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S-adenosylmethionine mediates inhibition of inflammatory response and changes in DNA methylation in human macrophages

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Pfalzer AC, Choi SW, Tammen SA, Park LK, Bottiglieri T, Parnell LD, Lamon-Fava S. S-adenosylmethionine mediates inhibition of inflammatory response and changes in DNA methylation in human macrophages. Physiol Genomics 46: 617–623, 2014. First published July 15, 2014; doi:10.1152/physiolgenomics.00056.2014.—S-adenosylmethionine (SAM), the unique methyl donor in DNA methylation, has been shown to lower lipopolysaccharide (LPS)-induced expression of the proinflammatory cytokine TNF-α and increase the expression of the anti-inflammatory cytokine IL-10 in macrophages. The aim of this study was to assess whether epigenetic mechanisms mediate the anti-inflammatory effects of SAM. Human monocytic THP1 cells were differentiated into macrophages and treated with 0, 500, or 1,000 μmol/l SAM for 24 h, followed by stimulation with LPS, TNFα and IL-10 expression levels were measured by real-time PCR, cellular concentrations of SAM and S-adenosylhomocysteine (SAH), a metabolite of SAM, were measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS), and DNA methylation was measured with LC-MS/MS and microarrays. Relative to control (0 μmol/l SAM), treatment with 500 μmol/l SAM caused a significant decrease in TNF-α expression (−45%, P < 0.05) and increase in IL-10 expression (+77%, P < 0.05). Treatment with 1,000 μmol/l SAM yielded no significant additional benefits. Relative to control, 500 μmol/l SAM increased cellular SAM concentrations twofold without changes in SAH, and 1,000 μmol/l SAM increased cellular SAM sixfold and SAH fourfold. Global DNA methylation increased 7% with 500 μmol/l SAM compared with control. Following treatment with 500 μmol/l SAM, DNA methylation microarray analysis identified 765 differentially methylated regions associated with 918 genes. Pathway analysis of these genes identified a biological network associated with cardiovascular disease, including a subset of genes that were differentially hypomethylated and whose expression levels were altered by SAM. Our data indicate that SAM modulates the expression of inflammatory genes in association with changes in specific gene promoter DNA methylation.

inflammation; macrophage; S-adenosylmethionine; S-adenosylhomocysteine; DNA methylation

INFLAMMATION PLAYS A CRITICAL ROLE in the initiation and progression of chronic conditions such as diabetes (28), cardiovascular disease (11, 34), osteoarthritis (19), and clinical depression (31). Moreover, it has been shown that inhibition of inflammation may prevent and/or alleviate insulin resistance (7) and atherosclerotic plaque formation (38). Therefore, anti-inflammatory compounds can have a significant impact on the prevention and/or treatment of these chronic diseases.

S-adenosylmethionine, or SAM, has been used for several years in the treatment of hepatic conditions and osteoarthritis (21) and more recently in the treatment of clinical depression (32). Of relevance, recent evidence indicates that SAM exerts anti-inflammatory effects by reducing the expression of the proinflammatory cytokine tumor necrosis factor-alpha (TNFα) (2) and increasing the expression of the anti-inflammatory cytokine interleukin 10 (IL-10) (30). SAM is the main cellular methyl donor involved in DNA, RNA, and histone methylation and therefore may modulate gene expression via epigenetic mechanisms. Methylation reactions generate S-adenosylhomocysteine (SAH) (36), a potent inhibitor of SAM-dependent methylation processes. SAM is also involved in the enzymatic reactions leading to polyamine biosynthesis and in the trans-sulfuration pathway producing the antioxidant glutathione. It has been suggested that SAM’s anti-inflammatory effects are in part mediated by altering the methylation and/or binding of histones to the promoter region of proinflammatory or anti-inflammatory cytokine genes (2). In addition, SAM was shown to alter the methylation of histone H3 and its binding to the promoter region of the short isoform of the phosphodiesterase 4B gene (PDE4B), thus affecting cAMP-mediated TNFα expression (18). These epigenetic modifications may contribute to the anti-inflammatory action of SAM. However, SAM has the potential to regulate gene expression also by affecting the methylation of CpG-islands of specific promoter regions. In the present study, we have evaluated the effects of SAM on global and site-specific DNA methylation and gene expression in the context of lipopolysaccharide (LPS)-stimulated inflammation in human macrophages. Our results demonstrate for the first time that SAM may modulate inflammation through the regulation of the methylation of specific promoter regions.

MATERIAL AND METHODS

Materials and reagents. The stabilized salt form of SAM, S-(5'-adenosyl)-L-methionine p-toluenesulfonate salt, and LPS were obtained from Sigma (St. Louis, MO). RPMI-1640 medium and other cell culture reagents were obtained from Life Technologies (Carlsbad, CA). TRIZol reagent, RNeasy kits, and SuperScript III First Strand Synthesis System kits were purchased from Life Technologies, and Power SYBR Green PCR Mastermix was purchased from Applied Biosystems (Life Technologies, Carlsbad, CA). Nuclease P1, venom...
phosphodiesterase I, and alkaline phosphatase, as well as ammonium bicarbonate and ammonium acetate used for the hydrolysis of DNA for liquid chromatography and tandem mass spectrometry (LC-MS/MS), were purchased from Sigma (St. Louis, MO). The stable isotopes [15N]2-deoxycytidine and (methyl-d3,ring-6-d1)-5-methyl-2-deoxycytidine used as internal standards were synthesized by Cambridge Isotopes Laboratories (Andover, MA). The magnetic methylated immunoprecipitation (MagMeDIP) kit was purchased from Diagenode (Denville, NJ), the whole genome amplification kit from Sigma (St. Louis, MO), and the QIAQuick clean-up kit from Qiagen (Beverly, MA). All 3×720K RefSeq Promoter and CpG Island arrays and microarray processing reagents were obtained from Roche-NimbleGen (Madison, WI).

Cell culture. The human monocyctic cells THP1 were obtained from the American Type Culture Collection (Rockville, MD) and were maintained in culture in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 4,500 mg/l glucose, 1 mM sodium pyruvate, 10 mM HEPES, 1,500 mg/l sodium bicarbonate, and 100 U/ml penicillin-streptomycin at 37°C in a 5% CO2 incubator. Cells were plated at a concentration of 1 × 10^6 cells/ml and differentiated into macrophages with 100 ng/ml phorbol 12-myristate 13-acetate for 72 h as previously described (10). Cells were then incubated into macrophages with 100 ng/ml phorbol 12-myristate 13-acetate and 100 U/ml penicillin-streptomycin at 37°C in a 5% CO2 incubator. Cells were then harvested for DNA and RNA isolation. Cell viability was assessed by Trypan blue staining to identify anypotential toxicity associated with SAM treatment. No toxicity (>95% viability, relative to control cells) was observed for up to 1,000 μM SAM.

Quantitative real-time PCR. Total RNA was isolated with TRIzol reagent and reverse-transcribed with SuperScript III First-Strand Synthesis System kits. mRNA levels of the reference gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and of the target genes (see below) were assayed by real-time PCR in an Applied Biosystems 7300 Real Time PCR system (V1.4 SDS software) using Power SYBR Green PCR Master Mix and the following primer sets: GAPDH AGGCGGGTGTCT AGTATGCT (forward), TGCTGTCTCAGCACCTTCTT (reverse); TNFα ATGGGCTTTCCGAAATTC (forward), GAGGCAACTGCA CACTCTCTCTT (reverse); IL-10 TAC TCTGAGGAAGGTGATG (forward), GCCGTGCTCTGTTCTTCC (reverse); α2 mimokine (C-C motif) ligand 2 (CCL2) TTCCAAAGA AAGCTGTTACCTTA (forward), TGCTGTCTCAGGTGTCCTCAT (reverse); C-C chemokine receptor type 2 (CCR2) ATGCTGCTCA CTCTGGTCTC (forward), TTATACACGGGCGAGACTTC TTGCT (reverse); and nuclear factor kappa light-chain enhancer of activated B cells (NFκB1) (p50) CCATATTGGAAGGCGGTAA (forward), TGCTTCCACATAGTTGCAGA (reverse). The relative quantification (ΔΔCT) method was used to determine the expression of the target genes, using GAPDH as the internal control.

SAM and SAH measurements. Intracellular SAM and SAH were measured by LC-MS/MS in Dr. Bottiglieri’s laboratory (Baylor Research Institute, Dallas, TX). Cells were treated as indicated above, then culture medium was aspirated, and the cell monolayer was washed with cold PBS. Cells were then treated with 100 mmol/l perchloric acid and immediately scraped and placed into microcentrifuge tubes. Cells were immediately frozen at −80°C until measurement. Cell pellets were reconstituted in 100 mmol/l NaOH prior to analysis. The concentrations of SAM and SAH were measured in cell lysates by a modification of an LC-MS/MS method as previously described (37).

Global DNA methylation. DNA was isolated by a phenol-chloroform extraction. DNA was then enzymatically hydrolyzed, and global methylation was analyzed by an LC-MS/MS assay as previously described (14).

Genome-wide DNA methylation. The location of differentially methylated regions in the genome was assessed by microarray analysis. In brief, DNA was randomly fragmented by sonication into 200–1,000 bp fragments and then immunoprecipitated with an antibody (Diagenode) against methylated cytosine (meDIP), resulting in samples enriched with methylcytosine 4. Methylcytosine-enriched samples were then fluorescently labeled (Cy5), and input control samples (not methylcytosine-enriched) were fluorescently labeled (Cy3), followed by competitive hybridization to a high throughput 3×750K NimbleGen array containing CpG islands and all annotated gene promoters in the human genome. Signals were detected with the NimbleGen microarray scanner MS 200 (16). The Comprehensive High-throughput Array for Relative Methylation (CHARM) method in R Studio previously described (3) was modified to analyze the meDIP DNA methylation arrays for control and 500 μmol/l SAM-treated cells (3, 27). Individual Z-scores per probe were calculated, and a minimum of three adjacent probes with P < 0.005 between groups were identified to contribute to a differentially methylated region (DMR). This analysis assumes that most regions in the genome are not differentially methylated; therefore, the SD of the null distribution is generated by the median absolute deviation of Z-scores across all array probes. Using the online software program Genomic Regions Enrichment of Annotations Tool (GREAT, version 2.0.2), we annotated these identified DMRs to particular genes. For this study, DMRs that were 5 kb upstream or 1 kb downstream of a gene’s transcription start site were mapped to that gene, and the nearest genes outside this range but within 1,000 kb are labeled as distal. Pathway assignments of the gene lists generated in GREAT was conducted by Ingenuity Pathway Analysis, or IPA (http://www.ingenuity.com).

Statistical analysis. All data are expressed as means ± SD obtained from at least three independent experiments conducted in duplicate. Statistical differences were assessed by one-way ANOVA and further analyzed by Fisher’s exact test. Differences between treatments were considered to be statistically significant at P < 0.05.

RESULTS

Effects of SAM on TNFα and IL-10 expression. The effects of 24 h SAM treatment on the LPS-stimulated expression of TNFα and IL-10 were assessed to establish THP1 cells as a model of human macrophage inflammatory response. LPS stimulation resulted in an average 170-fold increase in TNFα and 150-fold increase in IL-10 expression relative to unstimulated control cells (data not shown). In LPS-stimulated cells, 500 μmol/l SAM decreased TNFα expression by 45% (P < 0.05), and 1,000 μmol/l SAM decreased TNFα expression by 43% (P < 0.05), relative to control (0 μmol/l SAM) (Fig. 1A). Treatment with SAM also increased IL-10 expression levels by 77% at 500 μmol/l SAM concentration and 79% at 1,000 μmol/l SAM concentration (both P = 0.01), relative to control (Fig. 1B).

Effects of SAM treatment on intracellular SAM and SAH. The effects of 24 h SAM treatment on intracellular SAM and SAH concentrations were measured in two independent experiments. Relative to control, treatment with 500 μmol/l SAM increased cellular SAM concentrations approximately twofold, while 1,000 μmol/l SAM treatment resulted in a sixfold increase (Table 1). Although intracellular SAH concentrations did not significantly differ between control and 500 μmol/l SAM treatments, SAH levels increased more than fourfold in 1,000 μmol/l SAM-treated cells relative to control cells (Table 1).

Effects of SAM treatment on global methylation. To assess the effects of altered intracellular levels of SAM and SAH on DNA methylation, we measured global DNA methylation of THP1 cells treated with different SAM concentrations. Global DNA methylation was increased 6.6% in cells treated with 500...
μmol/l SAM relative to control cells (4.8% vs. 4.5%, respectively, \( P < 0.05 \)) (Fig. 2). No significant difference in global DNA methylation relative to control cells was observed in cells treated with 1,000 μmol/l SAM (4.6%).

**Effects of SAM treatment on genes associated with cardiovascular disease.** As 1,000 μmol/l SAM treatment did not alter global methylation, the remaining experiments on site-specific gene methylation were performed comparing cells treated with 500 μmol/l SAM to control cells. DNA isolated from THP1 macrophages was used in microarray analyses to identify differentially methylated CpG islands. Microarrays identified 765 DMRs between cells treated with 500 μmol/l SAM and control cells. These DMRs were annotated to 918 genes (Supplemental Table S1). The transcription start site for 628 of 765 DMRs between cells treated with 500 μmol/l SAM and control cells are shown in Fig. 5. Relative to control, 500 μmol/l SAM resulted in a 35% decrease in CCL2, 39% decrease in CCR2, and 25% decrease in NFKB1 (all \( P < 0.05 \)).

**DISCUSSION**

The link between inflammation and chronic diseases, such as cardiovascular disease, diabetes, and osteoarthritis, is well established, and thus the potential to regulate the inflammatory response, particularly through the use of small molecules, may have significant implications for reducing the risk or progression of these chronic conditions. The present study examined the effect of SAM on cytokine expression in human THP1 macrophage cells and provides suggestive evidence that SAM participates in genome-wide as well as gene-specific epigenetic modifications with concomitant changes in the expression of genes encoding specific inflammatory mediators. Notably, a significant effect of SAM on global and gene-specific methylation and on gene expression occurred within 24 h of exposure, suggesting that epigenetic regulation of gene expression may be modulated rapidly after nutritional or supplemental exposure. Our study clearly shows a significant reduction in LPS-stimulated expression of CCL2, CCR2, and NFKB1 in THP1 cells are shown in Fig. 5. Relative to control, 500 μmol/l SAM resulted in a 35% decrease in CCL2, 39% decrease in CCR2, and 25% decrease in NFKB1 (all \( P < 0.05 \)).

**Table 1. Effects of 24 h SAM treatment on THP-1 macrophage intracellular SAM and SAH levels**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>SAM, nmol/mg protein</th>
<th>SAH, nmol/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAM 0 μM</td>
<td>56.1</td>
<td>2.1</td>
</tr>
<tr>
<td>SAM 500 μM</td>
<td>90.3</td>
<td>2.2</td>
</tr>
<tr>
<td>SAM 1,000 μM</td>
<td>329.7</td>
<td>9.2</td>
</tr>
</tbody>
</table>

*Data are the means of 2 independent experiments. SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine.

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inhibitor of DNA methyltransferases (DNMT) (26). In the context of the increased SAM/SAH ratio in cells treated with 500 µmol/l SAM, we observed significant epigenetic changes. The lack of significant changes in DNA methylation with 1,000 µmol/l SAM is likely related to the fourfold increase in intracellular SAH associated with this treatment. Previous studies have demonstrated that elevations in SAH as opposed to decreases in SAM are more predictive of cellular methylation capacity and DNMT activity (9), suggesting that the inhibitory effect of SAH on DNMTs is stronger than the enhancing effect of SAM (9). Thus, the ratio of SAM to SAH may be the best predictor of methylation capacity and/or DNMT activity. Elevations of SAH are also associated with inflammatory disorders such as vascular disease (8). The fact that the transcriptional changes in TNFα and IL-10 at 1,000 µmol/l SAM mirrored those observed at 500 µmol/l SAM may indicate that the fluctuation in SAH levels did not affect the anti-inflammatory effect of SAM: future microarray methylation studies should provide information on the effects of different doses of SAM on specific gene methylation. SAM functions as the universal methyl donor and as such participates in epigenetic regulation of gene expression via methylation of DNA and histone proteins (29). SAM is also the methyl donor in many other cellular methyltransferase reactions, including RNA and protein methylation, synthesis, and detoxification processes (42). Interestingly, we found 10 genes encoding select methyltransferases and other proteins known to bind to SAM (44) to be differentially methylated after 24 h 500 µmol/l SAM treatment, and this may have consequences for feedback or regulatory mechanisms.

SAM has been shown to reduce TNFα mRNA levels and protein secretion in mouse RAW264.7 cells by affecting the methylation of the histone H3 binding to the TNFα promoter (2). On the other hand, another study has reported that the SAM-associated suppression of TNFα expression in RAW264.7 cells is mediated by an increased methylation of the histone H3 binding to the PDE4B gene, specifically affecting the PDE4b2 isoform (18). This histone modification reduces accessibility to the gene for transcription, thus leading to reduced expression of the phosphodiesterase enzyme, increased cellular cAMP levels, and eventually activation of the cAMP-dependent protein kinase A and reduces TNFα expression (18). The amplitude and breadth of the downstream effects of SAM provide further incentive to better understand the mechanism of action on DNA methylation.

To date, our study is the first to assess the effect of SAM on DNA methylation and, in particular, on the methylation of promoters of genes involved in inflammation. In our study, genome-wide DNA methylation was significantly increased by 500 µmol/l SAM. Our data indicating a reduction, though not significant, in genomic methylation with 1,000 µmol/l SAM (4.6%) compared with 500 µmol/l SAM (4.8%) are consistent with the hypothesis that elevations in SAH may be inhibiting DNMT activity. While microarray analyses of DNA isolated from control cells and cells treated with 500 µmol/l SAM revealed the impact of SAM on the methylation of a large number of genes, the majority of the gene regions affected were hypomethylated. Of particular relevance is the observation that SAM differentially hypomethylated regions associated with inflammatory genes. This suggests that the increase in genome-wide methylation may occur mostly in nonpromoter regions.

We restricted our analysis to genes already shown to be important TNFα regulators in human macrophages, and thus, it is possible that our microarray results included additional significant inflammatory regulators of biological relevance whose altered methylation patterns were not investigated further. Although our analysis did not identify changes in TNFα or IL-10 promoter methylation, we pursued the hypothesis that SAM may be mediating changes in the expression of these important cytokines indirectly. Therefore, we focused on the potential for CCL2, CCR2, and NFKB1 to be mediating the anti-inflammatory effects of SAM. CCL2, also referred to as monocyte chemoattractant protein 1, recruits immune cells to sites of inflammation and has been shown to positively regulate TNFα and negatively regulate IL-10 message levels (12).

Fig. 2. Effects of 24 h SAM treatment on THP1 macrophage global DNA methylation. Values represent the means ± SD of 3 independent experiments. Bars that do not share a letter differ significantly (P < 0.05).

![Global Methylation](image)

Fig. 3. Effects of 24 h SAM treatment on changes in site-specific DNA methylation in THP1 macrophages. The heat map generated by CHARM depicts the changes in relative methylation in 4 SAM-supplemented and 5 control samples.
Dewald et al. (12) demonstrated that epigenetic mechanism by which SAM regulates inflammation. Significantly suppressed message levels indicates a potential impairment of TNFα production (5). Furthermore, the circulating monocytes, recruiting monocytes to sites of inflammation, are the receptor for CCL2 and is present on the surface of circulating monocytes, recruiting monocytes to sites of inflammation (5). CCL2 is the receptor for CCR2 and is present on the surface of circulating monocytes, recruiting monocytes to sites of inflammation (5). Furthermore, the CCL2-CCR2 pathway, specifically CCR2 antagonists, is currently under clinical investigation as a therapy to alleviate inflammation associated with the progression of cardiovascular disease (5). Our identification that CCL2 and CCR2 are both hypomethylated and have significantly suppressed message levels indicates a potential epigenetic mechanism by which SAM regulates inflammation. Dewald et al. (12) demonstrated that Ccl2−/− mice had significantly impaired IL-1 and IL-6 production and TNFα secretion. Similarly, Goser et al. (20) found that Ccr2−/− mice had similar impairments in inflammatory response compared with Ccl2−/− mice. Interestingly, Feterowski et al. (13) have shown that CCR2 blockade has a more potent stimulatory effect on IL-10 production compared with its impairment of TNFα signaling. Altogether this evidence provides a potential mechanism by which changes in methylation within the CCR2 promoter region may regulate both TNFα and IL-10.

The NFKB transcription factors are well known as master regulators of the inflammatory response (4, 15). Although not identified as differentially methylated by SAM supplementation in our experiments, NFKB1 has been shown to be upregulated by CCL2 in human decidual stromal cells (45) and smooth muscle cells (39). NFKB1 directly regulates TNFα transcription through previously identified NFKB1 binding sites within the promoter region of TNFα (47). Furthermore, CXCL1 and SPP1, which were hypomethylated within their promoter region after SAM supplementation (Fig. 4), are also known regulators of the NFKB signaling pathway, and increases in CXCL1 and SPP1 activity are coupled with a corresponding increase in NFKB activity (41, 46). SPP1 also has been connected to the development of atherosclerotic plaques as it is differentially expressed in macrophages isolated from atherosclerotic plaques compared with peripheral macrophages (33). Although changes in message levels for CXCL1 and SPP1 were not measured, decreases in methylation have been associated with reductions in gene transcription (24).

Our results demonstrating a decrease in methylation status of inflammatory genes after SAM treatment are consistent with two recent findings regarding the relationship between changes in DNA methylation and gene expression. Although historically increases in gene-specific methylation have been associated with reductions in expression (23), more recent research suggests that hypermethylation can result in increases in expression (29) with the implication that hypomethylation may result in a reduction in expression. Although the mechanism is unclear, it is likely that SAM-mediated changes in methylation affect DNMT activity. In fact, 500 μM SAM treatment for 24 h has been shown to reduce DNMT activity (1). Reductions in DNMT 3a and/or 3b activity may reduce de novo methylation at particular promoter regions (25). Secondly, the location of methylation appears to regulate its transcriptional impact. Methylation within the promoter region is often associated with reductions in transcription (6), while methylation within the gene body has the opposite effect on gene transcription (23). However, even within the promoter region, the impact of methylation has variable transcriptional consequences depending on the “particular genomic and cellular context” of a given cell (24). Thus, our observations that reductions in CCL2 and CCR2 promoter methylation is coupled with a reduction in transcription is consistent with previous findings (24, 29).
Lastly, differentially methylated regions that were 5.0 kb upstream or 1.0 kb downstream of a gene’s transcription start site were annotated to that gene. Therefore, it is important to acknowledge the difficulty in assessing the transcriptional impact of particular CpG methylation sites. It is clear that methylation within a promoter region has a direct impact on its transcriptional activity (6), although there is increasing evidence that CpG shore methylation as well as methylation within the gene body also regulate transcription (22).

Our observation that high-dose SAM supplementation had no effect on global methylation yet had the same transcriptional impact on IL-10 and TNFα expression levels as low-dose SAM may indicate that the anti-inflammatory effects of SAM functions through changes in gene-specific or perhaps site-specific methylation. Recent work by Glier et al. (17) provides initial evidence that SAM may preferentially alter particular CpG islands. They found that in mice genetically engineered to produce varying concentrations of SAM, mice with lower levels of SAM had tissue-specific changes in DNA methylation (17). The findings that SAM results in tissue-specific modifications may provide preliminary support for the ability of SAM to preferentially regulate particular genes.

Since SAM is a metabolite of methionine, it may be assumed that methionine supplementation has similar anti-inflammatory effects. However, studies have shown an anti-inflammatory effect of methionine restriction in the diet (40), and this finding is possibly explained by the significant metabolic and physiological differences between the two compounds.

Our findings regarding the potential modification of inflammatory gene expression levels by epigenetic mechanisms may have significant implications for regulating inflammation. These findings may lay the groundwork for discovering a mechanism by which to safely manipulate IL-10 and TNFα, as well as a number of other known inflammatory mediators, which would have significant implications for chronic inflammatory conditions.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


