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Identificação de biomarcadores salivares e urinários para desenvolvimento de plataformas sustentáveis para diagnóstico e monitoramento do diabetes mellitus

DOUGLAS CARVALHO CAIXETA

UBERLÂNDIA – MG
2020

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Tese apresentada ao Programa de Pós-Graduação em Ciências da Saúde da Faculdade de Medicina da Universidade Federal de Uberlândia, como requisito parcial para a obtenção do título de Doutor em Ciências da Saúde.

Área de concentração: Ciências da Saúde.

Orientador: Robinson Sabino da Silva
Co-orientador: Foued Salmen Espindola

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Douglas Carvalho Caixeta

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Suplente: Profa. Dra. Renata Roland Teixeira - Universidade Federal de Uberlândia (UFU)

À minha família por toda a dedicação, carinho, amor e apoio á minha formação profissional

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"Todo caminho é o caminho certo. Tudo poderia ter sido qualquer outra coisa e teria sido igualmente importante"

Mr. Nobody

RESUMO

O diagnóstico e o monitoramento do diabetes mellitus (DM) pelo sangue é altamente preciso, entretanto é um procedimento invasivo, gera desconforto aos pacientes e possui um elevado custo tanto para o sistema de saúde como para os pacientes. O desenvolvimento de plataformas não invasivas alternativas com outras amostras biológicas de forma sustentável, com alta sensibilidade e custo reduzido podem colaborar com a vigilância de DM. Nesta tese, foram investigados possíveis biomarcadores salivares e urinários como potenciais alternativas para o diagnóstico e monitoramento do DM divididos em três estudos. Inicialmente, foi realizada análise com a ferramenta de espectroscopia de infravermelho com transformada de Fourier (FTIR) para avaliar os componentes urinários de ratos não diabéticos, diabéticos e diabéticos tratados com insulina. Em outro estudo, uma plataforma de FTIR foi utilizada em associação com a classificação de máquina de vetor de suporte para discriminação de sujeitos não-diabéticos e pacientes com DM tipo 2 por meio da análise de saliva. Além disso, foi realizada uma revisão sistemática e metanálise que avaliou a correlação entre a proteína alfa-2-macroglobulina (A2MG) salivar com a glicemia e hemoglobina glicada (HbA1c) para revisar sistematicamente a eficácia desta proteína para determinar os níveis de glicemia e HbA1c de pacientes com DM tipo 2. Em resumo, podemos concluir que a plataforma FTIR apresenta grande potencial para diagnóstico e monitoramento de pacientes diabéticos de forma não invasiva, sem utilização de reagente, e altamente sensível utilizando um baixo volume com preparação mínima de amostra (saliva ou urina). Além disto, os dados apresentam uma significativa correlação entre HbA1C e A2MG salivar que necessitam ser provados em estudos com grandes coortes para vigilância do diabetes pela saliva.

Palavras chave: Diabetes, saliva, urina, FTIR, biomarcador e diagnóstico.

ABSTRACT

The diagnosis and monitoring of diabetes mellitus (DM) by blood is highly accurate, however it is an invasive procedure, it causes discomfort to patients and has a high cost for both the health system and patients. The development of alternatives non-invasive platforms with other biological samples, which are sustainable, with high sensitivity and reduced cost, can collaborate to diabetes surveillance. In this thesis, potential salivary and urinary biomarkers were investigated as alternatives for the diagnosis and monitoring of DM divided in 3 studies. Firstly, an analysis was performed with the Fourie transform infrared spectroscopy (FTIR) tool to evaluate the urinary components of non-diabetic, diabetic and diabetic rats treated with insulin. In another study, the FTIR platform was used in association with the classification of the support vector machine (SVM) for discriminate non-diabetic subjects and type 2 diabetic patients using saliva. Besides that, a systematic review and meta-analysis was carried out to assess the correlation between the salivary alpha-2-macroglobulin (A2MG) protein with glycemia and glycated hemoglobin (HbA1c) to systematically review the effectiveness of this protein to determine the levels of glycemia and HbA1c in type 2 diabetic patients. Briefly, Here we outline the potential of the FTIR platform as an alternative tool without reagent, non-invasive and highly sensitive for screening and monitoring diabetic patients using a reduced volume and minimal sample preparation (saliva or urine). Besides, the data show a strong correlation between HbA1C and salivary A2MG that should be proved in large cohort of patients to monitoring diabetes using saliva.

Keywords: Diabetes, saliva, urine, FTIR, biomarker and diagnosis.

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LISTA DE ABREVIATURAS E SÍMBOLOS

ADA	Associação Americana de Diabetes
ATR	Reflexão total atenuada
ATR-FTIR	Espectroscopia de Reflexão Total Atenuada no Infravermelho com Transformada de Fourier
DM	Diabetes mellitus
DM1	Diabetes mellitus tipo 1
DM2	Diabetes mellitus tipo 2
DMG	Diabetes mellitus gestacional
FTIR	Espectroscopia de infravermelho por transformada de Fourier
HbA1c	Hemoglobina glicada
IDF	Federação Internacional de Diabetes
IR	Infravermelho
LADA	Diabetes latente autoimune do adulto
OMS	Organização Mundial da Saúde
SBD	Sociedade Brasileira de Diabetes
SUS	Sistema único de sáude
TNF-α	Fator de necrose tumoral alfa
TOTG	Teste Oral de Tolerância a Glicose

SUMÁRIO

1. INTRODUÇÃO

O diabetes mellitus (DM) é uma síndrome metabólica com característica epidêmica e apresenta altos custos de saúde para sua vigilância e tratamento. Dessa forma, se fazem necessárias intervenções por meio de políticas públicas com a finalidade de diagnóstico precoce, monitoramento e prevenção desta patologia. O DM é uma doença crônica definida pela disfunção na secreção e/ou ação da insulina que induz um estado de hiperglicemia, afetando vários tecidos periféricos (ASHCROFT; RORSMAN, 2012). Um controle rígido dos níveis glicêmicos é fundamental para reduzir a morbidade e mortalidade em pacientes diabéticos. Nesse contexto, métodos alternativos para avaliar o controle glicêmico são fundamentais para prevenir complicações a longo prazo e melhorar a qualidade de vida em indivíduos diabéticos (COPUR; ONAL; AFSAR; ORTIZ *et al.*, 2020; OKAMOTO; ANHÊ; SABINO-SILVA; MARQUES *et al.*, 2011).

O padrão ouro para o diagnóstico do DM é a análise de sangue e pode ser realizado com base em critérios de glicose plasmática (ADA, 2020c). A utilização de glicosímetro para availiar os níveis de glicose é usualmente a mais utilizada, sendo aplicado para triagem, diagnóstico e monitoramento do DM (SACKS; ARNOLD; BAKRIS; BRUNS *et al.*, 2011). Contudo, a necessidade de perfurar os dedos constantemente, várias vezes ao dia é uma inconveniência, que pode gerar dor e desenvolver calosidades nos dedos (HOLLAND; SMITH; ESKENAZI; BASTAKI, 2003; SACKS; ARNOLD; BAKRIS; BRUNS *et al.*, 2011). Os testes realizados por glicosímetro com tiras reativas de glicose são considerados dispositivos com alta precisão no controle glicêmico, entretanto, muitos pacientes possuem resistência ao se submeterem a esse procedimento diversas vezes ao dia, por ser invasivo e ter um elevado custo (SACKS; ARNOLD; BAKRIS; BRUNS *et al.*, 2011).

Diante disso, é necessária uma busca por novos métodos de diagnóstico e monitoramento para o DM, com avanços em pesquisas de biomarcadores utilizando diversos fluidos e tecidos biológicos associados com novas ferramentas metodológicas (STRIMBU; TAVEL, 2010). A saliva e a urina são potenciais candidatos para fluídos diagnósticos e de rastreamento, pois refletem o estado sistêmico do indivíduo podendo ser utilizados como fontes de biomarcadores para diversas doenças, incluindo o DM, sendo um substituto do sangue em testes laboratoriais para diversas patologias

(PISITKUN; JOHNSTONE; KNEPPER, 2006; RATHNAYAKE; AKERMAN; KLINGE; LUNDEGREN *et al.*, 2013).

A utilização da urina como fluído diagnóstico e de rastreamento possui algumas vantagens, pois é de fácil coleta, não-invasiva, não é necessário um profissional da área da saúde para a coleta, é excretada em grandes quantidades, além dos constituintes moleculares presentes na urina serem relativamente estáveis, devido ao armazenamento na bexiga (PISITKUN; JOHNSTONE; KNEPPER, 2006). De forma semelhante, a saliva apresenta as vantagens de ser não-invasiva, fácil armazenamento, fácil coleta, menor custo de coleta e menor risco de contaminação e manipulação quando comparado ao sangue (PFAFFE; COOPER-WHITE; BEYERLEIN; KOSTNER *et al.*, 2011).

Os biomarcadores urinários e salivares com potencial diagnóstico para o DM podem ser avaliados considerando a sua sensibilidade, especificidade e valores preditivos em relação à glicemia de jejum, teste oral de tolerância à glicose, glicosúria e os níveis de hemoglobina glicada (HbA1c) (Takada et al., 2013). Assim, a utilização desses fluídos como fluido de diagnóstico é um campo emergente para realizar o diagnóstico e monitoramento de doenças como o DM.

A espectroscopia de infravermelho com transformada de Fourier (FTIR) associada a reflectância total atenuada (ATR) é uma ferramenta bioanalítica adequada para fornecer informações de maneira rápida, não destrutiva, sem a utilização de reagentes, com alto rendimento e relativamente barata em comparação com outras plataformas de análise de componentes moleculares em biofluidos (BUTLER; BRENNAN; CAMERON; FINLAYSON *et al.*, 2019; CAIXETA; AGUIAR; CARDOSO-SOUSA; COELHO *et al.*, 2020). Dessa forma, o uso da urina e da saliva como triagem do DM poderiam reduzir os desfechos clínicos desfavoráveis desta patologia e aumentariam o número de mensurações diárias de controle glicêmico que poderiam ser usadas pelo médico para prescrição mais eficiente no tratamento da população diabética.

No presente estudo, nosso objetivo foi identificar biomarcadores espectrais urinários e salivares para desenvolvimento de plataformas de ATR-FTIR sustentáveis com potencial diagnóstico e monitoramento do diabetes mellitus. Adicionalmente, buscamos identificar um novo biomarcador salivar para detecção do diabetes por meio de uma revisão sistemática e metanálise,

2. FUNDAMENTAÇÃO TEÓRICA

2.1 Diabetes mellitus – aspectos gerais

Diabetes mellitus (DM) é uma doença endócrina decorrente de uma deficiência na secreção de insulina ou resistência à insulina resultando em hiperglicemia inapropriada (COMBETTES-SOUVERAIN; ISSAD, 1998; PADHI; NAYAK; BEHERA, 2020). As vias metabólicas que são mais afetadas neste processo são as de carboidratos e lipídios, ambas são essenciais para o fornecimento e armazenamento de energia para o organismo (SAVAGE; PETERSEN; SHULMAN, 2007; ZHENG; LEY; HU, 2017). Além disso, o metabolismo de proteínas é afetado nos limiares de síntese proteica, na transcrição gênica de alguns intermediários importantes do ciclo de Krebs e de proteínas intracelulares (TESSARI; CECCHET; COSMA; PURICELLI *et al.*, 2011). Embora no diabetes o organismo apresente um elevado índice glicêmico inapropriado, os tecidos periféricos não estão aptos para captar essa glicose excedente, pois apresentam disfunções nos transportadores de glicose, dessa forma, o organismo ativa as vias metabólicas para a sua produção e para obtenção de energia através do aumento da gliconeogênese, da lipólise e da produção de corpos cetónicos, agravando as complicações desta patologia (HOLT; HANLEY; AL-LAMKI, 2007; IM; KWON; KIM; KIM *et al.*, 2007; PADHI; NAYAK; BEHERA, 2020).

O aumento dos níveis de glicose no organismo podem gerar de forma progressiva complicações crônicas microvasculares e macrovasculares. Além disso, a produção excessiva de espécies reativas de oxigênio, alterações na degradação lipídica pela β-oxidação e diminuição do status antioxidante também são fatores críticos responsáveis pela progressão destas complicações (WANG; ZHU; CHEN; CHEN, 2018). Dentre as complicações microvasculares incluem: doenças oculares, como catarata, retinopatias e glaucoma (SHUKLA; TRIPATHY, 2020); doenças renais, como nefropatia e insuficiência renal crônica (VARGHESE; JIALAL, 2020); danos nos nervos periféricos que desencadeiam neuropatias, parestesias e dor. Já as complicações macrovasculares incluem: doenças cerebrovasculares, (BARBAGALLO; DOMINGUEZ, 2014; KIM, 2019) e cardiovasculares (aterosclerose, insuficiência cardíaca congestiva, doença arterial coronariana e doença arterial periférica) (PAUL; ALI; KATARE, 2020).

O DM é uma doença com características de epidemia, apresenta alta taxa de morbidade e mortalidade, resultando em custos significativos para manejo e tratamento (AZHAR; GILLANI; MOHIUDDIN; MAJEED, 2020). De acordo com o relatório da Federação Internacional de Diabetes (IDF) de 2019, 1 a cada 11 adultos têm DM, um total de 463 milhões de pessoas em todo o mundo, e aproximadamente 232 milhões de pessoas ainda não foram diagnosticadas (IDF, 2019; KHURSHEED; SINGH; WADHWA; KAPOOR *et al.*, 2019). Destaca-se que 126 milhões de diabéticos são maiores de 65 anos de idade (1 a cada 5 pessoas) e 79% dos diabéticos são de países de baixa e média renda. Projeta-se um incremento da população mundial com DM para 578 milhões em 2030 e para 700 milhões em 2045, caso não se tenha mudanças nos hábitos relacionados aos fatores de risco desta patologia para diminuir e/ou controlar o seu aumento (IDF, 2019).

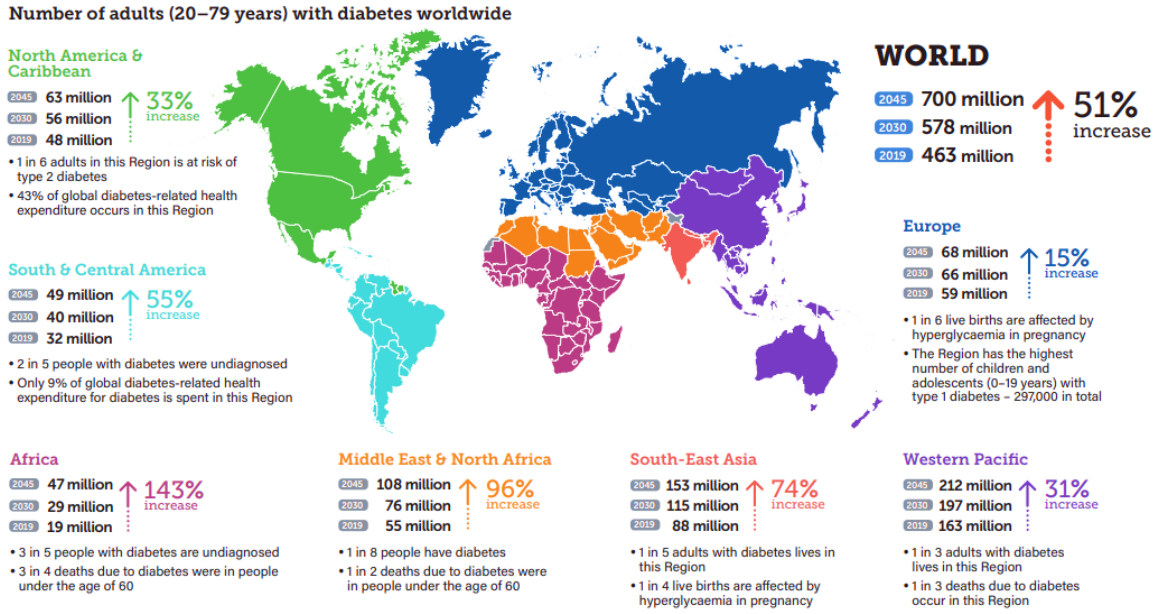

Figura 1. Estimativa mundial de diabetes mellitus em 2019 (IDF, 2019)

. Segundo a Organização Mundial de Saúde (OMS) e a Federação Internacional de Diabetes (IDF), no Brasil são 16,8 milhões de pessoas diagnosticadas com DM (1 a cada 9 pessoas). Além disso, a proporção de pessoas não diagnosticadas com DM é de cerca de 46% no Brasil (IDF, 2019; WHO, 2020). A mortalidade oficial relacionada com o DM foi de 135,2 mil pessoas em 2019, correspondendo a 55,6% de óbitos por DM registrados na América do Sul (IDF, 2019). O aumento do sedentarismo, obesidade, envelhecimento, maior urbanização, dieta inadequada e sobrevida dos pacientes diabéticos são alguns dos

fatores para a crescente prevalência de DM (MALTA; BERNAL; ISER; SZWARCWALD *et al.*, 2017; SHAW; SICREE; ZIMMET, 2010; ZHENG; LEY; HU, 2018). A partir desse contexto epidemiológico alarmante, é importante o desenvolvimento de estratégias de prevenção do DM que possuem o objetivo de reduzir o crescimento dessa síndrome metabólica (ASIF, 2014).

Devido a gravidade das complicações e por ser uma doença crônica, o DM é uma doença que exige um alto valor de investimento para o seu tratamento e controle para os sistemas públicos e privados de saúde (COSTA; FLOR; CAMPOS; OLIVEIRA *et al.*, 2017; MALTA; BERNAL; ISER; SZWARCWALD *et al.*, 2017). Desta forma, até 20% do gasto total com a saúde é direcionado para o DM, sendo um desafio para os sistemas de saúde e para um desenvolvimento econômico sustentável (SBD, 2020). Pacientes diabéticos possuem maiores taxas de hospitalizações quando comparado com pacientes que não possuem DM e a duração da hospitalização para uma mesma enfermidade também é maior (SBD, 2020). Estas hospitalizações consomem grande parte dos recursos de saúde, aproximadamente 55% dos custos na Europa, 44% nos Estados Unidos da América e 10% na América Latina. No Brasil, os resultados evidenciam que 15,3% dos custos hospitalares do Sistema Único de Saúde (SUS) entre 2008 a 2010 foram decorrentes do DM (ROSA; NITA; RACHED; DONATO *et al.*, 2014).

O diagnóstico precoce do DM torna-se necessário como forma de evitar várias complicações de curto e longo prazo, redução de gastos públicos e para melhorar a qualidade de vida dos diabéticos. O padrão ouro para o diagnóstico de DM é a análise de sangue, o que pode ser realizado com base em critérios de glicose plasmática, tanto a glicose plasmática em jejum ou o valor de glicose plasmática de 2 horas durante um teste de tolerância oral à glicose de 75g (TOTG), ou critérios de hemoglobina glicada (HbA1c) (SBD, 2020; ADA, 2020a), como apresentado na Tabela 1 abaixo:

Tabela 1. Parâmetros para diagnóstico de normoglicemia, pré-diabetes e diabetes adotados pela SBD.

	Glicose em jejum (mg/dL)	**TOTG (mg/dL)**	**Glicose ao acaso**	**HbA1c (%)**	**Observações**
Normoglicemia	< 100	< 140	-	< 5.7	OMS indica o valor de 110 mg/dL para normalidade da glicose em jejum
Pré-diabetes	≥ 100 e < 126	≥ 140 e < 200	-	≥ 5.7 e < 6.5	O valor positivo em qualquer um dos parâmetros confirma o diagnóstico de prédiabetes.
Diabetes	≥ 126	≥ 200	≥ 200 com sintomas inequívocos de hiperglicemia	≥ 6.5	Método de HbA1c deve ser o padronizado.

(Adaptado de SBD 2020)

Para se ter um diagnóstico de DM, o ideal é repetir os exames em que é confirmado a alteração dos parâmetros supracitados (ADA, 2020d). De acordo com a Associação Americana de Diabetes (ADA) e a OMS, o DM pode ser classificado em quatro categorias gerais, diabetes mellitus tipo 1 (DM1), diabetes mellitus tipo 2 (DM2), diabetes mellitus gestacional (DMG) e tipos especificos de diabetes em decorrência de outras causas (ADA, 2020a; WHO, 2020).

2.1.1 Diabetes Mellitus tipo 1

O DM1 é uma das doenças endócrino-metabólicas mais comuns durante a infância e adolescência (IDF, 2019). Estima-se que ocorre cerca de 79 mil novos casos de DM1 em crianças com 15 anos ou menos em todo mundo. No Brasil, a incidência é em torno de 10 casos de DM1 a cada 100 mil habitantes (IDF, 2019; SOUZA; KRAEMER; KOLISKI; CARREIRO *et al.*, 2020).

A etiologia do DM1 não é totalmente conhecida, mas se sabe que as principais causas são pela interação entre fatores genéticos, imunológicos e ambientais que desencadeiam a destruição das células β-pacreáticas pelas células T, ocorrendo disfunção na produção de insulina pelo organismo (IDF, 2019; ATKINSON; EISENBARTH, 2001). Entre os fatores genéticos, o sistema de antígeno leucocitário humano de classe II (HLA), HLA-DRB1, HLA-DQB1 e HLA-DQA1, contribui com 30-50% do risco genético de desenvolver DM1 (NOBLE; VALDES, 2011). A regulação e resposta imunológica também contribuem para a patogênese de DM1, com ação de anticorpos de células das ilhotas, auto-anticorpos de proteína tirosina fosfatase, autoanticorpos contra o transportador de zinco 8, auto-anticorpos de ácido glutâmico descarboxilase e auto-anticorpos de insulina como os principais marcadores de destruição imunológica das células β-pacreáticas (PELLEGRINO; CRINÒ; ROSADO; FIERABRACCI, 2019; TAPLIN; BARKER, 2008). Além disso, a associação com fatores ambientais, como infecções, dieta e microbiota intestinal podem contribuir com autoimunidade estabelecida pelo DM1 (REWERS; LUDVIGSSON, 2016).

Uma cascata de eventos imunológicos ocorrem na condição de DM1, como a infiltração e ativação de auto-anticorpos no pâncreas com a liberação de citocinas pró-inflamatórias, como o fator de necrose tumoral alfa (TNF-α), que se liga ao seu receptor de membrana das células β-pacreáticas, e ativam as vias de sinalização intracelular que terminam na indução de mecanismos pró-apoptóticos das células β-pacreáticas (CNOP; WELSH; JONAS; JÖRNS *et al.*, 2005; PIROT; CARDOZO; EIZIRIK, 2008)

O indivíduo com DM1 possui a necessidade de administrar insulina cronicamente, associado ou não com combinação de hipoglicemiantes orais, como forma de controlar a doença e evitar suas complicações. Em geral, a taxa de destruição das células β-pancreáticas é variável, porém é maior e mais rápida em crianças do que em adultos (SBD, 2020). A progressão em adultos é mais lenta, sendo denominada diabetes latente autoimune do adulto (LADA), em que esses indivíduos não possuem a necessidade de utilizar insulina inicialmente, entretanto possuem os auto-anticorpos contra as células β-pacreáticas (ANDERSEN, 2020; TUOMI; GROOP; ZIMMET; ROWLEY *et al.*, 1993). Entre os indivíduos com DM1, cerca de 10% deles são diagnosticados como LADA (LAUGESEN; ØSTERGAARD; LESLIE, 2015). Em geral, os sintomas clínicos do DM1 se manifestam quando mais de 70% da massa das células β-pacreáticas são destruídas (CNOP; WELSH; JONAS; JÖRNS *et al.*, 2005).

Atualmente, vários pesquisadores buscam formas de prevenir, interromper o avanço e/ou tentam reverter o DM1, uma vez que a expectativa de vida de indivíduos com essa doença é reduzida (RAWSHANI; SATTAR; FRANZÉN; RAWSHANI *et al.*, 2018). COUPER; HALLER; GREENBAUM; ZIEGLER *et al.* (2018) publicaram nas diretrizes de consenso de práticas clínicas da Sociedade Internacional de Diabetes Pediátrico e Adolescente estágios de DM1 sobre a evolução da doença, na perspectiva de identificação do estágio pré-sintomático anterior ao estabelecimento de DM1 até o diagnóstico clínico. Estes estágios são divididos em estágio 0, em que os indivíduos portadores de alelos com susceptibilidade de auto-anticorpos das células β-pancreáticas ainda não estão desenvolvidos; no estágio 1 ocorre o aparecimento de vários anticorpos das ilhotas pancreáticas em condição de normoglicemia; enquanto que o estágio 2 desenvolve avanço para disglicemia e por fim o estágio 3 com o início de DM1 sintomático. Essa compreensão da evolução do DM1 é importante para permitir terapias com o objetivo de desacelerar e prevenir os sintomas de DM1.

2.1.2 Diabetes Mellitus tipo 2

Alterações na ação da insulina são as causas do DM2, ocasionando uma resistência à insulina em órgãos alvos periféricos, com elevada produção de glicose hepática e disfunção dos adipócitos (DEFRONZO, 2004; TRIPATHI; SRIVASTAVA, 2006). Durante o estágio inicial, ocorre um aumento na secreção de insulina para manter a homeostase da glicose como medida compensatória, mas com o avanço da doença ocorre a diminuição da produção de insulina (GASTALDELLI, 2011; ISMAIL-BEIGI, 2012). Esta resistência à insulina e deficiência relativa da secreção de insulina gera um quadro de hiperglicemia característico de DM2 (OLOKOBA; OBATERU; OLOKOBA, 2012; ZHENG; LEY; HU, 2017).

O DM2 representa aproximadamente 90% dos diagnósticos de DM em todo mundo e a sua incidência é crescente com a idade, geralmente com diagnóstico a partir dos 40 anos (IDF, 2019). Porém cada vez mais crianças e adultos jovens são diagnosticados com DM2 devido ao estilo de vida, com alterações alimentares, obesidade e sedentarismo (SAMI; ANSARI; BUTT; HAMID, 2017).

De maneira geral, a resistência à insulina está associada ao aumento de ácidos graxos e das citocinas pró-inflamatórias, gerando uma disfunção no transporte de glicose e maior degradação de lipídeos (UNGER; ORCI, 2010). Como há uma resposta ou

produção inadequada de insulina, o organismo responde aumentando inadequadamente ao glucagon, contribuindo ainda mais para a hiperglicemia (EIZIRIK; PASQUALI, 2020). Além disso, a hiperglicemia crônica gera glicação não enzimática de proteínas e lipídios, causando danos em capilares sanguíneos da retina, rins e nervos periféricos (DARIYA; NAGARAJU, 2020). Em conjuntos, esses danos levam às complicações gerais de DM, como retinopatia, nefropatia e neuropatia.

Os sintomas de DM2 se manifestam de forma tardia e podem ser perceptíveis muitos anos após ser diagnosticada a doença (TRIPATHI; SRIVASTAVA, 2006). Vários sinais e sintomas são idênticos aos de DM1, porém, em sua maioria, são menos graves/severos (BUTLER; MISSELBROOK, 2020). Indivíduos com DM2 não-controlada por longo prazo podem ter infecções recorrentes, alterações de sensibilidade nas extremidades dos membros inferiores e superiores, úlceras diabéticas, disestesia, maior tempo de cicatrização e cetoacidose metabólica (SAPRA; BHANDARI, 2020). Doenças cardiovasculares desenvolvidas pela condição de DM2 são as maiores responsáveis pela morbidade de indivíduos com esta patologia, podendo chegar a mais de 80% das causas de morte por essa doença (EIZIRIK; PASQUALI, 2020; MARTÍN-TIMÓN; SEVILLANO-COLLANTES; SEGURA-GALINDO; DEL CAÑIZO-GÓMEZ, 2014).

O controle da glicemia previne comorbidades, o que reduz os índices de mortalidade, morbidade e redução da qualidade de vida em pacientes diabéticos (ADA, 2020b). Para isso, o controle da dieta, uma rotina de atividades físicas, cessação do tabagismo e controle da hipertensão e dislipidemia são fundamentais para a prevenção das complicações do DM2 (FORBES; COOPER, 2013).

Após a instalação do DM, alterações de hábitos alimentares e a prática de atividade física não são suficientes para ter um bom controle glicêmico, havendo a necessidade de associação medicamentosa, com efeitos benéficos para o tratamento do diabetes (ASIF, 2014). Diversas classes de drogas são utilizadas para tratar o DM2, entretanto, nenhuma delas permitiu estagnar a disfunção progressiva das células β-pancreáticas ao longo do tempo (ASIF, 2014).

2.1.3 Diabetes Mellitus Gestacional

O DM gestacional é caracterizado por uma intolerância a glicose e pela diminuição da sensibilidade à insulina em conjunto com uma incapacidade de

compensação deste hormônio pelo organismo (ADAM; RHEEDER, 2017; CHEN; WANG; JI; GE *et al.*, 2015). Neste caso, o diagnóstico ocorre pela primeira vez durante a gravidez, podendo ter início ou pré existência de DM, mas tendo o diagnóstico durante a gestação. Normalmente o desenvolvimento de DMG ocorre após o quarto/quinto mês de gestação, pois as alterações hormonais (estrogênio, progesterona, leptina, cortisol, lactogênio placentário e o hormônio de crescimento placentário) neste período interferem na insulina, promovendo um estado de resistência à insulina (PLOWS; STANLEY; BAKER; REYNOLDS *et al.*, 2018).

Segundo o estudo de PARSONS; BRELJE e SORENSON (1992), que avaliou a proliferação de células das ilhotas e a secreção de insulina durante a gestação, sugere-se que durante a gestação as mulheres compensam essas mudanças da insulina por meio da hipertrofia e hiperplasia das células β-pancreáticas, e com o aumento da secreção de insulina estimulada por glicose. Porém, as adaptações metabólicas normais durante a gravidez não ocorrem adequadamente em todas as gestações, podendo desenvolver DMG. Além disso, existe um risco de desenvolvimento de DM no feto, causado pelos elevados índices glicêmicos maternos, sendo uma das preocupações durante a gestação (CHEN; WANG; JI; GE *et al.*, 2015; KIM, 2010). É importante o acompanhamento através de exames bioquímicos, pois o DMG em sua maioria é assintomático. Os testes bioquímicos são realizados para determinar o valor da glicemia em jejum ao realizar o início do pré-natal e em conjunto com o TOTG entre as 24ª e 28ª semanas de gestação (BÜHLING; WINKEL; WOLF; KURZIDIM *et al.*, 2005).

A prevalência de DM gestacional tem crescido com os anos em decorrência do elevado índice da obesidade, avanço da idade materna e histórico familiar de DM (ADAM; RHEEDER, 2017) e cerca de 1 a 14% das gestações tem a probabilidade de desenvolver DMG (SBD, 2020). Esta patologia pode ser transitória ou perdurar mesmo após o parto, sendo um fator de risco para a ocorrência de DM2 no futuro, entre 30% a 50% das gestantes (SBD, 2020).

2.1.4 Outros tipos específicos de Diabetes Mellitus

Com o aumento de estudos e conhecimentos relacionados a patogênese do DM, cada vez mais se tem o aumento de categorias de tipos específicos de DM (ADA, 2020a). Sua classificação se torna mais específica e definitiva com a vantagem de um tratamento mais eficiente (GROSS; SILVEIRO; CAMARGO; REICHELT *et al.*, 2002). A

Organização Mundial de Saúde reconhece diversos outros tipos de DM, que são caraterizadas pela variedade de manifestações clínicas entre elas e são uma pequena parte do diagnóstico quando comparado aos demais tipos de DM (WHO, 2020). Essa classificação é dependente das causas que são responsáveis pela disfunção do metabolismo glicêmico (SBD, 2020).

Entre as dezenas de causas incluídas na lista de tipos específicos de DM, estão alguns distúrbios genéticos na função das células β-pancreáticas, como: MODY 1 - defeitos no gene HNF4A, MODY 2 - defeitos no gene GCK, MODY 3 - defeitos no gene HNF1A, MODY 4 - defeitos no gene IPF1, MODY 5 - defeitos no gene HNF1B, MODY 6 - defeitos no gene NEUROD1, DM mitocondrial e; defeitos genéticos na ação da insulina, entre outros (SBD, 2020; ADA, 2020a).

Diante disso, os testes moleculares ou celulares podem se tornar cada vez mais necessários em situações de diagnóstico com precisão para DM (FLANNICK; JOHANSSON; NJØLSTAD, 2016) e novas técnicas de rastreamento são necessárias para identificação de possíveis mutações antes mesmo do diagnóstico, sendo uma perspectiva futura com a necessidade de maiores investimentos (MELNIKOV; ROGOV; WANG; GNIRKE *et al.*, 2014).

Mesmo com o crescente avanço sobre o entendimento da fisiopatologia do DM, o fator de risco principal para as complicações relacionadas ao DM é ainda a falta de controle glicêmico pelos pacientes. Com isso, acreditamos na relevância das pesquisas sobre novas alternativas de monitoramento e diagnóstico precoce de DM com a finalidade de reduzir as complicações tardias desta patologia, além de diminuir os custos para os sistemas de saúde e melhorar a qualidade de vida dos pacientes diabéticos.

2.2 Biomarcadores – definição e suas aplicações

Biomarcadores são caracterizados como moléculas que possuem potencial de medida como indicadores de processos biológicos normais ou patológicos, ou ainda como preditores de resposta para uma intervenção terapêutica (NIH, 2001). No geral, os marcadores biológicos se sobrepõem entre os indivíduos saudáveis, dificultando a identificação de biomarcadores ideais, pois estes devem ter alta sensibilidade, especificidade e ter um bom valor preditivo (CALIFF, 2018). Além disso, preferencialmente devem ser de fácil acesso, simples coleta, custo mínimo e ter reprodutibilidade para que seja aplicado na prática clínica.

Os biomarcadores devem possuir eficácia e segurança observadas em estudos *in vitro,* amostras teciduais, estudos com modelos animais e ensaios clínicos de fase inicial, pois assim podem estabelecer prova de conceito para sua aplicabilidade. Dentre as aplicações dos biomarcadores, podemos incluir (CALIFF, 2018):

- como ferramenta de diagnóstico na indicação de que o paciente possui uma condição normal ou a doença, como por exemplo a alta concentração de glicose sanguínea no diagnóstico de DM (AZHAR; GILLANI; MOHIUDDIN; MAJEED, 2020).
- como ferramenta de localização e extensão de algumas doenças, pois assim é possível determinar o avanço da doença no organismo, como por exemplo avaliação de microRNAs no soro e urina para avaliar a progressão da doença renal crônica (NASCIMENTO; DOMINGUETI, 2019).
- como indicador de prognóstico de doenças, como por exemplo a associação de metabólitos como prognóstico e prevenção de DM (ZENG; TONG; TONG; YANG *et al.*, 2017).
- como monitoramento e previsão de resposta a uma intervenção, como por exemplo os níveis reduzidos de adiponectina para indicar maiores riscos de resistência à insulina e obesidade (TABÁK; CARSTENSEN; WITTE; BRUNNER *et al.*, 2012).

O crescente aumento de pesquisas com biomarcadores se tornou possível por meio de tecnologias para detecção, quantificação e a identificação de moléculas em conjunto com os conhecimentos moleculares e aplicações de técnicas em alta escala por meio de análises ômicas e espectroscópicas, possibilitando a identificação de biomarcadores para várias doenças (DOMAGALA-KULAWIK, 2019; GUO; HUANG; WANG; FENG, 2020). Apesar destes avanços, a média de introdução de novos biomarcadores na prática clínica é pequena, isso demonstra o quanto o processo é longo e difícil para a implantação de um novo biomarcador no contexto médico (SCHERF; BECKER; CHAN; HOJVAT, 2010).

Existem níveis de validade que devem ser considerados ao avaliar um biomarcador, o primeiro é a validade da medição. O possível biomarcador deve ser medido de forma objetiva e com alta reprodutibilidade, além disso com exatidão, ou seja, o possível biomarcador deve se correlacionar fortemente com o desfecho clínico. Outro nível de validação é a externa, em que o possível biomarcador necessita ter o alto poder

preditivo semelhante em outros tipos de estudos ou populações, sendo assim, ser possível estabelecer a sua validade (STRIMBU; TAVEL, 2010). Shariat et al. (2010) propuseram um processo de desenvolvimento de biomarcadores que possui cinco fases até a sua implementação (Tabela 2). A proposta destes autores não é uma diretriz oficial pois não existe uma regulamentação, mas este esquema propõe a sistematização de estudos relacionados a biomarcadores (SHARIAT; LOTAN; VICKERS; KARAKIEWICZ *et al.*, 2010).

Tabela 2 Proposta de sistematização de implementação de biomarcadores

Fase	Objetivos	Experimentação	Amostra
I	Hipótese sobre biomarcador	Pré-clínicos	Ensaios *in vitro* ou modelos animais
II	Classificação e nomeação dos biomarcadores	Discriminação entre os grupos (robustez)	Grupos de pacientes reduzidos
III	Teste em uma população pequena	Regras de predição (discriminação)	Grupos de pacientes maiores
IV	Validação do biomarcador	Funcionalidade	Grupos grande e diferente de pacientes
V	Avaliação do impacto social sobre o biomarcador	Relatório sobre a viabilidade de implementação do biomarcador	-

Adaptado de Shariat et al. (2010)

Existe uma necessidade no desenvolvimento de novos métodos de monitoramento e diagnóstico para várias doenças, e neste sentido, há um avanço em pesquisas de biomarcadores utilizando fluidos e tecidos biológicos para essa finalidade (DWIVEDI; PUROHIT; MISRA; PAREEK *et al.*, 2017). Componentes urinários e salivares são capazes de fornecer informações do estado fisiológico, podendo ser utilizados como meios de identificação e monitoramento de uma doença, como também para estabelecer um adequado tratamento sem a coleta de sangue (BARRATT; TOPHAM, 2007; IORGULESCU, 2009; NUNES; MUSSAVIRA; BINDHU, 2015; YU; RICH; FOREMAN; SMITH *et al.*, 2017).

2.2.1 Urina como fluido diagnóstico

A busca por moléculas como biomarcadores para doenças tem sido explorada em diversos fluidos corporais, incluindo a urina, que apresenta ser promissora na identificação de biomarcadores (BARRATT; TOPHAM, 2007; DE BOCK; DE SENY; MEUWIS; CHAPELLE *et al.*, 2010). A utilização da urina como fonte de biomarcadores não é uma prática recente, durante muito tempo a glicose presente na urina era avaliada se formigas eram atraídas pela mesma (LAKHTAKIA, 2013). A presença de albumina na urina tem sido utilizada como indicador de doenças renais (KANG; CHOI; SONG; HAN *et al.*, 2012). Portanto, a utilização da urina tem sido fundamental na medicina investigativa baseada em conhecimentos da fisiopatologia das doenças para identificar biomarcadores potenciais.

A urina é formada pelos néfrons, que são as unidade funcionais dos rins, por sua vez os néfrons são divididos em glomérulos e túbulos renais. O sangue arterial é direcionado sob alta pressão até os capilares do glomérulo, realizando a filtração com parte do plasma passando para a cápsula de Bowman (LAWRENCE; DOHERTY; DHANDA, 2018; OGOBUIRO; TUMA, 2020). O líquido filtrado não possui proteínas e elementos celulares, pois são incapazes de atravessar os vasos. Assim, a concentração de sais e moléculas orgânicas são semelhantes às encontradas no plasma (LAWRENCE; DOHERTY; DHANDA, 2018). Em seguida, o material filtrado passa para os túbulos renais e algumas substâncias são reabsorvidas para o sangue, como alguns íons, glicose, lactato e aminoácidos, enquanto que outras são secretadas do sangue para os túbulos renais distais, como íons, creatinina, ácido úrico e pequenas moléculas (LAWRENCE; DOHERTY; DHANDA, 2018; OGOBUIRO; TUMA, 2020; RONDON-BERRIOS; BERL, 2019). Portanto, a urina é formada por esses três processos renais, podendo ser resumida conforme o esquema abaixo:

Urina = filtração golemrular – reabsorção tubular + secreção tubular

A urina final é conduzida até os ureteres para a bexiga, e então armazenada até o momento de sua eliminação (OGOBUIRO; TUMA, 2020). Assim, a urina é formada por algumas biomoléculas do plasma, o que lhe confere informações de vários órgãos e também do próprio trato urogenital, tornando a urina um fluído importante para estudos relacionados com alterações sistêmicas de várias doenças (BLOCK; GENZEN, 2020;

PISITKUN; JOHNSTONE; KNEPPER, 2006). Cerca de 30% do proteoma da urina é devido a filtração do plasma glomerular e 70% se origina do trato urogenital, contendo assim uma variedade de biomarcadores que podem ser relacionados a saúde em geral (DECRAMER; GONZALEZ DE PEREDO; BREUIL; MISCHAK *et al.*, 2008).

A utilização da urina como fluído diagnóstico e rastreamento apresenta vantagens, pois é excretada em grandes quantidades, a coleta não requer métodos invasivos e não possui a necessidade de um profissional da área da saúde para realizar sua coleta, permite a amostragem repetida de um mesmo indivíduo para reprodutibilidade e o conteúdo dos componentes moleculares são relativamente estáveis devido ao armazenamento na bexiga, sendo um fator positivo na busca por biomarcadores (PISITKUN; JOHNSTONE; KNEPPER, 2006).

Contudo, a urina possui algumas variações entre as amostras individuais devido a fatores biológicos relacionados à saúde, idade, dieta, prática de atividade física e proteólise no armazenamento na bexiga (HARPOLE; DAVIS; ESPINA, 2016). Essa variabilidade também pode ocorrer devido a degradação das amostras no processo de coleta e armazenamento, sendo assim, as amostras de urina como diagnóstico e rastreamento possuem potenciais com diferentes vantagens e desvantagens de acordo com o método de coleta e respectivo teste clínico (PAREKH; KAO; MEONI; IPP *et al.*, 2007; THOMAS; SEXTON; BENSON; SUTPHEN *et al.*, 2010).

A urina matinal possui pequenas variações na concentração de proteína, enquanto que a segunda urina da manhã ou uma amostra urinária aleatória possuem menores taxas de proteólise devido o menor tempo armazenado na bexiga (JULIAN; SUZUKI; SUZUKI; TOMINO *et al.*, 2009). Já a urina de 24 horas pode indicar o reflexo do tipo e concentração da proteína ao longo do tempo e detectar disfunções no organismo, ajudando no diagnóstico, monitoramento e progresso de um determinado tratamento (CORDER; RATHI; LESLIE, 2020; RODELO-HAAD; ESQUIVIAS-MOTTA; AGÜERA; ALJAMA *et al.*, 2018). No mesmo sentido, ao avaliar a concentração de proteínas de um mesmo indivíduo ao longo do tempo indica que a urina de 24 horas possui menor desvio padrão entre os valores de proteínas individuais , como demostrado no estudo de THOMAS; SEXTON; BENSON; SUTPHEN *et al.* (2010) que avaliou a urina de 24 horas por 10 dias, e os resultados mostraram que os desvios padrão relativos foram mais baixos para a coleta de 24 horas (39%) e primeiras coletas da manhã (41%), enquanto as segundas amostras de urina do dia (54%) e coleta aleatória (61%) exibiram maiores variabilidades.

O potencial da urina para diagnóstico e rastreamento de diversas doenças sistêmicas já foi descrito para câncer de bexiga (SHARIAT; LOTAN; VICKERS; KARAKIEWICZ *et al.*, 2010), lesão renal aguda (SUN; LERMAN, 2020; WASUNG; CHAWLA; MADERO, 2015), doença renal crônica (WASUNG; CHAWLA; MADERO, 2015), câncer de próstata (KOTOVA; SAVOCHKINA, 2020), nefropatia diabética (THI; GIA; THI; THI *et al.*, 2020), esquistossomose (DIAB; TOLBA; GHAZALA; ABU-SHEASHA *et al.*, 2020), infecção congênita por citomegalovírus (CHIOPRIS; VERONESE; CUSENZA; PROCACCIANTI *et al.*, 2020), transplante renal (SWANSON; AZIZ; GARG; MOHAMED *et al.*, 2020), câncer de mama (LI; GUAN; FAN; CHING *et al.*, 2020) e entre outras patologias.

No DM, com o agravar dessa patologia, os glomérulos podem ser lesados e consequentemente pode resultar em albuminúria, glicosúria e diminuição da taxa de filtração glomerular (LIMAN; JIALAL, 2020; VARGHESE; JIALAL, 2020). Os rins de pacientes diabéticos são suscetíveis a alta concentração glicêmica, e assim são incapazes de reduzir os efeitos da hiperglicemia no interior das células. Diante disso, a urina apresenta potencial para indicar alterações provenientes da elevação de glicose ocasionada pelo DM.

2.2.2 Saliva como fluído diagnóstico

A saliva é um fluido biológico que é secretado pelas glândulas salivares maiores (parótida, submandibular e sublingual) e menores (centenas de glândulas acessórias na língua, palato e mucosas orais) (ZOLOTUKHIN, 2013). Sua composição majoritária é água, com 99%, e os outros 1% são de proteínas, lipídios, carbohidratos, DNA, RNA e componentes inorgânicos. A saliva é importante para a manutenção da saúde oral e sistêmica (LEITE; MARLOW; FERNANDES, 2013). Este fluído possui um papel relevante para mastigação, gustação, fonação, deglutição, digestão e proteção de tecidos mineralizados e mucosos (DE ALMEIDA PDEL; GREGIO; MACHADO; DE LIMA *et al.*, 2008; YOSHIZAWA; SCHAFER; SCHAFER; FARRELL *et al.*, 2013).

A saliva pode ser classificada em saliva total e saliva de glândula específica. A saliva total é um fluído misturado com com fluido crevicular, componentes da mucosa, bactérias, entre outros componentes, sendo frequentemente utilizado em estudos de disfunções sistêmicas, devido a sua origem ser praticamente das glândulas salivares

maiores. Em relação a saliva de glândula específica, são secreções usualmente utilizadas em patologias das próprias glândulas (ZOLOTUKHIN, 2013).

Diversos componentes do sangue podem atingir o líquido extracelular e em seguida serem transportados para o citoplasma das células das glândulas salivares ou para o lúmen acinar (MELVIN; YULE; SHUTTLEWORTH; BEGENISICH, 2005). Devido a esse transporte de moléculas por via transcelular ou paracelular, o fluido salivar contém informações moleculares sistêmicas que pode refletir o estado de saúde de um indivíduo, ou seja, é possível encontrar potenciais biomarcadores salivares para diversas patologias. Considerando que os componentes da saliva podem indicar alterações sistêmicas, a saliva apresenta potencial para ser um fluido alternativo ou substituto do sangue em testes laboratoriais para diagnóstico e monitoramento de doenças crônico-degenerativas ou infecciosas (RATHNAYAKE; AKERMAN; KLINGE; LUNDEGREN *et al.*, 2013).

A saliva apresenta algumas vantagens: coleta de forma fácil; não-invasiva (indolor e praticamente não tem desconforto); fácil armazenamento, manipulação e menor risco de contaminação ao ser comparada com o sangue; menor custo envolvido na coleta; permite múltiplas coletas e em praticamente qualquer faixa etária ou tipo de doença e não existe a necessidade de um profissional especializado e qualificado para a sua coleta (PFAFFE; COOPER-WHITE; BEYERLEIN; KOSTNER *et al.*, 2011).

O processo de coleta da saliva total pode ser de forma não-estimulada, que é a coleta passiva em um recipiente adequado, ou de forma estimulada em que se tem o auxílio de materiais absorventes, como o algodão ou polietileno, ou auxílio mastigatório, como pastilha elástica e ou parafina (CHOJNOWSKA; BARAN; WILINSKA; SIENICKA *et al.*, 2017; NAVAZESH, 1993; YOSHIZAWA; SCHAFER; SCHAFER; FARRELL *et al.*, 2013). Ao realizar a coleta de saliva não-estimulada em idosos, cuspir em um tubo pode ser um desafio, e o mesmo é válido para indívíduos que possuem xerostomia, como em pacientes com DM, dificultando a coleta por este método principalmente quando se exige maior quantidade de fluídos (VILLA; CONNELL; ABATI, 2014; YOSHIZAWA; SCHAFER; SCHAFER; FARRELL *et al.*, 2013). Isto evidencia a necessidade de testes diagnósticos com volume reduzido de amostras.

Na coleta de saliva específica de glândula, o procedimento ocorre pela sucção ou drenagem diretamente dos ductos salivares, sendo uma coleta mais complexa, invasiva e necessita de um profissional especializado (BHATTARAI; KIM; CHAE, 2018). Por esses motivos, este tipo de procedimento de coleta são poucos utilizados e a coleta de saliva total é mais conveniente (BHATTARAI; KIM; CHAE, 2018; CHOJNOWSKA;

BARAN; WILINSKA; SIENICKA *et al.*, 2017; PFAFFE; COOPER-WHITE; BEYERLEIN; KOSTNER *et al.*, 2011).

Contudo, atualmente a coleta de saliva não possui um critério sistematizado estabelecido para a coleta, o que restringe a reprodução de resultados nas investigações e pesquisas científicas. Desse modo, é possível encontrar algumas divergências devido ao método de coleta na quantificação de alguns componentes da saliva (CHOJNOWSKA; BARAN; WILINSKA; SIENICKA *et al.*, 2017; YOSHIZAWA; SCHAFER; SCHAFER; FARRELL *et al.*, 2013). Essa limitação pode ser minimizada ao comparar com as inúmeras vantagens da saliva como fluído diagnóstico e de rastreamento para várias doenças.

A saliva já foi descrita como potencial fonte de biomarcadores diagnósticos para diversas doenças, como doença de Alzheimer (FRANÇOIS; BULL; FENECH; LEIFERT, 2019), câncer oral (KHURSHID; ZAFAR; KHAN; NAJEEB *et al.*, 2018), síndrome de Sjörgenm (CHEN; CAO; LIN; OLSEN *et al.*, 2015), câncer de ovário (LEE; KIM; ZHOU; KIM *et al.*, 2012), câncer de pulmão (XIAO; ZHANG; ZHOU; LEE *et al.*, 2012), câncer de mama (FERREIRA; AGUIAR; SILVA; SANTOS *et al.*, 2020; ZHANG; XIAO; KARLAN; ZHOU *et al.*, 2010), malária (BUPPAN; PUTAPORNTIP; PATTANAWONG; SEETHAMCHAI *et al.*, 2010), Zika vírus (KHURSHID; ZAFAR; KHAN; MALI *et al.*, 2019), doença renal crônica (RODRIGUES; AGUIAR; CARDOSO-SOUSA; CAIXETA *et al.*, 2019), diabetes mellitus (CAIXETA; AGUIAR; CARDOSO-SOUSA; COELHO *et al.*, 2020; REZNICK; SHEHADEH; SHAFIR; NAGLER, 2006) e entre outras patologias.

Na condição diabética, a saliva diminui seu fluxo e ocorre alterações na composição salivar, incluindo o aumento dos níveis de glicose (BAJAJ; PRASAD; GUPTA; SINGH, 2012; RAO; REDDY; LU; DASARI *et al.*, 2009; SABINO-SILVA; OKAMOTO; DAVID-SILVA; MORI *et al.*, 2013). Devido a essas alterações, a saliva pode ser um fluido com potenciais biomarcadores para o estado hiperglicêmico (BALAJI; CHANDRASEKARAN; SUBRAMANIUM; FERNZ, 2017; CAIXETA; AGUIAR; CARDOSO-SOUSA; COELHO *et al.*, 2020; REZNICK; SHEHADEH; SHAFIR; NAGLER, 2006). A correlação entre a glicose salivar e as variáveis sanguínea (glicemia e HbA1c) possuem resultados divergentes na literatura. Alguns estudos apontam uma correlação alta e outros moderada entre esses indicadores (ABIKSHYEET; RAMESH; OZA, 2012; AMER; YOUSUF; SIDDQIUI; ALAM, 2001; KADASHETTI; BAAD; MALIK; SHIVAKUMAR *et al.*, 2015; MAHDAVI; HASHEMI; BOOSTANI;

ZOKAEE, 2012; PUTTASWAMY; PUTTABUDHI; RAJU, 2017) enquanto que outros estudos não observaram essa correlação (GUPTA; SINGH; PADMAVATHI; RAJAN *et al.*, 2015; SOARES; BATISTA-FILHO; PIMENTEL; PASSOS *et al.*, 2009; VASCONCELOS; SOARES; ALMEIDA; SOARES, 2010) ou observaram uma fraca correlação entre a glicose salivar e os indicadores de DM (DARWAZEH; MACFARLANE; MCCUISH; LAMEY, 1991). Segundo uma metanálise realizada por MASCARENHAS; FATELA e BARAHONA (2014), sugeriram que a glicose salivar poderia ser um potencial biomarcador com uma correlação significativa entre a concentração de glicose salivar e os valores de glicemia e HbA1c, apesar da heterogeneidade e fatores de confusão presentes nos estudos observacionais incluídos nesta metanálise. Adicionalmente, uma série de outros estudos tem buscado o diagnóstico de diabetes baseados na detecção de proteínas ou peptídeos específicos na doença e que possam apresentar correlações com a glicemia ou HbA1c, entre elas uma das principais candidatas é a alfa-2-macroglobulina (A2MG).

Apesar das vantagens mencionadas para o uso de biomarcadores salivares, ainda é um desafio a utilização deste fluido como diagnóstico, pois seus componentes estão em baixas concentrações. Contudo, esta limitação pode ser superada com o desenvolvimento de novas tecnologias e o aprimoramento das técnicas existentes. Uma alternativa seria o desenvolvimento de plataformas diagnósticas e de monitoramento utilizando análises espectroscópicas para o diagnóstico rápido, de baixo custo e não invasivo das patologias.

2.3 Espectroscopia de infravermelho por transformada de Fourier (FTIR)

A espectroscopia consiste nas técnicas que utilizam a interação da radiação eletromagnética com a matéria como forma de obtenção de informações químicas e físicas sobre o material analisado (BARTH, 2007). A espectroscopia de infravermelho faz uso da região do infravermelho do espectro eletromagnético, que corresponde ao comprimento de onda entre 0,78 μm e 1000 μm (12800 – 10 cm^{-1}). Sendo o comprimento de onda próximo de 0.78 μm a 2,5 μm (12800 a 4000 cm^{-1}), médio de 2,5 μm a 50 μm (4000 a 400 cm^{-1}) e distante de 50 μm a 1000 μm (400 a 30 cm^{-1}) (GAFFNEY; MARLEY; JONES, 2012) (Figura 2).

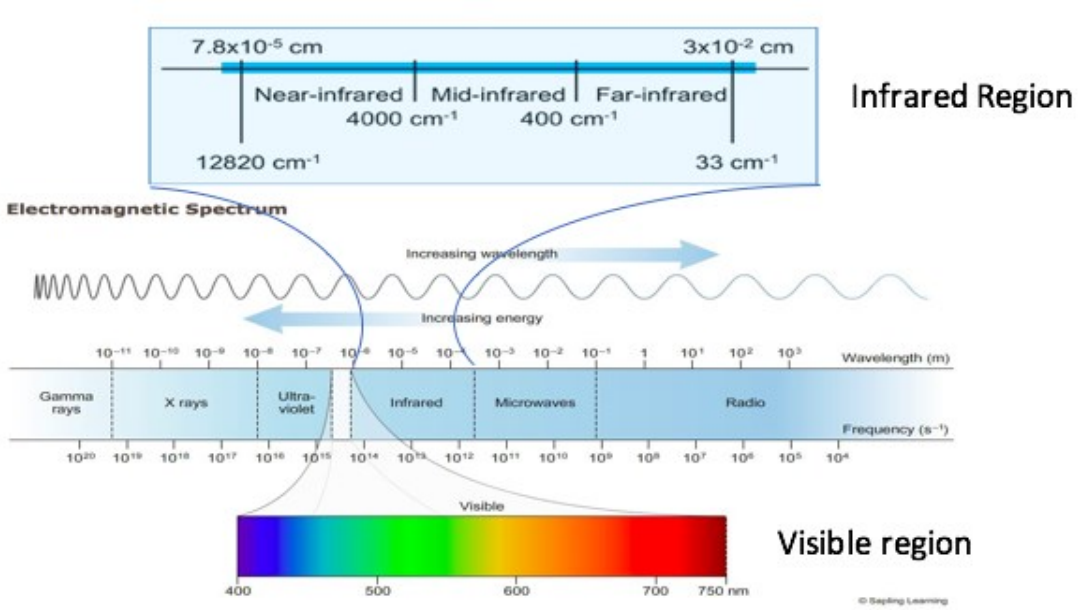

Figura 2. Espectro eletromagnético. Ilustração: Macmillan and LCGC's chromacademy

A radiação infravermelha é não ionizante e possui baixa energia, assim não ocorre transições eletrônicas na amostra. Usualmente o comprimento de onda é a grandeza para representar as análises espectroscópicas, sendo proporcional com a energia e frequência da onda eletromagnética. A técnica de espectroscopia de infravermelho fundamenta-se na absorção da radiação eletromagnética pela amostra. Ao movimentar, os átomos da molécula gera uma variação na distribuição de carga molecular, conforme a molécula gira ou vibra (momento dipolo), ocorrendo uma flutuação em seu momento dipolo, com a criação de um campo elétrico que interage com o campo associado à radiação. Se houver uma correspondência entre a frequência da radiação e a vibração natural da molécula, ocorre a absorção e isso altera a amplitude da vibração molecular. Isso também ocorre quando a rotação de moléculas assimétricas em torno de seus centros resulta em uma mudança no momento dipolo, que permite a interação com o campo de radiação. Assim, ao conhecer a frequência da radiação absorvida é possível identificar a molécula responsável pela absorção (BUNACIU; HOANG; ABOUL-ENEIN, 2017; SKOOG; HOLLER; CROUCH, 2017).

O espectro eletromagnético correspondente possui energia compatível com a vibração dos átomos de uma molécula, dessa forma, a espectroscopia no infravermelho é utilizada em estudos de modos vibracionais de amostras biológicas e materiais, pois cada espectro possui uma assinatura específica de cada componente presente na amostra

(BAKER; TREVISAN; BASSAN; BHARGAVA *et al.*, 2014; SEVERCAN; BOZKURT; GURBANOV; GORGULU, 2010).

Um dos equipamentos mais utilizados atualmente na espectroscopia no infravermelho são os com transformada de Fourier, que possuem em sua óptica o interferômetro de Michelson. Dessa forma, permite uma melhor resolução e qualidade do espectro promovendo o aumento da razão sinal/ruído e maior velocidade de varredura e aquisição dos espectros (AL-SAEED; KHALIL, 2012).

No interferômetro de Michelson, um *beamsplitter* (espelho semi-reflexivo) divide o feixe de radiação infravermelha em duas partes (perpendiculares entre si), sendo uma parte direcionada a um espelho fixo e a outra parte para um espelho móvel. Após a reflexão nos espelhos, ambos os feixes voltam a se combinar e atingem a amostra (AL-SAEED; KHALIL, 2009; 2012). A Figura 3 demonstra um esquema de funcionamento do espectrofotômetro de absorção no infravermelho por transformada de Fourier. A radiação emitida por uma fonte no infravermelho passa por um interferômetro antes de incidir na amostra. A radiação não absorvida pela amostra incide em um detector fotossensível que irá captar o sinal, transformando-o em um interferograma. Em seguida esse interferograma passa pela transformada de Fourier e é convertido em um escpectro de absorção óptica, que mostra intensidade de absorção em função do número de onda (relacionado à energia vibracional da molécula) (BASSAN, 2011; SKOOG; HOLLER; CROUCH, 2017). O processamento de dados usados no FTIR segue a lei de Bouguer-Beer-Lambert, ou simplesmente lei de Beer, relacionando a intensidade da banda à sua concentração (KHAUSTOVA; SHKURNIKOV; TONEVITSKY; ARTYUSHENKO *et al.*, 2010).

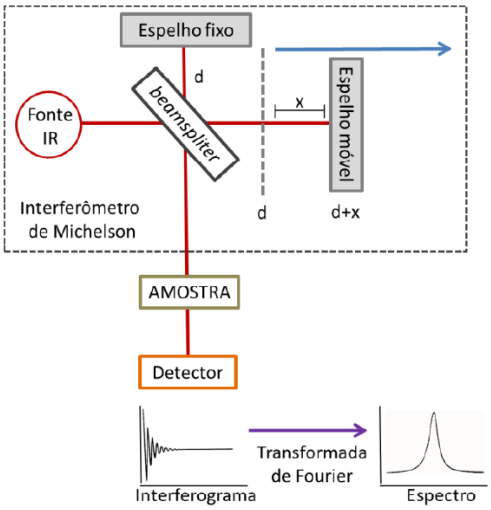

Figura 3. Esquema de funcionamento de um espectrofotômetro FTIR (Benetti, 2014).

A aquisição dos espectros pelo FTIR podem ser realizadas por transmissão, transflexão, reflexão difusa, reflexão total atenuada (ATR) e técnicas associadas a microscopia. As análises espectrais por reflexão geralmente são mais utilizadas em relação as de transmissão, uma vez que os espectros obtidos por transmissão devem ser corrigidos computacionalmente devido a ocorrência de interferências físicas (BEASLEY; BARTELINK; TAYLOR; MILLER, 2014; CHAN; KAZARIAN, 2013). Ao contrário da transmissão e transflexão, medidas no ATR não requerem substratos especiais e nenhuma ou pouca preparação da amostra, necessitando apenas que o material estudado (sólido ou líquido) seja colocado em contato direto com o elemento de reflexão interna, tornando o processo de análise mais rápido e simples, sendo mais indicado para processos de diagnósticos de desordens patológicas (DORLING; BAKER, 2013; TATULIAN, 2003).

A técnica de ATR consiste na reflexão de um feixe que incide em um ângulo crítico que passa de um meio mais denso (um cristal) para um menos denso (amostra), e uma pequena parcela da radiação incidente (onda evanescente) penetra na amostra (SILVERSTEIN; WEBSTER; KIEMLE, 2005). O esquema representativo do acessório ATR é representado na figura 4. O meio em que o feixe se propaga inicialmente é composto de um material de alto índice de refração, geralmente um cristal de diamante, seleneto de zinco ou germânio (SKOOG; HOLLER; CROUCH, 2017).

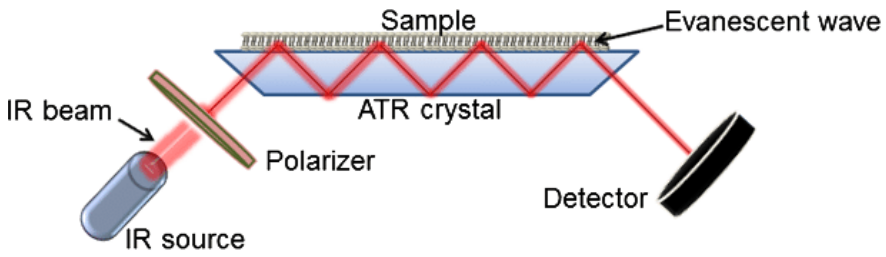

Figura 4. Representação esquemática do do sistema ATR-FTIR (AUSILI; SÁNCHEZ; GÓMEZ-FERNÁNDEZ, 2015)

As técnicas de espectroscopia vibracional possuem potenciais para fornecer informações para diagnóstico e rastreamento de doenças de forma não destrutiva, rápida, livre de reagentes, volume reduzido de amostras, alta especificidade e clinicamente relevantes (BAKER; TREVISAN; BASSAN; BHARGAVA *et al.*, 2014; BUNACIU; HOANG; ABOUL-ENEIN, 2017). Além disso, permite a utilização de fluídos e tecidos

biológicos e em conjunto com as análises univariadas e de quimiometria, tais medidas podem contribuir para o uso como uma ferramenta alternativa de avaliação diagnóstica desses materiais biológicos.

Os componentes bioquímicos (proteínas, lipídeos, ácidos nucleicos) possuem modos vibracionais na região do infravermelho médio (4000-400cm^{-1}) e são usualmente indicados como marcadores na identificação de alterações metabólicas decorrentes de diversas doenças, incluindo o DM (BUNACIU; HOANG; ABOUL-ENEIN, 2017; CAIXETA; AGUIAR; CARDOSO-SOUSA; COELHO *et al.*, 2020; SCOTT; RENAUD; KRISHNASAMY; MERIC *et al.*, 2010). Portanto, o uso da espectroscopia permite diversos benefícios em relação ao desenvolvimento de estratégias de intervenções terapêuticas precoces que possam minimizar as morbidades e reduzir a mortalidade, além de diminuir os recursos econômicos dos sistemas de saúde (BUNACIU; HOANG; ABOUL-ENEIN, 2017).

A espectroscopia de infravermelho (IR) é uma técnica crescente na caracterização de componentes em amostras biológicas e está cada vez mais presente como alternativa para diagnóstico e monitoramento de doenças (BELLISOLA; SORIO, 2012). Os compostos biológicos presentes nas amostras possuem um espectro ATR-FTIR único, pois cada molécula é caracterizada pela sua estrutura única, sendo representado as ligações estruturais no modo vibracional (OJEDA; DITTRICH, 2012; SEVERCAN; BOZKURT; GURBANOV; GORGULU, 2010). Como os modos vibracionais de IR são considerados impressões bioquímicas, a utilização da saliva e urina como fluídos diagnósticos podem relacionar a presença ou ausência de doenças. Desta forma, os espectros ATR-FTIR podem indicar informações quantitativas de analitos que possuem potencial para diagnóstico e triagem de diversas doenças (CAETANO JÚNIOR; STRIXINO; RANIERO, 2015; KHAUSTOVA; SHKURNIKOV; TONEVITSKY; ARTYUSHENKO *et al.*, 2010).

3. OBJETIVO

3.1 OBJETIVO GERAL

Identificar biomarcadores urinários e salivares para diagnóstico e rastreamento por espectrometria de reflexão total atenuada no infravermelho com transformada de Fourier (ATR-FTIR) e determinar a acurácia de um potencial biomarcador salivar proteico analisado por revisão sistemática e metanálise direcionados para o diagnóstico e monitoramento do diabetes mellitus.

3.2 OBJETIVOS ESPECÍFICOS

Artigo I:

- Demonstrar a capacidade da espectroscopia ATR-FTIR combinada com a análise univariada e multivariada de resolução de curva alternada (MCR-ALS) para monitorar parâmetros urinários de ratos não diabéticos e diabéticos, bem como animais diabéticos tratados com insulina.

Artigo II:

- Comparar os componentes salivares por espectroscopia de infravermelhos em indivíduos normoglicêmicos não diabéticos e pacientes diabéticos hiperglicêmicos tipo 2 para serem empregados como uma ferramenta alternativa de biomarcador salivar para rastreamento de diabetes mellitus.

Artigo III:

- Avaliar por revisão sistemática e metanálise se as concentrações da proteína alfa 2-macroglobulina salivar podem estar correlacionadas com os níveis de HbA1c e glicemia em pacientes com diabetes mellitus tipo 2.

4. ARTIGO I

ATR-FTIR AS A SUSTAINABLE AND LABEL-FREE TOOL TO DISCRIMINATE CHANGES PROMOTED BY DIABETES IN URINE

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ABSTRACT

The development of novel platforms to non-invasive continuous glucose monitoring applied in the screening and monitoring of diabetes is crucial to improve the diabetes surveillance system. ATR-FTIR spectroscopy in urine can be an alternative as a sustainable, label-free, fast, non-invasive and, high sensitive analysis to detected changes promoted by diabetes and insulin-treatment in urine. In this study, we used the ATR-FTIR tool to evaluate the urinary components of non-diabetic (ND), diabetic (D), and diabetic insulin-treated (D+I) rats. As expected, insulin treatment was capable to reverted glycemia, 24-h urine volume, and 24-h urine creatinine, urea, and glucose excretion promoted by diabetes. Several differences in the urine spectra of ND, D, and D+I were observed, the urea, creatinine, and glucose analytes are related to these changes. The PCA discriminated ND and D+I from D with an accuracy of ~99%. The PCA loadings associated with PC1 confirmed the urea and glucose vibrational modes as responsible for urine discrimination. The univariate analysis of second derivative spectra showed a high correlation ($r^{2:}$ 0.865, $p < 0.0001$) between the heigh of 1074 cm^{-1} vibrational mode with urinary glucose concentration. In order to estimate the amount of glucose present in the spectra, the MCR-ALS was applied and a higher predicted concentration of glucose in the urine was observed with a correlation of 78.9% when correlated with the urinary glucose concentration. ATR-FTIR associated with univariate and multivariate chemometric analysis was efficient in providing information on the status of urinary glucose with this innovative, non-invasive, and sustainable approach to diabetes surveillance.

Keywords: Diabetes, ATR-FTIR, urinary biomarkers, chemometrics analysis

1. Introduction

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by hyperglycemia related to damages in insulin secretion by pancreatic β-cells and/or reduced insulin action on target tissues [1]. It is estimated that 1 to 11 adults have DM, representing around 463 million people worldwide[2]. This endocrine disorder is a global health issue and presents higher health costs for surveillance and treatment. Tight control of glycemic levels is critical to reduce morbidity and mortality in diabetic patients. In this context, alternative methods to assess metabolic control are critical to prevent long-term complications and to improve the quality of life in individuals with diabetes [3, 4].

Diabetes affects multiple organs and it is the leading cause of kidney dysfunctions [5]. Polyuria and changes in urinary composition including glucose, creatinine, and urea were detected in diabetes. Diabetes treatment with optimal glycemic control is capable to suppress these diabetic urinary parameters [6, 7]. Urine is an attractive noninvasive fluid that has been frequently used to support clinical decisions for diabetic patients based on monitoring of the renal function [6].

Attenuated total reflection-Fourier transform infrared (ATR-FTIR) spectroscopy is a powerful bioanalytical tool suitable to provide a sustainable, label-free, rapid, non-destructive, and relatively low-cost molecular component analysis in biofluids [8-10]. Furthermore, FTIR technology is also available as portable devices, which is ideal for point of care medicine. ATR-FTIR is capable to measure vibrational modes resulting from carbohydrates, proteins, lipids, electrolytes, DNA, and RNA in unique measurement [8, 10]. The discrimination of healthy and pathological conditions, as well as the efficacy of treatments, has been widely performed by ATR-FTIR [11]. Urine based spectroscopy IR bands have already been applied successfully in several studies, for microproteinuria [12], bacteria causing urinary tract infections [13], endometrial and ovarian cancers [14], prostate cancer [15], drug-induced crystalluria [16], primary hyperoxaluria [17] and among other diseases [18]. In order to open new avenues for the use of urine infrared spectra as personalized medicine, suggesting a potential clinical implementation for screening or monitoring outcomes in diabetes.

Here, we hypothesized that ATR-FTIR is capable to identify changes in urinary molecules promoted by diabetes and insulin-treatment. Thus, the present study aims to demonstrate the ability of ATR-FTIR spectroscopy combined with univariate and multivariate curve resolution-alternating least square (MCR-ALS) analysis to monitor

urinary parameters of non-diabetic and diabetic rats, as well as insulin-treated diabetic animal.

2. Experimental section

2.1 Animal experiment

All experiments were conducted following recommendations of the Brazilian Society of Laboratory Animals Science (SBCAL) in the guide for the care and use of laboratory animals. Experimental procedures for the handling, use, and euthanasia were approved by the Ethics Committee for Animal Research of the Federal University of Uberlandia (UFU) (License #CEUA-UFU No. 013/2016) according to Ethical Principles adopted by the Brazilian College of Animal Experimentation (COBEA) and conformed to ARRIVE guidelines. All efforts were also performed to implement the principles of the 3Rs.

Male Wistar rats (~260g) were provided from the Center for Bioterism and Experimentation (REBIR) at the Federal University of Uberlandia. The animals were maintained under standard conditions (12-hour light/dark cycles, light on at 7 AM; 22 ± 2 °C; humidity ~60%) and were allowed free access to water and standard diet in a rodent facility.

Overnight-fasted animals received a single intraperitoneal injection (60 mg/kg) of streptozotocin (STZ) (Sigma-Aldrich, St. Louis, MO. USA) dissolved in 0.1 M citrate buffer (pH 4.5) to induce diabetes. Animals with hyperglycemia (>250 mg/dl) 48h later were considered diabetics (D). Non-diabetic (ND) animals received injection of NaCl 0.9% in similar volume (n = 8). Thus, diabetic animals were divided in placebo-treated Diabetic (D, n = 10) and diabetic treated with 6U insulin (D+I, n = 10). Subsequently, twenty-one days after diabetes induction, diabetic rats received treatment with vehicle (NaCl 0.9% in D rats) or with 6U of insulin [NPH insulin, Biobrás, MG, Brazil] (D+I) per day (2U at 8:30 a.m. and 4U at 5:30 p.m.) subcutaneously in D+I rats. ND animals also received vehicle (NaCl 0.9%) [19-21].

2.2 Urine Sample Collection and Preparation

On day 6 of treatment, the rats were maintained for 24-hours in metabolic cages to 24h-urine collection individually. The samples were collected, measured, processed, and stored at -80ºC until further analysis. Creatinine, urea, and glucose concentration in

the urine were measured using enzymatic assays following the manufacturer's instructions (Labtest Diagnostica SA, Brazil). Blood glucose levels obtained from the tail vein were measures in overnight-fasted animals using reactive strips for glucometer (Accu-Chek Performa, Roche Diagnostic Systems, Basel, Switzerland). Besides that, the variation of gain/loss body weight (Δ body weight) after STZ or placebo administration was analyzed. Animals were euthanized with an excessive anesthetic dose immediately after sample collections.

After that, urine samples were lyophilized (L101, Liotop®). This freeze-drying of the samples was performed to remove the strong water infrared light absorption from spectra that may mask the signal from the sample and may reduce the intensity of the urinary compounds under investigation [22, 23].

2.3 ATR-FTIR Spectroscopy and Data Analysis

Infrared spectra were acquired using an FTIR system (Nicolet 6700, Thermo Scientific, Waltham, MA, USA) on Attenuated Total Reflectance (ATR) mode over the range 400-4000 cm^{-1}. Spectra were recorded with a spectral resolution of 4 cm^{-1} and 100 scans per spectrum. FTIR spectra were smoothed via Savitzky-Golay filter (polynomial of second order in an eleven-point window), baseline corrected, and vector normalized prior spectra analysis. The fingerprint region (1800-900 cm^{-1}) was used as input to principal component analysis (PCA) and MCR-ALS techniques. All processed urinary spectra were subjected to the chemometric unsupervised method of principal component analysis (PCA) [24]. All pre-processing steps and spectral analyses were performed using Matlab® R2019b (MathWorks, Natick, MA, USA). Besides that, the second derivative was obtained by applying Savitzky-Golay algorithm with polynomial order 5 and 20 points of the window. The normalization of second derivatives was made by the norm, and the valley heights were used to indicate the absorbance of each urinary vibrational mode. The second derivative analysis was performed using Origin Pro 9.1 (OriginLab, Northampton, MA, USA) software.

2.4 Statistical Analysis

The normality of data distribution was analyzed for Kolmogorov-Smirnov test. The data of the valley intensity were analyzed using the one-way analysis of variance (ANOVA), followed by Tukey Multiple Comparison as a *post-hoc* test. Receiver Operating Characteristic (ROC) curve and Pearson correlation test were applied for all

second derivative peaks intensity. All these analyses were performed using the software GraphPad Prism (GraphPad Prism version 7.00 for Windows, GraphPad Software, San Diego, CA, USA). Only values of $p<0.05$ were considered significant and the results were expressed as mean ± S.D.

3. Results and Discussion

The DM and insulin treatment effects on animals were characterized by several parameters, which are shown in Table 1. As expected, a reduction in weight gain ($p<0.05$), an increase in plasmatic glycemic levels ($p<0.05$) and higher urinary volume ($p<0.05$) were observed in D compared with ND rats. In contrast, no change in the concentration of urinary creatinine was observed, however, 24 h urinary creatinine excretion was increased ($p<0.05$) in D compared to ND. In addition, the urinary urea concentration was decreased ($p<0.05$) in D compared to ND, and its level in 24 h urinary urea excretion was increased ($p<0.05$) in D than ND rats. Both glucose concentration ($p<0.05$) in urine and 24 h urinary glucose excretion were also higher ($p<0.05$) in D compared to ND.

Insulin treatment was profitable to increase weight gain ($p<0.05$) and reduced plasma glucose levels ($p<0.05$) and urinary volume ($p<0.05$) compared to diabetics rats. Creatinine levels in urine did not change, however, 24 h urinary creatinine excretion decreased ($p<0.05$) in D+I compared to D rats. Regarding urea concentration in urine, insulin treatment was not efficient to restore ($p>0.05$) this parameter. On the other hand, insulin treatment reduced ($p<0.05$) 24 h urinary urea excretion compared to D rats. As expected, the urinary glucose concentration and 24 h urinary glucose excretion were lower ($p<0.05$) in D+I compared to D rats (Table 1). Bearing in mind the classical effects of insulin treatment on urinary parameters, diabetic animals treated with insulin or placebo showed similar metabolic profile described in previous studies, in which urinary parameters were changed by diabetes induction, and those parameters were restored to normoglycemic profile with insulin treatment [19, 25-27].

Table 1. Effect of diabetes and insulin on body weight, glycemia, 24 h urinary volume, urinary creatinine concentration, 24 h urinary creatinine excretion, urinary urea concentration, 24 h urinary urea excretion, glycosuria and 24 h urinary glucose excretion.

Parameters	ND	D	D+I
Δ Body weight (g)	51.6±24.9	-20.1±37.4*	23.2±31.5#
Glycemia (mg/dL)	83.7±13.4	483.8±59.9*	102.4±100.8#
24 h urinary volume (mL)	23.2±10.4	124.2±31.0*	35.1±12.7#
urinary creatinine concentration (md/dL)	0.52±0.15	0.62±0.28	0.65±0.33
24 h urinary creatinine excretion (mg/24h)	12.6±8.2	76.7±40.0*	20.7±8.1#
urinary urea concentration (md/dL)	94.6±56.0	35.1±9.0*	38.4±15.5*
24 h urinary urea excretion (mg/24h)	20.9±14.9	44.0±16.6*	12.5±5.4#
Glycosuria (mg/dL)	24.7±20.3	367.8±62.8*	165.5±181.3*#
24 h urinary glucose excretion (mg/24h)	4.6±2.6	461.3±173.4*	51.9±58.3#

Values are expressed as mean ± S.D. *p < 0. 05 vs ND rats; #p < 0. 05 vs D rats. No diabetic rats (ND), Diabetic rats (D) and Diabetic rats with insulin treatment (D+I).

Urinary infrared spectra is a complex signature related to each infrared-active molecule in the biological mixture. Water is the main component of urine (over 95%) and due to its strong absorption bands in the mid-infrared region, the samples must be dry prior ATR-FTIR analysis in order to assess the signatures of molecules in lower concentrations [10]. Figure 1 shows the fingerprint region (900-1800 cm^{-1}) of spectra acquired from urine of non-diabetic and diabetic rats, as well as, the spectra of glucose, creatinine, and urea.

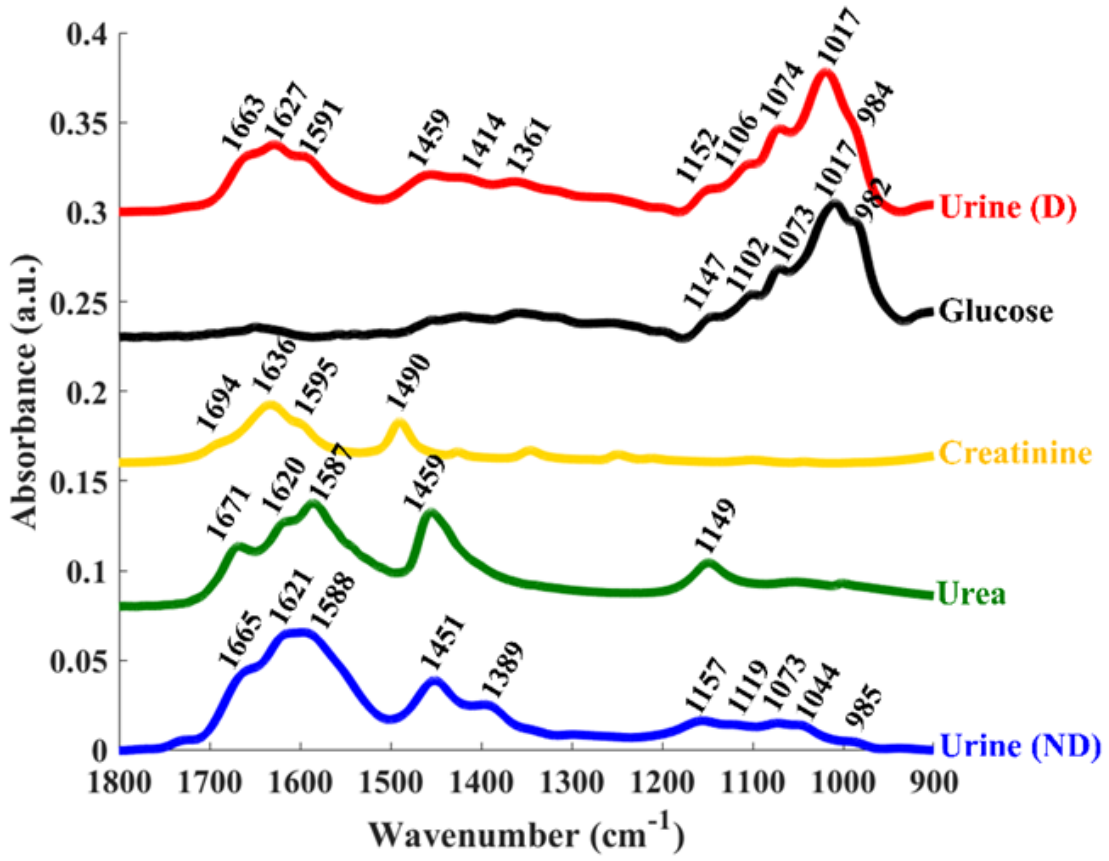

Figure 1. Fingerprint region (1800-900 cm^{-1}) of spectra of urine collected from healthy (ND, blue), diabetic (D, red), urea (green), creatinine (yellow), and glucose (black).

Spectral signatures observed in both urinary spectra in non-diabetic and diabetic conditions are the linear combination of the individual spectrum of the urinary constituents. Urea is an important component in urine and other constituents include creatinine, uric acid, organic and inorganic salts, proteins, hormones, and other metabolites. Spectra of urea and creatinine are also shown in Figure 1 due to their abundance in urine as well as their clinical relevance. IR spectrum of urea is relatively simple and has distinct bands peaking at 1149 cm^{-1} (NH rocking vibrations), 1459 cm^{-1} (antisymmetric CN stretch), 1587 cm^{-1} (CO stretch), 1620 cm^{-1} (antisymmetric NH bend), and 1671 cm^{-1} (symmetric NH bend) [28]. Creatinine spectrum depicts bands peaking at 1490 cm^{-1} (C=N stretch, CN stretch, NCH bend) and three bands between 1500-1700 cm^{-1} [29]. It is important to notice that urea and creatinine molecules strongly interact with water, therefore IR spectra of both compounds are different in aqueous solution and fully dried states. Oliver et al (2016) demonstrated that the antisymmetric C–N stretch

(νas(CN)) vibration from urea, which was found peaking at 1468 cm^{-1} in aqueous solution, shifts to 1464 cm^{-1} when urea is completely dry. However, two additional states of urea with νas(CN) peaking at 1454 cm^{-1} or 1443 cm^{-1} were also observed in partially hydrated samples [30]. Thus, IR spectra interpretation of such molecules must be performed with caution since the intermediate forms due to different hydration degree of molecules may be trapped when the whole urine is dried. According to Figure 1, bands peaking over 1400 cm^{-1} in spectra collected from urine of both healthy and diabetic rats are mainly due to urea and creatinine.

The exact constitution of urine varies with lifestyle and health status for each individual. Hence, some diseases can result in elevated levels of certain urine components. It is well known that diabetic patients present elevated glucose levels in urine (glycosuria). IR signatures of glucose are also shown in Figure 1 and it is mainly dominated by stretching C-O and C-O-C vibrations peaking in the low wavenumbers (900-1200 cm^{-1}) [31, 32]. The low wavenumber region of spectra from diabetic rats is dominated by vibrations related to glucose, which indicates elevated glycosuria.

Spectra from healthy and non-diabetic animals were subjected to PCA in order to evaluate any specific separation related to the development of diabetes. The resultant PCA scores plot (Figure 2.A) displayed a clear separation between spectral data acquired from healthy and diabetic animals. Scores related to healthy rats were grouped on the negative side of PC-1 axis, while diabetic animals were clustered on the positive side. The first principal component (PC-1), which accounts for 98% of the total explained variance (TEV), retains information responsible by the clustering pattern obtained in the scores plot. PC-1 loadings are illustrated in Figure 2.B. Positive loadings on bands peaking at the low wavenumber region indicate the high glucose levels in urine from diabetic rats. On the other hand, negative loadings were observed for bands associated with other urinary constituents, indicating higher urea and creatinine content in urine of non-diabetic animals.

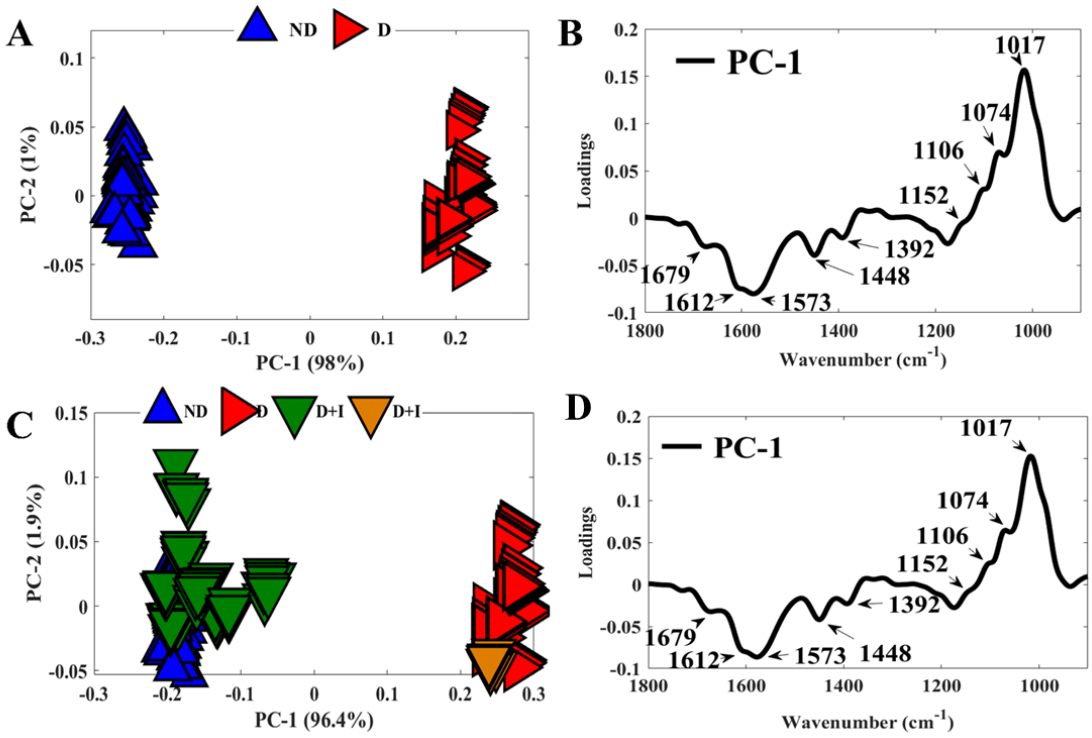

Figure 2. (A) and (B) show PCA scores plot and PC-1 loadings obtained by applying PCA to FTIR spectra of urine acquired from healthy and diabetic animals, respectively. (C) and (D) PCA results obtained for FTIR spectra of urine acquired from healthy, diabetic, and insulin-treated diabetic rats. One D+I animal with glycemia of 439 mg/dL was market in yellow due to reduced effectiveness to change diabetic metabolic profile.

Figure 3 illustrates averaged spectra calculated for each D+I animal (n = 10), as well as averaged spectra of signatures acquired from all D and ND rats. Most spectra from insulin-treated animals (90 %) presented glucose bands at levels comparable to healthy animals, indicating a positive response to the treatment. However, the spectrum from one diabetic animal that underwent to treatment presented bands indicating high glucose content, suggesting a reduced insulin response. Similar findings were evidenced by PCA applied to all datasets in ND, D, and D+I rats. PCA scores related to D+I animals were grouped on the same cluster of ND animals, indicating a positive response to insulin (Figure 2.C). On the other hand, PCA scores associated with the animal with high urinary glucose content were grouped within the cluster containing scores from untreated diabetic animals. PC-1 loadings are shown in Figure 2.D and illustrate similar findings to PC-1 loadings depicted in Figure 2.C. These findings indicate the ability of urinary ATR-FTIR

spectroscopy as a screening platform for diabetes as well as a tool to evaluate the metabolic response of diabetic patients to insulin treatment.

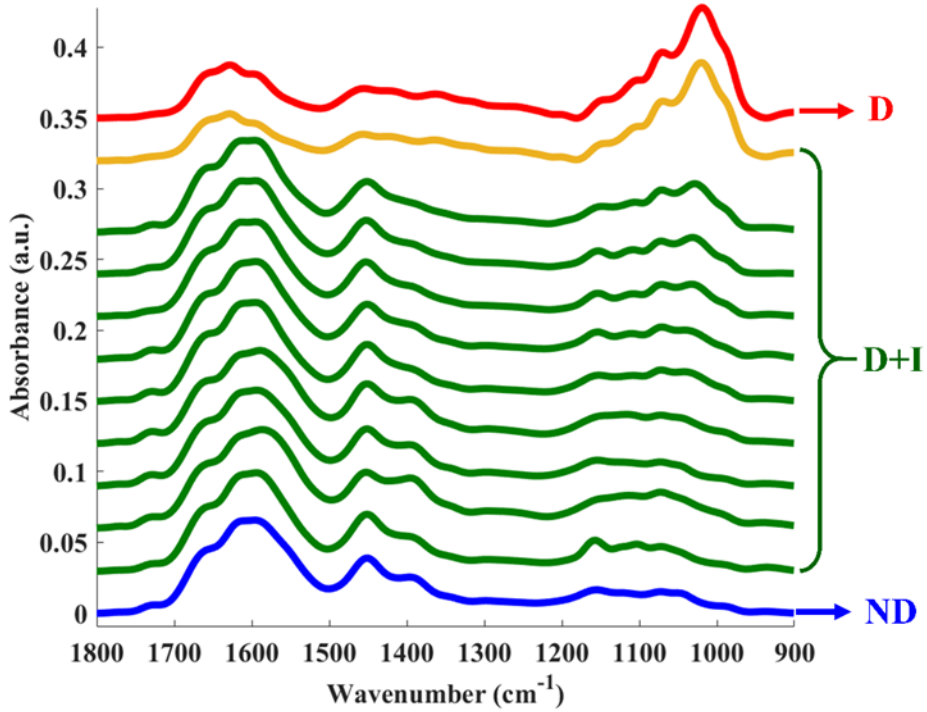

Figure 3. Averaged FTIR spectra calculated for each insulin-treated diabetic animal, as well as averaged spectra of signatures acquired from all diabetic (red) and non-diabetic rats (blue). Green lines relate to animals with positive response to insulin-treatment, while range line represents the animal with no response to insulin.

We also carried out the second derivative of ATR-FTIR spectrum of 900-1800 cm^{-1} to amplify the spectral variations of some urinary components (glucose, creatinine, and urea (Figure 4A) and ND, D and D+I rats (Figure 4B). The valley heights of urea at 1452 cm^{-1} was reduced ($p<0.05$) in the urine of D compared to ND rats, and the treatment with insulin was effective to increase ($p<0.05$) this valley heights compared to diabetic rats. Considering that sensitivity and specificity are basic characteristics to determine the accuracy of diagnostic and monitoring tests [33-35], ROC curve analyses were used to evaluate the potential of these spectral vibrational modes. In this context, we compared hyperglycemic (D) rats compared to ND and D+I, removing that sample of D+I due to the glycemia of 439 mg/dL, which indicates a diabetic metabolic profile. The area under the curve (AUC) of ROC analysis based on valley heights at 1552 cm^{-1} in the second

derivative was 1.0 ($p<0.0001$), and the best discrimination threshold value was 0.0207 with a sensitivity and specificity of 100%. However, no correlation was observed between this valley heights with urinary urea ($r= -0.51$, $p=0.798$) (Figure 5.A). The specific valley representing creatinine at 1490 cm^{-1} was also reduced in the urine of D rats ($p<0.05$) compared to ND and D+I. The AUC of ROC analysis based on valley heights at 1490 cm^{-1} in the second derivative was 0.958 ($p<0.0001$), and the cut-off value was 0.01105 with a sensitivity of 70% and specificity of 94%. No correlation was observed between the valley heights at 1490 cm^{-1} in second derivative with urinary creatinine ($r = -0.167$, $p = 0.405$) (Figure 5.B). The urinary valley heights of glucose at 1074 cm^{-1} was increased ($p<0.05$) in D compared to ND rats, and the treatment with insulin was effective to reduce ($p<0.05$) this valley heights compared to D rats. The area under the curve (AUC) of ROC analysis based on valley heights at 1490 cm^{-1} in the second derivative was 1.0 ($p<0.0001$), and the selected discrimination threshold value was 0.1371 with both sensitivity and specificity of 100%. The analysis of 1490 cm^{-1} valley heights with glycosuria showed a strong and positive correlation ($r=0.865$; $p<0.0001$) (Figure 5.C).

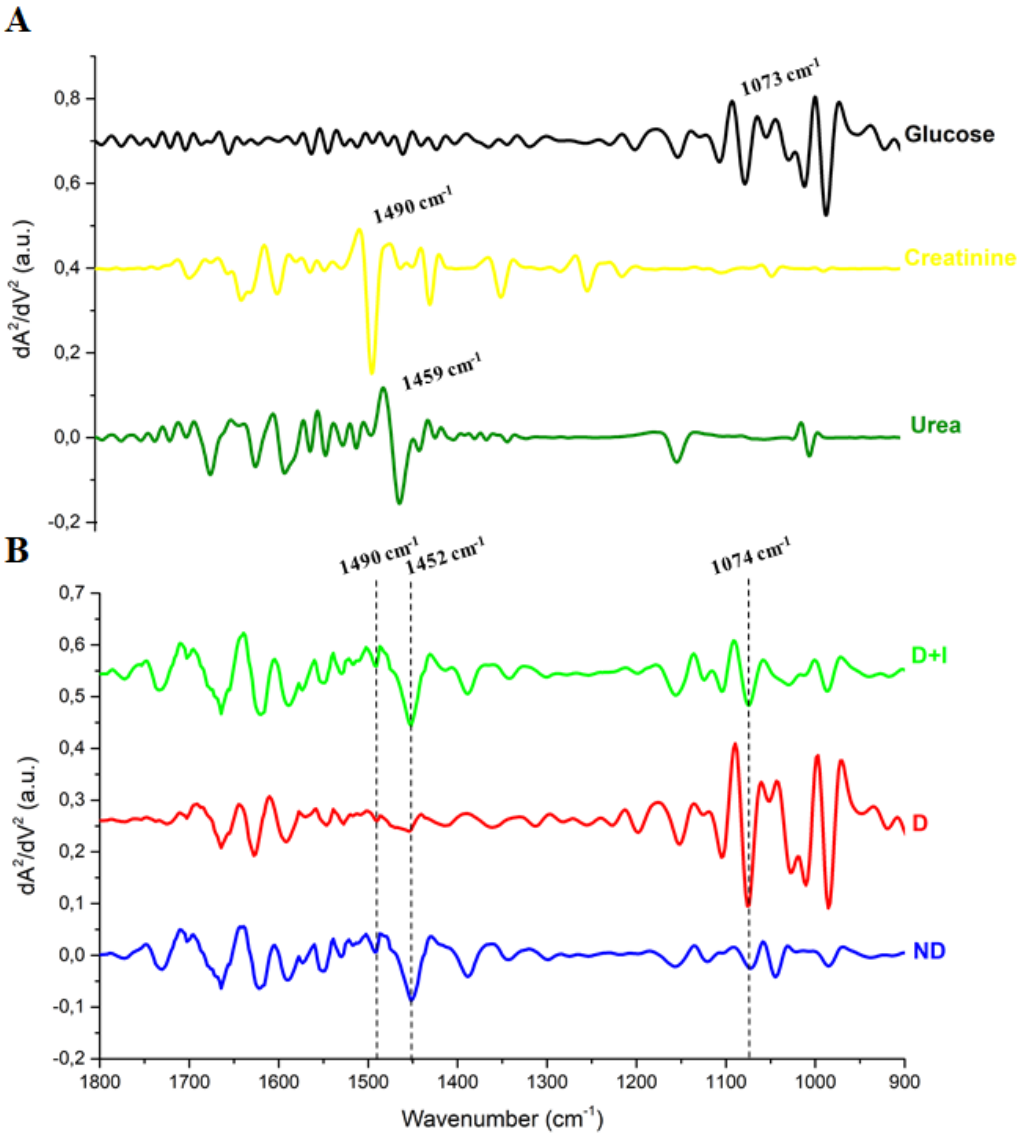

Figure 4. Fingerprint region (1800-900 cm^{-1}) of urinary spectra from glucose (black), creatinine (yellow) and urea (green) (A) and non-diabetic (ND, blue), diabetic (D, red) and insulin-treated diabetic (D+I) rats (B).

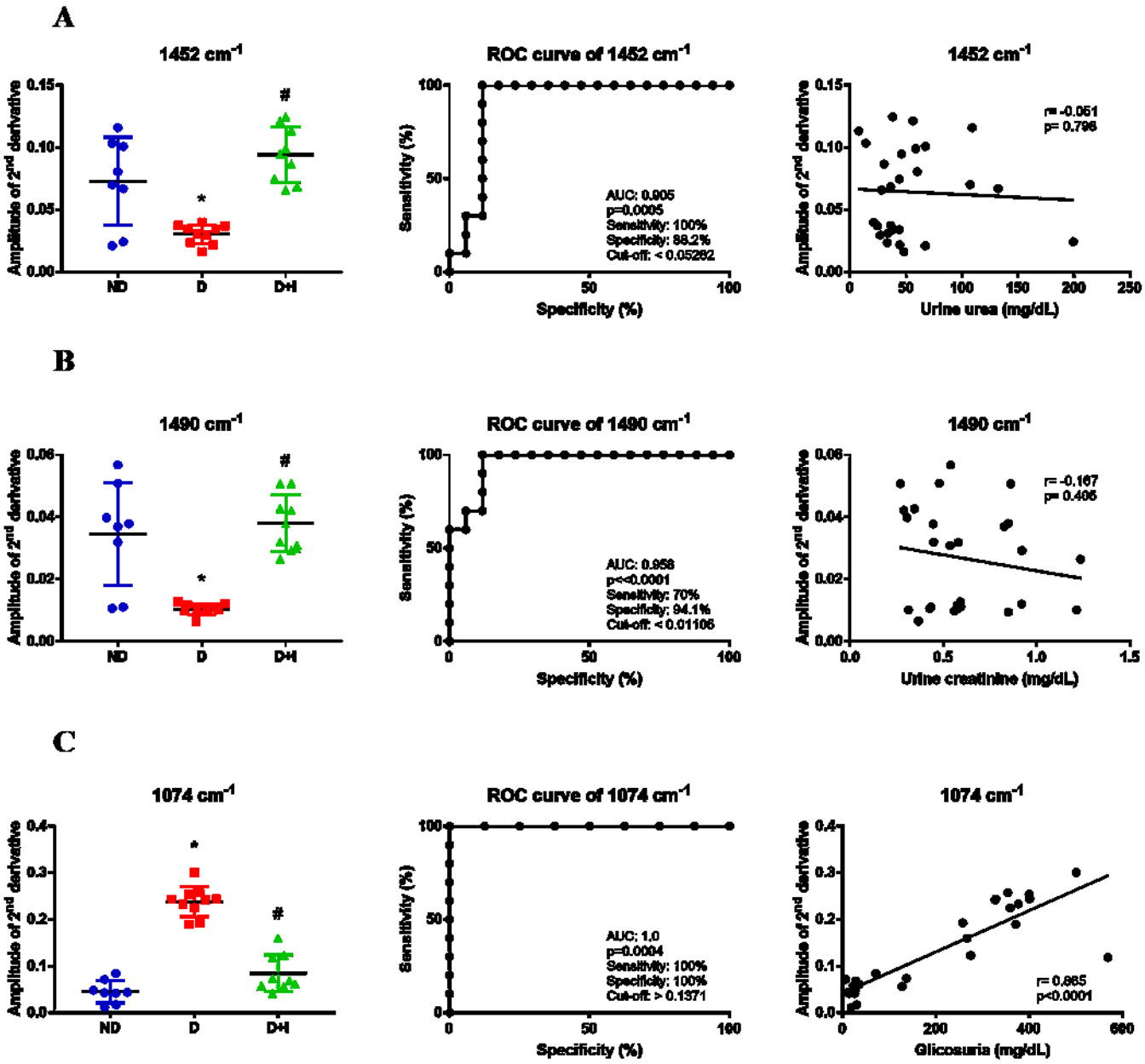

Figure 5. Amplitude of second derivative vibrational mode at 1452 cm^{-1}, ROC curve analysis to discriminate ND and D+I from D rats and Pearson correlation between urinary creatinine and amplitude of 1452 cm^{-1} (A); Amplitude of second derivative vibrational mode at 1490 cm^{-1}, ROC curve analysis to discriminate ND and D+I from D rats and Pearson correlation between urinary creatinine and amplitude of 1490 cm^{-1} (B); Amplitude of second derivative vibrational mode at 1074 cm^{-1}, ROC curve analysis to discriminate ND and D+I from D rats and Pearson correlation between glycosuria and amplitude of 1490 cm^{-1}.

MCR-ALS is a popular modeling method used to deconvolve spectra from mixtures into its individual components. Thus, MCR-ALS was applied to infrared urinary spectra of ND, D and D+I animals in order to estimate the relative concentrations of creatinine, urea, and glucose to the whole urine spectrum. MCR-ALS assumes that the recorded spectra are the weighted sum of pure spectra of the components present in the investigated mixture and returns the resolved spectrum of each compound as well as its concentration in the mixture spectrum. Figure 6.A shows a good correlation of FTIR spectrum of laboratory-grade commercial glucose (blue line) to the resolved spectrum obtained through MCR-ALS. Thus, this approach can be applied to estimate the relative concentration of glucose in urine spectra. On the other hand, MCR-ALS was not able to reproduce the signatures of urea and creatinine (data not shown), which may have occurred due to the different hydration degree of spectral signatures obtained from pure compounds compared to signatures of both molecules in urine. Figure 6.B shows a scatter plot of glucose concentration estimated by MCR-ALS plotted against glucose levels in urine obtained via enzymatic kits. Linear regression showed a positive correlation between glucose levels obtained through MCR-ALS and enzymatic kits (R2 = 0.7893).

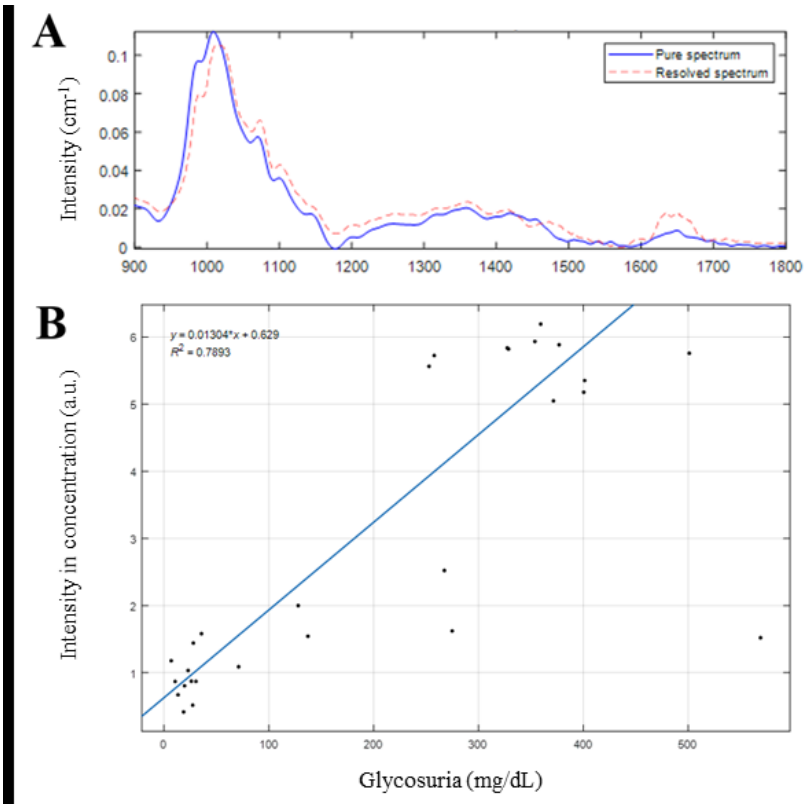

Figure 6. FTIR spectra of laboratory-grade commercial glucose (blue line) and resolved spectrum obtained through MCR-ALS (dashed red line) (A). Scatter plot of glucose concentration estimated by MCR-ALS plotted against glycosuria obtained via enzymatic kits.

Although the present study identified novel urinary infrared spectral biomarkers for screening and monitoring metabolic control of diabetes, further studies are needed to confirm these potential urinary biomarkers in humans and to evaluate the suitableness of ATR-platforms for the screening and monitoring diabetes in human urine. Besides, a limitation of the present study is the presence of diabetic animals with higher levels of blood glucose concentration, which is a typical outcome in streptozotocin-induced diabetic rats. Another limitation of urine samples are the changes in urinary components during the day due to changes in hydric behaviour and glycemic variation, which can restrict the detection of acute metabolic modulation. Altogether, these novel urinary infrared biomarkers open new perspectives for screening diabetes in triage analysis and for surveillance of metabolic control during insulin treatment. Bearing in mind the expressive changes in urinary spectra, this platform should be proved in large cohort of patients to triage and monitoring diabetes using urine.

4. Conclusion

In this study, ATR-FTIR spectroscopy was used to evaluate the urinary spectral differences between non-diabetics and insulin-treated diabetic than diabetic rats. The ATR-FTIR mean spectral analysis exhibited consistent changes related to urea, creatinine, and glucose from ND and D+I than D rats. PCA demonstrated discrimination between the ATR-FTIR spectra of urine with 99% of the cumulative variance of ND compared to D, and 98.3% compared all groups, include D+I. PCA loadings associated to PC1 indicated urine glucose as one of the components for this discrimination between groups. It was reinforced by the 100% of accuracy to discriminate ND and D+I than D rats using the valley heights of glucose at 1074 cm^{-1} as well as by the strong correlation ($r=0.865$; $p<0.0001$) between this vibrational mode with glycosuria. MCR-ALS analyses were also efficient in predicting glucose in the urine with a high relation between glucose in urine by enzymatic kit. In summary, these urinary results indicate that ATR-FTIR spectroscopy coupled with multivariate chemometric analysis has the potential to provide

information about the status of glucose in urine as a novel noninvasive approach to diabetes screening and monitoring.

5. Conflicts of interest

The authors declare that they have no conflicts of interest.

6. Acknowledgements

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5. Artigo II

SALIVARY ATR-FTIR SPECTROSCOPY COUPLED WITH SUPPORT VECTOR MACHINE CLASSIFICATION FOR SCREENING OF TYPE 2 DIABETES MELLITUS

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ABSTRACT

The diagnosis of diabetes mellitus (DM) performed by blood is highly accurate, however, it is an invasive, high cost, and painful procedure. In this context, the combination of ATR-FTIR spectroscopy and machine learning techniques in other biological samples has been used as an alternative tool to a non-invasive method, with high sensitivity, fast, inexpensive, and label-free for several diseases, including DM. In this study, we used the ATR-FTIR tool associated with the support vector machine (SVM) classifier in order to identify changes in salivary components to be used as alternative biomarkers for diagnosis of type 2 DM. The band area values of 2962 cm^{-1}, 1641 cm^{-1}, 1549 cm^{-1}, 1451 cm^{-1}, 1073 cm^{-1} were higher in hyperglycemic type 2 diabetic patients than non-diabetic subjects. The classification of salivary infrared spectra by support vector machine (SVM) showed a sensitivity of 93.3 % (42/45), specificity of 73.9% (17/23) and accuracy of 87% between non-diabetic subjects and uncontrolled type 2 diabetic patients. In summary, these data highlight the potential of ATR-FTIR platforms coupled with machine learning as a sustainable, reagent free, non-invasive, and highly sensitive tool to screening and monitoring diabetic patients using an ultra-low volume of saliva and minimal sample preparation.

Keywords: Diabetes, ATR-FTIR, SVM, salivary biomarkers, screening

1. Introduction

Diabetes mellitus (DM) is a metabolic disorder characterized by hyperglycemia and glucose intolerance, which are related to defects in insulin secretion and/or reduced effect of insulin on glucose uptake in peripheral tissues, as skeletal muscle, adipose tissue, and liver [1,2]. The severity of these comorbidities is strongly related to glycemic control and late diagnosis [3]. This endocrine disorder is a worldwide epidemic and presents a significant rate of morbidity, diabetes-associated mortality, and higher health costs for management and treatment [4]. According to the International Diabetes Federation (IDF) report of 2019, 1 to 11 adults have DM, a total of 463 million people worldwide, and proximally 232 million people still are undiagnosed [5]. A rapid and early diagnosis of DM becomes imperative to avoid several short-term and long-term complications and improve health-related quality of life.

The gold standard for diagnosis of DM is blood analysis and may be diagnosed based on plasma glucose criteria, either the fasting plasma glucose or the 2-hours plasma glucose value during a 75g oral glucose tolerance test (OGTT) or glycated hemoglobin (HbA1c) criteria [6]. The most widely used tool to assess DM progression is the glucose test strips for a point-of-care glucometer, which is currently feasible for screening, monitoring, and diagnosing diabetes by needle finger punctures [7]. However, the constant need of piercing the fingers several times daily is inconvenient, painful and may lead to the development of finger calluses, which may difficult blood collection [7, 8]. The glucose test strips for a point-of-care glucometer is considered a high accuracy device to monitor glycemia, however, many patients are reluctant to undergo this procedure several times per day due to its invasiveness and high costs.

Attenuated total reflection-Fourier transform infrared (ATR-FTIR) spectroscopy is a highly sensitive and reproducible technique capable to characterize the molecular fingerprint of a sample without extensive sample preparation [9]. It can provide label-free, sustainable, rapid, nondestructive, and cost-effective analyses of multiple components in biological samples [10-13]. In this context, ATR-FTIR provides an alternative to identify changes in carbohydrates, proteins, lipids, DNA, RNA promoted by diseases, which can indicate spectral infrared biomarkers that correlate com gold-standard clinical biomarkers [9, 14]. A minimally or non-invasive screening or diagnostic tests facilitate compliance and, as such, saliva collection is an attractive sample. An infrared diagnostic system for diabetes using saliva with linear discriminant analysis (LDA) showed an accuracy of 88.2%. This protocol was used a higher amount of sample dried with continuous airflow

and the saliva was inserted in barium fluoride (BaF2) windows, which limits sequential analysis with 30 min drying times for each sample and use a potentially toxic element [15]. In addition, the salivary monitoring of DM was successfully determined using the FTIR analyses along with the PCA method with 95.2% of accuracy in rats samples [16].

Here, we hypothesized that ATR-FTIR is capable to identify changes in salivary components to be used as a screening tool for DM. The present study aimed to compare infrared salivary components in normoglycemic non-diabetic subjects and hyperglycemic type 2 diabetic patients to be employed as an alternative salivary biomarker tool for DM screening.

2. Materials and methods

2.1 Ethical Aspects and Study Subjects

The study was conducted at the Clinics' Hospital of the Federal University of Uberlandia (HC-UFU, Uberlandia, Minas Gerais, Brazil). All experimental procedures were carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) and were approved by the Institutional Review Board of the Federal University of Uberlandia (protocol number 1.715.975). Written informed consent was obtained from all the participants of this study including controls and patients. Exclusion criteria were age below 18 years, cancer, autoimmune diseases and transplanted.

The study group included 79 subjects: 23 non-diabetic individuals and 45 uncontrolled type 2 diabetic patients. Non-diabetic subjects with glycemia < 100 were considered normoglycemic, and diabetics with glycemia > 126 mg/dL were considered uncontrolled type 2 diabetic patients with poor glycemic control. This classification is according to the American Diabetes Association (ADA)[6], World Health Organization (OMS)[17], and Diabetes Brazilian Society (SBD)[18].

2.2 Saliva Sample Collection and Preparation

Saliva samples were collected in Salivette® tubes (Sarstedt, Germany) for 3 minutes after blood collection for routine blood biochemical analyzes at HC-UFUThe saliva was recovered by centrifugation for 3000 rpm at 4ºC for 15 minutes, and the supernatant was collected and aliquoted. All samples were kept frozen at -80ºC until

analysis. Glycemia and HbA1C information were obtained by medical records and the blood collection was performed immediately after saliva collection.

2.3 ATR-FTIR Spectroscopy

The salivary spectra were performed in 4000-400cm^{-1} in ATR-FTR spectrophotometer Vertex 70 (Bruker Optics, Reinstetten, Germany) coupled to attenuated total reflectance (ATR) component. The crystal material in ATR unit was a diamond disc as an internal reflection element. The salivary pellicle penetration depth ranges between 0.1 and 2 μm and depends on the wavelength, incidence angle of the beam, and the refractive index of ATR-crystal material. Saliva (1μl) was directly dried at room temperature on ATR-crystal for 6 minutes and then the spectra were recorded. Before each sample analysis, the air spectrum was used as a background. Each spectrum was obtained in a room with a temperature around 22–23°C, 4 cm^{-1} of resolution, and 32 scans were performed.

2.4 Discrimination Analysis Method

The infrared spectral data analysis was divided into two stages: pre-processing and classification. Pre-processing consisted of aggregation, attribute selection, and data transformation. The arithmetic mean of the three spectral readings of each patient was performed in aggregation. The spectral data were truncated with the lipidic region (3050-2800) associated with the biofingerprint region (1800-900 cm^{-1}). Then, the Savitzky-Golay smoothing filter was applied to each spectrum followed by a first order derivative and pre-processed by vector normalization.

The classification was tested with state-of-the-art machine learning algorithms of feature extraction coupled to discriminant analysis tools. The Support Vector Machine (SVM) was selected based on better results during model training. To analyze the predictive performance of the machine learning algorithms, ten times stratified cross-validation was used. The samples were divided into ten subsets, with each iteration; nine of them were used to train the algorithm and one exclusively to test it, so that each subset was part of the test once. In addition, the procedure was repeated three times with changes in the samples configurations in these subsets to achieve a closer estimate of the real performance of the model, thus totaling thirty executions. To measure the results obtained, three performance measures consolidated in the literature were used: sensitivity, specificity, and accuracy. The sensitivity or true positive rate is the proportion of positives

(diabetic) that were correctly classified, and the specificity or true negative rate is the proportion of negatives (normoglycemic) that were correctly classified. The accuracy is defined as the total number of samples correctly classified considering true and false negatives [16, 19].

3. Results

Demographic and metabolic laboratorial data of non-diabetic and diabetic patients are described in table 1. Briefly, the body weight was higher ($p<0.05$) in uncontrolled type 2 diabetic patients than non-diabetic subjects. As expected, both glycemia and HbA1C were also higher in uncontrolled type 2 diabetic patients than non-diabetic subjects.

Table 1. Characterization of patients: gender, body weight, age range, glycemia, and glycated hemoglobin (HbA1C).

Parameters	Non-Diabetic	Type 2 diabetes mellitus
Gender (M/F)	10/13	26/19
Body weight (kg)	68.4±8.4	87.17±11.9*
Age range	20-76	27-82
Glycemia (mg/dL)	98.8±6.7	187.0±90.2*
HbA1C (%)	5.2±0.1	8.3±1.7*

Values are expressed as mean ± S.D; *p < 0. 05 vs Healthy.

The infrared salivary spectra of non-diabetic subjects and type 2 diabetic patients were shown in Figure 1. The band area values of 2962 cm^{-1}, 1641 cm^{-1}, 1549 cm^{-1}, 1451 cm^{-1} and 1073 cm^{-1} (B-F) were higher in type 2 diabetic patients than non-diabetic subjects.

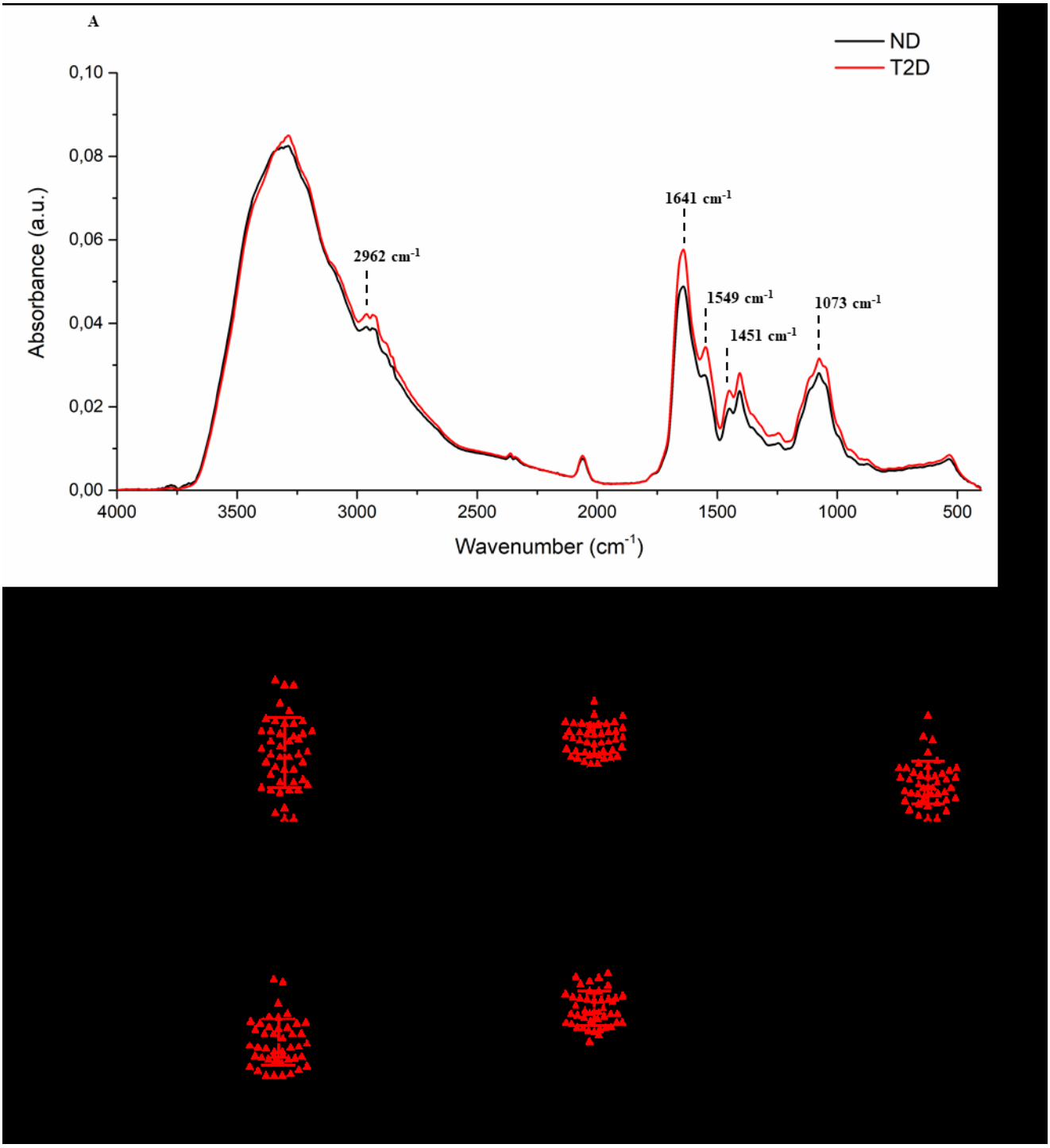

Figure 1. Representative average ATR-FTIR spectra (4000–400 cm^{-1}) in saliva of non-diabetic subjects and uncontrolled type 2 diabetic patients (A). Band area of 2962 cm^{-1} (B). Band area of 1641 cm^{-1} (C). Band area of 1549 cm^{-1} (D). Band area of 1451 cm^{-1} (E). Band area of 1073 cm^{-1}(F).

The classification of salivary infrared spectra by support vector machine (SVM) showed a sensitivity of 93.3 % (42/45), specificity of 73.9 (17/23), and accuracy of 87% between non-diabetic subjects and uncontrolled type 2 diabetic patients.

4. Discussion

The early diagnosis and adequate monitoring of diabetes can reduce the worst outcomes promoted by unappropriated hyperglycemia, which is critical to improve quality of life, to saving health care costs, reduce costs related to reduced work productivity, and inability to work as well as early mortality [20, 21]. Bearing in mind the progress of portable ATR-FTIR devices towards the clinic [9, 22], the development of non-invasive and sustainable reagent-free platforms to detect changes in salivary components of type 2 diabetic population has great potential to be applied in point-of-care, at-home, or decentralized laboratorial settings with reduced infrastructure [16].

The levels of glycemia and HbA1C have been considered as an effective clinical biomarkers for diagnosis and monitoring. These glucose monitoring strategies in the blood are the most widely used and provide an effective method for diabetes surveillance. However, the inconvenience of blood collection can reduce the continuous monitoring of metabolic control and reduce the detection of hyperglycemia intervals [23]. In this context, ATR-FTIR platform using alternative non-invasive specimens can be more amenable for clinical applications.

The discriminatory vibrational modes in saliva of hyperglycemic type 2 diabetic patients than non-diabetic subjects were related to: asymmetric stretching vibration of CH3 of lipids (2962 cm^{-1}); amide I (1641 cm^{-1}), amide II (1549 cm^{-1}); asymmetric CH3 bending modes of the methyl groups of proteins (1451 cm-1); and carbohydrates and glycosylated proteins (1073 cm^{-1}) [16, 24]. In fact, we previously showed that hypoinsulinemic animal model of diabetes showed changes in lipid, amide II and asymmetric CH3 bending modes of proteins [16]. The increase in the amide I and carbohydrates/protein glycosylation are in agreement with changes in protein and glucose concentrations in saliva [15, 25, 26].

From the point of view of the diagnostic or screening application, the sensitivity of 93.3% with adequate specificity represents the significant translational potential of this salivary platform to diabetes. Population-based diabetes screening could be an attractive intervention as a result of its prevalence, high medical and non-medical costs and, especially, for the long asymptomatic phase previous to diabetic symptoms. However, the

test cost affects the evaluation of cost-effectiveness. From a sustainable perspective, other issues in large population screening programs are the potential toxicity of the reagents and generation of health care waste [27, 28]. For example, ATR-FTIR analysis of blood analysis is frequently necessary to insert anticoagulants to avoid blood clotting [29]. The development and clinical assessment of a low-cost, sustainable, reagent free, non-invasive, and highly sensitive infrared platform using only 1 uL of saliva and minimal sample preparation could be a shift paradigm to detect undiagnosed diabetes and to increase the chance to treat diabetes early.

This exploratory clinical study supports the significant potential towards salivary screening platform for type 2 diabetes. Despite this recent biotechnological advance for diabetes screening, the predictive accuracy with respective sensitivity and specificity should be further validated in large clinical trials [30]. Besides, novel studies are also needed to evaluate operational challenges as access to ATR-FTIR devices and impact on medical decision making, which is outside the scope of present study [31]. In this context, the salivary ATR-FTIR spectroscopy coupled with SVM classification could provide a novel alternative for biomedical screening or monitoring.

5. Conclusion

The present study seeks to contribute to the application of ATR-FTIR platforms in medical and dentistry settings. Here, data indicate 2962 cm^{-1}, 1641 cm^{-1}, 1549 cm^{-1}, 1451 cm^{-1}, 1073 cm^{-1} as discriminatory vibrational modes of type 2 diabetes than normoglycemic controls. The very high sensitivity of 93.3% with an adequate specificity was obtained by SVM analysis. In summary, these data highlight the potential of ATR-FTIR platforms coupled with machine learning as a sustainable, reagent free, non-invasive, and highly sensitive tool to screening and monitoring diabetic patients.

6. Conflicts of interest

The authors declare that they have no conflicts of interest.

7. Acknowledgements

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Crook, G. P. Taylor, W. Barclay, N. Mughal, L. S. P. Moore, K. Jeffery and G. S. Cooke, *The Lancet. Microbe*, 2020, DOI: 10.1016/s2666-5247(20)30121-x.

6. Artigo III

ASSOCIATION OF THE ALPHA-2-MACROGLOBULIN WITH GLYCEMIC CONTROL OF TYPE 2 DIABETES MELLITUS: A SYSTEMATIC REVIEW AND META-ANALYSIS STUDY

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Abstract

Background: The traditional monitoring of glycemia is invasive and painful in diabetes, limiting appropriate testing frequency. The non-invasive diabetes diagnostic method is currently being developed to increase diabetic testing frequency. Salivary components are changed in type 2 diabetes (DM2) and several studies indicate that salivary alpha-2-macroglobulin (A2MG) levels are elevated in diabetic patients, which suggests a potential non-invasive biomarker for diabetes. **Objective:** This meta-analysis evaluated the correlation between salivary A2MG and glycemia / glycated haemoglobin (HbA1c) to systematically review the effectiveness of this protein to estimate glycemia and HbA1c of DM2 patients. **Methods:** Eight databases (PubMed, Scopus, LILACS, Web of Science, Embase, SciELO, OpenGrey, and OpenThesis) were used as research sources. The protocol was submitted on a PROSPERO database. We conducted a meta-analysis of peer-reviewed published studies that reported data regarding mean salivary A2MG and correlation between glycemia and/or HbA1c levels of DM2 (controlled and non-controlled) and non-diabetic individuals. The risk of bias of the studies selected was assessed with the "Joanna Briggs Institute Critical Appraisal tools for use in JBI Systematic Reviews". Pooled correlation coefficients were estimated using the Hunter-Schmidt method. Study estimates were weighted according to their sample size, and heterogeneity was calculated using the chi-squared statistic. **Results:** Four studies with DM2 patients were included in this meta-analysis after careful analysis of 1482 studies. Three studies compared A2MG to HbA1c and glycemia. Overall, the correlation between A2MG and HbA1c was strong (r=0.838). In contrast, the correlation between A2MG and glycemia was low (r=0.354). **Conclusion:** The strong association between HbA1C and salivary A2MG with a reduced level of certainty of evidence suggests the potential to this salivary protein to surrogate HbA1C after further evidence with large-scale studies.

Key words: Diagnostic, saliva, biomarker, diabetes mellitus, glycemia, HbA1C

1. Introduction

Type 2 diabetes mellitus (DM2) is a metabolic disorder caused by a combination of decreased insulin secretion and decreased insulin sensitivity in peripheral tissues, primarily in the liver, muscle, and adipose tissue as target organs [1,2]. Currently, the best characterized parameter available to screening, monitoring, and diagnosing of DM2 is glycemia levels and glycated hemoglobin-A1c (HbA1c), which reflects the glycemic control from the previous 2-3 months [3]. Different diagnostic tools such as glycemia, HbA1C, and oral glucose tolerance test (OGTT) are used in the diagnosis of diabetes. According to the ADA guidelines, the individuals with glycemia concentration of ≥126 mg/dL, HbA1C level ≥6.5%, and 2-hour plasma glucose value after 75 grams OGTT ≥200 mg/dL are considered as having diabetes [4]. Besides being invasive and painful, [5,6] the blood tests may lead to the development of finger calluses, poor peripheral circulation, and risk of infection [5]. However, the classical HbA1c tests dispend several reagents with a relative high cost and need some specific equipments [7], which reduces the availability of HbA1c tests in low- and middle-income countries despite its well-recognized the recognized capability to diabetes surveillance [8]. In this way, other types of biological samples to evaluate glycemic control, as salivary biomarkers, might be an attractive alternative for early detection and monitoring of DM2.

The major salivary glands secrete saliva in response to the autonomic nervous system [9]. We have shown that diabetes changes autonomic activity to salivary glands in both acinar and ductal cells, which reflects is salivary composition [10,11]. Human saliva contains a wide variety of proteins, including enzymes derived from salivary glands, blood, microorganisms, and gingival crevicular fluid [12]. For these reasons, saliva may contain novel biomarkers for some disease or be an alternative and more accessible source of biofluid for diagnostic and monitoring DM2. Diabetes mellitus affects the salivary composition and salivary flow due to microvascular alterations, neuropathies, and hormonal imbalances, or because to a combination of these factors [13]. In this context, both salivary sugars and glycosylated proteins were capable to discriminate hyperglycemic and normoglycemic conditions [14].

Alpha-2-macroglobulin (A2MG) is a glycoprotein produced by the liver and can be present in human blood plasma, cerebral spinal fluid, and saliva fluid [12,15]. The cage-like structure of α2M (720kDa) is formed by the assembly of four 180kDa subunits into two disulfide-linked dimers, which noncovalently associate to complete the tetrameric

quaternary structure of the protein [16]. The main function of alpha2M is to bait and trap proteinases, but also regulates the distribution and activity of many cytokines, hormones, growth factors, and other proteins [17].

Diabetics have elevated levels of A2MG in the blood, as shown in several studies [18-21]. Moreover, A2MG levels in plasma have been correlated with HbA1c percentages [22]. The high serum A2MG decreases the bioavailability of insulin, leading to impairment of blood sugar control [5,23]. Bencharit et al. (2013) and Rao et al. (2009) characterized the salivary proteomic in DM2 and found that A2MG was differentially increased in the saliva of uncontrolled diabetic subjects when compared with subjects in the prediabetic [24,25]. Furthermore, Aitken et al. (2015) and Chung et al. (2016) suggest that the level of salivary A2MG is an indicator of the degree of glycemic control in diabetic patients and represents a promising alternative method to evaluate this parameter [23,26]. In this way, saliva as a source of the A2MG and its association with glycemic control in DM2 can be a useful and promising alternative auxiliary method of diagnosis of DM2.

Thus, the present systematic review aims to answer the following guiding question: "A2MG salivary is related to the diagnostic and glycemic control parameters of patients with DM2?" The authors tested the following hypothesis: the concentrations of salivary A2MG are correlated to HbA1c and glycemia levels of blood in DM2 patients.

2. Material and methods

2.1 Protocol and registration

This systematic review was performed following the guideline for the preferred reporting items of systematic review and meta-analysis (PRISMA) [27] and the Cochrane guidelines [28]. The systematic review protocol was submitted in the database under PROSPERO 2020 CRD42020183831, available from: https://www.crd.york.ac.uk/prospero/display_record.php?ID=CRD42020183831.

2.2 Eligibly and exclusion criteria of the study

Studies were included if they were observational studies (cross-sectional) with patients of type 2 diabetes mellitus and assessed the correlation between salivary A2MG concentration and blood sugar level and/or serum HbA1c. Studies selections were without restriction of year and publication status (published, accepted/ahead of print articles).

Exclusion criteria included studies that: i) were not related to the objective, ii) review articles, iii) follow up studies or assess participants with other comorbid diseases, like patients with rheumatic diseases, terminal illnesses, chronic liver disease, chronic inflammatory processes in the oral cavity, chronic kidney disease of stage IV and V and autoimmune diseases; iv) not reported the procedures following the ethical standards.

2.3 Sources of information and search

We searched studies which evaluate salivary alpha-2 macroglobulin (A2MG) levels and serum glycemia and glycated haemoglobin (HbA1c) in type 2 diabetes mellitus in electronic databases of PubMed (including MedLine), Scopus, LILACS, Web of Science, Embase and SciELO databases were used as primary study sources. Besides that, OpenGrey and OpenThesis were used to partially capture the "gray literature". MeSH (Medical Subject Headings), DeCS (Health Sciences Descriptors), and Emtree (Embase Subject Headings) were used to search the descriptors. The boolean operators "and" and "or" were combined with the descriptors to improve the search strategy (Table 1). The bibliographic research was developed in November 2019. In addition, we also manually checked the reference sections of the eligible studies and indications by expert researchers for the possibility of any additional studies that might have been missed by the electronic search. E-mails were forwarded to three referral specialists for articles potentially eligible for this review.

Table 1. Strategies for database search.

Database	Search Strategy (November, 2019)	Results
PubMed (Best Match) http://www.ncbi.nlm.nih.gov/pubmed	((“Diabetes Mellitus Type 2” OR “Diabetes Mellitus, Noninsulin-Dependent” OR “Diabetes Mellitus, Non-Insulin-Dependent” OR “Diabetes Mellitus, Type II” OR “NIDDM” OR “Type 2 Diabetes” OR “DM2” OR “T2DM”) AND (“A2M protein, human” OR “α2-macroglobulin” OR “salivary α2-macroglobulin” OR “α2-MG” OR “alpha 2-macroglobulin” OR “A2MG”))	34
Scopus http://www.scopus.com/	("Diabetes Mellitus Type 2" OR "Diabetes Mellitus, Noninsulin-Dependent" OR "Diabetes Mellitus, Non-Insulin-Dependent" OR "Diabetes Mellitus, Type II" OR "NIDDM" OR "Type 2 Diabetes" OR "DM2" OR "T2DM") AND ("A2M protein, human" OR "α2-macroglobulin" OR "salivary α2-macroglobulin" OR "α2-MG" OR "alpha 2-macroglobulin" OR "A2MG")	617

LILACS http://lilacs.bvsalud.org/	((“Diabetes Mellitus Type 2” OR “Diabetes Mellitus, Noninsulin-Dependent” OR “Diabetes Mellitus, Non-Insulin-Dependent” OR “Diabetes Mellitus, Type II” OR “NIDDM” OR “Type 2 Diabetes” OR “DM2” OR “T2DM”) AND (“A2M protein, human” OR “α2-macroglobulin” OR “salivary α2-macroglobulin” OR “α2-MG” OR “alpha 2-macroglobulin” OR “A2MG”))	9
Web of Science http://apps.webofknowledge.com/	((“Diabetes Mellitus Type 2” OR “Diabetes Mellitus, Noninsulin-Dependent” OR “Diabetes Mellitus, Non-Insulin-Dependent” OR “Diabetes Mellitus, Type II” OR “NIDDM” OR “Type 2 Diabetes” OR “DM2” OR “T2DM”) AND (“A2M protein, human” OR “α2-macroglobulin” OR “salivary α2-macroglobulin” OR “α2-MG” OR “alpha 2-macroglobulin” OR “A2MG”))	16
Embase https://www.embase.com	('diabetes mellitus type 2'/exp OR 'diabetes mellitus type 2' OR 'diabetes mellitus, noninsulin-dependent' OR 'diabetes mellitus, non-insulin-dependent'/exp OR 'diabetes mellitus, non-insulin-dependent' OR 'diabetes mellitus, type ii'/exp OR 'diabetes mellitus, type ii' OR 'niddm'/exp OR 'niddm' OR 'type 2 diabetes'/exp OR 'type 2 diabetes' OR 'dm2' OR 't2dm'/exp OR 't2dm') AND ('a2m protein, human' OR 'α2-macroglobulin' OR 'salivary α2-macroglobulin' OR 'α2-mg' OR 'alpha 2-macroglobulin'/exp OR 'alpha 2-macroglobulin' OR 'a2mg')	70
SciELO https://www.scielo.org/	((“diabetes mellitus type 2” OR “diabetes mellitus, noninsulin-dependent” OR “diabetes mellitus, non-insulin-dependent” OR “diabetes mellitus, type ii” OR “niddm” OR “type 2 diabetes” OR “dm2” OR “t2dm”) AND (“a2m protein, human” OR “α2-macroglobulin” OR “salivary α2-macroglobulin” OR “α2-mg” OR “alpha 2-macroglobulin” OR “a2mg”))	38
OpenGrey http://www.opengrey.eu/	“Diabetes Mellitus Type 2” OR “Diabetes Mellitus, Noninsulin-Dependent” OR “Diabetes Mellitus, Non-Insulin-Dependent” OR “Diabetes Mellitus, Type II” OR “NIDDM” OR “Type 2 Diabetes” OR “DM2” OR “T2DM” AND “A2M protein, human” OR “α2-macroglobulin” OR “salivary α2-macroglobulin” OR “α2-MG” OR “alpha 2-macroglobulin” OR “A2MG”	781
OpenThesis http://www.openthesis.org/	((“Diabetes Mellitus Type 2” OR “Diabetes Mellitus, Noninsulin-Dependent” OR “Diabetes Mellitus, Non-Insulin-Dependent” OR “Diabetes Mellitus, Type II” OR “NIDDM” OR “Type 2 Diabetes” OR “DM2” OR “T2DM”) AND (“A2M protein, human” OR “α2-macroglobulin” OR “salivary α2-macroglobulin” OR “α2-MG” OR “alpha 2-macroglobulin” OR “A2MG”))	16
TOTAL		1581

2.4. Studies selections

The selection of studies was performed in four stages. Initially, a calibration exercise was performed to fits pre-specified eligibility criteria and applied them to a small sample of the studies (20%) retrieved to determine inter-examiner agreement. After achieving an appropriate level of concordance (Kappa ≥ 0,81), the reviewers *[Blinding]* performed a methodical analysis of all study titles independently. The disagreements between of inter-examiners were discussed with a third reviewer [*Blinding*] to reach consensus.

In the first stage were performed the identification of the studies obtained in databases. The data were exported to the EndNote Web™ software (Thomson Reuters, Toronto, Canada), in which duplicates were removed. The remaining results were exported to Microsoft Word™ 2016 (Microsoft™ Ltd, Washington, USA), in which the remaining duplicates were manually removed. In the second stage, all titles were analyzed independently to determine their relevance by both review authors, the names of authors and journals were not blinded. Titles not related to the topic were eliminated in this phase. Then, in the third stage, the abstracts were reviews to apply the exclusion criteria aforementioned. Titles in accordance with the aims of the present study without abstracts available were fully analyzed in the fourth stage. Besides that, expert investigator and studies eligible in the references were included for subsequent analyses. In the fourth stage, preliminary eligible studies had their full texts obtained and evaluated to verify whether they fulfilled the eligibility criteria, included the expert investigator and studies eligible in the references list.

2.5. Data collection

The two authors then independently accessed full-text copies of all eligible articles and collected data from each study using a plotted spreadsheet [*Blinding*]. Data extracted from identification of the studies included were author, year, country, DM2 population, average age, average age range, sex ratio, diagnosis, and collection period. In addition, were collected characteristics, preparation and measurement of the samples in the eligible studies (saliva collection, saliva collection criteria, saliva preparation, blood collection, A2MG measurement, glycemia measurement, and HbA1c measurement) and main results of the studies included (mean glycemia, mean HbA1c, mean A2MG, correlation salivary A2MG with glycemia, correlation salivary A2MG with HbA1c).

In order to ensure the consistency among reviewers, a calibration exercise was performed with both reviewers [*Blinding*], in which information was extracted jointly from an eligible study. Any disagreement between the reviewers was solved through discussions, and when both reviewers disagreed, a third one [*Blinding*] was consulted to make a final decision.

2.6. Risk of individual bias of the studies

The Joanna Briggs Institute Critical Appraisal Tools for use in JBI Systematic Reviews for observational studies (cross-sectional) [29] was used to assess the risk of bias and the individual quality of the studies selected. Two authors [*Blinding*] assessed independently each domain regarding their potential risk of bias, as recommended by the PRISMA statement [27]

Each study was categorized according to the percentage of positive answers to the questions corresponding to the assessment tool. The risk of bias was considered ***High*** when the study obtained 49% of "yes" answers, ***Moderate*** when the study obtained 50% to 69% of "yes" answers, and ***Low*** when the study reached more than 70% of "yes" score.

2.7 Statistical analyses

The correlation between A2MG and other two DM2 biomarkers (glycemia or HbA1c) was considered in the meta-analysis. Correlation coefficients were pooled using the Hunter-Schmidt method [30,31] and stratified according to the DM2 biomarker to which the A2MG was compared. Estimates using this method are weighted according to the sample size of each study. The correlation was considered perfect if coefficients were equal to 1 or -1; was considered strong if coefficients ranged between |0.7| and |0.9|; was considered moderate if coefficients ranged between |0.4| and |0.6|; was considered weak if coefficients ranged between |0.1| and |0.3|; and zero if coefficients were 0 [32].

The presence or absence of between-study heterogeneity was also calculated by the Hunter-Schmidt method using the chi-squared statistic [30,31]. A 5% significance level was considered in all analyses, which were all conducted using Stata 16.1 software (StataCorp LLC).

2.8 Certainty of evidence

Quality of evidence and strength of recommendation were assessed with the Grading of Recommendation, Assessment, Development, and Evaluation (GRADE) tool

[33] The GRADE pro GDT software (http://gdt.guidelinedevelopment.org) was used for summarizing the results. This assessment was based on study design, methodological limitations, inconsistency, indirect evidence, imprecision, and other considerations. The quality of evidence was characterized as high, moderate, low, or very low (Balshein et al., 2011).

3. Results

3.1. Study selection

During the first phase of study selection, 1581 results were found distributed in eight electronic databases, including the "gray literature". After removing the duplicate results, 1482 articles remained for the analysis of titles and abstracts. In this phase, after a detailed analysis of titles and abstracts, only 7 studies were eligible for the full-text analysis. The references of the 7 potentially eligible studies were also carefully evaluated and 1 additional article was selected. Besides that, one article was indicated by expert investigator, resulting in 9 studies for the full-text reading. After reading the full text, 5 studies did not fulfilled the inclusion criteria and were eliminated. Of these excluded studies, the Discher, Velcovsky, Federlin [34] is not related to the objective of this meta-analysis, two articles, Bencharit, Baxter, Carlson, Byrd, Mayo, Border, Kohltfarber, Urrutia, Howard-Williams, Offenbacher, Wu, Buse [24] and Rao, Reddy, Lu, Dasari, Krishnaprasad, Biggs, Roberts, Nagalla [25], are proteomic analysis study, the SENTHILMOHAN [35] is a review study and Chung, Hsu, Chen, Liu, Chang, Li, Huang, Shieh, Lee [26] is a follow up study, therefore, for these reasons have been removed. Thus, 4 studies were selected for qualitative and meta-analysis. Figure 1 reproduces the process of search, identification, inclusion, and exclusion of articles.

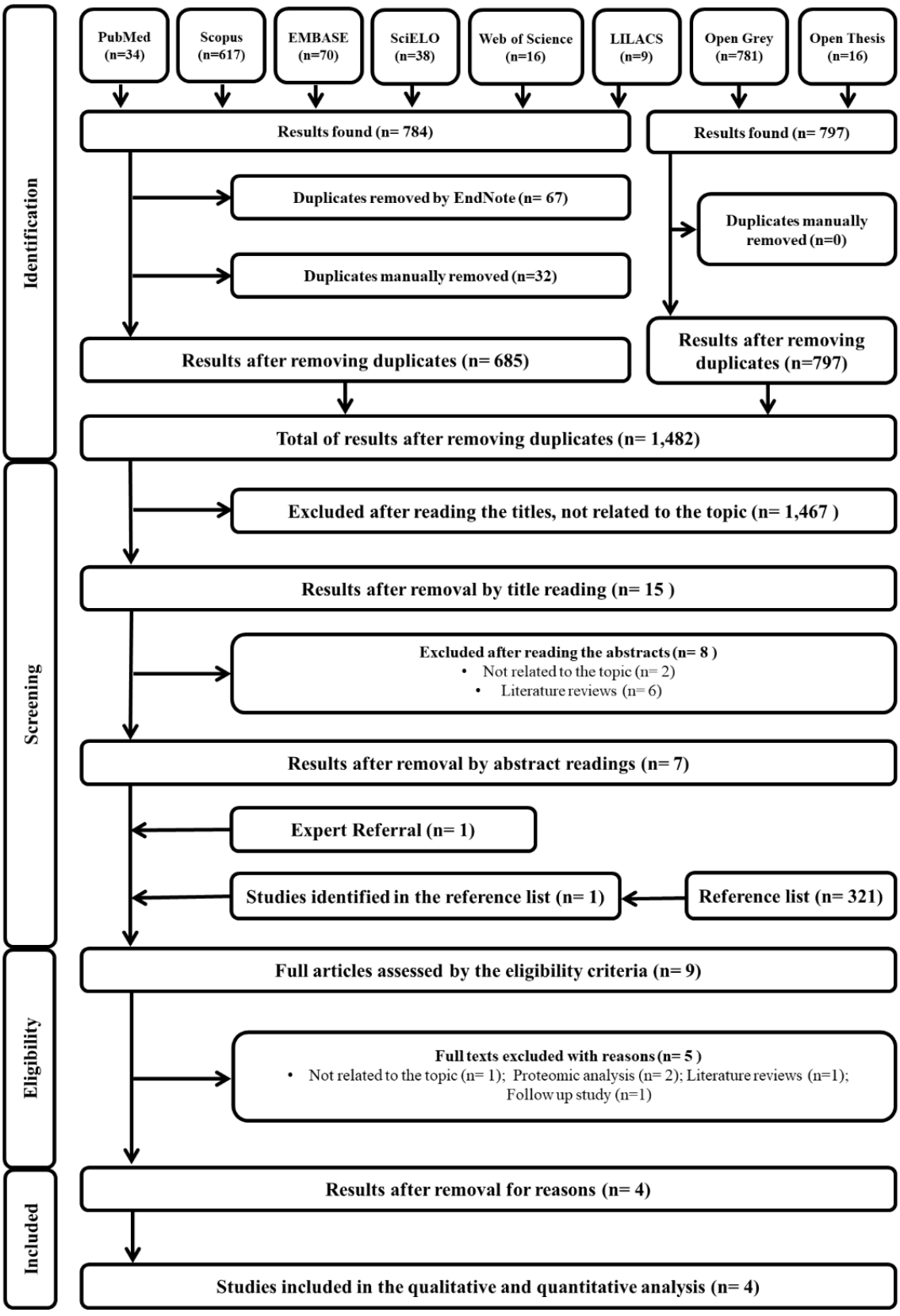

Figure. 1 Flow-chart showing the search strategy, identification, and inclusion/exclusion criteria used in the meta-analysis and systematic review

3.2. Study characteristics of eligible studies

The studies were published between 2015 and 2019 and were performed in Chile [23], China [36], Egypt, [37] and India [38]. All articles [23,36-38] were approved by the Ethics Committee of their respective institution or hospital and also reported that informed consent was obtained before the start of the study. None of the articles used STROBE checklist for cross-sectional studies. Other sources of information regarding demographic and characteristics of the population are available in Table 2. Characteristics, preparation, and measurement of the samples in the eligible studies are in Table 3.

Table 2. Characteristics on the population of the eligible studies included.

Author, year	Country	Type DM2 population	Control population	Average age (years)	Average age range (years)	Sex ratios	Diagnosis	Collection Period
Aitken et al. 2015	Chile	120 patients (75 patients uncontrolled glycemia and 45 patients having controlled glycemia)	NA	61.6 ± 10.1	31-79	32.5% ♂, 67.5% ♀	Patients with HbA1c levels <7% were classified as having adequate glycemic control and those with levels >7% were classified as having inadequate glycemic control	July 2013 to December 2013
Feng et al. 2015	China	116 patients DM2 and 60 patients IFG (Impaired Fasting Glucose)	60 healthy volunteers	DM2 (57 ± 12.3), IFG (55 ± 14.3), Control (51 ± 11.3)	Not reported	DM2 (54♂ / 62♀), IFG (27♂ / 33♀), Control (22♂ / 38♀)	American Diabetes Association in 2010 for DM2; IFG ≥ 7.0 mM (pre-diabetic); Fasting blood glucose ranged from 5.6-6.9 mM (control)	February 2011 to March 2012
Nsr-Allah et al. 2019	Egypt	40 patients (20 patients uncontrolled glycemia – group 1 and 20 patients controlled glycemia (group 2)	20 healthy volunteers (group 3)	Group 1 (49.75 ± 10.74). Group 2 (50.90 ± 10.54), Group 3 (48.9 ± 11.47)	23-65	Group 1 (7♂ / 13♀) Group 2 (9♂ / 11♀) Group 3 (13♂ / 7♀)	Patients with HbA1c levels <7% were classified as having adequate glycemic control and those with levels ≥ 7% were classified as having inadequate glycemic control. Group 3 included with fasting plasma glucose less than 100 mg/dl and HbA1c less than 5.7%.	April 2016 and June 2017
Rastogi, et al. 2019	India	87 patients (53 patients uncontrolled glycemia and 34 patients controlled glycemia	NA	52.4 ± 8.1	35-65	43♂, 44♀	Not reported	August 2018 to October 2018

Note: NA: Not applicable, ♀: women, ♂: men.

Table 3. Characteristics of the collection, preparation and measurement of the samples in the eligible studies included.

Author, year	Saliva collection	Saliva collection criteria	Saliva preparation	Blood collection	A2MG measurement	Glycemia measurement	HbA1c measurement
Aitken et al. 2015	NR	NR	NR	NR	ELISA (Salivary A2MG)	NA	The Variant II brand team Bio-Rad
Feng et al. 2015	Unstimulated	At 8 a.m., the subjects were asked to rinse their mouths thoroughly with water before breakfast.	Centrifuged at 2000g for 10 min at 4°C and the supernatant were used.	Venous blood was collected from the ulnar vein.	ELISA (Salivary A2MG) BNII automatic protein analyzer (Plasmatic A2MG)	DXC800 automatic biochemistry analyzer	NR
Nsr-Allah et al. 2019	Unstimulated	At 8 am, the patients were asked to rinse their mouths thoroughly with water before breakfast. They were then required to tilt their heads forward, and saliva was accumulated in the floor of the mouth for 2 min and collected into a sterile container.	Centrifuged at 2000g for 10 min and the supernatant were collected.	Venous blood after 10 h of fasting was collected.	ELISA (Salivary A2MG)	Hexokinase method by spectrophotometry on cobas 8000 by Roche Diagnostics GmbH D-68298	NR
Rastogi, et al. 2019	Unstimulated	They had to wash their mouths with tap water and spit two to three times, after which they had to spit saliva pooled in their mouths for the following 5 min into the sterile container.	Centrifuged at 3800rpm for 10 min at 4°C and the supernatant was collected.	NR	ELISA (Salivary A2MG)	NR	High Performance Liquid Chromatography (HPLC) method on Bio-Rad D-10

Note: NA: Not applicable; NR: Not reported

3.3 Risk of bias within studies

All studies presented a low risk of bias or high methodological quality. However, Rastogi, Kalra, Gowda [38] did not describe specific information about the population and the parameters to assist the diagnosis of diabetes. In this context, it was indicated as unclear in the risk of bias table (Table 4).

Table 4 - Risk of bias assessed by the Joanna Briggs Institute Critical Appraisal Tools for use in JBI Critical Appraisal Checklist for Analytical Cross-Sectional Studies (Moola S, et al. 2017).

Authors	Q1	Q2	Q3	Q4	Q5	Q6	Q7	Q8	% Yes	Risk
Aitken et al., 2015	√	√	√	√	√	√	√	√	100	Low
Feng et al., 2015	√	√	√	√	√	√	√	√	100	Low
Nsr-Allah et al., 2019	√	√	√	√	√	√	√	√	100	Low
Rastogi et al., 2019	√	U	√	U	√	√	√	√	75	Low

Q1. Were the criteria for inclusion in the sample clearly defined? Q2. Were the study subjects and the setting described in detail? Q3. Was the exposure measured in a valid and reliable way? Q4. Were objective, standard criteria used for measurement of the condition? Q5. Were confounding factors identified? Q6. Were strategies to deal with confounding factors stated? Q7. Were the outcomes measured in a valid and reliable way? Q8. Was appropriate statistical analysis used? √ - Yes; -- - No; NA – Not Applicable; U – Unclear.

3.4 Summary measures and synthesis of results

Table 5 describes the correlation of salivary A2MG with glycemia and/or HbA1c and the respective means/standard deviations of glycemia, HbA1c, and A2MG of the selected studies included in quantitative analysis. All studies were also included in the meta-analysis. However, only three studies compared A2MG to HbA1c [23,37,38], and three studies compared A2MG to glycemia [36-38].

The correlation between A2MG and HbA1c ranged between 0.722 and 0.977 for the three studies analyzed. Overall, the pooled correlation between these biomarkers was strong ($r = 0.838$; 95%CI: 0.719; 0.956; $p < 0.001$) (Figure 2). In contrast the pooled correlation between A2MG and glycemia was low ($r = 0.354$; 95% CI: 0.077; 0.630; $p = 0.006$). Both meta-analyses presented significant heterogeneity between study results ($p < 0.001$), however heterogeneity levels were higher for glycemia compared to HbA1c analysis.

Table 5. Summary of the main results of the studies included in the quantitative analysis.

Study	Mean glycemia	Mean HbA1c	Mean A2MG	Correlation salivary A2MG with glycemia	Correlation salivary A2MG with HbA1c
Aitken et al. 2015	NA	HbA1c >7% (62.5%) HbA1c <7% (37.5%)	NR	NA	r = 0.7748, P < 0.0001
Feng et al. 2015	DM2 (10.08±2.44 mM); IFG (6.58±0.24 mM); Control (5.01±0.41 mM)	DM2 (8.7±1.7%); IFG (5.8±1.1%); Control (5.7±0.7%)	Salivary A2MG (ng/mL) DM2 (192.6±65.3), IFG (158.1±60.1), Control (134.8±63.2) Plasmatic A2MG (g/L) DM2 (1.70±0.55); IFG (1.57±0.36); Control (1.54±0.38)	DM2 (r = 0.12, P = 0.199)	NA
Nsr-Allah et al. 2019	Group 1 (172.20±26.52 mg/dL) Group 2 (100.65±21.30 mg/dL) Group 3 (90.95±8.66 mg/dL)	Group 1 (9.02±1.38%) Group 2 (6.20±0.61%) Group 3 (5.35±0.44%)	Salivary A2MG (ng/mL) Group 1 (820.65±190.17), Group 2 (331±98.01), Group 3 (146.90±42.01)	Group 1 (r=0.586, P < 0.05); Group 2 (r=0.146, P = 0.539); Group 3 (r=0.650 ,P < 0.05); All subjects (r=0.788, P < 0.001)	Group 1 (r-0.778, P < 0.001); Group 2 (r=0.666, P < 0.05); Group 3 (r=0.474, P < 0.05); All subjects (r=0.927, P < 0.001)*
Rastogi, et al. 2019	Uncontrolled glycemia (290.58±96.126 mg/dL) Controlled glycemia (172.83±39.955 mg/dL)	HbA1c >7% (60.9%) HbA1c <7% (39%)	Salivary A2MG (ng/mL) Uncontrolled glycemia (2017.42±575.133), Controlled glycemia (772.54±118.324)	r = 0.660, P < 0.001	r = 0.977, P < 0.001

Note: NA: Not applicable; NR: Not reported

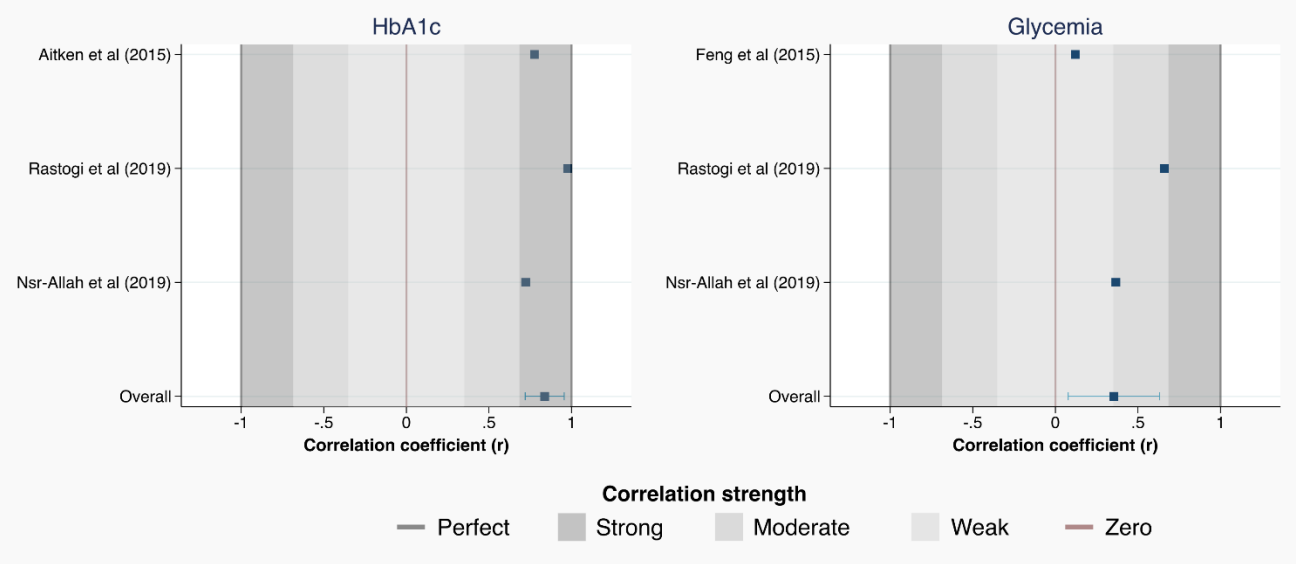

Figure 2. Correlation between salivary A2MG with HbA1c and Glycemia.

3.5 Certainty of evidence

The GRADE tool [33] assessed two outcomes. Both outcomes (correlation between A2MG and HbA1c and correlation between A2MG and glycemia) were categorized as a very low level of certainty, which means the true effect is likely to be substantially different from the estimate of effect. Table 6 shows more details on each outcome.

Table 6. Grading of Recommendations Assessment, Development, and Evaluation (GRADE) Summary of Findings Table for the Outcomes of the Systematic Review and Meta-Analysis.

Quality Assessment							Summary of Results		Importance
Number of studies	**Study Design**	**Methodological Limitations**	**Inconsistency**	**Indirectness**	**Imprecision**	**Others considerations**	**Number of participants**	**Effect** Random *r* (95% CI)	**Certainty of evidence**	
Outcome 1: Correlation between A2MG and HbA1c										
3	Cross-sectional studies	Not serious[a]	serious[b]	Not serious[c]	Serious[d]	none	247	0.838 (0.719; 0.956)	⨁ VERY LOW	Critical
Outcome 2: Correlation between A2MG and Glycemia										
3	Cross-sectional studies	Not serious[a]	serious[b]	Not serious[c]	Serious[d]	none	243	0.354 (0.077; 0.630)	⨁ VERY LOW	Critical

[a] All studies presented low risk of bias
[b] The effect estimate presented significant heterogeneity ($p < 0.001$).
[c] Evidence stems from studies with the population suitable for PICO
[d] The number of participants is too low and do not reach the optimal information size (n=400).

GRADE Working Group grades of evidence
High certainty: We are very confident that the true effect lies close to that of the estimate of the effect.
Moderate certainty: We are moderately confident in the effect estimate: The true effect is likely to be close to the estimate of the effect, but there is a possibility that it is substantially different.
Low certainty: Our confidence in the effect estimate is limited: The true effect may be substantially different from the estimate of the effect.
Very low certainty: We have very little confidence in the effect estimate: The true effect is likely to be substantially different from the estimate of effect.

4. Discussion

We have performed a systematic review to evaluate if the increase in salivary A2MG concentration is correlated to HbA1c and glycemia levels of blood in DM2 patients. Herein, we showed a strong correlation between salivary A2MG with HbA1c with a low level of certainty, indicating the need to perform new studies and a potential application in salivary platforms. However, the low association between A2MG with glycemia levels suggest that A2MG is not an accurate salivary protein to surrogate glycemic tests.

Considering that glycemia reflects the blood glucose levels in the moment of the analysis, this test fault to express the glucose control over prolonged periods [39]. HbA1c test has been recommended to assess variations in glucose tolerance in type 2 diabetic patients for testing and monitoring diabetes [8]. In addition, HbA1c can be performed at any time of the day, without worrying about the fasting state to indicate an average plasma concentration of two to three months, which predicts the half-life of red blood cells [40,41]. However, the classical HbA1c tests dispend several reagents with a relative high cost and needs some specific equipments [7], which reduces the availability of HbA1c tests in low- and middle-income countries despite its well-recognized the recognized capability to diabetes surveillance [8]. Moreover, several biological factors such as clinical conditions that alter erythropoiesis, glycation rate, erythrocyte destruction, and analytical interferences such as hyperbilirubinaemia, carbamylated hemoglobin, certain medications, and hemoglobin variants affect the alteration cutoff values of HbA1C test [42]. Our findings in this meta-analysis confirm the hypothesis that A2MG present a strong correlation with HbA1c test ($r = 0.838$).

In this context, the presented association between salivary A2MG with HbA1C levels indicates saliva as a promising alternative auxiliary method to diagnose and monitoring of diabetes. Among the advantages, saliva is simple to collect, non-invasive, convenient to store and, compared to blood, requires less handling during clinical procedures. In this context, future studies should be carried out in order to verify the clinical applicability of salivary A2MG to surrogate HbA1C in the diagnostic and monitoring analysis with non-diabetic and type 2-diabetic subjects.

This systematic review has some limitations. The absence of a control group in the included studies (Aitken et al., 2015 and Rastogi et al., 2019) could be considered a limitation; however, the analysis with uncontrolled hyperglycemic subjects and subjects

with type 2 diabetes sub-optimally controlled is also clinically relevant. In addition, GRADE found that there is a reduced level of certainty of evidence especially due to the number of the participants is lower than 400 in the meta-analysis. More studies with larger populations should be carried out in order to minimize imprecision including non-hyperglycemic, uncontrolled diabetic, and controlled diabetic subjects. Although the HbA1c reflects the average blood glucose levels during the previous ~75 days, we consider as a limitation the absence of the mean duration of diabetes in the included studies. Finally, the absence of systematic reviews and meta-analysis in this field increases the importance and timeliness of this meta-analysis. In the future, it will be important to define the predictive power of salivary A2MG to estimate HbA1c levels.

In conclusion, we have shown a strong association between HbA1C and saliva levels of A2MG in non-diabetic and DM2 subjects. On the other hand, the meta-analysis suggests a very low correlation between glycemia and salivary A2MG. Due to the reduced level of certainty of evidence further large-scale studies are needed to recommend salivary A2MG levels as a surrogate for HbA1C.

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6. Conflicts of interest

The authors have no conflicts of interest

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