The role of neurohumoral modulation in fracture healing

Valorisation

Martijn Hofman

As presented in the introduction of this thesis, the process of fracture healing and bone regeneration is an extremely complex process in which, besides the musculoskeletal system, other systems such as the neurological, vascular and immune systems play an important role. An immense amount of biological factors from each of these systems interact with each other and contribute to the regeneration of bony defects. With this thesis, we aimed to evaluate the influence of neuro-humoral modulation in the fracture healing process, without pretending that the evaluation is complete, hence to just lift a tip of the veil.

The valorisation of results from experimental research, in the way Karl Marx originally conceptualised this theoretical concept of 'Verwertung' in his critique of political economy, is not that obvious because the results of experimental studies often cannot directly be implemented in daily lives or in medical treatments and thus will not generate money. Therefore, in this valorisation, we describe the value our results have or could have for further research and possibly in the long run for clinical applications.

The results from our review described in **chapter 2**, the retrospective study described in **chapter 3** and the results of the literature study described in the general discussion (**chapter 10**) about newer studies on the impact of TBI on fracture healing are all concerned with the influence of TBI on the fracture healing process. On one hand, we could demonstrate that the majority of the experimental studies tend to provide evidence for the positive influence of TBI on fracture healing; on the other hand, the clinical studies, most of which are retrospective, could not generally subscribe these findings. The value of these results should be such that the scientific community is triggered to further investigate this phenomenon and discover the pathophysiologic mechanisms that explain the relation between certain biologic factors released after TBI and the bone regeneration process. Moreover, the results shown in our studies could be an impulse to instigate a large randomised, prospective, clinical study to find clinical support for these experimental findings.

In **chapter 4**, we demonstrated that the blockage of the NK1-receptor for substance P has a considerable influence on gene expression and bone strength throughout the fracture healing process. A logical next step in the research on the influence of neurotransmitters in fracture healing would be to administer substance P in small animal fracture healing and/or disturbed fracture healing models to evaluate its direct influence on the fracture healing process. Substance P is a biomarker, which is set free after TBI; in addition to causing pro-inflammatory effects, it causes increased vascular permeability, brain oedema and functional deficits after TBI [1]. Lorente et al. showed that the substance P levels in serum are correlated to the severity and mortality of TBI [2]. In this context, it would be very interesting to evaluate the dynamics of substance P and fracture healing in a small animal fracture model with concomitant TBI. After further experimental research on the function of substance P, it also could become a biomarker for fracture healing in patients because the concentration of substance P might be associated with the quality of fracture healing.

In **chapter 5**, we proved the CatWalk gait analysis system to be an outstanding tool to assess both static and dynamic gait parameters in a non-invasive, longitudinal manner in an experimental small animal model of fracture healing. In our opinion, the CatWalk system has the potential to become the gold standard for gait analyses

in small animal fracture models. Because more than 50% of experimental animal fracture models are performed with mice and rats, the use of this system would significantly improve the knowledge about behavioural and locomotor recovery after lower extremity fractures.

To date, the most important tools for diagnosing disturbed fracture healing are clinical and radiological findings. Although research on possible biomarkers that can be used as predictors for non-union development is promising, achieving consensus is very difficult because the evidence available is heterogeneous [3]. The results of our study described in **chapter 6** most importantly indicate a significantly lower ornithine concentration and arginase-1 expression in the bone marrow of patients developing non-unions. This was the first study to recognise these possible biomarkers (i.e. arginine, ornithine and iNOS) that could be used as predictors of the outcome of the autologous bone grafting procedure by RIA in cases of non-union. As the exact treatment and the time point of this non-union treatment are still under discussion, and the socio-economic impact of disturbed fracture healing is immense, finding biomarkers, which can predict the prognosis of the fracture healing process and the outcome of certain procedures performed in cases of non-unions, will be very valuable. Therefore, this study could be an excellent starting point to further investigate these biomarkers in the fracture healing process and to determine certain cut-off points for the different biomarkers, based on which the prognosis of fracture healing and non-union treatment could be estimated.

During the last years, the intercellular communication through vesicles loaded with different proteins, mRNAs and miRNAs, known as exosomes, MVs, and apoptotic bodies, is gaining interest in fracture healing research. In this context, we showed in an *in vitro* study, described in **chapter 7**, that MVs isolated after a femoral fracture were time-dependently incorporated in osteoblasts and concentrated around the nucleus. These MVs from trauma plasma increased the proliferation and viability of osteoblasts, particularly in the late phase (i.e. two weeks post-fracture) of fracture healing. The fracture healing process involves a complex network of signal transduction between a variety of cells. Demonstrating the regulating effect of MVs on fracture healing by increasing the proliferation and viability of osteoblasts is just a first step in understanding the role that MVs might play in the fracture healing process. The characteristics of the exact role of MVs in the intercellular communication between cells in the fracture healing process are still to be discovered in future research, including the origin, the composition and the target cells of these MVs as well as the mechanism of action of the different constituents within these MVs. If these questions are answered, the way in which the responsible contents of MVs can be used to help in the diagnostics or treatment of fractures should be addressed. Possible applications could be the adjustment of the content or local application of MVs to alter the fracture healing process. Because the current exosome isolation methods such as ultracentrifugation and ultrafiltration only provide a low exosome yield [4], the challenge of obtaining sufficient amounts of exosomes should be overcome.

In our small animal neutrophil studies, demonstrated in **chapter 8 and 9**, we showed that three days after intramedullary nailing and fracture induction, the concentration

of circulating neutrophils as well as the neutrophil activation, characterised by a change of integrin expression on their surface, decreased. One theory explaining this decrease is that the decrease expresses the homing of neutrophils into the fracture haematoma during the inflammatory stage of fracture healing, wherein they clear fracture debris and initiate further steps in the normal fracture healing process. Another theory, supported by our findings in **chapter 9**, is that this decrease is attributable to an increased extravasation of neutrophils not only into the fracture haematoma but also into peripheral tissues, such as the lungs, potentially causing tissue damage (i.e. ARDS/ALI). In future studies the number of circulatory neutrophils should be compared to the number of neutrophils in the fracture haematoma and the pulmonary pool after fracture. All in all, the total range of functions of the neutrophils as activators and regulators of different cell processes is still to be discovered in future studies.

We also demonstrated, in **chapter 8**, the increased heterogeneity of the blood neutrophil pool during the restoration phase after fracture, with a new subset of unique $CD11b^{high}/CD11a^{high}$ neutrophils present in the post-inflammatory neutrophil pool. Because it is not yet clear if these subsets belong to separate developing lineages or embody certain activation states of a common precursor, this finding offers a basis for further research on the exact origin of this novel subtype and on novel immunotherapeutic strategies to modulate neutrophil homeostasis after fractures or in cases of disturbed fracture healing.

Similarly to several former studies that have shown the increased pulmonary neutrophil influx after trauma, in **chapter 9**, we demonstrated a transient increase in pulmonary neutrophil deposition and a contemporary increase in the activation status of the pulmonary neutrophil pool after an intramedullary stabilised femur fracture. Furthermore, we showed the striking differences in the activation status of the neutrophils belonging to the pulmonary parenchymal compartment and those belonging to the broncho-alveolar compartment. This qualitative description of pulmonary neutrophil populations and their characteristics should incite further research on the influence of different neutrophil subsets on the development of pulmonary complications and on the possibility to use these neutrophil cell-surface receptors as markers for neutrophil activation status.

REFERENCES

1. Donkin JJ, Nimmo AJ, Cernak I, Blumbergs PC, Vink R. Substance P is associated with the development of brain edema and functional deficits after traumatic brain injury. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism* 2009; **29**(8): 1388-98.

2. Lorente L, Martin MM, Almeida T, et al. Serum substance P levels are associated with severity and mortality in patients with severe traumatic brain injury. *Critical care* 2015; **19**: 192.

3. Sousa CP, Dias IR, Lopez-Pena M, et al. Bone turnover markers for early detection of fracture healing disturbances: A review of the scientific literature. *Anais da Academia Brasileira de Ciencias* 2015; **87**(2): 1049-61.

4. Peinado H, Aleckovic M, Lavotshkin S, et al. Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. *Nature medicine* 2012; **18**(6): 883-91

.