Único de Saúde [23].

A pandemia introduziu preocupações extras aos profissionais e usuários dos serviços de saúde bucal, entre eles os pacientes com diagnóstico de câncer de cabeça e pescoço, considerando que: 1. alguns pacientes não estão preparados emocional ou psicologicamente para receber tratamentos que mudam a vida; 2. os pacientes diagnosticados com câncer ou com suspeita de lesões malignas devem ser monitorados constantemente e; 3. a falta de acompanhamento dentário pode levar a um aumento de casos não diagnosticados, causando danos futuros ao paciente devido ao atraso no diagnóstico [24].

O uso das TIC para fornecer atendimento odontológico remotamente pode permitir que as ESB realizem triagem para atendimentos odontológicos de emergência e urgência, que evitem a ida desnecessária de usuários as unidades de saúde e forneçam serviços não essenciais, evitando contato próximo com os usuários. Por outro lado, aqueles que não oferecem este tipo de serviço devem continuar cumprindo sua obrigação profissional contínua de responder às perguntas e não abandonar os pacientes [25].

Uma rapid review com recomendações para a reabertura de serviços odontológicos, concluiu que 94% das fontes fornecem informações sobre como agrupar pacientes, principalmente por telefone, para incluir a avaliação de risco

do status potencial de COVID-19 (por exemplo, COVID-19 positivo, suspeito de COVID-19 , necessidade / proteção especial assintomática), o que pode contribuir para o rastreamento e telemonitoramento de usuários doentes (sintomáticos ou assintomáticos) e seus contactantes. Alguns estudos também recomendam estratégias como a triagem de temperatura na recepção, consultas por telefone e vídeo, triagem por tela telefônica de todos os pacientes em busca de sinais ou sintomas de doença respiratória e avaliação sistemática do paciente no momento do check-in nas clínicas odontológicas [26].

Discussão

As diversas especialidades que realizam procedimentos ambulatoriais envolvendo a região de cabeça e pescoço, tais como odontologia, otorrinolaringologia e oftalmologia, sofreram um “lockdown” com a instalação de uma nova pandemia, a COVID-19. Tal decisão foi pautada em dois pilares: 1) os atendimentos ambulatoriais relacionados a tais especialidades apresentam alto risco de contágio visto o íntimo contato do profissional de saúde com aerossóis potencialmente contaminados; 2) dificuldade na aquisição de equipamentos de proteção individual, que possibilitariam uma redução da exposição operador-paciente [35].

Para mitigar o risco de contágio e ainda estimular medidas de distanciamento social, alternativas ao atendimento presencial rapidamente se mostram elegíveis. Em todo o mundo grande parte das profissões tem revisto suas práticas e na área da saúde, os atendimentos em telessaúde são citados pela literatura como alternativa assertiva e viável para garantir o acesso à rede de saúde pelos pacientes [27].

No Brasil, medidas emergenciais foram tomadas, visando adequar o processo de trabalho dos serviços de saúde à nova realidade imposta pela pandemia. A maioria dos conselhos adotaram uma abordagem cautelosa a fim de garantir o cuidado em saúde mediado pelas tecnologias de informação e comunicação, mas restringindo as atividades possíveis. No caso do CFM, mesmo que restringido ao momento atual, a resolução permite a realização de teleconsultas, diagnóstico, telemonitoramento, teleinterconsulta e teleorientação [36].

No que diz respeito ao CFO, tardiamente, foi publicada normativa que tornou expressamente vedado o exercício da odontologia à distância, mediado por tecnologias, para fins de consulta, diagnóstico, prescrição e elaboração de plano de tratamento odontológico. Entretanto, foram admitidas atividades de telemonitoramento e teleorientação, desde que não sejam realizadas por centrais de atendimento ou qualquer outro meio que centralize o recebimento de demandas e as distribua automaticamente [30].

Além disso, através da normativa, foi vedada às operadoras de planos de saúde odontológicos e demais pessoas jurídicas, a veiculação de publicidade e propaganda utilizando o termo “Teleodontologia”, termo internacional que engloba outras expressões aqui discutidas na odontologia e que serão estratégicas para a sua retomada no “novo mundo”, principalmente no contexto da Atenção Primária à Saúde (APS). Essa segregação do termo “Teleodontologia” tira o Brasil de um cenário mundial, no qual se discute o cuidado em saúde bucal mediado por tecnologia em diversos níveis de atenção à saúde e cabe à academia e aos grupos que militam e estudam telessaúde e teleodontologia no Brasil resistir e reafirmar a Teleodontologia como área do conhecimento e ferramenta de cuidado em saúde.

Ainda que a resolução do CFO necessite ser revista e precise contemplar as necessidades do mundo trans e pós-pandemia, mesmo com todas as limitações, esta resolução já permite uma retomada da odontologia com as ferramentas do telemonitoramento. Se monitorar é vigiar, a equipe de saúde bucal pode realizar “exames clínico, mediado por tecnologia, com finalidade epidemiológica”, então é urgente que se desenvolva ferramentas capazes de fazer triagem de risco, busca ativa do paciente com suspeita de câncer de boca, cuidado remoto dos acamados e dos pacientes com necessidades especiais, no sentido de “orientar” familiares e usuários com uso de TIC. Ainda, respeitando os limites impostos pela resolução do CFO, é possível que as equipes realizem “escuta inicial”, mediada por tecnologia, a fim de orientar o usuário, realizar pré-triagem, organizar agenda e o fluxo nas unidades para evitar acúmulo dos pacientes em salas de espera [30,35]. Vale ressaltar ainda que os gestores vão enfrentar problemas para registrar parte desses procedimentos nos sistemas de informação, já que muitos deles são classificados como “consultas”.

Desde o início da pandemia, observamos constantemente cálculos em todos os veículos de comunicação sobre a necessidade de leitos de UTI, o que colabora com o entendimento da população sobre a gravidade do problema. Entretanto, apesar de o Protocolo do Ministério da Saúde informar que 80% dos casos serão atendidos na APS [37], pouco tem sido discutido sobre o impacto na APS, que é o nível de atenção à saúde em que os profissionais atuam próximo aos territórios, às famílias que ali residem e aos determinantes sociais [38]. É na APS que podemos conscientizar as pessoas sobre sua responsabilidade social no momento que estamos vivendo, que podemos monitorar os casos suspeitos e seus contactantes, mesmo com a disponibilidade de poucos testes para diagnóstico, e organizar atividades de promoção de saúde [39]. Ademais, a Nota Técnica Nº 9/2020-CGSB/DESF/SAPS/MS afirma que a ESB "os profissionais de saúde bucal, como corresponsáveis pelo cuidado da população e integrantes das equipes multiprofissionais, deverão compor a equipe que realizará as ações do FAST-TRACK COVID-19*" [35], entretanto, os gestores municipais têm enfrentado dificuldade à medida que não há como registrar a produção desses profissionais nos sistemas de informação, tendo em vista que não houve ajustes, por parte do Ministério da Saúde, nesses sistemas.

Diante da paralisação dos atendimentos eletivos, cabe uma reflexão: "Qual o significado do cuidado odontológico na saúde do usuário?" A Odontologia poderá deixar de atender os pacientes com doenças crônicas (cardíacos, diabéticos, tabagistas e etilistas)? Como fazer busca ativa de câncer de boca no contexto do isolamento social? Como fazer atividades do Programa de Saúde na Escola (PSE) sem aulas presenciais?

Não há dúvidas que a Teleodontologia pode ser uma ferramenta potente para retomada segura do cuidado em saúde bucal no Sistema Único de Saúde, já que no contexto da saúde pública temos que planejar as ações com base nos princípios da universalidade, equidade e integralidade.

No Rio Grande do Sul, por exemplo, há um serviço muito eficiente disponível aos dentistas do Sistema Único de Saúde, chamado "EstomatoNet", que recebe demanda de dentistas e médicos da atenção primária, com envio de perguntas, dados clínicos e até fotos, para auxiliar no diagnóstico e na condução dos casos [40]. Este mesmo grupo chegou a usar a plataforma Whatsapp para

trocar informação entre os profissionais da atenção básica e os teleconsultores do programa telessaúde redes do Rio Grande do Sul [41].

Ainda sobre o programa Telessaúde Brasil Redes, é importante reforçar que houve grande investimento nos últimos anos para disseminação de Núcleos pelo território nacional e atualmente o Sistema Único de Saúde conta com uma malha qualificada e capilarizada que pode ser utilizada para impulsionar a teleodontologia no Sistema Único de Saúde, em todos os níveis de atenção, com ênfase para a atenção primária à saúde, que é o nível capaz de realizar medidas de prevenção, promoção e monitoramento dos grupos de risco.

No Núcleo Telessaúde Redes Unifesp, as perguntas sobre saúde bucal representam 32,9% de todas teleconsultorias, portanto a odontologia não é desprezível e merece ser observada pelo Conselho que regula o exercício da Odontologia, pelos tomadores de decisão e formuladores de políticas públicas de saúde, no contexto da teleodontologia. No que diz respeito à “Proposta de apoio emergencial à coordenação da saúde bucal do Estado de São Paulo diante da pandemia do COVID19”, observa-se que os trabalhadores do SUS estão dispostos e motivados a discutir e participar de atividades de educação permanente que respondam às demandas de qualificação das ESB em face da pandemia da COVID-19, à medida que a gestão estadual mostra compromisso com seus trabalhadores e grande articulação com coordenadores municipais de saúde bucal e demonstram preocupação com a séria situação que a odontologia enfrenta no SUS. Em relação às dúvidas dos trabalhadores e gestores do SUS, observa-se que a maioria se concentra na biossegurança, o que é natural, à medida que a Odontologia é umas das profissões que mais sofre com este tema na sua prática profissional em face das rotas de transmissão do novo coronavírus.

Há fortes evidências no manejo de doenças crônicas em outras áreas da saúde, em campos distintos como a psiquiatria e a dermatologia. Exames com alta sensibilidade e especificidade demonstram a eficácia do teleatendimento em áreas clínicas e que realizam procedimentos ambulatoriais [42]. No âmbito da prática de telecardiológica, há relação eficaz na resolutividade dos casos atendidos, de alta e baixa complexidade, com melhoria na atenção da saúde de regiões onde tal atenção foi instalada. Estudos mostram que a telemedicina apresenta aumento da adesão do tratamento por parte dos pacientes, além de importante

redução nos custos da atenção terciária. A implementação de teleatendimentos no âmbito das especialidades pode representar uma economia de até 3546 euros para hospitais e centros de referência [43]. A relação de custo-morbidade que as especialidades médicas vivenciam apresentam um limiar de tolerância menor do que os da realidade odontológica, mesmo assim, a prática da Teleodontologia ainda não se mostra tão sedimentado quanto a Telemedicina.

Considerações finais

Em um contexto global complexo, caracterizado pela maior crise sanitária contemporânea imposta pela pandemia causada pelo novo coronavírus, somada, no Brasil, com uma grave crise política, o atraso no diagnóstico e acúmulos de necessidades são problemas que podem causar um impacto financeiro e social importante para os serviços de saúde e seus usuários, e o uso da Teleodontologia tem um grande potencial de enfrentamento, pois permite a redução de barreiras geográficas em um país com dimensões territoriais extensas, bem como o fortalecimento da APS. O Sistema Único de Saúde tem compromisso constitucional com a universalidade do acesso, observando a equidade das ações e a integralidade do cuidado, portanto, cabe aos trabalhadores e gestores deste sistema dar assistência, que no contexto da pandemia só pode ser viável com uso das TIC. O telemonitoramento já permite uma retomada parcial do cuidado em saúde bucal da APS no SUS, mas é preciso que se revise a portaria com inclusão de procedimentos tais como consulta e prescrição, para aumentar as possibilidades da atuação dos profissionais do SUS. Vale ressaltar que as TIC podem ser grandes aliadas para aumentar acesso, neste mundo cada vez mais digital, lutar contra esta realidade pode significar um atraso que vai ser cobrado pela história.

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CAPÍTULO 7

aPDT for oral decontamination of hospitalized patients with COVID 19

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aPDT for oral decontamination of hospitalized patients with COVID 19

ABSTRACT

Emerging severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2) variants may have an impact on the virus's transmissibility and pathogenicity and an increased risk of reinfection. Antimicrobial photodynamic therapy (aPDT) is a promising technique to decontaminate the oral cavity to minimize and inactivate microorganisms' load. This article reports through a case series, a proposal for efficient oral decontamination for hospitalized patients with COVID 19 using aPDT. Samples of oral tissues were obtained after aPDT and analyzed using two methods of RT-qPCR to elucidate qualitative and quantitative viral profiles of SARS-CoV-2 RNA in the oral cavity. There was a reduction of viral load in the oral cavity immediately and one hour after the use of aPDT. This method could be a good option to decontaminate the oral cavity to minimize and inactivate microorganism load.

Keywords: COVID-19; SARS-CoV-2; Photodynamic therapies; oral decontamination

aPDT for oral decontamination of hospitalized patients with COVID 19

Background

SARS-CoV-2 has already been detected in several organs and tissues, including oral tissues and saliva. The role of saliva in disease spread may be associated with viral replication within salivary gland cells that are reservoirs for SARS-CoV-2 [1]

In addition, patients with COVID-19 may undergo long periods of hospitalization and often need oral care. In intensive care units, oral decontamination needs to be effective to prevent oral bacteremia, viremia, and co-infections. The association of oral bacteremia and viremia with systemic manifestations has been reported in a patient with heart disease and COVID-19 [2]. The clinical management of these patients represents a great challenge for healthcare workers, especially when treatments involve exposure to saliva and/or blood. A fact that places them at greater risk of contamination and virus transmission [3].

Photodynamic therapy (aPDT) is an effective alternative method for decontamination of the oral cavity, as it forms reactive oxygen species that can inactivate enveloped and non-envelope DNA and RNA viruses, which suggest their promising potential against SARS-Cov-2 [4] in reducing the risk of contamination for dentists and patients.

In vitro studies confirmed the effectiveness of aPDT antiviral activity against SARS-CoV-2 [4], but no *in vivo* study was conducted with aPDT and SARS-CoV-2. This article reports through a case series, a proposal for efficient oral decontamination for hospitalized patients with COVID 19 using aPDT.

Case Reports

A 42-year-old white man presented himself to the Emergency Military Police Hospital, with a history of nausea, vomiting, cough, and fever (38.8°C). A chest computed tomography scan was performed revealing a pulmonary consolidation (>50%). A diagnosis of pulmonary infection was made. Another 82-year-old white man presented himself to the Emergency Department with a

history of worsening dyspnoea and dizziness. A diagnosis of acute decompensation of chronic heart failure precipitated by pulmonary infection was made. Both men were hospitalized because of the deteriorating respiratory pattern and real-time polymerase reverse transcriptase chain reaction (PCR) confirmed COVID-19.

Oral care and decontamination are important to avoid bacteremia. During the COVID-19 pandemic, these measures can prevent co-infections that could aggravate the general condition. Our protocol was approved by the Committee of Ethics of the Medical Center PMESP (nº. 43125520.7.0000.8847), and the patients signed a consent term. The photosensitizer was prepared by diluting (1.5g/L) curcumin solution (Empório Medicinal Farmácia de Manipulação- São Paulo- Brazil) in dimethylsulfoxide (0.1%) and then in distilled water (980ml), to obtain the final curcumin solution (30 mg/L) [5].

The oral cavity of both patients was rinsed with 10 ml of curcumin final solution for the 30s, and cotton rolls wet with the solution were placed in the upper left superior region for 5 min (Fig 1A). The buccal and gingival surface were irradiated with a blue LED (ECEL ®, RD-7, 455 ± 30nm, 900mW/cm^2) with a cylindrical diffuser tip, (89 mm in length and 6.73 mm in diameter) for 5 minutes (Poly Wireless, Kavo-Brazil) (Fig 1B). The buccal and gingival mucosa surface of the left posterior region were scraped with a sterile punch (Kolplast ci LTDA, Brazil) three times (before therapy, immediately after, and 1 hour after aPDT).

The samples were frozen and stored at -80°C (Fig 1C-D). RT qPCR reactions were performed as previously described [1] All samples were evaluated by the CDC human RNase P (RP) and Charité molecular assays for detection of SARS-CoV-2. The results regarding the effects of aPDT in the Ct values obtained to detect SARS-CoV-2 target genes were descriptive. As the viral load and the Ct values are inversely correlated, we considered a high viral load when Ct≤25, intermediate Ct 26-30, and low Ct ≥31 [6]. Samples from all patients and evaluated periods showed good quality. A detailed description of the Ct values obtained in each patient is shown in Table 1. The patients showed an substantial decrease increase in the Ct values immediately and 1h after aPDT, indicating a decrease in the viral load in the oral cavity. The results regarding the Ct values were very similar using Chaerité and CDC protocols. The patients were followed up for 48h and no side effects of aPDT were observed or reported.

Fig 1: A. Cotton rolls wet with curcumin solution placed in the upper left superior region; B. Blue LED device positioned in the patient's mouth to perform the aPDT technique; C-D. Samples were collected and immediately stored at -80°C.

Table 1: Description of the Ct values mean obtained by Charité and CD protocols for SARS-COV-2 detection in patients treated with aPDT at different time points.

Groups/ Patients	Charité protocol (Ct)			CDC protocol (Ct)		
	Before	Immediatlly after	1h after	Before	Immediatlly after	1h after
Patient 1	17.3	28.8	30.4	17.5	28.1	27.3
Patient 2	26.8	26.3	34.3	25.3	23.3	35.6

DISCUSSION

aPDT has emerged as a promising technique to reduce antimicrobial-resistant pathogens [4]. Our study showed there was an increase in Ct values in the oral cavity immediately andor 1 hour after a single section of aPDT. compared

to baseline. Furthermore, no adverse effects were reported until 48h after the procedure.

Antimicrobial PDT with methylene blue has been used in different procedures, however, in oral disinfection curcumin has been investigated and presented promising results. It has the advantage of being a colorless natural substance, combined with a LED light, safe for the oral tissues, has low cost, and can promote the reduction of microorganisms in a similar way to traditional 1-minute mouthwash with 0,12% chlorhexidine [7,8].

Our results are corroborated by the findings of Santezi et al. (2016) which demonstrated that aPDT reduced the microbial load, and has no side effect on taste, teeth color, burning sensation, or mucosal desquamation. Oral itching was observed only in a few volunteers in the Araújo et al. (2012) study. These aspects are important principally in patients hospitalized with COVID-19, which may have lesions in the oral cavity due to immunosuppression.

The main targets of the curcumin are external structures of the microorganisms, the adhesion of the photosensitizer is sufficient for its destruction when activated by light. aPDT also has a crucial role in the minimal risk of resistance development, which provides an advantage over the mutation ability of SARS-CoV-2 [4] and conventional antimicrobials [7].

COVID-19 determined paradigm shifts for healthcare workers, whose repercussions have not yet been dimensioned. aPDT is a promising technique for clinical use in the pandemic era, but further clinical trials must establish a viable, effective, and safe oral decontamination protocol for patients infected with SARS-CoV-2.

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CAPÍTULO 8

Chlorhexidine mouth rinse against SARS-CoV-2– Case series

Submission in progress

Abstract

The new coronavirus has high transmissibility and virulence, which led to the COVID-19 pandemic. The main form of contagion is through respiratory droplets and saliva, thus, the effectiveness of oral decontamination methods are important strategies for public health in order to minimize and inactivate microorganisms previously of dental procedures. This study reports through a case series, a proposal for oral decontamination for hospitalized patients with COVID-19 using chlorhexidine mouthwash to reduce salivary viral load that would reduce the risk of spreading SARS-CoV-2 through aerosols during dental procedures. Three (3) patients get mouth rinsed for 30s with 0.12% chlorhexidine gluconate (CHLX). The buccal and gingival mucosa surface of the left posterior region were sampled to get a cell block specimen (cells, saliva, and biofilm) with a sterile punch, before, immediately after, and 1h after the CHLX, with the idea to understand the viral kinetics. Samples of oral tissues were analyzed using two protocols of RT-qPCR to elucidate qualitative viral profile of SARS-CoV-2 RNA in the oral cavity. Two patients showed an increase in the CT values immediately and 1h after CHLX, suggesting a decrease in the viral load in the samples region. In one patient, the CT was negative after CHLX but after 1h, the CT values decreased. The use of CHLX mouthwash increase de CT value in two cases, suggesting a slightly reduction of viral load. In one case there was an viral load catch up after the CHLX application. More studies are needed to understand if tradicional mouthwash can be an effective and sustaintable measure to promote viral-oral decontamination.

Keywords: COVID-19; SARS-CoV-2; Chlorhexidine Gluconate; Mouthwash; Oral decontamination.

Introduction

The World Health Organization (WHO) declared a pandemic for the coronavirus (SARS-CoV-2) that causes a severe acute respiratory syndrome. The virus and the emergence of its new variants have generated enormous challenges in the clinical management of the disease due to the increase in transmissibility and virulence, as well as the worsening of clinical conditions (Amato et al. 2020; Carrouel et al. 2021; Casillas Santana et al. 2021; Fernandes Matuck et al., 2021; Matuck et al., 2021; Yoon et al. 2020; WHO, 2021). Based on the currently available evidence, the overall risk related to Omicron remains very high levels of transmission, leading to rapid spread in the world with higher levels of incidence than previously seen in this pandemic. Current strategies and measures are recommended by WHO to reduce transmission through established are crucial aspects of the global strategy to avoid the occurrence of mutations that have negative public health implications (WHO, 2021).

SARS-CoV-2 has been detected in several tissues and organs, including salivary glands and oral tissues (Casillas Santana et al. 2021; Fernandes Matuck et al., 2021; Matuck et al., 2021; Yoon et al. 2020, N Huang et al. 2021). Saliva is the on of the main responsible for the spread of the disease, due to viral replication within salivary gland cells that are reservoirs for SARS-CoV-2 (Fernandes Matuck et al. 2021), and it's main form of contagion is respiratory droplets and saliva disseminated by coughing, sneezing, breathing, or talking (Amato et al. 2020; Carrouel et al. 2021; Casillas Santana et al. 2021; Fernandes Matuck et al., 2021)

Oral procedures in individuals affected by COVID 19 represents a great challenge for healthcare workers, specially for dentists, who cannot keep the distance and are directly exposed to saliva and blood during treatment, placing dentistry as one of the health care areas that poses a greater risk of contamination. (Amato et al. 2020; Carrouel et al. 2021; Casillas Santana et al. 2021; Moreira et al., 2020; Estrich et al., 2020). In addition, patients who are in intensive care units often need oral care and dental procedures. These patients may undergo long periods of hospitalization, and thus, oral decontamination protocols needs to be effective in order to reduce the spread of the virus and prevent oral bacteremia, viremia, and co-infections (Estrich et al., 2020;

Brignardello-Petersen et al., 2019). The association of oral bacteremia and viremia with systemic manifestations after dental procedures has been reported in patients with heart disease and COVID-19 (Moreira et al., 2020).

Several mouthwashs containing antiviral molecules and other compounds could be of interest against SARS-CoV-2, such as hydrogen peroxide (H2O2), chlorhexidine (CHLX), cyclodextrin, Citrox, or essential oils (Carrouel et al. 2020). In addition to this, there are drawbacks, especially in terms of microbial resistance, which requires investigations have focused on the search for alternative methods for oral decontamination (Brignardello-Petersen et al., 2019; Morimoto et al. 2022).

Currently, 0.12% chlorhexidine digluconate is the most widely used antiseptic for oral disinfection and is a broad spectrum antimicrobial agent, have been known to have effective antiviral activity, mainly against lipid-enveloped viruses, but not against nonenveloped viruses (Carrouel et al. 2020). However, there is a controverse if it's use in reducing the risk of spreading SARS-CoV-2 through aerosols, as instructed the Guidelines for the Diagnosis and Treatment of Novel Coronavirus Pneumonia (5th edition) release by the National Health Commission of the Republic of China affirmed that CHLX as a mouthrinse may not be effiective to kill SARS-CoV-2 (Peng et al. 2020). Only one study focused on the effect on SARS-CoV-2, Yoon et al. (2020) evaluated the viral load in the saliva of two hospitalized patients with COVID-19 that used CHLX mouthwash. A transient decrease in the viral load was observed for 2h post gargling, but it increased again after that, something expected for viral infections.

Although, even though CHLX is the most used as a mouthwash in dental practice, doubts remain about its effectiveness to reduce the risk of spreading SARS-CoV-2 through aerosols during dental procedures (Peng et al. 2020). Thus, given the importance of the topic, its controversies and the scarcity of clinical studies, there is an urgent need for studies that address the gap that exists even for CHLX, which is the "gold standard" among mouthrinses, that it is in fact a good option to decontaminate the oral cavity to minimize and inactivate SARS-CoV-2 for oral decontamination before dental procedures in hospitalized patients in a consistant way. (Carrouel et al. 2021; Peng et al. 2020).

Case report

This study was approved by the Committee of Ethics of the Medical Center PMESP (nº. 43125520.7.0000.8847), each participant signed a consent term.

All patients reported below were hospitalized in Military Police Hospital since the real-time polymerase reverse transcriptase chain reaction (PCR) confirmed COVID-19. A 57-year-old man, with a history of smoking, alcohol consumption, without pulmonary involvement, but with thrombosis in the region of the stent in the arm and cardiac pain. Another 58-year-old man presented the diagnosis of pulmonary infection, and difficulty breathing. A third man, 68-year-old also arrived at the hospital with a history of migraine, body aches, sneezing, cough, dyspnea and fever (38.7ºC). A chest computed tomography scan was performed and a diagnosis of pulmonary infection was confirmed.

Patients rinsed the mouth with 15 ml of 0.12% CHLX for 30s (Periogard-Colgate). The buccal and gingival mucosa surface of the left posterior region were sampled to get a cell block specimen with a sterile punch (Kolplast ci LTDA, Brazil) before, immediately after, and 1 hour after CHLX mouthwash.

The samples were frozen and stored at -80ºC. RT qPCR reactions were performed as previously described (Fernandes Matuck et al., 2021; Matuck et al., 2021). All samples were evaluated by the CDC human RNase P (RP) and Charité molecular assays for detection of SARS-CoV-2 (Corman et al. 2020; To et al. 2020; WHO, 2020; CDC, 2020).The results regarding the effects of CHLX in the Ct values obtained to detect SARS-CoV-2 target genes were descriptive.).. A detailed description of the Ct values obtained in each patient is shown in Table 1. Two patients showed an increase in the Ct values immediately and ahour after CHLX, suggesting a decrease in the viral load in the oral cavity. In one patient, the Ct value was undetectable immediately after CHLX, after 1h the Ct values decreased, suggesting a increasing of the viral load. The results regarding the Ct values were very similar using Charité and CDC protocols. Samples from all patients and evaluated periods showed detectable Ct values for the RNAse P gene (Ct <40), indicating the presence of the human RNase P and good quality. The patients were followed up for 48h and no side effects of CHLX were observed or reported.

Table 1: Description of the Ct values mean obtained by Charité and CD protocols for SARS-COV-2 detection in patients treated with CHLX mouthwash at different time points.

Groups/ Patients CHLX	Charité protocol (Ct)			CDC protocol (Ct)		
	Before	Immediatlly after	1h after	Before	Immediatlly after	1h after
Patient 1	28	not detected	25.7	29.6	not detected	22.7
Patient 2	19.3	31.7	29.9	22.4	28.4	30.6
Patient 3	31.6	36.3	38.7	29.3	33.5	35.8

DISCUSSION

Oral decontamination is an important procedure to avoid bacteraemia and viremia specially in hospitalized patients with COVID-19. There are currently no recommendations from the Ministries of Health or the World Health Organization (WHO) for the use of mouthwashs in patients with COVID-19 for oral decontamination previously dental procedures to minimize risks that patients and dentists are exposed during clinical treatment. Available guidance is not based on evidence of the clinical efficacy of pre-procedure (?) mouthwashs to reduce SARS-CoV-2 viral loads or to prevent transmission, but rather on the clinical efficacy of mouthwashs only on other viruses (Carrouel *et al*. 2021).

CHLX is often used in hospitalized patients, however, there is a controverse if it's use in reducing the risk of spreading SARS-CoV-2 through aerosols (Peng *et al*. 2020). Furthermore, it has also been associated with bacterial resistance, there is no evidence of its effectiveness against spores, and the routine use may cause alteration in taste and staining of the teeth and tongue (Carrouel et al. 2021; Yoon et al., 2020).

In our study, two patients showed an increase in the Ct values immediately and 1h after CHLX, suggesting a decrease in the viral load in the oral cavity. In one patient, the Ct value was undetectable immediately after CHLX but after 1h, the Ct values decreased. the first two patients are in agreement with Yoon et al. (2020) that evaluated the viral load in the saliva of two patients with COVID-19 that used CHLX mouthwash, and the viral load decresead for 2 h.

Also, one study using antimicrobial photodynamic therapy (a-PDT) reported the similar antiviral activity against SARS-CoV-2 in hospitalized patients, with a similar behaviour in the Ct values through the evaluated periods in this study (Morimoto et al., 2022).

Thus, these results can be clarified based on the findings by Matuck et al. [5], who found the presence of SARS-CoV-2 in periodontal and epithelial cells, which have the enzyme-converting angiotensin receptor 2 (ACE2), which allows the entry of the virus into cells, not suffering the effective action of the decontamination methods used. Added to this, research has demonstrated viral replication within salivary gland cells, which are considered a reservoir for SARS-CoV-2 (Matuck et al., 2021, N Huang et al , 2021).

The repercussions of the COVID-19 pandemic have not yet been dimensioned, consequently paradigm shifts for the dental and medical community are necessary, and given this new scenario of gradual resumption of dental care, oral decontamination protocols before dental procedures should be better established in order to promote infection control in dental practice during the COVID-19 pandemic.

These findings encourage the routine use of CHLX mouthwash before the dental procedures for hospitalized patients with COVID 19. Thus, it is essential that further research address this knowledge gap, with the need to expand studies of the action of oral decontamination methods on SARS-CoV-2, through increased sampling, to provide greater safety and establishment of effective protocol. CHLX reduced the viral load for until 1h, and has a great advantage since its use is easy, safe to the oral tissues, and has low cost (Carrouel et al. 2021; Yoon et al. 2020; Brignardello-Petersen et al., 2019).

CONCLUSION

Traditional 30s mouthwash with 0,12% CHLX seems to promote the reduction of SARS-COV-2, been an potential viable, and safe oral decontamination protocol for hospitalized patients before dental procedures. Further clinical trials must establish to understand the real effectiveness of CHLX as an oral decontaminatpr an protocols for patients infected with SARS-CoV-2.

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Author contributions statement

SM contributed to conception, design, data acquisition, interpretation, and drafted and critically revised the manuscript. BFM contributed to design, interpretation, data acquisition and analysis, and drafted and critically revised the manuscript. JLAR contributed to acquisition, interpretation, drafted and critically revised the manuscript. MFDSR contributed to interpretation, data analysis, and drafted and critically revised the manuscript. MSM contributed to conception, design, interpretation, and drafted and critically revised the manuscript. KMR contributed to design, interpretation, and drafted and critically revised the manuscript. DPR contributed to interpretation, and drafted and critically revised the manuscript.

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CAPÍTULO 9

Autopsy bridges a forthcoming era of oral-systemic health investigations

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Autopsy bridges a forthcoming era of oral-systemic health investigations

A medical autopsy is a surgical method to investigate and understand the primary cause of death and associated diseases that contribute to the fatality [1]. Autopsies were one of the most antique procedures described in medical history, the concern of physicians about disease courses motivates the observational study of dead bodies, initially, in an external examination. Subsequently, in gross analysis of systems and organs[2].

The first report of a deceased body study came from 300 BC, from Aristoteles school, and Alexandria medical school [3] culminating in a long journey of anatomists and pathologists - Galeno 200AC, DaVinci 1500, Morgagni, and in the beginning of 19th century Rikotansk and Virchow reached the autopsy technique used nowadays.

Autopsies were first described as a pathology tool to understand the physiopathology of severe and acute diseases. In the 15th century, the centralization of autopsies in medical universities brought to the procedure a teaching drapery[4], once medical schools in France and Italy used routine autopsies in the study of *anatomy art*. The surgical specialties benefited from the autopsy technique to practice surgical access and techniques that might be harmful to be executed in a patient by a student [5].

The use of autopsies as a structured tool, not only for teaching purposes, but also to diagnose death causes in hospital and home care, have increased the number of analyzed cases from an incipient number to more than 25% percent of all deaths around Europe ([6]WHO 2020.) Some specialties used the autopsy to determine important changes in the course of diseases, and one of the highest impact alteration seen during these evolution was described in atheroesclerotic disease.[7, 8, 9,10]. The embolus process triggered by the rupture of a coronary plaque was first elucidated by a post-mortem examinations and histopathological studies of heart vessels in patients that deceased by cardiovascular diseases.

The physiopathology understanding of atheroscletic disease enables a whole modification in treatment approach. The evolution of histology and biomolecular methods allowed us to understand the inflammatory component of

cardiovascular disease, changing completely the use of medications to prevent the formation of new plaques.

Rationality the use of autopsies are like a fundamental pillar on diseases understanding, it’s the first step behind a trinomial that permeates all the clinical medical specialties OBSERVATION - COMPREHEND - TREATMENT

The surgical specialties, dentistry included, have the trend to initiate this trinomial in treatment, or even biomaterials studies, and let for the last step the understanding of histopathology process. A great example of this rational logic dynamic is the COVID-19, the first touch with this new disease is the observational moment of seeing patients with severe acute respiratory syndrome, a pneumonia of an unknown cause, and that became a routine in a cluster of patients. The second moment was the comprehensive process, using four respiratory tracts of patients and performing biomolecular tests to evaluate the causal agent [11], and lastly creating treatments (support or curative). On the other hand, we have some dental processes, like cervical non-carious lesions, that treatment is way beyond the comprehensive science. The wide range of desensibilization and restorative material to eliminate symptoms are highly developed and widespread through dental professionals, but which patient is going to develop the disease is in an embrionary stage of the observational process. We still don't know what is the periodont participation, or even if the ligamentary fibers have an influence on impact absorption of occlusal stress? The pulp contributes to a highly mineralization of dynamic that favors the crack of enamel structure?

The majority of these questions are difficult to answer because it's hard to understand the whole situation of these meticulous masticatory system without an observational study of all the components integrated in a contextual view. Researchers should understand, not only the oral mastigatory tissues, but the whole body status and systemic diseases interactions.

The use of autopsies could be enhanced in these situations, for the possibility of removing an entire structure, like the completely alveolar process, gingiva, a glossectomy, hemimandibulectomy, TMJ, complete Waldeyer ring, tongue with neural and vascular complex. The autopsy make possible a complete sample of oral niches, with theirs neighborhoods and site interactions, not only stritcly related to he gastro intestinal system, but also with the respiratory tract(Figure1).

Autopsies were used in dentistry, generic, in cases reports or short reports, but not substantially in research itself; I the early 80's the majority of the studies are related with tissue characterizations and de demosntrations of anatomic sites and celular discovery. Some progretions had happened with the use of immunohistochemistry techniques and some providential studies that embased how periodontal disease can act interact with alveolar bone process, and how far can inflamattory response reach in a cellular level. But the with the adjvent of the biomolecular strategies there was no increase in the number of studies using new technologies.

Recent studies, using autopsies samples, showed the presence of p. Gengivalis in the amyloid plaque formation in Azlheimer disease (AD) patients. The methodology was related to brain specimens obtained in autopsies procedures, but not from gingival tissues from these same patients [12] . The initial idea of a potential link between periodontal disease and AD appeared after several observational and association studies. Not surprisingly, other several studies previously published had suggested that gingipain inhibitors or periodontal treatment could mitigate the appearance of beta-amyloid in brain tissues of AD patients . The literature is sparse when we talk about periodontal disease and gingival conditions analyzed by an axis in the whole patient. Recent the gum-gut axis was explored to evaluate the inflammatory component that can circulate through gingival and circulatory tissues, to evaluate circulating periodontopathogenic organisms, atherosclerotic diseases (13), bowel disease (14), grafted (Tx) patients, DM. that a subject that oral medicine comprehend as central to understand these axis(table1).

It's notice that the most productive centers that publisehd papers with autopsies are those that have in routine some oral tissues in the autopsy routine. Using the Virchow's /Rokitansky technique the removal of tongue and sublingual salivary glands are usual. The use of these autopsy routine samples to understand how tongue tissues can interact with other diseases it's easier in this centers. Not only that, but the fact that autopsy techniques or oral pathologists are used to deal with oral tissues makes easy to sample other tissues when new research projects are created.

Periodontal disease is a complex and multifactorial status, the relation between the bone/gingival inflammation, attachment loss and other systemic conditions still uncertain, once the prevalence of predisposing factor, such as, poor hygiene conditions, smoke, age and diet are convergent with a lot of other prevalent disease, like diabetes, COPD or coronary diseases. Making periodontal disease a two-way contributor, sometimes systemic conditions can modify periodontal status and sometimes periodontal pathogens can contribute to the establishment of systemic disease (15)

Studies using autopsy contribute, in some way, to great advance to understand these relations. The literature showed that p. gingivalis, typically found in gingival tissue of patients with periodontal disease, was found during autopsies procedures in patients with AD.

In aneurysm, during a study that evaluate the presence of endodontic and periodontal bacterias, seven autopsies were included, the PCRs are positive for these relation in more than a third of the cases, immunohistochemistry for autopsies cases are highly positive for CD14 and TLR-2.[16]
A classic study realized in the 90's, observed the progression of periodontal disease *in locu* ,using tissues from deceased bodies, to understand the extension that inflammatory infiltration can reach.

That work can be revisited with all the biomolecular arsenal that are available nowadays. Some important questions can be raised, such as, the perception of the disease and the relation with alveolar bone tissue, how far the inflammasomes can be in the mineral and soft tissue or how it can change inflammatory signature in bloodstream and other organs, such as, kidneys and heart[17].

The physiopathology of periodontitis itself are based in a autopsy work, in early 70’s the idea that subgingival plaque could contribute to the attachment loss, appears after an observational study of six autopsied patients, the thickness of these plaque showed to be strictly related to how much of bone loss can be found in studied patients[18]

The understanding of how periodontal ligament can work and contribute to attachment loss, or inflammatory response, still uncertain. Sampling these region in human is almost impossible, the use of autopsies where somewhat more

brod(?) tissue can be removed for further analyses open a new screen to understand how physical stretch can be transcript in several periodontal outcomes. [19]

Autopsies were used in some case reports, to create a link between fatal diseases and periodontal findings, even in these situations the number of publications are not wide, probably due to the restricted number of dentists and oral pathologists working in autopsies centers/non-forensic around the world. [20]

Implants can be reviewed in physical tests and biomolecular ones, following the same logic, once we can access samples with whole alveolar bone, and the interface between implants and bone. Wook-jin seong et al. used a single corpse, they performed a maxillectomy and a total mandibulectomy, in a patient that presented 5 mandibular implants, and performed several tests[21]. Not only physical evaluation can be performed, but we can evaluate histomorphometric[22] and biomolecular in those tissues. The long term survival from implants could be observed in a new optical, once we can understand how the intraosseous micro-environment responds to mastigation and chronic inflammation in spatial omics. Bone regenerative procedures may be one of the most interesting fields, once the cell response front of a biomaterial can generate several forms of reactive process, in different times[23]. The utilization of post-mortem biopsies makes possible how each biomaterial responds, similar ways are used *in vivo* works. The autopsies let the science to evaluate in humans, retrospective studies, and several researches used in animals, after euthanasia.

Decay and autopsy

Decay it's one of the most prevalent diseases in human beings, the most prevalent oral health condition, and impacts directly to the quality of life quality. The early diagnosis is still the gold standard to treat patients with caries disease. A multifactorial disease, as it be, needs to be understood in a generic overview. The use of autopsies can, not only make it possible to observe the relation with stateside tissues, but allow the research to extrapolate some complementary examens, such as radiographs, tomographies, MRI and USG.

Standardized radiographs are used as a potential tool to diagnose, but the specificity of these kinds of exams is still unknown; some authors showed that the capacity of radiography creates several overtreatments.[24]

The use of death patients allows us to use different patterns of radiation, and exposition, in a tooth that is still vital, in a physiological relation with the adjacent tissues. Beyond that, we can use other imaginological exams, such as tomographies, helicoidal and cone beam, and magnetic resonance to accurate even more the diagnosis question.

Other useful tool for autopsies and decays, is the tooth development, sometimes autopsies are performed in fetus or newborn (NB), allowing us to analyze how systemic conditions, from mother and babies can lead to a developmental disorder, such as, molar incisor hypomineralization (MIH). Much is speculated on how mother-baby interaction can contribute to MIH, once a NB is autopsied, the pathologist has in hand all the systemic exams, from mother during the gestational period, to understand the cause of death. Other tooth development diseases can be understand in these optical

COVID-19

In recent experience, the use of autopsies provides consistent collaboration. Our group used samples from covid-19 deceased patients, to evidence the presence and the replication ability of SARS-CoV-2 in gingival tissues and salivary glands. The acces of this kind of sample demands a special training and familiarity to the alterations that are commonly seen in the oral cavity in dead patients. Besides that, the oral research facility needs to behave an autopsy structure in it. Once dental research programs do not use these as a research tool.

General pathology autopsies usually use knives and fasteners to completely remove organs, but when we are talking about head and neck situation, some protocol needs to be followed, for ethical or legal convenience, we are not able to remove all tissues, deceased bodies going to be veiled and buried, the family will recognize the body to pay your last condolence[Figure 2].

Minimally invasive autopsy can contribute in some way to solving the problem of removing samples from head and neck tissues without create damage to facial recognition or ethical problems. The use of image apparatus, like tomography, nasofibroscopy tube, attached to a monitor or even a smartphone, or ultrasonography makes possible to reach internal areas and the oral and nasal cavity to sample specific tissues from respiratory tract and adjacent tissues, that are the mainly entrance for SARS-CoV-2 in the human body. The use of these tissues were extremely useful to elucidate how saliva contribute to contamination, and in which way the oral cavity tissues interact with the SARS-CoV-2.

Future

The appearance of new diseases seems to be something that humanity will live with, in the last two decades the number of new pandemias are becoming more and more common. The establishment of new autopsies centers where dentists are present must be a goal for dental science, to contribute substantially to health science with all the knowledge that are restricted to dentistry, concepts like minimally invasive procedures and biosecurity are part of dentistry day by day.

The presence of that professional could contribute to other diseases, like aforesaid, creating a biobanking of oral tissues, making possible contributions for future studies on severe chronic diseases that presented oral manifestations, improving and re-visiting older studies using the biomolecular technology. The partnership between oral/ dental research centers, dental schools and autopsy centers are highly indicated to initiate a culture of autopsy use contributing to surgical technique learning of students and being the basis of oral/dental disease research and knowledge

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Figure 1: Autopsy Can Facilitate Oral-Systemic Investigations

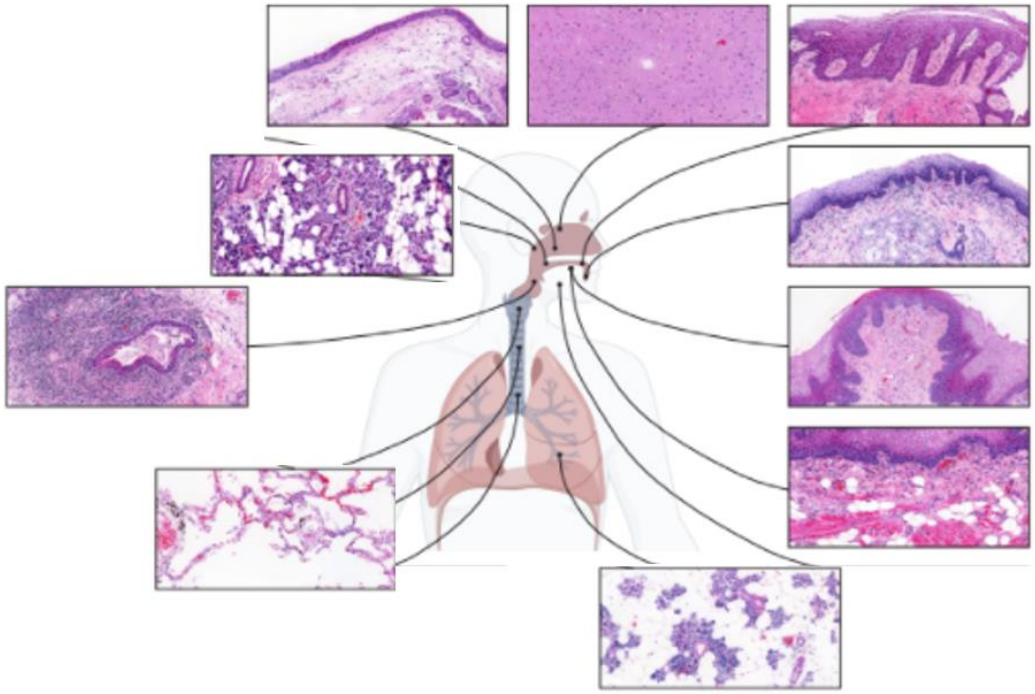

Figure 2. *Conventional autopsy*. A Tongue and Pharynx were dissected for further histopathologic analysis. Part of the autopsy block was removed to tongue evaluation. – *Minimmaly invasive autopsy* B. Nasofibroscope probe for oro-nasal analysis. C. Two health care professionals performing minimmaly invasive autopsy fibroscope guided – no need to body resections D. Minimmaly invasive autopsy- ultrasound guided - parotid being sampled by true cut needle 14G E. Samples obtained after minimmaly invasive autopsy

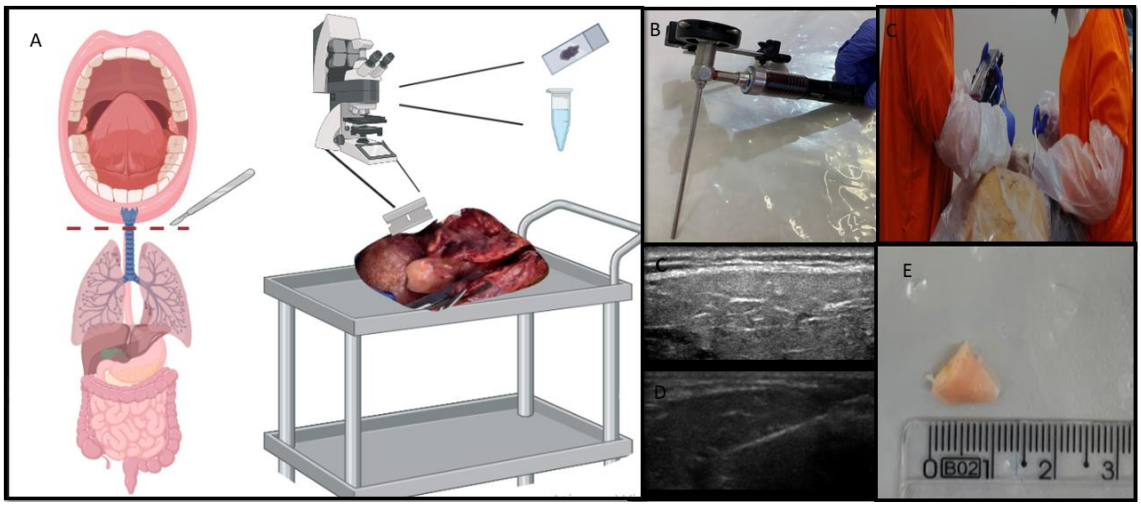

Table 1. Literature review of autopsy papers in dental sciences

Studies	Title	Sample type	Donors	Pub date	Area of interest	Where	Methology
Risa Bandou , Hiroaki Ichioka, Masataka Kawamoto , Hiroshi Ikegaya	Utilization of oral check-up data of autopsy cases	observational	403	2021	Epidemiology	Japan	Observational
Fernanda de Paula 1, Tathyane Harumi Nakajima Teshima 2, Ricardo Hsieh 1, Milena Monteiro Souza 3, Claudia Malheiros Coutinho-Camillo 4, Marcello Menta Simonsen Nico 1 3, Silvia Vanessa Lourenco 1	The expression of water channel proteins during human salivary gland development: a topographic study of aquaporins 1, 3 and 5	Parotid, submandibular	20	2017	Histology	Brazil	HE and IHC
Ágatha Nagli de Mello Gomes 1, Maria Aparecida Nagai 2 3, Silvia Vanessa Lourenço 4, Cláudia Malheiros Coutinho-Camillo 1	Apoptosis and proliferation during human salivary gland development	tongue, ans salivary glands	50	2019	Histology	Brazil	He and IHC
E Nyström 1, K E Kahnberg, T Albrektsson	Treatment of the severely resorbed maxillae with bone graft and titanium implants: histologic review of autopsy specimens	Maxila	1	1993	Implant	Sweden	HE
J M Whittaker, R A James, J Lozada, C Cordova, D J GaRey	Histological response and clinical evaluation of heterograft and allograft materials in the elevation of the maxillary sinus for the preparation of endosteal dental implant sites. Simultaneous sinus elevation and root form implantation: an eight-month autopsy report	Maxilla	1	1989	Implants	USA	HE
K Donath	Pathogenesis of bony pocket formation around dental implants	Bone Crest	1	1992	Implants	German	HE
Sungtae Kim, Kyung-Seok Hu, Ui-Won Jung	Reosseointegration After Regenerative Surgical Therapy Using a Synthetic Bone Substitute for Peri-implantitis: Human Autopsy Study	Alveolar bone and implants0	1	2018	Implants	China	HE and CT
Tatsuro Otani 1, Shinobu Ikeda, Htay Lwin, Tomio Arai, Masaaki Muramatsu, Motoji Sawabe	Polymorphisms of the formylpeptide receptor gene (FPR1) and susceptibility to stomach cancer in 1531 consecutive autopsy cases	Gastric Cancer	1531	2011	Oral Medicine	Japan	PCR
Mikko J Pyysalo 1, Liisa M Pyysalo, Tanja Pessi, Pekka J Karhunen, Juha E Öhman	The connection between ruptured cerebral aneurysms and odontogenic bacteria	Aneurism	7	2013	Oral Medicine	Finland	PCR
Anne-Mari Louhelainen 1, Joonas Aho 1, Sari Tuomisto 1, Janne Aittoniemi 2, Risto Vuento 2, Pekka J Karhunen 3, Tanja Pessi 4	Oral bacterial DNA findings in pericardial fluid	Pericardial fluid	22	2014	Oral Medicine	Finland	PCR
Bárbara Bellocchio Bertoldo 1, Renata Margarida Etchebehere 2, Taíssa Cássia de Souza Furtado 3, Juliana Barbosa de Faria 1, Camilla Beatriz Silva 4, Márcia Fernandes de Araújo 1, Denise Bertulucci Rocha Rodrigues 1 3 4 5, Sanivia Aparecida de Lima Pereira 1 3 4 5	Lingual salivary gland hypertrophy and decreased acinar density in chagasic patients without megaesophagus	tongue	27	2019	Oral medicine	Brazil	He and IHC
E Pedersen 1, K Andersen, B Melsen	Tooth displacement analysed on human autopsy material by means of a strain gauge technique	Mandible	3	1991	Ortho	Denmark	radiography and tensiometry
H Wehrbein 1, W Bauer, P R Diedrich	Gingival invagination area after space closure: a histologic study	Maxila	1	1995	Ortho	German	HE
H Wehrbein 1, R A Fuhrmann, P R Diedrich	Human histologic tissue response after long-term orthodontic tooth movement	Maxila	1	1995	Ortho	German	HE
H Wehrbein , W Bauer, P Diedrich	Mandibular incisors, alveolar bone, and symphysis after orthodontic treatment. A retrospective study	Mandible	1	1996	Ortho	German	macroscopic, radiologic, and HE
Gustavo Hauber Gameiro , Jorge Eugenio Bocchiardo , Michel Dalstra , Paolo Maria Cattaneo	Individualization of the three-piece base arch mechanics according to various periodontal support levels: A finite element analysis	Maxilla	1	2021	Ortho	Denmark	Finite elent analyses
B A Wright, F Fenwick	Candidiasis and atrophic tongue lesions	Tongue	46	1981	Pathology	UK	HE and histochemistry
N Vander val, I Van der Val	Candida Albicans In Median rhomboid glossitis: a post mortem study	Tongue	100	1986	Pathology	Netherlan ds	HE and Histochesmitry
N Hashimoto 1, K Kurihara, H Yamasaki, S Ohba, H Sakai, S Yoshida	Pathological characteristics of metastatic carcinoma in the human mandible	Mandible	62	1987	Pathology	Japan	HE and Roentgenographic
Y Takeda, H Yamamoto	Iron deposits in the human labial minor salivary glands: a postmortem study	Minor salivary glands	195	1989	Pathology	Japan	HE
Fernandes Matuck B, Dolhnikoff M, Maia GVA, Isaac Sendyk D, Zarpellon A, Costa Gomes S, Duarte-Neto AN, Rebello Pinho JR, Gomes-Gouvêa MS, Sousa SCOM, Mauad T, Saldiva PHDN, Braz-Silva PH, da Silva LFF.	Periodontal tissues are targets for Sars-Cov-2: a post-mortem study.	Gingiva	8	2020	Pathology	Brazil	PCR, HE

S V Sowmya 1, Roopa S Rao 1, E Vinesh 2, Chandini Rajkumar 3, Prasanna Nichat 4, Prem B Karthick 5, Thilla S Vinothkumar 6, Girish Chandra 7, Snehashish Ghosh 8, A Thirumal Raj 9, Shankargouda Patil 1	Histopathological Changes in Oral Tissues Induced by Pesticide Poisoning: A Pilot study	Tongue, buccal mucosa	10	2021	Pathology	India	H&E analyzies
Zarpellon A, Matuck BF, Dolhnikoff M, Duarte-Neto AN, Maia G, Gomes SC, Sendyk DI, Souza SCOM, Mauad T, Saldiva PHN, Braz-Silva PH, da Silva LFF.	Oral lesions and SARS-CoV-2: A postmortem study.	tongue, gingiva, buccal mucosa, lips, hard and soft palte	30	2021	Pathology	Brazil	IHC and H&E
Matuck BF, Dolhnikoff M, Duarte-Neto AN, Maia G, Gomes SC, Sendyk DI, Zarpellon A, de Andrade NP, Monteiro RA, Pinho JRR, Gomes-Gouvêa MS, Souza SC, Kanamura C, Mauad T, Saldiva PHN, Braz-Silva PH, Caldini EG, da Silva LFF.	Salivary glands are a target for SARS-CoV-2: a source for saliva contamination.	Parotid, submandibular gland and minor salivary gland (lower lip)	25	2021	Pathology	Brazil	EM, IHC, PCR, H&E
Huang N, Pérez P, Kato T, Mikami Y, Okuda K, Gilmore RC, Conde CD, Gasmi B, Stein S, Beach M, Pelayo E, Maldonado JO, Lafont BA, Jang SI, Nasir N, Padilla RJ, Murrah VA, Maile R, Lovell W, Wallet SM, Bowman NM, Meinig SL, Wolfgang MC, Choudhury SN, Novotny M, Aevermann BD, Scheuermann RH, Cannon G, Anderson CW, Lee RE, Marchesan JT, Bush M, Freire M, Kimple AJ, Herr DL, Rabin J, Grazioli A, Das S, French BN, Pranzatelli T, Chiorini JA, Kleiner DE, Pittaluga S, Hewitt SM, Burbelo PD, Chertow D; NIH COVID-19 Autopsy Consortium; HCA Oral and Craniofacial Biological Network, Frank K, Lee J, Boucher RC, Teichmann SA, Warner BM, Byrd KM.	SARS-CoV-2 infection of the oral cavity and saliva.	minor salivary glands, tongue, buccal mucosa, gingiva, palatine tonsils	18	2021	Pathology	US	IHS, FISH, PCR, IHC, HE,
Yasuhiro Sakashita 1 2 3, Tomoyasu Matsubara 1 4, Tadayuki Takata 1 5, Zen-Ichi Tanei 1 6, Atsuko Motoda 1 4, Mikihiro Yamazaki 1 7, Ito Kawakami 1 8, Renpei Sengoku 1 7, Yuko Saito 1, Tomio Arai 2, Masahito Yamada 3, Shigeo Murayama 1 9	Lewy pathology of the submandibular gland in Lewy body disease: A report of autopsy cases	Submandibular gland	64	2021	Pathology	Japan	HE and IHC
Dickson W L Wong 1, Barbara M Klinkhammer 1, Sonja Djudjaj 1, Sophia Villwock 1, M Cherelle Timm 1, Eva M Buhl 1 2, Sophie Wucherpfennig 1, Claudio Cacchi 1, Till Braunschweig 1, Ruth Knüchel-Clarke 1, Danny Jonigk 3 4, Christopher Werlein 4, Roman D Bülow 1, Edgar Dahl 1, Saskia von Stillfried 1, Peter Boor 1 2 5	Multisystemic Cellular Tropism of SARS-CoV-2 in Autopsies of COVID-19 Patients	tonsils, salivary glands	8	2021	Pathology	German	ISH
Felipe Paiva Fonseca , Johanna Pamela Latta Moreira , O P Almeida , Pablo Agustin Vargas , Thais Mauad 2	Neuroepithelial structures associated with neurogenous subgemmal plaque of the tongue: an autopsy finding	Tongue	1	2015	Pathology	Brazil	HE and IHC
GONDAK, R. ; MAUAD, T. ; SCHULTZ, L. ; SOARES, F. A. ; KOWALSKI, L. P. ; VARGAS, P.A	Decreased CD1a, CD83 and factor XIIIa dendritic cells in cervical lymph nodes and palatine tonsils of AIDS patients	Tongue	53	2014	Pathology	Brazil	He and IHC
SILVA, Andréia Aparecida da ; Bingle, L. ; SPEIGHT, P. M. ; Bingle, C.D. ; MAUAD, T. ; SILVA, L. F. ; VARGAS, P. A.	PLUNC protein expression in major salivary glands of HIV infected patients. Oral Disease	Salivary glands	45	2011	PAthology	Brazil	He and IHC
ROCHA, Lilia Alves ; VARGAS, P. A. ; SILVA, L. F. ; LEON, Jorge Esquiche ; SANTOS, A. B. ; HIEMSTRA, P. S. ; MAUAD, T.	Expression of Secretory Leukocyte Proteinase Inhibitor in the submandibular glands of AIDS patients	Salivary glands	36	2008	Pathology	Brazil	He and IHC
Moskow, B. S., & Poison, A. M.	Histologic studies on the extension of the inflammatory infiltrate in human periodontitis.	Jaws and gingiva	350	1991	Perio	USA	HE
B S Moskow 1	A histomorphologic study of the effects of periodontal inflammation on the maxillary sinus mucosa	Jaws	20	1992	Perio	USA	HE
J S Vacek 1, M E Gher, D A Assad, A C Richardson, L I Giambarresi	The dimensions of the human dentogingival junction	Jaws- gingiva	10	1994	Perio	USA	Histomorphometric
R Rautemaa 1, S Meri	Protection of gingival epithelium against complement-mediated damage by strong expression of the membrane attack complex inhibitor protectin (CD59)	Gingiva	2	1996	Perio	Finland	Imunofluorescence
C E Uden 1, S Ganatra, R A Reinhardt, K D Patil	Histology near periodontitis osteoclasts	Alveolar crest and gingiva	13	1998	Perio	USA	HE
H K Kuramitsu 1, M Qi, I C Kang, W Chen	Role for periodontal bacteria in cardiovascular diseases	Fibrous caps	381	2001	Perio	USA	ELISA and Is Vitro simulation
G R Riviere 1, K H Riviere, K S Smith	Molecular and immunological evidence of oral Treponema in the human brain and their association with Alzheimer's disease	Brain	34	2002	Perio	USA	PCR

Hideki Ohyama 1, Keiji Nakasho, Koji Yamanegi, Yuichiro Noiri, Ayako Kuhara, Nahoko Kato-Kogoe, Naoko Yamada, Masaki Hata, Fusanori Nishimura, Shigeyuki Ebisu, Nobuyuki Terada	An unusual autopsy case of pyogenic liver abscess caused by periodontal bacteria	biliary tract, portal vein and hepatic artery	1	2009	Perio	Japan	IHC
Lambert K Sørensen 1, Jørgen B Hasselstrøm 2, Line S Larsen 3, Dorthe A Bindslev 4	Entrapment of drugs in dental calculus - Detection validation based on test results from post-mortem investigations	Dental Calculus	10	2021	Perio/Forensics	Denmark	ultra-high-performance liquid chromatography-tandem mass spectrometry method using pneumatically assisted electrospray ionisation (UHPLC-ESI-MS/MS)
K Tanimoto 1, A Petersson, M Rohlin, L G Hansson, C C Johansen	Comparison of computed with conventional tomography in the evaluation of temporomandibular joint disease: a study of autopsy specimens	TMJ	15	1989	Radiology	Sweden	Ct and Macro
K Tanimoto 1, A Petersson, M Rohlin, L G Hansson, C C Johansen	Comparison of computed with conventional tomography in the evaluation of temporomandibular joint disease: a study of autopsy specimens	TMJ	15	1990	Radiology	Sweden	CT and Macro
G Landini 1	Videodensitometrical study of the alveolar bone crest in periodontal disease	Bone crest	25	1991	Radiology	Japan	microradiography and videodensitometry
C Lindh 1, A Petersson, M Rohlin	Assessment of the trabecular pattern before endosseous implant treatment: diagnostic outcome of periapical radiography in the mandible	Mandible	7	1996	Radiology	Sweden	Radiographs
S E Widmalm, P L Westesson, I K Kim, F J Pereira Jr, H Lundh, M M Tasaki	Temporomandibular joint pathosis related to sex, age, and dentition in autopsy material	TMJ	224	1994	TMJ	USA	Macroscopic

CAPITULO 10 – CONSIDERAÇÕES FINAIS

Considerações finais

A utilização de diversas metodologias para estabelecer um padrão de evidência tem importante papel na construção do conhecimento científico. Estudos relacionando achados histopatológicos e biomoleculares frente a infecções respiratórias, na cavidade oral, ainda são muito escassos. Conhecer a interação entre esses patógenos e os tecidos da cavidade oral parece, de maneira assertiva, ser um caminho para entender melhor os padrões de contaminação e os mecanismos de disseminação dessas doenças.

Ao longo dos últimos dois anos, no contexto de um momento trágico para toda a humanidade, o presente projeto buscou aliar a disponibilidade de material biológico num contexto de uma crise epidemiológica relevante, à colaboração com pesquisadores e laboratórios de diferentes áreas com diferentes expertises clínicas, fisiopatológicas e moleculares, para compor, através de pequenas peças, um quadro significativo da distribuição e dos possíveis papéis do SARS-CoV-2 neste processo. O quadro retratado ao longo deste caminho está descrito a seguir e pode ser determinado não necessariamente na cronologia das publicações, mas na compreensão do processo como um todo.

O epitélio da cavidade oral mostrou-se um reservatório para a presença do SARS-Cov-2, tendo não só a capacidade de armazenamento, como também, proliferativa para o vírus.

A literatura tem convergido para esse mesmo entendimento. Análises de células únicas e de subpopulações mostraram a presença e a replicação do vírus em tecidos da cavidade oral, transformando assim a saliva como um vetor de transmissão por si só, inclusive em pacientes assintomáticos.

Entender os mecanismos utilizados pelos vírus para entrar nas células e reconhecer a presença deles nos tecidos epiteliais é um ponto de partida inicial para essa análise.

A utilização de autópsias para a obtenção desses tecidos se mostra bastante efetiva no que diz respeito a entender todo o processo, uma vez que não se limita a um tecido específico, mas à combinação de tecidos de diferentes órgãos que possibilitam a compreensão integral dos achados em tecidos específicos e o que representam (ou representaram) para o paciente como um

todo. Assim foi possível entender como os tecidos da cavidade oral se comportaram frente a diferentes reações do órgão alvo. Particularmente, no caso de um vírus respiratório como o SARS-CoV-2 foi possível avaliar as manifestações orais de forma contextualizada e conhecer a gravidade do acometimento pulmonar e das vias aéreas dos pacientes.

Apesar de todas as possibilidades de correlação, não foi possível determinar que o SARS-CoV-2, por si só, seja capaz de gerar lesões e manifestações bucais clinicamente evidentes como agente primário. Porém, foi possível compreender que, assim como ocorre em outros órgãos, a deterioração da saúde geral e as complicações associadas à gravidade do quadro nos órgãos alvo centrais (incluindo o longo tempo de internação) parecem exercer mais influência sobre o aparecimento de lesões orais do que a infecção pelo SARS-CoV-2 *per se*.

A autópsia sempre foi considerada um método de estudo que possibilita a análise integral do indivíduo e do processo de doença. Isso é extremamente bem aplicado do ponto de vista da integração entre diferentes especialidades médicas. Ao longo deste projeto, porém, foi possível demonstrar que as técnicas para a obtenção de amostras da região de cabeça e pescoço e a presença de profissionais da odontologia para a execução destas técnicas em serviços de autópsia podem incrementar esse campo de pesquisa, assim como dar embasamento para a rede de saúde pública e a gestão para uma retomada segura dos atendimentos em saúde bucal em um momento crítico do sistema. Também ressalta a importância de novos protocolos de biossegurança de atendimento para uma profissão que trabalha com fluidos da cavidade nasal e oral, seja de forma direta com o contato de gotículas de saliva, ou com a presença de sprays de aerossol jogados no ar durante a realização de procedimentos ambulatoriais.

As autópsias desempenharam papel fundamental no conhecimento da humanidade sobre a saúde e a doença desde tempos imemoriais. Desde a manipulação dos cadáveres na idade antiga, passando pelas dissecções “às escuras” durante a idade média, chegando às enormes contribuições patológicas na idade moderna e contemporânea. Um procedimento muito antigo, que ao longo dos séculos foi compreendido de maneiras diferentes, e que continua a ser reinventado, modernizado e hoje se torna multiprofissional, mantendo seu

objetivo primordial intacto: garantir à humanidade mais conhecimento sobre si mesma, sobre seu processo de saúde e doença e contribuir no sentido de monitorar, compreender e garantir as bases morfológicas para o conhecimento clínico do diagnóstico e tratamento.

Perspectivas futuras

Os trabalhos do nosso grupo se estendem em direção a entender como a cavidade oral se comporta com infecções com o H1N1, influenza A e B, vírus sincicial respiratório, rinovíroses e adenoviroses. Assim como, vigilar as alterações de novas variantes da COVID-19 na cavidade oral.

O entendimento de como outras infecções respiratórias virais se comportam na cavidade oral é um importante passo para entender a diferença no coeficiente de disseminação, visto que o do SARS-CoV-2 é maior do que de outras doenças respiratórias.

O mesmo vale para a resposta imune e o comportamento celular exercido na mucosa oral. Visto seu contato inicial com o patógeno, os mecanismos de egressão do vírus de dentro da célula para o resto do organismo, em um momento inicial das doenças respiratórias parece ser um campo novo a ser estudado.

Nosso grupo de trabalho concretizou uma parceria importante para o desenvolvimento de novas técnicas de biologia molecular utilizando o nosso biobanco criado durante a execução desse projeto. Um contrato de transferência de material foi executado entre a FMUSP e o NIDCR – NIH (National Institute of Health), assim como, no âmbito pessoal, o pós-graduando envolvido na execução desses projetos irá realizar um pós-doutorado no laboratório da American Dental Association Scientific Research Innovation dentro do NIH.

A realização de técnicas de análise de isoformas de RNA com resoluções em célula única, assim como avaliações fenotípicas espaciais do microambiente tecidual oral, poderão implementar de forma mais acurada o objetivo de entender como os tecidos se comportam frente a infecções virais e sua relação com as vias aéreas superiores.

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Anexo 1

USP - HOSPITAL DAS CLÍNICAS DA FACULDADE DE MEDICINA DA UNIVERSIDADE DE SÃO PAULO - HCFMUSP

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Análise da presença do SARS-CoV-2 em glândulas salivares e tecidos periodontais: uma avaliação biomolecular, imunohistoqúimica e de ultra-estrutura em casos fatais da COVID19

Pesquisador: Luiz Fernando Ferraz da Silva

Área Temática:

Versão: 1

CAAE: 45276721.4.0000.0068

Instituição Proponente: Hospital das Clínicas da Faculdade de Medicina da USP

Patrocinador Principal: FUNDACAO DE AMPARO A PESQUISA DO ESTADO DE SAO PAULO

DADOS DO PARECER

Número do Parecer: 4.687.643

Apresentação do Projeto:

Projeto bem escrito e delineado. Projeto que visa a análise da presença do SARS-CoV-2 em tecido salivar glandular e periodontal. Faz parte de um projeto maior com parecer ja aprovado (CAAE: 30364720.0.0000.0068). O procedimento será realizado em pacientes com testes positivos, do tipo Rt-PCR de COVID-19, e óbito no Complexo HCFMUSP. A autópsia será
realizada com autorização dos familiares, seguindo TCLE aprovado pelo CONEP com parecer 30364720.0.0000.0068. A Autópsia será realizada
nas dependências do PISA (Plataforma de Imagem na Sala de Autópsia da FMUSP), por uma equipe composta por dois profissionais previamente
treinados pela equipe de autopsia minimamente invasiva do PISA. A equipe será composta por ao menos um dentista e um técnico ou auxiliar de
autópsia Os corpos serão embalados com plástico protetor e submetidos a Tomografia Computadorizada a região de cabeça e pescoço, cujo
protocolo já foi previamente testado em projeto da mesma instituição intitulado "Estudos da COVID-19 fatal por meio da autópsia minimamente
invasiva guiada por ultrassom" cujo pesquisador principal é a professora Marisa Dolhnikoff com parecer número 30364720.0.0000.0068.A coleta
será realizada em 30 cadáveres do sexo masculino ou feminino com idade entre 6 e 80 anos. A

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biópsia post-mortem será realizada por punção de
órgãos-alvo guiada por ultrassom ou sonda fibro-optica, por meio de sistema com agulhas Tru-CutRsemi automáticas coaxiais de 14G, com 20 cm
de comprimento. Os seguintes órgãos serão biopsiados: parótida, glândula submandibular, glândula salivar menor – removida da região de mucosa
labial inferior e tecidos periodontais, sendo este caracterizado pela ameia mesial do primeiro molar superior. Na sua ausência o próximo espaço
periodontal coletado será do dente adjacente ao espaço protético Utiliza-se equipamento portátil de ultrassonografia (US) SonoSite MTurboR(Fujifilm, Bothell, WA, USA) com transdutores banda larga multifrequenciais C60x (5-2 MHz Convexo) e HFL38X (13-6 MHz Linear) e
imagens padrão DICOMR. E ótica Karl-Storz Optical, Tuttlingen –German, associado a smartphone do tipo iphone com aplicativo M-scope para
visualização direta das amostras periodontais e de glândulas salivares menores.Serão coletados um mínimo de 5 fragmentos adequados de cada
órgão alvo, os fragmentos terão espessura conforme protocolo padronizado pela autópsia minimamente invasiva [30]Um fragmento será
armazenado em nitrogênio liquido, Os demais fragmentos colocados em frascos individuais com formol a 10% para posterior análise histopatológica
e de ultraestrutura. As amostras serão submetidas a análise histopatológica, assim como, reações de imunohistoquímica para SARS-CoV-2, ACE2,
TMPRSS2 e FURINA. A caracterização da inflamação será feita a partir da realização das reações de IHC para CD4, CD8 CD68, IL6, IL10.As
Amostras congeladas serão submetidas a reação de PCR para SARS-CoV-2 e análise da ultraestrutura por microscopia eletrônica de transmissão.

Objetivo da Pesquisa:

Identificar a presença do SARS-CoV-2 nas glândulas salivares e tecidos periodontais de casos fatais de COVID-19; Caracterizar as alterações celulares do sistema glandular salivar de pacientes que morreram em decorrência da COVID-19; Quantificar a
expressão tecidual dos receptores ACE2, TMPRSS e Furina nos tecidos glandulares e periodontais de pacientes que morreram em decorrência da
COVID-19 e casos controles; Caracterizar as alterações da ultraestrutura das células acinares, ductais e miofibroblastos infectadas pelo SARSCoV-2; Caracterizar as alterações da ultraestrutura

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dos Fibroblastos e queratinócitos dos tecidos periodontais infectados pelo SARS-CoV-2; Quantificar a expressão imunohistoquímica do antígeno Anti-SARS-CoV-2 nas amostras coletadas de periodonto e glândulas salivares.

Avaliação dos Riscos e Benefícios:

Riscos: Não há riscos aos cadáveres, os procedimentos realizados são inerentes a autópsia minimamente invasiva já realizada no centro de pesquisa em casos advindo do hospital das clinicas da faculdade de medicina da universidade de São Paulo

Benefícios: A Identificação do vírus em tecidos glandulares e periodontais, podem explicar a presença da alta carga viral no fluido salivar em diferentes estágios da doença. Sendo este um órgão alvo de entrada do vírus no corpo humano, assim como, uma importante via de contaminação pessoa-pessoa.

Comentários e Considerações sobre a Pesquisa:

Pesquisa bem descrita e detalhada a respeito da pesquisa do SARS-CoV-2 em tecidos. Pesquisa será realizada por equipe treinada em relação a autopsia minimamente invasiva. Apresenta descrição detalhada da coleta do material e do processamento do mesmo.

Considerações sobre os Termos de apresentação obrigatória:

Apresenta TCLE já aprovado em projeto anterior pela CONEP (CAAE: 30364720.0.0000.0068), sendo bem descrito, e de forma simples ao familiar/ responsável legal. Apresenta todos os itens que um TCLE deve apresentar, e deixa bem explícitos os benefícios da pesquisa.

Conclusões ou Pendências e Lista de Inadequações:

Pesquisa importante para o entendimento da fisiopatologia do vírus SARS-CoV-2 nos tecidos que apresentam os recepetores ACE2. Apresenta financiamento já aprovado para a elaboração do mesmo (aprovação de projeto anterior CAAE: 30364720.0.0000.0068). Projeto bem descrito tanto em relação a coleta do meterial e sua análise.

Considerações Finais a critério do CEP:

Em conformidade com a Resolução CNS nº 466/12 – cabe ao pesquisador: a) desenvolver o projeto conforme delineado;b) elaborar e apresentar relatórios parciais e final; c)apresentar dados solicitados pelo CEP, a qualquer momento; d) manter em arquivo sob sua guarda, por 5 anos da pesquisa, contendo fichas individuais e todos os demais documentos recomendados pelo CEP; e) encaminhar os resultados para publicação, com os devidos créditos aos pesquisadores associados e ao pessoal técnico participante do projeto; f) justificar perante ao CEP interrupção do projeto ou a não publicação dos resultados.

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Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1697899.pdf	05/04/2021 10:15:11		Aceito
Outros	docdigital.pdf	05/04/2021 10:14:39	Luiz Fernando Ferraz da Silva	Aceito
Cronograma	cronograma.pdf	05/04/2021 10:13:20	Luiz Fernando Ferraz da Silva	Aceito
Declaração de Instituição e Infraestrutura	custos.pdf	05/04/2021 10:12:32	Luiz Fernando Ferraz da Silva	Aceito
Declaração de Pesquisadores	CartaCompromissoDoutorado.pdf	05/04/2021 10:11:20	Luiz Fernando Ferraz da Silva	Aceito
Declaração de Instituição e Infraestrutura	SVO.pdf	05/04/2021 10:10:03	Luiz Fernando Ferraz da Silva	Aceito
Folha de Rosto	folhaderosto.pdf	30/03/2021 11:26:20	Luiz Fernando Ferraz da Silva	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE2.docx	30/03/2021 11:21:43	Luiz Fernando Ferraz da Silva	Aceito
Projeto Detalhado / Brochura Investigador	projetodocMatuckBurnsglsalivar.docx	05/02/2021 21:11:09	Luiz Fernando Ferraz da Silva	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

SAO PAULO, 03 de Maio de 2021

Assinado por:
ALFREDO JOSE MANSUR
(Coordenador(a))

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Anexo 2

HOSPITAL DAS CLÍNICAS DA FACULDADE DE MEDICINA DA UNIVERSIDADE DE SÃO PAULO-HCFMUSP

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

DADOS DA PESQUISA

Título da pesquisa - **Estudos da COVID-19 fatal por meio da autópsia minimamente invasiva guiada por ultrassom**

Pesquisador principal – **Marisa Dolhnikoff**
Cargo / Função – **Professora do Departamento de Patologia da FMUSP**
Inscrição Conselho Regional de Medicina (CRM): **54500**
Unidade do HCFMUSP: **FMUSP, Departamento de Patologia**
Departamento/Instituto – **Departamento de Patologia/ Faculdade de Medicina da USP**

Convidamos o(a) Sra. para participar desta pesquisa "Estudos da COVID-19 fatal por meio da autópsia minimamente invasiva guiada por ultrassom".

Uma pessoa de sua família ou sob sua responsabilidade faleceu e os médicos que a atenderam solicitaram a realização de uma autópsia para esclarecer melhor todas as possíveis causas da morte. Atualmente, em alguns países já se realizam autópsias associadas aos exames de imagem, como por exemplo, ultrassonografia e tomografia, os quais têm contribuído para explicar melhor os motivos da morte desta pessoa.

O Hospital das Clínicas-FMUSP está implantando aqui a autópsia minimamente invasiva dirigida pela ultrassonografia (AMI-US), que consiste na coleta de tecido de vários órgãos para avaliação microscópica, utilizando-se uma punção por agulha, sem necessidade de realização de autópsia com abertura do corpo. Em casos específicos, ou seja, quando houver suspeita de eventos cardiovasculares com comprometimento de vasos de grande ou médio calibre, incluindo tromboembolismo pulmonar e infarto agudo do miocárdio, será realizada abertura de até 3,0 cm no tórax para retirada de fragmentos maiores de coração e pulmão. A avaliação do tecido coletado permitirá complementar os resultados obtidos pelos exames clínico-laboratoriais no diagnóstico da causa da morte e comorbidades e propiciará o estudo da patogenia dessa nova doença (COVID-19). Os tecidos coletados serão armazenados de forma adequada sob minha responsabilidade, e irão fazer parte de um biorrepositórios de covid19. O biorrepositório é uma coleção de material biológico humano, coletado e armazenado ao longo da execução do estudo, conforme o regulamento e normas técnicas, éticas e operacionais pré-definidas, sem fins comerciais.

Por isso, solicitamos sua autorização para o estudo do tecido coletado pela Autópsia Minimamente Invasiva e do sangue coletado para exames laboratoriais e para o armazenamento dos tecidos em nosso biorrepositório para estudos futuros.

Embora não haja benefícios diretos para a pessoa, a análise do tecido coletado irá contribuir para o entendimento dos mecanismos envolvidos no desenvolvimento da COVID-19.

Rubrica do sujeito de pesquisa ou responsável

_____ Rubrica do pesquisador

_____

Hospital das Clinicas da Faculdade De Medicina da USP	_____ Rubrica do Participante da Pesquisa /Representante Legal	_____ Rubrica do Investigador Responsável

Em qualquer etapa do estudo, você terá acesso aos profissionais responsáveis para esclarecimento de eventuais dúvidas. A investigadora coordenadora do estudo é a professora Marisa Dolhnikoff do Departamento de Patologia da FMUSP. Ela pode ser encontrada na Av. Dr. Arnaldo, 455 – 1º andar – sala 1155 no telefone (11) 3061-8521. Se você tiver alguma consideração ou dúvida sobre a ética da pesquisa, entre em contato com o Comitê de Ética em Pesquisa (CEP) – Rua Ovídio Pires de Campos, 225 – 5º andar – tel: (11) 2661-7585, (11) 2661- 1548, (11) 2661-1549, das 7 às 16h de segunda a sexta feira ou por e-mail: cappesq.adm@hc.fm.usp.br

Mesmo tendo fornecido seu consentimento, você poderá retirá-lo a qualquer momento. Nesta situação o tecido coletado não será utilizado para pesquisa.

Todos os dados serão utilizados exclusivamente para fins de pesquisa e as informações de identificação serão mantidas sob sigilo, garantindo a confidencialidade. Os dados serão analisados em conjunto com outros pacientes, não sendo divulgada a identificação de nenhum paciente em particular seja por nome ou imagem que permita sua identificação.

Informamos ainda que não há despesas adicionais para os familiares / responsáveis em qualquer fase do estudo, incluindo os exames realizados. Também não há compensação financeira relacionada à participação.

Os familiares/responsáveis têm direito de serem mantidos atualizados sobre os resultados parciais do estudo.

O material obtido da autópsia minimamente invasivo será armazenado em um biorrepositório e será analisado pelo grupo de pesquisa deste estudo e de seu colaborador no exterior, respeitando as normas éticas previstas em lei *(Resolução CNS nº 441 de 2011, itens 1.II e 2.II).*

Porém pode acontecer que futuramente seja interessante usar estas amostras para outros trabalhos de pesquisa. Neste caso, há necessidade de consultá-lo para autorizar o uso deste material?

(.....) SIM. Eu quero ser consultado para autorizar ou não cada pesquisa futura com o material.

(....) NÃO. Eu dispenso a autorização para cada pesquisa e estou informado (a) que a Comitê de Ética em Pesquisa do Hospital das Clínicas (CAPPesq) irá examinar a nova pesquisa e decidir sobre a utilização ou não do material.

Acredito ter sido suficientemente informado a respeito das informações que li ou que foram lidas para mim, descrevendo a pesquisa "Estudos da COVID-19 fatal por meio da autópsia minimamente invasiva guiada por ultrassom".

Estudos da COVID-19 fatal por meio da autópsia minimamente invasiva guiada por ultrassom	Confidencial	
Termo de Consentimento Livre e Esclarecido versão 1 de de de		

Nome do pesquisador: **Marisa Dolhnikoff**	_____ Rubrica do Participante da Pesquisa /Representante Legal	_____ Rubrica do Investigador Responsável
Hospital das Clinicas da Faculdade De Medicina da USP		

Eu discuti com o Dra Marisa Dolhnikoff sobre a minha decisão em participar nesse estudo. Ficaram claros para mim quais são os propósitos do estudo, os procedimentos a serem realizados, seus desconfortos e riscos, as garantias de confidencialidade e de esclarecimentos permanentes. Ficou claro também que a autorização para a inclusão de meu familiar é isenta de despesas e que tenho o direito de ser mantido atualizado sobre os resultados parciais do estudo.

Concordo voluntariamente em participar deste estudo, assino este termo de consentimento e recebo uma via rubricada pelo pesquisador.

Data____/____/_____
Assinatura do participante /representante legal

Nome do participante/representante legal

Data ____/____/_____
Assinatura do responsável pelo estudo

<table>
<tr><td>Estudos da COVID-19 fatal por meio da
autópsia minimamente invasiva guiada por ultrassom</td><td colspan="2">Confidencial</td></tr>
<tr><td>Termo de Consentimento Livre e Esclarecido versão 1 de de de</td><td rowspan="3">_____
Rubrica do Participante da Pesquisa /Representante Legal</td><td rowspan="3">_____
Rubrica do Investigador Responsável</td></tr>
<tr><td>Nome do pesquisador: Marisa Dolhnikoff</td></tr>
<tr><td>Hospital das Clinicas da Faculdade De Medicina da USP</td></tr>
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