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PROGRAMA DE PÓS-GRADUAÇÃO EM DIAGNÓSTICO GENÉTICO E MOLECULAR

UTILIZAÇÃO DO DNA PLASMÁTICO COMO MARCADOR PROGNÓSTICO NO TRAUMATISMO CRÂNIO-ENCEFÁLICO GRAVE EM HUMANOS

Dissertação submetida ao Programa de Pós Graduação em Diagnóstico Genético e Molecular da Universidade Luterana do Brasil, para obtenção do Título de Mestre em Diagnóstico Genético e Molecular.

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RESUMO

O trauma crânio – encefálico (TCE) é causa freqüente de mortalidade e atualmente são utilizadas apenas medidas clínicas no estabelecimento de sua gravidade e desfecho. Os marcadores preditivos já utilizados na rotina médica como prognósticos no TCE grave incluem a idade, o grau de coma inicial (estabelecido pela *Glasgow coma scale* - GCS) e o *status* neurológico no desfecho (aferido pela escala *Glasgow outcome Scale* - GOS). Apesar do significativo impacto sócio-econômico causado pelo TCE grave, os marcadores preditivos atualmente empregados têm eficácia restrita. O presente trabalho teve como objetivo avaliar as concentrações de DNA livre de célula circulante no plasma de pacientes que sofreram traumatismo crânio encefálico grave e buscar correlacioná-las ao prognóstico dos pacientes. A quantificação se deu através da amplificação do gene da β-globina pelo método de PCR em tempo real. Foram analisadas amostras de plasma coletadas na entrada dos pacientes na unidade de tratamento intensivo e 24 h após. Foi demonstrado que as concentrações de DNA livre circulante no plasma foram significativamente maiores do que a dos controles. Na primeira coleta não foi encontrada correlação entre as maiores concentrações de DNA no plasma e o desfecho. Porém, no 2° tempo amostral (24 h após a entrada do estudo) as concentrações de DNA se correlacionaram com a mortalidade, independente da presença de lesões associadas, indicando que concentrações que persistem elevadas podem constituir um marcador de prognóstico.

ABSTRACT

Traumatic brain injury (TBI) is a frequent cause of death, and to date only clinical measures are used to establish its severity and outcome. The predictive markers of outcome, that have being used as routine after severe TBI, include age, the initial coma level (established by the Glasgow coma scale- GCS), and the outcome neurological status (assessed by the Glasgow outcome scale-GOS). Despite the significant social-economic impact caused by severe TBI, the predictive markers currently used have restricted efficacy. In this study we aimed to investigate DNA concentrations in plasma of patients after severe traumatic brain injury and its relationships with outcome. Plasma DNA was measured using a real-time quantitative PCR assay for the β-globin gene. We analyzed plasma samples collected at the intensive care unit admission and 24 h later. Cell-free plasma DNA concentrations were significantly higher in TBI patients than in the control group. At study entry, no correlation was found between higher plasma DNA concentrations and outcome. However, at the second sampling time (24 h after study entry) DNA concentrations correlate with mortality, despite the presence of extracranial associated injuries, suggesting that persistent high concentrations may constitute a predictive marker of outcome.

CAPÍTULO I

1. INTRODUÇÃO

1.1 Traumatismo crânio-encefálico (TCE)

Cerca de 300 pessoas morrem por dia, no Brasil, em decorrência de trauma, sendo esse a principal causa de morte na faixa de 1 – 44 anos. Em pacientes poli-traumatizados, freqüentemente a cabeça é a região do corpo mais afetada, contribuindo em metade de todas as mortes. Quase 75% das vítimas fatais de acidentes de trânsito têm evidências *post-mortem* de dano cerebral (Brocker *et al.*, 1991; Valadka & Narayan, 1996).

A medida clínica mais utilizada no estabelecimento da severidade e desfecho (predição de mortalidade) no traumatismo crânio-encefálico (TCE) é a *Glasgow Coma Scale* (GCS). Essa escala fornece uma medida quantitativa do nível de consciência, que é baseada na avaliação da abertura ocular, da resposta verbal e da melhor resposta motora. O TCE é classificado conforme sua gravidade em: leve (GCS 13-15), moderado (GCS 9-12) e grave (GCS 3-8). Nos EUA, dos TCE de pacientes que chegam vivos aos hospitais: 80% são leves; 10% moderados e 10% graves. A mortalidade decorrente do TCE grave pode chegar a 80%. Seqüelas permanentes ocorrem em 10% dos sobreviventes de TCE leve, em 66% nos casos de TCE moderado e 100% nos casos de TCE grave (Kraus *et al.*, 1996; Valadka & Narayan, 1996). Alguns marcadores preditivos já são utilizados na rotina médica como prognóstico do grau de seqüela no TCE grave: o grau de coma inicial (estabelecido pela GCS), o *status* neurológico no desfecho (aferido pela escala de *Glasgow Outcome Scale* - GOS) e a idade. Outras características associadas com pior prognóstico são: pupilas fixas, ausência ou alteração grave dos movimentos oculares e padrões anormais de resposta motora (Ghajar, 2000).

Apesar do significativo impacto sócio-econômico causado pelo TCE grave, os marcadores preditivos atualmente empregados na rotina médica têm eficácia restrita. Apesar de suas limitações a escala GOS tem sido a variável preditiva mais utilizada. A avaliação precoce da severidade do dano neural e de

deterioração cerebral do paciente com TCE grave freqüentemente é dificultada durante a internação do paciente na UTI de trauma, uma vez que esses pacientes geralmente estão sedados e em ventilação mecânica. Além disso, as variáveis clínicas de deterioração neurológica no paciente com TCE grave podem ser tardias, diminuindo a janela terapêutica para o emprego de estratégias mais invasivas de tratamento. Assim, marcadores preditivos moleculares poderão ter grande impacto nas decisões terapêuticas no TCE, permitindo a estratificação dos pacientes de maior risco, indicando quais indivíduos se beneficiarão de condutas mais agressivas (Raabe *et al.*, 1998; Ghajar, 2000; Faden, 2002; Petzold *et al.*, 2002; Townend *et al.*, 2002). Diversos marcadores têm sido investigados como preditores de desfecho no TCE, dentre os quais: a proteína S100B, a enolase neuronal e a creatina-quinase-BB (Skogseid *et al.*, 1992; Raabe *et al.*, 1999; Berger *et al.*, 2002; Woertgen *et al.*, 2002; Savola *et al.*, 2004).

1.2 Utilização de ácidos nucléicos circulantes para diagnóstico de enfermidades

A detecção de ácidos nucléicos circulantes tem sido explorada com o objetivo de se obter alternativas de diagnóstico não invasivo para uma variedade de condições clínicas. Baseado em técnicas moleculares, principalmente PCR em tempo real, o DNA livre circulante pode ser detectado e quantificado no soro ou plasma, tanto através de um marcador nuclear como o gene da β-globina (Lo *et al.*, 1998a; Lo *et al.*, 2000; Lam *et al.*, 2003) quanto por marcadores específicos fetais (Lo *et al.*, 1997; Lo *et al.*, 1998a), tumorais (Gautschi *et al.*, 2004) ou mitocondriais (Lam *et al.*, 2004).

O procedimento de PCR em tempo real utiliza um termociclador combinado a um fluorímetro com a capacidade de monitorar opticamente o progresso da amplificação a cada ciclo de PCR (detecção direta do produto de amplificação - amplicon). Assim, quanto maior a concentração de DNA alvo, em ciclos mais precoces ele será detectado, permitindo assim uma inferência quantitativa (Livak *et al.*, 1995; Heid *et al.*, 1996; Lunge *et al.*, 2002).

1.2.1 Ácidos nucléicos circulantes x Câncer

Como o DNA livre provavelmente poderia ser detectado em doenças associadas à destruição de tecido, atenção passou a ser dada a esse novo marcador. A partir dos anos 70, a presença de elevados níveis de DNA no plasma e soro de pacientes com câncer passou a ser reconhecida (Leon *et al.*, 1977), e foi demonstrado que esse DNA exibia alterações relacionadas a tumores (Stroun *et al.*, 1989). A confirmação de que tumores podem liberar DNA para a circulação se deu através da detecção de mutações oncogênicas no DNA plasmático (Sorenson *et al.*, 1994; Vasioukhin *et al.*, 1994). Posteriormente, foi demonstrada a presença, no soro ou plasma de pacientes com diversos tipos de câncer, de várias alterações genéticas e epigenéticas, como:

- mutações no gene K-ras (Anker *et al.*, 1997; Mulcahy *et al.*, 1998; Yamada *et al.*, 1998; Sorenson 2000; Kopreski *et al.*, 2000),
- mutações no gene p53 (Mayall *et al.*, 1998; Silva *et al.*, 1999; Gonzalez *et al.*, 2000; Jackson *et al.*, 2001;),
- alterações em regiões microssatélite (Sozzi *et al.*, 1999; Bruhn *et al.*, 2000; Gonzalez *et al.*, 2000; Shaw *et al.*, 2000; Taback *et al.*, 2001; Utting *et al.*, 2002),
- alterações no padrão de metilação (Esteller *et al.*, 1999; Tsou *et al.*, 2002)
- mutações no DNA mitocondrial (Jeronimo *et al.*, 2001; Nomoto *et al.*, 2002).

Em geral, além da possibilidade de diagnóstico não invasivo, esses estudos buscaram correlacionar os níveis de DNA circulante ao estágio da doença, à presença de metástases, ao prognóstico, à resposta terapêutica e à recaída.

Uma nova perspectiva de grande interesse científico é a avaliação de RNA livre de célula para fins de aplicação prognóstica e diagnóstica. Apesar da grande suscetibilidade à degradação, a presença do RNA circulante já foi detectada em diversos tipos de câncer (Kopreski *et al.*, 1999; Chen *et al.*, 2000; Fleischhacker *et al.*, 2001; Kopreski *et al.*, 2001; Silva *et al.*, 2002; Schmidt *et al.*, 2005). A distinção

da análise de RNA da de DNA, é que a primeira permite analisar a expressão de distintas proteínas. Como diferentes tipos de tumores expressam um diferente repertório de proteínas, os respectivos mRNAs encontrados no soro ou plasma podem ser usados como marcadores tumorais específicos.

1.2.2 Ácidos nucléicos circulantes x DNA fetal

Outra promissora possibilidade de diagnóstico não invasivo surgiu com a descoberta de que DNA fetal livre de célula é encontrado no soro e plasma materno (Lo *et al*., 1997). O DNA fetal foi detectado já no primeiro trimestre de gestação (a partir da 7ª semana), com concentrações crescentes ao longo do tempo gestacional (Lo *et al*., 1998a). Foi demonstrado que, após o parto, o DNA fetal é rapidamente depurado do plasma materno (Lo *et al*., 1999a), a grande maioria das mulheres estudadas não apresentou níveis detectáveis 2 horas após o nascimento, sendo a meia-vida do DNA fetal estimada em 16,3 min.

O primeiro marcador utilizado para a detecção de DNA fetal foi o cromossomo Y (Lo *et al.,* 1997; Lo *et al*., 1998a), pois seqüências específicas desse cromossomo, ausentes no genoma da mulher, poderiam ser facilmente amplificadas e detectadas como sendo de origem fetal, se presentes no soro ou plasma materno. No entanto, esse marcador só pode ser utilizado no caso de fetos do sexo masculino, o que limita estudos de quantificação importantes já que níveis aumentados de DNA fetal na circulação materna têm sido associados a diversas complicações da gravidez. Porém, dada à facilidade da identificação do DNA masculino, uma importante aplicação é a identificação de fetos do sexo masculino com risco de doenças ligadas ao cromossomo X (Costa *et al.,* 2002; Honda *et al*., 2002). Diversos marcadores, também úteis no caso de fetos femininos, têm sido investigados, como: polimorfismos autossômicos (Pertl *et al*., 2000; Li *et al*., 2004), marcadores epigenéticos fetais (Poon *et al*., 2002) e mRNA de genes expressos na placenta (Ng *et al*., 2003a).

A análise do plasma materno também é útil para a determinação pré-natal não invasiva do RhD fetal no caso de mães RhD negativo (Faas *et al*., 1998; Lo *et al*., 1998b; Zhong *et al*., 2000a; Brojer *et al.,*2005). Outras aplicações genéticas,

do DNA fetal na circulação materna, incluem o diagnóstico de: fibrose cística (Gonzalez-Gonzáles *et al*., 2002; Nasis *et al*., 2004), Coréia de Huntington (Gonzalez-Gonzáles *et al*., 2003), hiperplasia adrenal congênita (Rijnders *et al*., 2001, Chiu *et al*., 2002a) e beta talassemia (Chiu *et al*., 2002b).

Alterações na quantidade de DNA fetal circulante no soro ou plasma materno foram descritas em diversas situações. Muitos estudos demonstraram aumentos significativos nos níveis de DNA fetal em amostras de sangue obtidas de mulheres com preeclampsia (Lo *et al*., 1999b; Zhong *et al*., 2001; Swinkels *et al*., 2002; Hahn & Holzgreve, 2002), assim como de mulheres que desenvolveriam preeclampsia, as quais apresentaram duas fases de elevação que precederam os sintomas clínicos (Leung *et al*., 2001). Quantidades elevadas de DNA fetal também foram descritas em mulheres grávidas de fetos com trissomia do 21 (Lo *et al*.,1999c; Zhong *et al*., 2000b; Lee *et al*., 2002) e trissomia do 13, mas não do 18 (Wataganara *et al*., 2003). Além destas aplicações, níveis aumentados de DNA fetal também foram sugeridos como indicadores de parto prematuro. Leung *et al.* (1998) encontraram aumentos significativos nas concentrações de DNA fetal em mulheres que tiveram partos prematuros, entre as 26° e 34° semanas de gestação.

Além do DNA circulante, atenção tem sido dada ao RNA presente na circulação. Poon *et al*. (2000) demonstraram a presença de RNA fetal no plasma de mulheres grávidas, através da detecção do mRNA de um gene expresso no cromossomo Y. Estudo desenvolvido por Tsui *et al*. (2002) demonstrou que o mRNA circulante é estável, provavelmente protegido por associação a partículas, e, assim, esses resultados sugerem que seqüências de mRNA originadas do feto ou da placenta podem ser detectadas no soro ou plasma materno se processadas apropriadamente.

Na busca por um marcador fetal independente do sexo, Ng *et al*. (2003a) demonstraram que um mRNA expresso pela placenta é detectável no plasma materno. Também foi demonstrado que a análise do mRNA plasmático pode ser usada na detecção de algumas desordens associadas à gravidez, como preeclampsia e certas aneuploidias (Ng *et al*., 2003b; Ng *et al*., 2004).

1.2.3 DNA circulante x trauma

O DNA genômico circulante também se mostrou alterado em situações de emergência médica, como derrame cerebral (Rainer *et al*., 2003) e trauma (Lo *et al*., 2000; Lam *et al*., 2003). Esses estudos procuraram estabelecer o nível de DNA circulante como indicador de severidade clínica. Foi sugerido que o DNA presente no plasma ou soro é um potencial marcador prognóstico, já que foi demonstrada correlação entre os níveis de DNA e a gravidade das lesões, assim como com o desenvolvimento de complicações pós-traumáticas (Lo *et al*., 2000; Lam *et al*., 2003). Concentrações de DNA mitocondrial no plasma de pacientes após a ocorrência de trauma também foram avaliadas (Lam *et al*., 2004) e o padrão encontrado foi similar ao do DNA genômico, sugerindo que ambos sejam liberados através do mesmo mecanismo.

O mecanismo pelo qual o DNA livre circulante aumenta após trauma não está bem estabelecido. As altas concentrações precoces, observadas logo após a lesão, sugerem que o DNA extracelular se origine do dano ao tecido (necrose), enquanto mecanismos de apoptose podem contribuir para aumentos persistentes, além da redução (*clearance*) de DNA prejudicada provavelmente por comprometimento dos órgãos responsáveis devido à inflamação sistêmica (Lam *et al*., 2003).

2. JUSTIFICATIVA E OBJETIVOS

O trauma crânio - encefálico é causa freqüente de mortalidade. Atualmente são utilizadas apenas medidas clínicas no estabelecimento de sua gravidade e desfecho. A análise laboratorial de diversas proteínas tem sido investigada para determinação de prognóstico, no entanto, nenhuma delas tem sua utilização estabelecida na prática clínica. Recentemente, tem-se investigado a utilização de DNA livre de célula circulante no plasma como marcador diagnóstico e prognóstico em uma variedade de condições clínicas. Assim, esse estudo tem como objetivo avaliar a eficiência deste parâmetro em pacientes que sofreram traumatismo crânio encefálico grave, quantificando o DNA plasmático na tentativa de estabelecer uma relação entre as concentrações obtidas e o prognóstico.

CAPÍTULO II

Role of plasma DNA as a predictive marker of fatal outcome following severe head injury in males

Este artigo refere-se ao estudo de quantificação do DNA livre de célula circulante no plasma de pacientes que sofreram traumatismo crânio-encefálico grave como marcador prognóstico e será submetido à revista Journal of Neurotrauma.

Key words: plasma DNA, prognostic marker, traumatic brain injury, real time PCR

Abstract

Trauma is the leading cause of death in people under 45 years of age and up to half of the fatalities is caused by head injury. Prediction of outcome is one of the major problems concerning head injury. The wide range of conditions associated and the relatively variable predictive value of clinical assessments complicate the identification of patients at risk. Recently, investigations have been performed on the potential use of circulating cell-free DNA in the plasma or serum for clinical diagnosis, prognosis and monitoring of a variety of conditions. Significant increases of circulating DNA in the plasma of trauma patients have been reported. In this study we aimed to investigate DNA concentrations in plasma after severe traumatic brain injury (TBI) and the relationships with outcome, in two different sampling times. We studied 41 male victims of severe traumatic brain injury, 16 patients presented isolate severe TBI and 25 had multitrauma with severe TBI. Control blood samples were obtained from 13 healthy male volunteers. Plasma DNA was measured by a real-time PCR assay for the β-globin gene. The mean time for first sampling (study entry) was 11.7 ± 5.2 h after injury, subsequent DNA determinations were performed 24 h after study entry. Mean plasma DNA concentrations were significantly increased in TBI patients (347704 and 110009 kilogenomes-equivalents/L, at study entry and 24 h later respectively) compared with the control group (3029 kilogenomes-equivalents/L) ($p < 0.05$). Significant correlation between higher plasma DNA concentrations determined 24 h after study entry and fatal outcome was observed ($p = 0.043$). Otherwise, no correlation was found at study entry ($p = 0.0851$). There was no significant correlation between plasma DNA concentrations at the second sampling time and the presence of associated extracranial injuries ($p = 0.4684$). High plasma DNA concentrations at second sampling time predicted fatal outcome with a sensitivity of 67 % and specificity of 76 % considering a cut-off value of 77883 kilogenomes-equivalents/L. The present study confirms that severe TBI is associated with elevated plasma levels of DNA, and suggests that persistent elevations correlate with mortality.

Introduction

Trauma is the leading cause of death in people under 45 years of age worldwide and, within this group, up to half of the fatalities is caused by head injury (Gennarelli, 1993; Jennett, 1998). Prediction of outcome is one of the major problems concerning severe head injury, since accurate identification of patients at greater risk highly depends on an effective predictor. The Glasgow Coma Scale (GCS) score has been frequently used as one of the most important predictors of outcome after head injury (Balestreri *et al.*, 2004) and remains a key measure in neurological assessment in these cases. In most studies, classification of the severity of the trauma is still based on the admission GCS. According to this scale, a score less than or equal to 8 is the traditional criterion for differentiating between severe and moderate to mild head injury, and patients' management is frequently dependent on this initial classification. Otherwise, Marion & Carlier (1994) analyzed the difficulty of determining the initial GCS in a repeatable and reproducible manner.

Additionally, the Glasgow Outcome Scale (GOS) has become the most widely used scale for assessing outcome after head injury and nontraumatic acute brain insults (Wilson *et al.*, 1998). However, the GOS is increasingly recognized as having important shortcoming (Wilson *et al.*, 1998) and early assessment of patients' brain damage may be quite difficult during stay at the intensive care unit (ICU). Therefore, the wide range of conditions associated and the relatively variable predictive value of clinical assessments complicate the identification of patients at risk for development of secondary injury (high intracranial pressure) and fatal outcome (Gahjar, 2000).

Clearly, a practical and sensitive marker is needed to identify these patients as early as possible. If a biomarker were found to be related to outcome, then earlier identification and intervention would be possible (Townend *et al.*, 2002). Thus, treatment of acute brain injuries caused by trauma would benefit from improvements in diagnosis at the onset of secondary brain damage. Rapid identification of patients especially at risk of mortality could indicate earlier the need of higher risk therapy strategies (Coplin, 2001).

Consequently, increasing effort is being devoted to the development of biochemical surrogate markers for brain damage. Among the most extensively studied potential markers for brain damage are the proteins S100B, C-tau, neuron specifc enolase and Hsp 70 (Zemlan *et al*., 2002; Townend *et al*., 2002; Petzold *et al*., 2002; Savola *et al*., 2004; Herrmann *et al*., 2005; Da Rocha *et al.,* 2005). However, neither of these proteins has been established yet as a broadly applicable surrogate marker for brain damage.

Recently, investigations have been performed on the potential use of circulating cell-free DNA in the plasma or serum for clinical diagnosis, prognosis and monitoring of a variety of conditions. Tumor, fetus and donor-derived DNA sequences have been detected in the plasma and serum of cancer patients, pregnant woman and transplant recipients, respectively (Lo *et al*., 1997; Lo *et al*., 1998a; Lo *et al*., 1998b; Johnson *&* Lo, 2002; Wang *et al*., 2003). Significant increases of circulating DNA in the plasma of trauma patients have been reported and found to be a potentially good marker for risk stratification of minor, moderate and severely injured patients. The increased plasma concentrations of DNA were correlated with injury severity and development of posttraumatic complications (Lo *et al*., 2000; Lam *et al*., 2003). Plasma DNA concentrations were also related to stroke severity (Rainer *et al*., 2003).

Therefore, the quantification of plasma DNA may be a useful marker for monitoring diverse types of tissue damage. In this study we aimed to investigate whether nuclear DNA concentrations in plasma after severe traumatic brain injury are correlated to primary outcome (survival or death).

Materials and Methods

Patients

This study was a secondary analysis of archival plasma samples obtained from 41 male patients victims of severe traumatic brain injury. Ethical approval for the study protocol was granted by the Medical Research Ethics Board of the Hospital Municipal de Porto Alegre, Hospital Cristo Redentor and Universidade Luterana do Brasil. Due to unconsciousness of the patients, informed consent was obtained from patients' relatives who were instructed about the purpose of blood sampling. Patients with GCS 3-8 at the emergency room admission and without previous history of neurological or psychiatric disease were recruited. Sixteen patients presented isolate severe TBI and 25 patients had multitrauma with severe TBI.

Only the patients transferred to the ICU within 24 h of the head injury were included in the study. Clinical outcome variables of severe TBI comprised: survival, time for ICU discharge, and neurological assessment using the GOS at the ICU discharge. At study entry (trauma ICU admission), the circulatory function and GCS scores were monitored and APACHE II scores were determined. All patients were sedated and mechanically ventilated. Corticosteroids were not administered. Previous studies established gender differences in the pathophysiology of and outcome after acute neurological injury (Roof & Hall, 2000) or systemic trauma (Jarrar *et al.*, 2000). Lesser susceptibility to postischemic and posttraumatic brain injury in females has been observed (Roof & Hall, 2000; Jarrar *et al.*, 2000). Thus, to avoid interference of possible sex-dependent differences in outcome following brain trauma, only males were enrolled in the study. Control blood samples were obtained from 13 healthy male volunteers without history of brain damage.

Blood sampling

Peripheral blood was collected into heparin containing tubes at admission in the ICU (study entry) and 24h later (if the patient was still in the ICU). Blood samples were centrifuged at 3,000 rpm for 10 minutes, the plasma was removed (with great care taken not to disturb the pellet), and stored at -20℃ until further

processing. Blood samples from the control group were processed in the same way.

DNA extraction from plasma samples

DNA from plasma samples was extracted according to the protocol developed by Boom *et al*. (1990). Briefly, 100 uL of plasma samples were lysed in 900 uL of a GuSCN buffer. After lysis, nucleic acids were bound to silica particles and subsequently washed with several solvents (a GuSCN-containing wash buffer, 70% ethanol and acetone) in consecutive steps. After being dried, the nucleic acids were released from the silica particles in 50 uL of elution buffer.

Real-time quantitative PCR

Theoretical and practical aspects of real-time quantitative PCR were described by Heid *et al*. (1996).

Real-time quantitative PCR analysis was performed using a PE Applied Biosystems 7000 Sequence Detector System. The amplification and product reporting system was based on the 5' nuclease assay (Taqman assay) (Holland *et al*., 1991).

Plasma DNA was measured using a real-time quantitative PCR assay for the β-globin gene (Lo *et al*., 1998a). The β-globin Taqman system consisted of the amplification primers beta-globin-354F (5'-GTG CAC CTG ACT CCT GAG GAG A-3'), beta-globin-455R (5'-CCT TGA TAC CAA CCT GCC CAG-3'), and a dual-labeled fluorescent probe beta-globin-402T [5'-(FAM)AAG GTG AAC GTG GAT GAA GTT GGT GG (TAMRA)-3'] (Lo *et al*., 1998a).

The expression of quantitative results as kilogenome-equivalents/L was described previously (Lo *et al*., 1998a). One genome-equivalent was defined as the amount of a target sequence contained in a single diploid human cell.

Statistical analysis

Group means values were compared using Kruskal-Wallis analysis followed by Dunn´s posttest. The correlation between: (I) plasma DNA concentrations and severe TBI primary outcome (dead/alive); (II) plasma DNA concentrations and APACHE II scores; (III) plasma DNA concentrations and multitrauma (present/not present); was assessed using Spearman's method for non-parametric data. The extent to which the DNA concentrations differed between individuals surviving or dying in the ICU after severe TBI was assessed using receiver operator characteristics (ROC) plots. The ROC plot is obtained by calculating the sensitivity and specificity for every distinct observed data value, and plotting sensitivity against 1 - specificity. The ROC curve was used to evaluate the optimal cut-off values measured 24 h after study entry for prediction of unfavorable outcome. A cut-off point on the curves was chosen to attain the best compromise between sensitivity and specificity for death in the ICU.

Results

Characteristics of the TBI population stratified for the primary outcome measure (survivors/nonsurvivors) are shown in Table 1. The median age was similar between groups, 32 years (range 23-41) in the control group and 34 years (range 18-64) in the severe TBI group (Table 1). Among the patients, there were no significant differences concerning age, initial GCS, first blood sampling time, hemodynamic status, total serum protein or proportion of associated injuries (multitrauma victims) between survivors and nonsurvivors (Table 1). Severe TBI was associated with a 48.8% mortality rate, mostly (13/20, 65%) occurring within 72 h (Table 1, Fig 1A). The median time between the traumatic event and death was 3 days (range 1-15). In contrast, in the survivors group, the median time between trauma and ICU discharge was 11 days (range 2-39) (Table 1), and most patients (16/21, 76%) were severely disabled (GOS < 4) at ICU discharge. Nonsurvivors presented, at study entry, significant higher APACHE II scores than survivors (18.3 ± 4.8 and 11.9 ± 3.8, mean ± S.D., for nonsurvivors and survivors, respectively) (Table 1). Further, there was a significant correlation between higher APACHE II scores and mortality (Spearman's rho = 0.6283 $p < 0.0001$) (Fig. 1B). GCS scores at study entry were, generally, the major determinant of higher APACHE II scores. Sixty one percent of the patients presented multitrauma, especially with chest and extremity associated injuries (Table 1). However, there was no correlation between the presence of associated extracranial injuries and outcome (GOS) (Spearman's rho = 0.2539, $p = 0.1188$) (Fig. 1C).

The plasma concentration of DNA was estimated for all patients at study entry. The mean time for first sampling (study entry) was 11.7 ± 5.2 h (mean ± S.D) after injury (Table 1). Then, in the TBI group, subsequent DNA determinations were performed 24 h after study entry (if the patient survived). In the group of nonsurvivors, 75 % (15/20) of the patients survived until the second sample collection. The control group presented a mean DNA concentration of 3029.7 ± 595.87 kilogenomes-equivalents/L (mean ±S.E.M.). Mean plasma DNA concentrations were significantly increased in TBI patients (347704 ± 184929 and 110009 ± 26206 kilogenomes-equivalents/L, mean ± S.E.M., at study entry and

24h later respectively) compared with the control group (3029 kilogenomes-equivalents/L)(Fig 2A). The mean plasma DNA concentrations of the TBI patients categorized by survival or death, at study entry and 24h later were 95520 ± 38647; 65964 ± 19098; 612497 ± 372477; 171673 ± 54087 kilogenomes-equivalents/L (mean ± S.E.M., DNA concentrations determined at study entry and 24h later for patients who survived and for patients who died respectively). There was a significant difference between the control group and all groups of patients (Dunn's post test $p < 0.05$) (Fig. 2B). There was no significant correlation between higher plasma DNA concentrations determined at study entry and fatal outcome (Spearman's rho = 0.2722 p = 0.0851). However, there was a significant correlation between higher DNA concentrations and fatal outcome 24h after study entry (Spearman's rho = 0.3390 p = 0.043) (Figs. 3A and 3B). Noteworthy, there was no significant correlation between higher plasma DNA concentrations 24h after study entry and either higher APACHE II scores (>15) (Spearman's rho = 0.2899, p = 0.1137) or the presence of associated extracranial injuries (Spearman's rho = 0.1248, p = 0.4684) (Figs. 4A and 4B). ROC curve was plotted (Fig. 5). A cut-off point that would ensure the detection of the highest proportion of individuals with fatal outcome with the least compromise of specificity was chosen. Therefore, a cut-off point of 77883.5 kilogenomes-equivalents/L plasma DNA concentration 24 h after study entry was chosen (Fig. 5). The sensitivity and specificity of plasma DNA, 24 h after study entry, in predicting mortality according to the cutoff point was 67% and 76%, respectively. Thus, the overall mortality rate in the severe TBI group was 48.8%, with most deaths occurring in the first 72 h after injury, and high plasma DNA concentrations (> 77883.50) within 48 h after hospital admission was associated with an increased death risk despite the presence of associated extracerebral injuries.

Discussion

In this study we evaluated the role of plasmatic cell-free DNA as a predictive marker of fatal outcome in adult male patients with severe TBI. As in other studies (Lo *et al*., 2000; Lam *et al*., 2003; Lam *et al*., 2004) all trauma patients had elevated concentrations of DNA in plasma compared with healthy individuals. The precise mechanism by which DNA is released into bloodstream remains uncertain, since both necrosis and apoptosis have been observed in this scenario (Fackelmayer *et al*., 2001). Decreased efficiency of DNA clearance owing to systemic inflammation after trauma may also play a role in the increase of cell-free DNA. Previous studies (Lo *et al*., 2000; Lam *et al*., 2003) found that the increases in plasma DNA were related to injury severity and development of post traumatic complications. In accordance with these studies, we demonstrated that significantly higher concentrations of plasmatic DNA at the second sampling time (mean time 35. 7 ± 5.2 h after trauma) correlated with mortality.

This study investigated victims of either isolated severe TBI or severe TBI with associated extracranial lesions. The most prevalent associated injuries detected were pulmonary contusions and extremity injuries. We found no correlation between the presence of associated extracranial injuries and outcome (GOS) or plasma DNA concentrations. This result is in agreement with Leone *et al*. (2003) that suggested that pulmonary contusion does not appear to increase the morbidity and mortality of multiple trauma patients with head trauma.

Lam *et al*. (2003) reported considerable variation in the degree of early plasma DNA increases. Accordingly, in our first sampling time (mean 11.7 ± 5.2 h) DNA concentrations presented significant variation, possibly affecting correlation with primary outcome. These variations were probably caused by unspecific increases due to DNA release from multiple sites of primary injuries. Neurological damage after severe TBI does not all occur immediately at the moment of impact (primary injury), but evolves afterwards (secondary injury) (Ghajar, 2000). Most secondary brain injury is caused by brain swelling, with an increase in intracranial pressure and a subsequent decrease in cerebral perfusion leading to ischemia. Patients with severe TBI have a significant risk of brain swelling, and if this sequel

is not prevented or treated properly, it can exacerbate brain damage and increase the risk of death (Ghajar, 2000). Later and persistent increases of plasma DNA correlate with mortality following severe TBI despite the presence of associated extracranial injuries. At this time point, persistent increases of plasma DNA may indicate sustained cell damage and evolving secondary brain injury, which is the leading cause of in hospital deaths after TBI (Ghajar, 2000; Finfer & Cohen, 2001).

As expected, higher initial APACHE II scores were significantly correlated with fatal outcome (Zagara *et al*., 1992; Lai *et al*., 1998). However, we could not find a correlation between plasma DNA concentrations and APACHE II scores. Accordingly, Wijeratne *et al*. (2004), that evaluated the prognostic role of cell-free plasma DNA in the prediction of clinical outcome in the intensive treatment unit and, also, did not find correlation between plasma DNA concentrations and APACHE II scores, whereas they found that median DNA concentration in nonsurvivors was 2.3 fold greater than that in survivors.

In the present study, high plasma DNA concentrations at second sampling time (mean time 35.7h after injury) predicted fatal outcome with a sensitivity of 67% and specificity of 76% considering a cut-off value of 77883.5 kilogenomes-equivalents/L. However, at study entry (mean time 11.7h), the results were not predictive. Thus, time from injury must be considered as an important factor to be assessed concerning the predictive value of plasma DNA following severe TBI. Determining the correct and timely diagnosis of deterioration of a patient with TBI is also a challenge for specific neuro-markers with previously established clinical value, such as S100β protein (Rothoerl *et al*., 1999; Raabe *et al*., 1999; Regner *et al*., 2001; Petzold *et al*., 2002).

Lam *et al*. (2003) demonstrated a temporal profile of DNA concentrations in the plasma of patients after trauma with different levels of injury. Considering daily changes, although they found considerable individual variation at study entry, by the second day, mean plasma DNA concentration fell 42%. We observed a similar pattern, since at study entry DNA concentrations also showed great variation (mean concentration 347704.46 kilogenomes-equivalents/L) and fell 68% after 24 hours (mean concentration 110009.78 kilogenomes-equivalents/L). Indeed, in

agreement with Lam *et al*. (2003) that reported that persistent plasma DNA increases coincided with organ failure, we established a positive correlation between mortality and DNA concentrations at our second sampling time (mean time 35.7h after injury). In a previous study that investigated healthy pregnant women, it was demonstrated that DNA has a rapid clearance from plasma, the mean half-life was estimated to be 16.3 minutes (range 4-30 min) (Lo *et al*., 1999). Although, after trauma, clearance mechanisms may be impaired, the short half-life suggests that serial analysis may be useful for monitoring posttraumatic disease processes, as was shown by Lam *et al*. (2003). However, in severe TBI, the ideal temporal profile for plasma DNA prediction of mortality seems to be after the initial 24 h after injury, but has to be further elucidated.

The present study presents some limitations in the establishment of plasma DNA as a predictive marker of outcome after TBI. Early after primary injury, plasma DNA concentrations showed great variation among patients, probably due to different sources of DNA release and therefore probably do not specifically relate to sustained or evolving brain damage. At the second sampling time (mean time 35.7h after injury) higher plasma DNA concentrations correlated with mortality, and could be reflecting sustained cell damage due to secondary neurological deterioration and complication. However, we could not establish a direct correlation between cerebral tissue destruction and plasma DNA concentrations. In this sense, it would also have been important to cross-validate the findings for plasma DNA with other paraclinical measures. A shortcoming of the present study is that image analysis was not defined as a primary outcome measure and therefore was not standardized.

Previous studies have established gender differences in the pathophysiology of and outcome after acute neurological injury. Lesser susceptibility to postischemic and posttraumatic brain injury in females has been observed, and it was suggested that the greater neuroprotection afforded to female is likely due to hormonal factors (Roof and Hall, 2000). For that reason, a homogeneous group of patients was evaluated in the present study to validate a cut-off value for plasma DNA. This established cut-off value now needs to be

validated in a larger and heterogeneous group of patients, which would more closely reflect the situation in a general trauma emergency service.

In conclusion, the present study suggests that severe TBI is associated with elevated plasma levels of DNA regardless of the presence of associated extracranial injuries, and that persistent elevations of plasma DNA correlate with mortality. Thus, plasma DNA concentrations may constitute a predictor of unfavorable outcome in severe TBI in males. However, further research is needed in attempt to establish the optimal sampling time and the precise predictive value of plasmatic DNA after TBI in a larger and heterogeneous group of patients.

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Table 1- Characteristics of the traumatic brain injury study population (TBI) and stratified for the primary outcome measure (survivors/nonsurvivors).

	TBI	survivors	nonsurvivors
Age, median (range),yrs	34 (18-64)	33 (18-64)	41.5 (19-61)
First sampling time following injury (study entry), mean (±S.D)	11.7(5.2)	12.8 (5.6)	10.4 (4.6)
Total serum protein (mg/ml), mean (S.D)	52.4 (9.6)	54.9 (7.7)	49.7 (10.8)
GCS at emergency room admission, mean (S.D)	5.6 (1.7)	5.7 (1.6)	5.5 (1.7)
GCS at study entry, mean (S.D)	5.7 (1.9)	6.5 (1.8)	5 (1.9)
Systolic blood pressure, mean (S.D)	129 (28.3)	136.5 (20.3)	121.1 (33.5)
APACHE II scores, mean (S.D)	15 (5.3)	11.9 (3.8)	18.3 (4.8)
Mechanism of injury (%)			
Motor vehicle accident	46.3	42.9	50.0
Auto-pedestrian	24.4	28.6	20.0
Fall	19.5	19.0	20.0
Assault	9.8	9.5	10.0
Craniotomy, n (%)	13 (31.7)	4 (19)	9 (45)
Multitrauma (%)	61.0	76.2	45.0
Discharged from ICU, n (%)	21(51.2)	-	-
Time between event and ICU discharge, median (range), days		11 (2-39)	-
GOS at discharge from ICU, mean (S.D)		2.9 (0.7)	-
Mortality, n (%)	20 (48.8)	-	-
Time between event and death, median (range), days		-	3 (1-15)

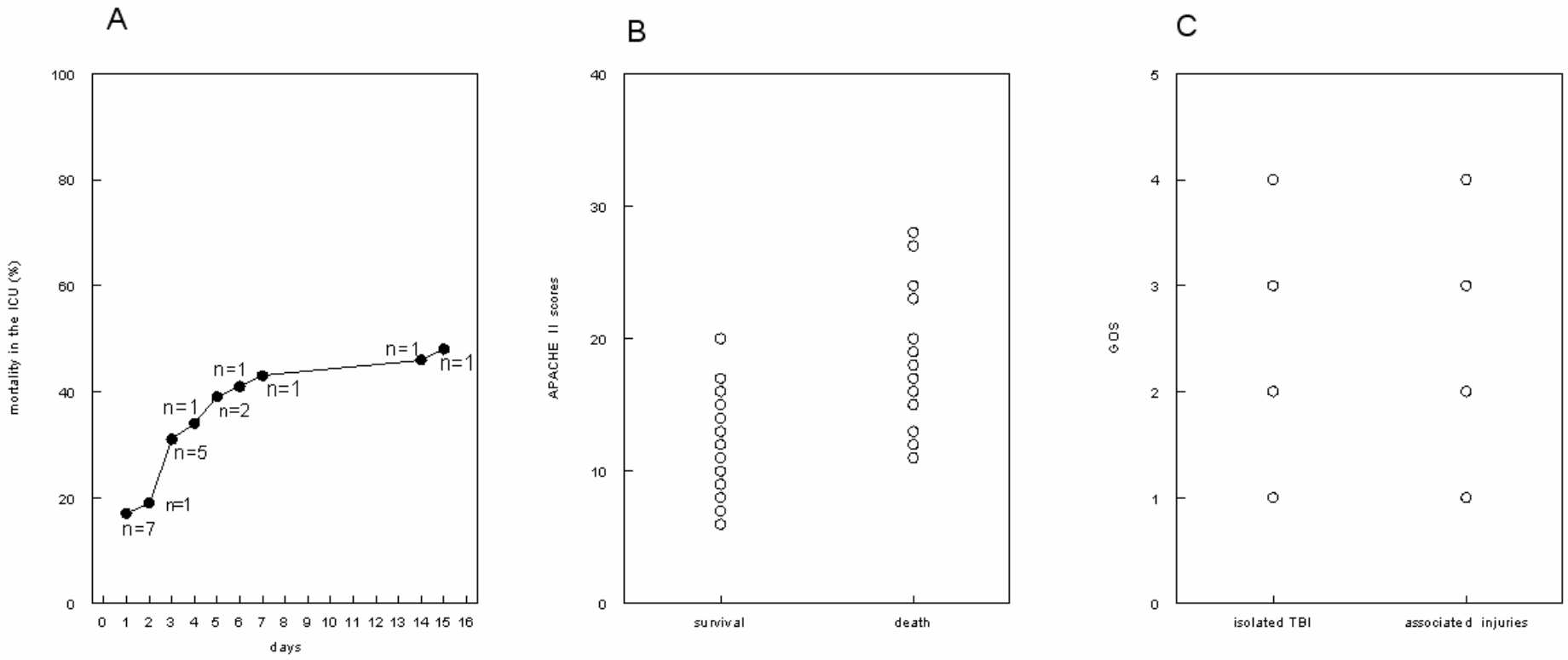

Figure 1. Outcome following severe traumatic brain injury (TBI) (A) and correlation between APACHE II scores and survival (B) or GOS and multitrauma (C). TBI individuals were grouped according to outcome: ICU discharge (n = 21) or death (n = 20). There was a significant correlation between APACHE II scores and survival (Spearman's rho = 0.6283, p < 0.0001) and no correlation between GOS and the presence of multitrauma (Spearman's rho = 0.2539, p = 0.1188).

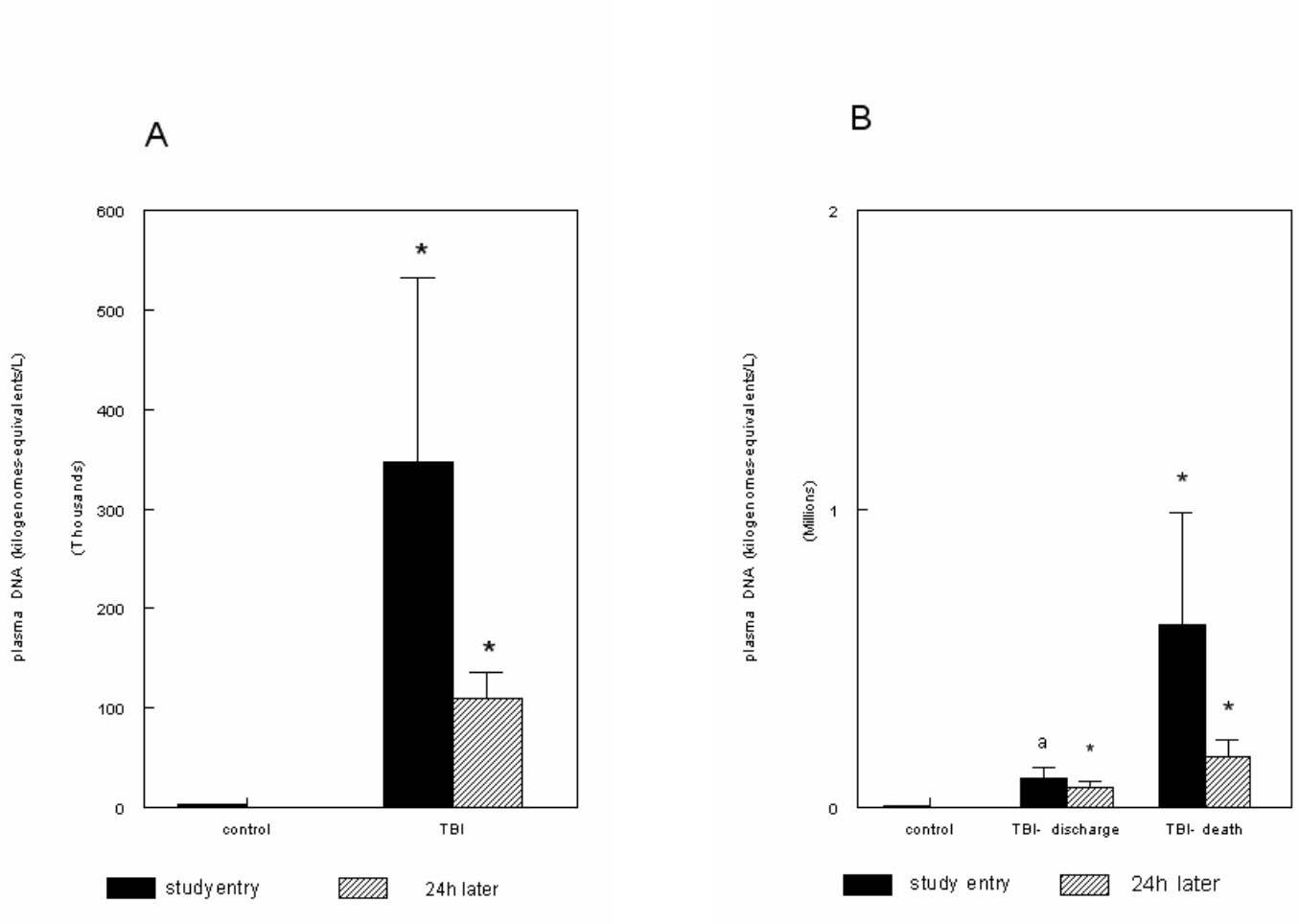

Figure 2. DNA concentrations in the plasma of control and severe traumatic brain injury (TBI) individuals (A), and TBI individuals grouped according to outcome: ICU discharge (n = 21) or death (n = 20)(B). Plasma DNA concentrations were determined at study entry and 24 h later in the TBI groups. Values represent mean ± S.E.M.

* Significantly different from control group ($p < 0.001$, Kruskal-Wallis, followed by Dunn's test).
a Significantly different from control group ($p < 0.01$, Kruskal-Wallis, followed by Dunn's test).

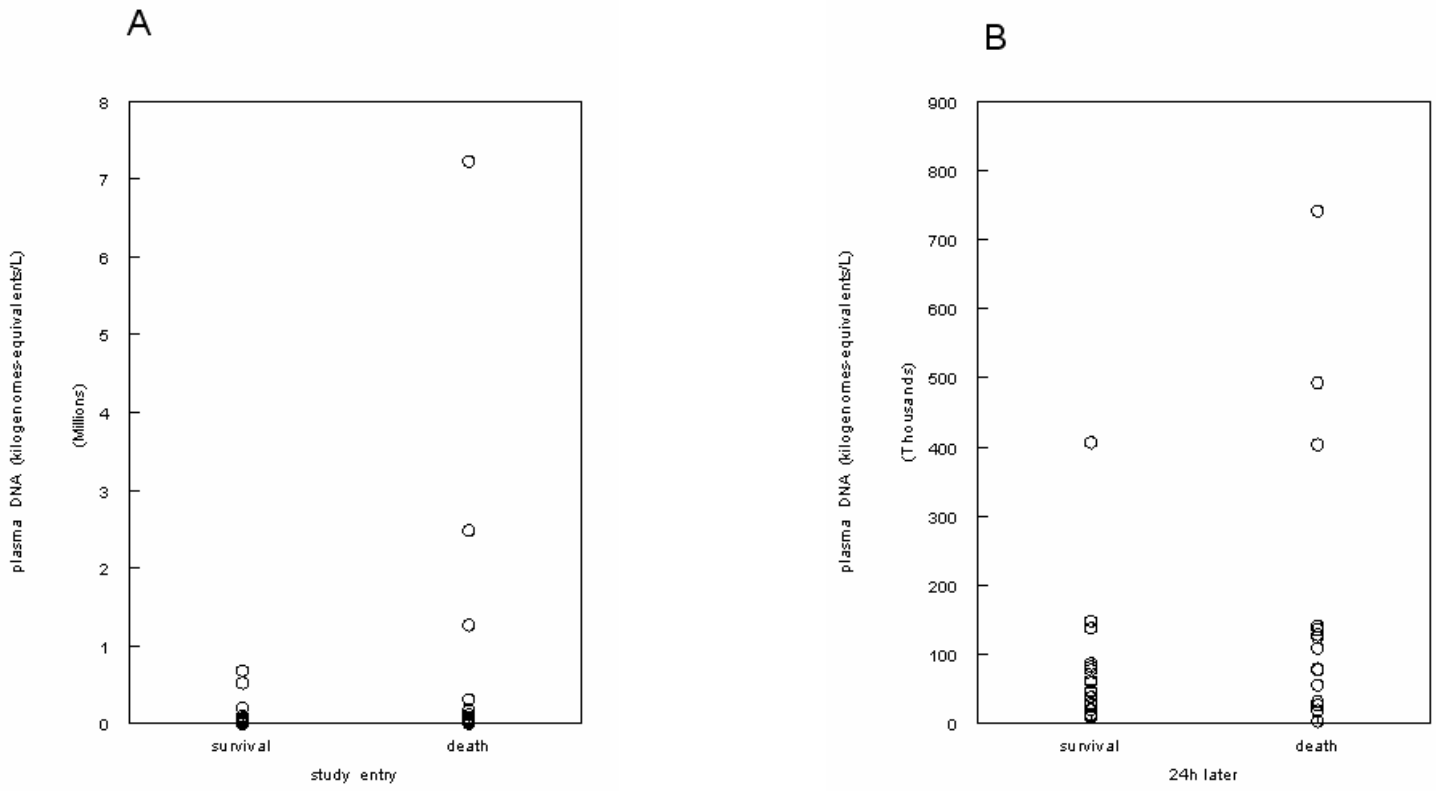

Figure 3. Correlation between mortality and plasma DNA levels at study entry (A) and 24 h later (B) following severe traumatic brain injury (TBI). TBI individuals were grouped according to outcome: survival (n = 21) or death (n = 20). There is a significant correlation between mortality and high plasma DNA levels 24 h after study entry (Spearman's rho=0.3390, p=0.0431).

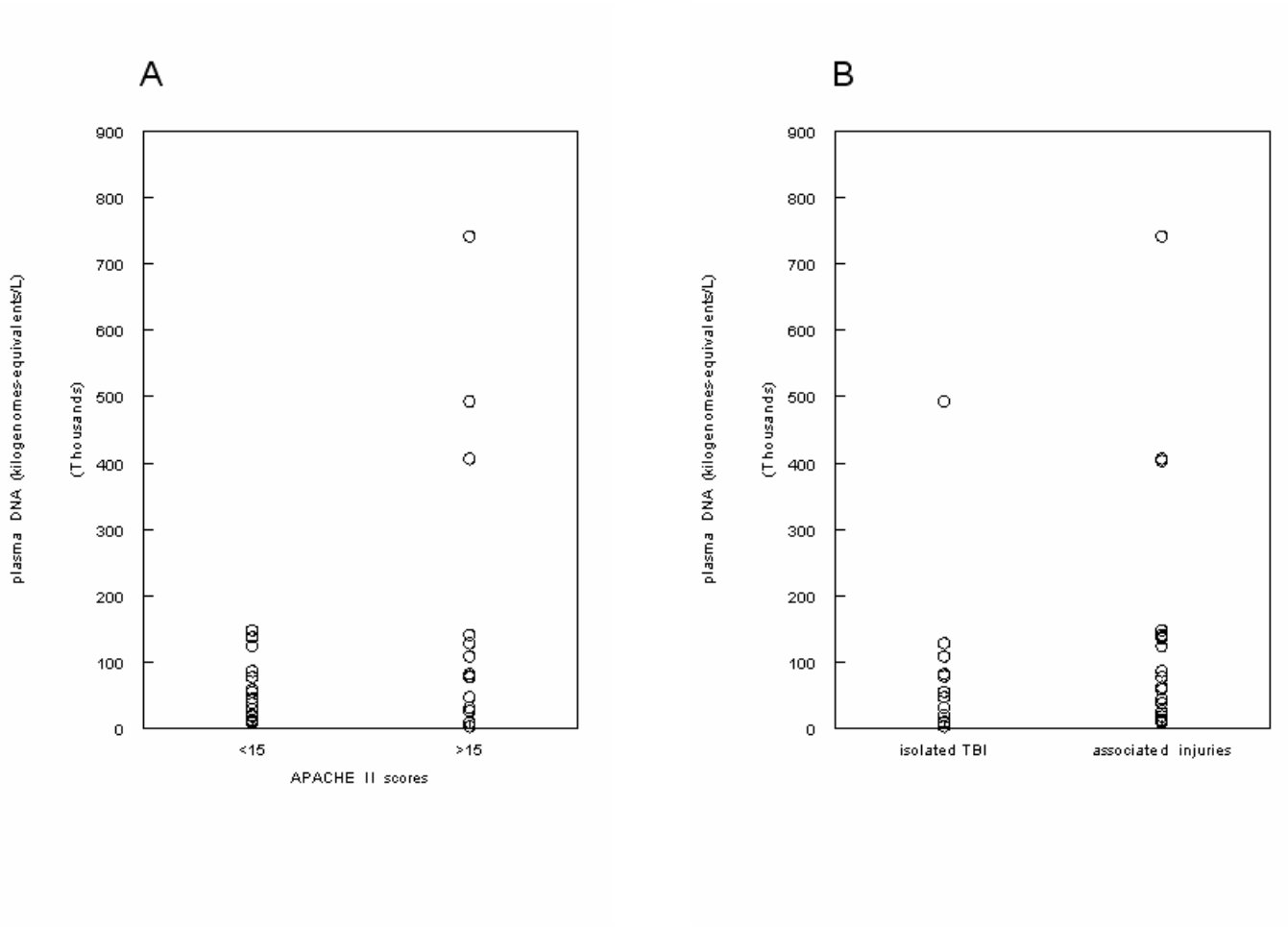

Figure 4. Correlation between plasma DNA levels 24h after study entry and either APACHE II scores (A) or presence of associated extracerebral injuries (B). There was no correlation between DNA levels and either APACHE II scores (Spearman's rho = 0.2899 p = 0.1137) or presence of associated extracerebral injuries (Spearman's rho = - 0.1248 p = 0.4684).

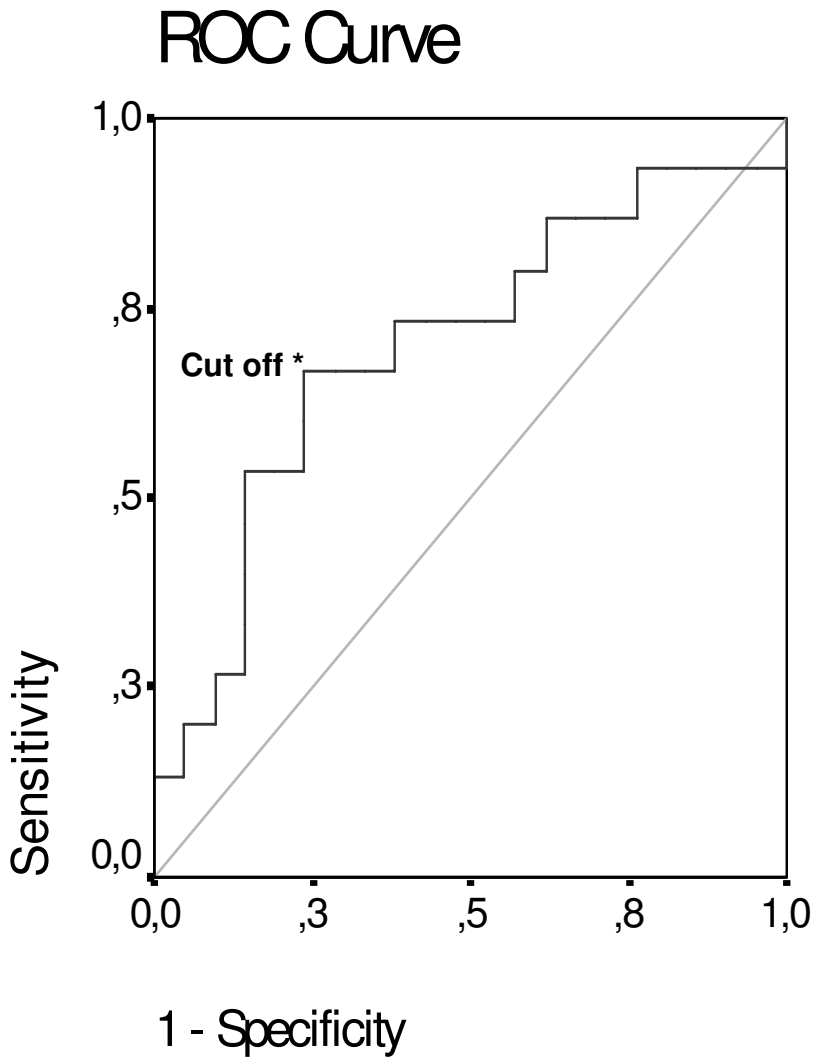

Figure 5. Receiver operator characteristics curves (ROC) of DNA concentrations for predicting fatal outcome 24 h after study entry. The most appropriate cutoff for sensitivity and specificity is indicated and equals 77883.5. The sensitivity and specificity of plasma DNA in predicting mortality according to cutoff point was 67% and 76%, respectively.

CAPÍTULO III

1) PERSPECTIVAS

A avaliação de ácidos nucléicos plasmáticos tem se mostrado uma promissora ferramenta diagnóstica e prognóstica para diversas situações clínicas. Embora os métodos sejam eficazes e rápidos, necessitam de padronização para uso clínico e apresentam custos consideravelmente elevados quando comparados com técnicas bioquímicas.

Métodos moleculares para quantificação de ácidos nucléicos já são amplamente utilizados para fins diagnósticos, prognósticos e de avaliação terapêutica através da determinação de carga viral. A detecção no plasma de DNA do vírus da hepatite B e Citomegalovíurs (CMV), e RNA do vírus da hepatite C e HIV são utilizados como marcadores de infecção viral (Berger e Preiser, 2002). Os métodos de quantificação do genoma desses patógenos, em poucos anos, passaram de ferramentas de pesquisa a fundamentais marcadores diagnósticos correlacionados com doença clínica em grupos de risco (CMV), marcadores prognósticos (HIV) e marcadores terapêuticos (HIV, HCV e HBV) (Berger e Preiser, 2002).

De forma muito semelhante, utilizando os mesmos equipamentos e a mesma tecnologia, existem várias possibilidades de aprimoramento na aplicação de DNA e RNA livres circulantes com objetivos de diagnóstico e monitoramento nas diversas áreas em que têm sido pesquisados. O DNA fetal, por exemplo, é abundantemente produzido e rapidamente metabolizado, fornecendo um quadro dinâmico dos eventos que ocorrem durante a gravidez. Esse fato estimulou o estudo de diversas aplicações clínicas, como detecção de aneuploidias, identificação de pré-eclampsia, diagnóstico não invasivo do genótipo RH e de alguns polimorfismos. Espera-se que novos desenvolvimentos nessa área e a determinação de marcadores moleculares efetivos tornem viável a utilização rotineira e difundida de métodos de diagnóstico pré-natal baseados em ácidos nucléicos circulantes para desordens comuns na gravidez. E, talvez futuramente, com a tecnologia de microarranjos, se possa explorar várias regiões do genoma fetal de modo não invasivo.

Também nos estudos de câncer os resultados são promissores e devem estimular novas pesquisas para pacientes em risco. A tendência é comparar os testes moleculares com os convencionais testes clínicos, pois se faz necessário o desenvolvimento de métodos sensíveis e específicos de diagnóstico não invasivo para detecção precoce, avaliação prognóstica e terapêutica, e determinação de reincidência. Como DNA e RNA circulantes são facilmente acessados e parecem exibir as alterações genéticas presentes no tumor sem apresentar a possibilidade de erro por amostragem como nos diagnósticos histológicos, sua utilização é potencial no diagnóstico e monitoramento do câncer.

No caso do trauma, estudos anteriores com quantificação de DNA plasmático, após a ocorrência de traumas em diferentes níveis de severidade (Lo *et al*., 2000; Lam *et al*., 2003), demonstraram sua correlação com a gravidade das lesões e com o desenvolvimento de complicações pós-traumáticas. Sendo, portanto, possivelmente útil a sua aplicação na estratificação de pacientes em risco e no acompanhamento do processo pós-traumático, o que poderia contribuir nas decisões quanto a intervenções apropriadas. No presente estudo, com pacientes que sofreram traumatismo crânio-encefálico (TCE) grave, foram encontrados padrões muito semelhantes aos estudos anteriores, indicando a possível utilidade deste marcador também para TCE.

Entretanto, algumas limitações devem ser consideradas. O DNA é um marcador inespecífico, liberado de qualquer célula que sofrer necrose, portanto quantidades elevadas não podem ser diretamente associadas ao dano neural. Ainda assim, concentrações persistentemente elevadas, correlacionadas à mortalidade, provavelmente estejam associadas à lesão secundária, que constitui a maior causa de mortalidade após TCE (Ghajar, 2000). A avaliação de diagnósticos de imagem pode ser uma estratégia para se tentar correlacionar a extensão da lesão cerebral com os aumentos de DNA na circulação.

A inexistência de correlação no primeiro tempo amostral (embora se tenha observado tendência) ocorreu pela grande variação nas concentrações de DNA obtidas. Essa elevada variação está possivelmente relacionada com a liberação de diferentes focos de lesão, já que vítimas de TCE com freqüência apresentam

multitrauma com lesões menos significativas, que liberam DNA, mas que não podem ser correlacionadas à mortalidade. Porém, a variação de tempo entre a ocorrência do trauma e a primeira coleta de sangue também pode ter contribuído para a maior variabilidade. Estudos anteriores (Lo *et al.*, 2000; Lam *et al.*, 2003), nos quais a faixa de tempo não foi tão ampla, obtiveram correlações já nos tempos amostrais precoces (o que é importante para a estratificação de risco), ainda que com concentrações de DNA consideravelmente variadas. Talvez, se diminuída a variação de tempo entre o trauma e a primeira amostragem, a variabilidade das concentrações observadas fosse reduzida e se conseguisse obter correlação, mesmo que se obtivesse variação considerável devido à liberação de diversos focos de lesão. Já para a segunda amostragem esse fator temporal possivelmente não seja interferente, pois estabilizadas as lesões menos significativas, aquelas que persistem provavelmente se correlacionam com a mortalidade.

Outras possíveis causas de variabilidade nas concentrações poderiam ser o tempo entre coleta e processamento, ou o tempo de armazenamento das amostras. No entanto, têm-se relatos de que a concentração de DNA no plasma não é alterada quando as amostras de sangue são mantidas por 8h em temperatura ambiente ou por 24h a 4°C (Jung *et al.*, 2003). E também de que não há prejuízo à quantificação pela utilização de amostras de soro arquivadas, desde que o armazenamento seja sob temperatura controlada (Lee *et al.*, 2002; Wataganara *et al.*, 2003).

Uma grande perspectiva de estudo seria a avaliação da concentração no plasma de um mRNA codificado por um gene ativo somente em células neurais. Essa estratégia possibilitaria o desenvolvimento de um marcador mais específico de dano neural.

2) CONCLUSÕES

● As concentrações de DNA livre circulante no plasma de pacientes que sofreram TCE grave foram significativamente maiores do que a dos controles.

● No 2° tempo amostral as concentrações de DNA se correlacionaram com a mortalidade, independente da presença de lesões associadas, indicando que concentrações elevadas podem constituir um marcador de prognóstico desfavorável.

● Após o TCE, o tempo ideal de amostragem para a utilização do DNA plasmático como fator indicativo de mortalidade deve ser melhor estabelecido, com maior padronização nos tempos de coleta.

● A inespecificidade do marcador deve ser considerada como uma limitação do método, pois não se estabeleceu uma relação direta entre as concentrações de DNA e a destruição de tecido cerebral.

● Futuras pesquisas devem ser desenvolvidas para o estabelecimento de um preciso valor preditivo para o DNA plasmático após o TCE em um grupo maior e mais heterogêneo de pacientes.

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