IMAGE: A MAP OF THE STARS OF THE ORION CONSTELLATION

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Drug Poisoning: Toxicological Emergency

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ABSTRACT

The use of medications in conventional dosages guarantees their mechanism of action and provides the expected biological effects. Clinical trials and research on substances approved by the national health agency justify their clinical efficacy and demonstrate the risk-benefit of the medication, but they also contain some information on the toxic effects on the body when used in doses above the recommended. A bibliographic review was conducted targeting some classes of medications, such as antipsychotics, SSRI antidepressants (serotonin reuptake inhibitors), tricyclic and tetracyclic antidepressants, lithium, benzodiazepines, nonsteroidal anti-inflammatory drugs (NSAIDs), anticholinergics, digitalis, opiates, opioids and thyroid hormone, to update knowledge on drug poisoning protocols established by other institutions that are references in the subject, making it possible to identify symptoms and define treatments for these cases. Thus, in drug poisoning, irreversible effects can be observed in the short and long term, and even death if not treated appropriately.

Keywords: drug poisoning, toxicological emergency, overdose.

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Drug Poisoning: Toxicological Emergency

Intoxicação Por Medicamentos: Emergência Toxicológica

Guilherme Campos da Luz[α], Stella de Bortoli[σ] & Sinvaldo Baglie[ρ]

RESUMO

A utilização de medicamentos em doses posológicas convencionais garante seu mecanismo de ação e proporciona os efeitos biológicos esperados. Os ensaios clínicos e pesquisas acerca das substâncias aprovadas pelo órgão sanitário nacional justificam sua eficácia clínica e demonstram o risco-benefício do medicamento, porém também possuem algumas informações sobre os efeitos tóxicos sobre o organismo quando utilizados em dosagens acima do recomendado. Foi realizada uma revisão bibliográfica direcionada para algumas classes de medicamentos, como antipsicóticos, antidepressivos ISRS (inibidores da recaptação de serotonina), antidepressivos tricíclicos e tetracíclicos, lítio, benzodiazepínicos, anti-inflamatórios não esteroidais (AINES), anticolinérgicos, digitálico, opiáceos, opioides e hormônio da tireóide, para atualizar o conhecimento sobre protocolos de intoxicação por medicamentos estabelecidos por outras instituições que são referência no tema, possibilitando identificar sintomas e definir tratamentos para esses casos. Desse modo, observa-se na intoxicação medicamentosa efeitos irreversíveis a curto e longo prazo e até mesmo a óbito se não for tratada de maneira adequada. Os protocolos disponíveis utilizados em centros e unidades de referência nesse tipo de atendimento descrevem a identificação de possíveis síndromes tóxicas associadas, o manejo dos sintomas, tratamento sintomático e o antídoto que pode ser utilizado. Assim sendo, as pesquisas clínicas e a farmacovigilância contribuem na elucidação e compreensão dos efeitos toxicológicos, sendo uma fonte atualizada a partir da disponibilidade de informações que ocorrem na prática hospitalar.

Palavras-Chave: intoxicação medicamentosa, emergência toxicológica, superdosagem.

ABSTRACT

The use of medications in conventional dosages guarantees their mechanism of action and provides the expected biological effects. Clinical trials and research on substances approved by the national health agency justify their clinical efficacy and demonstrate the risk-benefit of the medication, but they also contain some information on the toxic effects on the body when used in doses above the recommended dose. A bibliographic review was conducted targeting some classes of medications, such as antipsychotics, SSRI antidepressants (serotonin reuptake inhibitors), tricyclic and tetracyclic antidepressants, lithium, benzodiazepines, nonsteroidal anti-inflammatory drugs (NSAIDs), anticholinergics, digitalis, opiates, opioids and thyroid hormone, to update knowledge on drug poisoning protocols established by other institutions that are references in the subject, making it possible to identify symptoms and define treatments for these cases. Thus, in drug poisoning, irreversible effects can be observed in the short and long term, and even death if not treated appropriately. The available protocols used in reference centers and units for this type

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of care describe the identification of possible associated toxic syndromes, symptom management, symptomatic treatment and the antidote that can be used. Therefore, clinical research and pharmacovigilance contribute to the elucidation and understanding of toxicological effects, serving as an updated source based on information generated in hospital practice.

Keywords: drug poisoning, toxicological emergency, overdose.

RESUMEN

El uso de medicamentos en dosis convencionales garantiza su mecanismo de acción y proporciona los efectos biológicos esperados. Los ensayos clínicos y las investigaciones sobre sustancias aprobadas por el organismo nacional de salud justifican su eficacia clínica y demuestran el riesgo-beneficio del medicamento, pero también contienen alguna información sobre los efectos tóxicos en el organismo cuando se utilizan en dosis superiores a las recomendadas. Se llevó a cabo una revisión de la literatura sobre algunas clases de medicamentos, como los antipsicóticos, los antidepresivos ISRS (inhibidores de la recaptación de serotonina), los antidepresivos tricíclicos y tetracíclicos, el litio, las benzodiazepinas, los antiinflamatorios no esteroideos (AINE), los anticolinérgicos, los digitálicos, opiáceos, opioides y hormona tiroidea, para actualizar el conocimiento sobre los protocolos de intoxicación por medicamentos establecidos por otras instituciones referentes en el tema, permitiendo identificar síntomas y definir tratamientos para estos casos. Así, en las intoxicaciones por medicamentos se observan efectos irreversibles a corto y largo plazo e incluso la muerte si no se tratan adecuadamente. Los protocolos disponibles y utilizados en los centros y unidades de referencia en este tipo de atención describen la identificación de posibles síndromes tóxicos asociados, Los ensayos clínicos y las investigaciones *que se puede utilizar. Por tanto, la investigación clínica y la farmacovigilancia contribuyen al esclarecimiento y comprensión de los efectos toxicológicos, siendo una fuente actualizada en función de la disponibilidad de información que se produce en la práctica hospitalaria.*

Palabras Clave: intoxicación por drogas, emergencia toxicológica, sobredosis.

I. INTRODUÇÃO

O tratamento de pacientes em situações que envolvem intoxicação grave por medicamentos deve ser tratado como prioridade no atendimento hospitalar. As situações de exposição que envolvem risco à vida devem ser analisadas conforme protocolos de vias aéreas, respiração, circulação e incapacidade (HUMPHRIES, 2024). Para avaliação inicial do paciente deve ser verificado o histórico através da investigação da exposição, tentando obter dados sobre as doenças crônicas, medicações de uso contínuo, acesso a substâncias, solicitar amostra em caso de acompanhantes (MOWRY, 2014).

Algumas substâncias possuem antídotos específicos, nos quais o agente tóxico ou os seus metabólitos têm alta toxicidade ou resultam em complicações com risco à vida. (HUMPHRIES, 2024).

Os incidentes envolvendo intoxicações por substâncias químicas exógenas (agrotóxicos, gases tóxicos e medicamentos) são de notificação compulsória semanal, de acordo com a portaria do Ministério da Saúde 1.061 de 2020 (BRASIL, 2020). O registro da ocorrência de intoxicações exógenas é considerado de notificação obrigatória, auxiliando em políticas públicas locais, regionais e nacionais quanto ao manejo e atualização das informações dos medicamentos (BRASIL, 2011). A intoxicação medicamentosa aparece como um problema social ao tempo que a medicalização nos tempos atuais se faz necessária para os crescentes problemas de saúde.

II. OBJETIVOS

O objetivo deste trabalho foi realizar uma revisão bibliográfica de protocolos e informações citadas na literatura sobre a toxicologia de classes determinadas de medicamentos e assim corroborar sua aplicação na prática hospitalar atual.

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III. METODOLOGIA

Foi realizada a revisão bibliográfica através das bases de dados disponíveis nos periódicos da CAPES, Google acadêmico, Scielo, Scopus, Science Direct e Pubmed. Para realizar as buscas, foram utilizadas como palavras-chave as seguintes expressões em português e inglês: "Toxicologia clínica", "intoxicação por medicamentos", "protocolos de intoxicação medicamentosa", "intoxicações intencionais e não intencionais por medicamentos", "superdosagens por medicame- ntos". A pesquisa foi direcionada para as determinadas classes farmacológicas: antipsicóticos, antidepressivos inibidores da recaptação de serotonina, antidepressivos tricíclicos e tetracíclicos, lítio, benzodiazepínicos, anti-inflamatórios não esteroidais (AINES), anticolinérgicos, digitálico, opiáceos, opioides e hormônio da tireoide. Foram utilizados somente artigos de revisão (review art Para a elaboração e análise das informações compiladas, foi utilizado o programa Microsoft Office Word® 2016, versão 2405. A partir do levantamento, os dados foram organizados e descritos de modo a obter um material para o atendimento de pacientes com sinais e sintomas de intoxicação por determinados medicamentos.

IV. RESULTADOS E DISCUSSÕES

O atendimento inicial para pacientes com suspeita de intoxicação exógena por medicamentos deve ser tratado como potencialmente fatal. Na coleta de informações pode ser utilizado a estratégia dos "5W's", obtendo os dados do paciente acerca de doenças, medicações de uso contínuo, (*Who* – Quem?), a substância e a quantidade utilizada, (*What* – O quê?), tempo de exposição (*When* – Quando?), local da ocorrência, (*Where* - Onde?) e motivo da exposição, identificando possível tentativa de suicídio, homicídio, acidente ou abuso de medicamentos (*Why* -Por quê?) (NELSON, 2011). Os casos suspeitos de intoxicação exógena devem ser notificados de acordo com a Portaria MS/GM 104 de 25 de janeiro de 2011, através da ficha de investigação exógena disponível eletronicamente através do Sistema Nacional de Agravos de Notificação (SINAN).

Nos casos de intoxicação confirmada, o campo 66 da ficha de notificação deve ser preenchido conforme o agente causador da intoxicação, seguida do código da classe ou substância estabelecidos e descritos especificamente, para intoxicações por ocorrência acidental (T 36.) e intoxicações por exposição intencional (X 60.). A identificação clínica de uma síndrome tóxica pode auxiliar no diagnóstico através da sintomatologia apresentada pelo paciente, como descrito no Quadro 1.

Quadro 1: Características Das Síndromes Tóxicas

Síndromes Tóxicas	Sinais Vitais	Pupilas	SNC	Outros Sistemas	Agentes Tóxicoss
Hipnótica sedativa narcótica	Hipotermia Hipotensão Bradicardia Bradipneia	Miose	Depressão SNC Depressão respiratória	Hiporreflexia Edema pulmonar	Barbitúricos Benzodiazepínicos Opioides
Colinérgica	Hipotermia Hipotensão Bradicardia Bradipneia	Miose	Confusão mental Convulsões Coma	Sialorreia intensa Sudorese Lacrimejamento Náusea/vômito Dispneia Broncoconstrição	Nicotina Neostigmina Fisostigmina Piridostigmina
Anticolinérgica	Hipertermia Hipertensão Taquicardia Taquipneia	Midríase	Agitação Alucinação Delírio Convulsões	Retenção urinária Mioclonias Convulsões Mucosas secas	Atropínicos Anti-histamínicos Antidepressivos tricíclicos

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Simpatomimética	Hipertermia Hipertensão Taquicardia Hiperpneia	Midríase	Agitação Alucinações Paranoia Convulsões	Tremores Hiperreflexia Diaforese	Teofilina Efedrina Cafeína Cocaína Anfetamina
Extrapiramidal	Não característico	Midríase	Sonolência Crise oculógira	Tremores Hipertonia muscular Opistótono Trismo	Antipsicóticos (Haloperidol, Aripiprazol, Risperidona, Sulpirida) Fenotiazínicos Antieméticos (Metoclopramida. Bromoprida, Droperidol)
Serotoninérgica	Hipertermia	Midríase, pupilas	Delírio	Mioclonias	Selegilina
	Taquicardia Taquipneia	não reativas	Agitação	Hiperreflexia Hipertonia muscular	Lítio Trazodona Antidepressivos tricíclicos (Amitriptilina, Imipramina), Inibidores da recaptação de serotonina e noradrenalina (Venlafaxina)

Fonte: Atendimento inicial das intoxicações agudas. Coordenadoria de Vigilância em Saúde. <cvs.saude.sp.gov.br/up/MANUAL DE TOXICOLOGIA CLÍNICA - COVISA 2017.pdf> 2017. Acesso em: 13 mai. 2024.

Alguns métodos tradicionais podem ser empregados em situações de intoxicação. A descontaminação cutânea, visa retirar roupas impregnadas com o agente tóxico e lavar a superfície exposta com água em abundância; nos casos de contaminação ocular deve ser instilado uma ou duas gotas de colírio anestésico no olho afetado e proceder a lavagem com soro fisiológico 0,9% ou água filtrada, sempre da região medial do olho para a região externa; para o sistema gastrointestinal depende da substância ingerida, do tempo ingerido e a gravidade do caso. A lavagem gástrica e administração de carvão ativado devem ser realizadas considerando alguns fatores, como ausência de sonolência ou torpor, na ingestão de quantidades potencialmente tóxicas de alguma substância, na ingestão recente de 1h a 2h de exposição e nos casos que envolvam a diminuição do trânsito gastrointestinal (SILVA, 2021).

A lavagem gástrica, pode ser empregada e consiste na infusão e posterior aspiração de solução de cloreto de sódio 0,9% (soro fisiológico) através de sonda nasogástrica, infundindo e retirando o volume de SF até que retorne límpido. O volume recomendado varia de acordo com a faixa etária, para crianças 10mL/kg por infusão até o volume total de 4L a 5L para crianças em idade escolar, 2 a 3L para lactentes e 0,5L recém-nascidos; para adultos recomenda-se 250mL por vez até um volume total de 6 L a 8L. A administração de carvão ativado tem o efeito adsorvente impedindo a absorção da substância pelo organismo, pode ser utilizado após a lavagem gástrica ou como medida única de descontaminação. A dosagem para crianças 1g/kg em uma suspensão com água ou de solução de cloreto de sódio 0,9% na proporção de 4-8mL/g. Para adultos deve ser utilizado 50g em 250mL de água ou de solução de cloreto de sódio 0,9% (BRASIL, 2017).

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4.1 Antipsicóticos

Os antipsicóticos podem ser classificados como típicos ou atípicos diferenciando a seletividade no seu mecanismo de ação, como antagonistas dos receptores dopaminérgicos (D1 e D2), serotoninérgicos (5-HT), alfa-1 adrenérgicos, M1 (colinérgicos) e H1 (histaminérgicos). No quadro 2, está demonstrado a classificação farmacológica dos antipsicóticos.

Quadro 2: Classificação Farmacológica Dos Antipsicóticos

Princípio ativo	Tipo	Dose terapêutica diária em adultos (mg)	Toxicidade e efeitos adversos
Aripiprazol	Atípico	10 - 30	A, E, H, Q
Clozapina	Atípico	25 - 900	A, H
Olanzapina	Atípico	5 - 20	A, E, H
Pimozida	Atípico	2 - 10	E, H
Quetiapina	Atípico	150 - 750	A, E, H, Q
Ziprazidona	Atípico	60 - 160	A, E, H, Q
Clorpromazina	Fenotiazina (típico)	200 - 800	A, E, H, Q
Droperidol	Butirofenona (típico)	2 - 10	E, Q
Flufenazina	Fenotiazina (típico)	2,5 - 20	E, A
Haloperidol	Butirofenona (típico)	1 - 100	E, Q
Tioridazina	Fenotiazina (típico)	150 - 300	A, E, H
Tiotixeno	Tioxanteno (típico)	5 - 60	E
Trifluperazina	Fenotiazina (típico)	1 - 40	E

A= Efeito anticolinérgico; E=reações extrapira- midais; H= hipotensão; Q= aumento do sinal QT Fonte: As bases farmacológicas da terapêutica. Disponívelem:http://accessmedicine. Mhmedical. com/content. Aspx? bookid=374 & Sectionid= 41266222. Acesso em: 13 mai. 2024.

A descontaminação deve ser feita com lavagem gástrica com solução de cloreto de sódio 0,9% até uma hora após a ingestão e administração de carvão ativado através de sonda nasogástrica após lavagem com solução fisiológica, nos casos da ingestão em pequenas doses pode ser administrado somente o carvão ativado, excluindo a etapa de lavagem gástrica (MEYER, 2011).

4.1.1 Tratamento

Pode ser realizado o tratamento sintomático nos casos que forem diagnosticados síndrome extrapiramidal, convulsões ou síndrome neuroléptica maligna, conforme demonstrado no Quadro 3.

Quadro 3: Tratamento Sintomático Para Intoxicação Por Antipsicóticos

Sintomatologia tóxica	Tratamento
Síndrome extrapiramidal	Biperideno Adultos: 3 a 5mg a cada 6 horas Crianças: 0,06 a 0,1mg/kg a cada 6 horas se necessário
Convulsão	Benzodiazepínicos Adultos: até 30 mg parceladamente Crianças: até 0,3 a 0,5 mg/kg
Síndrome Neuroléptica Maligna	Dantroleno: 2,5 mg/kg até um máximo de 10mg/kg/dia. Pode ser dividido em 4 doses ao dia Bromocriptina: 2,5mg a 10mg; 3 a 4 vezes ao dia. Amantadina: 100mg a 200mg; 2 vezes ao dia.

Fonte: Adaptado de: Pharmacotherapy of Psychosis and Mania. Disponível em: < http://accessmedicine .mhmedical. com/content.aspx?bookid=374 & Sectionid=41266222>. Acesso em: 10 jun. 2024.

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Os casos suspeitos de intoxicação por antipsicóticos devem ser notificados na ficha de investigação e ser preenchido o campo 66 da FIIE, conforme os agentes envolvidos: intoxicação por antipsicóticos neurolépticos derivados da fenotiazina -T43.3; intoxicação por neurolépticos tipos butirofenona e tioxanteno – T43.4 e intoxicação por outros antipsicóticos e neurolépticos e os não especificados – T43.5.

4.2 Antidepressivos Inibidores Seletivos Da Recaptação De Serotonina (Isrs)

Os medicamentos desta classe possuem índice terapêutico considerado alto e doses acima de 10 vezes a dose terapêutica pode ser bem tolerada se comparada aos antidepressivos tricíclicos (DEMARCHI, 2020).

Nos casos de intoxicação leve a moderada pode resultar em ataxia e letargia, porém nos casos graves pode ser identificado bradicardia, hipotensão e rebaixamento do nível de consciência. Não é indicado lavagem gástrica nos casos de pequena ingesta, podendo ser administrado somente carvão ativado 1g/kg por via oral. Em intoxicações com grande quantidade, deve ser realizado lavagem gástrica (até 1 hora após a ingestão) com solução de cloreto de sódio 0,9% e administrado carvão ativado (MARCOS, 2019).

4.2.1 Tratamento

O tratamento consiste no controle dos sintomas pós exposição, podendo ocorrer arritmias do tipo taquicardia, prolongamento do intervalo QRS> 100 mseg, devendo ser administrado bicarbonato de sódio 1 a 2 mEq/kg IV em bolus e repetir se necessário. A síndrome serotoninérgica deve ser considerada nos casos graves, o resfriamento corporal com medidas físicas é apropriado, pois os antitérmicos não surtem efeito pois o aumento da temperatura está relacionado à hiperatividade muscular, quando a temperatura for > 40°C, estão indicadas, sedação com benzodiazepínicos e intubação orotraqueal. Nos casos refratários a essas medidas pode ser administrado ciproeptadina 12 mg via oral ou sonda inicialmente e 4 mg por hora por 3 a 4 doses (AMARAL, 2021). Nos casos de intoxicação confirmada deve ser preenchido o campo 66 da ficha de notificação como intoxicação por outros antidepressivos e os não especificados T43.2.

4.3 Antidepressivos Tricíclicos E Tetracíclicos

São utilizados no tratamento da depressão, dor crônica, transtorno de déficit de atenção. As doses acima de 10 -20mg/kg representam risco à vida, na intoxicação leve a moderada os sintomas clínicos que podem se manifestar são: sonolência, sedação, taquicardia, alucinações e midríase (MENOLLI, 2020).

4.3.1 Tratamento

A descontaminação pode ser feita com a lavagem gástrica precoce até 1 hora após a ingestão, quando há ingestão de pequenas doses, pode-se optar pelo uso somente do carvão ativado, nos casos graves pode-se optar por administrar carvão ativado em dose múltipla, repetindo 1 a 3 vezes nas primeiras 12 horas podendo também associar laxantes. O prolongamento do intervalo QRS >100 mseg ou arritmias ventriculares pode predizer convulsões e arritmias, podendo ser administrado bicarbonato de sódio 1 a 2 mEq/kg EV em bolus, mantendo o pH entre 7,45 e 7,55. Os benzodiazepínicos são a primeira escolha para controle de convulsões, considerando fenobarbital, fenitoína e bloqueadores neuromusculares como outras opções a serem consideradas (MURTA, 2022).

Os casos confirmados devem ser preenchidos a ficha de notificação como intoxicação por antidepressivos tricíclicos e tetracíclicos, T43.0.

4.4 LÍTIO

O carbonato de lítio é utilizado como agente estabilizador de humor no transtorno bipolar. As doses tóxicas podem ser muito próximas à dose terapêutica em pacientes em uso crônico da medicação. As intoxicações agudas leves a moderadas podem levar ao aparecimento de sintomas gastrointestinais, na intoxicação crônica prevalece sintomas neurológicos, podem ocorrer náusea, vômitos, diarreia, nistagmo, desidratação, tremores, rigidez, ataxia, agitação, coma e síndrome serotoninérgica. (MECE, 2022). Pode

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haver sintomas importantes mesmo em concentrações terapêuticas, conforme descrito na Tabela 1.

Tabela 1: Concentrações Séricas De Lítio E Seu Impacto Clínico

Concentração de lítio (mEq/L)	Níveis clínicos
0,6 a1,2	Nível terapêutico
1,2 a 2,5	Reações leves a moderadas
2,5 a 4,0	Efeitos mais graves
Acima de 4,0	Efeitos graves em SNC e risco de morte

Fonte: Adaptado de: atendimento inicial das intoxicações agudas. Coordenadoria de Vigilância em Saúde. <cvs.saude.sp.gov.br/up/MANUAL DE TOXICOLOGIA CLÍNICA - COVISA 2017.pdf> 2017. Acesso em: 17 jun. 2024.

4.4.1 Tratamento

O tratamento de suporte consiste na hidratação com solução salina fisiológica, facilitando a eliminação de lítio e correção de distúrbios hidroeletrolíticos. A lavagem gástrica tem benefício somente nas intoxicações agudas em até 1 a 2 horas após a ingestão, o carvão ativado não tem a capacidade de adsorver a substância. A irrigação intestinal com polietilenoglicol pode ser utilizada na ingestão em grande quantidade. Ainda pode-se optar pela hemodiálise como método de eliminação nos pacientes com manifestações neurológicas importantes, insuficiência renal e níveis séricos altos (geralmente acima de 3,5 mEq/L) ou quando níveis em ascensão mesmo após hidratação agressiva. Nove horas de hemodiálise removem aproximadamente 60% dos estoques totais de lítio, sendo necessário nova dosagem 12 horas após diálise, com o objetivo de alcançar concentrações de 1 mEq/L,devendo ser repetida a hemodiálise se necessário (PIRES, 2022).

Nos casos de intoxicação confirmada deve ser preenchido o campo 66 da ficha de notificação como intoxicação por outras drogas psicotrópicas não classificadas em outra parte, T43.8.

4.5 Benzodiazepínicos

A dose tóxica pode ser variável, visto que são substâncias com índice terapêutico alto, a ingestão de altas doses por usuários crônicos podem não produzir efeitos previsíveis devido ao desenvolvimento de tolerância. Nos casos de intoxicação leve podem aparecer sonolência e sedação, na intoxicação grave estão presentes os seguintes sintomas: depressão respiratória, coma, hipotensão e hipotermia (CHAPACAIS, 2020),

4.5.1 Tratamento

A descontaminação pode ser feita com a administração de carvão ativado 1g/kg, a lavagem gástrica não é necessária em pequenas e moderadas quantidades. O antídoto utilizado é o flumazenil, na dose de 0,1 a 0,2 mg IV em 15 a 30 segundos, podendo ser repetido em até 1mg. Nas intoxicações graves, pode ser necessária a infusão contínua de 0,1 a 1mg/h (TOLEDO, 2021). Nos casos de intoxicação confirmada deve ser preenchido o campo 66 da ficha de notificação como intoxicação por benzodiazepinas – T42.2.

4.6 Anti-Inflamatórios Não Esteróides (Aines)

São medicamentos que agem farmacologicamente por inibição da ciclo-oxigenase (COX 1 e COX-2) levando a uma redução de prostaglandinas e consequentemente diminuindo a dor e o processo inflamatório. A dose tóxica pode variar entre 5 a 10 vezes a dose terapêutica, conforme demonstrado no Quadro 4.

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Quadro 4: Características farmacológicas dos anti-inflamatórios não esteroidais

Substância	Dose máxima diária (mg)	Meia-vida plasmática (horas)	Observações
Ácido acetilsalicílico	3000 – 4000	Doses terapêuticas 12h. intoxicação: até 20 h	-
Diclofenaco	400	1 – 2	-
Ibuprofeno	3200	2	Overdose pode levar a coma, acidose metabólica e depressão respiratória.
Paracetamol	4000	2,4 – 4,0	-
Dipirona	4000	7	-
Indometacina	200	2,6 – 11,2	-
Cetoprofeno	300	1,5 – 4,0	Overdose pode levar a coma, depressão respiratória e convulsão
Cetorolaco	40 – VO 60 – 120 EV	4 – 10	Alto risco de insuficiência renal aguda
Ácido mefenâmico	1000	2 – 4	Overdose pode levar a espasmos e convulsões
Naproxeno	1500	13	-
Fenilbutazona	600	70	Overdose pode levar a convulsões e acidose
Sulindaco	400	11 – 19	Apresenta recirculação entero-hepática
Meloxicam	15	15 – 20	-
Tenoxicam	40	60 – 75	-
Etoricoxib	90	22	-
Piroxicam	20	50	Overdose pode levar a convulsão e coma
Celecoxibe	Adultos: 400 Crianças 100 - 200	8 – 12	-

Fonte: Adaptado de: atendimento inicial das intoxicações agudas. Coordenadoria de Vigilância em Saúde. <cvs.saude.sp.gov.br/up/MANUAL DE TOXICOLOGIA CLÍNICA - COVISA 2017.pdf> 2017. Acesso em: 24 jun. 2024.

4.6.1 Tratamento

As medidas de suporte devem consistir na hidratação adequada, monitoramento dos sinais vitais e administração de oxigênio suplementar, quando necessário. Em casos de intoxicação leve o tratamento com protetores de mucosa (inibidores da bomba de prótons, antagonistas do receptor H_2) podem ser suficientes, nos casos de ingestão de altas doses de meloxicam, diclofenaco e piroxicam, o uso de doses repetidas (16 a 24g/dia) de colestiramina tem demonstrado eficácia. Dentre os AINES, destaca-se o paracetamol por ter como antídoto a N-acetilcisteína, por aumentar os níveis de glutationa hepática, fazendo com que aumente a ação desintoxicante. O antídoto deve ser iniciado 8h após a ingestão.

Os regimes de administração devem ser feitos da seguinte forma: via oral - esquema de 72 horas, uma dose de ataque 140 mg/kg diluído a 5% em suco e manutenção com 17 doses de 70mg/kg com intervalos de 4 horas. Para a via endovenosa - esquema de 20 horas dividido em 3 fases: 150 mg/kg diluído em 200mL de solução glicofisiológica 5%, sendo administrado em 15 a 60 minutos; 50mg/kg diluído em 500mL de solução glicofisiológica, sendo administrado em 4

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horas e 100mg/kg diluído em 1000 mL de solução glicofisiológica 5%, sendo administrado em 6 horas (SOUZA, 2021).

Nos casos de intoxicação confirmada deve ser preenchido o campo 66 da ficha de notificação como intoxicação por salicilatos – T39.0 ou intoxicação por outros agentes anti-inflamatórios não-esteroides – T39.3.

4.7 Anticolinérgicos

Os medicamentos com este efeito englobam uma série de efeitos farmacológicos: antipsicóticos, antiespasmódicos e antidepressivos. O mecanismo de ação consiste na inibição da acetilcolina por competição em receptores muscarínicos periféricos e centrais. A dose potencialmente letal da atropina é de 10 mg em adultos. Nas intoxicações graves pode haver delírio, psicose, alucinações, bradicardia, convulsões, hipertermia e coma, devendo ser monitorado o ritmo cardíaco, eletrólitos, CPK, gasometria e glicemia (NERY, 2019).

4.7.1 Tratamento

A fisostigmina pode ser utilizada como um antídoto para intoxicações graves por anticolinérgicos, demonstrando ser eficaz e segura na prática clínica, a dose endovenosa pode variar entre 0,5mg a 6mg, podendo ser repetido após 40 minutos, se necessário. O tratamento sintomático é definido pelos sinais clínicos predominantes, no caso de agitação ou delirium pode ser administrado Diazepam EV 5 a 10mg em 1 a 3 minutos e para hipertermia com medidas de resfriamento corporal (HOFFMAN, 2015; CROWELL, 1967).

Nos casos de intoxicação confirmada deve ser preenchido o campo 66 da ficha de notificação como intoxicação por outros parassimpaticolíticos (anticolinérgicos e antimuscarínicos) e espasmolíticos não classificados em outra parte – T44.3.

4.9 Digitálico

A digoxina, principal representante desta classe, possui baixo índice terapêutico, fazendo com que a dose terapêutica efetiva permaneça sempre próxima a dose tóxica, para adultos 2 a 3mg pode desenvolver sintomas de intoxicação, doses acima de 5mg relacionados a toxicidade grave e maior que 10mg são potencialmente fatais. As manifestações clínicas nas intoxicações graves incluem bloqueio atrioventricular, vômitos choque, hiperpotassemia e arritmias ventriculares. A determinação laboratorial quantitativa de digoxina pode ser utilizada, níveis maiores que 2 ng/mL devem ser considerados de risco juntamente com os sinais de intoxicação, considerando os sintomas clínicos apresentados pelo paciente (HACK, 2015; BEAULIEU, 2024).

4.9.1 Tratamento

O antídoto disponível nos casos de intoxicação grave são os anticorpos anti- digoxina, sendo indicado nos seguintes casos: concentração sérica de potássio maior que 5,0 mEq/L, arritmias ventriculares, bradicardias progressivas e não resposta à terapia convencional. A dose varia conforme a quantidade ingerida, uma ampola (38mg) neutraliza 0,5mg de digoxina ou digitoxina, sendo administrado em infusão lenta em 30 minutos (ARQUEIRO, 2024).

O tratamento sintomático é baseado na correção dos distúrbios eletrolíticos (manter o potássio acima da normalidade, o magnésio no nível médio e cálcio em nível inferior), bradicardia pode ser tratada com atropina, nos casos de arritmia cardíaca a cardioversão elétrica deve ser evitada (ORNILLO, 2020)

Nos casos de intoxicação confirmada deve ser preenchido o campo 66 da ficha de notificação como intoxicação por glicosídeos estimulantes do coração e substâncias de ação semelhante – T46.0.

V. OPIÁCEOS E OPIOIDES

Os opiáceos são substâncias presentes na papoula do ópio e opioides são sintetizados para se obter efeitos semelhantes às substâncias opiáceas. São utilizados como analgésicos e estão associados a outros efeitos, incluindo euforia e dependência em curto prazo. A dependência de opioides,

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em curto prazo. A dependência de opioides, toxicidade aguda e a morte estão se tornando cada vez mais problemática no mundo.

Os opioides exógenos e endógenos atuam nos quatro principais receptores opióides (μ, δ, κ e opioid receptor-like 1), entre os sintomas de intoxicação e efeitos adversos, estão presentes a alteração de humor, sonolência, coma, bradicardia, arritmias, depressão respiratória e rigidez muscular (LAYNE, 2024).

5.1 Tratamento

A dose tóxica depende do agente envolvido, podendo ser utilizado como antídoto naloxona para reverter quadro de depressão respiratória, na dose 0,4 a 2 mg EV, podendo ser repetida a cada 2 a 3 minutos até a dose máxima de 10mg. Para pacientes com quadro de apneia a dose inicial deve ser 2 mg, para substâncias com ação prolongada pode ser feita a infusão contínua, a dose sugerida é de 2/3 da dose necessária para reverter a depressão respiratória (LEAL, 2020).

Nos casos de intoxicação confirmada deve ser preenchida a ficha de notificação como transtornos mentais e comportamentais devido ao uso de opiáceos - intoxicação aguda – F 11.0; transtornos mentais e comportamentais devido ao uso de opiáceos – uso nocivo para a saúde – F 11.1; transtornos mentais e comportamentais devido ao uso de opiáceos – síndrome de dependência – F11.2. Para os casos acidentais ou quando ocorre o óbito, deve ser preenchido o campo 66 da ficha de notificação de acordo com o agente envolvido, intoxicação por ópio – T 40.0; intoxicação por outros opiáceos - T 40.2 (codeína e morfina); intoxicação por metadona – T 40.3 ou intoxicação por outros narcóticos sintéticos - T 40.4 (meperidina).

5.2 Hormônio Tireoidiano

A principal substância utilizada para deficiências funcionais da glândula tireoide é a levotiroxina, sendo aplicada nos casos de hipotireoidismo. A reposição hormonal sintética com levotiroxina é recomendada na dose de manutenção de 1,6μg/kg, sendo ajustada a cada oito semanas, dependendo dos resultados dos exames de dosagem de TSH, T3 e T4 (Benabdelkamel, et al 2023). A intensidade das manifestações clínicas depende da concentração de T_3 e T_4, conforme demonstrado no Quadro 5.

Quadro 5: Exames Laboratoriais Diagnósticos Para Avaliação Da Função Da Tireoide

Exame zaboratorial	Valores normais	Observações
TSH	0,5 – 5,0 μLUI/mL	-
T3 livre	0,2 – 0,52 ng/dL	Aumento em casos de hipertireoidismo ou intoxicações exógenas (T3 ou T4)
T4 livre	0,7 – 1,86 ng/dL	Aumento em casos de hipertireoidismo ou intoxica- ções exógenas com levotiroxina

Adaptado de: BOUCHARD, N. C. Thyroid and antithyroid medications. Goldfrank's toxicologic emergencies; 2015. Acesso em: 25 ago. 2024.

5.1.1 Tratamento

Para doses superiores a 4000mcg, pode ser realizado a lavagem gástrica ou descontaminação com carvão ativado, nos casos de intoxicação aguda. O tratamento consiste no manejo de sintomas, incluindo hidratação, intubação orotraqueal considerando rebaixamento do nível de consciência, alteração do estado mental e excesso de estimulação adrenérgica. Pode ser utilizado beta-bloqueador, como o propranolol para o tratamento de arritmias e taquicardia. Para os casos de agitação psicomotora, a administração de benzodiazepínicos e barbitúricos deve ser benéfica. A administração de drogas que atuam no hipertireoidismo, como propitiluracil, pode ser administrado na dose de 25mg a cada 6h ou 8h ou metimazol 30 mg a cada 24 horas pode auxiliar impedindo a conversão de T4 para T3. Estudos tem demonstrado efetividade dos medicamentos utilizados para hipertireoidismo, nos casos de intoxicação (BEZERRA, 2020). Nos casos de intoxicação confirmada deve ser preenchida a

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ficha de notificação como intoxicação por hormônios tireoidianos e seus derivados – T38.1.

VI. CONCLUSÃO

As intoxicações por medicamentos devem ser tratadas como prioridade nas emergências devido ao alto risco de óbito. A toxicidade das substâncias está relacionada com a quantidade ingerida, tempo de exposição e a via utilizada. O preenchimento das fichas de notificação obrigatória é fundamental para estabelecer a incidência e prevalência de casos e suas localidades. O conhecimento interno hospitalar de situações que envolvam intoxicações por medicamentos é essencial para que se possa adquirir antídotos e outros fármacos necessários na estabilização e tratamento do paciente, evitando mortes e a piora clínica.

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Expression of Glioblastoma Molecular Markers and Sensitivity to Innate Immune Peptides LL-37 and Protegrin-1 as Predictors of Chemotherapy Response

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ABSTRACT

Glioblastoma (GBM) is one of the most aggressive and difficult-to-treat tumours in humans. The low efficacy of treatment is due to the molecular and cellular heterogeneity of the tumour, as well as disruption of biochemical mechanisms of the innate immune system, including peptides such as cathelicidin LL-37 and protegrin-1 (PG-1).

The Objective: to establish the effect of the combined use of LL-37, PG-1 and chemotherapy drugs on the expression of p53, GFAP, ATRX, Ki-67, TF, PDPN and EGFR proteins in GBM patient cell cultures, as well as their relationship to overall survival (OS) and life expectancy (LE) of patients.

Keywords: glioblastoma, antimicrobial peptides, LL-37, Protegrin-1, chemotherapy response, immunohistochemistry, tumour biomarkers, cell culture, EGFR signaling, patient-derived glioma cells.

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Expression of Glioblastoma Molecular Markers and Sensitivity to Innate Immune Peptides LL-37 and Protegrin-1 as Predictors of Chemotherapy Response

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ABSTRACT

Glioblastoma (GBM) is one of the most aggressive and difficult-to-treat tumours in humans. The low efficacy of treatment is due to the molecular and cellular heterogeneity of the tumour, as well as disruption of biochemical mechanisms of the innate immune system, including peptides such as cathelicidin LL-37 and protegrin-1 (PG-1).

The Objective: to establish the effect of the combined use of LL-37, PG-1 and chemotherapy drugs on the expression of p53, GFAP, ATRX, Ki-67, TF, PDPN and EGFR proteins in GBM patient cell cultures, as well as their relationship to overall survival (OS) and life expectancy (LE) of patients.

Materials and Methods: Cells were isolated from GBM biopsies obtained from patients (n = 30) and grown under standard conditions for 2 days. The cells were treated with different doses of the following chemotherapy drugs: temozolomide (TMZ), doxorubicin (DOX), carboplatin (CARB), cisplatin (CIS), etoposide (ETO) as well as peptides LL-37, PG-1, to determine the 50% inhibitory concentration (IC_{50}) using the MTT assay. The expression of p53, isocitrate dehydrogenase-1 (IDH1), glial fibrillary acidic protein (GFAP), ATP helicase chromatin (ATRX), proliferation antigen (Ki-67), transferrin (TF), podoplanin (PDPN), and epidermal growth factor receptor (EGFR) proteins in GBM cells was analysed using immunohistochemical (IHC) staining with specific antibodies. Associations between these proteins and OS were analysed using Graph Pad Prism 8.0 software.

Results: Statistically significant correlations were found between the expression of TF (r= -0.556, p=0.04), EGFR (r=0.799, p=0.03) and Ki-67 (r= 0.651 p=0.002) and TF (r=-0.899, p=0.004) in GBM cells and the LL-37, PG-1 IC_{50} values of respectively. The low LL-37 IC_{50} (less than 7 μM) and the high expression of p53 protein (20%, 12 vs 6.5 months, p=0.0037), GFAP (40%, 12 vs 9 months, p=0.0019) were associated with a longer life expectancy for the patients. In contrast, low sensitivity of GBM cells to LL-37 (higher 7 μM) and high expression of Ki-67 (15% or higher) were associated with a shorter life expectancy for the patients (10 vs 14 months, p=0.0452). Conversely, low EGFR expression (below 40%) and a low LL-37 IC_{50} were associated with a longer life expectancy (12 vs 6.5 months, p=0.0031). High sensitivity of GBM cells to PG-1 (less than 8 μM) and high expression of proteins p53 (20%, 12 vs 8 months, p=0.0126), GFAP (40%, 24 vs 10 months, p=0.0042), ATRX (higher 15%, 8 vs 14 months, p=0.0346), as well as low expression of Ki-67 (below 15%, 8 vs 14 months, p=0.0346) and EGFR (below 40%, 8 vs 12 months, p=0.0124) were associated with an increase life expectancy. Low EGFR expression (below 40%) and low sensitivity to PG-1 were

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associated with longer life expectancy (12 vs 8 months, p=0.0124).

Conclusions: The observed associations between the expression of p53, GFAP, Ki-67, and EGFR proteins in GBM cells, as well as ATRX expression and LL-37, PG-1 IC_{50} with OS of the patients, may potentially be used to develop a chemoimmunotherapeutic scheme for evaluating the efficacy of treatment in GBM patients.

Keywords: glioblastoma, antimicrobial peptides, LL-37, Protegrin-1, chemotherapy response, immunohistochemistry, tumour biomarkers, cell culture, EGFR signaling, patient-derived glioma cells.

I. INTRODUCTION

Glioblastoma (GBM) is one of the most aggressive and intractable tumours in humans [1]. The overall 5-year survival rate (OS) of patients in the United States is 6.9% and life expectancy is only 8 months, with relapses occurring in 100% of cases [2]. GBM therapy protocols include surgical resection of the tumour followed by radio- and chemotherapy with temozolomide (TMZ) [3]. It is assumed that the main reason for the low effectiveness of GBM therapy is molecular and cellular heterogeneity of the tumour and its microenvironment.

GBMs include tumor and stem cells that differ in their degree of differentiation, proliferative activity, ability to invade, metastasis, DNA repair mechanisms, and expression of multidrug-resistant proteins. The most common molecular alterations in GBM involve: wild-type and mutant forms of isocitrate dehydrogenase (IDH-wt, IDH-mut), methylation of the *MGMT* gene promoter (O6-methylguanine DNA methyl-transferase), ATRX, Ki-67, GFAP as well as mutations in genes encoding proteins such as: p53 (TP53), EGFR, and others [4]. Another reason associated with the progression of GBM and its resistance to therapy is the disruption of biochemical and molecular-cellular mechanisms of anti-cancer immunity. Cytotoxic T- and B-lymphocytes, plasmocytic cells and antibodies secreted by them, as well as proteins and peptides of innate immunity, such as defensins, cathelicidins, peroxidases, lactoferrin and lysozyme participate in its formation [5,6]. As components of the GBM microenvironment, they can determine the individual sensitivity of tumour cells to anti-cancer therapy. In this regard, the authors selected two peptides with different structures from the cathelicidin family: cathelicidin LL-37, with an α–helical structure, from human neutrophil azurophil granules, and protegrin-1 (PG-1), a peptide with a β-hairpin conformation, from pig neutrophils, to study their anti-cancer cer activity.

The Objective: to establish the effect of the combined use of LL-37, PG-1 and chemotherapy drugs on the expression of p53, GFAP, ATRX, Ki-67, TF, PDPN and EGFR proteins in GBM patient cell cultures, as well as relationship their to overall survival (OS) and life expectancy (LE) of patients.

II. MATERIAL AND METHODS

2.1 Patients

The study was conducted on 30 patients with GBM who were treated at the Polenov Neurosurgical Institute of the Almazov Centre (St. Petersburg, Russia) between 2021 and 2025. All patients underwent magnetic resonance imaging (MRI), tumor resection, and histological examination in a pathology laboratory. All patients underwent magnetic resonance imaging (MRI), tumour resection, and histological examination in a pathology laboratory. All patients received chemotherapy with TMZ or cisplatin, carboplatin, or etoposide for 2-8 courses from 2021 to 2025 at the Napalkov State Budgetary Healthcare Institution «Saint-Petersburg clinical scientific and practical center for specialised types of medical care (oncological)».

Inclusion Criteria: patients over 18 years old, primary histological diagnosis of GBM grade IV, availability of survival data and IHC expression markers.

Exclusion Criteria: patients under 18 years old, histological other brain tumour types than GBM,

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or unstable hemodynamics, severe somatic disease course, lack of life expectancy data or IHC markers. The primary endpoint of the study was neurosurgical surgery to remove the GBM. The final endpoint of the study was biological death.

The study was approved by the local ethics committee at the Institute of Experimental Medicine (no. 4/25 dated 25 December 2025).

2.2 Cell Culture

In sterile laminar flow conditions, GBM biopsies were cut into small fragments and treated with 0.25% trypsin-EDTA solution (Sigma-Aldrich, USA) at 37°C for 5 minutes. Isolated cells were counted, and 1×10^4 cells were transferred to each well of a 96-well plate (TPP, Switzerland), to which Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum (FCS) (Sigma Aldrich, USA) was added and incubated at 37.0°C and 5.0% CO_2 for two days [7].

2.3 MTT Analysis

To assess the anti-cancer effects of chemotherapy drugs, LL-37 and PG-1 on GBM cells, an MTT assay was performed [8]. We prepared 2-10-fold dilutions of chemotherapy drugs and a 2-fold dilution of LL-37 and PG-1 at a volume of 50 µl of DMEM (Table 1), which were added to each well of cell culture plates containing GBM cells. The plates were incubated at 37.0°C and 5.0% CO_2 for 24 hours. Then, 25 µl of MTT solution (5 mg/mL) was added to each well and left for 3 hours under the same conditions. At the end of the incubation, 50 µl of isopropanol (0.04 N) was added to all the wells. HCl was mixed, and the optical density of the solutions was measured using a SpectraMax 250 flat-bed spectrophotometer at wavelengths of 540 and 590 nm. The anti-cancer effect of the drug was determined by comparing the optical density of the wells containing GBM cells to those of positive and negative control wells, according to formula 1:

$$DC(\%) = \frac{OD(control)-OD(test)}{(OD(control)-OD(0\%\,VC))}\times 100 \qquad (1)$$

Where DC (%) is the percentage of dead cells, OD (test) is the optical density of wells with cells when a drug is added at a given dose, OD(0% VC) is the OD of the control wells with the nutrient medium, and OD (control) is the density of the cells in the wells without the addition of any drugs.

2.4 Determination of IC_{50} for Chemotherapy Drugs, LL-37 and PG-1

The *in vitro* anti-cancer effects of chemotherapy drugs and LL-37, PG-1 were evaluated based on the calculation of their 50% inhibitory concentration (IC_{50}). GBM cells were incubated with doxorubicin (DOX, Doxorubicin-LANS®, 2 mg/ml, Veropharm, Russia), etoposide (ETO, 20 mg/ml, Ebewe Pharma, Austria), carboplatin (CARB, Carboplatin-LANs®, 10 mg/ml, Veropharm, Russia), temozolomide (TMZ, Temodal capsules, 100 mg, Orion Pharma, Finland), cisplatin (CIS, Cisplatin-LANS®, 0.5 mg/ml, Veropharm, Russia), as well as human cathelicidin LL-37 (LL-37, Anaspec, USA), and porcine protegrin-1 (PG-1, SynPep, USA). The cells were incubated with each drug at different concentrations, as shown in Table 1.

Table 1: The doses of chemotherapy drugs and LL-37, PG-1 used to calculate IC_{50}

Drugs	Dose, µM
DOX	7.3; 18.4; 36.8; 73.6; 460.0; 920.0
CARB	134; 269; 673; 1 350; 2.690; 26.900
TMZ	155; 386; 773; 1.550; 5.150; 15.500
CIS	16.1; 33.2; 83; 166; 332; 830; 1.660
ETO	0.8; 1.6; 3.3; 6.7; 13.5; 27
LL-37	1.0; 2.0; 4.0; 8.0; 16.0; 32.0
PG-1	2.0; 4.0; 8.0; 16.0; 32.0; 64.0

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2.5 Immunohistochemical Analysis

GBM samples from 30 patients were immunohistochemically (IHC) tested for the expression of IDH1, GFAP, p53 (TP53), EGFR, Ki-67, ATRX, and PDPN in GBM tissues. The samples were fixed in 10% formalin and poured into paraffin blocks. Sections of 5 microns were prepared and de-waxed in xylene. They were then transferred to a 0.3% hydrogen peroxide solution with methanol for 30 minutes, after which they were washed in a phosphate buffer. All sections were incubated with primary antibodies for 12 hours to detect proteins. The primary antibodies used were IDH1-R132H (clone HMab-1.5 µg/ml, Dako, Denmark) [9], p53 (clone DO7, 1:50, Dako, Denmark), EGFR (clone EP38Y, 1:100, Abcam plc, UK), and GFAP (ASTRO6, 1-2 µg/ml, ThermoFisher, USA), Ki-67/MIB-1 (1:100, Immunotech, Germany), ATRX (0.5 mg/ml, Sigma-Aldrich, USA) [9,10,11]. Immunohistochemical analysis of ATRX, p53, GFAP, EGFR, and Ki-67/MIB-1 was performed using the streptavidin-biotin-peroxidase method with the LSAB2 kit from Dako Glostrup (Denmark) [12,13].

2.6 Statistical Analysis

All experiments were performed in triplicate. The data are presented as an average and standard deviation, and were considered significant at $p < 0.05$. A nonparametric Mann–Whitney U test was used to compare differences between two independent groups with a small sample size (<30) [14]. Descriptive statistics and Kaplan–Meier survival analysis were performed using GraphPad Prism version 8.0.1 (21/09/2020, San Diego, CA, USA).

III. RESULTS

First, an IHC staining was conducted to examine the expression levels of marker proteins p53, GFAP, ATRX, Ki-67, and EGFR in GBM cells from patients as well as their in vitro sensitivity to chemotherapy drugs, LL-37 and PG-1 (Table 2).

Table 2: Determination of the Effectiveness of Chemotherapy and Peptides on Gbm Cells of the Patients

ID patients	IC_{50}, µM						
	DOX	CARB	TMZ	CIS	ETO	LL-37	PG-1
11081	290.4	29,431.0	16,179.5	2,448.4	27.0	10.3	16.0
11961	3,350.3	39,792.9	43,539.3	11,919.7	86.5	32.2	123.6
6770	850.0	4,000.0	14,000.0	10,90.0	26.3	9.5	8.7
7934	50.9	2,000.0	7,491.0	200.0	7.5	2.0	1.2
49142	548.3	2,708.4	11,056.0	776.0	11.4	6.6	7.4
25873	560.0	888.8	8,619.2	300.0	8.9	24.1	30.1
57595	16.9	3,093.6	194.5	1,682.3	7.5	8.3	8.6
55068	546.5	27,574.5	4,789.5	11,04.8	11.8	6.4	3.9
15159	179.2	116.4	436.8	698.1	11.4	32.1	15.8
62642	20.3	42,495.1	24,015.7	1,158.5	32.3	28.1	34.3
60886	278.8	4,498.0	2,174.3	>1,660.0	6.3	1.1	1.2
18871	2,682.8	24,031.9	11,976.9	1,776.4	30.9	24.3	23.8
114495	3,350.3	39,792.9	43,539.3	965.8	86.5	26.8	35.4
10677	1,180.1	20,471.8	1,309.1	2,448.4	3.4	3.5	7.4
1401	920.0	5,136.5	611.8	261.2	10.3	4.0	16.0
19872	2,682.8	24,031.9	11,976.9	1,776.3	30.9	24.3	23.8
8989	817.1	20,195.2	14,486.0	1,218.8	26.3	9.7	12.1
20939	920.0	17,861.9	15,500.0	476.5	38.0	11.8	19.3
39114	3,458.6	25,000.0	12,282.1	1,824.2	32.8	26.8	26.2
40906	1,083.2	38,147.6	1,4961.7	1,596.1	58.9	20.8	19.4
48993	–	1,126.8	15,407.5	120.4	38.7	3.1	14.1
48307	1,260.3	20,852.7	1,510.7	1,280.8	9.5	5.7	6.2
9439	1,513.2	26,116.5	22,206.3	1,784.9	41.3	7.2	0.8

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10448	478.7	24,237.2	14,659.1	1,299.0	3.4	1.9	4.3
27980	733.4	2,223.4	5,345.6	835.3	9.3	17.5	1.9
12645	483.6	2,605.4	5,258.3	729.8	7.0	7.2	3.9
7593	1,123.9	2,110.4	14,905.5	298.9	26.8	8.6	13.2

All patients were categorized as wild-type (IDH1-wt). This data allowed for a correlation analysis between the expression of these markers in GBM cells and chemotherapy drugs, LL-37 and PG-1 IC_{50} values, Table 3.

Table 3: Correlations Between the Expression of Markers in GBM Cells and the Chemotherapy Drugs, LL-37 and PG-1 IC_{50}

Drugs	ATRX	GFAP	Ki-67	P53	EGFR	PDPN	TF
DOX	0.576 p=0.019	-0.584 p=0.01	-0.101 p=0.615	-0.231 p=0.446	0.395 p=0.438	0.400 p=0.251	-0.421 p=0.224
CARB	0.221 p=0.411	-0.075 p=0.300	0.455 p=0.016	0.231 p=0.448	-0.337 p=0.513	0.247 p=0.490	-0.494 p=0.146
TMZ	0.586 p=0.017	-0.580 p=0.002	0.093 p=0.643	-0.203 p=0.504	0.367 p=0.474	-0.197 p=0.586	-0.627 p=0.04
CIS	0.094 p=0.728	-0.436 p=0.02	0.179 p=0.368	-0.142 p=0.642	0.237 p=0.651	0.413 p=0.235	-0.433 p=0.210
ETO	0.486 p=0.05	-0.358 p=0.06	0.392 p=0.04	-0.185 p=0.544	0.418 p=0.409	0.036 p=0.921	-0.719 p=0.019
LL-37	0.483 p=0.05	-0.320 p=0.103	0.268 p=0.176	-0.191 p=0.532	0.799 p=0.03	0.293 p=0.411	-0.556. p=0.04
PG-1	0.190 p=0.480	-0.331 p=0.09	0.651 p=0.002	-0.244 p=0.420	0.550 p=0.08	-0.277 p=0.437	-0.899 p=0.004

Note: Statistically Significant Correlations (P<0.05) are Indicated in Bold.

Statistically significant positive correlations were found between the degree of Ki-67 expression and IC_{50} in GBM cells for CARB (r=0.455, p=0.016), ETO (r=0.392, p=0.04), ATRX and DOX (r=0.576, p=0.019), TMZ (r=0.586, p=0.017). All these data show that the expression of Ki-67 and ATRX is associated with an increase in the chemotherapy drugs IC_{50} and the development of GBM chemoresistance. Statistically significant negative correlations were also found between the expression levels of GFAP and TMZ IC_{50} values (r=-0.580, p=0.002), DOX (r=-0.584, p=0.01); TF expression and TMZ IC_{50} (r=-0.627, p=0.04) and ETO (r=-0.719, p=0.019). The revealed correlations indicate the involvement of GFAP and TF in increasing the chemosensitivity of GBM cells. Statistically significant negative and positive correlations were found between the expression of TF (r=-0.556, p=0.04) and EGFR (r=0.799, p=0.03), and LL-37 IC_{50}. It indicates the involvement of these proteins in increasing and decreasing the sensitivity of GBM cells to a peptide. A significant positive correlation was found between PG-1 IC_{50} and Ki-67 expression (r=0.651, p=0.002). It shows that the expression of this marker is associated with an increase of PG-1 IC_{50} and the development of resistance to PG-1 in GBM. There was also a statistically significant negative correlation between PG-1 IC_{50} and TF expression (r= -0.899, p=0.04), indicating the involvement of TF in increasing sensitivity to the peptide in GBMs.

In the next stage, the associations between marker expression and the sensitivity of GBM cells to chemotherapy drugs, LL-37 and PG-1 with the OS of the patients were examined, Figures 1,2. We previously studied the sensitivity of GBM to chemotherapy drugs [15], and in this paper, we found that high DOX IC_{50} (13 versus 7.5 months; p=0.0446) and CARB (12 versus 5 months; p=0.0015) are associated with increased patient survival compared to low drug levels. In contrast, patients with high sensitivity to cisplatin (CIS)

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had more extended life expectancy (LE) than those with low sensitivity (12 vs 7 months, p=0.0293).

It should be noted that when combining LL-37 and PG-1 IC_{50} with the protein expression levels in GBM cells, significant associations between these indicators and the life expectancy of patients were revealed. The graphs in Figures 1A and 1B show that high sensitivity of GBM cells to LL-37 (20%, LE 12 vs 6.5 months, p=0.0037) and high p53 expression (40% vs. 9 months, p=0.0019) are associated with a longer life expectancy for patients. Interestingly, high expression of ATRX (>15%) and low LL-37 IC_{50} (<7 μM) was associated with a more extended lifespan (12 vs. 6.5 months, p=0.0009), as compared to low expression of ATRX and a high value of the peptide, Fig. 1C. In contrast, low sensitivity to GBM cells of LL-37 (>7 μM), and high Ki-67 expression (>15%), were associated with shorter survival time in patients (9 vs. 12 months; p=0.05), Fig. D. Also, lower EGFR expression (<40%) and lower LL-37 IC_{50} were associated with more extended survival time (12 versus 6.6 months, p=0.031), Fig. 1E. The median life expectancy of patients with a low LL-37 IC_{50} (less than 7 μM) was statistically significantly higher than that of patients with high LL-37 IC_{50} (LE 14.0 versus 3 months, p=0.0147), Fig. 1F.

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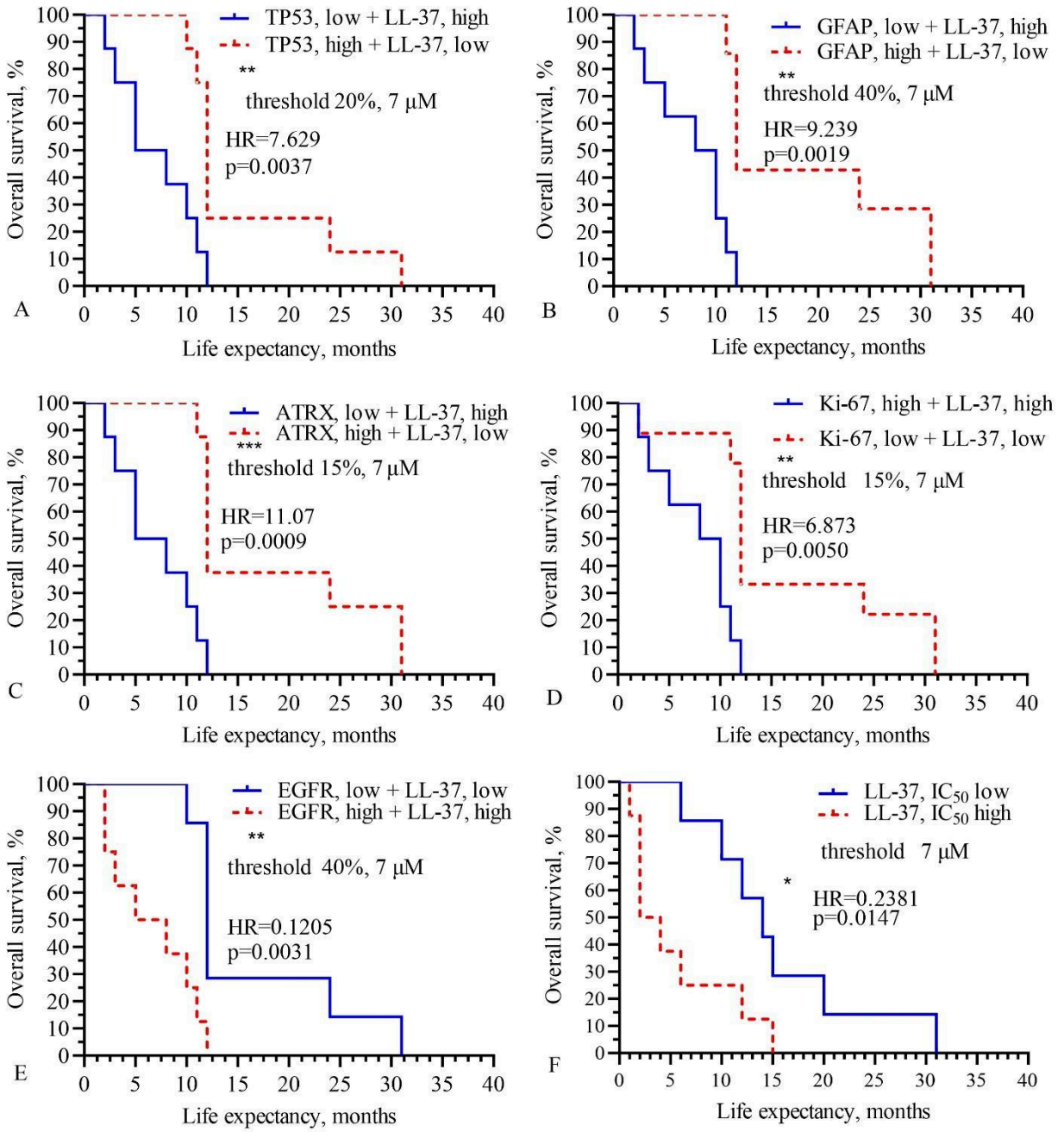

Fig. 1: Associations between the combinations of LL-37 IC_{50} and expression of A) p53, B) GFAP, C) ATRX, D) Ki-67 and E) EGFR proteins in GBM cells and OS of patients. F) Association between LL-37 IC_{50} and OS of GBM patients. Mantel-Cox test, χ^2, *p<0.05, **p<0.01 and ***p<0.001 indicate statistically significant associations of the OS for GBM patients with the combination of the proteins` expression and LL-37 IC_{50}.

High sensitivity of GBM cells to PG-1 (less than 8 µM) and high expression of p53 proteins (20%, LE 12 vs. 8 months, p=0.0126), GFAP (40%, LE. 24 vs. 10 months, p=0.0042), ATRX (>15%, LE 8 vs. 14 months, p=0.0346), as well as low expression Ki-67 (<15% LE 8 vs 14 months, p=0.0346) and EGFR (less than 40%, LE 8 vs 12 months, p=0.0124), were associated with increased OS in patients, Fig. 2.

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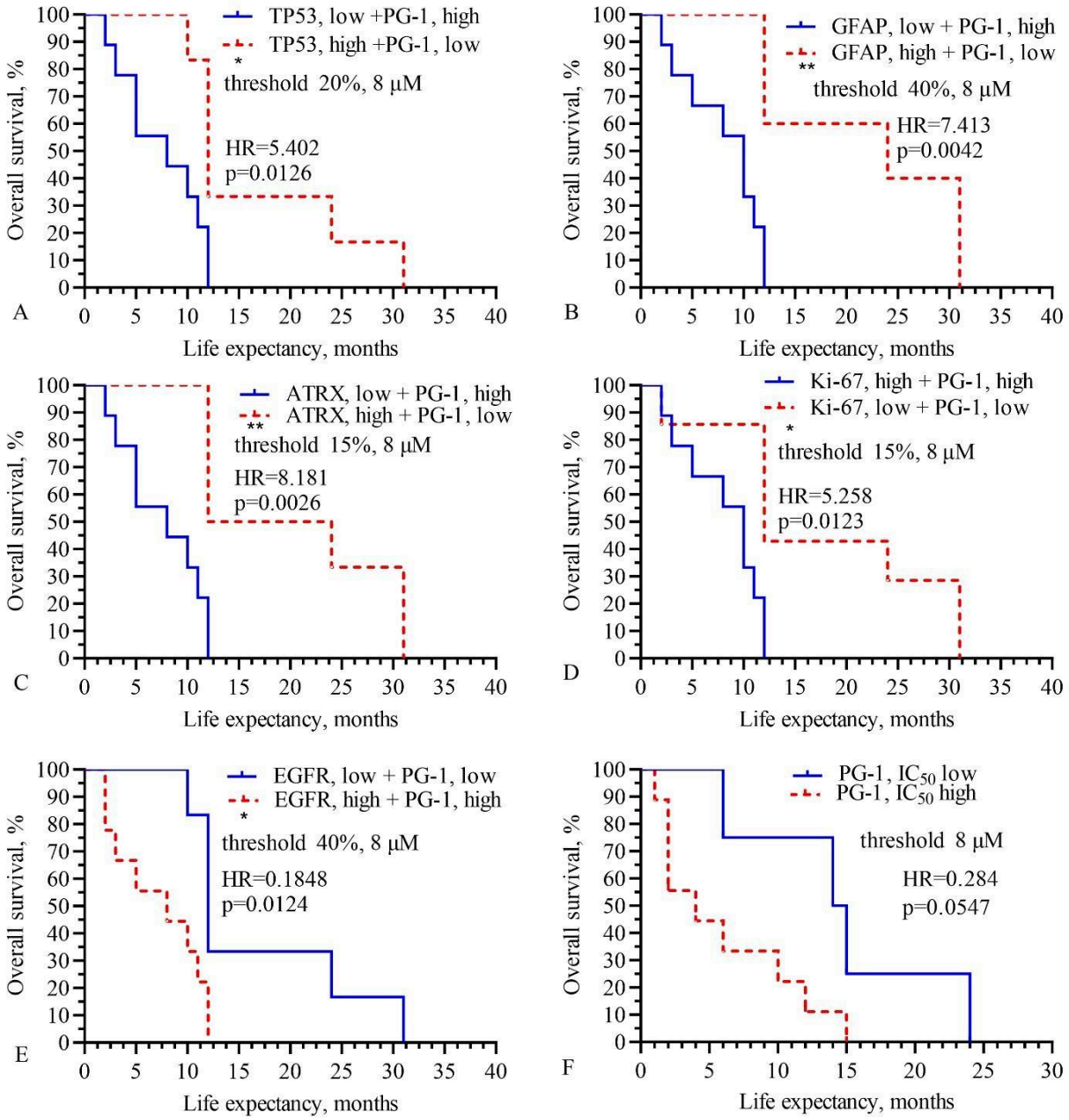

Fig. 2: Associations of the combination of PG-1 IC_{50} and the expression of: A) p53, B) GFAP, C) ATRX, D) Ki-67, D) EGFR proteins in GBM cells with OS of the patients and E) Associations of PG-1 IC_{50} and the OS of the GBM patients. Mantel-Cox test, χ^2, *p<0.05, **p<0.01 and indicate statistically significant associations of the OS for the GBM patients with the combination of the proteins` expression and PG-1 IC_{50}.

Low EGFR expression (less than 40%) and low sensitivity to PG-1 were associated with more extended LE (12 vs. 8 months, p = 0.0124), Fig. 2D. The LE of patients with high sensitivity to GBM cells for PG-1 (8 µM threshold, 14.5 vs. 4 months, p=0.05) was on the verge of a statistical difference, Fig. 2F.

IV. DISCUSSION

The positive correlations between the values of LL-37, PG-1 IC_{50}, in combination with EGFR and Ki-67 expression in GBM cells, can be explained by the fact that tumour cells with peptide resistance have chemoresistance combined with cell proliferation and tumour progression [16]. On

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the other hand, the negative correlation between the LL-37, PG-1 IC_{50} and TF expression indicates the involvement of this iron-transported protein in ferroptosis. This is confirmed by the fact that a high expression of TfR2 receptors on GBM cells correlates with a higher sensitivity of GBM to TMZ and the OS of patients [17,18]. The association of high expression of p53, GFAP, and ATRX with GBM cells, and low of LL-37 IC_{50}, with increased OS in patients, can be partly explained by the mechanisms of action of these proteins and their effects on therapy. For example, the p53 protein is known to activate apoptosis mechanisms in GBM cells [19], therefore, its expression is associated with a decrease in tumour volume and an increase in OS of patients (p=0.0399) [20]. The GFAP protein is involved in the differentiation of neurons and glial cells. Therefore, its high expression in GBM cells is associated with a higher OS (p=0.0022) in patients than in individuals whose tumours are negative for the expression of this protein [21]. Gulten G. et al. showed that among 83 patients with GBM, a decrease in ATRX expression was associated with a life expectancy of 17.25 ± 2.95 months (median 15 months). In contrast, patients without a decrease in ATRX expression had an average life expectancy of only 11.66 ± 1.43 months, with a median of 8 months [22]. On the other, the expression of Ki-67 nuclear antigen is associated with the number of tumour cell divisions. Therefore, its number less than 15% is significantly correlated with a higher OS in patients (p=0.005) [23]. Also, since EGFR is involved in the proliferation and resistance of GBM cells to therapeutic effects, its high expression correlates with low life expectancy for patients [24]. The cytotoxic effect of LL-37 on GBM cells can be explained by the presence of lysine residues with a positive charge in the peptide molecule. This was confirmed by a study by Guo X et al., in which the authors replaced lysine residues in neutral amino acid peptides TsAP1 and TsAP2 from the Brazilian yellow scorpion, *Tityus serrulatus*. These changes significantly enhanced the anti-cancer activity of these peptides [25].

The anti-cancer effect of LL-37 has also been established in other cancer models. For example, it was found by Mader J.S. et al that LL-37 induces apoptosis in Jurkat T-cell leukaemia cells as a result of the activation and translocation of mitochondrial AIF factor into the nucleus, where it induces chromatin condensation and DNA fragmentation [26]. This mechanism suggests the involvement of LL-37 in epigenetic modifications of DNA histones [27]. Fan R et al. found that LL-37 has a cytotoxic effect on colorectal cancer HCT116 cells and, in combination with docetaxel, suppresses angiogenesis and increases survival of BALB/c nude mice [28].

V. CONCLUSIONS

The results of the study indicate a statistically significant correlation between the expression of TF, EGFR and LL-37 IC_{50} in GBM cells, as well as the expression of Ki-67 and TF and PG-1 IC_{50}, indicating involvement of these marker proteins in modulating sensitivity of GBM to LL-37, PG-1. Associations were found between combinations of p53, GFAP, Ki-67, EGFR, ATRX proteins expression and LL-37, PG-1 IC_{50} in GBM cells with OS of patients, which could be used to predict efficacy of chemoimmunotherapy in GBM patients.

Conflict of Interest: The authors declare no conflict of interest.

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Compliance with Patient Rights and Principles Of Bioethics

The study protocol was approved by the local ethics committee of the Federal State Budgetary Scientific Institution "Institute of Experimental Medicine" No. 4/25 dated December 25. 2025.

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Effectiveness and Safety of Treatment of Double Chin with Combined Enzymatic Therapy

Diana Forero, Carlos Lloreda, Ana Toro, María Cristina Cuello & Jorge López Berroa

ABSTRACT

Introduction: Excess fat in the submental area (FSA) is associated with a negative impact on facial harmony and self-perception, often leading to decreased patient satisfaction with appearance and increased demand for aesthetic interventions.

Methods: in this prospective, multicentric, cohort study Pbserum HA 1.5 Medium, a combined enzymatic therapy (collagenases G/H PB220, lyase PB72K, lipase PB500), together with non cross-linked high molecular weight hyaluronic acid (HMW-HA)), was used for treating FSA in healthy adults according to a recommended protocol. Primary efficacy outcome was the variation of FSA in every visit, quantified by a scale ranging from 0 (no localized FSA) to 4 (extreme submental convexity). Patient-reported outcomes were also considered.

Results: 33 patients (median age: 44; range 21-72; 79% females) were evaluated at visit 1; 87.8% completed the study. At baseline, excess FSA scored ≤ 2 was present in 53.3% of patients. After two treatment sessions, proportion increased to 75.8%. Proportion of patients with an FSA score of 4 was reduced from 26.67% to 6.9%. FSA scale was significantly reduced from baseline to final visit ($p<0.05$). Regarding safety, 36 % of the patients reported adverse effects, which included itching (23%), pain (23%), edema (15%), isolated rash (8%), dysphonia (8%) and discomfort (8%).

Keywords: double chin, collagenase, lyase, lipase, hyaluronic acid, combined enzymatic treatment.

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Effectiveness and Safety of Treatment of Double Chin with Combined Enzymatic Therapy

Diana Forero[α], Carlos Lloreda[σ], Ana Toro[ρ], María Cristina Cuello[Ↄ] & Jorge López Berroa[¥]

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Conclusions: Pbserum HA 1.5 Medium was associated with significant reduction of excess FSA in a three-week period, favorable patient-reported outcomes and a good tolerability profile.

Keywords: double chin, collagenase, lyase, lipase, hyaluronic acid, combined enzymatic treatment.

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I. INTRODUCTION

Beauty standards increasingly favor a healthy and youthful appearance [1]. Both the shape and the contour of the chin play a relevant role in facial aesthetics, and fat accumulation in the submental area (FSA, commonly known as “double chin”) has been associated with a negative impact on appearance and self-perception [2]. While the accumulation of fat in this region is often associated with elevated body mass index (BMI) and lifestyle factors, genetic predisposition and age-related changes in soft tissue distribution can also contribute to its development. Moreover, FSA can be resistant to conventional weight loss strategies, such as caloric restriction and physical activity, due to the localized nature of fat deposits and the structural characteristics of submental fat pads [3]. This resistance to reduction through lifestyle modification underscores the need for targeted therapeutic approaches aimed specifically at submental contouring.

Several therapeutical strategies have been developed to address localized fat deposits [1]. These include pharmacologic treatment (e.g. local injection of deoxycholic acid) [4] surgical rejuvenation techniques such as cervicoplasty [5], targeted liposuction [2] and energy-based devices including laser and radiofrequency [6]. These treatments focus on tightening the submental skin and improve contour. However, several of these strategies require surgical procedures, which carry

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inherent risks. By contrast, enzymatic therapy represents a novel and promising advancement in aesthetic medicine. It targets specific extracellular components with high affinity and specificity [7], enabled by advances in recombinant technology and genetic engineering [8]. These enzymes are characterized by their high purity levels and can be produced in large quantities.

A combined approach involving high molecular weight (>400 kDa) hyaluronic acid (HMW-HA), collagenase, lipase and lyase has emerged as an attractive strategy for managing FSA. HMW-HA is a volumizing molecule that hydrates tissues, facilitates enzyme diffusion across the extracellular matrix, and acts as a physical barrier that helps modulate inflammatory responses [9]. Collagenase G/H degrades non-functional collagen fibers [10]. Lipase contributes by breaking down adipose tissue [11]. And lyase hydrolyzes proteoglycans and increase skin tissue permeability [12,13]. Each component plays a distinct role, but together, their actions are synergistic.

The main goal of our study was to evaluate the efficacy of Pbserum HA 1.5 Medium for the treatment of FSA in an outpatient clinical setting.

II. PATIENTS AND METHODS

2.1 Study Design

A prospective, quasi-experimental cohort study was performed in Colombia. Inclusion criteria were: (a) age between 18 and 70 years (b) proper understanding of the clinical study; (c) basal good clinical and mental health status, as determined by local main investigator; (d) availability to regular visits to the research center; (e) willing to participate in the study. Exclusion criteria were: (a) history of allergic reaction to HA, collagenases, lipases and/or lyases; (b) history of malignancies; (c) use of any treatment for fat accumulation; (d) current participation in another clinical trial; (e) history of comorbidities that could reduce treatment adherence; (f) pregnancy or breastfeeding.

Before entering the study, all patients underwent a complete history and physical examination. They received detailed information regarding the study's objectives, potential risks and benefits, and their rights as subjects. They provided written informed consent, which included authorization for product application, photographic documentation, data processing, and the publication of collected data and images.

2.2 Product Characteristics

Pbserum HA 1.5 Medium (Proteos Biotech, S.L.) includes 1,5 mL of sterile 0.1% HA, obtained by fermentation and purification from *Streptococcus equi* var *zooepidermicus;* a lyophilized vial containing specific proportions of Lipase, Collagenase G/H and Lyase and a vial with normal sterile saline (18 mL). After reconstitution of the lyophilized enzyme cocktail with 1.5 mL of HA, a necessary volume of reconstitution buffer was added according to the area to be treated.

2.3 Application Protocol

Visits were scheduled in Day 1 (visit 1), Day 8 (visit 2) and Day 22 (visit 3). Pbserum HA 1.5 Medium was administered according to the standard dose per area (1 mL/cm^2) by deep infiltration with retro-injection in the adipose tissue in specific points (Figure 1) in Day 1(visit 1) and Day 8 (visit 2). A final evaluation visit (visit 3) was scheduled on Day 22, fifteen days after the last application, to assess the final outcomes of the study. In all visits, FSA was quantified, and a photographic registry was obtained.

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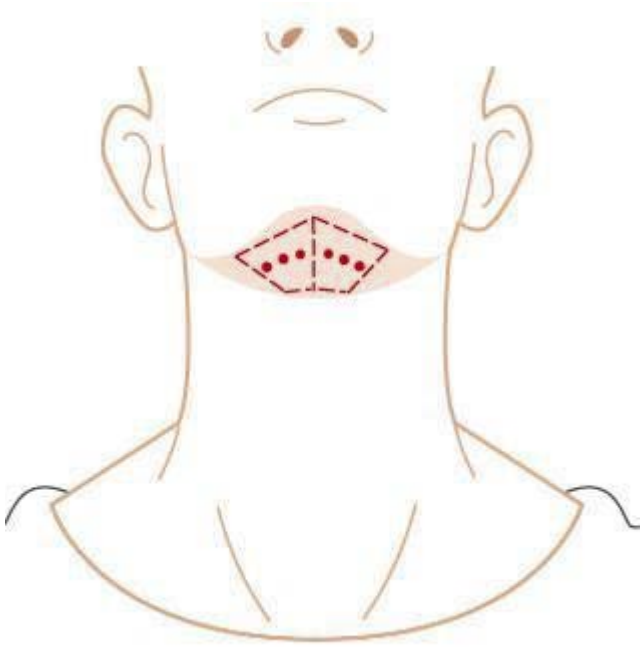

Figure 1: Application Points in the FSA

2.4 Study Outcomes

The primary objective of the study was to evaluate the efficacy of subcutaneous enzyme cocktail for the reduction of FSA in a controlled outpatient setting as measured by the variation of FSA severity assessed by the investigator at each visit using a validated grading scale ranging from 0 (no localized FSA) to 4 (extreme submental convexity due to FSA) (Table 1).

Secondary endpoints included (a) Patient satisfaction, quantified by 7-point Likert scales in a survey with two domains (global impression and organoleptic characteristics); (b) Patient's perception of efficacy, quantified by 5-point Likert scale covering three domains (subjective FSA reduction, flaccidity reduction and mandibular profile); and (c) safety and tolerability, evaluated through adverse event reporting and patient-reported outcomes.

Table 1: FSA Scale

Scale	0	1	2	3	4
Submental convexity	Absent	Mild	Moderate	Severe	Extreme
Description	No evident localized FSA	Minimal localized FSA	Prominent localized FSA	Marked localized FSA	Extreme submental convexity

2.5 Statistical Analysis

A sample size of 30 participants with a dropout rate of 10% was estimated. A descriptive analysis of the quantitative variables was performed, including parameters of central tendency and variation to define the variables distribution.

The Wilcoxon Signed-Ranks test was used to compare FSA scores variation in all time points. Specific stats packages of R software (*wilcox.test* and *lme* functions) were used. The significance value established for all statistical tests was $p < 0.05$.

III. RESULTS

Thirty-three patients were evaluated at baseline (visit 1), and 87.8% (n = 29) completed the study.

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Pbserum HA 1.5 Medium was administered at all prespecified injection sites in each participant without any protocol deviations. The median age of participants was 44 years (range: 21 to 72) and 79% were female.

The primary efficacy endpoint was the change in excess FSA. During the first visit, an FSA score ≤ 2 was present in 53.3% of patients. After two treatment sessions, this proportion increased to 75.8% (Figure 2). The proportion of patients with a maximum FSA score of 4 was reduced from 26.67% at visit 1 to 6.9% at the final follow-up visit. Overall, FSA scale was significantly reduced from baseline to the end of the study ($p < 0.05$, Wilcoxon Signed-Rank test) (Figure 3).

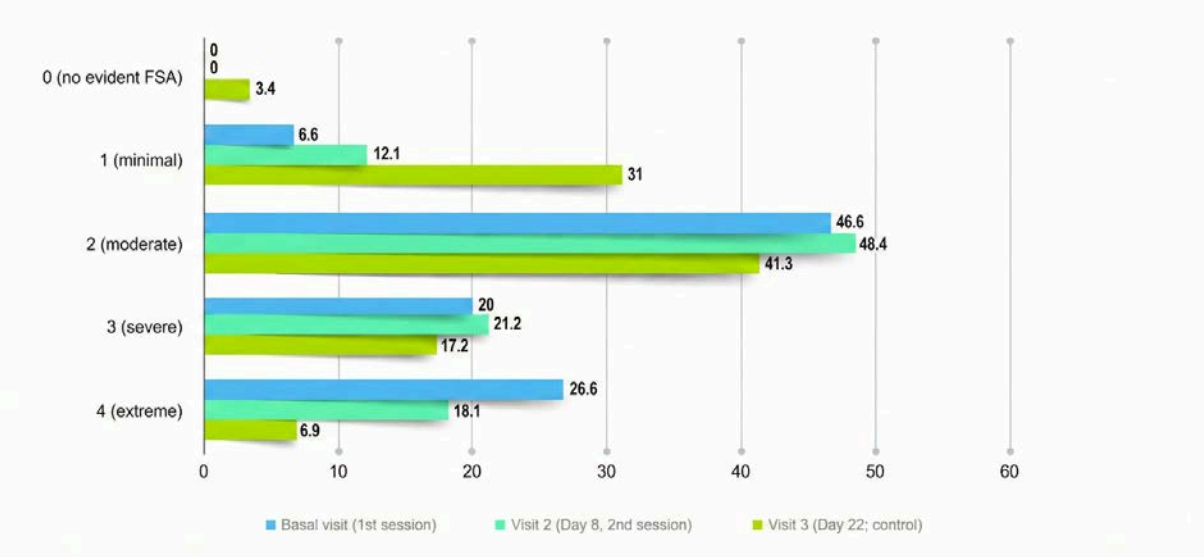

Figure 2: Distribution (%) of Patients According to FSA Score in Each Visit

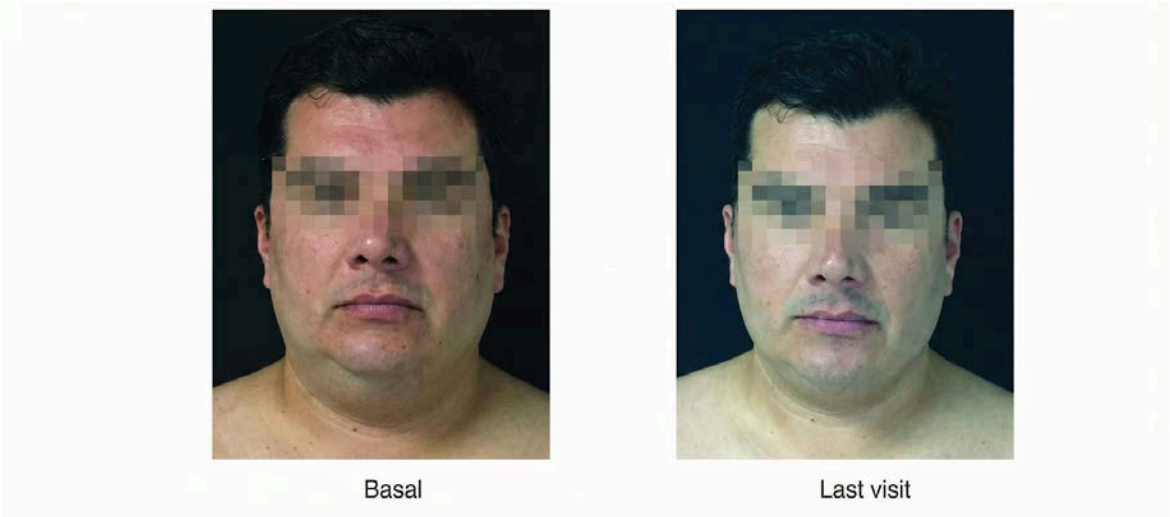

Figure 3: Reduction of Excess Fat in the Submental Area in a Male Patient

Regarding patient endpoint, 100 % of participants felt satisfied with the combined enzymatic treatment (50%, 43% and 7% liked it "very much", "moderate" and "somewhat", respectively), in the "global impression" domain. Organoleptic properties were also assessed using Likert scales and are summarized in Figure 4. Patients were also asked if they would use the product again and 83% responded affirmatively. \

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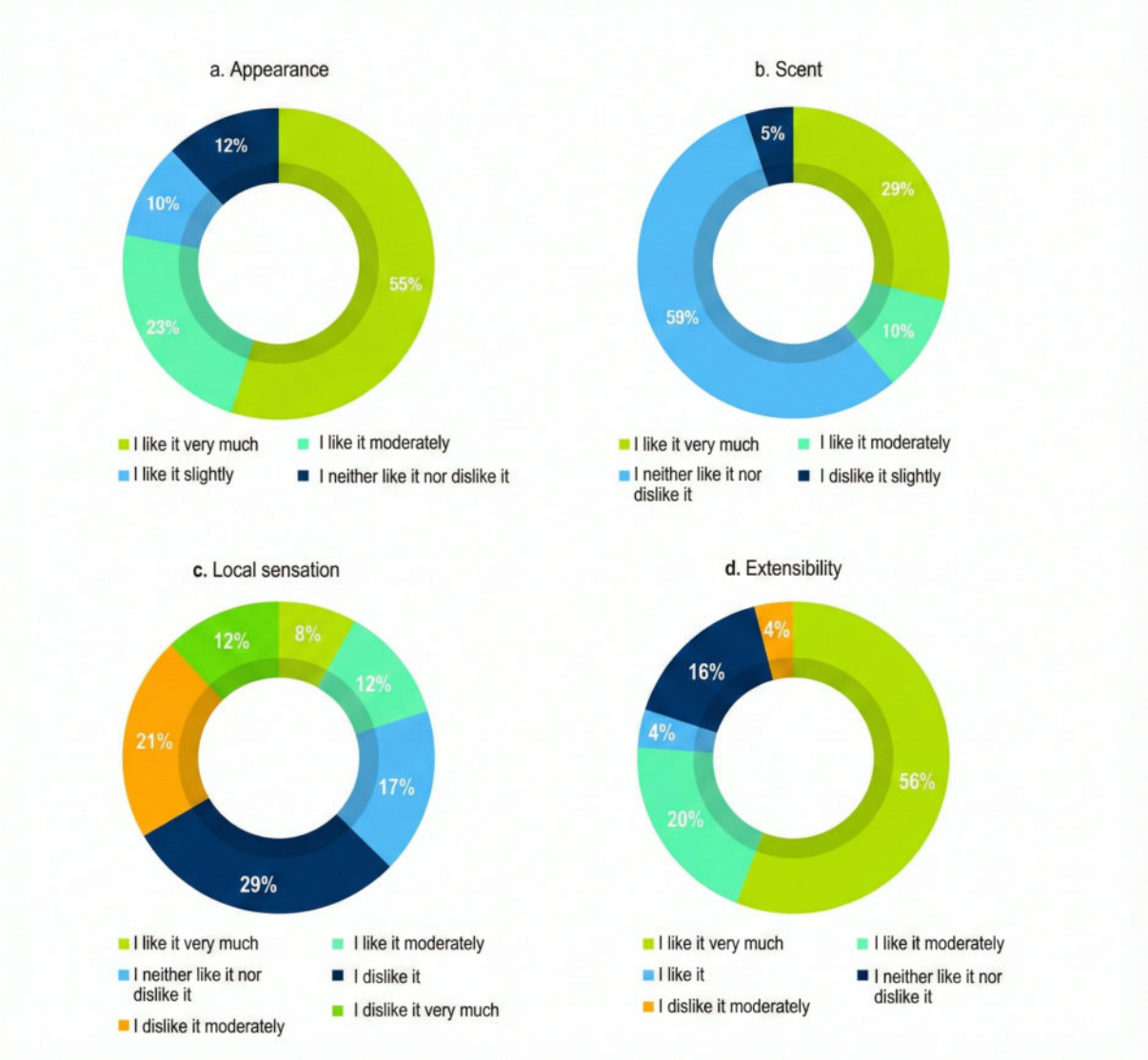

Figure 4: Subjective Opinion about Organoleptic Properties of Ha 1.5 Medium

Patient's perceptions on efficacy revealed that 38% of patients reported subjective improvement in FSA volume, 77% reported reduced flaccidity, and 83% noted improvement in mandibular contour.

Pbserum HA 1.5 Medium™ was well tolerated and 64% of participants did not refer any adverse event. The remaining patients described mild itching (23%), pain (23%), edema (15%), isolated rash (8%), dysphonia (8%) and discomfort (8%).

IV. DISCUSSION

Two sessions of Pbserum HA 1.5 Medium were associated with a significant reduction of excess FSA in a three-week period. This favorable outcome was accompanied by high patient-reported satisfaction and a good tolerability profile. Our findings are consistent with those reported by Jabbour et al. (2024), who observed a submental fat reduction of more than 10% in 9 out of 10 patients treated with the recombinant enzyme cocktail. Furthermore, 9 out of 10 participants expressed overall satisfaction with the treatment, highlighting both its efficacy and patient acceptability [14]. This high level of patient satisfaction with the use of recombinant enzymes for the management of skin laxity and localized fat accumulation in facial and body areas has also been documented in a recent multicenter study involving 229 cases [15].

Neck rejuvenation strategies, including those targeting the chin area, are progressively evolving to address the clinical and esthetic consequences

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of aging. These include changes in skin quality, muscle tone, sun-induced damage, or fat accumulation resulting from weight fluctuations or hormonal changes [16-18]. Specifically, interest in jowl and jawline rejuvenation procedures is increasing; according to a 2017 report of the American Society for Dermatologic Surgery, 73% of respondents of a consumer survey reported that they were somewhat to extremely bothered by excess of FSA [19].

FSA is located between the skin and the platysma muscle, and is invested by the superficial cervical fascia. FSA thickness may differ depending on the patient´s body habits and weight, typically forming a triangular shape with the apex at the hyoid and base at the mandibular line [16]. It is worth noting that the superficial fat compartments of midface tend to descend, contributing to the loss of jawline definition [19]. The cocktail of recombinant enzymes acts on different specific targets in these patients. Lipase can address accumulated FSA by degrading triglycerides to smaller, easily diffusible molecules (glycerol and free fatty acids). Recombinant lipases activity is not modulated by cofactors and are associated with a broad substrate specificity [11,20]. Subcutaneous injections of collagenase in minipigs were also seen to induce a decrease in the thickness of adipose tissue [21]. In addition, collagenase G/H degrades collagen fibers and induces multiple scissions in the collagen triple helix, leading to an efficient collagenolysis process [22,10]. Lyase can hydrolyze local proteoglycans and facilitates the penetration and spread of collagenases G and H through dense extracellular structures [12]. Hyaluronic acid plays a key role in adipocyte differentiation. It has been shown that hyaluronic acid accumulates during adipocyte maturation and is associated with increased expression of adipogenic markers [23]. HMW-HA facilitates also enzyme diffusion across the extracellular matrix and could help with the chin contouring and modulating inflammatory processes.

This significant efficacy outcome in terms of FSA reduction was associated with benefits in most patient-related outcomes, including satisfaction and several subjective perceptions. The use and importance of patient-reported outcomes is increasing across all medical specialties, including aesthetic medicine; they will help clinicians better understand the goals and expectations of new patient segments [24,25]. In our study, all patients felt satisfied with the treatment and most of them would use the product again and perceived a subjective reduction of flaccidity with improvement of mandibular profile. The latter is especially important, considered that anatomic transition of the lower third of the face to the neck (also known as “neck-face interface”) is fundamental to the overall facial aesthetic [16].

A range of injectable therapies are employed in aesthetic medicine to address the accumulation of facial subcutaneous adipose tissue (FSA) [26]. Aminophylline is administered via subcutaneous injections to promote lipolysis by increasing intracellular cyclic adenosine monophosphate (cAMP) levels and antagonizing adenosine receptors [27]. Hypotonic pharmacological lipo-dissolution relies on the pressure-assisted injection of compounds to disrupt adipocytes [28]. Glycerophosphorylcholine, a precursor to choline, is proposed to stimulate lipid metabolism [29]. In contrast, the combination of phosphatidylcholine and deoxycholate, although commonly used, has some concerns about its safety and limited clinical evidence [30]. Deoxycholic acid is approved by the Food and Drug Administration (FDA) for FSA reduction and its mechanism of action is linked to focal adipocyte lysis. Nevertheless, such cell membrane lysis as main pharmacological action is associated with a local tissue response followed by macrophage infiltration in order to remove cellular debris [31]. According to data from a recent metanalysis including five randomized controlled trial (n = 1838), withdrawals due to adverse events in patients receiving local deoxycholic acid treatment ranged from 6.8% to 8.3%, without statistical differences related to the injected doses [4]. Among adverse events, pain (73.3% to 82.7%), anesthesia/numbness (46% to 60.8%) and swelling/edema (38.6% to 49.8%) were significantly more common than with placebo [4]. In our cohort of patients with excess FSA treated with Pbserum HA 1.5 Medium,

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adverse events were reported by 36% of participants and both pain and edema were less frequent, as reported by 23% and 15% of treated patients, respectively.

Our study has several limitations, including a small sample size and the possibility of bias related with inter-observer variability in the application of the FSA score. However, some important strengths are highlighted, including a low dropout rate and the consideration of both physician and patient-reported outcomes.

We conclude that Pbserum HA 1.5 Medium is an effective and well tolerated treatment for patients with excess FSA, with evident favorable short-term outcomes.

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Conflict of Interest

Jorge López Berroa is an employee of Proteos Biotech. Diana Forero, Carlos Lloreda, Ana Toro and María Cristina Cuello have no conflict of interest to disclose.

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Evolution of EU Legislation Governing the Presence of Undesirable Substances in Animal Feeds

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ABSTRACT

The legislative framework for undesirable substances in EU territory is explained at first with parallel commenting on the political dimension involved in this issue as regards the sector of animal feeding. Then, a brief account on developments of Community legislation in this field is given, with paying particular emphasis on the delicate subject of "dilution principle". In turn, reference is made to methods available for the analysis of undesirable substances in feeds. Furthermore, the article focuses on two groups of undesirable substances, i.e. firstly mycotoxins, where reference is made to legislative developments in this issue as well as prevention measures, and secondly, dioxins where a look is thrown to the historical background in this subject, while focusing on the dioxin episode in Belgium but also on subsequent findings. The legislative tools to confront problems of undesirable substances presence in the sector of animal nutrition is discussed, concluding with the importance of environmental dimension. Finally, reference is made to developments in Community Legislation for dioxins and mycotoxins during the last decade.

Keywords: EU legislation, animal feeds, undesirable substances, dioxins, mycotoxins.

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Evolution of EU Legislation Governing the Presence of Undesirable Substances in Animal Feeds

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ABSTRACT

The legislative framework for undesirable substances in EU territory is explained at first with parallel commenting on the political dimension involved in this issue as regards the sector of animal feeding. Then, a brief account on developments of Community legislation in this field is given, with paying particular emphasis on the delicate subject of "dilution principle". In turn, reference is made to methods available for the analysis of undesirable substances in feeds. Furthermore, the article focuses on two groups of undesirable substances, i.e. firstly mycotoxins, where reference is made to legislative developments in this issue as well as prevention measures, and secondly, dioxins where a look is thrown to the historical background in this subject, while focusing on the dioxin episode in Belgium but also on subsequent findings. The legislative tools to confront problems of undesirable substances presence in the sector of animal nutrition is discussed, concluding with the importance of environmental dimension. Finally, reference is made to developments in Community Legislation for dioxins and mycotoxins during the last decade.

Keywords: EU legislation, animal feeds, undesirable substances, dioxins, mycotoxins.

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I. INTRODUCTION

The primary purpose of agriculture is to produce food for human consumption, either directly from plants or through the animals' organism. The EU has focused its attention on protecting consumers, animals, and the environment by introducing transparency in the food production sector through the General Food Law (EU, 2002a). In modern agricultural practices, the concept of maximizing profits by minimizing costs through waste recycling is widespread, making the contamination of agricultural products with undesirable substances appear almost inevitable. In this respect, the BSE (Bovine Spongiform Encephalopathy) scandal emerged (Zoiopoulos, 2011; Zoiopoulos & Drosinos, 2010). Furthermore, with developments in the applications of biotechnology in animal nutrition (Zoiopoulos, 2004) new problems emerged with GM (Genetically Modified) animal feeds (Zoiopoulos, 1998a; 1998b; Zoiopoulos and Natskoulis, 2013).

The risk of food contamination from toxic substances in feed was reviewed by Kan and Meijer (2007), while the transfer of chemical substances from feed to animal products was examined by Leeman et al. (2007). Undesirable substances such as dioxins, mycotoxins, heavy metals, pesticides, and veterinary drugs are almost unavoidable in the environment. At this point, we would like to underline that in Community legislation the term "toxic substance" is rather not in use but that of "undesirable substance", since a substance could be undesirable - i.e. to adversely affect the organoleptic properties of a product, such as colour - but without causing harm to animal health.

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In recent years, increasing attention has been paid to consumer risks arising from the presence of toxic substances in animal feed. This was prompted by various livestock products being contaminated with environmental pollutants. The most well-known examples include the contamination of animal products with dioxins as a result of industrial activities. Additionally, feed has occasionally been found adulterated with hormones, antibiotics, dioxins, and other chemical substances, either intentionally or through poor agricultural or industrial practices. The use of pesticides is an example of "controlled contamination" of crops which can, however, reach the consumer. Furthermore, feed contamination can occur in a more or less biological manner, as in the case of mycotoxins due to, for example, improper storage of feed raw materials.

There are several proposals for the categorization and classification of contaminants or undesirable substances along the food chain (SCAN, 2003), distinguishing between ions and elements, mycotoxins and microbial products, organic pollutants and botanical impurities. D'Mello (2003) differentiates the main groups of contaminants as follows: biotoxins (plant origin, bacterial pathogens and toxins, mycotoxins, etc.) and anthropogenic contaminants (pesticides, dioxins, veterinary drugs, etc.). Finally, Flachowsky and Danicke (2005) classify contaminants found in livestock nutrition into 8 groups as follows: 1. Heavy metals or other inorganic contaminants; 2. Naturally occurring toxic plant substances; 3. Microorganism products such as mycotoxins; 4. Human-origin contaminants; 5. Industrial, exhaust emission, and urban waste contaminants; 6. Fertilizer residues; 7. Plant production processing aids, and 8. Veterinary drugs.

This paper addresses the extensive network of Community legislation covering the presence of undesirable substances in animal feeds. After reporting on legislative developments, the text focuses on two prominent feed contaminants, specifically mycotoxins and dioxins. It should be stressed, however, that reference to provisions of Community legislation in this article in no way substitutes for the legislation itself, as formulated in the Official Journal of the European Union.

II. LEGISLATIVE FRAMEWORK OF UNDESIRABLE SUBSTANCES IN ANIMAL FEEDS

Problems in the field of animal nutrition are global in nature.

2.1. The Political Dimension

The global trade of animal feeds involves massive volumes and enormous sums of money, and consequently, significant economic interests. Among the most important agricultural commodities traded in international markets are feedstuffs such as maize, the world's leading cereal grain, and soya bean, the world's leading oilseed. These are used exclusively as raw materials in animal nutrition. However, following the GATT agreement (General Agreement on Tariffs and Trade) and its evolution into the WTO (World Trade Organization), which brought profound changes to international trade tariffs, it is said that one way to impose restrictions on the import of cheap products in a country, that are competitive with its own, is the quality of the products themselves. Quality, however, is a concept interwoven with the presence of undesirable substances. The whole issue tends to become more difficult, if not particularly delicate to resolve, when one considers that toxic substances may be present, such as heavy metals measured in ppm, i.e., Kg 10^{-6}, and dioxins measured in ppt or Kg 10^{-12}.

2.2. Evolution of Community legislation

EU legislation on undesirable substances in animal feed constitutes a particularly critical area. The first piece of legislation adopted in this field was Directive 74/63 (EEC, 1974), which aimed at defining Maximum Permitted Levels (MPLs) in ppm (mg/kg) for various types and categories of feed for different livestock species to secure the health of animals and humans consuming their products. The Annex of this Directive contained three sections: 1. Substances (ions or elements) such as As, Pb, F, Hg, nitrates, etc.; 2.

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Microorganism products, particularly aflatoxin B1 from fungus *Aspergillus flavus*, as well as other naturally occurring undesirable substances like hydrocyanic acid, free gossypol, theobromine, mustard oil, etc.; and 3. Botanical impurities such as weed seeds (*Lolium*, *Datura*, etc.).

Over the years, one more Annex was added to Directive 74/63, which concerned the definition of MPLs for undesirable substances, particularly aflatoxin B1 in raw materials, mainly oilseed cakes and meals. Following certain preceding amendments and the recasting of various pieces of legislation necessitated by the General Food Law (Regulation 178/2002), Directive 2002/32 (EU, 2002b) on undesirable substances in animal feeds was adopted. To give an example of an undesirable substance in Annex I of this Directive, the case of heavy metal cadmium (Cd) is selected, the content of which should not exceed 1 ppm in feed materials of vegetable origin, 2 ppm in those of animal origin, 10 ppm in phosphates, and 15 ppm in premixes.

Furthermore, for a uniform approach to cases of elevated levels of undesirable substances, "action thresholds" were established to trigger off investigations aimed at identifying the sources of undesirable substances in Member States. Moreover, Directive 1999/29 (EU, 1999) contained a safeguard clause, that is, when a Member State has grounds to believe that an MPL set in Annex I, or when an undesirable substance not included in it, poses a risk to animal or human health or the environment, then the Member State may temporarily reduce or establish a new MPL, while simultaneously notifying the Commission.

Also, a new development in the legislation on undesirable substances in animal feed is the adoption of Community rules covering the issue of inevitable carry-over of authorized coccidiostats and histomonostats not purposely put in the feed, with their subsequent presence in produced foods. This was achieved through the issuing of Directive 2009/8 (EU, 2009b). Subsequently, the European Commission, in order to cover also the issue of the presence of residues of these substances in products of animal origin and thus protect public health, adopted Regulation 124/2009 (EU, 2009c), setting MPLs for the presence of these substances in foods. Finally, Directive 2002/32 contains a provision resolving at the time the long-pending debate on the "dilution principle" regarding contaminated feeds.

2.3 The Dilution Principle

With Directive 77/101 (EEC, 1977) a list for "straight feeds" was adopted, while with Directive 92/87 (EEC, 1992) another list was published, this one for "raw materials" or "ingredients" of "compound" feeds or mixtures. A paradox appears here, namely the existence of two lists for the same feed. This distinction is "artificial" or even "political". To simplify, "straight feed" was the feed intended directly for the farmer, while 'raw material' was the same feed intended for the feed industry. This distinction was made to overcome problems regarding the use of feed containing an undesirable substance exceeding the MPL set in Directive 74/63. The reason is that a recognized feed industry possesses the appropriate scientific staff and equipment to detect, identify, and measure the content of an undesirable substance that exceeds the MPL set for that feed in Annex I, but remains within the upper limit set in Annex II for the corresponding raw material. This enables the feed industry to dilute the feed with others free of this undesirable substance, so that the final mixture administered to the animal remains within the maximum limit set for complete or complementary feeds for a specific species or physiological state of the animal.

Ultimately, the possibility of "dilution" for excessively contaminated feeds was abolished by the consolidated Directive 2002/32, which had a major impact on international feed trade. The relevant provision in Article 5 of this Directive states: "Member States shall ensure that products intended for animal feed which contain levels of an undesirable substance exceeding the MPL set in Annex I for these feeds cannot be mixed for dilution purposes with the same or other products intended for animal feeding". Nevertheless, Article 8 of the same Directive opens a "window" regarding the possibility of defining criteria for detoxification processes of contaminated feed and in any case, Member States must secure that

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measures are taken to guarantee the correct application of the accepted procedure for contaminated products destined for animal feeding. A significant work on the detoxification of feeds has been edited by Flachowsky (2006).

2.4 Methods of Analysis

As early as 1971, the then EEC adopted analytical methods for the official control of undesirable substances in animal feeds. Additionally, in 1976, the EEC issued methods for the sampling of feeds for control purposes via Directive 76/371 (EEC, 1976a). The first group of undesirable substances covered by official control methods included hydrocyanic acid, mustard oil, theobromine, lupin alkaloids, and trypsin inhibitor (EEC, 1971). Subsequently, the EEC issued official methods for the determination of gossypol in cottonseed oil by-products (EEC, 1972). It later issued an official method for the determination of aflatoxin B1 in feeds, which was based on TLC (Thin Layer Chromatography) (EEC, 1976b). Much later, when the MPL for aflatoxin B1 in complementary feeds for dairy cows was set at 5 ppb - below the lower detection limit of the TLC method (which was 10 ppb) - a method based on HPLC was introduced.

At this point, we would like to note that the subject of classical, rapid, but also modern emerging techniques for the analysis of mycotoxins has been reviewed by Krska and Welzig (2006). Furthermore, Binder (2007), in reviewing this subject, states that the requirements for fast results created rapid test systems which often prove satisfactory, though in certain situations it may be necessary to combine them with validated chromatographic techniques. In this spirit, a variety of immunological methods exist, such as ELISA or radioimmunoassay (RIA). Specifically, ELISA test kits are considered to have high throughput for feed samples, requiring small sample amounts and short analysis times (less than 1 hour, or even as short as 15 minutes). However, ELISA results for certain substrates should only be considered reliable if the kits have been validated for the corresponding agricultural commodities—for example, feed shipments on vessels involving bulk quantities, where the issue of sampling is rather critical. Finally, Directive 2002/70 (EU, 2002c) was published for the determination of dioxins and dioxin-like PCBs in feed. It should also be emphasized that, following the recasting of Community Directives as a consequence of the fundamental Regulation 178/2002 for food and feed, the consolidated and updated Regulation 152/2009 (EU, 2009a) was issued in the field of official analytical methods for feeds.

2.5 Developments at Community level

With Regulation 178/2002 of the European Parliament and of the Council, EFSA (European Food Safety Authority) was established. The EFSA CONTAM group, specifically the EFSA Panel on Contaminants in the Food Chain, completed its final Opinion on a series of 30 risk assessments undertaken over a period of 5 years in the field of undesirable substances in animal feed (EFSA, 2011a). These Opinions were provided following a request from the European Commission to EFSA to study the potential risks associated with animal and human health due to the presence of these undesirable substances in feeds. In most cases, the EFSA CONTAM group at the time did not identify any risk to animal health resulting from feed consumption at maximum permitted levels, provided that good agricultural practices were followed. However, adverse effects on animal health could not be excluded, such as from the mycotoxin deoxynivalenol for pigs or gossypol for sheep. Indicatively, Opinions were provided by the EFSA CONTAM panel on risks to animal and human health from γ-HCH and other hexachlorocyclohexanes (EFSA, 2005), tropane alkaloids from the weed *Datura spp*. (EFSA, 2008a), gossypol (EFSA, 2008b), and the mycotoxins zearalenone (EFSA, 2011b) and phomopsins (EFSA, 2012) due to the presence of these undesirable substances in food and feed.

III. MYCOTOXINS

Mycotoxins are chemical compounds produced by certain fungi. Many such compounds exist, but only a few are regularly found in food and feed. Nevertheless, those actually encountered in food and feed are of great importance for human and animal health. In fact, mycotoxins are secondary

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metabolites produced by fungi, which cause a toxic reaction in the animal when ingested with food. The most widely distributed fungi of this type belong to the genera *Fusarium*, *Aspergillus*, and *Penicillium*. These fungi produce mycotoxins and contaminate food and feed through mycelium growth before and during harvest, or during storage (Bhatnagar et al., 2004). For practical reasons, in the feed manufacturing industry, aflatoxins, ochratoxins, zearalenone, and fumonisins are of particular interest, and the extent of damage caused by each category much depends on the species of the fungus. Aflatoxin B1 is considered the primary hepato-carcinogenic mycotoxin for animals. Trout, ducklings, and piglets are the most sensitive animals (Weidenborner, 2001). The setting of limits and regulatory provisions for mycotoxins can be influenced by several factors (Binder, 2007; Egmond and Jonker, 2004). These factors, which are of both, scientific and social nature, are as follows: 1) availability of toxicological data, 2) availability of data on occurrence in various agricultural commodities, 3) knowledge of the distribution of mycotoxin concentrations within a feed lot, 4) availability of analytical methods, 5) national legislation, and 6) the need for adequate market supply of food.

3.1 Evolution of Community Legislation

A report on the decision-making process, as well as an overview of developments in Community legislation on mycotoxins in the food and feed sector, has been published by Verstraete (2006). Regarding animal feed specifically, the legislation refers only to aflatoxin B1, starting from Directive 74/63 and extending to Directive 2002/32. The MPL for aflatoxin B1 in feed materials is 0.02 mg/kg (20 ppb), while for dairy mixtures for cows, it is 0.005 mg/kg (5 ppb). Community legislation (Directive 2002/32) does not define MPLs for mycotoxins other than aflatoxin B1 in feed. However, Commission Recommendation 2006/576 (EU, 2006a) provides recommendations for monitoring the presence of toxins deoxynivalenol, zearalenone, ochratoxin A, T-2 and HT-2, as well as fumonisins, in products intended for animal nutrition. Finally, Patterson (2004) provides the MPLs for various mycotoxins in animal feed in both the USA and Canada.

Recent developments in EU legislation for mycotoxins in animal feeds have focused on three main pillars: tightening the criteria for detoxifying contaminated feeds, standardizing the use of mycotoxins-detoxifying additives, and converting long standing recommendations in mandatory legal limits for specific toxins. More specifically, Regulation 2015/786 mandates that any process used to reduce mycotoxins must be scientifically validated and shown it does not result in toxic residues or alter the feeds' safety. In addition, this Regulation reinforced that mixing highly contaminated with clean feed to lower the average toxins levels is strictly forbidden (EU, 2015). Recent scientific opinions from EFSA Risk Assessments have paved the way for legislative updates. In fact, it concluded that current levels of Ochratoxin A (OTA) in feeds pose a low risk to poultry and pigs, but monitoring remains a priority due to potential kidney toxicity (EU, 2023). In addition, regarding T-2 and HT-2 mycotoxins, EU is currently reviewing the guidance values for these toxins in feeds to determine whether they should be converted to mandatory maximum limits under Directive 2002/32/EC.

3.2 Prevention of Mycotoxins

When the elimination of mycotoxins from feed raw materials is not possible due to adverse weather conditions prior to harvest or improper storage, then therapeutic and preventive strategies must be utilized to minimize production losses and ensure the safety and quality of animal products. Preventive strategies include organic and inorganic adsorbents, which minimize intestinal absorption of mycotoxins and help their excretion via faeces.

Binder (2007) states that management practices aimed at maximizing plant yields can substantially reduce mycotoxin contamination. These include the use of adapted varieties, appropriate fertilization, weed control, necessary irrigation, and crop rotation (Edwards, 2004). However, even the better management strategies cannot eliminate mycotoxin contamination in

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years favourable for plant disease development. For post-harvest mycotoxin control, it is necessary to consider the prevention of conditions favouring fungal growth with subsequent toxin production, such as the water activity of stored products, temperature, seed condition, air composition between grains, microbial interactions, and the presence of chemical or biological preservatives (Shapira and Paster, 2004). Furthermore, Commission Recommendation 2006/583 (EU, 2006b) was issued, recommending measures for the prevention and reduction of *Fusarium* toxins in cereal grains and their products.

The subject of environmental conditions affecting the presence of mycotoxins in feeds was reviewed by Sanchis (2004), while the control of mycotoxins during storage and detoxification techniques were reviewed by Shapira and Paster (2004). Finally, the subject of mycotoxin control was studied by Jenning (2004), and the control of mycotoxins in animal feeds by Patterson (2004) as well as Natskoulis and Zoiopoulos (2014a).

Furthermore, due to the advancement in analytical techniques on (multi)mycotoxins determination, new concerns are rising regarding the toxicity of mycotoxins. With more sufficient "weapons" available, scientific community recently investigates, on the one hand the toxicity of emerging mycotoxins, not studied or regulated by legislation yet, but apparently present in our food chain (EFSA, 2014), and on the other hand the additive toxicological effect of co-occurring mycotoxins in a product (Schuchardt et al., 2014).

IV. DIOXINS

One of the most serious contaminants of animal feeds is dioxins. Under the name "dioxins" lies a large number of compounds, some of which are highly toxic and confirmed carcinogens (Tuomisto et al., 1999). They are formed as by-products in a number of industrial and thermal processes and enter the environment in various ways, the most important of which is through the release of polychlorinated biphenyls (PCBs) into the atmosphere, during which two classes of compounds called dioxins - specifically polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) - are formed as by-products during synthesis. The majority of environmental pollution with dioxins originates from the energy production sector and industrial activity. Certain PCBs have properties similar to those of dioxins and are often referred to as dioxin-like PCBs and considered together with dioxins. Something over 20 years ago, the EU had the bad experience of a series of incidents involving dioxin contamination of the food chain. It is clear that one source of human exposure to dioxins is food (Furst et al., 1992), with food of animal origin being the primary source of contamination. It also appears that, for dioxins, food contamination is directly proportional to feed contamination. Therefore, if one wishes to reduce dioxin contamination in the food chain, it is important to adopt dioxin control measures in animal feeds.

4.1. Historical Background

A long history of accidents has occurred resulting in human exposure to dioxins (Covaci et al., 2008), the most notable of which were the rice oil poisonings in Yusho, Japan in 1968 (Tanabe et al., 1989) and Yucheng, Taiwan in 1979 (Soong and Ling, 1997), respectively. Other accidents, less known to the public, include the contamination of French cheese in 1970 with agricultural machinery engine oil, the poisoning of poultry in France in 1970 from plastic netting wire contaminated with dioxins, and contaminated pig feed in piggeries of Montana, USA in 1979 (Lock and Powell, 2008). Although it was neither the largest nor the most serious, the Belgian "PCB-dioxin" crisis was one of the most widely publicized food poisoning incidents in the media.

Covaci et al. (2008) state that the literature is 'generous' with episodes of environmental food contamination by dioxins, such as the dioxin-contaminated waste from Philips Duphar in the Netherlands in 1965. Also, the dioxin contamination of Agent Orange over large areas during the Vietnam War (Schecter et al., 2006a). Additionally, the dioxin contamination of poultry feed in New York in 1971 and Wilmington, USA, in 1972, as well as the large-scale environmental pollution following the incident in Seveso, Italy,

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when a chemical plant exploded and released 2,3,7,8-tetrachloro-dibenzo-p-dioxin (TCDDs) into the environment - a group of the most toxic synthetic compounds the humanity has ever known (Alaluusua et al., 2004). Furthermore, the "burial" of barrels with dioxin-contaminated residues in Lekkerkerk, Netherlands, in 1980, and the various adverse incidents that took place between 1996 and 2002 in Germany, Brazil, the USA, and other countries, where animal feeds (kaolinitic clay, citrus pulp, etc.) were contaminated with dioxins (Schecter et al., 2006b).

Thus, in 1998, in Germany, elevated dioxin levels were found in cow's milk. This was due to an ingredient in the dairy mixture, specifically dried citrus pulp of Brazilian origin (Malisch, 2000), which had been exported to a number of EU countries. The exact cause of the contamination was slow to be identified. Initially, it was attributed to an agent added to the fuel to increase efficiency during the pulp dehydration process. However, the exact cause was later found to be the limestone used to raise the pH and facilitate the removal of water from the hydrophilic pectins of the fresh citrus pulp during the dehydration process.

Immediately after the incident, the EU began to address the issue of dioxin contamination in feed seriously. First, it took measures to discard the contaminated pulp, and second, it introduced for the first time an MPL for dioxins in dried citrus pulp in Directive 1999/29 on undesirable substances in animal feed, at a level of 500 pg. This was the first time it was noted that, not only in Europe but worldwide, very few laboratories were capable of performing reliable dioxins analyses covering the entire range of its constituent congeners.

4.2. The Dioxins Incident

While the case of "Mad Cow Disease" in Britain in 1996 became known as a "scandal" - as it is rumoured to have resulted from the deliberate action of the meat industry to compress the production costs of meat-and-bone meal, followed by an initial State attempt for a cover-up - the 1999 dioxin event in Belgium became known as an "episode" because it was due to an accident. Thus, fat contaminated with dioxins was incorporated, due to negligence (unintentionally), into the rations of various farm animals, mainly poultry (Broeckaert and Bernard, 2000). Egg hatchability dropped dramatically and egg production fell to approximately 30% (Covaci et al., 2008). It was found that waste oil from transformers, heavily contaminated with PCBs, was mistakenly mixed with used frying oils from collection bins intended for incorporation into animal feeds after refining. It appears that extensive waste recycling is a rather vulnerable area that can lead to feed contamination.

Scheprens et al. (2001) provide a brief but accurate report on the Belgian dioxin episode. These authors state that in 1999, approximately 30 kg of polychlorinated biphenyls (PCBs) and 1 kg of dioxins entered the food chain via about 1,500 tons of feed containing 60 tons of contaminated fat from a Belgian fat-melting company. This episode drew global interest both within and outside Belgium and forced the Belgian government to take drastic measures to protect public health, including a large-scale food control program with PCBs and dioxins measurements in over 20,000 and 450 samples of feeds and fat, respectively (Bernard et al., 1999).

Furthermore, Van Larebeke et al. (2001) provide an extensive description of the Belgian PCBs and dioxins episode. These authors write that in Belgium, about 20 companies collect animal fat from slaughterhouses, melt it, and sell it to feed industries. It is common practice in Belgium to add domestic waste fat collected at community recycling centres to this material. In January 1999, at the Flemish fat-melting company Verkest, 40-50 kg of mineral oil containing polychlorinated biphenyls (PCBs, most probably oil from worn-out old transformers coming from a waste recycling centre) was mixed with fat sent to 10 feed industries. Between January 15 and 31, 500 tons of animal fat containing approximately 60-80 tons of contaminated fat with 40-50 kg of PCBs and nearly 1 kg of dioxins, were distributed to poultry farms and to a lesser extent, rabbit farms, cattle farms, and piggeries, mainly in

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Belgium. However, small quantities were exported to the Netherlands, France, and Germany. The 500 tons of contaminated feed represented a limited percentage of the total amount of feeds produced and used in Belgium, which was estimated to exceed 28,000 tons per week. The aforementioned authors also report the pathological conditions first recorded in February 1999. These included a decrease in egg production and hatchability, and an epidemic of chick edema disease.

Covaci et al. (2008), approximately ten years after the event, provide a detailed review of the Belgian PCBs/dioxins crisis. These authors conclude that the consequences of this food crisis were: 1) the introduction of standards for PCBs in feeds in Belgium and, in 2002, harmonized standards in the EU, as well as for food of animal origin, 2) systematic national controls on food of animal origin, and 3) the creation of a Federal Agency for the Safety of foods in Belgium. The risk for human health from this major food incident was assessed with controversial results. It was suggested that, since only a small proportion of the food chain was contaminated, large-scale adverse effects on the Belgian population are unlikely. However, another assessment suggests that neurotoxic effects in infants, as well as behavioural impacts, might be observed. The same authors concluded that poor crisis management by the government had dramatic political and economic consequences (Authors' Note: On June 9, 1999, the causes of the crisis were announced, and the following day the Jean-Luc Dehaene government fell). However, this episode made both politicians and the public aware that food safety constitutes an issue of top priority. Furthermore, the PCBs crisis imposed a system for effective and rapid surveillance of the food chain, as well as risk communication, through the issuing of reliable and transparent Regulations, leading to a reduction in risk levels. Finally, it rendered the introduction of Community MPLs for PCBs and dioxins urgent.

4.3. Subsequent Findings

Immediately after the incident in Belgium, still in 1999, another case of dioxins contamination was discovered in Austria involving a kaolinitic clay used as an additive, specifically as a pelleting agent or binder in animal feeds. This agent had been mined in Germany. The EU took immediate measures to address the problem by adding a footnote to the relevant legislation, setting the MPL for certain binding agents at 500 pg/kg. Another incident with dioxins in feed took place in 2000. Dioxins were found in certain premixes containing choline chloride of Spanish origin. This substance is classified as a provitamin and is used as a feed additive. However, the problem was not the pure choline itself, but rather the carrier of vegetable origin which, although declared as corn cob meal, was actually sawdust, apparently from wood treated with an insecticidal preservative (eventually pentachlorophenol was detected).

Another case of dioxin contamination in the feed sector refers to two products from the USA, in which dioxin contamination was detected in 2002 in Ireland. These products are administered to pigs as a copper source but also contained peat as an appetite enhancer, and imports of these preparations into the EU were banned. Finally, exactly 12 years after the Belgian episode, another serious incident of dioxins contamination in an agricultural product, specifically meat, took place in Germany. And one should think that Germany is considered one of the strictest countries in the Community regarding controls in animal production. It seems that the phenomenon of recycling, which is endemic to food crises, it also recycles the concept of "dioxin episode".

4.4. Legislative Tools

Two main tools were available at the time within EU animal nutrition legislation to cope with the major dioxins' episode involving recycled contaminated oil in Belgium. First, the Decision on prohibited ingredients in animal nutrition (EEC, 1991), and second, Directive 1999/29 on undesirable substances in feeds. However, legislative efforts to include used frying oil in the list of prohibited feed materials were unsuccessful, as the majority of Member States believed that frying oil itself was not the cause of the problem, but rather the improper collection method that allowed PCB-contaminated

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transformer oil to enter the food chain. Instead, the implementation of strict conditions was proposed to the industry, including GMP (Good Manufacturing Practice) and HACCP (Hazard Analysis and Critical Control Points).

The second alternative solution for the EU was to set MPLs for dioxins in the Directive on undesirable substances in animal feeds, in order to control the circulation of feed materials with dioxins exceeding this level. One might suggest that the existing MPL of 500 ppt, established for dried orange pulp, should be extended to cover all feed, but this is risky due to the fluctuation of dioxins levels in various raw materials. This proved extremely difficult in practice, as a number of unexpected obstacles emerged during the investigation of the consequences of implementing such MPLs. The EU delayed for two and a half years before adopting MPLs for dioxins in all types of animal feeds and did so with the footnote that the proposed levels should be reviewed later following further scientific evidence.

Recent research has transitioned from single toxicity assessments to complex interdisciplinary studies, notably adapting the "One Health" framework to link environment, animal, and human health (Houlihan et al., 2025). Major surveys in Ireland (2016-2018) and Belgium (2014-2024) have tracked dioxins levels in human milk, showing a downward temporal trend in exposure due to tighter regulatory bans (Anjelkovic et al., 2024; Houlihan et al., 2021). Furthermore, research post-2015 has heavily targeted the reduction of dioxins formation during waste processing by using sulphur oxide (Li, 2024), whereas new environmental risks have emerged with the burning of improperly managed health care waste (Muyise et al., 2024). Finally, major developments in EU legislation on dioxins after 2015 have been driven by 2018 EFSA's Scientific Opinion that drastically lowered the safety threshold for human exposure, in other words lowering TWI (Tolerable Weekly Intake) (EFSA, 2018). This scientific shift triggered off a wave of new maximum levels for various food categories and more stringent industrial monitoring (EU 2022; EU 2023; EU, 2024).

4.5 Problems In Animal Nutrition Practice

After the dioxins episode in Belgium in 1999 and before taking measures for MPLs, the EU conducted a study to obtain indications for the background of dioxins levels in various animal feeds. However, the result of this study was unexpected: the natural level of dioxins in feed materials of marine origin from "closed seas", particularly from the Baltic Sea - which were considered of the highest quality in chemical composition and protein content - was remarkably high. In fact, the dioxin level in fish oil was much higher compared to fishmeal due to the lipophilic nature of these pollutants. European fishmeal and fish oil, which were found with an average dioxin content of the order of 1.2 and 4.8 ng/kg on Dry Matter basis, respectively (SCAN, 2000), were more heavily contaminated than those originating from the South Pacific Ocean (Chile or Peru), which contained 0.14 and 0.61 ng/kg DM, respectively. The problem was mainly concentrated on feeds for fish farming (Tacon, 1993), as approximately 20% of the total global production of fishmeal and fish oil was used in fish farming (EUROSTAT, 1999).

Generally, rations for livestock (cattle, sheep and goats, pigs, poultry) are formulated at a protein level of about 15-20%, whereas the protein required for the nutrition of carnivorous fish is about three times higher. The fishmeal content in these can reach 70%. Fish oil has a higher energy content and is also a good source of essential ω-3 type fatty acids. To provide the required nutrients to farmed fish, it is necessary that almost 2/3 of their ration consists of fishmeal and fish oil. Fish farming constitutes a growing sector in the EU, and this requires delicate handling of the situation so that not to jeopardize the future of this promising sector.

Ultimately, the EU adopted dioxin MPLs for various types of feed (9 in total), but these varied among different feed materials; for example, 0.75 ng/kg for feed materials of plant origin, 2.25 for fishmeal, and 6.0 ng/kg for fish oil, meaning the latter values are 3 and 8 times higher than those for plant-based feed materials. The EU also proposed setting both target and action levels for

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dioxins in plant origin feed materials (in the updated Directive 2002/32).

4.6 The Environmental Dimension

There is a school of thought in Europe advocating that the problem of dioxin contamination in animal feed cannot be solved solely by setting MPLs and controlling the circulation of feeds, and that these efforts are time-consuming and expensive, as the cost of a full dioxin analysis is very high. The quantities of feeds traded are immense, and one wonders "who is going to pay the price for dioxins analyses on a daily basis?". Environmental pollution with dioxins is mainly caused by atmospheric emissions from various sources such as municipal wastes incineration, chemical production, etc. (EU, 2000, 2002d). Taking into account the impact of environmental pollution in the case of animal feeds, measures should be taken aiming at the general reduction of the atmospheric dioxins load. Therefore, there is a view that the problem of dioxin pollution should be addressed at its true source, namely at the environmental level. However, this is primarily a matter of political decision. Some thoughts on the issue of dioxins in animal feeds have been expressed twelve years ago (Natskoulis and Zoiopoulos, 2014b).

V. CONCLUSIONS

Although progress has been made by developing alternative, i.e. cleaner, forms of practising animal production without the use of chemicals, like the so called "Organic Livestock Farming" (Zoiopoulos and Hdjigeorgiou, 2013; Zoiopoulos and Natskoulis, 2025) there are questions still pending in the field of presence of undesirable substances related to animal feeds. Initially, it must be emphasized that the possibility of applying the "dilution principle" regarding undesirable substances in the animal feed sector has been eliminated from Community legislation. However, a "window" has been opened regarding the possibility of defining criteria for the detoxification process of feeds and, in this spirit, efforts should, among others, focus on the field of mycotoxins prevention. Regarding dioxins, fishmeal and especially fish oil are the most heavily contaminated feed materials and fish farming is the most critical sector. Raw materials, especially those that are products from recycling, must be controlled for their quality and safety. The application of GAP (Good Agricultural Practice), GMP (Good Manufacturing Practice), and HACCP principles should be generalized in the production and processing of feed materials. Control programs should also be executed at European level in the field of raw material contamination. Furthermore, carry-over coefficients for dioxins from the environment, and particularly from the soil, into animal tissues and products should be investigated. In this spirit, the work of Verstraete (2011) is significant regarding the risk management of undesirable substances in animal feed, following the updated risk assessments conducted within the Community territory. Finally, environmental pollution with dioxins, which subsequently enter the food chain, is an important problem on which the scientific community should focus its attention, but the issue of environmental pollution is primarily political. However, we can be optimistic that solutions will be found within the European Union territory, despite the complexity of the matter and the involved difficulties.

Conflict of Interest

The authors have no conflict of interest.

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