UNIVERSITÀ DEGLI STUDI DI TORINO

Technical Report on the Detection by RT-PCR of pro-inflammatory contaminants

Customer: Titanmed srl

C O N F I D E N T I A L

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Aim

The aim of the work was to measure the presence of endotoxins adherent to the implant surface. To this scope, we used a recently developed in vitro protocol so as to evaluate the expression of some key genes involved in the inflammatory process in cultured macrophages. Tests were carried out with the following three samples:

one benchmark implant "Nobel Biocare MKIII Groovy RP";

one test implant "IMPIANTO con lotto 002978 con trattamento superficiale TU Tecom Implantology" which was delivered on 23rd April 2015 (Delivery note number: 978);

and a control condition obtained using endotoxin-free plastic.

Materials and Methods

The samples analysed were the following:

- Nobel Biocare MKIII Groovy RP ref. 32131
- IMPIANTO con lotto 002978 con trattamento superficiale TU Tecom Implantology
- Plastic

The samples were received within sealed blisters perfectly packed. A vertical flow hood was used to ensure sterile handling. The amount of adherent endotoxins was measured recurring to gene expression by RT-PCR. In particular, the transcript levels of five key genes of the inflammatory response to endotoxin in macrophages J774A-1 were quantified:

- Interleukin 1 beta (IL-1),
- Interleukin 6 (IL-6),
- Tumor Necrosis Factor alfa (TNFa),
- Monocyte chemotactic protein-1 (MCP-1),
- Cyclooxygenase 2 (COX-2).

Following a recent scientific publication (Morra et. 2012), in which the authors correlate the

expression of the considered genes to the presence of endotoxin contamination, we adopted the following analytical method. The expression of these genes at short times (4h) is controlled by the level of adherent endotoxins and is independent from the surface topography.

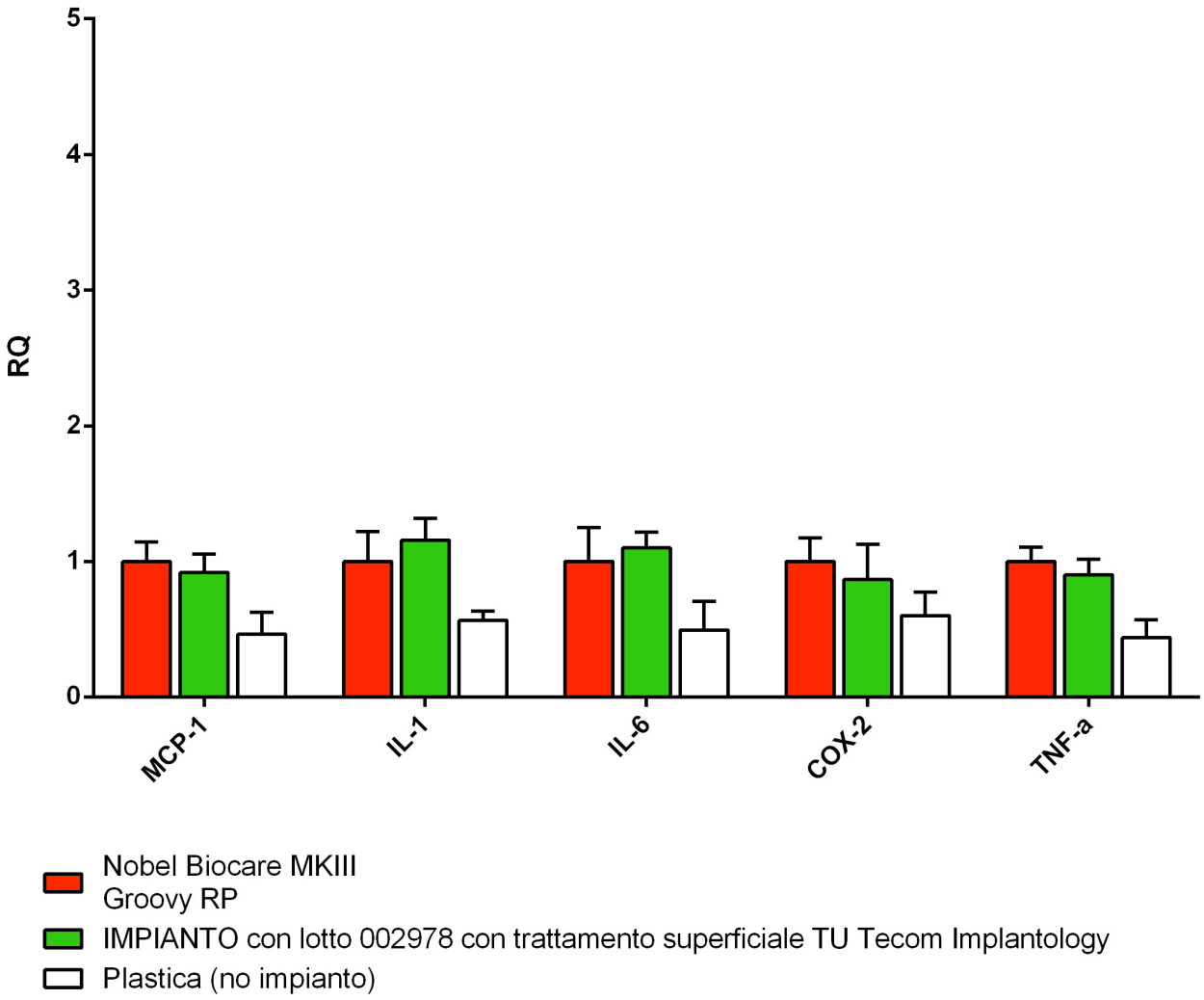
Based on this observation, the amount of endotoxins adherent to the implant surfaces can be measured by analyzing the transcriptional response in macrophages J774A-1. The measurements were carried out as follows: a suspension of 2 x 105 cells ± 0:15 J774A-1 (cultured in DMEM (Gibco®) containing L-glutamine 200um (Gibco®, Life TechnologiesTM) and 10% Foetal Bovine Serum (FBS, Gibco ®, Life TechnologiesTM), penicillin and streptomycin) was introduced in cell culture sterile polystyrene plates (12 well multiwell plates, Corning) containing the samples. The gene expression analysis was performed using real-time reverse transcription PCR (qRT-PCR). Total RNA was extracted after 4 h, using the Purelink® RNA Isolation Kit (Ambion®, Life TechnologiesTM). The quality of RNA was assessed by checking that the A260 / A280 ratio was 2.0 ± 0.2 in order to avoid any DNA contamination and controlling the A260 / A230 which provide an index of protein contamination). The RNA was then reverse transcribed to obtain cDNA using the kit High-Capacity cDNA Reverse Transcription KitTM (Applied BiosystemsTM). The relative quantification of the genes was obtained using TaqMan® probes specific for each gene and 18S as a reference gene. The amplification reactions were performed in duplicate recurring to 7900H Fast Real-Time PCR System (Applied BiosystemsTM) according to the manufacturer's instructions. The graphs of gene expression were obtained by normalizing the data with the dedicated software, according to the standard method ΔCt.

Result

The quantification of gene expression is shown in the graph. For each gene, the first bar shows the results of an implant "Nobel Biocare MKIII Groovy RP ref. 32131 ", the second bar shows the data obtained on your test implant “IMPIANTO con lotto 002978 con trattamento superficiale TU Tecom Implantology”, the third bar shows the results obtained on plastic alone sample.

In particular, data show the expression levels of the considered genes- normalized to the control

gene "18S" - and related to the sample "Nobel Biocare MKIII Groovy RP ref. 32131 "which is set as value 1. Standard error bars are also reported in the graph.



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Conclusion

In conclusion, this report shows that dental implant tested “IMPIANTO con lotto 002978 con trattamento superficiale TU Tecom Implantology” does not lead to a significant over-expression of pro-inflammatory genes in macrophages J774A-1 compared to implant "Nobel Biocare MKIII Groovy RP ref. 32131" suggesting a very low amount of adherent endotoxin. We can therefore argue that there is no pro-inflammatory difference between the implants analysed.

Turin, 30th April 2015

Sincerely,

Federico Mussano

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