Potential Effect of Metabolic Acidosis on β2-Microglobulin Generation: In Vivo and In Vitro Studies¹

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ABSTRACT

β2-Microglobulin (β2M) is responsible for dialysis-associated amyloidosis. Levels of β2M in plasma increase during chronic renal failure; however, retention does not appear to be the sole mechanism responsible. The effect of metabolic acidosis on β2M production was examined. Thirty-six patients with stable chronic renal insufficiency, 12 uremic patients before their first dialysis, 8 hemodialysis patients who were assigned to acetate or bicarbonate dialysate before their first dialysis, 8 hemodialysis patients who were assigned to acetate or bicarbonate dialysate and then crossed over to the alternative regimen, and 6 normal subjects given NH₄Cl to initiate metabolic acidosis were studied. In vitro studies in the human myeloid cell line U 937 were also performed. β2M protein was measured with ELISA, β2M mRNA was measured with reverse transcription polymerase chain reaction, and the U 937 cells were studied at two pH levels with FACScan flow cytometry. The cells were exposed in vitro up to 60 min in a buffered incubation medium to either pH 5.10 or pH 7.34. An inverse correlation was found between β2M and bicarbonate concentrations in plasma in the stable chronic renal failure patients (r = -0.54; P < 0.05) and in the uremic patients before their first dialysis (r = -0.72; P < 0.05). In hemodialysis patients, blood pH and plasma bicarbonate values were lower (P < 0.05) and β2M concentrations in plasma were higher (P < 0.05) with acetate than with bicarbonate dialysate. In normal men, NH₄Cl resulted in an increase (P < 0.05) in β2M mRNA expression in lymphocytes by an average factor of 1.5 (range, 1.1 to 1.8). In U 937 cells, the cell surface expression of β2M and HLA Class I heavy chain assembled with β2M decreased at low pH compared with normal pH. Concomitantly, an increase in β2M release into the supernatant was observed, possibly as the result of β2M dissociation from cell surface HLA Class I complex. The results suggest that metabolic acidosis may enhance cellular β2M generation and release.

Key Words: β2-Microglobulin, metabolic acidosis, healthy volunteers, uremia, dialysis, amyloidosis

β2-Microglobulin (β2M)-induced amyloidosis is presently recognized as a frequent and major complication in long-term hemodialysis patients (1,2). The factors involved in its pathogenesis are not yet well understood. The constant elevation of β2M levels in plasma as the result of renal retention is a prerequisite for dialysis-associated amyloidosis; however, decreased renal excretion may not be the sole explanation. Other factors may also play a role, such as the duration of chronic renal failure, the type of dialysis technique, and the patients’ age (3).

Attention has been recently directed at the type of dialysis membrane used. The contact of circulating blood with the dialysis membrane may lead to the generation of various serum complement components, cytokines, oxygen radicals, and proteases that contribute to β2M generation and amyloid formation (2). Furthermore, the passage of bacterial endotoxins across the dialysis membrane from the blood compartment may engender similar effects (4). In addition, the β2M structure may be modified, contributing to β2M transformation into amyloid fibrils (5,6). Even though extensive in vitro and ex vivo evidence has been provided in favor of an enhanced β2M production by cellulose dialysis membranes and dialysis-associated factors, clear-cut evidence for their role in vivo is still lacking, probably because of methodological problems (7–9). Moreover, an increase in plasma β2M is not restricted to patients with renal failure. Inflammatory diseases, including connective tissue diseases, rheumatoid arthritis, AIDS, and malignancies, are also accompanied by a more modest elevation of plasma β2M (10) in response to various inflammatory mediators (11,12).

The purpose of these studies was to investigate the possibility that a hitherto unexplored factor, namely metabolic acidosis, might increase the generation or release of β2M. The suggestion that acidosis might play a role came to us by a chance observation made...
during another study in which we observed that metabolic acidosis increased sodium-hydrogen exchanger (NHE)-1 gene expression (13). In that study, we made the serendipitous observation that not only NHE-1, but also \( \beta 2M \), gene expression was increased compared with the “housekeeping” gene glyceraldehyde phosphate dehydrogenase (GAPDH) in lymphocytes.

**METHODS**

**In Vivo Studies**

Chronic Renal Failure Patients and Normal Study Subjects. Two groups of patients with chronic renal failure who did not yet require dialysis were studied. Group 1 consisted of 36 white patients with different stages of stable chronic renal failure. There were 20 men and 16 women with a mean age of 48 ± 20 (SD) yr. Group 2 included 12 other white patients with end-stage chronic renal disease, who were all studied the day before their first intermittent chronic hemodialysis session. These seven men and five women had a mean age of 52 ± 21 yr.

No patient in Group 1 or 2 had clinical evidence of cardiopulmonary decompensation, none had respiratory acidosis, inflammatory disease, or malignancy, and none was diabetic. The underlying nephropathies were similar in Groups 1 and 2 and consisted of chronic glomerulonephritis \( (N = 11) \), reflux nephropathy \( (N = 3) \), chronic interstitial nephritis \( (N = 6) \), polycystic kidney disease \( (N = 14) \), hypertensive nephrosclerosis \( (N = 10) \), and unknown renal disease \( (N = 4) \). The patients were advised to consume a very limited low-protein diet. The patients regularly were prescribed one or several of the following medications: antihypertensive drugs, calcium carbonate, vitamin D derivatives, sodium-containing phosphate binders, and sodium bicarbonate.

Group 3 consisted of eight anticoagulant or dialysis output <50 mL/day) chronic hemodialysis patients. Four were men and four were women, with a mean age of 40 ± 7 yr. They had been treated with hemodialysis for 42 ± 12 months, with three 4-h weekly dialysis treatments with polyacrylamide membranes. The patients were assigned to either acetate (four patients) or bicarbonate (four patients) dialysate. The acetate concentration of the dialysis fluid was 37 mM, and the bicarbonate concentration was 32 mM. After 6 wk, the alternative regimen was given for another 6 wk. A \( \text{Kt/V} \) of 1.1 was maintained throughout. All of the patients were in good condition, none was diabetic, and none had inflammatory or neoplastic disease. Two patients had chronic glomerulonephritis, two had interstitial nephritis, two had polycystic disease, and two had reflux nephropathy. The patients received one or several of the following medications: antihypertensive agents, vitamin D derivatives, \( \text{CaCO}_3 \), and erthropoietin.

Blood was drawn in Groups 1 and 2 patients in the fasted state between 7:00 and 9:00 a.m. In Group 3 patients, blood was withdrawn from the dialysis fistula immediately before a dialysis session was started. Blood \( \text{pH} \), plasma creatinine, bicarbonate (\( \text{HCO}_3 \)), and \( \beta 2M \) were measured with an \( \text{H}^+ \)-specific \( \text{pH} \) electrode (Radiometer, Copenhagen, Denmark), standard automated methods, and an immunoenzymatic method with IMX apparatus and a commercially available kit (Abbott Laboratories, Wiesbaden, Germany) for \( \beta 2M \), after appropriate dilution of the samples when necessary, to \( \beta 2M \) concentrations of 1 to 3 mg/L. The interassay coefficient of variation for \( \beta 2M \) measurements was <5%.

Six normal male subjects (Group 4) were studied in the course of an investigation on the effect of metabolic acidosis on \( \text{NHE-1} \) gene expression (13). They volunteered to ingest 30 500-mg \( \text{NH}_4\text{Cl} \) capsules in six divided doses for a total of 5 days as described elsewhere (14, 15). On the morning of the first day (before \( \text{NH}_4\text{Cl} \) ingestion) and again after the fifth day, arterial venous blood specimens (16) were obtained. Lymphocytes were isolated from blood specimens according to a previously described method (15). The protocols were approved by the institutional review boards of the various institutions involved, and informed consent was obtained from all subjects.

Quantification of \( \beta 2M \) mRNA. The preparation of RNA and the synthesis of cDNA were conducted as described in full detail previously (13). Oligonucleotides used for polymerase chain reaction (PCR) were synthesized on an Applied Biosystems DNA Synthesizer and purified by HPLC. Primers specific for human \( \beta 2M \) sequences were 5'-ACCCCCACT-GAAAGAGTTA-3' (nucleotides 1544 to 1563; sense strand) and 5'-ATCTTCAACCCCTCATGATG-3' (nucleotides 2253 to 2262 and 3508 to 3517; antisense strand). The resulting PCR product is 120 base pairs long (17). Human GAPDH sequences were amplified with the primers 5'-ATCAAGAAGGTT-GGGTGAAG-3' (residues 768 to 758; sense strand) and 5'-ACCTCTGGAGGCCCCATGT-3' (residues 985 to 1002; antisense strand), giving rise to a 235-base-pair product (18).

After reverse transcription, PCR was performed as described previously (13). The reaction was carried out in a volume of 50 mL containing 20 mM Tris-HCl (pH 8.0); 50 mM KCl; 0.1 mg/mL bovine serum albumin (BSA); 1.2 mM MgCl\(_2\); 1 mM dithiothreitol; dATP, dGTP, and dTTP (200 \( \mu \text{M each} \)); 10 \( \mu \text{mol} \) dCTP; 5% (vol/vol) deionized formamide; 100 pmol of the sense and antisense primers, respectively; and 2 \( \mu \text{L} \) of cDNA (corresponding to 100 ng of RNA). After preincubation at 94°C for 3 min, 2 \( \mu \text{L} \) of [\( \alpha ^{32}\text{P} \)]dCTP (3,000 Ci/mol) and 2.5 U of Taq DNA polymerase (BRL, Gairthersburg, MD) were added. The mixture was overlaid with 40 mL of mineral oil and amplified in an automatic Thermal-Cycler (Biomed, Schloss Ditfurth/Theres, Germany). The amplification profile involved denaturation at 94°C for 30 s, primer annealing at 55°C for 1 min, and extension at 72°C for 2 min. Within the primer-specific exponential regions of the amplification reaction, the procedure was stopped after every second cycle and five aliquots of 6 mL each were taken from the reaction mixture and stored at 4°C. A negative control containing water instead of cDNA was included in each experiment. Amplification of \( \beta 2M \) and GAPDH-specific sequences was started with identical amounts of cDNA. The PCR products were separated on 8% polyacrylamide gels and stained with ethidium bromide. The bands corresponding to \( \text{2M} \) and GAPDH, respectively, were excised. Fragments comparable in size were cut from the lane with the negative control for the determination of background levels of \( ^{32}\text{P} \). The radioactivity counted in these samples was subtracted from the counts in the specific bands. The incorporated radioactivity was measured by liquid scintillation counting in 2 mL of Universal Scintillation Cocktail (Roth, Karlsruhe, Germany).

The amount of radioactivity recovered from the gel bands was plotted on a log scale against the number of PCR cycles. Every point in the graph represents the average value of an experiment performed in duplicate and corrected for the background levels of \( ^{32}\text{P} \). Semilogarithmic plots of the radioactivity in the specific PCR products versus cycle numbers yielded straight lines throughout. The coefficients obtained from linear regression analysis were used for the calculation of the amount of radioactivity at cycles 16 and 19 for \( \beta 2M \) and GAPDH, respectively. These cycle numbers lie within the middle of the determined ranges.
TABLE 1. Serum creatinine, HCO₃, and β2M concentrations in stable chronic renal failure patients (Group 1) and uremic patients (Group 2) before their first dialysis (mean ± SD)

<table>
<thead>
<tr>
<th>Group</th>
<th>Creatinine (µM)</th>
<th>HCO₃ (mM)</th>
<th>β2M (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (N = 36)</td>
<td>791 ± 303a</td>
<td>21 ± 4.8</td>
<td>13.5 ± 3.7</td>
</tr>
<tr>
<td>2 (N = 12)</td>
<td>947 ± 177</td>
<td>17.7 ± 5.7</td>
<td>13.9 ± 3.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group</th>
<th>Correlation Coefficient Between Parameters</th>
<th>Creatinine</th>
<th>HCO₃</th>
<th>β2M</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>β2M versus Creatinine</td>
<td>-0.65b</td>
<td>-0.55b</td>
<td>-0.54c</td>
</tr>
<tr>
<td></td>
<td>HCO₃ versus Creatinine</td>
<td>-0.51d</td>
<td>-0.58c</td>
<td>-0.72c</td>
</tr>
</tbody>
</table>

α P < 0.05, Group 1 versus Group 2.
β P < 0.001.
γ P < 0.01.
δ P = not significant.
ε P < 0.05.

In Vitro Studies

A human myeloid cell line (U 937) was cultured in RPMI 1640 medium (GIBCO, Grand Island, NY), supplemented with 10% heat-inactivated fetal bovine serum. After lysis with 10 mL of sterile phosphate-buffered saline (PBS), 5 × 10⁶ cells were resuspended in two different media with a pH of either 5.10 or 7.34. The low-pH solution consisted of 0.2 M citric acid-Na₂HPO₄ buffer containing 1% BSA. The normal pH solution consisted of standard phosphate-buffered physiologic saline with 1% BSA.

The cells were resuspended with 0.5 mL of either one or the other of the above solutions and incubated at 4°C for 1, 6, or 60 min. Thereafter, the incubation procedure was stopped with ice-cold PBS and cell suspensions were centrifuged. Supernatants were lyophilized, and β2M concentrations were determined with a commercially available ELISA (Pharmacia, Uppsala, Sweden). Cell surface antigens were measured by flow cytometry with monoclonal antibodies: anti-β2M HB-149, anti-HLA Class I heavy chain assembled (Immunotech, Marseille-Luminy, Marseille, France) with β2M (W6/32), and anti-immunoglobulin (IgG1 isotypic negative control). For this purpose, 10⁶ cells in PBS containing 1% BSA and 0.1% sodium azide were first incubated for 30 min at 4°C with 1 mg/mL heat-inactivated goat IgG to block Fcγ receptors. Cells were then incubated with appropriate dilutions of specific monoclonal antibodies, followed by fluorescein isothiocyanate-conjugated F(ab)² fragments of goat anti-mouse IgG. After fixation with 1% formaldehyde, the cells were analyzed for fluorescence intensity with a FACSScan flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA) with light scatter gate set.

Statistical Analyses

Data are expressed as means ± SD. Univariate linear regression analysis was used as appropriate. Comparison between data was made by a t test for paired or unpaired data, respectively. For nonparametric comparisons, data were compared with the two-tailed Wilcoxon's signed rank test for paired samples.

RESULTS

Chronic Renal Failure Patients (Groups 1 and 2)

Table 1 shows the mean (±SD) concentrations of creatinine, HCO₃, and β2M in the plasma of patients with different degrees of stable chronic renal failure (Group 1) and of patients with end-stage chronic renal failure immediately before their first dialysis session (Group 2). By definition, the mean creatinine level of Group 2 patients was greater than that of Group 1 patients, although there was no significant difference between the other two variables.

In Group 1 patients, significant correlations existed between plasma values of β2M and creatinine (direct relationship), of HCO₃ and creatinine (inverse relationship), and of HCO₃ and β2M (inverse relationship). In Group 2 patients, there was also a significant inverse relationship between plasma HCO₃ and β2M (Figure 1), even though there was no relationship between plasma β2M and the serum creatinine concentration. In this group, an inverse relationship was nonetheless observed between plasma HCO₃ and the creatinine concentration. A multivariate regression analysis could not be applied because of the relatively limited sample size in Group 2.

Hemodialysis Patients (Group 3)

Table 2 shows mean±SD blood pH and plasma
TABLE 2. Parameters in eight hemodialysis patients (Group 3) with either acetate or bicarbonate dialysate for 6 wk, respectively (mean ± SD)

<table>
<thead>
<tr>
<th>Dialysate</th>
<th>Blood pH</th>
<th>HCO₃⁻ (mM)</th>
<th>β2M (mg/L)</th>
<th>Creatinine (μM)</th>
<th>Na (mM)</th>
<th>K (mM)</th>
<th>Total Ca (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate</td>
<td>7.29 ± 2.97&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17.9 ± 2.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>35.5 ± 6.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>933 ± 167&lt;sup&gt;c&lt;/sup&gt;</td>
<td>138 ± 4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5.4 ± 0.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.3 ± 0.20&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Bicarbonate</td>
<td>7.32 ± 3.25</td>
<td>21.0 ± 2.8</td>
<td>32.9 ± 5.6</td>
<td>898 ± 167</td>
<td>138 ± 3</td>
<td>5.0 ± 1.3</td>
<td>2.3 ± 0.25</td>
</tr>
</tbody>
</table>

<sup>a</sup> Acetate versus bicarbonate group: P < 0.01.
<sup>b</sup> Acetate versus bicarbonate group: P < 0.05.
<sup>c</sup> Acetate versus bicarbonate group: P = not significant.

Creatinine, HCO₃⁻, and β2M values of the hemodialysis patients before and after treatment with either acetate or bicarbonate dialysate, respectively. Blood pH and HCO₃⁻ concentrations were significantly lower with acetate than with bicarbonate dialysate, whereas plasma β2M values were significantly higher with acetate than with bicarbonate dialysate. Creatinine, sodium, potassium, total calcium, and Kt/V (not shown) were not different with the two treatments. No inverse relationship was found between plasma HCO₃⁻ and β2M in this group; however, the number of patients was relatively small.

Healthy Volunteers (Group 4)

Figure 2a displays typical amplification profiles for β2M mRNA in lymphocytes before and during metabolic acidosis (blood pH, 7.40 ± 0.01 versus 7.28 ± 0.04; P < 0.05). The amplification was linear within cycles 12 to 20. Also evident is the distinct parallel shift toward increased β2M mRNA levels in lymphocytes prepared from an individual during metabolic acidosis. Figure 2b displays the amounts of β2M-specific PCR products before (control) and during metabolic acidosis in six different individuals. On average, β2M-specific mRNA increased by a factor of 1.51 ± 0.27 (P < 0.05, acidosis versus control). GAPDH-specific mRNA products, on the other hand, did not change significantly during metabolic acidosis (data published in Reference 13).

In Vitro Studies

Figure 3 shows the concentrations of β2M determinations in the supernatant of the incubation medium of human myeloid U 937 cells after they had been exposed to either low or normal pH for 1, 5, or 60 min. Table 3 contains the flow cytometry measurements of the respective amounts on the U 937 cell surface of β2M, HLA Class I heavy chain assembled with β2M, and IgG1. After exposure of the cells to low pH compared with normal pH, a greater release of β2M into the incubation medium was observed at the 60-min incubation time. Moreover, the amount of cell surface β2M and HLA Class I heavy chain assembled with β2M, but not that of control IgG1, was lower at low than at normal pH for each time studied.

DISCUSSION

This study provides the first clinical and experimental evidence in favor of the notion that metabolic acidosis may enhance cellular β2M generation and release in humans. First, in patients with stable chronic renal failure, HCO₃⁻ and β2M were correlated, whereas in end-stage renal failure patients before their first dialysis, the degree of metabolic acidosis was correlated with the concentration of β2M in plasma in spite of the absence of a relationship between plasma β2M and creatinine values. Second, in chronic hemodialysis patients, the switch from bicarbonate to acetate dialysate was associated with a significant increase in β2M concentrations in plasma, together with a decrease in blood pH and HCO₃⁻ concentrations in plasma. Third, in normal healthy volunteers, an experimentally induced metabolic acidosis led to an increase in lymphocyte β2M mRNA expression. Finally, in in vitro experiments with human myeloid U 937 cells, diminishing the pH of the incubation solution was associated with an acute decrease in β2M expression on the cell surface and an acute release of β2M into the incubation medium.

Several factors are currently incriminated in the increase of plasma β2M observed in various disease states. Clearly, renal insufficiency is the most prominent cause through the retention of β2M secondary to diminished glomerular filtration and tubular catabolism of the protein (2, 19). Enhanced β2M generation and/or release during the course of various inflammatory and neoplastic disease states is another commonly incriminated cause (10), subsequent to stimulation by interferons and cytokines (11,12). The protein is formed in all types of eukaryotic cells because it is an obligatory component of the HLA Class I major histocompatibility complex. Controversy remains about the relative contribution of different cell types and organs to the systemic generation of β2M in the organism. It appears that the liver represents a major source (20).

To the best of our knowledge, acidosis has never been suspected to play a role in β2M production in vivo. There are only two previous reports that we were able to identify that dealt with a possible pH effect on cellular β2M release in vitro. The first report is the
study by Polakova et al. (21), who performed short-term incubation experiments in which blood-borne cells were exposed to a solution with the extremely low pH of 3 for up to 6 min. They used immunoprecipitation and RIA methodology in their study. Polakova et al. (21) observed a decrease in the amount of free β2M, β2M associated with HLA Class I heavy chain, and HLA Class I heavy chains associated with β2M on the cell surface. Moreover, the concentration of β2M in the incubation medium increased during the same time period. In another study, Hochman et al. (22) also showed a dissociation of β2M from the HLA Class I complex after the exposure of cells in vitro to pH values of 5.0 to 5.5.

In this study, we proceeded to similar in vitro experiments. We decreased the pH of the incubation solution to a value of 5.10. Moreover, the exposure of cells to low or normal pH was prolonged up to 60 min. We were able to confirm the above in vitro findings. The acute decrease of free β2M expression and of HLA Class I heavy chain–associated β2M expression on the cell surface and the acute increase of β2M concentra-
tion in the incubation medium at pH 5.10 relative to pH 7.34 suggest a dissociation of the protein from the HLA Class I complex and a release of β2M into the environment.

On the other hand, the ex vivo finding of an increased β2M mRNA expression in lymphocytes isolated from healthy volunteers with an experimentally induced metabolic acidosis is consistent with an increased generation of β2M, after the induction of a more prolonged acid state. The observation in our end-stage renal failure patients (Group 2) of an inverse relationship between β2M and bicarbonate levels in plasma, in the absence of a significant relationship between β2M and creatinine concentrations in plasma at this stage of severe renal insufficiency, would be consistent with enhanced β2M generation and/or enhanced β2M release, as well as the β2M retention due to decreased renal function. Similarly, the higher plasma β2M values in hemodialysis patients in the presence, rather than in the absence, of metabolic acidosis, may be best explained by enhanced generation or release. However, an additional possibility remains a direct effect of acetate on β2M synthesis. In a recent study, Anderson et al. (23) examined the effect of acetate versus bicarbonate dialysate on various cytokines, growth factors, and serum β2M. They found that serum β2M values were 81 mg/mL after acetate dialysis compared with 73 mg/mL after bicarbonate dialysis. This numerical difference was not statistically significant; however, the exposure to the different dialysates was for only 1 wk in their study. In our study, the exposure to the different dialysates was for 6 wk.

We cannot offer much insight into the mechanisms by which metabolic acidosis may promote increased β2M gene expression and increased β2M synthesis on the basis of our data. However, metabolic acidosis is known to increase the expression of other genes. For instance, we observed earlier that metabolic acidosis increases Na+/H+ antiport activity in lymphocytes and caused increased production of NHE-1 mRNA (13). Consistent with these findings are the observations of Corry et al. (24), who found increased Na+/H+ exchanger protein in the erythrocytes of uremic patients with metabolic acidosis. Moe et al. (25,26) were able to confirm that mouse renal cortical tubular cells and an opossum kidney cell line responded to a 24-h in vitro metabolic acidosis with an almost twofold increase in Na+/H+ exchange activity. This increase in antiport activity was accompanied by a threefold increase in antiport mRNA. These results could be confirmed by feeding rats with a diet that induced metabolic acidosis. Metabolic acidosis may contribute to the progression of chronic renal disease in a variety of ways (27,28). We believe that our findings suggest a role for increased expression of yet to be identified genes in this process.

Our findings obviously do not minimize the major importance of the failing kidney itself in increased β2M levels in plasma, as shown by the relation of plasma β2M and creatinine in Group 1 patients in this study, who had less advanced degrees of chronic renal insufficiency. The relative contribution of metabolic acidosis to the variable but increased β2M levels in plasma in patients with chronic renal failure remains to be defined. Further, the role of metabolic acidosis in the increased β2M production occurring in other disease states warrants investigation. Because metabolic acidosis is amenable to treatment, the findings may have therapeutic implications.


