



## ORIGINAL ARTICLE

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## Relationship between percentage of regulatory T-cells and dental amalgam fillings.

**Abstract:** Introduction: Regulatory T-cells are the main component of peripheral tolerance and their level is decreased in autoimmunity. In dental amalgam, a mixture of metals is used as a restorative material. During daily activities, these metals are ingested and affect renal, neurosensory and immune systems. Studies have demonstrated an increased risk of autoimmune diseases in patients with dental amalgam fillings. It was hypothesized that the percentage of regulatory T-cells decreases in individuals with amalgam fillings. Therefore this study was designed to determine and compare the percentage of regulatory T-cells in individuals with and without amalgam fillings. Material and Methods: This was a cross-sectional study. Subjects were divided into two groups with each group consisting of 40 individuals. Group I (study group) comprised individuals with amalgam fillings, and Group II (control group), individuals without amalgam fillings in their teeth. Blood samples of all the participants were collected and tagged with CD4-FITC, CD25-PE and CD127-PerCP-Cy monoclonal antibodies for the detection of regulatory T-cells, FACSCalibur was used for this purpose. Results: The percentage of regulatory T-cells in the control group was high ( $77.77 \pm 5.54\%$ ) compared to the study group ( $76.09 \pm 7.68\%$ ), however, on comparison, the difference was not statistically significant ( $p=0.25$ ). Conclusion: Dental amalgam fillings did not show a declining effect on the percentage of regulatory T-cells.

**Keywords:** Regulatory T-cells, Peripheral tolerance, Autoimmunity, Dental amalgam.

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### INTRODUCTION.

Immune regulation is a complex mechanism and this complexity has focused attention on regulatory T-cells, which play a vital role in maintaining the balance between immunity and tolerance. In humans, regulatory T-cells are a distinct population of CD4 cells that constitute 5–10% of peripheral CD25<sup>+</sup>CD4<sup>+</sup> cells. The most important function of regulatory T-cells is to prevent the development of autoimmune diseases. A number of these diseases are also associated with alterations in the number

and functions of regulatory T-cells<sup>1-3</sup>.

Dental amalgam is the most widely used restorative material in dentistry. The main constituents of dental amalgam are mercury, silver, copper, tin and zinc<sup>4</sup>. The use of dental amalgam as a restorative material has been controversial since the third decade of the twentieth century<sup>5</sup>. Constant release of Hg and other constituents of dental amalgam fillings during the routine activities of brushing, chewing, eating and teeth grinding has been reported<sup>6,7</sup>. Individuals with fillings have high levels of

amalgam constituents in the saliva, blood and faeces<sup>8</sup>. High concentrations of Hg and other constituents of amalgam are linked with dysfunction of the nervous, gastrointestinal, haematologic, reproductive, immune and respiratory systems<sup>9,10</sup>.

The immune system is affected by dental amalgam because it continuously releases mercury as described earlier. When the human body is exposed to mercury, there is a significant change in the levels of blood lymphocytes and their subsets. Serum immunoglobulin levels are also affected by mercury. Due to its high mercury content, dental amalgam has been associated with increased incidence of autoimmune diseases such as systemic lupus erythematosus, Crohn's disease, lichen planus and endometriosis<sup>11,12</sup>.

It has been hypothesized that amalgam fillings downregulate the number of regulatory T-cells, which may cause autoimmune diseases. Therefore, this study was designed to determine and compare the percentage of regulatory T-cells in subjects with and without dental amalgam fillings.

## MATERIALS AND METHODS.

### Subjects

This was a cross-sectional study that comprised 80 individuals divided into two groups of 40 subjects each, Group I with amalgam restorations and Group II without amalgam restorations. The study was approved by the Ethical Review Committee and Advanced Studies and Research Board of the University of Health Sciences, Lahore, Pakistan. Informed written consent was obtained from all the participants of the study. Apparently healthy individuals on the basis of clinical history, physical examination and complete blood count (CBC) of either gender between 20 and 30 years of age were recruited from the Department of Restorative & Operative Dentistry, Nishtar Dental College, Multan, Pakistan, between January and June 2014.

On intraoral examination, the number of dental fillings was noted by two expert restorative dentists. Only patients with amalgam restorations were included in the study group, and subjects without dental fillings were

taken as the control group. To overcome other possible confounders, age- and sex-matched participants with an equal number in both groups were selected. From each individual, 5ml of venous blood was collected and transported to the Department of Immunology, University of Health Sciences, Lahore, in an ice pack.

### Peripheral Blood Mononuclear Cell Isolation and Cell Staining

Fluorescein isothiocyanate (FITC)-tagged mAb against CD4, phycoerythrin (PE)-tagged mAb against CD25 and peridinin-chlorophyll-protein Cy (PerCP-Cy)-tagged mAb against CD127 were used for immunophenotyping. All the antibodies were purchased from Becton Dickinson (BD) Pharmingen™, USA.

Immunostaining was performed by lyse-wash technique. One hundred  $\mu$ L of anti-coagulated blood was taken in two separate polypropylene tubes. Twenty  $\mu$ L of each monoclonal antibody was added to one tube and 20  $\mu$ L of isotype control to the other. Both were incubated in the dark at room temperature (18°C-25°C) for 15 minutes. Two ml of BD FACS lysing solution (BD Pharmingen™, USA) were added to each tube and then mixed. The tubes were reincubated in the dark at room temperature for 10 minutes<sup>13</sup>. Each of these tubes was centrifuged at 3200 rpm for 10 minutes and the supernatant was discarded. The pellet of the cells in each tube was broken and the cells were washed twice by adding 2ml of sheath fluid. In the end, cells were resuspended in 0.5ml of sheath fluid with 2% paraformaldehyde<sup>14</sup>.

Cells were analyzed with a FACSCalibur 4-color analyser (BD Pharmingen™, USA). Fluorescence attributable to FITC, PE and PerCP-Cy-labelled mAb was determined using excitation by a 15mW, 488nm, argon-ion blue laser. Before running the samples, the machine was calibrated and fluorescent signal compensation was performed using CellQuest Pro software (BD Pharmingen™, USA) and CaliBRITE beads (BD Pharmingen™, USA).

CD4 and CD25 positive cells were defined using qua-

drants of double-positive cells after the exclusion of non-specific binding by using isotype control. CD4<sup>+</sup>CD25<sup>hi</sup> regulatory T-cells were determined as reported by Ammirati *et al.*<sup>15</sup>. The brightest 2% cut-off was taken because these cells were FOXP3<sup>+</sup> and had regulatory properties<sup>16</sup>. The geometric mean and standard deviation (SD) of these demarcation channels were calculated for the study population. Mean±2SD was set as a cut-off above which all fluorescent cells were considered CD25<sup>hi</sup> (CD25<sup>hi</sup> as R3). CD25<sup>hi</sup> cells in R3 were enumerated using gate statistics and their percentage was calculated.

A demarcation was made between CD127<sup>lo</sup> and CD127 rest by using a single parameter along with signal intensity on a histogram in the gated CD4<sup>+</sup>CD25<sup>hi</sup> population. The non-specific binding and CD127<sup>hi</sup> population were excluded by the isotype control and signal intensity. The percentage of CD127<sup>lo</sup> was determined by histogram statistics. The percentage of CD127<sup>lo</sup> cells was determined from the CD4<sup>+</sup>CD25<sup>hi</sup> population<sup>17</sup>. All the lab procedures were carried out under the supervision of two immunologists.

#### Data analysis

The Statistical Package for Social Sciences 20.0 (IBM, USA) was used to analyze the data. The mean±SD of

age, number of amalgam fillings, number of filled surfaces of teeth and duration of amalgam fillings of the study group were determined. The mean±SD of age and percentage of regulatory T-cells were determined and compared for both groups<sup>18</sup>. The Student's t-test was used for comparison of normally distributed regulatory T-cells (CD4<sup>+</sup>CD25<sup>hi</sup>CD127<sup>lo</sup>) and the Mann-Whitney U test was used for the not normally distributed population. A value of p≤0.05 was considered as statistically significant. The data were analyzed in consultation with the official biostatistician of the University of Health Sciences, Lahore, Pakistan.

#### RESULTS.

The mean±SD of age, number and percentage of males and females in both the study groups are given in Table 1.

The mean age of patients with amalgam fillings was higher than that of patients who did not have amalgam fillings. The percentage of CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>lo</sup> cells was higher in control subjects without amalgam fillings than in patients with amalgam fillings. The Student's t-test found no statistical difference between the mean of the percentage of CD<sup>+</sup>CD<sup>hi</sup>CD127<sup>lo</sup> cells in the control and study groups (Table 2).

**Table 1.** Demographic data of the studied subjects.

Variables	Control group (n=40)	Study group (n=40)
Male (n, %)	24 (60%)	27 (68%)
Female (n, %)	16 (40%)	13 (32%)
Age (Mean±SD)	24.40± 2.65	25.15± 2.76

**Table 2.** Number, mean ±SD of age, dental fillings, duration of amalgam fillings, regulatory T cells and their comparison between control and study groups.

	Study group (n=40)	Control group (n=40)	p-value
Male/Female, (n)	27/13	24/16	0.48
Age, years ( mean±SD)	25.15±2.76	24.40± 2.65	0.22
Dental fillings (n, mean±SD)	2.60±.98		
Filled surfaces (mean±SD)	3.88±1.63		Not applicable
Duration of dental amalgam (months) (mean±SD)	54.45±18.94		
CD4 <sup>+</sup> CD25 <sup>hi</sup> CD127 <sup>lo</sup> (mean ±SD)	76.09%±7.68%	77.77%± 5.54%	0.25

## DISCUSSION.

The current study demonstrated higher percentages of CD4<sup>+</sup>CD25<sup>hi</sup>CD127<sup>lo</sup> regulatory T-cells in the control group than in the study group. This is in line with Loftenius *et al.* who determined levels of T-cells, helper T-cells, cytotoxic T-cells and B cells before and after the removal of amalgam fillings in 10 healthy individuals and observed no change in the number of circulating T-cells<sup>19</sup>. Although they did not study regulatory T-cells, the level of other lymphocytes is somewhat similar to the findings of this study.

The results of this study are consistent with those of Osorio *et al.* They reported no alteration in the percentage of lymphocyte subsets before or after the placement of amalgam restorations<sup>20</sup>. Wilhelm *et al.* were also unable to detect any changes in the total or relative counts of granulocytes, NK cells, B cells, cytotoxic T-cells and helper T-cells in patients with amalgam fillings<sup>21</sup>. The results of these above-mentioned studies agree with this study in that the levels of various populations of cells are not perturbed by the presence of dental amalgam fillings.

Shenker *et al.* also established a slight but transient increase in lymphocyte percentage in the New England Children's Amalgam Trial that normalized on the next assessment at six months. Although the researcher was able to detect some shift in the level of lymphocytes, it was for a very short period and the cause of this transient variation could have been the body's response to foreign substances, which returned to normal after a short period of time<sup>22</sup>.

However, Di Pietro *et al.* were able to prove damage and a decrease in the number of lymphocytes due to dental amalgam. They suggested a relationship between the number of dental fillings and the degree of damage to the lymphocytes, which is not in line with the current study<sup>23</sup>. The probable reason for this discrepancy is the increased number and longer duration of dental amalgam fillings in their oral cavity.

Park *et al.* determined lower numbers of CD4, NK and B cells in the control group than in Hg-exposed individuals. The small sample size and differences in the cell sorting staining technique might be the reason for the contrast in the results<sup>24</sup>. Eggleston and co-workers reported an increase in the percentage of T-cells from 47%

to 73% after the removal of dental amalgam fillings and a decline in the proportion of T-cells from 73% to 55% after the reinsertion of dental amalgam fillings, which differs from the outcomes of this study<sup>25</sup>. A possible reason could be that they only studied three individuals and used a conventional slide method to count T-cells.

The researchers failed to observe any correlation between the duration of dental amalgam fillings and the number of regulatory T-cells in this study. This is in line with Shenker *et al.* who also could not deduce a relationship between neutrophils, monocytes, NK cells, helper T-cells, cytotoxic T-cells, B cells and duration of amalgam fillings<sup>22</sup>.

The results of this study are not in line with Di Pietro *et al.* who reported that a long exposure to dental amalgam causes damage to lymphocytes<sup>23</sup>. It is strongly believed that a lack of control of confounding variables such as daily intake of alcohol, smoking and a positive history of allergy in a few patients is responsible for the contradictory results compared to the prevailing study.

In this study, the number of dental amalgam fillings and the surfaces filled did not correlate with the percentage of regulatory T-cells, which coincides with other studies<sup>22</sup>. However, Angela Di Pietro *et al.* noted damage to lymphocytes from an increased number of fillings. A lack of some essential information such as a history of smoking and allergy to Hg could be plausible reasons for the alteration in the results compared to the recent study<sup>23</sup>.

Considering the prevalence of dental amalgam fillings, our findings are similar to those of Angela Di Pietro *et al.* Their study population also included more females with dental fillings than males<sup>23</sup>. The probable reason for this finding is that females are more concerned about their dental health than males.

The small sample size, the absence of standardization of the composition of dental amalgam and no information about the serum level of mercury in both the control and study groups are some of the possible limitations. More specific markers for regulatory T-cells, e.g. foxp3 and markers of immunosuppression such as TGF- $\beta$ , IL-10 and CTLA-4, were not sought. Therefore, further studies are suggested to overcome the limitations of this study.

## CONCLUSION.

No differences in the percentage of regulatory T-cells in subjects with and without dental amalgam fillings were demonstrated, which indicates that dental amalgam fillings do not cause a decline of regulatory T-cells.

## Relación entre el porcentaje de células T reguladoras y obturaciones de amalgama.

**Resumen:** Introducción: Las células T reguladoras son el principal componente de la tolerancia periférica y su nivel se reduce en la autoinmunidad. En las obturaciones de amalgama, una mezcla de metales se utiliza como un material de restauración. Durante las actividades diarias, estos metales se ingieren y afectan el sistema renal, neurosensorial e inmunológico. Los estudios han demostrado un aumento del riesgo de enfermedades autoinmunes en pacientes con amalgamas dentales. Se planteó la hipótesis que el porcentaje de células T reguladoras disminuye en individuos con obturaciones de amalgama. Por tanto, este estudio fue diseñado para determinar y comparar el porcentaje de células T reguladoras en individuos con y sin obturaciones de amalgama. Material y Métodos: Se realizó un

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estudio de corte transversal. Los sujetos fueron divididos en dos grupos, cada uno con 40 individuos. El grupo I (de estudio) estuvo conformado por individuos con obturaciones de amalgama y el grupo II (de control) por individuos sin obturaciones de amalgama. Se colectaron muestras de sangre, las que fueron marcadas con anticuerpos monoclonales CD4 FITC, CD25 PE y CD127 PerCP-C para detectar las células T reguladoras, se utilizó FACSCalibur para este propósito. Resultados: El porcentaje de células T reguladoras en el grupo control fue alta ( $77,77 \pm 5,54\%$ ) en comparación con el grupo de estudio ( $76,09 \pm 7,68\%$ ), pero esta diferencia no fue estadísticamente significativa ( $p=0,25$ ). Conclusión: Las obturaciones de amalgama no se asociaron con una disminución en el porcentaje de células T reguladoras.

**Palabras clave:** *Células T reguladoras, Tolerancia periférica, Autoinmunidad, Amalgama dental.*

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