On Platelet-Rich Plasma

in Reconstructive Dental Implant Surgery

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To Katja,
Beata, Hedvig and Tobias
With love

To my mother
Who would have been delighted

This thesis represents number 35 in a series of investigations on implants, hard tissue and the locomotor apparatus originating from the Department of Biomaterials, Institute for Clinical Sciences at the Sahlgrenska Academy, Göteborg University, Sweden.

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31. **Pia Bolind DDS, 2004**. On 606 retrieved oral and cranio-facial implants. An analysis of consecutively received human specimens. Thesis defended 17.12. 2004. Ext. examin: Professor A. Piattelli.
32. **Patricia Miranda Burgos DDS, 2006.** On the influence of micro-and macroscopic surface modifications on bone integration of titanium implants.Thesis defended 1.9. 2006. Ext. examin: Professor A. Piattelli.
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34. **Anna Göransson DDS, 2006.** On Possibly Bioactive CP Titanium Surfaces. To be defended 8.12. 2006 Ext. examin.: Professor B. Melsen.
35. **Andreas Thor DDS, 2006**. On platelet-rich plasma in reconstructive dental implant surgery. To be defended 8.12. 2006. Ext. examin.: Professor E.M. Pinholt.

Abstract

Background Severe athrophy of the edentulous maxilla may require augmentation before implants can be placed. Autogenous bone has been used for reconstruction in block or in particulated form. Platelet-rich plasma (PRP) has been suggested to enhance the healing of bone grafts, as activated platelets release autogenous growth factors (GFs) into the wound healing site. Additionally, the GFs of PRP are suggested to enhance the integration of implants into bone. However, controversies exist in the literature with respect to the effect of combining PRP with bone grafts, or implants, as the concept has been evaluated in different study models with a wide range of results.

Aims The first two Papers presented, evaluates the effects of PRP in conjunction with autogenous bone grafts and subsequent installation of implants. Paper III explores the thrombogenic properties *in vitro* of titanium in whole blood and PRP and also evaluates the potential effect of a fluoride titanium surface modification regarding the thrombotic response. Furthermore, a recently developed surgical procedure is evaluated in Paper IV, where simultaneous sinus mucosal lining elevation and installation of implants is performed without the addition of any graft material. Finally in Paper V, an attempt to correlate platelet count, GF release in PRP and its effect on bone formation is performed in a canine peri-implant defect model, where additionally, the modified surface from Paper III is further evaluated.

Materials & Methods In Paper I, 19 patients were subjected to autogenous bone grafting from the iliac crest to the maxillary sinus with or without PRP in a *split mouth* setting. Implants were installed (n=152) after 6 months of healing. Patients were followed with Resonance Frequency Analysis (RFA) and radiological follow-up up to 1 year after loading of implants. 3 months after grafting, biopsies were retrieved and micro implants installed in the grafted site, left to heal for 3 months and thereafter collected with surrounding bone, simultaneously with installation of dental implants. Biopsies from 3 and 6 months were evaluated regarding new bone formation and bone-implant contact in Paper II.

In Paper III, *in vitro* tests with the heparinised slide chamber model were performed. In this model, the tested biomaterial is the only part of a secluded chamber that is not furnished with heparin, and therefore the tested surface is allowed to cause thrombotic reactions in e.g. blood or PRP, that subsequently can be quantified regarding e.g. generation of thrombin and platelet activation.

Paper IV consisted of 20 patients, followed clinically and with radiographs for a minimum of 1 year. These patients were all subjected to elevation of the sinus mucosal lining, where a bone window was cut out in the sinus wall and replaced after installation of implants (n=44) consequently tenting the mucosal lining.

In Paper V, 6 dogs were used. Peri-implant defects were created in the mandibles and implants with and without a fluoride titanium surface were installed. PRP or whole blood thereafter filled the defects before closing of the surrounding soft tissues and left to heal for 5 weeks before collection of samples for histomorphometric evaluations.

Results Paper I showed an overall survival rate of 98.7 % after 1 year in function and stable marginal conditions regardless use of PRP or not. RFA disclosed significantly higher values for the PRP side at abutment connection after 6 months but not at the 1 year follow up. Early bone healing was enhanced with PRP as evaluated in biopsies collected from grafts after 3 months of healing, however, no differences were found in biopsies with micro implants after 6 months.

Whole blood showed a stronger activation of the coagulation system, in Paper III, and a fluoride modification of a titanium surface seemed to augment the effect.

In Paper IV, the survival rate of implants was 97.7% after a minimum of 1 year of evaluation and the average bone gain was 6.51 mm. Marked bone formation was observed around implants also when installed in diminutive amounts of bone.

In Paper V, the use of PRP added no significant value to the healing of defects. Regardless of PRP or blood in the defects, a fluoride titanium surface modification enhanced the bone healing significantly.

Conclusions This thesis supports the use of PRP in augmentation with particulated autogenous bone due to enhanced early healing and enhanced handling abilities. The use of PRP and implants in combination can not be supported as a result of *in vitro* and experimental animal studies performed in this thesis. Implant surface characteristics seems to be more important. Bone grafts may be obleviated during sinus lift surgery if the described method is used and will result not only in acceptable results of implant integration, but also in minimising morbidity of patients.

Keywords: autogenous bone graft, bone formation, coagulation, clinical study, complement, dental implant, experimental study, growth factors, platelet activation, platelet-rich plasma, sinus lift surgery, thrombogenicity

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This thesis is based on the following papers, which will be referred to by their Roman numerals (I-V):

I. **Thor A, Wannfors K, Sennerby L, Rasmusson L.** Reconstruction of the severely resorbed maxilla with autogenous bone, platelet-rich plasma and implants: 1-year results of a controlled prospective 5-year study.
Clin Impl Dent Rel Res 2005;4:151-160

II. **Thor A, Franke.Stenport V, Johansson C, Rasmusson L.** Early bone formation in human bone grafts treated with platelet-rich plasma.
Int J Oral & Maxillofac Surg 2006, Accepted.

III. **Thor A, Rasmusson L, Wennerberg A, Thomsen P, Hirsch J-M, Nilsson B, Hong J.** The role of whole blood in thrombin generation in contact with various titanium surfaces.
Biomaterials 2006, In press.

IV. **Thor A, Sennerby L, Hirsch J-M, Rasmusson L.** Bone formation at the maxillary sinus floor following simultaneous elevation of the mucosal lining and implant installation without graft material –an evaluation of 20 patients treated with 44 Astra Tech implants.
J Oral & Maxillofac Surg 2006, In press.

V. **Thor A, Hong J, Zellin G, Sennerby L, Rasmusson L.** Correlation of platelet growth factor release in jawbone defect repair – a study in the dog mandible.
Submitted.

Abbreviations

Following abbreviations are used in the thesis and Papers

ACS	Absorbable collagen sponge
ALP	Alcaline phosphatase
ACP	Acid phosphatase
ADP	Adenosin diphosphate
AT	Anti-thrombin
BHA	Bovine hydroxyapatite
BIC	Bone-implant contact
BMP	Bone morphogeneic protein
BMU	Bone metabolising unit
BPBM	Bovine porous bone mineral
BSA	Bovine serum albumin
BSU	Bone structural unit
β-TG	Beta-thromboglobulin
CC	Cancellous chips
CPD	Citrate phosphate dextrose
CT	Computed tomography
Ct	Calcitonin
DBM	Demineralised bone matrix
DBBM	Deproteinised bovine bone matrix
DFDB	Demineralised freeze dried bone
EIA	Enzyme-immuno assay
ELISA	Enzyme-linked immunosorbent assay
FDB	Freeze dried bone
FG	Fibrin glue
FGF	Fibroblast growth factor
FMB	Freeze dried mineralized bone
GDF	Growth and differentiation factor
GF	Growth factor
GH	Growth hormone
GTR	Guided tissue regeneration
HA	Hydroxyapatite
HBM	High bone mass
HMWK	High molecular weight kininogen

IGF	Insulin-like growth factor
IL	Interleukin
ISQ	Implant stability quotient
MAC	Membrane attack complex
MSC	Marrow stromal cells
OP	Osteogenic protein
OPG	Osteoprotegrin
Osx	Osterix
PAL	Probing attachment level
PD	Probing depth
PDGF	Platelet derived growth factor
PAR	Protease-activated receptor
PBS	Phosphate buffered saline
PDL	Periodontal ligament
PL	Phospholipids
PLF	Platelet growth factors
PK	Prekallikrein
PMMA	Polymethyl methacrylate
PPP	Platelet-poor plasma
PRF	Platelet-rich fibrin
PRGF	Plasma rich in growth factors
PRP	Platelet-rich plasma
PTH	Parathyroid hormone
RANK	Receptor activator of nuclear factor KB
RANKL	Receptor activator of nuclear factor KB Ligand
RFA	Resonance frequency analysis
rhBMP	recombinant human bone morphogeneic protein
ROI	region of interest
Runx2	Runt-related transcription factor 2
SOST	Sclerostin
TCP	Tricalcium phosphate
TF	Tissue factor
Ti	Titanium
TiN	Titanium nitride
TAT	Thrombin anti-thrombin
TBA	Trabecular bone area
TGF-β	Transforming growth factor beta
TNF	Tumor necrosis factor
VEGF	Vascular endothelial growth factor

CONTENTS

Preface

Oral disability caused by edentulism, has been an ordeal for mankind for a very long time. Over the centuries until present, there are testimonies throughout the history. For example, the first President of the United States, George Washington, suffered immensely from his loss of teeth and needed pain-relief with laudanum for his problems[1]. His tooth and alveolar bone loss are well known through texts and illustrations and his appearance, as seen on the front side of the one-dollar bill, was altered by his overextended dentures. The efforts by the most skilful dentists of that era can be seen as a carved wooden prosthesis at the National Museum of Dentistry, University of Maryland, Baltimore, Maryland, USA.

In our time, even though the research discoveries and inventions by Brånemark[2] and Schroeder[3], founders of modern implantology, have helped millions of people, there is still a significant part of the population that suffer. This is due to lack of availability of treatment resources and financing, but also because there are still limitations in our arsenal of implant treatment modalities.

The idea and initiation of this work came while working on a patient that needed augmentation from the iliac crest to the severely resorbed maxilla. At that time, the piece of block bone from the iliac crest was adjusted to the maxilla through carving with maxillofacial burs and saws. To instead particulate the bone and to sculpt the graft seemed more efficient if combined with the concept of platelet-rich plasma (PRP) [4, 5], and so the thought process was under way.

The idea for this thesis was, after some promising initial patient experiences[6-8], to explore and to validate the method of particulated bone and PRP in patients. Furthermore, we wanted to investigate the use of PRP to enhance bone healing around dental implants and to experimentally study the initial thrombotic events of PRP and whole blood on an implant surface in an in vitro model.

Background

Augmentation procedures for edentulous jaws in combination with endosseous implants have been research issues for over 30 years [9]. The lack of jawbone volume and the consequence of unfavourable forces that act on the implant supra-construction, have been problems to clinicians and patients. The edentulous mandible, often atrophied, has most often

been treatable due to the bone still available between the mental foramina. However, the severely resorbed edentulous maxilla, except from being considered more difficult to treat with long-term implants [10, 11], has other limitations due to anatomical landmarks such as the nasal cavity, maxillary sinuses and the incisor canal. The resorption pattern of the maxilla varies; in some cases the height of the remaining bone is the limiting factor, in others the width [12] [13]. Over the years many techniques have been introduced to gain bone volume and quality for placement of oral implants for oral rehabilitation. The gold standard is still autogenous bone, but intensive research strives to simplify the augmentation procedures, constantly looking for alternative methods. One of the adjunctive measures in bone augmentation is platelet-rich plasma (PRP). This concept for clinical use was introduced by Marx et al. in a paper published in 1998 [14] and this thesis focuses on the use of PRP in dental implantology.

Bone

Origin and formation

Bone is indeed a living and adaptive connective tissue and except from giving mechanical support also a reservoir of Ca-ions (97%) for the organism[15]. Disturbances of bone turnover can lead to problems, also affecting the outcome of implant treatment, such as osteoporosis[16, 17]. The bone cells and the proteins in the extracellular matrix (proteoglycan ground substance and predominantly type I collagen) packed with mainly hydroxyapatite crystals, give bone tissue its strength and some elasticity. Bone consists of mineral, collagen, non-collagenous proteins, water and lipids[18].

From a developmental view, the craniofacial skeleton (including maxilla and mandible) is formed from the neural crest, as opposed to the axial and appendicular skeleton that are formed from the sclerotomes of the somites and the lateral plate mesoderm, respectively[19]. Intramembranous and endochondral bone-formation are the two ways in which bone is formed during development. The craniofacial skeleton is formed by intramembranous ossification whereby mineral is directly deposited in a mesenchymal connective tissue. In intramembranous

ossification the mesenchymal cells are differentiated into osteoblasts directly. In the second way, endochondral bone formation, e.g. the long bones, the skull base, vertebrae and the pelvis, goes through a hyaline cartilaginous model before being ossified and hypertrophy of chondrocytes initiates the maturation followed by matrix erosion. The remaining cartilage matrix mineralizes and chondrocytes regress and die. The cartilage model is entered by primitive mesenchymal stem cells via invading blood vessels. From there, the stem cells populate the calcified cartilage model and differentiate to osteoblasts or haematopoetic tissue giving rise to bone formation on evolving bone trabeculae, eventually also to cortical or cancellous bone[20].

The matrix

Understanding of the extracellular matrix content and its interactions with bone cells are of vital importance. In addition to the collagenous matrix proteins (e.g. osteopontin, bone sialoprotein, osteonectin and bone acidic glycoprotein-75) where type I collagen (90 % of the organic matrix) is the most abundant, there are a number of non-collagenous matrix proteins[18]. These proteins were discovered to be vital for the mineralization process after they were extracted from bone experimentally in vitro; without these proteins no mineralization of bone could take place [21]. Today, they are known to contribute to the organization of the extracellular matrix, the control of cell-cell and cell-matrix interactions and to regulate signalling to bone cells. Osteocalcin, another non-collagenous protein, is a good marker of bone forming activity in whole blood. A great amount of GFs are also laid down in the matrix, such as IGF-I, IGF-II and TGF- β, all important for later remodelling of the bone. For a review, see[18].

The cells

The osteoblast, the bone lining cell, the osteocyte and the osteoclast are the four cell types found in bone. The three first named cells are derived from mesenchymal osteoprogenitor cells found in bone marrow and periosteum[16]. The osteoclasts are formed by giant multinucleated cells[22].

The osteoblast and the bone lining cell

This cell is responsible for bone formation through secretion of the organic components of the bone matrix. The preosteoblast is a mesenchymal cell found in various locations such as periosteum, endosteum and bone marrow[16]. The osteoblast is through its origin as a mesenchymal precursor cell related to other cells, such as the fibroblast. The same mesenchymal cells also give rise to chondrocytes, myoblasts, adipocytes and tendon cells[23]. One important difference is that the osteoblast produces bone matrix in a polarized way other than the fibroblast that produces matrix around the whole cell. Marrow stromal cells (MSCs), from the iliac crest or femoral bone marrow, have been used for bone regeneration after being differentiated

in vitro for osteogenesis. After cultivating MSCs to osteoblast-like cells, the possibility for direct autologous reimplantation or *ex vivo* expansion and reimplantation on a adequate scaffold has been suggested for bony reconstruction[24].

The osteoblasts are located on the surface of the bone where they form a syncytium and secrete the organic components of the bone matrix. A thin zone of osteoid is present between the osteoblasts and the mineralized bone[22]. They are important in bone regulation as they regulate the differentiation and activity of osteoclasts. Osteoblasts also produce growth factors such as TGF-β and bone morphogeneic proteins (BMPs), which will be entrapped in the bone matrix and eventually, released by osteoclastic activity, affect active osteoblast precursor cells. Parathyroid hormone (PTH) is said to stimulate differentiation of osteoprogenitor cells systemically. Of the matrix proteins produced by osteoblasts, some are likely to be involved in regulation of bone cell adhesion, migration, proliferation and/or differentiation. Furthermore, they take part in the osteoid formation through supplying proteins like sialoprotein and regulating enzymes like alkaline phosphatase (ALP), a membrane-bound marker of osteoblast differentiation[16]. ALP is said to be the enzyme that mostly marks high bone-forming activity in the osteoblast. High intracellular levels of this enzyme can be visualized by enzymatic staining [25] and like osteocalcin, ALP is a good systemic indicator in whole blood for bone-forming activity. After differentiation and secretion of matrix some osteoblasts are embedded in bone matrix and become osteocytes as well as some that go through apoptosis. It has been suggested that GFs, TGF-β and interleukin-6 (IL-6), may have antiapoptopic effects. The life span of an active osteoblast is estimated to 3 months[26].

The bone lining cells, or surface osteoblasts, are flattened differentiated cells, mainly derived from osteoblasts that cover non remodelling bone surfaces and are connected with osteocytes through cell processes into the bone. It is possible for these bone lining cells to be activated and to differentiate into osteogenic cells. The bone lining cells are also suggested to take part in the homeostasis of mineral through control of bone fluids and ions[27].

The osteocyte

Once the osteoblast is entrapped in bone it is called an osteocyte and from there it communicates with other osteocytes and with surface osteoblasts through gap junctions, extending out with cell processes placed in canaliculi in the mineralized tissue. The reason for why some osteoblasts are entrapped and others remain bone matrix-producing osteoblasts is still unknown. The osteocyte is probably responsible for receiving and modulating signals of variations of mechanical loading on the bone mass and consequently for the adaptation of bone tissue at the microscopic level through communication with bone producing osteoblasts[28]. Osteocytes are the most numerous cells in mature bone.They are, however difficult to observe being closed off into their lacuno-canalicular system in the bone mass[15]. The time span for a motile osteoblast to become an entrapped osteocyte takes about 3 days. The stellate or dendritic-shaped osteocyte is reduced to 30 % of the size of the osteoblast origin. The life

expectancy of an osteocyte is believed to vary widely, but a half-life time of 25 years has been proposed[15].

The osteoclast

This cell is responsible for bone resorption and takes part in the calcium homeostasis of the body. The multi-nucleated osteoclast is found and formed, in smaller numbers compared to other bone cells, on the surface of the bone. The origin of the osteoclast is haematopoietic and is derived from mononucleated cells in bone marrow or from the spleen. Different from the previously discussed cells, the osteoclast belongs to the leukocyte family, related to monocytes and macrophages. The osteoclast is motile, but since it is only formed on the bone surfaces, it is not found in the blood circulation. Research in various pathological conditions has given a greater knowledge of the osteoclast in later years. Three hormones are exemplified as being important for influencing the osteoclastic activity to control serum calcium: parathyroid hormone (PTH), $1,25(OH)_2$-vitamin D3 and calcitonin (Ct). However, this action is due to receptors present for these hormones on the osteoblasts and the indirect regulating influence thereby from the osteoblasts on the osteoclasts [22], i.e. a coupled function.

The interaction between osteoblasts and osteoclasts; remodelling of bone.

The knowledge of extracellular hydroxyapatite crystal formation is still sparse. The osteoblast is thought to have an influence on mineralization through two ways. Firstly, ALP produces phosphate ions, necessary for formation of hydroxyapatite. Secondly, ALP through osteoblasts degrades pyrophosphate, a substance necessary in all connective tissue for regulating excessive mineralization. In that way, mineral can be laid down in the osteoid.
The osteoblast establishes cell-cell contact with the osteoclast progenitor cell.This results in the differentiated multinucleated osteoclast and takes place in the periosteal/endosteal areas[22]. The system of the cell membrane-bound cytokine RANKL and the receptor RANK on the osteoclast-progenitor cell is activated, giving a signal to formation of osteoclasts. Indirect or direct inhibitory systems of osteoclast activation are also present. Osteoblasts are known to produce osteoprotegerin (OPG) which can inhibit RANK activation indirectly through blocking of RANKL on the osteoblast. Calcitonin and oestrogen are also inhibitors of osteoclastic activity directly on receptors on the osteoclast-progenitor cell.
In short, once the area about to be resorbed by the osteoclast is pointed out (through e.g. systemic PTH activation of receptors expressed on the osteoblast), the latent osteoclast (now created between fusion of osteoclast progenitors and osteclast precursor cells) is stimulated by the osteoblasts. At the same time, the osteoblast degrades the thin osteoid covering the bone through proteolytic enzymes[22]. The osteoblast now retracts from the bone surface leaving space for the osteoclast to adhere to the bone surface and start the resorption. In the so called sealing zone, the resorption takes place through acidic degradation of mineral and

matrix in lacunae. After 3 weeks the osteoclast moves away to the next site. During this time, the incorporated GFs (e.g. TGF-β and IGF-I and II) are released and in turn stimulate osteoblasts to start laying down new bone in the area; however a process that takes several months depending on species.

Bone regeneration

Bone heals without leaving a scar unlike other connective tissues and the primary point in healing of bone is to fill up a created defect or to re-establish continuity. The bone repair in bone grafts or fracture healing or in situations with a biomaterial, i.e. a dental implant, may display obvious similarities[29]. However, there are reasons to believe that the titanium implant, present in an implant defect model, causes a different set of initial reactions in early bone repair.

As Davies and Hosseini (2000) has pointed out, the different events in healing of a wound in bone can be divided in haemostasis, formation of granulation tissue, osteoconduction, wound contraction (including retention of the clot to a biomaterial surface) and bone formation (in two ways, de novo bone formation and appositional growth)[29]. A haemorrhage in the bone caused by a trauma, i.e. a fracture or an implant burr, causes a blood clot of various sizes. The traumatized blood vessels are constricted and platelets act upon the fibrin clot with retractional forces in order to reduce the size of the clot through condensation of the fibrin mesh. Necrosis, due to ischemia of the traumatized bone, is a fact beyond the intact circulation and vessels tries to anastomose where possible through Volkmann's canals. Many factors in the clot, such as leukotrienes and chemoattractants, including PDGF and TGF-β, attract leukocytes. The clot is now degrading through the actions (phagocytosis of debris, bacteria and damaged tissue) of initially neutrofils and later macrophages[30]. An acid environment through the degradation of the clot by macrophages and a low level of oxygen in the centre of the clot causes angiogenesis by endothelial cells and also attraction and proliferation of cells capable of collagen matrix synthesis. This fibrous vascular tissue is called granulation tissue, due to endothelial proliferation at a rate of 80 μm per day, measured in a soft tissue model[31]. Migration of osteogenic cells into the area (osteoconduction) is now made possible and once the cells stop migrating they polarize and are named osteoblasts and secrete matrix.

Wound contraction, as in clot retraction, is the result of contractile forces of migrating cells (such as fibroblasts or osteogenic cells)[32]on the extracellular matrix produced and is an important stage in efficient wound healing. Parallel to this, the concept of *clot retention* to an implant surface is important as pointed out by Davies and Hosseini (2000) and Davies[29, 33]. The ability of an implant surface to retain an adherent clot and to exceed the tractional forces acted upon the clot by the migrating cells, can as a result be a determining factor for successful implant integration[29].

Important findings in bone formation has been made through studies in the bone chamber model, where the sequence has been possible to observe on a day to day basis[34-36].

Bone formation in healing and remodeling situations in a 3-dimensional matrix or on a surface (e.g. biomaterial or a bone surface), is characterized by osteoconduction through the matrix, *de novo bone formation* and appositional growth. Undifferentiated osteogenic cells infiltrate the matrix as well as colonize the surface of a bone or a biomaterial, such as an implant. These cells lead the way towards the target and in doing so some cells stop to produce matrix as osteoblasts and eventually get incorporated in the bone as osteocytes. To continue bone formation, not only as bone spicules advancing the bone formation through the tissue, a small amount of proliferation among osteoblasts take place to make up for the loss of cells due to osteocyte formation.

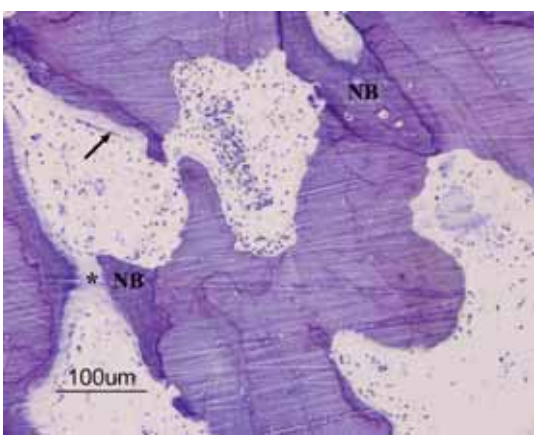
If the osteogenic undifferentiated osteoconducting cells are the frontrunners of bone formation through the defect, the active osteoblasts makes the base of the forming bone wider with continuous secretion of osteoid – *appositional bone formation*. The spicules eventually fuse to become trabeculae of bone. More osteogenic cells line up at the surface of the bone and continue to secrete osteoid, increasing the size of the trabeculae. A very rapid bone formation will result in woven bone and will be more asynchronous as opposed to the lamellar bone formation, which is a slower and more coordinated way of producing bone by the secreting osteoblasts. Appositional bone formation is seen also during growth, due to periosteal enlargement or deposition of bone beneath the periosteum. Through studies in the titanium bone chamber by Albrektson and co-workers, the rate of bone formation has been observed and it is said that osteoconductive formation is 30 to 50 times faster (up to 50 μm per day) than appositional growth. Osteoconductive growth is faster because bone can be formed in many locations simultaneously in the general direction of growth[29, 36].

Osteoid is replaced by mineralized woven bone after 1-3 days. A more ordered state of bone thereafter replaces the woven bone. This bone is organized in lamellae around the vascular canal (Haversian canal) supplying the bone with nutrients and oxygen. These units of lamellae are called osteons and usually run parallel along the long axis of the bone. In compact bone, the border of an osteon is noticeable due to different collagen fibre density in the perimeter that borders to the next structural unit. In appositional growth, the *primary osteon* is deposited from the perimeter to the inside towards the central Haversian canal including the capillaries and nerve fibre. The *secondary osteon* is thereafter initiated through osteoclastic activity by progenitor cells from the blood vessel in the Haversian canal and probably osteocytes in the area (*creeping substitution*). After the resorption phase of the new canal, the Bone-Metabolizing Unit (BMU) lays down bone in concentric layers. These units cut through the bone as cones and so remodels the bone throughout life. As the secondary osteons run parallel to the axis of the long bone, interconnecting transverse vascular channels, Volkmann's canals, are formed. In cancellous bone, the histological appearance of the trabeculae is different with bone-structural units (BSUs), like wall formations, separated by so-called cement lines (collagen free interface) forming the trabeculae.

Healing of autogenous free bone grafts

Autogenous bone is grafted in cortical, cancellous or cortico-cancellous form and can be placed onto the recipient bed either as a piece, *en bloc,* or particulated. Either way, the transplanted bone can, on one hand, be regarded as a mainly a piece of partial necrotic tissue that has to go through various stages of resorption and later act as a scaffold for new bone formation under a variably long time. On the other hand, a swift and gentle handling of the graft with the potential of cell survival, may lead to revitalisation of the graft *in situ*. Since osteocytes are dependent on vascular supply on a distance not further away than 0.1 mm [29], a cortical bone graft only have a small potential to exhibit surviving cells (cellular pools on endosteal and periosteal surfaces). The cells of a cancellous graft may be more prone to survival due to the structure of the graft and possible diffusion of nutrients and revascularisation from the recipient bed. Both the concepts of *osteoconduction*, where gradually new bone is formed around the resorbing graft and *osteoinduction*, where proteins are released that are capable of stimulating osteoblasts or preosteoblasts to new bone formation, are present in this form of healing. In most aspects, the healing of bone grafts exposes the same events as healing of a fracture. Several factors may be important, to various degrees, for the incorporation of an autogenous bone graft as discussed by Alberius et al.[37], i.e. the embryonic origin of the graft, the rate and extent of revascularization, structural and biomechanical differences, rigid fixation of the graft to the recipient site, graft orientation and contents of local growth factors.

Burchardt[38] has pointed out three histological differences that cancellous and cortical autografts display: "i) cancellous grafts are revascularised more rapidly and completely than cortical grafts; ii) creeping substitution of cancellous bone initially involves an appositional bone formation phase, followed by a resorptive phase, whereas cortical grafts undergo a reverse creeping substitution process; iii) cancellous grafts tend to repair completely with time, whereas cortical grafts remain as admixtures of necrotic and viable bone. Physiologic skeletal metabolic factors influence the rate, amount, and completeness of bone repair and graft incorporation. The mechanical strengths of cancellous and cortical grafts are correlated with their respective repair processes: cancellous grafts tend to be strengthened first, whereas cortical grafts are weakened"[38].



In a rabbit tibia model with a titanium chamber, Albrektsson studied the repair in vivo of cancellous and cortical bone grafts[34, 35]. Some basic and important findings, on which the above statement by Burchardt is founded, were presented. Surgical trauma to a graft influences the survival of cells in the graft and due to this, a prolonged time until revascularization and

start of remodelling could be seen in the more traumatized grafts (in average between 7 days for carefully handled grafts and up to 15 days for more traumatised grafts). A bone graft, placed on a bone surface is very dependent on the blood supply from the recipient site, which of course will be smaller compared to a graft placed in a defect within the skeletal envelope. Cancellous bone grafts exhibit a faster rate of revascularization than cortical bone grafts (maximum 0.2-0.4 mm/day and 0.15-0.30 mm/day, respectively). In the exact laboratory model of the rabbit tibia where a bone block with the chamber included, was cut out, rotated and replaced, the development of new vessels was evident 5-8 days after grafting. The remodeling started after 3 weeks. However, two grafts displayed end-to-end anastomose of vessels as these could be seen functioning even after grafting, due to the vital microscopic observation of the same vessels (>30μm in diameter) before grafting. Start of remodelling at one week was observed[39].

The embryologic origin of a bone graft has been discussed extensively after human clinical and experimental observations in many different animal models made clear that membranous bone (i.e. cranial bone), was preferable to endochondral bone (i.e. iliac crest bone) grafts due to less resorption over time[40-44]. Faster revascularization of cancellous over cortical bone graft was confirmed by different workers[45-47] and the question arose if not the different micro-architecture of the grafted bone (relative cortical and cancellous composition) was the true explanation of different graft volumetric stability and revascularization during healing[48-50].

In a rabbit cranial model separating the composite cortical and cancellous bone from each other, pure cortical membranous and endochondral, as well as pure cancellous endochondral bone graft was placed as an onlay graft on the outside of the rabbit cranium. The cancellous bone resorbed almost totally after 16 weeks (the longest observation period) in comparison with the two cortical bone grafts, who had lost 50 % of their initial bone volume. There was no difference in comparing the embryonic origin of the cortical graft and it was also concluded that a graft placed under the periosteum will be resorbed mostly in its projection and less in width[48]. The same authors also describe the slow change in character of a dense cortical bone graft into a more cortico-cancellous type when placed on a bone surface in the cranio-facial skeleton. Micro computed analysis could confirm the decrease of mineralized bone content and increased internal graft surface area (more trabeculated bone), progressively resembling the recipient bone[49]. Soft tissue pressure from the periosteum and healing of a flap covering the graft is therefore also an important factor increasing osteoclastic activity, as shown if the recipient bed was altered by preoperative tissue expansion, lowering the pressure from the periosteum[51, 52].

Rigid fixation of a block bone graft is important to healing since there probably is a limit to the motion accepted by invading progenitor cells, as they can differentiate in various directions to soft tissue forming cells (fibroblasts) or bone producing cells (osteoblasts). Studies have shown a greater graft survival when properly fixated to the recipient site[53, 54].

Studies by Gordh and Alberius[55] concludes that a unicortical cortico-cancellous bone graft is best placed with the cancellous part against the recipient site and the cortical part acting as a barrier and "space-keeper" against the pressure from the flap. A bi-cortical bone graft may cause more resorption to the recipient site. Exposure of the underlying marrow by cortical perforations was also found to facilitate revascularisation. However, the adaptation of a block bone may be difficult to the recipient site, i.e. the human maxilla. Therefore, block bone can be particulated in a mill and easily placed[6, 8, 56, 57] into groves and pits additionally reducing the potential risk of soft tissue in-growth between the recipient site and graft.
The use of particulated bone in sinus-inlay situations is extensively documented[58, 59] as well as particulated grafts for mandibular [5, 14, 60-62] and maxillary reconstructions[57, 63, 64]. The bone harvested for particulation for maxillofacial reconstructive purposes is taken from intra-oral sites as the posterior lateral part of the mandible, or extra-oral sites as the iliac crest. The bone chips created may therefore be of various densities due to ratio of cortical or cancellous content, but will be transplanted in a paste-like condition after milling soaked in whole blood.

Other arguments for particulating bone are the possibility for faster vascular in-growth and the accomplishment of a more homogenous and dense graft compared to the often used cortico-cancellous bone onlay graft from the iliac crest.

However, the volumetric stability of the particulated graft has been questioned[65] and especially if placed outside the skeletal envelope[64]. Particle size, quality and viability after various methods to collect the graft and the resulting osteoconductive capability has been addressed but this has mostly been discussed regarding periodontal surgery and xenografts[66-68]. In an orthopaedic reamer designed study, the ALP activity of the bone chips collected was studied[69]. Using this design, it was concluded that the bone particles from the procedure contained vital osteoblasts. More vital cells could also be found in un-milled and cancellous bone than in milled or cortical bone. Based on a primate study, the particle size was recommended to exceed 125µm, due to the risk of macrophage removal without osteogenic result[66] if the chips were to be smaller. Sharp instruments were also recommended for a good result as prolonged heating and mode of collecting the bone chips seemed to play an important role for graft vitality[70-72].

Vascularised bone grafts

Instant blood circulation between a vessel pedicle and recipient vessel (e.g. the facial artery and vein), is achieved in the graft with micro-vascular surgery if successful. Large bone segments from the fibula or iliac crest can be transplanted and together with various amounts of soft tissue restore form and function after trauma or ablative surgery. The healing of a micro vascular bone graft resembles fracture healing and the graft is stabilized with plates and screws (osteosynthesis). This advanced technique was proven successful in a maxillary reconstruction study, where meticulous pre-operative planning was performed by the reconstructive team. In

short, the protocol included prefabrication of the grafted bone *in situ* of the fibula with split-thickness grafting of skin 6 weeks before transfer, insertion of dental implants positioned with a drilling template in a preplanned position in the fibula, and thereafter transferred to the recipient site in the maxilla and fixed with plates and screws[73, 74].

Bone substitutes

Due to limited supply and morbidity, patients often refrain from autogenous bone grafts and therefore alternatives are looked upon[75]. Some of these alternative bone substitutes are commonly used and are often mixed with autogenous bone for volume preservation of the graft, e.g. in sinus augmentations[76].
Urist[77] has described the allogenous graft (allograft or allogeneic) as a graft derived from bone tissue from the same species but containing no viable cells.
The allografts are removed, or remodelled in a similar fashion as autogenous bone grafts, but there may be a potential risk of immunological reactions from remaining proteins in the graft[78]. Frozen[79], freeze dried mineralized (FMB) or demineralised freeze dried bone (DFDB)[80] are examples of these grafts. Pinholt et al.[81] explored the osteoinductive capacity of demineralised and lyophilized dentin and bone implants and found some osteoinductive capacity in rats in one study, but not in another rat study[82] and a goat study[83], respectively.
Alloplastic grafts (allogenous or allogeneic) are synthetically derived, contain no proteins and are osteoconductive only. Examples of these grafts are hydroxyapatite[84], "bioactive" glass[85], tricalciumphosphate (TCP)[86] and calcium sulphate (plaster of Paris)[87].
Xenografts (xenogenous or xenogeneic) grafts are very commonly used in reconstructive implantology today. They are derived from bone tissue from animals of various species and proteins have been extracted for immunological safety. Bovine hydroxyapatite (BHA), marketed as Bio-Oss®, is an example of a xenograft that has been described in many papers[88-90].

Systemic and local regulation of bone metabolism- proteins, hormones and growth factors

The continuous remodelling of the human adult skeleton in approximately two million microscopic resorption/formation sites is influenced by systemic factors whose mechanism also can be affected with different drugs to treat bone-related diseases such as osteoporosis[91]. Bone formation is slow compared to bone resorption; 3 months of bone formation will follow the resorptive phase of 2-3 weeks. *Coupling* means that events like resorption and formation follow each other up and down in intensity. Due to different rates in resorption and formation, negative changes in bone homeostasis may therefore be a result of alterations in stimulatory factors listed below[91].
Intermittent administration of parathyroid hormone (PTH) has been proven on a long term basis, in different experimental models, to restore bone mass and strength through enhanced

formation of trabecular and cortical bone in animal models[91, 92]. This treatment has also been proven to be the only pharmacological treatment for increasing bone formation in humans[91]. Neer et al. injected PTH subcutaneously once-daily in post-menopausal women for treatment of postmenopausal osteoporosis[93]. However, if given continuously, PTH stimulates bone resorption[94], but this intermittent administration decreased the risk for osteoporotic women of vertebral and non-vertebral fractures and PTH also increased vertebral, femoral, and total-body bone mineral density if administered this way. Steady state in bone homeostasis is explained by feed-back mechanisms, where PTH and calcium levels in plasma is one, and sex hormones and mechanical feedback signals are other examples[91].
Together with PTH, vitamin D (named $1,25(OH)_2$vitamin D or calcitriol) is responsible for calcium regulation in plasma through bone resorption, intestinal absorption and renal reabsorption.

Calcitonin, a thyroid hormone produced by the parafollicular cells of the thyroid glands, is regarded as a pharmacological inhibitor of bone resorption. Studies on mice, lacking the gene for calcitonin, suggests the protein hormone to be an inhibitor of bone remodelling as these mice displayed a phenotype with high bone mass (HBM) caused by high bone formation and normal bone resorption[95]. Other thyroid hormones, as tri-iodothyronine (T_3) and thyroxine (T_4), are also essential for normal skeletal growth and maintenance of bone mass in adulthood; hypo-thyroidism results in impaired bone formation and reduced bone mass and thyrotoxicosis in increased bone formation.

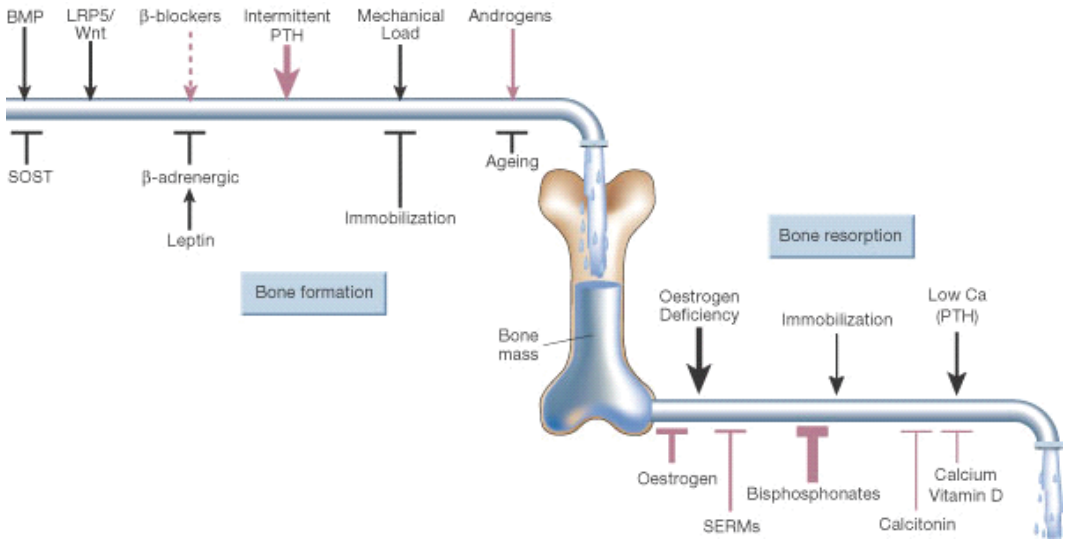
Glucocorticoids have recently been proven in mice to act directly on osteoclasts. Osteoclast and osteoblast precursors are reduced after administration of glucocorticoids but the effect is not the same on differentiated osteoclasts and osteoblasts. An imbalance develops between the cells in favour of osteoclasts, as the lifespan of these cells is increased in contrast to osteoblasts, resulting in reduced bone density over time[96].

Growth hormone (GH) from the anterior part of the pituitary gland, is proposed to be important in bone mass maintenance through direct action on osteoblasts or indirect via liver-mediated increased levels in plasma of insulin-like growth factors (IGF)-I and II[97]. The connection between GH and IGF has been studied in patients with anorexia nervosa (AN), who exhibit raised levels of GH but reduced IGF-levels. A combination therapy, but not alone, of bone anabolic and anti-resorptive therapy with recombinant IGF-I and oestrogen has resulted in reduced bone mineral density in adult women with AN[98, 99].
Insulin is produced in the β-cells of the pancreas and is essential for bone growth. This fact has especially been noticed in studies on children with type 1 diabetes[100, 101].

Sex hormones are regarded as important as regulators of bone mass, together with calcium availability and mechanical usage of the skeleton. Androgens, oestrogens and progestins are the steroids of this group. Gonadectomy in either sex causes increased bone remodelling, bone resorption and a relative deficit in bone formation[102].

Oestrogen inhibits bone resorption by reducing osteoclast number. Oestrogen-deficiency is worldwide a huge problem estimated to afflict 200 million women. Approximately 14 billion

dollars are spent in the US on treating osteoporotic fractures each year[103]. Mechanisms of bone homeostasis are complex, since not all menopausal women develop osteoporosis. Hormonal imbalance resulting in reduced cortical bone mass may also affect men with lowered levels of e.g. testosterone[104].



Schematic representation of the servo system that maintains bone mass at steady-state levels. Physiological (blue) and pharmacological (orange) stimulators and inhibitors of bone formation and resorption are listed. The relative impact, where known, is represented by the thickness of the arrows. Solid lines are current therapies and dotted lines putative ones. Abbreviations: BMP, bone morphogenetic protein(s); SOST, sclerostin; LRP5, low-density lipoprotein (LDL)-receptor-related protein 5; PTH, parathyroid hormone; SERM, selective oestrogen-receptor modulator.

From Harada S, Rodan GA. Control of osteoblast function and regulation of bone mass. Nature 2003;423:349-55.

The central nervous system has also been proposed to play a major role in bone formation through leptin signalling pathways[105]. Leptin, a protein hormone, was discovered in the study of obese mutant mice. Leptin is produced in adipose tissue and is a key player in regulating energy intake and energy expenditure; binding of leptin to the hypothalamus signals that the body has had enough to eat. Lack of the leptin gene will cause severe obesity. Through very complicated pathways, as proposed by different groups, via direct action on osteoblasts or/and through central nervous system mechanisms, leptin represses bone formation and reduces bone mass[106, 107]. The role of leptin in bone homeostasis and bone formation was found by Takeda et al.[107] to be effected by the sympathetic nervous system. Using this new knowledge, propanolol, a b-adrenergic antagonist, has been shown in mice to have stimulatory effects on fracture repair[108] and this has been proposed to be an interesting new way in treating osteoporosis[91].

Through genetic mutations in animals and humans and methods to map these changes, different transcriptional factors important for osteogenesis (closely related to chondrogenesis) have been identified and further explored. These factors include runt-related transcription factor 2 (Runx2) and Osterix (Osx). Runx2 is also important for chondrogenesis. Genetically deficient mice for Runx2 and Osx will develop a cartilaginous skeleton. The lack of Osx-gene will give a perfect cartilaginous skeleton without osteblasts; the idea is that Osx is a mediator inferior of Runx2 which plays the role of inducing osteoblastic differentiation in bipotential chondro-osteo progenitor cells[91, 109-111]. Genetic methods used in disclosing hereditary skeletal disorders have also discovered genes responsible for bone regulation. Two examples of such genes are LRP5 and sclerostin (SOST)[91]. LRP5 is responsible for encoding a protein responsible for HBM alterations in individuals with very dense bone[112]. SOST is a gene, expressed mainly in bone and cartilage, which decreases bone formation supposedly by suppressing bone morphogenetic protein (BMP) activity. Mutations that have lead to SOST inactivation in humans results in HBM and sclerosteosis (elevated bone formation and otherwise healthy)[113].

Growth factors in bone and in fracture healing

GFs are present in the bone matrix and plasma in small concentrations, but execute important actions. GFs binds to trans-membrane receptors molecules on the cell and a transduction of information from the GF to the cell via cytoplasmic cascade reactions, result in transcription of mRNA, and subsequent intra- and extracellular actions[114].

Levander[115] already in 1938 observed ectopic bone formation around periosteal- and surface layer-free bone grafts in non-skeletal sites and Urist could much later verify that protein extracts from demineralised bone matrix were able to induce bone formation[116] and named it BMP in 1971[117]. BMP activity is not species specific and its activity closely related to the delivery matrix used in combination with the BMP [118, 119]. BMPs are not only capable of inducing bone and cartilage but are also important regulators of morphogenesis during development[120, 121]. The BMPs form a sub-group of the TGF-β super family, which is a large group of proteins that affect cell growth, migration, and differentiation including regulatory roles in tissue homeostasis and repair in adult organisms[122, 123], and at least 30 BMPs have been identified. At least BMP-2 to BMP-8 are osteogenic[124]. The members of the TGF-β super family are termed BMPs, osteogenic proteins (OPs), cartilage-derived morphogenetic proteins and growth and differentiation factors (GDFs) as well as BMP-like molecules from different species[125]. BMP secretion from cells has been suggested to work in three ways: local immediate action, binding to extracellular antagonists at the site of secretion or, finally, interaction with extracellular matrix proteins that serve to enhance BMP action on target cells. Important research has been made on the cell receptors of BMP, for a review see[126], and in vitro mesenchymal stem cells (MSCs) have a great number of BMP receptors

making osteoblast differentiation possible from MSCs. Noggin, gremlin, follistatin and sclerostin are BMP antagonists, synthesized by MSCs as they differentiate into osteoblasts during development, and capable of blocking osteogenesis. These factors are important also in normal bone regulation in blocking BMP-activity and since not all BMPs induce bone formation, the highly osteogenic capacity of, e.g. BMP-9, has been proposed to be explained by the lack of binding of these regulatory factors to this particular BMP. Among the bone forming functions of the osteoblast, a negative feedback mechanism is said to act on BMP activity by the osteoblast. Osteoblasts secrete both BMP and its antagonists and therefore this balance during remodelling of bone is an important area of research [127].

Another member of the TGF-β super family is the transforming growth factors (TGF-β). TGF-β is found in highest concentration in platelets[128] but in total, bone is the quantitatively the most abundant source (200 μm/kg tissue) [129]. TGF-β is produced by osteoblasts and thus stimulates the expression of bone matrix proteins[130] as well as decreases the degrading activity of the matrix by enzymes such as metalloproteinase[131]. There are five isoforms[122] of the cytokine with various effects, but in contrast to BMP, TGF-β does not induce ectopic bone formation[132]. TGF-β induces differentiation or proliferation of osteoblastic cells and further inhibits the formation of osteoclast precursors and even, in greater concentrations, displays inhibitory effects on osteoclasts[133].

Smads are the signalling pathways for the TGF-β family from the membrane of the effector cell to the nucleus[134]. These proteins have been found in a great number of species and therefore scientists have been able to use more simple models to understand the transcription events of genes taking place in the nucleus of the affected cell after stimulation by these cytokines[135]. TGF-β-release (TGF-β1, -β2, -β3) is abundant in fracture healing where these factors are dissolved as well as BMPs 1-8 and GDF-1, -5, -8, -10[136].

During fracture healing, a long list of signalling molecules is important. These can be categorised into three groups: i) the *pro-inflammatory cytokines* (interleukin-1 (IL-1), interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNF-α)), ii) *the TGF-β super family* (BMPs, TGF-βs) *and other growth factors* (platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), insulin-like growth factor (IGF) I andII, iii) *the angiogenic factors* (vascular endothelial growth factor (VEGF), angiopoietin 1 and 2, including the metalloproteinases (degrades bone and cartilage and enables vessel invasion))[137].

The cytokines IL-1, IL-6 and TNF-α, are early in the repair cascade. They are secreted by macrophages and mesenchymal cells present in the periosteum and respond to injury with a peak expression during the first 24 hours, but are also seen active in the cartilaginous and remodelling phase of the fracture. These factors have chemotactic effects on inflammatory cells, enhances cellular matrix synthesis and stimulates angiogenesis[137, 138].

A large source of TGF-β is found in platelets released during degranulation and this is also true for PDGF. PDGF is a potent mitogen for mesenchymal cells from e.g. the periosteal

layer. PDGF is also synthesized by monocytes, macrophages, endothelial cells and as well by osteoblasts[139]. The PDGF is a dimer of two polypeptide chains, A and B, and these chains may form either a heterodimer or a homodimer. Out of the three PDGFs (PDGF AB, AA or BB) is the PDGF BB is the most potent. In the early stages of fracture healing, PDGF is a powerful chemotactic agent for inflammatory cells and a stimulus for osteoblasts and macrophages[132].

Monocytes, macrophages, mesenchymal cells, chondrocytes and osteoblasts produce fibroblast growth factors. FGF is important in chondrogenesis and bone resorption. The target cells are mesenchymal and epithelial cells as well as chondrocytes and osteoblasts. Two forms, α-FGF and β-FGF, are present whereas the former is responsible for chondrocyte proliferation and the latter, more potent form, for maturation of chondrocytes and in addition, plays a role in bone resorption in the fracture healing process[137].

The role of IGFs in bone formation has been disputed[140, 141].

Growth hormone regulates insulin-like growth factors in plasma; general sources of IGF are the bone matrix, endothelial cells, osteoblasts and chondrocytes[132, 142]. Out of the two isoforms, IGF-I is more potent and is involved in bone matrix formation whereas IGF II acts later in endochondral bone formation. The insulin-like growth factor-binding proteins (IGFBPs) modulate the action of IGF in a cell-specific manner[143].

During late phases of fracture healing, i.e. endochondral ossification and even bone remodelling, the matrix metalloproteinases degrades cartilage and bone. This allows angiogenic factors in possibly two pathways to regulate vessel in-growth, namely the vascular-endothelial growth factor dependent pathway and the angiopoietin-dependent pathway[144].

VEGF is found in 4 isoforms (A, B, C, D) and the protein is produced by several cells such as macrophages, smooth muscle cells (SMCs) and osteoblasts. Hypoxia is proven in vitro to stimulate VEGF production by SMCs and osteoblasts[145, 146]. VEGF induces migration and proliferation of endothelial cells through the use of different integrins, transmembrane adhesion proteins, that indicates a connection to matrix for optimal response[147]. VEGF also induces relaxation in the cell-to-cell contacts of endothelial cells resulting in hyperpermeability of blood vessels and these stimulated endothelial cells also produce matrix degrading enzymes inducing migration of the cells[147]. Recently, VEGF has been shown to be an important factor for enhancing and directing stem cell motility[148].

Blood

Whole blood

Circulating human blood is composed of plasma and cells, predominantly red blood cells (RBCs or erythrocytes) and platelets (thrombocytes), but also a smaller number

(ratio 1/500 RBCs) of white blood cells (WBCs or leukocytes). The plasma makes up for 55% of the total blood volume. If the clotting factors have been removed from plasma, the solution is called serum. The plasma has a faint straw colour and is composed of water, blood proteins, inorganic salts and nutrients. Furthermore, it serves as a transport medium for lipids, glucose, metabolic end products, carbon dioxide and oxygen[149]. Two important capacities of blood is coagulation and messenger functions.

Red blood cells

The RBCs are shaped as biconcave disks with a mean diameter of 8μm. Their primary task is to transport haemoglobin from the lungs to the tissues in the body. Their role in haemostasis has been debated but observations and studies in thrombocytopenic patients, who had improved bleeding times after RBC transfusions, pointed towards the positive effects of the RBC, also later confirmed in studies[150-152]. Their role has been explained by changes of viscosity in blood, where e.g. a higher hematocrit (RBCs/ 1 volume blood, mean 0.45) can direct the platelets to the periphery of the vessels so that more platelets are available for activation (see below) from the endothelium[150]. The signalling between platelets and red blood cells is interesting and has been explored during the last 50 years. Hellem studied platelets increased adhesion on glass beads by addition of RBCs[153]. The ability by RBCs to enhance platelet aggregation[154] and enhance platelet degranulation[155] has been confirmed in studies. The pore size between the fibrils in the fibrin network is also increased by the presence of RBCs, which may have effects on the ability for cells to migrate and metabolites to be transported through the clot[156]. Later research has disclosed the coagulation stimulatory effects of erythrocyte membrane in activating coagulation factors such as factor IX[157].

White blood cells

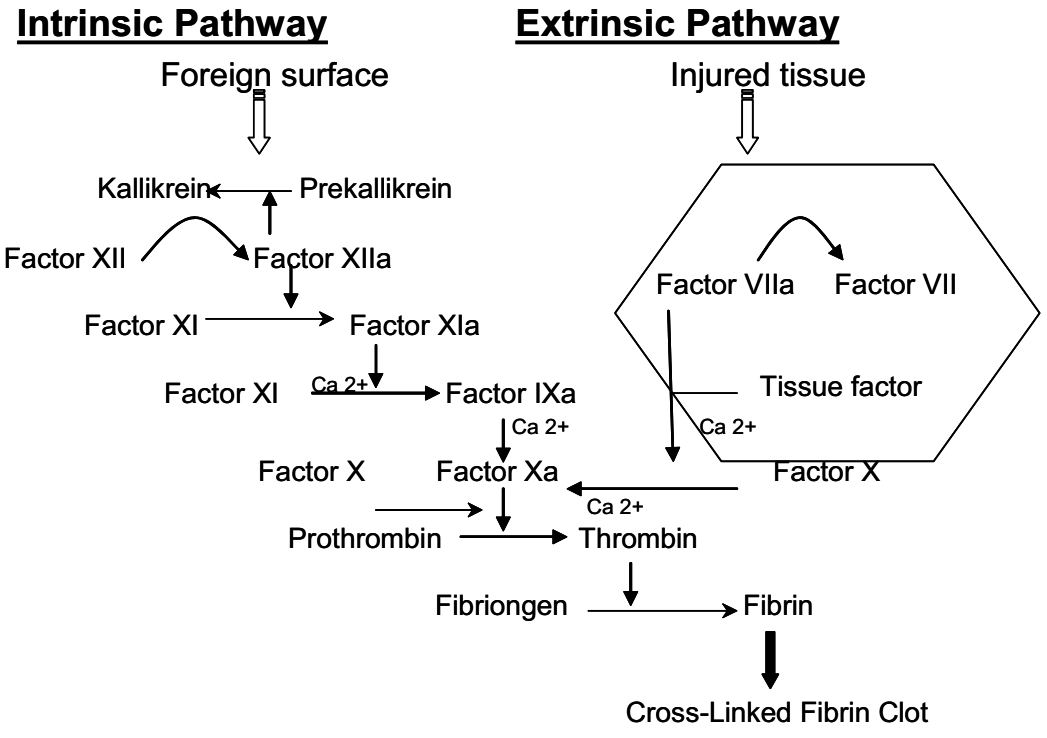
White blood cells are found in three groups: the polymorphonuclear granulocytes (PMN), monocytes and lymphocytes. WBCs, seen in centrifugated blood as a "buffy coat" layer between the red blood cells and plasma, are involved in part in the immune system and act as host response to infectious disease. Their role in inflammation and activation is also of great importance. Pathogens release chemotactic factors that attract available leukocytes in the circulation and the activated endothelium interacts with ligands in the membrane glycoproteins of leukocytes, further enhanced by immunoglobulin adhesion molecules on endothelial cells and integrins on leukocytes. The binding of leukocytes to one another results in further activation of other leukocytes[158, 159]. The activated WBCs crawl on the endothelium (also called "roll on"). The leukocytes thereafter exhibit their unique ability of diapedesis, where activated endothelium allows WBCs to pass in between the endothelial cells. This passage is regulated by junctional adhesion molecules on endothelial cells and possibly also on WBSs. WBCs can

therefore move through to the tissues to execute their defence against pathogens in the inflammatory response[158].

Platelets

Platelets are the second most abundant unit in blood (un-nucleated fragments of bone marrow megakaryocytes, 1.5 to 3.0 μm in diameter) [29, 160]. Their role in haemostasis is central and they are vital to wound healing and inflammation. In a resting state, they circulate in blood for 9-10 days. The granules of platelets, released upon activation, are at least of three types; α-*granules, dense core granules and lysosymes*.

The dense granules contain nucleotides such as adenine and guanine, amines such as serotonin and histamin and cations. α-granules are well known in regenerative surgery literature as they contain **I**) *adhesion molecules* (P-selectin, Platelet endothelial cell adhesion molecule-1, Glycoprotein IIb/IIIa, von Willebrand factor, Thrombospondin-1, Vitronectin, Fibronectin), **II**) *mitogenic factors* (PDGF, VEGF, TGF-β), **III**) *coagulation factors* (Fibrinogen, Plasminogen, Protein S, Kininogens, Factors V, VII, XI, XIII) and finally, **IV**) *protease inhibitors* (C1 inhibitor, Plasminogen activator inhibitor-1, Tissue factor pathway inhibitor).



A simplified overview of the coagulation system.

Lysosomes contains glycosidases, proteases and cationic proteins (for review see, [160]). Many activators of platelets are known (some produced by platelets themselves); a few of them are: collagen, thrombin, thromboxane A_2, adenosin phosphate, P-selectin and molecules that ligand to the protease-activated receptors (PAR), of which 3 out of four identified are expressed on platelets[160, 161]. Inhibitors of platelet activation work through the blockade of different receptors on the platelet surface. Inhibitors are e.g. several of the coagulation factors, ADP receptor inhibitors and aspirin[162].

Humoral systems

The coagulation as a protein based cascade and also a cell based series of events

The prevailing idea of the coagulation cascade as plasma protein dependent, activated by the intrinsic or the extrinsic pathway into a common final pathway[163, 164], has been complemented in recent years by a cellular based model where coagulation is regulated by properties of cell surfaces[165, 166]. The "cascade"-model of coagulation describes the coagulation as a series of proteolytic reactions where subsequent cleavage of proteases takes place in a series of events. Platelets has been looked upon as important in providing negatively charged phospholipids (PLs) on the cell membranes, so that coagulation complexes can assemble on the platelet cell surfaces[165].

The *intrinsic pathway* is activated on negatively charged foreign surfaces when F XII is bound. Active F XII (F XIIa) cleaves a complex formed by high molecular weight kininogen (HMWK) and prekallikrein (PK). The generated kallikrein converts more F XII to F XIIa. Thereafter, F XIIa activates F XI, subsequently resulting in F IX activation (F IXa). F IXa forms a complex with activated F VIII (F VIIIa), exposed phospholipids (from platelets and erythrocytes) and activates F X (Xa). This reaction is dependent on Cl^{2+}. This step in the cascade is part of the common pathway and is also shared by the *extrinsic pathway or tissue factor pathway*[167]. It has been proposed that the role of the intrinsic pathway may be to amplify the coagulation triggered by the tissue factor (TF) pathway. The idea of this pathway came when it was shown that thrombin can directly activate F XI on the surface of platelets[168]. Therefore, the first step with the complex of F XII, HMWK and PK was thought not to be necessary for activation of the coagulation[167, 169]. The extrinsic pathway is based on TF activation, e.g. present in injured tissue in vessel walls, activated endothelium or monocytes[162]. TF is a membrane-bound glycoprotein and the cellular receptor for F VI/VII, probably essential for life as mice embryos without TF die in utero[170]. As the membrane bound TF in cells from an injured vessel wall is exposed to plasma, cells can bind FVII. This event is also here dependent on PL and Cl^{2+}[171]. Bound to a cell membrane, the F VIIa-TF-complex is thought to be the most potent activator of the coagulation[172]. This

complex then activates F IX and F X as an end to the extrinsic pathway where it meets the intrinsic and common pathway[165, 172].

The common pathway displays activated F X as its first step. The F Xa-F Va-complex (with PL and Cl^{2+}) then cleaves prothrombin to thrombin. Thrombin subsequently cleaves the soluble fibrinogen into insoluble fibrin monomers. The polymerization of the monomers into a gel is the final stage of the coagulation process.

Early generated thrombin has the ability to feed back into the cascade system to activate F V and F VIII[173, 174]. The knowledge of thrombin generation on the platelet surfaces has led to a cell-based model of coagulation[165, 175]. In this way, the in vivo events of coagulation can be better understood, as the cascade model is more descriptive of the in vitro events of coagulation. In the cell-based model, three phases occur on different cell surfaces. *Initiation* of coagulation takes place on TF-bearing cells such as a fibroblast. *Amplification* occurs on the platelet when it is activated, and last, *propagation* at the surface of the activated platelet. If strong enough stimuli are present, the coagulation starts and thrombin feeds back to enhance the reaction even further. In the amplification stage, enough coagulation factors accumulate on the platelet so that, in the final stage, the sufficient amount of thrombin is produced[165].

Two binding sites for thrombin on platelets that send a signal for activation are glycoprotein (GP) Ib/IX-receptors[176] and protease-activated receptors (PAR)[177], but there are possibly others[165]. Additionally, platelets have been shown to have receptors for fibrinogen (GPIIb/IIIa)[178] and F X (effector-protease receptor-1, EPR-1)[179] as well as possibly F XI, F IX and F VIII[165].

The fibrin formation results in fibres that aggregate to form a mesh and the fibrin mesh is stabilized by cross-linking catalysed by the thrombin-activated F XIIIa[180].

Regulation of the coagulation

Partly, as a result of the cell-surface mediated coagulation process, there are built in limitations, regulatory mechanisms or inhibitors, to excess coagulation in vivo. Endothelial cells displays specialized anti-coagulant features and are thought to be relatives to the platelets, as they can express similar regulatory receptors, such as GPIb and PAR for thrombin[165]. Activation of coagulation can only take place on activated cells and platelets, where negatively charged PLs are present and where the receptors, presented above, are available[172]. To avoid massive fibrin deposition, the system allows only activation at the site of vessel injury and the system must be shut off when enough fibrin to seal the wound has been produced.

TF-availability is limited to the site of vascular injury only, and additionally, there are regulatory pathways: TF pathway inhibitor (TFPI), generation of antithrombin (AT), the protein C anticoagulant pathway and inhibitors of the intrinsic pathway[172]. The TFPI quickly inhibits the TF-VIIa complex and is released from endothelial cells and the effect is a rapid inhibition of the extrinsic pathway[181]. Antithrombin (AT) can rapidly bind and neutralize F IXa, Xa,

TF-VIIa-complex and thrombin. AT is a glycoprotein produced in the liver. Intact endothelium is non-thrombogenic due to heparan sulphate on the surface of the vascular lumen. These molecules, with heparin like structure on the vessel walls, act as a co-factor for AT in inhibiting coagulation[162, 172]. The stable complex of thrombin-antithrombin (TAT), which rapidly is cleared from circulation, can be used as a reflection of thrombin production in vivo and in vitro[182-184].

The protein C dependent pathway is a key mechanism in anticoagulation. Protein C is a vitamin-K-dependent plasma protein circulating in an inactive state. To become activated, protein C binds to a transmembrane receptor on endothelial cells and this is thrombin dependent. Thrombomodulin, another receptor of endothelial cells, binds thrombin, and this complex activates protein C.

Protein S, a cofactor of activated protein C and also vitamin-K-dependent and a plasma protein, must be present for activated protein C to act as an inhibitor of coagulation. This complex can inactivate F Va and VIIIa and as a result, thrombin has "shut down itself" and its production[172].

Inhibitors of the intrinsic system are C1-inhibitor, which inhibits factor XIIa, antitrypsin that inhibit F XIa, and α_2-Macroglobulin that acts as a secondary inhibitor to e.g. thrombin[172].

Fibrinolysis limits clot formation by cleaving formed fibrin into fibrin degradation products and the responsible enzyme is plasmin. Its inactive form is called plasminogen and this circulating molecule has activators and inhibitors and is essential in controlling fibrinolysis.
Activators of plasminogen are F XIIa, F XIa, kallikrein, tissue plasminogen activator (tPA). tPA is the most important for in vivo activation and is produced by endothelial cells[185]. The most important inhibitor of active plasmin is α_2-antiplasmin[186].

The complement system

This system consists of more than 35 soluble and cell-bound proteins, complement (C), with primary goal of host defence by disrupting the membrane of the target cells. Three biochemical pathways are responsible for activation of the complement system; the classical, the alternative and the mannose-binding lectin (MBL) pathways. These three pathways finally converge into a common pathway and form the membrane attack complex (MAC). The classical pathway is activated by the formation of antigen-antibody complexes. Complement 3 (C3) triggers the alternative pathway and allows triggering by e.g. biomaterials. The MBL is activated independently by antibodies and also triggered by MBL binding to carbohydrates. Through a sequence of complex formations of complement at the end of the common pathway the MAC is formed and finally inserts into the cell membrane. For an extensive review, see [187].

Biomaterials

Biomaterials are used in medical devices in contact with living tissues and biological fluids that are intended for diagnosis, treatment, augmentation or replacement of tissues, organs or functions of the body. A search on the term "biomaterials", on one of the available databases PubMed (National Library of Medicine and the National Institutes of health), in October 2006, gives 44 749 hits. It is an extensive field, but could be divided into four categories, namely: ceramics, composites, metals and polymers. It is beyond the scope of this review to penetrate all of these materials. However, some examples are important to mention. Hydroxyapatite can be placed in the first group of ceramics, as it is said to be a bioactive ceramic used for implant coating. Composites are often used in restorative dentistry and prosthodontics[188]. Polymers are used for fabrication of prostheses and in devices for extracorporeal circulation and vascular grafts. Polyvinyl chloride (PVC) for soft tubes (with added plasticizer)[189] and poly methyl methacrylate (PMMA)[190] for bone cement are often used polymers. Alloys of different metals have been widely used as biomaterials, such as cobalt-chromium alloys and iron-chromium-nickel alloys.

Contact between blood and a biomaterial

Biomaterials activate the coagulation system through the intrinsic pathway, as shown by Hong et al.[183]. The importance of negatively charged surfaces to initiate this reaction has also been pointed out[191]. This initial reaction involves the proteins high molecular weight kininogen (HMWK), F XII, F XI and Prekallikrein; already touched on under *Humoral systems*, above.

Titanium

Titanium (Ti) is today the most successful clinical implant metal used, especially in dental implantology[192]. It is also an example of a material that displays good biocompatibility. However, a materials ability of being biocompatible can be different depending on the actual local tissue, e.g. successful implantation of titanium in bone[193] or titanium placed in blood, where it may be thrombogenic and represent a risk[183].

Ti is found as number 22 in the periodic table, closely related to Zirconium (Zr). Ti is a very strong metal, comparable to steel, but with a lower density. Therefore, it has been used industrially in many fields of construction. 95% of the world consumption of titanium is produced as TiO_2 and used in a wide variety of applications such as ingredients in paints, cements and even toothpaste. As an implantable biomaterial, Ti is resistant to corrosion in saline solutions and resistant to acids. This may be explained by the fact that the natural state of titanium with

a non-modified surface is highly reactive and will instantly form a thin (2-17 nm) oxide film when exposed to oxygen in air[194, 195]. The thickness and character of the oxide film is then responsible for the implantation characteristics. The surface is slightly negatively charged at physiological pH and has low solubility, favourable for tissue contact and initiation of coagulation.

Surface modifications of dental implants

One focus of today in dental implantology is the design of the implant[196], another focus (perhaps more important) on modifications of the titanium surface[197-199]. The success rates for implant treatments have reached very high levels, even in patients with deficient bone volume and problematic quality of bone[193, 200-202]. Albrektsson et al.[203] disscussed factors for successful use of dental implants. Adequate implant material, design and surface quality, correct validation of the bone, delicate surgical technique and appropriate loading conditions for the implants were all stated as important. The implant surface may be characterized by its mechanical, topographical and physiochemical properties and the techniques of surface alterations are vast[204], now even including photolithotopography[205] and lasers[206].

Ellingsen et al.[195] summarised the importance of surface aspects: the techniques of analysing the surface of an implant are different from analysing the bulk material; the surface is the reactive part of the implant and strive to reach stabilising bonds with neighbouring atoms in the tissue (a fact that explains the success of implants but also has to be considered a risk of contamination of the surface); the properties of the surface directs the events after contact with biological components resulting in protein adsorption, cascade reactions, and cell behaviour); description of surface properties (such as chemical composition, structure, roughness, wettability, Electro-optics and mechanics) has been made possible through surface-sensitive techniques, and one key research area for the future is surface properties and their interaction with biological processes.

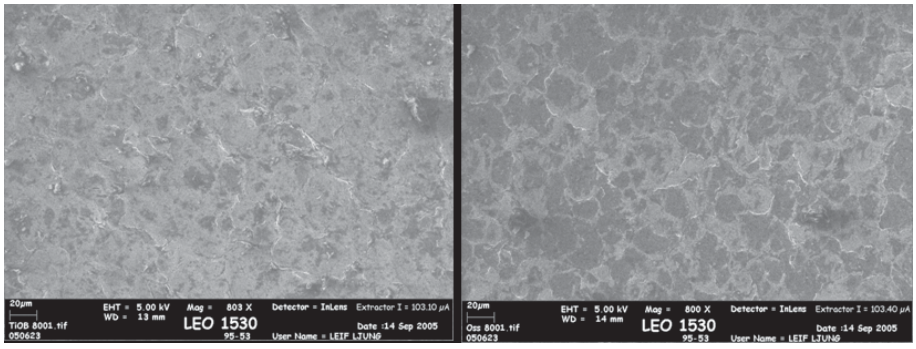
The description of all modifications of titanium surfaces is beyond the scope of this text, but the methods of modifications of the titanium used in the present thesis have to be addressed. The machined or turned titanium surface, was introduced in research and in clinical use by the Brånemark group[2], followed by several authors[193]. Due to the production it has a surface with grooves and valleys mostly oriented along the machining marks[195] and presenting a thin oxide layer of TiO_2 (complemented by small amounts of TiO and TiO_2O_3) that will slowly grow thicker over time. The surface oxide may in turn be modified by heat treatment, gel-derived oxidation and electrochemical oxidation[195]. However, it is the inertness, or stability of the TiO_2, which is important for the (lack of) tissue response.

Blasting is another example of a mechanical treatment of titanium that has been investigated for about 30 years[207], and in clinical use for almost 20 years[208]. The influence

of TiO_2-grit blasting of titanium was evaluated by Wennerberg in her thesis from 1996[209]. Here, the implant community was further introduced to surface characteristics and that an optimal surface roughness seemed to have advantages on integration of implants into bone[210-215]. It was disclosed that a too smooth surface could result in poor cellular adhesion and clinical failure and that a too "rough" surface with large irregularities, could cause a disturbed bone response and possible ionic leakage leading to clinical failure as well.

Wennerberg used a series of parameters to describe the surface topography which of five are relevant to this thesis: Sa = average height deviation, measured in µm, Sds = density of summits, a spatial descriptive parameter measured in $1/\mu m^2$; Ssk = skewness, a description of the height frequency distribution, where a positive value indicates more peaks than valleys and a negative value more valleys than peaks; Sdr = developed surface area, a hybrid parameter taking into account variation in height and spatial distribution and measure surface enlargement compared to a flat reference plane, the value is given in %; and, finally, Sci= core fluid retention index, a functional parameter, measuring the fluid retention index where a high value indicates a good fluid retention in the core zone. Value ranges are typically seen from 0 to slightly more than 2. A result from this thesis and the experimental work therein, that has been used extensively as a reference when introducing new modified surfaces, are the optimal characteristics of a blasted surface created in the thesis, by blasting with a particle size of 25 to 75 µm. The S_a-value (1 to 1.5 µm) and S_{dr} (1.4 to 1.5) are two examples of those parameters.

Ivanoff et al.[216], in a study with micro-implants in patients, could verify previous studies and showed a significantly greater bone-to-implant contact (BIC) for the test (TiO_2-grit blasted) rougher micro-implants, than the control smoother (machined) micro-implants.



Hydrofluoric acid etched TiO_2-grit blasted titanium has been introduced as modified implant surface[195, 217] and is a modification of the titanium oxide layer by using low concentrations of hydrogen fluoride. Fluoride (F) has effects in vitro by increasing the mitogenic action of bone cells and proliferation of osteoprogenitor cells[218, 219]. F further increases the remodelling of bone in a domestic pig-model[220] and also the trabecular bone density in skeletons of osteoporotic women[221, 222]. The action of fluoride has been proposed to be explained by the fact that F increases the intracellular levels of calcium[223]. Elevated calcium levels have been associated with cell proliferation. F is a modulator of the intrinsic formation

of GFs produced by osteoprogenitor cells and therefore a catalyst of bone formation. Phosphate affinity of the modified surface was said to be higher than to non-modified control in a study where implants were immersed in a radioactive calcium-phosphate solution and thereafter checked for radioactive uptake[224]. This ability was argued to be important for quick bone formation on the implants after insertion into bone[195]. Cooper et al.[225] found greater osteoblastic differentiation from human MSCs and expression of bone sialoprotein and BMP-2, when studying MSCs on TiO_2-grit blasted titanium disks with and without fluoride ion modification. Parallel in vivo experiments revealed a significant greater BIC for test implants compared to control in a rat tibia model.

If the F-ion modified surface can be characterized as a bioactive surface, referring to bioactivity as "characteristic of an implant material which allows it to form a bond with living tissues"[226], so is the hydroxyapatite-coated (HA) titanium surface[227]. Plasma spraying of $CaPO_4$ onto the titanium surface is mostly chosen for dental implants. The properties of interest of this surface used in e.g. dental implants are the similarities to bone mineral, the ability to form carbonate hydroxyapatite on the surface when installed in bone, promotion of a strong interface between material and bone, provision of a suitable scaffold for bone formation as well as the ability to bind and concentrate endogenous BMPs[195, 228]. These surfaces has however, been used with caution clinically[229] due to observed degradation of coatings and proposed macrophage-induced resorption found experimentally[230] as well as the more long term findings between HA and machined surfaces from the same group, where the machined surface displayed a significant higher percentage of bone around the implants installed in a rabbit tibia[231]. To deal with the problems of this surface, otherwise regarded as interesting, a new and ultra-thin (0.1 μm) surface modification with crystalline $CaPO_4$, has been introduced and has drawn attention as a promising new surface modification. The intact interface between this surface and bone has recently been illustrated for the first time by a new method of focused ion beam microscopy[232].

Integration of titanium implants in bone and autogenous bone grafts

The ability of integration of dental titanium implants into bone is almost common knowledge and has been published on extensively[2, 3, 233-238]. Implantology of today is so successful that some researchers have focused on finding clues to explain failures and try to identify the factors that may influence the course of osseintegration of implants[201, 202]. The interface between the surface and surrounding tissues of an implant is not yet fully explored and continues to attract attention from scientists that can present refined methods of interface studies[232].

Pioneering work was done by the groups of Brånemark[2] and Schroeder[3]. The "direct bone-to-titanium contact" found by the Göteborg group of Brånemark, further initiated studies by Albrektsson et al.[239] of the interface zone. Important work in this field was done by Sennerby and co-workers[240], showing fast formation of woven bone originating from the surrounding endosteal bone surfaces towards the surface of the rabbit, tibial implant. Remodelling of the interface was found to be near completion in about 90 days in this species. Sennerby et al.[241] also addressed the differences of implant healing characteristics of cancellous and cortical bone and found a correlation between the amount of cortical bone and biomechanical properties with removal torque measurements. Studies[239, 242, 243] revealed that the thin, separating zone between the implant and the bone was mineralized in some and unmineralized in other parts and generally less than 500 nm in width. These studies indicated a mechanically built stability, rather than a true bond between the bone and the implant surface. With rougher surfaces the bone to implant contact has been proven to increase with a peak at about double the roughness of the original Brånemark implant [212, 216]. Studies of retrieved implants from humans has been performed by Albrektsson et al.[236] and Bolind et al.[244]. The latter study demonstrated a lower percentage of bone-to-implant contact for unloaded compared to loaded implants. The possibility to achieve bone anchorage of clinical implants in irradiated tissue was also studied by this group[245]. Implants with longer healing times demonstrated the possibility of achieving integration even in this compromised patient group.

Franke Stenport[246] experimentally studied the effects of GFs (i.e. growth hormone, enamel matrix derative, fibroblast factor-4s or bone morphogenetic protein-7) used in conjunction with press fit titanium implants installed in bone in bone of rabbits and dogs. Results suggested that GFs was of little use in the ordinary clinical situation and that the additive effect of these GFs on bone formation during healing were low.

The work of Sul and co-workers has led the interface studies into a new era with the suggestion of a hypothesis of a biochemical bonding instantly starting between bioactive implants and bone and secondly, a mechanical interlocking developing over time[247]. Magnesium ion incorporated, oxidized test implants were compared to machined, turned implants in a rabbit tibia model. The results for the test group were interesting with faster integration and stronger bond to bone compared to controls. The mechanical tests showed breakage of the bond for test implants in bone as compared to the controls, where the interface between implant and surface was the weakest point, tested with removal turning forces to unscrew the implant out of the bone[248].

Regarding integration of implants in bone grafts, important findings has been made with the use of test micro implants inserted in grafted autogenous bone[7, 249-251]. In an elegant study by Lundgren et al.[249], three healing periods of inserted micro implants into autogenous block bone could be evaluated. At grafting, two micro implants were placed and after six months of healing, one was taken out with the surrounding bone by a trephine burr. Simultaneously, another additional micro implant was placed, leaving two implants to integrate

until re-entry at installation of abutments at 12 months after bone grafting. The histological findings regarding the micro implants supported ($P<0.05$) a delayed insertion of implants in the grafted bone, with respect to values of degree of BIC, bone area within implant threads and newly formed bone of total micro implant specimen area.

Wannfors et al. tested the 1-stage procedure (simultaneous block bone graft and installation of implants in the floor of the maxillary sinus) versus the 2-stage procedure (particulated bone in the maxillary sinus floor left to heal for 6 months) of sinus inlay grafts. 20 patients in each group received 76 and 74 machined implants, respectively. Additional 72 and 76 implants were placed in non-grafted bone in these patients groups, respectively. The results demonstrated a significantly lower risk for failure in the non-grafted sites and twice the risk for failure in 2-stage procedures compared to if the implants were placed at a second stage in a healed particulated bone graft. However, Johanssons'[252] thesis concluded that predictable results could be achieved, regardless of 1- or 2-stage grafting procedures, regarding implant and bridge stability in his study group of maxillary edentulous patients.
A recent study[253] of retrieved implants from grafted patients, illustrated that bone grafts are remodelled only after a long time, in that the specimens evaluated showed a variation of viability regarding cell content in osteocyte lacunae. Furthermore, loaded implants disclosed a higher level of bone-implant contact than unloaded implants.

The concept of platelet-rich plasma in reconstructive surgery

Reviews of PRP in the literature

The role of platelets in tissue wound healing has been described previously in this text, but is also reviewed by Anitua et al.[254] and Soffer et al.[255]. Further reviews on the subject are available; some enthusiastic[254, 256-259] and some more critical[59, 255, 260-263]. PRP was introduced primarily by Tayapongsak et al.[5], Whitman et al.[264] and Marx et al.[4] to the maxillofacial and dental community. Other reviews of platelet-rich plasma in different medical fields and sciences are available, such as in foot and ankle surgery[265, 266], knee surgery[267], spine fusion surgery[268] thoracic and general surgery[269, 270]. Other fields where autologous platelets have been used are in plastic surgery[271, 272], healing of skin and diabetic ulcers[273-275], eye surgery[276] and in sports medicine with tendon and ligament repair[277, 278]. Grageda[261] pointed out the need of intensive R&D and suggested standardized research protocols in a review, evidently feeling frustration over the diverging results and methods used to evaluate, in this case, PRP and bone grafts.

Background

In haematological and immunological research, PRP and platelet-poor plasma (PPP) has been explored for about 50 years[279]. The clinical use of PRP has evolved from the idea of fibrin glue (FG) [280, 281], which is used to control bleeding and adhere and seal tissues together during surgical interventions. The role of fibrin has recently been complemented by findings that it acts as a protector of the clot, preventing leukocytes from degrading it prematurely. FG can be prepared from platelet-rich plasma or by mixing concentrated fibrinogen solutions with thrombin. In the literature, the addition of platelets to this preparation has evolved into different terms, such as PRP, PRP-gel, platelet gel, PRP-clot, plasma rich in growth factors (PRGF) and eventually in a modified concept of platelet rich fibrin (PRF)[282-287]. FG can be prepared through fibrinogen precipitation (separation by centrifugation of blood elements and concentration of fibrinogen), cryoprecipitation (fibrinogen is concentrated from plasma by freezing and thaw cycles) or by different added chemical to precipitation (ammonium sulphate, ethanol or polyethylene glycol). There has been a wide range of applications described for FG, similar to areas where PRP has been used, including fracture repair, tendon repair, nerve sealing, bone grafting and in spine surgery[280].

The proposed benefits of PRP in clinical use (sinus grafting as model) was recently reviewed by Boyapati et al.[59]. PRP is proposed to improve handling of particulate grafts, facilitate graft placement and stability, improve rate and quality of vascular in-growth, increase bone regeneration, enhance soft tissue healing and present mitogenic effects on necessary cells. It is an inexpensive and readily available source of growth factors and a natural biologic sealant without the risk of disease transmission.

Preparation of PRP

Based on the separation technique with centrifugation of whole blood used in haematology, PRP can be prepared today in the operating room during surgery. Blood is drawn from a large peripheral vein of the patient, which is anti-coagulated with citrate phosphate dextrose (CPD) and differential centrifugation is achieved using a first "hard spin" that separates the PPP from the RBCs and PRP. The second spin, "soft spin", then separates the RBCs from the PRP. The components in blood with the highest specific gravity are found on the bottom of the container/ tube filled with plasma. The platelets are found in a small "pellet" that has to be dispersed out evenly in the plasma before use. Calcium chloride and thrombin are subsequently added for re-calcification and initiation of clot formation.

Devices and validation

Devices used today are small, table mounted centrifugation appliances[288-290], where different amounts of PRP can be collected[267, 291-301]. Early use of PRP was based on preparation of PRP in advance with help from the haematological laboratory in the hospital[4, 5, 264, 302]. Of the devices used in maxillofacial reconstruction, there have been studies regarding the result of the processes for attaining the PRP regarding platelet count[289, 290, 303-305] and growth factor content[297, 298, 302, 306-308]. The obvious risk in preparation of the PRP is that the platelets are prematurely activated during the process.
Work from Weibrich et al.[302, 307, 309, 310] disclosed more facts about PRP content than only platelet counts, also previously explored by Landesberg et al.[297]. In these studies, they evaluated GFs contents and levels in different preparations[302, 307, 309, 311] and also explored differences of sex and age[310]. Generally, PRP showed variations in growth factor levels between individuals, but not depending on age or sex [310].

Using a rabbit model, Weibrich et al. tried to explore the optimal concentration of platelets needed to regenerate bone in vivo and found advantageous biological effects of PRP with a platelet concentration of approximately 1,000,000/µl[312]. Okuda et al.[298] in 2003, found a correlation between platelet count and GF levels of PDGF and TGF-β1 in their PRP, which was an important finding for further research. A recent study by Leitner et al.[289] validated the efficacy of four available systems and regarded the compared systems as efficient and functioning in gaining high platelet counts and subsequent release of PDGF over 5 days. The correlation between platelet counts, GF levels and tissue generation can probably be regarded as the key area of later years research, effectuated in different models[313, 314].

Studies on PRP

The effects of PRP have been evaluated in many different ways, but with conflicting results. The lack of evidence for the efficacy of PRP from the first human and animal studies and low scientific value of many studies[59] have challenged researchers to leave human and animal studies for more basic research and in vitro experimental models. To review the attempts of evaluation of PRP during the last 8 years, since the article by Marx et al.[4] was published in 1998, is almost an impossible task. The search term "platelet-rich plasma" alone generates close to 4000 articles on PubMed, but this number includes the research on platelets over the last 50 years as previously mentioned[315]. However, to comprehend the accumulated papers published and available in English in abstract form or as a whole paper, they must in some way be divided into different categories. In this thesis, papers are primarily placed in categories of experimental (including "tissue engineered bone"), animal and human studies, and thereafter in subgroups of e.g. cell-lines, animal species or locations such as sinus grafting in humans.

Experimental in vivo and in vitro studies

If the GFs of PRP can be considered to be a natural mix in proportions, resulting in favourable interaction between each other and on regeneration on hard and soft tissue, is the question to be answered. PRP application in cellular *periodontal research* has been studied extensively by different groups of researchers[298, 316-318], investigating the influence of PRP on osteoblastic and epithelial cells, gingival fibroblasts and periodontal ligament (PDL) cells. It has been proposed that PRP modulates cell proliferation in a cell specific manner. The effects of PRP were similar to the effect of TGF-β in one study[298]. In this study, it was also shown that PRP stimulated DNA synthesis and increased cell division of osteoblastic cells, but in contrast, suppressed epithelial cell division. PRP also stimulated DNA synthesis in gingival fibroblasts and periodontal ligament cells.

Levels of PDGF-AB and TGF-β1 in PRP were evaluated by enzyme-linked immunosorbent assay (ELISA) kits and found in high amounts [298]. The same group studied gel formation and collagen synthesis in cultures of PDL or osteoblastic MG63 cell cultures after addition of PRP. PRP changed cell shape and up-regulated type I collagen synthesis in the extracellular matrix[319] of the cells. Furthermore they found some unidentified factors in PRP that seemed to increase ALP activity in human PDL cell cultures[317]. Finally, they found promotion of mineralization in rat PDL cells by the addition of PRP in an osteogenic medium[317]. These findings and effects combined were explained to mediate the ability of PRP to promote regeneration of periodontal and skeletal tissues.

Furthermore, Annunziata et al.[320] studied the effects of incubation of PRP on primary human periodontal ligament cells, gingival fibroblasts and keratinocytes. Growth stimulation of PDL cells over time was shown, as well as an increase in ALP activity and type I collagen formation. PRP also enhanced proliferation of human gingival fibroblasts and inhibited growth of human keratinocytes by 40%. The same group found significant value of using PRP in preclinical expansion of bone marrow stromal cells (BMSCs) preceding transplantation and bone engineering[321].

Osteoblastic cell lines and PRP

Kanno et al.[322] evaluated the proliferation and differentiation effects of PRP on two human osteosarcoma cell lines (HOS and SaOS-2). These cells were suppressed during initial growth by the presence of PRP (measured by ALP activity), but when cells were matured to more osteoblast like cells, ALP activity was enhanced. Semi quantitative reverse-transcription polymerase chain reaction analyses showed a favourable effect of PRP on collagenous matrix production, in that PRP enhanced the levels of procollagen type I, osteopontin, osteoprotegerin, and core binding factor alpha 1 (cbfa1) mRNA (a regulator of osteoblast differentiation)[322].

Ferreira et al.[323], could confirm that PRP promotes osteoblast proliferation when they studied increasing concentrations of PRP, with or without bovine fetal serum, on human

fetal osteoblast (hFOB1.19) cell proliferation. Frechette et al.[306], also using hFOB1.19 and additional human umbilical vein endothelial cells (HUVEC) in their model, found strong promotion of cell division in these cells from the supernatants of activated PRP. Furthermore, ELISA assays indicated that GF concentrations varied between individuals. Previous studies by this group has pointed to that calcium and thrombin regulate growth factor release[324, 325], with the higher concentration of $CACl_2$ (14.3 mg/ml) and thrombin (142.8 U/ml) tested giving the most powerful degranulation and GF release[325].

The human osteoblastic cell line SaOS-2 has receptors for PDGF-β and TGF-β, as shown in 2006, by Celotti et al.[326]. By blocking these receptors with antibodies (s.c. immunoneutralization) they were able to show the specific role of these growth factors (PDGF-β stimulating cell migration and TGF-β inhibiting cell proliferation) and that activated PRP added in different concentrations to SaOS-2 cells resulted in chemotaxis and cell proliferation. They also concluded that the actions of PRP on these cells were dose dependent, a fact also confirmed (2006) in a similar study by Ogino et al.[327]. Ogino et al.[328] previously found that cultured rat bone marrow cells are stimulated by PRP regarding proliferation, but suppressed with respect to differentiation. In turn, their results have been confirmed by Dolder et al.[314], whose results showed that PRP stimulated initial cell growth, however had no effect on ALP activity (as an indicator for differentiation).

Interestingly, Graziani et al.[329] recently showed that different concentrations of PRP preparations exert a dose-specific effect on cell proliferation in human cultured oral fibroblasts and osteoblasts. Optimal results were observed when platelet concentration increased 2.5 times from basic platelet count. Higher concentrations (maximum 5.5 x concentration) reduced proliferation and osteoblast function.

In a series of studies where human trabecular bone-derived cells and the osteogenic cell line MC3T3-E1 were used, Gruber et al.[330-332] could show that PRP increased migration and proliferation but decreased osteogenic differentiation in these cells in vitro. They also saw that the differentiation of osteogenic cells in the presence of BMPs was temporarily suppressed by PRP and recommended that this fact must be taken into account in future regeneration strategies.

Is there evidence of better angiogenesis with PRP?

A particulated bone graft with the addition of PRP is well vascularised and homogenous in bone density, which clearly can be seen during e.g. implant installation[6, 8, 57]. Endothelial cell proliferation has been found to be increased in cellular research under the influence of PRP[306, 333]. Kilian et al.[334] found in a pig defect model, that hydroxyapatite enriched with platelet growth factors (PLF) led to an up-regulation of VEGF-receptor 1 synthesis.

Histologically, they found some enhancement of new vessel formation with higher sprouting activity compared with HA without PLF filling defects.

Lucarelli et al.[335] found that PRP enhanced the proliferation and expansion of human stromal stem cells to osteoblasts and that they, after the influence of PRP were withdrawn, could start mineralize the matrix. These cells, together with PRP, looked upon as potent providers of VEGF, were investigated further to evaluate if this combination was able to increase allograft of collagen integration in a defect model in sheep[336]. Collagen only was used as control. Histomorphometric analyses, including studies on new vascularisation, showed substantial new bone formation and vascularisation in the combination allograft study group.

Anitua et al.[337] used PRP and tendon cells in culture and found enhanced proliferation of the cells, and could conclude that synthesis of VEGF by tendon cells was significantly higher for supernatants from PRP than from PPP. Using a gastric ulcer model for studying healing mechanisms, Wallace et al.[338] found that the accelerated healing induced by platelets could be reversed by immunoneutralization of VEGF and thus concluded that the presentation of VEGF by platelets at the site of injury is crucial in the healing process.

Evidently there is data accumulating supporting the positive effects of PRP on vascularisation in healing processes in different models.

Further findings in vitro and in vivo systems

Brodke et al.[339] used demineralized bone and cancellous chips (DBM-CC) enriched with osteoprogenitor cells and clotted with or without PRP, to heal canine femoral critical-size segmental defects and evaluated this with histology and radiology (micro-CT). Autologous bone grafts were used as controls and the result disclosed that DBM-CC and osteoprogenitor cell enriched grafts were comparable to autologous grafts. PRP added a non-statistical positive effect to the DBM-CC and osteoprogenitor cell enriched grafts compared to the same grafts without PRP. However, Dallari et al.[340] recently showed that the combination of bone marrow stem cells, freeze dried bone allograft and PRP permitted acceleration in bone healing and bone remodelling processes in a critical size defect on rabbits with a healing period of 2 weeks.

PRP and artificial implantable materials, have been tested with our without cell cultures, such as collagen sponge[341-343] and gelatin hydrogel. Pryor et al.[342, 343]found no effect of PRP, soak-loaded onto an absorbable collagen sponge (ACS) on bone formation in a rat calvarium defect model. Apornmaeklong et al.[341] used a collagen three dimensional rat marrow cell culture system to describe the effects and differences of action regarding PRP and rhBMP-2. Again it was shown that PRP enhances cell proliferation and suppresses differentiation and that rhBMP-2 has the opposite effect, inducing high ALP activity and mineral deposition; something Soffer et al.[344] could observe when seeing alterations in the effect of PRP on cultured bone cells over time. Low concentrations of PRP was, furthermore, found to

stimulate the viability of alveolar bone cells in vitro, whereas high concentrations of PRP in the culture medium suppressed the proliferation of cells[345]. The release of GFs PDGF and TGF-β has also been shown to be pH-dependent in that no activation was found at pH 5.0 but an activation was found at normal and alkaline pH[346]. Luengo Gimeno et al.[347] found that a 5% CaCl concentration (tested range 5-50 %) was most effective in activating PRP in a rabbit model. Using the rabbit ulna as model, Hokugo et al.[295] used gelatin hydrogel as a carrier for GFs in PRP and found release of GFs as the hydrogel progressively degraded. The effects of the tested combination PRP/gelatin carrier on bone formation in a rabbit ulnae defect model was significantly greater than the control defects of fibrin incorporating PRP, empty gelatin hydrogel, and free PRP.

The effect of PRP on osseointegration has been addressed after topical application, or site preparation with PRP[312, 348-352], as well as peri-implant defect studies involving PRP[87, 353-356]. Bone defects in different species have been supplemented with PRP and then grafted with autologous bone [357-361] and allologous bone [334, 336, 362-372]. Extraction socket defects was filled with allograft material[373]. Resection gaps in mandibles and long bones have been grafted with autogenous bone and allografts in combination with PRP[374-378]. Sinus augmentations, recently reviewed by Boyapati and Wang[59], have been performed and evaluated[294, 379-385]. PRP has further been used in distraction osteogenesis as an adjuvant[386]. Furthermore, PRP has been tested for bone forming activity in extra skeletal sites, alone and in combination with other products[368, 387, 388] and also tested as a delivery model for BMP[389]. Interesting models including PRP, e.g. with autogenous grafting in irradiated bone[357] and fracture healing in diabetic mice [390] have also been used for evaluating the effects of PRP.

PRP with implants

Fontana et al.[351] found a higher amount of peri-implant bone volume after inserting PRP and laminar test implants into rat tibial sites. Fuerst et al.[348] defined platelet rich in growth factors (PRGF), as the supernatant from washed, thrombin-activated, allogenic, platelet-rich plasma obtained after centrifugation, and used this solution with implants with success to increase BIC. Zechner et al.[352] also advocated the benefits of topical application of implants before insertion, and saw effects in early healing. On the other hand, Weibrich et al.[312] failed to see any benefit on BIC from PRP in their trial in rabbits. In a recent study[391], 18 titanium non-coated AlO grit-blasted and acid etched, and 18 magnetron-sputtered calcium phosphate (CaP) coated titanium (1 μm thick layer of CaP) implants were placed in trabecular bone in the femoral condyles in six goats. PRP was used as a test modality, as some implants were dipped in PRP before insertion and other implants were placed into sites where PRP-gel had been previously inserted. Results and conclusions were that CaP coated implants rendered

higher values for BIC than the rough implants used as control. No additive value for PRP was found except at an early healing stage.

Peri-implant defects and PRP

A general extensive literature overview on marginal defects around implants has been presented by Boticelli (2006)[392]. Yamada et al.[356] found similar results of tissue engineered bone and autologous bone after grafting and installation of implants in two stages. BICvalues for implants were comparable between the sides in that study. Kim et al.[87, 349] used freeze-dried bone and dentin-like plaster of Paris material as filler material with and without PRP and found better results with respect to BIC with the addition of PRP.
Sanchez et al.[354, 355, 393] failed to see more than a low effect of PRP combined with demineralised freeze-dried bone in 3-walls defects in the mandible of dogs. In another study by Casati et al[353], a buccal dehiscence implant defect model was used in dogs and the defects were randomly filled with PRP or blood as control. No effects on regeneration of that type of defect could be seen. Ito et al.[394], used precultured dog mesenchymal stem cells (dMSCs), attained via an iliac bone biopsy 4 weeks before implantation of the cells, together with PRP and fibrin in a peri-implant defect. They concluded the experiment successful with BIC of 25%, 49% and 53% after 2, 4 and 8 weeks, respectively, when using this combination.

Autogenous bone and PRP in defects

Tissue engineered bone, as described above using dMSCs and PRP, was also used by Ito et al.[361] in defects in dogs and was compared to autogenous bone, bone substitute (Bio-Oss®), PRP only or unfilled defects as control. Histology and mechanical properties were investigated and the tissue engineered bone was here superior with well matured bone and better hardness test values than the other groups and the control after 2 weeks.
Results in PRP research are conflicting and that may be illustrated by studies of Choi et al.[359] and Gerard et al.[360]. Choi et al. saw a delayed remodelling in autogenous bone grafts with PRP in dog mandibles compared to grafts without; Gerard et al. saw early effects (up to 2 months) on graft healing, also in a similar set up in dog mandibles. They explained the early enhanced healing by increased removal of the non-viable grafted bone and increased amount of new bone formation. Furthermore, PRP was said not to change the rate at which new bone was formed.

Earlier, in 2002, Aghaloo et al.[358] found no improvement in adding PRP to autogenous bone grafts in a critical size defect in a rabbit calvarial defect model. In contradiction, the same group later found a positive effect of PRP and Bio-Oss®; but in another study they failed to show a radiographic or histomorphometric increase in bone formation with the addition of PRP to either FMB or FDDB in non-critical-sized defects in the rabbit cranium[395, 396].

Allografts and PRP in defects

The released GFs from platelets in PRP need receptors on target cells to execute their actions, as stated earlier in this text. Despite this fact, many bone substitutes have been used together with PRP and the results have varied.
Positive effects were found in two studies of PRP together with β-tricalcium phosphate (β-TCP) in a dog defect model[370, 371]. New bone formation was accelerated and the authors recommended the use of PRP together with β-TCP. Yazawa et al.[362] could confirm an enhanced effect by PRP on the healing of β-TCP in a bicortical calvarial defect model in rabbits. In extraction sockets in dogs, Suba et al.[373] used β-TCP allografts (Cerasorb®) as filler and found en enhanced effect on bone healing with PRP.

Recently, this positive effect on healing could not be repeated in a rat calvarial defect model with a PRP/HA/β-TCP-filling of defects, examined after 4 weeks [369]. Roldan et al.[368] and Wiltfang et al.[363] both failed to see any positive additive effect from allografts and PRP on bone healing in defect models of rats and mini-pigs, respectively. However, the latter study confirmed the early effects of PRP on healing, when combined with autogenous bone in the defect. Schlegel et al.[367] observed *early* accelerated healing after 2 weeks from addition of PRP to autogenous bone, in a defect model in the forehead of adult pigs. Collagen was also tested as a grafting material with or without PRP, and surprisingly, collagen alone was found to reach higher mineralization values than autogenous bone after 4 and 6 weeks. The authors could also, in summary, see that the differences had levelled out after 12 weeks of healing in all groups. Fuerst et al.[372] grafted collagen type1 to mandibular defects in mini pigs and added PRGF to collagen in the test sites. The analysis revealed an inflammatory delay in bone healing in the test defects and a better result was therefore found with collagen only.

Thorwath et al.[397] (2006) found that PRP modulates the sequential expression of bone matrix proteins in a study in calvarial defects in pigs. Collagen type 1 expression was enhanced in defects grafted with autogenous bone, +/- PRP after 2 weeks, compared with defects filled with +/- PRP and deproteinized bovine bone matrix (DBBM). Osteopontin and osteonectin were enhanced in expression in nearly all groups, for autogenous bone as well as for DBBM, after 4 weeks. As a third step in the mineralization process, as the authors resembled the bone matrix expression with de novo bone formation, osteocalcin expression was at its peak at 12 weeks for autogenous bone. PRP was shown only to statistically enhance bone formation in the autogenous bone group after 2 weeks in the higher concentration (6.5 x increase from whole blood, vs. the group with 4.1 x increase) of PRP and seemed to suppress formation after 4 weeks. The lack of osteoinductive capacity for both PRP and DBBM was thereby demonstrated, as reported by these authors.

Lucarelli et al.[336] added stromal cells to bone allografts (collagen) and PRP and found a positive effect in this set up. Collagen was used as control in these defects made in the

metatarsal bone of sheep. These authors also correlated the better graft incorporation with better vascular in-growth in the defects.

Resection-gap defects with PRP

A positive result from 2002 by Fennis et al.[374] reported in enhanced bone formation in autogenous bone grafting with PRP in a goat mandible resection-defect model. This first report included radiology and clinical follow-up, but the next study[375], confirmed the results with histology. In an additional study[376], a radiated autogenous scaffold for carrying the particulated bone was found to negatively influence the result of graft incorporation.
Sarkar et al.[378] found, in a tibial diaphysis defect model in sheep, that PRP and a collagen sponge was not enough to heal the defect differently than the control of collagen alone.

Resection-gap defects with fibrin glue

Thorn et al.[398] reviewed and described the preparation of autologous fibrin glue made preoperatively from PRP of the patient in the blood bank. A concentrated fibrinogen solution together with thrombin, also prepared from the PRP of the patient, reached 12 times higher levels of fibrinogen and 8 times higher concentration of GFs in the fibrin glue compared to PRP. They reported successful use on eight patients but supplied no controlled data in this report. A resection gap dog study by Huh et al.[377] supplied evidence of significantly better particulate graft incorporation by the use of this fibrin glue.

Sinus inlay studies with PRP

Grafting of the maxillary sinuses is commonly performed as an augmentation procedure in patients, and animals have been used to compare effects of grafting material with PRP. Since split-mouth studies are elegantly performed in this set up, with two independent sides and the animal being its own control, several studies have been undertaken. Boyapati and Wang[59]reviewed these studies and concluded critically that "drawbacks of the current body of literature included inconsistent study designs, absence of documentation regarding platelet yields, and the use of animal models with questionable clinical application". The studies were divided into positive[379, 384] and negative reports[294, 380, 381, 385] on PRP-efficacy. The positive reports were performed in pigs and rabbits and revealed in the first study, a transient effect of PRP in bone healing and no effect of PRP in combination with HA[379]. The second study found that MSCs and PRP reached similar results as PRP and autogenous bone, but unfortunately did not include any negative controls to the tested methods[384].
Jakse et al.[381] and Butterfield et al.[294] used autogenous iliac crest bone and PRP in a controlled split-mouth set up in sheep and rabbits, respectively, but failed to see any differences. Grageda et al.[380] used bone allografts and PRP without success in a sheep sinus model and

Roldan et al.[385] found better results for the control side with rhBMP-7 than for the test side with a mix of autogenous bone, Bio-Oss ® and PRP. Recently in 2006, Klongnoi et al.[382] found no additive effects from PRP used with fluorohydroxyapatite (Algipore®) and a bioglass (Biogran ®), in a simultaneous implant/sinus lift setting. BIC was evaluated and no differences were found over time compared to negative controls without PRP.

Other findings regarding PRP in animal models

Jung et al.[389] found non-significantly higher amount of newly formed bone in calvarial defects of rabbits, filled with PRP in comparison with fibrin or blood. Fibrin and PRP were tested as delivery models for rhBMP-2 and these combinations increased bone formation considerably in the defects.

Calvarial bone of sheep was used in an experiment of distraction osteogenesis[386]. The study revealed that effect of PRP was shown when immediate distraction of the bone segment took place and distraction was started immediately after application of PRP in the distraction gap (latency period of 0 days instead of 5 days).

PRP probably lack the ability of ectopic bone formation in extra skeletal sites as shown by Roldan et al.[368], but may be an additive in combination with other materials in advanced tissue engineering experiments of abdominal implants in dogs[388]. Decreased expression of PDGF, βFGF, and TGF-β was seen by Aghaloo et al.[357] in radiated rabbit tibia. The same GFs were expressed significantly more when PRP was added to autogenous bone grafting in a rabbit cranial defect. This fact was argued by the authors to be a way of improving graft incorporation in radiated compromised sites in patients.

To study effects of improved healing in diabetic rats, PRP was percutaneously delivered into the experimental fracture site. Gandhi et al.[390] found that cellular proliferation and chondrogenesis were improved as was the end result of healing at termination of the test as shown by improved mechanical strength of the healed bone in the fracture sites.

Human studies on PRP

The general impression when reviewing the concept of PRP is that results vary whether there is support for PRP efficacy or not. Grageda[261] proposed a standardized research protocol including quantification of platelet yield in whole blood and PRP, quantification of GFs with available assays and the use of contra lateral control groups and histomorphometric specimen analysis. The lack of correlation studies between GF levels and histomorphometric analysis results has also been discussed[59]. The human studies reviewed below are reported in a similar way as were the animal studies. PRP has been studied extensively in human models. Unfortunately, many papers lack controls and are merely case reports and will not be reviewed here.

Periodontal defects and PRP

Periodontal defects have been resolved with guided tissue regeneration (GTR) by the use of membranes. Bovine porous bone mineral (BPBM) and PRP has been used together with GTR and thereafter evaluated by several authors[399-402]. However, the effect of PRP is difficult to establish, as all studies show good effects of healing of the defects in almost all groups irrespective if test or control. Ouyang et al.[403] experienced additive effects with PRP to BPBM in intrabony defects in a small group of patients. Okuda et al.[318] used HA and PRP, versus HA and saline and found better regenerative effects in the test group of patients after 1 year. In the treatment of gingival recession defects with a coronally advanced flap, no effect of PRP was found by Huang et al.[404].

Simon et al.[405] used PRP in a study on 14 patients after removing their mandibular 3rd molars on one side and randomly assigning them to either test or control group with 7 patients in each group. PRP was inserted into the extraction sockets after removal of teeth. Clinical parameters such as mouth opening, use of analgesics and swelling were evaluated, despite these being of unknown relevance with respect PRP efficacy. However, by radiography better bone formation was observed already after 1 week, an observation that together with other favourable evidence of soft tissue healing made the authors recommend the use of PRP in this clinical situation. However, the relevance of this paper is however questionable since no histomorphometric evaluation was made.

Sammartino et al.[406], in a somewhat better designed study in 18 patients, surgically extracted bilateral mesioangular positioned 3rd molars, that preoperatively had a probing depth (PD) $\geq$ 7.5 mm and a probing attachment level (PAL) of $\geq$ 6 mm. They used PRP in the wound in half of the sites in a split mouth design. With PRP, results showed a significant ($P<0.05$) reduction in PD and PAL for the test sites. Unfortunately, only re-entry procedures with bone biopsies were performed in sites with PRP and no data on bone formation, other than "considerable observable bone regeneration", was reported.

PRP with implants

Based on a rather small amount of observations, Anitua[407] reported that he saw positive effects on bone integration and adjunctive soft tissue healing in patients after introducing PRP into implant sites before installation. Monov et al.[408] used a split mouth setting, where PRP was inserted into the implant site in the posterior mandible before placement of implants. The same procedure was performed in the other site, but here no PRP was added. The implants were followed with RFA from installation up to 44 days. No differences between sides were seen. Additionally, the authors reported that the values of implant stability quotient (ISQ) between days 0 and 4 were reduced significantly in both groups.

Sinus augmentation and PRP

The posterior edentulous maxilla has been a problematic region for installing implants due to its limited amount of remaining bone. The initial reports on the decreased healing time for bone grafts with PRP[4, 407] and the morbidity found in autogenous grafting[409, 410], has set off many investigators to report on sinus augmentation and PRP. However, most of the papers are case report series without controls[411-416].
More confounding is the variation of allografts [408, 411, 412, 414-419] that has been used together with PRP. Raghoebar et al.[420] stated that the use of allografts in sinus augmentations with PRP as an adjuvant was not likely to show any positive results as the GFs in PRP lacked cellular element to act upon. Therefore, researchers have also included autogenous bone for cellular supply[408].

Wiltfang et al.[421] studied the effect of PRP to enhance bone formation and resorption of β-TCP (Cerasorb®, ceramic granules of 1000-2000μm) in sinus augmentations in 39 patients. Randomization resulted in 17 sinuses that were included in the test group with β-TCP and PRP vs. 18 sinuses with β-TCP alone as control. 10 more sinuses were grafted with the test protocol but were excluded from the study because of sinus membrane lacerations and low concentration factor of PRP (<3). Mean concentration factor of PRP was 4.1. According to the authors the bone formation in the test group was 38% (mean, range 32-43%) and 29% (mean, range 25-37 %) in the control. No difference in degradation of β-TCP was seen between the groups. The authors concluded that PRP was not beneficial in sinus augmentations if target cells such as osteoblasts or osteocytes are not present.

Raghoebar et al.[420] succeeded in performing a small but well designed study of PRP in combination with autogenous bone grafts from the iliac crest in 5 patients and a randomized split-mouth design. With a trephine, 3 months after reconstruction, bone biopsies were harvested and evaluated with microradiographs and histomorphometry. TGF-βlevels were not altered by the PRP procedure as tested in serum before and after the procedure. No effect on addition of PRP could be seen in the analysed bone biopsies after 3 months.

Oyama et al.[422] used autogenous grafting with PRP in cleft patients and had a group of patients grafted with autogenous bone and fibrin glue as control. They concluded by CT scan evaluation on a PC that PRP added bone grafts had a significantly better volume after 5-6 months. Their included number of patients was small, so conclusions must be regarded with caution. The group of Ueda and co-workers[423-425], reported on tissue engineered bone and implants and proposed beneficial effects of PRP in extra-corporal expansion of MSCs from dogs, and they have furthermore transplanted injectable bone (MSCs, PRP and β-TCP) into human sinuses and alveolar clefts. They concluded that PRP has a role in this form of translational research.

PRP in larger bone defects.

Marx et al.[4] reported on 88 patients with mandibular continuity defects who were randomized for treatment with autogenous bone grafts with or without the addition of PRP. At baseline, a monoclonal antibody study indicated that the sequestered platelets and the particulated iliac donor bone matched each other, showing levels of TGF-β and PDGF present in the PRP preparation and receptors positive for TGF-β and PDGF in the bone graft. After the final consolidation time of 6 months, dental implants were placed with at least one implant per grafted area. This allowed for obtaining cores of bone for histomorphometric analyses. Monoclonal antibody staining confirmed the presence of TGF-β-positive but not PDGF-positive cells. The histomorphometric analyses revealed more trabecular bone area (TBA) in the PRP group (74, 0±11 %) compared to the bone graft group without PRP (55,1±8 %) and the native mandible (38,9±6 %). Furthermore, panoramic radiographs were used to subjectively evaluate maturation of the grafts after 2, 4 and 6 months. When comparing test and control groups, the authors observed a twice more rapid maturation of the PRP treated defects.

Merkx et al.[426] reported on 8 patients with malignant mandibular tumours, where six of the patients were successfully reconstructed by this concept with reconstruction plates bridging the resection gap. Two additional patients were reported on; one died and the other had an unsuccessful graft with only fibrous tissue formed in the resection. In a consecutive report from this group[427] on 11 patients with ameloblastomas that were removed through a resection osteotomy, 6 of the cortical parts of the mandible were used as a cortical scaffold after 50 GY of radiation and replaced together with particulated autogenous bone and PRP. The other 5 patients had titanium plates and bone to reconstruct their mandible defects. This immediate reconstruction procedure rendered satisfactory results assessed by a quality of life study from the 7 patients that were free from complications. The other 4 patients (all some of the first in the series) in the study had problems postoperatively, e.g. fracture of the scaffold and infections, indicating a learning curve of the technique.

A recent report of preliminary data on the use of PRP in non-union cases after orthopaedic longbone trauma cases, states that the use of BMP-7 is far more effective than PRP in a non grafting protocol[428].

Robiony et al.[429] combined PRP and autogenous bone in a vertical distraction gap after a latent period of 15 days, and successfully distracted 3 patients for later placements of implants. Kitoh et al.[430] used marrow-derived MSCs and PRP to enhance distraction in femora and tibiae of patients.

The effects of the PRP concept have been extensively evaluated in different models. Indeed, conflicting results have been reported both from human trials and animal studies. Some results point to the need of target cells for GFs in PRP. Moreover, the effect in humans may be beneficial only in early healing of bone and possibly soft tissues. Researchers have tended to use more differentiating basic experimental models, e.g. cell cultures, and found effects on early events as cell proliferation and mitogenic effects. A lot of clarifying scientific work has been published since the introduction of PRP in reconstructive surgery. However, there is further need for experimental and clinical studies on the interactions of GFs in tissue healing.

Also, controversy exists with respect to the clinical use of combining PRP with bone grafts for enhancement of bone healing. Furthermore, contradictory results on the use of PRP in combination with dental implant surgery exist. Therefore, these controversies inspired me to the investigations presented in this thesis.

Containers with activated PRP and autogenous particulated bone.

AIMS

The aims of the present thesis were

1. To evaluate if PRP in conjunction with grafting of particulated autogenous bone to the edentulous maxilla could improve the integration and clinical function of dental implants in edentulous patients.
2. To evaluate the degree of new bone formation in grafted human maxillary sinus biopsies with and without PRP and micro-implants installed after 3 months of healing and retrieved after additional 3 months of healing.
3. To evaluate the thrombogenic properties of whole blood and PRP in contact with titanium.
4. To evaluate the thrombogenic properties of whole blood in contact with modified titanium surfaces
5. To evaluate a simultaneous implant installation with sinus elevation procedures without the use of grafted bone or bone substitutes.
6. To evaluate the bone regenerative capacity of PRP using whole blood as a control in a peri-implant defect model in dogs.
7. To evaluate the effect of a fluoride surface modification in the bone healing of a peri-implant defect in dogs.

MATERIAL AND METHODS

Reconstruction of the severely resorbed maxilla with PRP (Paper I).
Simultaneous sinus membrane elevation and implant installation without the addition of bone grafts (Paper IV).

Patients and study outlines

Paper I
19 patients (2 men and 17 women, mean age 58 years, range 35-75 years) were consecutively included in the study. The patients were consecutively recruited from patients referred to the Maxillofacial Unit, Stockholm Söder Hospital, Stockholm, Sweden, for implant treatment in the totally edentulous maxilla during the study period from June 1999 to March 2001.

Paper IV
20 patients (11 women and 9 men, with a mean age of 59 years, range 19 -78 years) were consecutively included from November 2001 to June 2004 with the latest follow-up in October 2005. The patients had been referred to the department of Oral and Maxillofacial Surgery, University Hospital, Uppsala, Sweden, for dental implant treatment in the posterior maxilla.

Presurgical examination, inclusion and exclusion criteria

Paper I
The patients in both Paper I and IV were pre-surgically evaluated by clinical and radiographic examinations. CT scans were used as well as panoramic radiographs and revealed healthy conditions of the maxillary sinuses in all study subjects (Papers I and IV). In Paper I, patients that were to be subjected to bone grafting in general anaesthesia, had to meet the following criteria: smoking less than 10 cigarettes/day, not abusing alcohol, 20 to 75 years of age, no medical contraindications for surgery and/or general anaesthesia using the American Society of Anaesthetists (ASA) standards and signed informed consent to participate in the study.
Patients in paper IV were included if they agreed to participate in the study and were healthy. Patients were included if their edentulous maxilla exhibited a bone height of less than 5 mm under the maxillary sinuses (paper I & IV), and/or a width of the alveolar crest less than 3 mm in the area planned for placement of dental implants (Paper I).
In Paper I, 12 patients and in Paper IV 2 patients were smokers before entering the study. Patients were all instructed to stop smoking, or reduce cigarette consumption to below 5 cigarettes per day if not possible to completely refrain from smoking.

Implants and abutments

A total of 196 titanium screw-shaped implants with a surface modified by TiO_2-grit blasting were used in Papers I and IV.
In Paper I, 152 implants with Ø3.5 mm (TiOblast™, Astra Tech AB, Mölndal, Sweden) were placed. In Paper IV, a total of 44 dental implants with either, Ø4.5 mm or 5 mm (n=40) (Fixture ST™ Astra Tech AB, Mölndal, Sweden) or Ø3.5 mm (n=4) Fixture Microthread™ Astra Tech AB, Mölndal, Sweden) were installed. The Fixture Microthread™ implant is provided with a cervical area with threads of a smaller dimension than the major threaded part of the implant. The Astra Tech Fixture ST™ is conical in its most cervical part of 5.5 mm, and this part is likewise designed with micro-threads in this area**.**
Healing abutments were placed after six months (Papers I and IV), except for five patients of the study group in Paper IV, where a one-stage implant procedure was performed.

Surgery and experimental protocol

Preparation of PRP (Paper I)

All patients were subjected to peroperative withdrawal of 450 ml of whole blood from a peripheral vein of the arm or foot before a preparation of PRP was made using a Sequestra 1000® gradient density cell separator (Medtronic, Minneapolis, MN, USA) in the operating room. 63 ml of Citrate phosphate dextrose (CPD) (Terumo Corp., Japan) was added to achieve anticoagulation and the blood was then separated into PRP and red blood cells collected in platelet poor plasma (PPP) as described by Marx et al**.**[4]. The cell separation was done in two steps, with the first at 5600 rpm and the second at 2400 rpm to finally extract the platelets in a concentrated form in plasma. The platelet count (x 10^9/L), was preoperatively recorded in venous whole blood upon admission of the patient to the hospital as well as after the preparation in the PRP. A sample of PRP from the patients was submitted for machine platelet count. The PPP with red blood cells was transfused back to the patients during surgery and the PRP (approximately 60 ml) was temporarily saved in its blood bag. Autologous thrombin was attained by adding 0.33 ml of CaCl (18 mg/ml) (Apoteksbolaget, Umeå, Sweden) to 10 ml of anticoagulated PRP in a small glass bowl. After approximately 6 minutes a gel was formed. The gel was then gently squeezed and the solution slowly extracted from it was used as autologous thrombin. By means of initiating a clot formation, the ratio of 4 ml of PRP was mixed with 1 ml of the autologous thrombin in a 10 ml syringe, and 1 ml of air for mixing the components. After approximately 2 minutes the syringe contained a gel that then could be used together with the bone.

Paper I

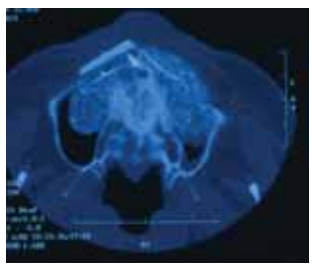
Harvesting of autogenous bone

In general anaesthesia, bone was harvested (after additional administration of local anaesthesia), from the anterior iliac crest of the right (n=17) or left (n=2) side, starting 2 cm posterior of the anterior superior iliac spine, and thus obtaining a cortico-cancellous bone graft. The graft technique aimed at leaving the lateral part of the crest intact, thus using the medial cortical part and obtaining the cancellous bone through that entry. Bovine collagen, Lyostypt© (B. Braun Surgical GmbH, Melsungen, Germany) was used as a coagulum stabilizer on the open bone surface and an activated drain was used. The incision was closed in layers. The bone graft taken from the anterior iliac crest was particulated in a bone mill (Tessier Osseous Microtome, Stryker Leibinger, Freiburg, Germany) into particles of 3x3 mm and then mixed with PRP in the container of the bone mill.

Reconstruction of the maxilla

All 19 patients were subjected to a bilateral inlay and onlay bone grafting procedure. Local anaesthesia (2% Xylocaine Dental with epinephrine 1:50,000, Dentsply Pharmaceutical, York, PA, USA) was induced to complement the general anaesthesia.

Both maxillary sinuses were grafted with particulated autogenous bone, one side with adjunctive PRP (test) and the other side without (control). The anterior maxilla was grafted with either particulated bone graft with PRP (test) or with blocks of bone secured with two 1.7 mm titanium screws (6-13 mm length) without PRP (control). In addition, where needed, 10 of the patients received particulated bone grafts to the floor of the nose, on the one side with (test), on the other side without (control) adjunctive PRP.



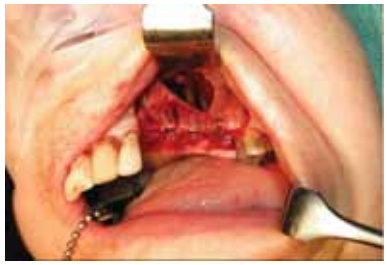
Paper IV

Sinus membrane elevation and insertion of implants

All patients were operated under local anaesthesia (2% Xylocaine Dental with epinephrine 1:50,000, Dentsply Pharmaceutical, York, PA, USA).

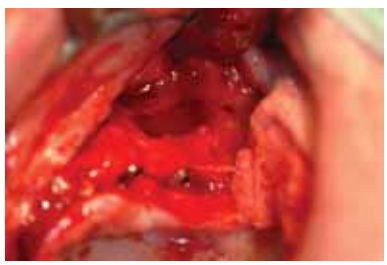
The posterior maxillary edentulous area and the maxillary sinus wall were exposed via a crest incision and a buccal mucoperiosteal flap was raised. Osteotomy in a rectangular fashion was made with a sagittal saw (KaVo Dental GmbH, Biberach, Germany) with a 5 mm wide rectangular blade (Aesculap AG & Co., Tuttlingen, Germany) in the anterior wall of the

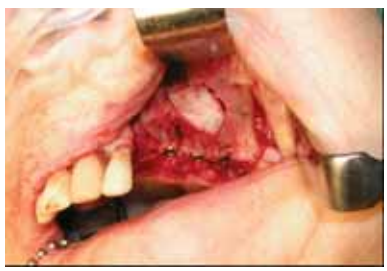
sinus, 5-6 millimetres cranial to the intended implant site. In denser cortex of the area of the infrazygomatic crest, an angulation of the bone cut was performed in the sinus wall in order to simplify the repositioning of the bony window at the end of the procedure. With small sharp elevators the cortical bony window was dissected free and gently removed from the underlying sinus mucosal lining of the anterior sinus wall.



The cut out bone piece was put aside and stored in a sterile saline compress. Where tears in the sinus mucosal lining appeared, they were recorded and attempts were made to repair them, using a set of nerve repair instruments and absorbable sutures (Vicryl 6-0, Ethicon Inc., Somerville, New Jersey, USA). In these cases, small holes were drilled in the bone above the cortical window through which the sutures were passed in order to elevate the mucosal lining to that level. With angulated elevators of various dimensions, the sinus lift was accomplished in all directions from the entrance of the cortical window. In cases of tears of the mucosal lining, the elevation was more extensive, especially in the posterior direction of the sinus cavity, in order to facilitate the repair and suturing. After the elevation, implants were installed in the residual sub-antral bone.

The remaining bone height was recorded with a depth gauge (Depth Gauge Fixture™ , Astra Tech, Mölndal, Sweden) during surgery. Primary implant stability was recorded in a manual way, but the stabilizing wrench was used when removing the implant carrier in all cases. Care was also taken not to use cooling of saline during the placement of the implants in thin bone (< 4 mm). This was to prevent the irrigation from removing bone fragments from the drilling procedure and blood needed for the formation of a coagulum in the sinus cavity and around the implants. In order to achieve implant stability in the cases of small remaining levels of vertical sub-antral bone, the protocol of installing the Astra Tech Fixture ST™ dental implant was modified in its last preparation step. The level to which the final conical burr was inserted into the bone was less (1-2 mm) than the standard recommended protocol in cases with only 2-3 mm residual bone. Thus, a slightly smaller hole for the implant was prepared and the implant could be placed with pressure, utilizing the effect of the conical design and the retention of the micro-threads in the superior part, adding an extra effect to the placement and thereby achieving sufficient primary stability. The compartment around the implants under the sinus mucosal lining in the sinus floor was filled with blood and the cortical window was thereafter repositioned and the incision closed with non-resorbable sutures (Ethibond Excel, Ethicon Inc., Somerville, New Jersey, USA).





Postoperative care; sedation, antibiotics and analgesics

Paper I

Antibiotics were given per-operatively at the initiation of bone graft surgery with benzyl-penicillin (3g x 3) or clindamycin (600 mg x 3) and for the first 24 hours following. For the next ten days following surgery, either phenoxymethyl-penicillin (1g x 3) and metronidazole (400 mg x 3) or clindamycin (300 mg x 3) was prescribed.
At the time of the installation of dental implants after 6 months, the patients routinely received 2 g of phenoxmethylpenicillin preoperatively and postoperatively 1g three times daily for five days (one patient received clindamycin (600 mg x 3) due to previous allergic symptoms from phenoxmethylpenicillin). Analgesics were prescribed with paracetamol and codein or with a non-steroidal anti-inflammatory drug for one to two weeks postoperatively following the surgeries.

Paper IV

Preoperative oral sedation with midazolam, 5-12.5 mg (Dormicum, Roche AB, Stockholm, Sweden) was used when required. Preoperative antibiotics were administered orally 45 min prior to surgery; patients routinely received 2 g of phenoxmethylpenicillin or 600 mg of clindamycin (when allergic to phenoxmethylpenicillin).

Analgesics were prescribed with paracetamol and codein or with a non-steroidal anti-inflammatory drug for one to two weeks postoperatively following the surgery. Antibiotic treatment was continued for five days postoperatively with phenoxmethylpenicillin 1 g (or clindamycin, 300 mg) three times daily.

Follow up and analyses

Paper I

Dentures were not worn during the first month following the grafting procedure and for 10 days after implant placement. Before use they were adjusted and softly relined with Viscogel® , Dentsply, York, PA, USA).The patients were finally examined after one year of loading.

Paper IV

Patients were instructed not to blow their nose and to use nasal spray saline for 14 days after surgery. Dentures were not allowed for 7-10 days following the surgery. Patients were reviewed and sutures taken out after 7-10 days and any postoperative problems were recorded.

Clinical and radiographic examinations

Paper I

Patients were initially monitored every week for the first month post surgery after bone grafting and implant installation. They were seen again at 3 months and any complications or problems were recorded. The patients were instructed in oral hygiene by their prosthodontists and checked regularly.

Parallel intra-oral techniques were used for radiographic examinations and carried out at the Department of Dental Radiology, Eastman Institute, Stockholm, Sweden. Care was taken to get a clear image of the threads on both sides of the implants.
Marginal bone level was measured at baseline (after completion of prosthetic treatment) and after 1 year in function. The radiographs were put on a light desk captured by a computer connected digital camera and stored in a PC with a picture analyzing system
(NIH Image analysis program, National Institute of Health, USA)**.** Contrast and light were automatically optimized where after the marginal bone level related to the reference point was measured on the mesial and distal aspect of each implant. The reference point was the uppermost point of the vertical part of the implant. A mean value was calculated for each implant.

Paper IV

Clinical and radiological follow-up was performed (1) at baseline after surgery, (2) at abutment connection and (3) annually up to four years following implant installation.

Periapical radiographs and orthopantomograms were used for measurements of (i) the height of the residual alveolar bone at each implant site, (ii) the height of newly formed bone in the maxillary sinus in relation to each implant. The measurements were made manually with a millimetre scale on the radiographs with assistance of digital orthopantomograms, since axial projections of implants in the maxillary sinus region were difficult to obtain due to the loss of alveolar crest and the nearby obstructing hard palate. The cervical micro-threaded area of the implant represents 5.5 mm and this was used as a reference point during measurement.

Resonance Frequency analysis (RFA) Paper I

The stability of the dental implants was assessed by resonance frequency analysis OSSTELL™(Integration Diagnostics AB, Göteborg, Sweden) according to Meredith et al.[431] A transducer was attached to the implants and measurements were made in ISQ

units (Implant Stability Quotient where 1 ISQ corresponds to 50 Hz). The instrument gives a value between 1 and 100 ISQ, where 1 is the lowest and 100 the highest degree of stability and a value over 50 is considered with a stable implant. The transducer beam generates a vibration over a range of frequencies. The maximal amplitude of the vibrations analyzed describes the resonance frequency of the system. A stiff and stable system will generate a high resonance frequency.

The same investigator (AT) performed all RFA measurements and the same RFA equipment was used at all times: at implant installation, abutment connection and after one year of loading, when supra-structures were removed before carrying out the measurements.

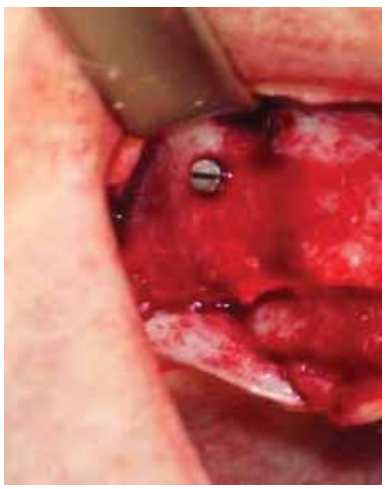
Histologic study of biopsies maxillae grafted with PRP (paper II).

Patients

The present material comprised of biopsies from 11 patients. The patients were selected from the same patient group as described previously in Paper I. Selection was based on the results of platelet counts and concentration factor in PRP (see Paper I). The patients (10 women and 1 man) had a median age of 55 years (range 36-72) at the time of bone graft surgery.

PRP and platelet count

The numbers and results from the preparation of platelet-rich plasma for the study group used in Paper I was used in this study.



Surgery and experimental protocol

3 months after reconstruction, two micro-implants were inserted bilaterally in the grafted maxillas, in a separate session where also bone biopsies were collected. Micro-implants were placed in the area of the cortical window that had been grafted earlier with bone. To get samples from the early phase of healing, biopsies were taken in the same area, bilaterally, with a 3.1 mm trephine at the same time as placement of the micro-implants. A burr with the same diameter as the implants was used before manually

inserting the implants. There was no contact between the micro-implant and the residual alveolar ridge. Three months later, (at placement of ordinary implants six months after bone grafting) the micro-implants were retrieved with the same type of trephine drill used before, with an inner diameter of 3.1 mm.

Specimen preparation

All biopsies and implants with surrounding tissues were immersed in appropriate fixative, further processed and finally embedded in methyl methacrylate resin (Technovit 9100 VLC, Kulzer Wehrheim, Germany). Undecalcified cut and ground sections were prepared with the Exakt® sawing machine and grinding equipment as previously described[432, 433]**.** Sections of a thickness of 10-15 mm were stained with our routine histological staining method, i.e. 1% toluidine blue mixed with 1% pyronin-G.

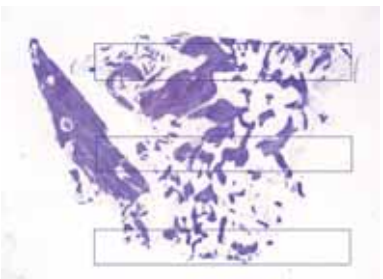
Histology

Quantitative and qualitative investigations of the routinely stained sections were performed directly in the eye-piece of a Leitz Aristoplan® microscope (Leitz, Wetzlar, Germany). The computer based histomorphometric evaluation was made by using Leitz Microvid® equipment that is connected to the microscope.
As an additional staining method, attempts were done to investigate the presence of bone remodeling activity (alkaline- (ALP) and acidic phosphatase (ACP) activity) with an enzyme-histochemical staining technique[25].

Quantitative histomorphometrical evaluations

Evaluation of biopsies retrieved after 3 months of healing:

The percentage of new and old bone in a region of interest (ROI) at three selected positions (an identical checked pattern area with positions medial and most bilateral with the specimen in a horizontal position) on the biopsies was evaluated. A mean value for the entire biopsy was calculated.



Evaluation of biopsies with micro-implants retrieved after 3 months of healing (6 months after bone grafting):

Histologic specimens from a total number of 11 patients were used for histomorphometric evaluations.

Measurements of the percentage of bone-to-metal contact and bone area in all threads and in the three best consecutive threads were calculated. "Mirror image" measurements of the percentage of bone in the out folded thread area, was calculated in the three best consecutive threads. The percentage of total bone in a ROI around the entire implant was measured.
Evaluation of percentages of new bone and old bone in a ROI at three selected positions around the implant was measured on all implants. A mean value for the entire implant was calculated.

In vitro study of the thrombogenic properties of whole blood, PPP and PRP in contact with various titanium surfaces (Paper III).

Slide chamber model

The slide chamber, previously described by Hong et al[434], contains two wells with a volume of 1.65 mL. These chambers are made of two polymethyl methachrylate (PMMA) rings (height 5 mm, outer diameter of 25 mm and an inner diameter of 19 mm) glued to a microscope slide of PMMA. The device is heparin coated (see below). Test surfaces can be placed on top of the cylinders and temporarily fixed with clips, giving two chambers that can be filled with plasma, PRP or blood. To prevent leakage during incubation, two thin rubber O-rings are placed between the cylinder and the test material surface.

Heparin treatment

The slide chamber, tubes and micropipette tips used in this Paper were furnished with Corline heparin surface (Corline Systems AB, Uppsala, Sweden) according to the manufacturers' recommendation. The surface concentration of heparin was 0.5μg/cm^2.

Biomaterial surfaces and sample preparation

Surfaces of titanium were produced, in the form of 25 x 25 mm flat squares (2-3 mm thick), from a mirror polished titanium surface of Ti-13Nb-13Zr alloy with 13 wt. % Nb and 13 wt.% Zr, developed for hip replacement and other prosthesis[435].
For the blood compatibility evaluation of the clinically used material, circular discs with a diameter of 25 mm were produced in commercially pure (c. p.) titanium and machine cut. All together, test discs of titanium (mTi), hydroxyapatite coated titanium (HA) and two commercially

available surfaces, TiO_2-gritblasted titanium (TiOB) and TiO_2 grit-blasted titanium and hydrofluoric acid treated (TiOB-F) were tested. Pieces of polyvinyl chloride (PVC) were used as a reference test surface. HA coated Ti discs were prepared in a radiofrequency (RF) magnetron sputtering apparatus. Sputtering was performed with a HA sputtering target (HC CAM, Leiden, The Netherlands) in a mixture of argon and reactive gases, resulting in 0.1 um calcium phosphate coating thickness.

Discs with TiOblast™ (TiOB) and Osseospeed™ (TiOB-F) surfaces were kindly prepared and provided by Astra Tech AB, Mölndal, Sweden. The exact preparation sequence of these surfaces is proprietary of the company and not known.

The surface topography was measured with an interferometer (MicroXam, Phase-Shift, Arizona, USA). Five different parameters were used to describe the surface topography; Sa = average height deviation, measured in μm, Sds = density of summits, a spatial descriptive parameter measured in $1/\mu m^2$, Ssk = skewness, a description of the height frequency distribution, a positive value indicates more peaks than valleys, negative value more valleys than peaks, Sdr = developed surface area, a hybrid parameter taking into account variation in height and spatial distribution and measure surface enlargement compared to a flat reference plane, the value is given in %, Sci= core fluid retention index, a functional parameter, measuring the fluid retention index, a high value indicates a good fluid retention in the core zone. Values of this index ranges typically from 0 to slightly more than 2.

Preparation of whole blood and plasma

For whole blood, PRP and PPP experiments 40-50 mL of whole blood was collected from healthy volunteers (n=4) in a heparinised 50 mL Falcon® tube (Becton Dickinson, USA) containing soluble heparin giving a final concentration of 1.0 IU heparin /mL (Bio Iberica, Spain).

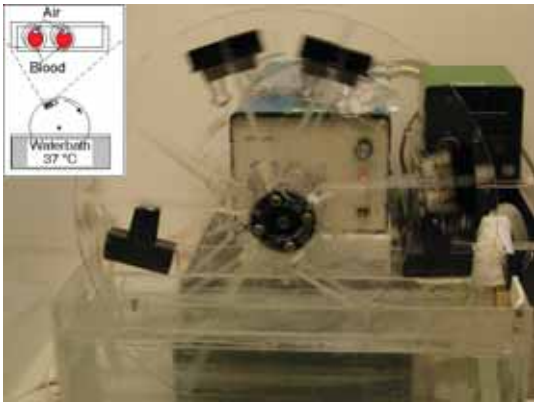
To prepare platelet-rich plasma (PRP) the blood was centrifuged for 15 min at 180 x g.

Platelet-poor plasma (PPP) was prepared from the supernatant of PRP by additional double centrifugation at 3200 x g for 5 min.

For evaluation of the clinically used material, whole blood was collected from healthy volunteers (n=5) in a heparinised 50 mL Falcon ® tube (Becton Dickinson, USA) containing soluble heparin giving a final concentration of 1.0 IU heparin /mL (Bio Iberica, Spain).

All preparations were collected using heparin-coated tubes and micropipette tips.

Experimental protocol



Pieces of titanium or pieces with titanium with modified surfaces were attached with two clips as a lid creating two circular chambers in the experiment that had two parts. First, the wells of the slide chamber were filled with 1.3 mL of blood, PRP and PPP. In the second experiment, *whole blood* was used. After closure, the device was rotated for 60 min vertically at 22 rpm in a 37 °C water bath in both experiments. After incubation, the blood was mixed with EDTA or citrate to give final concentrations of 4 mM or 13 mM, respectively. Prior to centrifugation, the EDTA samples were analyzed for leukocytes and platelets on a Coulter A^{C}T diff™ haematology analyzer (Coulter Corporation Miami, FL, USA). The EDTA-treated blood was centrifuged at 2200 x g for 10 min at +4 °C, and the citrated blood was double-centrifuged (10 min at 1000 x g and 10 min at 10 000 x g) at +4 °C within 30 min. The plasma samples were collected and stored at -70°C.

Analyses of blood and plasma samples

Enzyme immunoassays (EIA)

Phosphate buffered saline (PBS) containing 1% (w/v) bovine serum albumin (BSA) and 0.05% (v/v) Tween 20 (polysorbate detergent) was used as working buffer and PBS containing 0.05% (v/v) Tween 20 as washing buffer in all EIAs.
Commercially available EIAs were used the detection of thrombin-antithrombin (TAT) in plasma (μg per L) and β-Thromboglobulin (β-TG) in citrated plasma (units per mL)
For determination of F XIIa-AT, F XIIa-C1 INH, F XIa-AT and F XIa-C1 INH (nanomol per L) complexes in plasma, an EIA described by Sanchez J et al.[436] was used.
For measurement of C3a (nanograms per mL) in plasma an EIA described by Nilsson Ekdahl et al.[437] was used.
sC5b-9 (arbitrary units per mL) in plasma was detected using a modification of the EIA described by Mollnes et al.[438].

Correlation of platelet growth factor release from PRP and bone formation in a peri-implant defect in dogs
(Paper V)

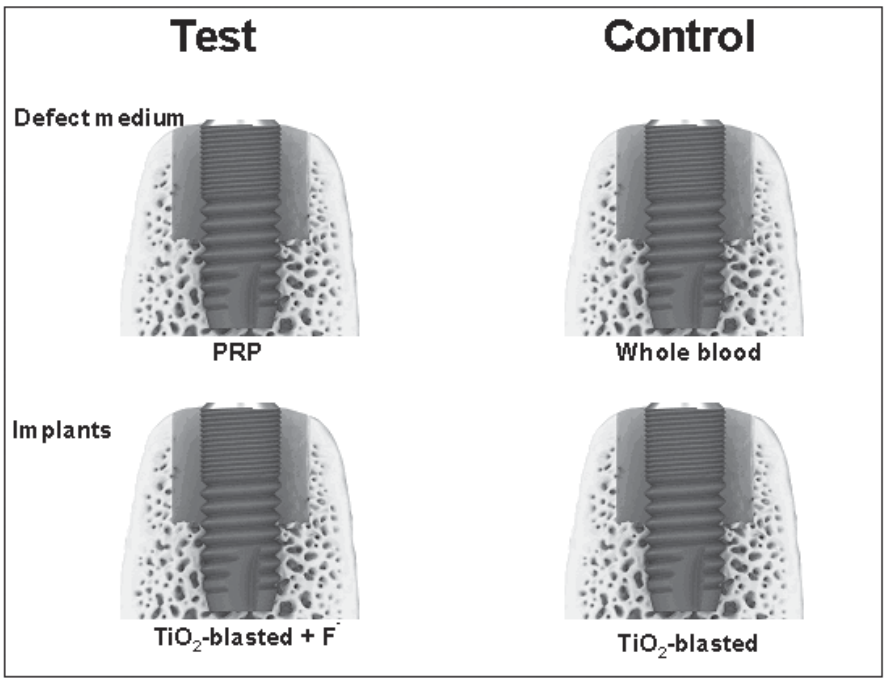
Animals and anaesthesia and postoperative medication

Six female (age 2 years) Labrador Retriever dogs weighing between 25.5 to 30.0 kg were used in the study. The Regional Ethical Committee for Animal Research at the Sahlgrenska Academy, Göteborg University, approved the study protocol.

As pre-surgical sedative, 0.05 mg/kg i.m. of acepromazin diluted with 0.9 % NaCl was given. General anaesthesia induced with propofol 4 mg/kg i.v., and continued by endotracheal intubation and maintained by isofluran. Buprenorphin, 0.018 mg/kg i.m., was used as analgesics. Local anaesthesia was given at the experimental site of surgery. Antibiotic treatment was continued twice daily for 8 days with 10 mg/kg amoxicillin/clavulanic acid.

Implants

The test implants were TiO_2-grit-blasted and hydrofluoric acid-treated (Osseospeed™ , Astra Tech AB, Mölndal, Sweden) whereas control implants, were TiO_2-grit-blasted only (Microthread ™, Astra Tech AB, Mölndal, Sweden). All implants were of the same dimension, 3.5 mm in diameter and 9 mm in length.

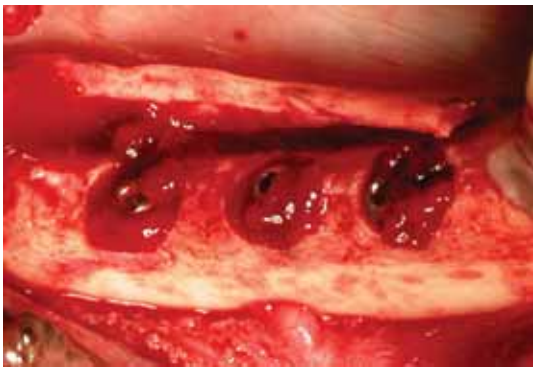


Preparation of PRP and blood counts.

Simultaneously with the experimental site preparation, 50 cc of venous whole blood was drawn from the radial vein of each dog. The blood was separated in a commercially available centrifuge (PCCS®, 3i Implant Innovations, Palm Beach, FL, USA) using the manufacturers' instructions. Citrate phosphate dextrose at a ratio of 1 ml to 10 ml blood was added subsequently to achieve anticoagulation. After separation, a platelet concentrate was collected and the coagulation was initiated with 10% calcium chloride. Samples of activated PRP was collected and stored in -70° C pending analysis. At the time of surgery for the respective dog, a blood sample was collected and the peripheral platelet count in whole blood was recorded as well as platelet count in the PRP. Values for leukocytes and erythrocytes were also recorded. Cell-counts were made mechanically at CaniLabEqui-Lab, Halmstad, Sweden.

Surgery, experimental protocols and delivery of PRP.

Three months prior to the start of the experiment mandibular premolars were extracted in order to prepare for later installation of implants.



At experimental surgery, mucoperiosteal flaps were elevated in both sides of the mandible. Three implant defect sites were prepared in the right and left side, respectively, with a trephine drill of a diameter of 6.0 mm. The implants were installed and cower-screws were connected central in the defects using a 3.2 mm drill. The remaining stabilising bone for the implants was 4 mm. The defect width, from implant surface to the bone wall, was estimated to 1.25 mm. PRP and whole blood was randomly administered to the defects of either the right or the left side of the mandible. The positions of the test-implants as well as the sites of the control-implants were then matched, so that half of the number of the test and control implants, respectively, were placed at PRP as well as whole blood sides of the mandible. Delivery of PRP to the experimental sites was done through a syringe immediately after the implants were installed on the PRP-side. On the whole blood side (control) the defects were left until they were passively filled by medullar bleeding. The defect areas were thereafter checked for fill of PRP/blood before the incision was closed with resorbable sutures.

Correlation of platelet growth factor release from PRP and bone formation in a peri-implant defect in dogs (Paper V)

Specimen preparation

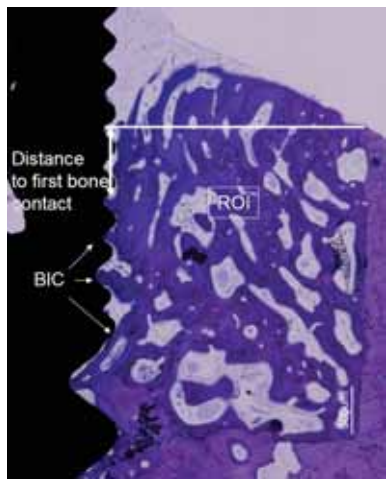
After five weeks of healing, the animals were euthanized by an overdose of pentobarbital.

Implants and surrounding bone from the mandibles were retrieved *en bloc* and fixed by immersion in 4% buffered formaldehyde. Dehydration of the specimens were performed by an increasing and graded series of ethanol and subsequently infiltrated and polymerized in light-curing resin (Technovit® 7200 VCL, Heraeus Kulzer GmbH & Co., Wehrheim, Germany). Ground sections, approximately 20 mm thick, were taken through the central part of the block and along the longitudinal axis of the implants (buccal to lingual of the mandible) and prepared by sawing and grinding as in Paper II. The sections were stained with toluidine blue, 1%.

Analyses

Histological examination

Examinations of each section were performed in a Nikon Eclipse 80i microscope (Teknooptik AB, Huddinge, Sweden) equipped with an Easy Image 2000 system (Teknooptik AB, Huddinge, Sweden) for histologic evaluation. All measurements were made on both sides of the 36 implants. Morphometric calculations of new bone formation in the defect, in a region of interest (ROI), were registered in all specimens (of total area of ROI in %). The outlining of the ROI was defined by i) the two defect walls (bottom and side), ii) a line from the most superior point of the bone envelope at the defect wall (perpendicular to the implant) to the implant and iii) the implant surface. Calculation of the bone-to-implant-contact (BIC) for the implant in the ROI (as specified above) of the defect was made. It was expressed as percentage of the total implant surface in contact with newly formed bone from the superior top of defect as defined above, to the bottom of the defect. A histometric measurement of the distance from the superior border of the ROI perpendicular to the implant to the first point of new bone on the surface of the implant was also executed.



Detection of GF release in PRP

Commercially available EIAs were used for detection of β-Thromboglobulin (β-TG), PDGF, VEGF and TGF-β in PRP after activation of PRP in the experiment. EIAs for β-TG, PDGF, VEGF were primarily for human analysis and for TGF-β a combined ELISA-kit for Mouse/Rat/Porcine/Canine TGF-β1 was used. For more details, see Paper V.

Statistics Papers I-V

In paper I, the Wilcoxon signed-rank test was used for pairwise comparison between test and control implants. The Spearman correlation test was used for correlations.

For Paper II, calculations of group means and standard deviations were carried out for all measurements. Wilcoxon signed-rank test was used for paired comparisons within each patient. $P < 0.05$ was considered to be significant.

In paper III, results were statistically evaluated using one factor ANOVA. A conservative post hoc test, Bonferroni/Dunn was applied to determine statistical significance between materials. The results were expressed as mean ± SE.

In paper IV, descriptive statistics was used and the correlation of results was confirmed with linear regression analysis.

In Paper V, mean values and standard deviations for observations in each dog were calculated. Wilcoxon signed-rank test was used and a $P<0.05$ was considered statistically significant. Paired Student's t-test was additionally used for the histomorphometric measurements.

SUMMARY OF PAPERS AND RESULTS

Papers I and II were collaboration between the Maxillofacial Unit, Stockholm Söder Hospital and Department of Biomaterials at Göteborg University. Work on Paper III was performed at Rudbeck laboratories, Department of Oncology, Radiology and Clinical Immunology, Uppsala University and Department of Biomaterials at Göteborg University. Paper IV was executed at Department of Surgical Sciences; Oral & Maxillofacial Surgery, Uppsala University Hospital. Finally, Paper V was prepared at Department of Biomaterials at Göteborg University.

Paper I

This paper addresses the question if PRP may enhance the integration of implants installed in bone that has been grafted to reconstruct severely resorbed maxillas after 6 months of healing.

The general impression of the procedure was positive and resulted in a high implant survival rate (98.7%) evaluated after 1 year of function. 152 implants were placed in 19 patients (8 implants in each patient) and only two implants were lost, both before loading of implants. Morbidity from iliac crest bone harvesting was considerable, but resolved after various time in the patients and no abnormal sequelae could be noted at 1-year follow-up from the donor site. Infections following bone grafting were not seen, but two patients had localised (posterior sites) infections after implant placement that could be resolved with drainage and antibiotics.A marked resorption of the bone graft was generally seen at the time of fixture installation at both test and control sides. At the follow-up after 1 year, smokers were reduced from 12 out of 19 patients prior to the study, to four patients still smoking at this follow-up.

Platelet counts

Platelet counts and concentration of platelets are seen in the Table below. Towards the end of the patient series, the concentration of platelets in PRP was more consistent in reaching the appropriate levels of x 4.

Radiographic findings

The marginal bone level measurements showed a tendency of less resorption on the test (PRP) side but the difference was not significant (see Tables below).

Table showing platelet counts in Paper 1.

Patient	Preoperative	PRP	X concentration
1	201	355	1,75
2	236	223	0,94
3	Unkno wn	Unkno wn	-
4	187	33	0,18
5	240	491	2,05
6	336	43	0,13
7	288	528	1,83
8	268	704	2,63
9	328	963	2,94
10	176	314	1,78
11	302	360	1,19
12	345	646	1,87
13	197	907	4,60
14	358	1378	3,85
15	301	616	2,05
16	247	677	2,74
17	262	1181	4,51
18	294	1509	5,13
19	260	997	3,83
Mean First 9 Last 9	**258**	**662**	**2,57 1,3 3,3**

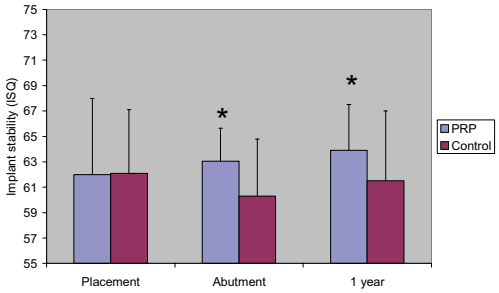
Results of radiographic measurements in Paper 1.

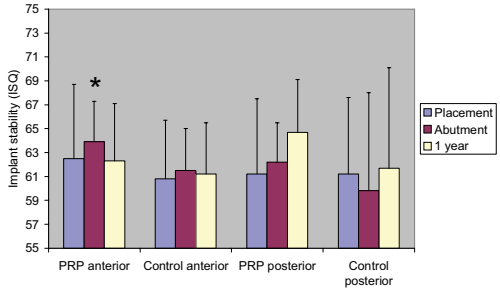
Mean marginal bone level resorption overall	**Right mm control**	**Left mm PRP**
Baseline	**1.47 ± 1.71**	**1.29 ± 1.90**
1-year follow-up	**2,00 ± 0,90**	**1,83 ± 1,11**

Mean marginal bone level resorption post. region	**Right mm control**	**Left mm PRP**
Baseline	**2,72 ± 1,0**	**2,70 ± 0,9**
1-year follow-up	**3,85 ± 0,8**	**3,71 ± 0.9**

RFA findings

Overall RFA measurements revealed a statistically significant higher stability for implants at the PRP sides at the time of abutment connection ($P<0.05$) and after 1 year of loading. Comparison between implants placed in the anterior maxilla showed a significantly higher stability at the PRP side at abutment connection ($P<0.05$). No differences were seen for implants placed in the posterior regions, representing implants in grafted maxillary sinuses.





Paper II

This paper reported the histologic findings from the 3 months biopsies and the results from biopsies with micro-implants six months post grafting with autogenous bone and PRP in the patient study group.

Platelet counts

The platelet counts for the study group where biopsies were possible to evaluate in either three months or biopsies with micro-implants at six months post bone graft surgery (n=9) are shown in this Table .

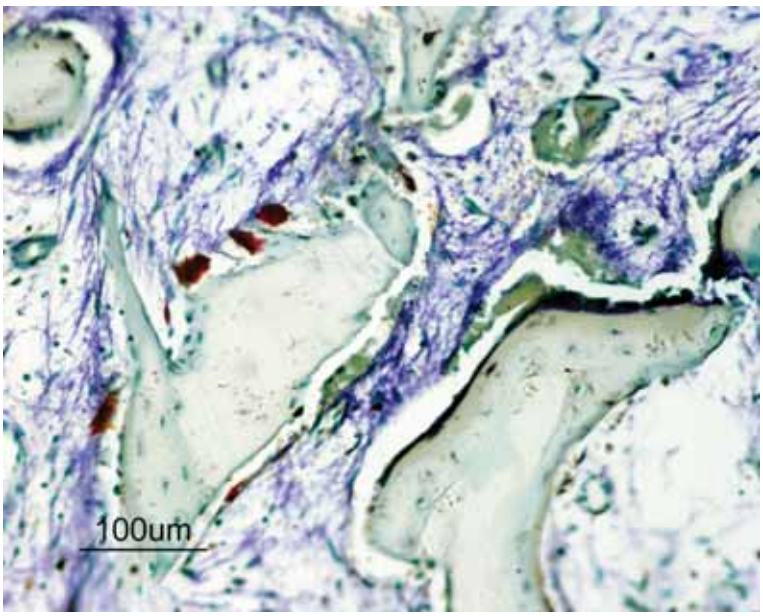
Patient	Preop. platelet counts	Perop. platelet counts	X concentration
1	240	491	2.0
2	268	704	2.6
3	328	963	2.9
4	197	907	4.6
5	358	1378	3.8
6	247	677	2.7
7	262	1181	4.5
8	294	1509	5.1
9	260	997	3.8
Mean (n=9)	273	979	3.6

General and qualitative description of bone biopsies

In general, the bone formation seemed to be more active (larger areas of immature bone / osteoid) in the bone biopsies from the PRP treated site (for details, see paper 2). This observation was later confirmed quantitatively (see below). For the micro-implants and surrounding tissue the impression of no qualitative differences could be observed. All samples demonstrated an ongoing bone tissue formation with sharp and clear demarcation lines between old and new bone tissue.

Enzyme-histochemical staining

The attempt to detect bone remodeling activities by applying an enzyme-histochemical staining method for alkaline- (ALP) and acidic phosphatase (ACP) worked out successfully, with the former stained blue and the latter showing an intense, red staining. ALP is referred to bone formation (osteoblasts, osteoid and osteocytes) and ACP is related to osteoclast activity if located on bone surfaces.



Positivity for ACP was also observed in the soft tissue areas, this is referred to being a marker of macrophage activity. The PRP treated site sections revealed large blue stained smeared-out areas also in the soft tissue regions. This feature could not be observed with the routine histological staining method.

Quantitative histomorphometry

Evaluation of biopsies retrieved after 3 months of healing:

Due to difficulties in harvesting biopsies after 3 months of healing (resulting in some incoherent specimen), samples from 7 patients (14 biopsies) could be evaluated histomorphometrically.

The mean value of the newly formed bone for the PRP treated group was 22 ± 9 % (range 8-35 %) while for the non-PRP treated samples the numbers were 11 ± 3 % (range 8-16 %). This difference was significant in favour for the PRP prepared samples (*P*=0.03).

There was no difference in the amount of old bone removal between the PRP and control biopsies (see Table below).

Bone biopsies	Old bone	New bone	Statistics
Test PRP (n=7)	13 ±7 (2-21)	22 ±9 (8-35)	p= 0.176
Control (n=7)	20 ±11 (9-37)	11 ±3 (8-16)	p= 0.043
Statistics	p= 0.063	p=0.028	

This Table shows the results of the histomorphometrical data from the 3 months bone biopsies. Mean values, standard deviations and ranges are exposed for calculation of the percentage of old- and newly formed bone in the biopsies.

In Paper II, the amount of new bone formation in test and control samples of the 3 months study group were combined with the achieved level of concentration of platelets in PRP and graphically illustrated. This was done to address the question of a potential correlation between platelet count and new bone formation (see Table 5 in Paper II).

Evaluation of biopsies with micro implants retrieved after 3 months of healing (6 months after bone grafting):

In two patients out of the original 11 included, biopsies were not possible to evaluate histomorphometrically, which implies that a total number of 9 patients (18 sections) were evaluated.

No differences were demonstrated in any of the histomorphometry parameters when comparing the PRP treated groups to the control groups. For example, the mean value of new bone formation was 14 ± 7 % (range 4-25 %) for the PRP group, while for the non-PRP group the numbers were 13 ± 6 %(range 4-21 %). For details, please see Paper II, Table 6.

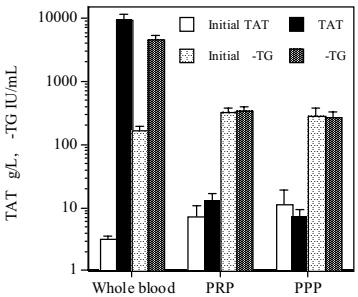
Paper III

The paper showed that the use of titanium in blood in this in vitro slide chamber model will generate a highly thrombotic response in activation of coagulation factors and release of GFs. The values in whole blood were significantly higher than those of PRP and PPP. In whole blood, a surface modification such as hydrofluoric acid treated grit blasted titanium, may even augment the response as shown in this paper.

Part 1

Evaluation of the thrombogenic response of whole blood, PRP and platelet poor plasma (PPP) in contact with titanium.

In the slide chamber model, whole blood in contact with Ti alloy resulted in the binding of platelets to the material surface and in the generation of thrombin-antithrombin (TAT) complexes. TAT levels in blood increased 1000 fold compared with PRP and PPP, in which almost no increase of TAT could be detected. In addition, the platelet activation showed a similar pattern with a 15 fold higher release of β-TG in whole blood.



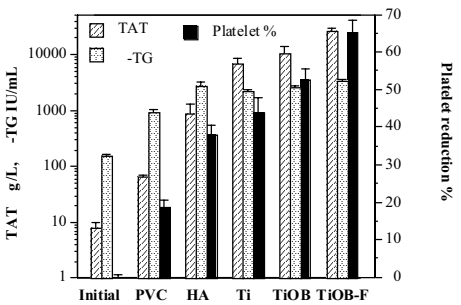
Part 2

Evaluation of the thrombogenic response of whole blood in contact with various titanium surface modifications.

The topographical modifications resulted in two surfaces with a minimally rough surface (Sa 0-1μm), the turned, machined titanium (mTi) and the hydroxyapatite (HA) samples and two moderately rough, TiO_2-blasted and fluoride modified surfaces (Sa 1-2μm). For details, see Table 2 in Paper III.

TAT

The results from part 1 led to the next part of the experiment, where the thrombogenic response of clinically used surfaces, such as hydroxyapatite (HA), turned, machined titanium (mTi), TiO_2 grit-blasted titanium (TiOB) and fluoride ion modified grit-blasted titanium (TiOB-F) were analyzed. The fluoride-modified surface (TiOB-F), showed pronounced TAT generation (3300-fold) compared with



TiOB (1300-fold), mTi (800-fold) and HA (100-fold). Values of TAT for TiOB-F were significantly higher than those for the other three ($P<0.0001$).

Binding of platelets to test surfaces

The platelet binding reflected as platelet consumption exhibits a pattern similar to the thrombin generation with the most extensive reduction of the platelet count with TiOB-F. The platelet reduction with TiOB-F was significantly higher compared with mTi and HA ($P<0.0001$).

Platelet activation

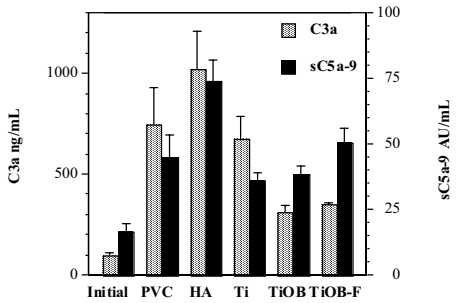
The platelet activation showed a different pattern with a 22-fold release of β-TG with TiOB-F compared to baseline values. With TiOB, mTi and HA, the β-TG values increased 15-fold over the initial level. The values of β-TG with TiOB-F was significantly higher than those for the mTi ($P<0.001$).

Coagulation factors and activation of the intrinsic pathway

Generation of FXIIa-C1INH, F XIIa-AT and F XIa-AT complexes was most pronounced with TiOB-F compared to the other materials. In all three complexes the generation was significantly higher with TiOB-F ($P<0.0001$).

Activation of complement.

Results of C3a and sC5b-9 are illustrated in the Table seen to the right. Levels were lower forTiOB and TiOB-F compared to the other surfaces.



Paper IV

This study was conducted to evaluate a recently developed surgical procedure where simultaneous sinus mucosal lining elevation and installation of dental implants was performed without any graft material. Blood was allowed to clot around inserted implants in the sinus bottom and the bone window was replaced to shut off the compartment where bone formation eventually took place.
In general, this procedure was well received by patients, experiencing results that were achieved easier than the patients studied in paper I. The morbidity was low and healing times shortened. This is a clinical paper evaluating the described surgical method and results were accumulated from the 20 patients during a mean follow-up of 27.5 months (range14 to 45 months). During the study period, only one implant was removed, giving an implant survival rate of 97.7 %.

Technical considerations

Perforations of the maxillary sinus mucosal lining occurred in 11 of the 27 operated sinuses (41%). Firm primary stability was achieved for all implants at installation with bone levels in residual bone of 2-9 mm, using a modified surgical protocol and the characteristics of a conical designed implant with micro-threads.

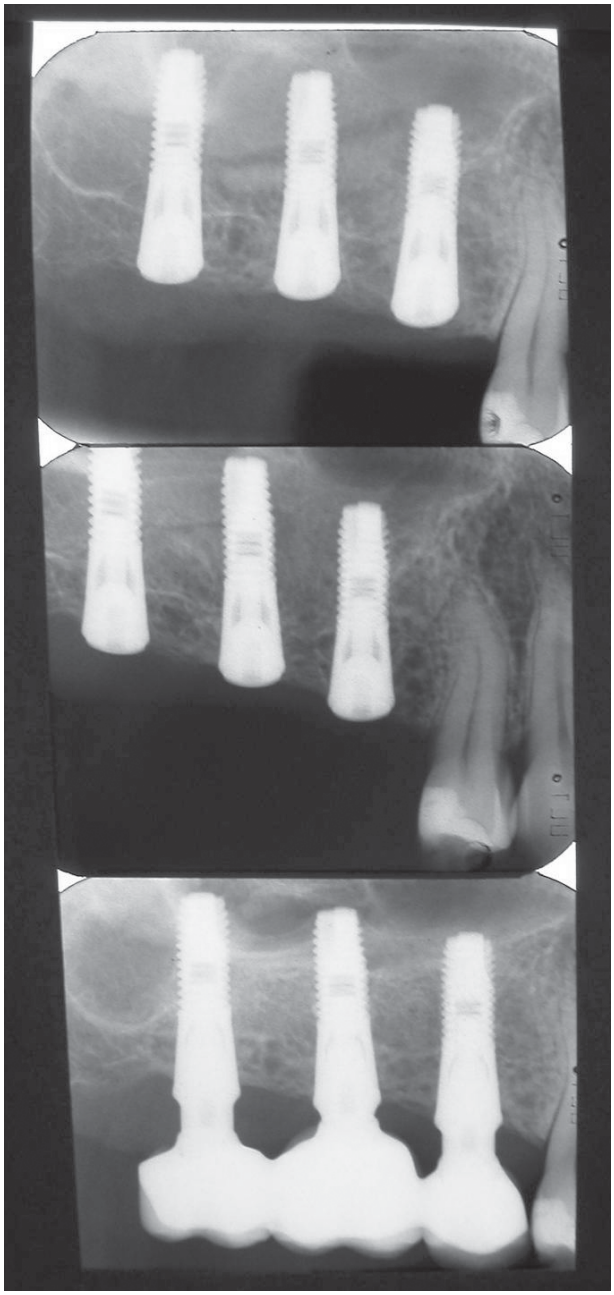
Bone gain

The average gain of bone at the sinus floor was 6.51 mm (SD = 2.49, 44 implants) including all measured implants after a minimum of 1 year follow up. Analysis of results also showed marked bone formation around long implants. Bone gain was also significant when the residual bone below the sinus was diminutive (see Table below).

Table Mean average bone gain and number of observations (not patients) for each combination of fixture length and bone baseline (All observations)

Bone baseline	Fixture length					Mean bone gain
	9	11	13	15	Tot	
2.0	0	2	4	1	7	7.00
2.5	0	0	1	1	2	10.00[1]
3.0	0	2	3	4	9	7.89
4.0	1	1	2	1	5	6.60
4.5	0	0	0	1	1	10.00
5.0	0	1	2	5	8	5.56
5.5	0	0	0	1	1	9.50
6.0	0	0	2	0	2	6.00
7.0	1	0	1	1	3	4.67
8.0	0	0	2	0	2	4.50
8.5	0	0	1	0	1	4.00
9.0	0	0	1	2	3	4.67
Tot	2	6	19	17	44	6.51 (std=2.492)
Mean bone gain	3.50	5.58	6.76	6.94		

[1] Based on only one value since BONE GAINED is missing for the combination BONE BASELINE=2.5 and FIX. LENGTH=15.



Radiological outcome in one of the study patients, from top to bottom: baseline, three months and one year postoperatively

Paper V

In the final study of this thesis, an attempt to correlate platelet count and GF release in PRP and its effect on bone formation was performed in a peri-implant defect model in dogs. Furthermore, a surface modification, used in Paper III, was tested with whole blood and PRP to evaluate its effect on bone formation in the defect.

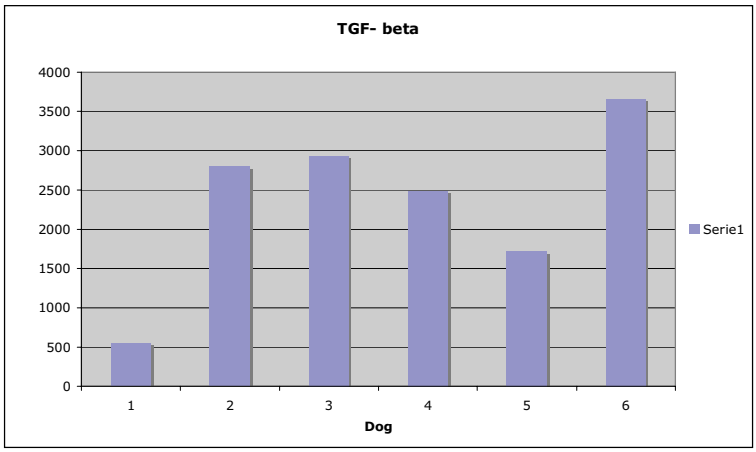
Blood and PRP cell counts

Samples from the PRP in the experiment revealed that white blood cell (WBC) count after sequestration was lowered in four out of six dogs, whereas in two dogs the levels were slightly increased. Red blood cell (RBC) counts showed reduction of erythrocytes in the PRP to a mean level of 5.3% of the initial value of whole blood. Reduction of Haemoglobin levels in PRP corresponded well with RBC; 3.8 % was found in PRP. A mean volume of 7.8 ml of PRP was collected in the six animals. Mean preoperative platelet count was 255 (range 217-290) and after concentration 1055 (range 343-1343), giving a mean concentration increase of 424%. In one of the six animals, the sequestration of platelets was low, with only 18% increase from the basal platelet count.

Detection of Growth Factors

TGF-β was detected and values were found to correlate to the platelet count found in PRP for each dog respectively (r^2=0.857). The immunoassays used for β-TG and PDGF did not show any detectable levels. No cross-reactions were seen for the antibodies used in these ELISAs. Only a weak cross-reactivity was seen for VEGF.

Table showing measured values for TGF-β, as given in pg/mL.



Histomorphometry

The regeneration pattern in the defects was directed from both the bottom and the lateral wall (corner) of the defect in an angle towards the top of the implant. A marginal bone resorption was seen in most of the specimens. The distance from the lateral top point of the implant, at the first micro-thread, to the most superior point of the bone envelope had a mean value of 1.23 mm (SD ±0.75mm). The distance from the same superior measuring-point of the implant, the first micro-thread, to the bottom of the defect was measured to a mean of 4.86 mm (SD ± 1.05 mm).

New bone formation in defect

There was a statistically significant difference ($P = 0.03$) found between the implant surfaces used, in favour of the TiOB-F implants regardless of blood or PRP in the defects. Approximately 50% of the ROI in the defect was filled with new bone after 5 weeks of healing. No significant difference was observed when comparing PRP and whole blood regarding new bone formation

Bone in contact with implant within ROI.

A mean of more than 20 % (21.3-22.3%) of the implant surface in ROI was in contact with newly formed bone. The influence of surfaces revealed no significant difference between PRP and whole blood.

Distance to first bone contact on implant in ROI.

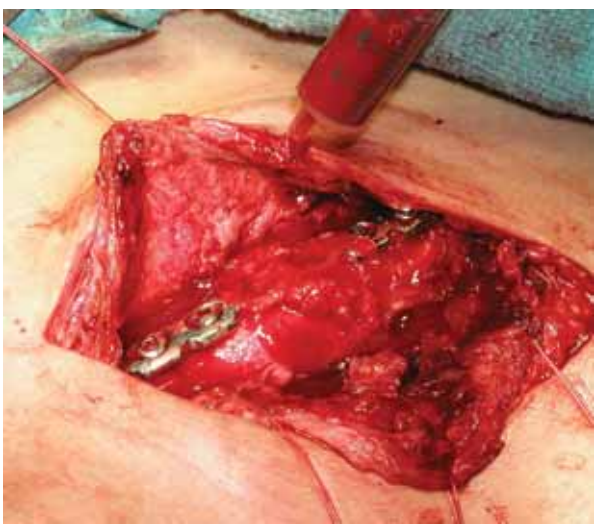
PRP did not show a difference compared to whole blood in this measurement. However, the TiOB-F surface implant used in the defects exhibited a closer distance to the marginal bone level along the implant compared to the control (in both PRP and blood combined, $P = 0.0511$). In those samples, the bone had contact higher up on the surface of the TiOB-F implants closer to the marginal reference point ($P = 0.019$). No significant difference was found between surfaces in the PRP-group.

DISCUSSION

The "dental implant and maxillo-facial community" quickly adopted Platelet-rich plasma as a promising concept, after its introduction by Marx et al.[4] in 1998. The idea of an autologous source of GFs to increase the rate of bone formation around installed implants or in healing of bone grafts was for many an irresistible idea. The concept was very early promoted by commercial companies, thus marketing office-based platelet concentrate systems versus the larger, previously used, institutional-based cell saver-type devices. These smaller centrifuges were subsequently evaluated in the literature, as presented in the overview earlier. Scientific focus was in the beginning on platelet counts, leading further to evaluation of GF levels in the final PRP-product. After this was accomplished, the correlation of GF levels in PRP with tisse healing has come into focus, but the optimal level considering platelet counts correlated to effect in e.g. bone formation is still debated. GFs interact with target cells, and the testing of one sole factor might not end up with the desired result of e.g. bone formation or soft tissue healing.

The work in this thesis, parallels well the evolution of PRP-research over the last eight years, namely from clinical studies and observations in humans, to the experimental settings in vitro or in vivo models. Therefore, the results presented in this thesis are based on five papers, performed over 5 years.

Parallel to the work in this thesis, many fields of medicine have been engaged in PRP-research. As an example, a project together with an orthopaedic shoulder surgeon (Jan Nowak, Dep't. Of Orthopaedics, Uppsala University Hospital) started with the myself involved. Bone grafting in revisions of clavicle non-union cases following fractures, was complemented with PRP. However, in this very challenging setting, moderate results have been accomplished so far. The very high biomechanical forces that acts on the shoulder region including the clavicle when reconstructed, is the primary issue to address, and probably demands more of a correct calculation of choosing the right plate and screws for successful healing and less on the possible percentage of new bone formation increase added by PRP [439]. This holds true also in mandibular reconstruction, where also very potent forces act upon the bone grafted area and have to be taken into account. However, in compromised situations and patients, all efforts have to be considered for successful healing of reconstructions of hard and soft tissues.



Surgical considerations on Paper I and IV

A frequently asked question from colleagues has been whether or not PRP is *worthwhile*?

There is obviously not a simple answer; the literature is most contradictory as reviewed earlier. Regarding bone healing with autogenous bone grafts, this thesis support to the use of PRP together with particulated bone, but the effect may be at the short term only (Paper II). The effects of PRP may also have contributed to the efficient soft tissue-healing seen in the study group from Paper I, where however no data could be presented regarding the differences with or without PRP. Regarding the use of PRP in conjunction with placing of implants, the results of Paper III and Paper V do not support this combination.

In general, follow-up of the grafting procedures in Paper I were free from serious events in the patients and the infection rate was very low. An observation from the first study was that particulating the bone made the handling of the grafts easier, which has been previously advocated in other studies[4, 293, 413].

Sinus lifts have been performed and reported on for over a quarter of a century[58] and are a routine procedures prior to installing implants in the posterior part of the maxilla. High success rates can be reached with a 2-stage grafting procedure as well as grafting installation of implants simultaneously (with either autogenous bone and bone substitutes, or a combination). In Paper IV, the focus was on two important topics: the importance of whole blood in contact with a titanium surface and the reduction of morbidity when the additional grafting was left out. How efficient the method used in Paper I ever was, the morbidity of autogenous bone grafting was still very considerable for the patients. Therefore, strengthened by results of Paper III, it was exciting to see the results of Paper IV, where successful use of whole blood without grafting material was combined with implant installation. Bone formation paralleled the integration of the implants, saved the patients time and minimised their suffering from an additional site of grafting. In Paper IV, we could confirm the results from other similar studies with this fairly novel technique[440-443].

The clinical studies in this thesis (I and IV) represent two very contrasting conditions for the patient study groups involved. Implant survival is high in both investigations after a minimum observation period of one year, but the way they were reconstructed were the very opposite. The evolution of methods to treat patients with less morbidity is of great interest to the surgeon involved, and for reconstruction of the posterior edentulous maxilla, the "Lundgren-method"[442] used in Paper IV, is a step forward. Significant evidence for this method was recently presented by Palma et al.[443] in a primate experimental model aimed at comparing the histologic outcomes of sinus membrane elevation and simultaneous placement of implants with and without adjunctive autogenous bone grafts. In that study, the results showed no differences between membrane elevation with grafting and the non-grafting technique regarding implant stability (RFA at installation and after 6 months), and histomorphometric measurements such as BIC, bone area in threads and bone area within and outside implant threads. The

researchers also found a difference in the degree of healing (BIC and bone area within threads were enhanced) when using a modified oxidized surface, compared to a turned surface of the control implant. The findings in our Paper IV and in previous studies[441, 442] are supported by results in this well designed study (unfortunately with too few studied animals).

Furthermore, Paper I aimed at reconstructing the entire atrophied maxilla including the anterior part, before installing implants. The study described the surgical method of particulating the bone graft also as an onlay in the anterior part of the maxilla, and the method worked out well for the outcome of patients. However, the study may more illustrate the advantages of a surgical method of particulating the bone graft, rather than the use of PRP, as the results were similar in the control side of the split-mouth setting.

The volumetric changes of these bone grafts over time are not yet evaluated. The question of difference in the resulting bone volume between a healed block graft and a corresponding particulated one (after 6 months of healing) is interesting, since one could argue the superior revascularisation ability and density of the particulated graft vs. the block graft fixed with plates and screws. The difference in width in anterior grafting of the maxilla and the remaining bone in the sinus are therefore two different issues. Johansson et al.[444] performed volumetric analyses in ten edentulous patients grafted with anterior block bone onlays and particulated bone sinus inlays. The changes of bone volume in the study were dramatic, as the inlays were reduced by an average of 49.5 % and onlays with 47% of the initial bone volume after 6-7 months of healing. The coefficient of variation was 0.79 in the onlay group and 0.38 in the inlay group. The predictability of grafting with autogenous bone in these situations could, therefore, be considered to be very low. The need for adjunctive materials or techniques to sinus lifts have evolved, e.g. mix of the autogenous bone with bone substitutes[445] or PRP added to autogenous grafting. Methods to control the resorption in width in onlay grafting also need to be more elucidated.

In the paper by Palma et al.[443], the osteoinductive potential of the Schneiderian membrane, or the mucosal lining, was demonstrated in the dog model used. Healing time in this study was 6 months. De novo bone formation was found in close contact with the membrane in the elevation only group. It was also demonstrated that the use of an implant with a modified oxidized titanium surface, resulted in far better results than the turned control in both of the study groups (membrane elevation with or without bone grafts). In the membrane elevation only group, BIC was 37.3 % (SD 8.5) for oxidized implants and 14.3 % (SD 15.1) for turned implants. In the bone graft and membrane elevation group, the corresponding figures were 44.7 % (SD 12.9) and 17.6 % (10.1), respectively. Bone area in threads was in favour of oxidized implants in the membrane elevation group and oxidized implants also displayed slightly higher figures in the bone grafts and membrane elevation group. Total bone area, or bone area in a rectangle outside the implant, was 45.4 % (SD 4.6) for *both types* of implants combined in the membrane elevation group and 38.5 % (SD11.6) in the combined graft-membrane elevation group. However, no statistical analysis was performed due to a low number of animals. Bone formation was also different in the implant groups. The oxidized

surface stimulated direct bone formation on the surface, whereas the turned implants integrated with bone trabeculae growing from the periphery towards the surface. Interestingly, addition of bone to the sinus compartment resulted in less bone formation. Bone particles could in the microscope bee seen encapsulated by fibrous tissue. The amount of bone in the sinus cavity floor where the implants were installed was cortical in character and provided an initial mean of 2.2 mm (SD ±1.1) of bone.

In Paper IV of this thesis, remaining bone height in the patient study group varied because of the anatomy of the maxillary sinus, as more bone can be found to the anterior. However, 23 out of 44 implants were placed in bone of 2-4 mm in height. The resulting bone gain was pronounced in the sites where baseline bone was small. Palma et al. saw a collapse of the Schneiderian membrane in between the implants in both groups; this seems also to be the case in patients of Paper IV and also of the bone grafted patients in Paper I (radiological observation in study I, no data provided here).

The occurrence of perforation of the sinus mucosal lining during sinus lift surgery is also of importance. The incidence of perforations of the membrane in Paper I was 12/38 and in Paper IV, 11/27 (32% vs. 41%, respectively). This difference may be accounted to the use of a round burr when making the bone window in the anterior sinus wall in Paper I and an oscillating saw in Paper IV. In the latter Paper, the importance of a replaceable window (with the need of a small cut in the bone) is stressed and with this technique, lacerations of the membrane may more often occur during dissection when the bone window is freed from the mucosal lining. However, the impact on results in both Papers remains unclear. A potential advantage with PRP in this situation could be the power of the clot to maintain the added graft material.

Favourable sinus health (based on radiology and anamnestic data) in follow-up were, however, also evident in all patients of both Papers I and IV. Jung et al.[446] recently evaluated the significance of perforation of the Schneiderian membrane during implant installation in the sinus floor. Implants were allowed to penetrate up in the maxillary sinuses of eight dogs. The implants were placed so that 2, 4 or 8 mm of the implant surface could be seen in the bottom of the sinus, as observed through the bone window and the intentionally made laceration of the membrane. The laceration was later closed with fibrin glue. The dogs were killed after 6 months of healing. No signs of sinus disorder were seen in the dogs, also verified with CT-scans after six months. Implants penetrating with 2 mm into the sinus showed overgrowth with a new membrane. This new covering membrane (called a functional barrier) was not seen in the 4 and 8 mm groups, but the membrane was here found, without inflammatory signs, more to the base of the implants with direct attachment to the titanium implant surface. Debris was collected on the exposed implant surfaces of the implants with 4 and 8 mm exposed in the sinus. However, this did not seem to alter the integration in bone, which was without objection in all implant sites regardless of length of implant in sinus, or regarding the non-inflammatory situation in the neighbouring mucosal lining. This study supports the findings of Paper IV,

where the perforations were comparably frequent. The maxillary mucosal lining in humans has a thickness between 0.13 and 0.5 mm (compared to 0.6 and 1.5 mm in the dog)[447]. If a perforation occurs, it might not be devastating to the operation. The amount of bone formed from the procedure may probably be less, if no attempt is done to repair the laceration.

Cessation of smoking was recommended for patients entering the studies in Paper I and IV. At the 1-year follow up, only 4 of 12 patients in Paper I was still smokers. This fact is a positive health side effect that has to be recognised when treating patients with dental implants.

Esposito et al.[201] reviewed and identified the biological factors associated with failures of oral implants: "medical status of the patient, smoking, bone quality, bone grafting, irradiation therapy, parafunctions, operator experience, degree of surgical trauma, bacterial contamination, lack of preoperative antibiotics, immediate loading, non-submerged procedure, number of implants supporting a prosthesis, implant surface characteristics and design". Levin et al.[448], who reported on a patient group of 81 patients that had a single molar replaced with implants over a ten year period (mean follow-up time of 36 months), found no correlation between smoking habits and failure or complications. Roos-Jansaker et al.[449] discovered a significantly increased amount of peri-implant lesions around implants in smokers, but could see a significant number of losses in a patient group of 218 patients seen after 9-14 years after implant placement[11].

Jemt and Hager[450] identified a group of "implant cluster-failure" patients (17 out of 1267 patients) in a retrospective study. The included patients had failing implant-supported prostheses in the edentulous maxilla within the first 3 years of follow-up. Apart from bone quantity, that was a significant contributor to loss of implants, smoking habits and bone-loss related to periodontitis in the lower jaw were more frequent in the study group, but however not in a significant number.

In previous studies where grafting of the alveolar processes has been undertaken, the need for smoke reduction has been advocated[78, 252]. Levin et al.[451] reviewed 143 operations over an 8 year period where 64 onlay bone grafts and 79 sinus lift operations were performed. Complications in onlay bone grafting (e.g. haematoma and swelling) were more frequent in smokers (50 vs. 23.1 %), as well as there was a correlation found between smoking and post-operative problems. These problems were regarded as major, when grafts were found mobile or exposed through the gingiva. In the sinus lift group, however, smoking did not influence the results.The long-term survival rates of implants placed simultaneously with sinus grafting in smokers and non-smokers were prospectively compared by Peleg et al.[452]. In two large patient groups of 226 smokers compared to control of 505 non-smokers, the negative influence of smoking on cumulative survival of implants was not verified statistically. However, these authors advocated a smoking cessation protocol. In another study of simultaneous implant installation and sinus lift without graft material in periodontally compromised patients, smoking doubled the risk of implant loss long term over 10 years[440].

Granström[453] evaluated implant treatment in irradiated, oncologic "head and neck"

patients. Of 107 irradiated patients, 55 were smokers and 6 were ex-smokers. This study did, however, not show a convincing significant relation between groups regarding implant survival rate and smoking (smoking amount $P>0.30$ and smoking time $P=0.28$).

Considerations on methods and analyses, Paper I and IV.

An obvious weakness of Paper I was the lack of randomisation between test and control sides. However, the amount of bone placed in each sinus and on the anterior part of the maxilla of test and control sides were registered. Slightly more bone was placed in the test side (Table 4 in Paper I). The implant lengths can be seen in Table 5 in Paper I (8 implants for each patient) and there were no major differences between the numbers in length when comparing test and control.

Only two implants were lost, both recognised as mobile and removed at abutment connection after 6 months of healing, in the two male patients in Paper I. The 1-year control worked out well for all patients; presently in 2006, half of the patients have been seen and followed up for the four to five year control. No further implant losses have been encountered in these patients.

RFA was a fairly novel technique of evaluating implant stability at the initiation of study I. Different transducers had to be manufactured to fit the situations in the three occasions of measuring; at implant placement and at abutment placement and with the different permanent abutments in place at the 1-year control. The plot analysis for all implants followed the general conception of RFA[431, 454], with decreasing stability for initially very stable implants over 1 year, and increasing stability for functionally less stable implants followed over the first year after placement. The placement of the implants was slightly superficial to the bone, in fear of interference from the marginal bone on the measurement procedure in recording RFA. This might have influenced the results in a way, as an early RFA study by Rasmusson et al.[455]disclosed the importance of the marginal bone covering the implant, and that longer implants will not increase the positive results of RFA.

Interestingly, a correlation between the RFA measurements and the histomorphometric data of bone anchorage could not be established in a dog mandible study, recently published by Schliephake et al.[456]. In Paper I, significantly higher values were found in implants placed in autogenous bone treated with PRP at abutment and 1-year control. If however, the anterior and posterior maxilla were evaluated, the only significant finding was for the PRP grafted bone placed in the anterior part of the maxilla (Figures 5 and 6, Paper I). The posterior sites, with implants placed in grafted sinuses with or without PRP, displayed no beneficial effect of PRP compared to control. Regarding the marginal conditions evaluated with radiology, no significant contribution was made of PRP on the amount of bone resorption around implants.

Lundgren et al. (2004) confirmed bone formation in their study by pre- and postoperative CT. They used RFA (average residual bone height 7 mm, range 4-10 mm) for stability measurements and this resulted in mean ISQ values of 65, 66 and 64 at placement, abutment

connection and at 1- year follow up. In Paper IV, the mean residual bone height was 4.6 mm (range 2-9 mm). RFA was not used in this paper, but would be interesting to perform in a similar study group, where values of remaining bone height (as in PaperIV) are lower compared to the ones in the study by Lundgren et al.[442]. In Paper IV, radiological control was done with dental films *and* orthopantomograms, due to the projection problems with dental films in a resorbed maxilla leading to interference with the hard palate for the placement of the film. The handling of digitally computed radiographs from the modern orthopantomogram allows for analysis of the results comparing new bone with surrounding mature bone (however no data provided in the Paper IV). More definite radiological analysis and a histological evaluation of the bone formed would have strengthened the study, but the surgical method as whole is supported also elsewhere[443], as previously discussed, by histology and radiological evidence.

The concentration of platelets in patients in the first study group in Paper I displayed a wide range. Only in the second part of the patients, the method delivered the expected concentration rates and this affected the possibility to analyse the whole group of patients in Paper II. The wide range of results from attaining PRP, may be due to a learning curve among staff involved. Methods of today are better validated and deliver a level of platelets in PRP of expected values of 4-5 times the baseline count in whole blood. The author has compared the achieved results of the Medtronics Sequestra 1000 gradient density cell separator used in study I and the 3i Platelet Concentration Collection System (PCCS) and published them in another Paper[8]. The mean concentration in PRP was 2.8 and 4.3, respectively for each system, indicating the superiority of the latter system in this aspect.

In contrast, the group of Weibrich et al. [301] could see differences in comparing systems (Blood bank and Curasan PRP Kit) regarding PRP platelet counts, WBC counts and GF levels. Efficient concentration of platelets (Blood bank) accounted for the high levels of TGF-β found in PRP and high levels of leukocytes found in another system (Curasan) accounted for high levels of PDGF-AB. Similar levels of IGF-I were found, but was speculated to only reflect the similar amount of plasma provided in PRP of each approach. The importance of analysing the total contents regarding blood cells of the actual PRP used has therefore eventually become very important in the literature.

Paper II

Since the results of platelet concentration in Paper I affected the outcome of the available samples for analysis in Paper II, the numbers were reduced. However, interesting results of the histomorphometric analysis were at hand. The 3 months biopsies with PRP demonstrated a significantly higher mean value for newly formed bone (P=0.03) compared to control. The amount of "old bone removal" in the PRP biopsies was higher (lower values of the presence of old bone in Table 4 of Paper II). However, not significant (P=0.063), these values are supportive of findings by Gerard et al.[360], who in a mandibular defect model in dogs saw that "early enhanced healing (by PRP) occurred by increasing the amount of non-viable grafted

bone that was removed and increasing the amount of new bone that was formed. PRP did not change the rate at which new bone was formed, and no increase in trabecular density was realized in these grafts." Furthermore, the evaluation of the micro-implants inserted at 3 months and retrieved at 6 months In Paper II, showed that similar results in bone healing were reached between test and control sides at this time.

Considering the design of Paper II, it may have been wiser to have left out the micro-implants and only secure a second bone biopsy, without micro-implants, at 6 months. The insertion into particulated, somewhat immature, bone grafts at 3 months was difficult, as the corticalisation of the bone graft was very not complete. The histological analysis of these samples taken out at 6 months was further complicated by the surgical trauma to the bone, caused by the burr inserted into the 3-months healed bone graft when placing the micro-implants. Therefore, the difficulty was evident in observing the different bone structures of old bone and new bone formation adjacent to the micro-implant, when studying the light microscopic slides after retrieving samples at 6 months.

A lot of effort by the involved laboratories was put into the new enzymatic analysis[25] of bone biopsies at 3 months. However, samples were too few for quantification and allow comparisons of results between test and control. Results from PRP samples were encouraging with bone remodelling activity found in bone and adjacent tissue. The staining used, indicated the presence of both ALP and ACP. ALP positvity (stained blue) was found on bone surfaces (osteoid and osteoblastic activity) as well as inside bone (osteocyte activity) and in soft tissues in adjacent cavities. ACP (stained red) was found on bone surfaces, indicating osteoblastic activity. Another interesting aspect of further studies would be the evaluation of vessel in growth in the bone graft treated with PRP; this was not accomplished in this study.

Paper III

The slide chamber model offers a unique *in vitro* possibility for the testing of the thrombogenic properties of biomaterials due to the coating technique with heparin (offered by Corline Systems, Uppsala). Clues to the effect of PRP combined with materials used in implantology can be gathered with this technique, as the first encountering moment between the implant and the tissues of the body is with whole blood.

In this paper, the thrombogenic properties of PRP were evaluated. Using the generation of TAT (thrombin formation) and the release of β-TG (activation of platelets) to illustrate this, we could show that PRP and PPP were inferior to whole blood in this in vitro model. Furthermore, the activation of the contact system as reflected by formation of several coagulation factor-inhibitor complexes was also best pronounced in whole blood.

Experimental removal of the RBCs in this study, achieved in the preparation of PRP and also PPP, therefore resulted in an inferior thrombogenic response compared to whole blood. We argued that erythrocytes may be active in the amplification of thrombin generation and be triggers of the coagulation system as the cells expose negatively charged phospholipids in their cell membranes and elastase on the membrane[157, 457]. A sufficient generation of thrombin is important for the onset of the coagulation and also for osteogenesis, as it exhibits a differential motogenic effect *in vitro* on osteogenic cells depending on their differentiation state[458]. Kark et al.[459] also showed that products released from thrombin-activated platelets increases the migration and proliferation of of osteogenic cultures of bone marrow cells. The response to a titanium surface starts with a low-grade generation of thrombin. This generation is triggered by F XII, is erythrocyte dependent, and activates platelets. Thereafter follows the activation of the platelet-dependent loop that augments further thrombin generation. The release of β-TG in whole blood correlates with the release of PDGF.

Mineralisation in osteoblastic cells is affected in a positive manner by short initial exposure of PDGF, as a continuous exposure is inhibitory to the cells[460]. Consequently, it is probably important to include RBCs in PRP, and to evaluate also effect from leukocytes; however the effect in humans may be different as compared to our and others *in vitro* studies.

Paper III and IV are kindred to each other, in a sense that using the thrombogenic properties of the whole blood with titanium is effected in the secluded maxillary sinus compartment in the patients of Paper IV. We tried to discuss the relevance of this in Paper III and to connect basic research with clinical practise (of importance to both sides of researhers). Furthermore, in the second part of Paper III, we explored different titanium surfaces and evaluated their ability to activate blood coagulation. New modifications of titanium surfaces[195] are frequently presented by implant companies. The TiO_2 grit blasted and hydrofluoric acid etched surface that exhibited the most interesting results in our study is an example of relevant ideas from basic bone biologic science that seem to have a positive effect on osseointegration in the real situation. We speculated that the mechanisms behind the pronounced activation of coagulation in this modification depended on increased negative charge on the fluoride modified test surface.

The differences in topography at the nanostructure level between the two similar surfaces (TiO_2 grit blasted, with and without hydrofluoric acid) may also be of importance, but is unknown by the author.

Paper V

The effects of PRP regarding new bone formation or BIC in the dog peri-implant defect model used were not convincing in this Paper. On the contrary, the modified implant surface seemed to play a more important role than the use of either PRP or whole blood in the defect. If our observations are correct, this study supports the findings of Paper III. This directs us to the importance of surface modifications of implants, currently a well investigated topic.
The findings of this study are in corroboration with Casati et al.[353] that in study found no effect of adding PRP to buccal dehiscence-type defects around implants placed in the mandibles of dogs.

The animal model in Paper V has been documented extensively[392]. We did however encounter serious problems in our ambition to estimate the release levels of the different GFs with commercially available EIAs in activated PRP during our experiment. To make progress in this area, the use of customised EIAs for the species engaged must probably be constructed. The use of other functional assays, such as the Cytometric bead array (CBA) technique[461] or the Luminex suspension array technique[462], where many proteins may be detected and quantified simultaneously, may be of value in later studies. However, with the TGF-β ELISA-kit, it was possible to do a minor correlation observation between platelet counts and release of this GF.

Considerations on PRP in general

As there are possibilities with the use of PRP, there are also stated limitations of this method.

As found in the results of Paper II, PRP as an additive procedure in autogenous bone grafting may be of short term benefits[332, 360, 418]. The method requires living target cells to impose some beneficial action. The osteoinductive capacity of PRP is low or non-existing as stated in several articles[4, 59, 379, 420, 421] and also shown in our Paper V.

Since the studies with high scientific value in this field are performed in a wide range of models, the evaluation of the potential benefit for patients is proven difficult. The first step towards knowledge in this field was the discussion of platelet counts. Reports on this topic have stated that there is a "window" of therapy, where the level of GFs is optimal. This may vary between species; a low number of platelets may be without effect on bone healing and a high number may be inhibitory (platelet counts of 1,000,000/mL were recommended for bone regeneration around implants in one dog study)[312]. The method has drawn heavy attention from the industry of medical technology. The devices used must be properly validated as indicated in studies on this topic, since the processing of PRP may alter the contents of the final product immensely, e.g. premature activation of platelets and different cell counts regarding WBCs and RBCs. In daily practise, there is also a need for education of assisting staff and considerable financial aspects as the treatment is expensive.

Furthermore, a debate of the safety issue of using bovine thrombin has been presented in the literature. Initial reports on the risk of PRP to impose immunological cross-reactions[463] from antibovine antibodies with human clotting factors[464] motivated a discussion in the literature. The bovine thrombin is no longer in major use, since the autologous thrombin may be extracted from PRP using a glass container, CaCl and a small piece of the bone graft, where a gel is formed and contracts. The exudate around the contracted clot contains the thrombin needed[465]. Alternatives to bovine and human thrombin are present[296, 466, 467] including the polysaccharide chitosan (modified hyaluronic acid and e.g. extracted from shells of shrimps).

The action of thrombin activation on PRP has been subject for several studies including gel formation characteristics[468] and thrombin concentration effects on GF release[467]. The effect of systemic drugs on platelets is of relevance to this area of research, but will not be penetrated in detail here. However, the use of antiplatelet substances (prostaglandin E1, aspirin, apyrase) in concentrating platelet GFs in PRP was used and resulted in a 400% increase[469] in the release of TGF-β and PDGF. Munsterhjelm et al.[470, 471] investigated the effect of commonly used analgesics on platelets and found that high doses of paracetamol and a combination of diclofenac and paracetamol can cause platelet inhibition and may increase risk of surgical bleeding.

In conclusion, and as a result of the overview presented earlier, results from studies are conflicting and ambiguous. Grageda[261] proposed more conformed studies when evaluating PRP and bone grafts. It is easy to jump between conclusions of PRP studies, but cautiousness is important in evaluating results made in cellular, animal and human research models.
An outlook into the future may be that the relationship between the potential effects of PRP in a dose dependent way on cell proliferation in experimental cell cultures may be further evaluated. PRP is said to be a potential inhibitor of rhBMP-2 action as PRP *enhances cell proliferation and migration* of cells *in vitro* but *decreases the osteogenic differentiation*[330-332]. On the other hand, BMP-2 led to an opposite cell response and induced the highest ALP activity and mineral deposition in a cell culture[341]. The clinical use of rhBMP-2 is however far from common, as a large project concerning the use of rhBMP-2 in an absorbable collagen sponge for sinus augmentation, only was a partial success[472]. Availability and alterations in the product are problems that have to be further developed.
Another focus for research, as stated previously, will be further development of bioactive implants, a fact partly supported in this thesis.

CONLUSIONS

1. PRP in conjunction with grafting of particulated bone to the severely resorbed edentulous maxilla did not improve the integration and clinical function of implants in patients after 1 year of follow-up.

2. Significantly more new bone was observed in biopsies of maxillary sinuses grafted with autogenous bone and PRP compared to controls without PRP after 3 months. No differences were found in biopsies with bone and micro implants, installed after 3 months of healing and retrieved after additional 3 months of healing.

3. *Whole blood, not PRP*, displayed significantly stronger activation of the coagulation system, with respect to generation of thrombin and platelet activation, tested *in vitro* in a heparinised slide chamber model.

4. A modification of the titanium surface with hydrofluoric acid, tested in *whole blood* in the slide chamber, showed a significantly pronounced thrombogenic response with respect to generation of thrombin, platelet consumption and activation markers of the intrinsic coagulation system. In addition, activation of the complement system was significantly lower with this surface compared to controls.

5. Consistent bone formation was found around implants in patients after sinus mucosal lining elevation and simultaneous implant installation without grafting of autogenous bone evaluated after a minimum of 1 year.

6. PRP displayed no significant difference in bone regeneration around implants in a peri-implant defect model in dogs compared to whole blood.

7. A significantly higher amount of bone fill was found at implants with a hydrofluoric acid treated surface compared to untreated control implants in a peri-implant defect model in dogs.

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