Diploma thesis

Evaluation of vitamin D and bone markers in patients with hepatitis B infection with and without tenofovir treatment

Submitted by
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Affidavit

Herewith I, Martina Erath, declare that I have written the present diploma thesis fully on my own without any assistance of third parties. Furthermore, I confirm that no sources have been used in the preparation of the thesis other than those indicated in the thesis itself.

Graz, February 2016 Martina Erath eh

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Abbreviations

AFP	Alpha fetoprotein
ALT	Alanine aminotransferase
AP	Alkaline phosphatase
APC	Antigen-presenting cell
AST	Aspartate aminotransferase
BMD	Bone mineral density
BMPs	Bone morphogenetic proteins
BMU	Basic multicellular unit
bp	Base pairs
BSAP	Bone-specific alkaline phosphatase
BSP	Bone sialoprotein
BTMs	Bone turnover markers
cccDNA	Covalently closed circular DNA
CD	Cluster of differentiation
CHB	Chronic hepatitis B (infection)
CTLs	Cytotoxic T lymphocytes
CTX	Carboxyterminal cross-linked telopeptide of type I collagen
DD	Differential diagnosis
DKK1	Dickkopf-related protein 1
DNA	Deoxyribonucleic acid
DPD	Deoxypyridinoline
ECL	Electrochemiluminescence
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ETV	Entecavir
FGF	Fibroblast growth factor
FSH	Follicle stimulating hormone
GCs	Glucocorticoids
GGHL	Glycosyl-galactosyl-hydroxylysine
GH	Growth hormone
GHL	Galactosyl-hydroxylysine
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HAV	Hepatitis A virus
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HDV	Hepatitis D virus
HBcAg	Hepatitis B core antigen
HBeAg	Hepatitis B e antigen
HBsAg	Hepatitis B surface antigen
HCC	Hepatocellular carcinoma
HIV	Human immunodeficiency virus
HLG	Hydroxylysine-glycosides
HPLC	High-performance liquid chromatography

Hyp	Hydroxyproline
IFNa	Interferon alpha
IGFs	Insulin-like growth factors
IL	Interleukin
IU/ml	International units per milliliter
M-CSF	Monocyte/macrophage colony-stimulating factor
MHC	Major histocompatibility complex
MSC	Mesenchymal stem cell
ng	nanogram
n.s.	not significant
NAs	Nucleos(t)ide analogues
NF-κB	Nuclear factor κB
NTX	Amino-terminal cross-linked telopeptide of type I collagen
OC	Osteocalcin
OPG	Osteoprotegerin
ORF	Open reading frame
P1CP	Carboxy-terminal propeptide of type 1 procollagen
P1NP	Amino-terminal propeptide of type 1 procollagen
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PEG-IFNa	Pegylated Interferon alpha
pg	picogram
pgRNA	Pregenomic ribonucleic acid
PTH	Parathyroid hormone
PTHrP	Parathyroid hormone-related protein
PYD	Pyridinoline
RANK	Receptor activator of nuclear factor-κB
RANKL	Receptor activator of nuclear factor κB ligand
rcDNA	Relaxed circular DNA
SCL	Sclerostin
SVP	Subviral particles
TDF	Tenofovir disoproxil fumarate
TGF-β	Transforming growth factor-β
TNF-α	Tumor necrosis factor α
TRAP	Tartrate-resistant acid phosphatase
TSH	Thyroid-stimulating hormone
ULN	Upper limit of normal
VEGF	Vascular endothelial growth factor
WHO	World health organization

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1 ABSTRACT

1.1 Abstract English

Background: Increased bone turnover rates as well as vitamin D deficiency and insufficiency have been observed in HIV-infected individuals receiving tenofovir disoproxil fumarate (TDF). There is a lack of data regarding the effects of TDF use on bone markers and vitamin D in HBV-infected individuals.

Objectives: The aim of the present pilot study was to evaluate bone markers and vitamin D levels in HBV-infected patients with and without TDF therapy.

Material and methods: A total of 41 HBV-infected patients attending the Division of Gastroenterology and Hepatology at the Medical University of Graz were enrolled including 23/41 patients with a serum HBV DNA level of ≥2000 IU/ml eligible for antiviral treatment (TDF Group) and 18/41 patients with a serum HBV DNA level of <2000 IU/ml (Non-TDF Group). TDF Group members received a daily dose of 300mg TDF. In this pilot study, the levels of 25-hydroxyvitamin D [25(OH)D], C-terminal cross-linked telopeptide of type I collagen (CTX), osteocalcin (OC), procollagen type I N-terminal propeptide (P1NP), and osteoprotegerin (OPG) were determined at baseline (BL) and followed-up three months thereafter (FU).

Results: In TDF Group members, significant increases in serum levels of CTX (+46%) and OC (+24%) were observed three months after TDF initiation. High rates of vitamin D deficiency and insufficiency were found in both study groups at BL (TDF 87%, Non-TDF 61%) and at FU (TDF 87%, Non-TDF 67%), especially when blood was drawn in the winter season. There were no significant changes in both study groups regarding the serum levels of P1NP and OPG.

Conclusion: Given the high rates of vitamin D deficiency and insufficiency found in both study groups it might be reasonable to screen HBV-infected individuals for hypovitaminosis D and to consider vitamin D and calcium supplementation. Prospective studies are required to evaluate whether the early increase of bone turnover observed in the present study is a short-term effect or a predictor of future bone loss.

1.2 Abstract German

Hintergrund: Sowohl erhöhte Knochenumsätze als auch Vitamin D–Mangelzustände wurden bei HIV-infizierten Personen unter Therapie mit Tenofovir Disoproxil Fumarat (TDF) beobachtet. Die Datenlage bezüglich der Auswirkungen von TDF auf Knochenmarker und Vitamin D bei HBV-infizierten Personen ist spärlich.

Ziel: Das Ziel der vorliegenden Pilotstudie war die Evaluierung von Knochenmarkern und Vitamin D bei HBV-infizierten PatientInnen mit und ohne TDF-Gabe.

Material und Methoden: Insgesamt umfasste die Studie 41 HBV-infizierte PatientInnen der Abteilung für Gastroenterologie und Hepatologie an der Medizinischen Universität Graz, darunter 23/41 mit einer HBV-Serumkonzentration von ≥2000 IU/ml und Indikation zur antiviralen Therapie (TDF Gruppe) und 18/41 mit einer HBV-Serumkonzentration von <2000 IU/ml (Non-TDF Gruppe). Mitglieder der TDF Gruppe erhielten eine tägliche Dosis von 300 mg TDF. In dieser Pilotstudie wurden die Parameter 25-Hydroxyvitamin D_3 [25(OH)D_3], Carboxyterminales Kollagen Typ I-Telopeptid (CTX), Osteocalcin (OC), Prokollagen Typ 1 Aminoterminales-Propeptid (P1NP) sowie Osteoprotegerin (OPG) zu Studienbeginn (BL) sowie nach drei Monaten (FU) bestimmt.

Ergebnisse: Innerhalb der TDF Gruppe zeigten sich signifikante Erhöhungen der Serumspiegel von CTX (+46%) und OC (+24%) nach dreimonatiger Behandlung mit TDF. In beiden Studiengruppen konnte sowohl zur BL (TDF 87%, Non-TDF 61%) als auch zur FU (TDF 87%, Non-TDF 67%) eine hohe Rate an Vitamin-D Mangelzuständen beobachtet werden, insbesondere im Winter. Bezüglich der Parameter P1NP und OPG ergaben sich keine signifikanten Veränderungen innerhalb beider Studiengruppen.

Fazit: Angesichts der hohen Rate an Vitamin D-Mangelzuständen könnte es sinnvoll sein, HBV-infizierte Personen bezüglich Vitamin D-Mangel zu screenen und bei Bedarf Vitamin D und Kalzium zu supplementieren. Prospektive Studien sind erforderlich, um zu beurteilen, ob es sich bei der beobachteten Steigerung des Knochenumsatzes um einen vorübergehenden Effekt handelt, oder ob diese Veränderungen prädiktiv für einen zukünftigen Knochenverlust sind.

2 INTRODUCTION

2.1 The Hepatitis B Virus

Hepatitis B virus (HBV) is the prototype of the hepadnavirus family, which includes the genus orthohepadnavirus of mammals and the genus avihepadnavirus of birds. Hepadnaviruses are characterized by hepatotropism, a high degree of host specificity, and the ability to cause acute and chronic hepatitis B (CHB) infection (1). Some facts about HBV infection are summarized in Table 1.

Table 1
Facts about HBV infection.

- ✓ More than 2 billion people have been infected with HBV.
- ✓ 350 to 400 million people are chronic HBV carriers.
- ✓ 70 to 90% of infants and 5 to 10% of adults infected with HBV remain chronically infected.
- ✓ CHB infection causes 60 to 80% of the world´s primary liver cancer.
- ✓ Up to 1.2 million deaths per year are related to CHB infection.

2.1.1 Epidemiology and geographical prevalence

Approximately two billion people, almost one third of the world´s population, show serological evidence of past or present HBV infection making it to one of the most common infectious diseases globally. It is estimated that 350 to 400 million people suffer from chronic HBV (CHB) infection, which can lead to cirrhosis, liver failure, and hepatocellular carcinoma (HCC) (Fig. 1) (2). The risk of developing liver cirrhosis and HCC is 15 to 40% among CHB patients. About 60 to 80% of all HCC cases worldwide are caused by HBV. Each year 500,000 to 1.2 million people die due to the consequences of CHB – the 10^{th} leading cause of death worldwide. In Europe, 1.0 million people are estimated to be infected with HBV of which 90,000 will develop CHB and 22,000 are at risk to die from cirrhosis or HCC.

Fig. 1. Stages of liver damage due to CHB infection (www.livercancerfree.org).

The prevalence of CHB ranges from 0.1 to 20% depending on geographical location. A distinction is made between areas with high (HBsAg seroprevalence >8%), intermediate (HBsAg seroprevalence from 2 to 7%), and low endemicity (HBsAg seroprevalence <2%) (Fig. 2) (3). Furthermore there is an association between endemicity and characteristic of infection (Table 2).

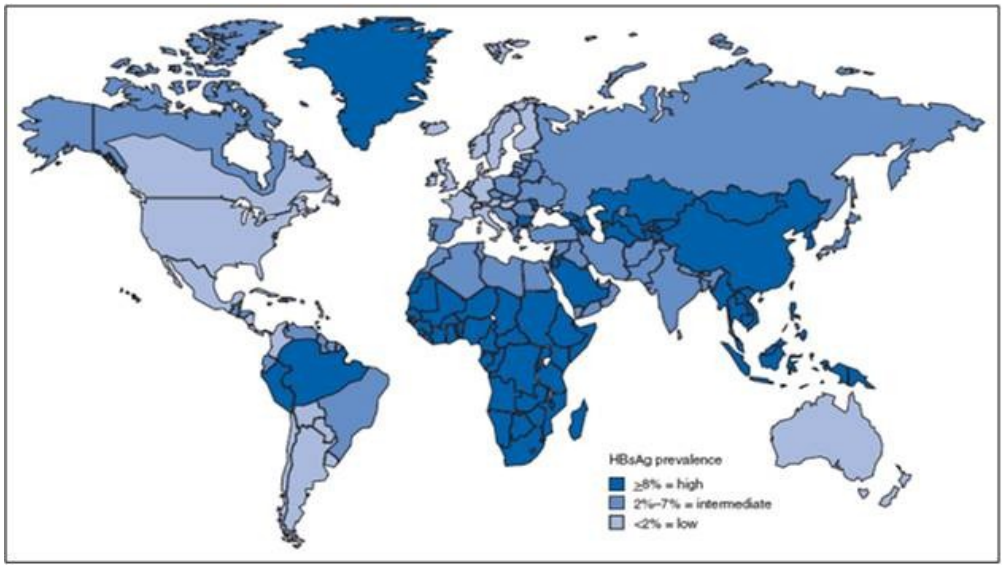


Fig. 2. Worldwide HBsAg prevalence, from (5).

Regions with low socio-economic standards including South East Asia, China, sub-Saharan Africa, and the western Amazon Basin show a high endemicity of HBV infection. 70 to 95% of the population have past or present serological evidence of HBV infection and 8% are chronic HBV carriers (2). The fact that many highly endemic countries are densely populated makes that nearly 60% of the world´s population lives in highly endemic areas (6). The degree of HBV endemicity

correlates with the predominant mode of transmission: Most infections in these areas are acquired perinatally or during infancy with a more than 90% risk of becoming chronic leading to high rates of liver cirrhosis and HCC in adults. There is only little evidence of acute disease because most infections in children are asymptomatic (2).

Regions such as Eastern and Southern Europe, the Middle East, parts of Central Asia, Japan, and parts of South America are moderately endemic – 10 to 60% of the population show serological evidence of previous HBV infection with a rate of chronic carriers ranging between 2 and 7%. Many infections occur in adolescents and adults leading to a higher evidence of acute HBV infection in comparison to highly endemic regions. Countries with intermediate endemicity have a mix of different routes of transmission: perinatal, horizontal, sexual, and health-care-related (2,7).

Regions with high socio-economic standards including the United States, Northern and Western Europe, and Australia are low endemic. Within these areas, the overall infection rate is 5 to 7%, with 0.5 to 2% being chronic carriers. Most HBV infections occur in adolescents or young adults in the main affecting well-defined high-risk groups including injection drug users, men who have sex with men (MSM), and health care workers (2,7).

Table 2
Associations between endemicity and characteristics of HBV infection, adapted from (2).

	High endemicity	Intermediate endemicity	Low endemicity
Chronic infection	>8%	2 – 7%	0.5 – 2%
Past infection	70 – 95%	10 – 60%	5 – 7%
Perinatal infection	Common	Uncommon	Rare
Early childhood infection	Very common	Common	Rare
Adult infection	Uncommon	Common	Very common

2.1.2 Morphology

The infectious HBV virion is approximately 42 nm in diameter and consists of a nucleocapsid containing the viral DNA genome and an envelope (Fig. 3).

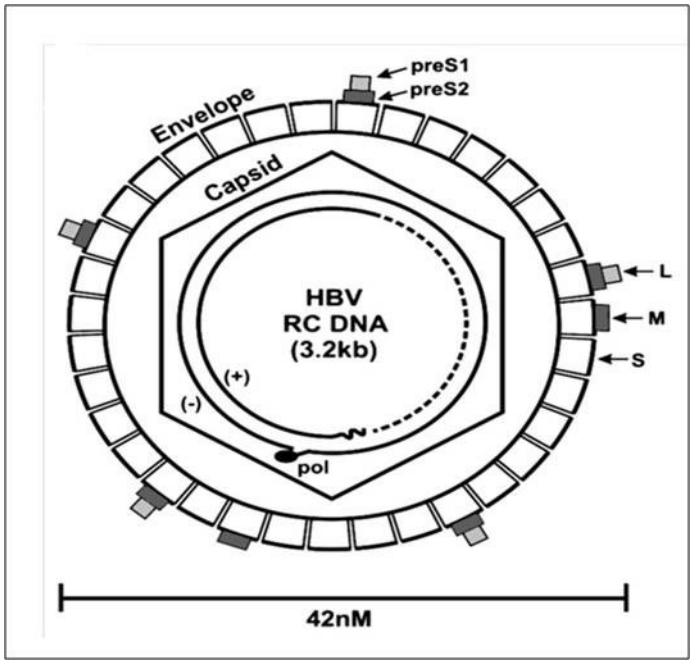


Fig. 3. The structure of HBV, from (8).

HBV nucleocapsid

The icosahedral nucleocapsid is 30 to 32 nm in diameter and contains the viral genomic DNA which is 3182 to 3248 bp in length according to the HBV genotype (9). The nucleocapsid is formed by homodimerization of two core proteins (HBcAg) followed by dimer oligomerization (10,11). An intermolecular four-helix bundle and a disulfide bond within the bundle are responsible for the stability of the capsid protein dimers (9,12). HBcAg is composed of 183 or 185 amino acid residues and shows two domains including the core domain, which is essential for the assembly of the capsid and the protamine domain, which plays an important role during the encapsidation process (8–11). The capsid shell has pores enabling the diffusion of deoxyribonucleotides during the synthesis of the viral DNA genome (10,11). Right after its formation the nucleocapsid encloses the pregenomic RNA (pgRNA), whereas secreted virions show circular, partially double-stranded DNA. This finding has led to the assumption that the viral DNA synthesis leads to structural changes in the

nucleocapsid acting as start signal for envelopment, whereas immature RNA-containing nucleocapsids are excluded from the budding process (Fig. 4) (11,13,14).

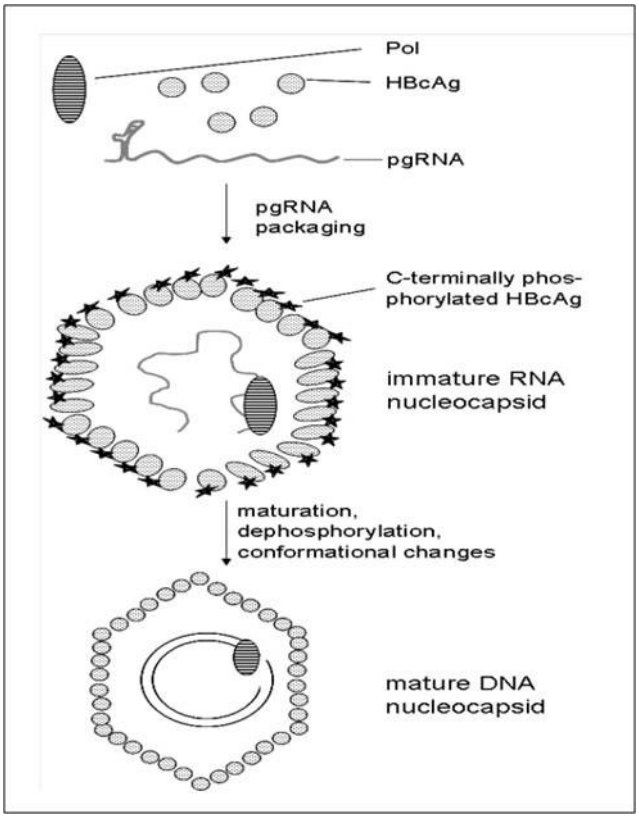


Fig. 4. Genome packaging and nucleocapsid maturation, from (14).

HBV envelope

The mechanism of envelopment is still not fully understood. It is assumed that the virions are formed by budding of the nucleocapsids through an intracellular membrane, most likely the post-endoplasmic reticulum (ER)/pre-Golgi membrane. After budding, the nucleocapsid is surrounded by a host-derived lipid bilayer containing three viral envelope proteins referred to as small (S), middle (M), and large (L) envelope protein which are synthesized in the ER (13). Because of three different start codons but one common stop codon these proteins share the carboxyl(C)-terminal S-domain and only differ by the length of their amino(N)-terminal domains (11). The major component, the S envelope protein, is also called HBsAg. The M protein shows an N-terminal addition of a sequence named preS2. It is well known that the absence of the M protein does not have any consequences concerning the viral morphogenesis or functionality but its exact function remains

unclear (10,11). The L protein shows the addition of a sequence named preS1 on the N-terminus of the M protein which is necessary for the recruitment of mature viral nucleocapsids for virion budding and the recognition of host cell receptors for viral cell entry (Fig. 5) (11). The suppression of L or S protein inhibits virion formation. In the absence of L protein the nucleocapsids return to the nucleus in order to amplify the covalently closed circular DNA (cccDNA) whereas the presence of L protein leads to the envelopment and secretion of enveloped virions (10).

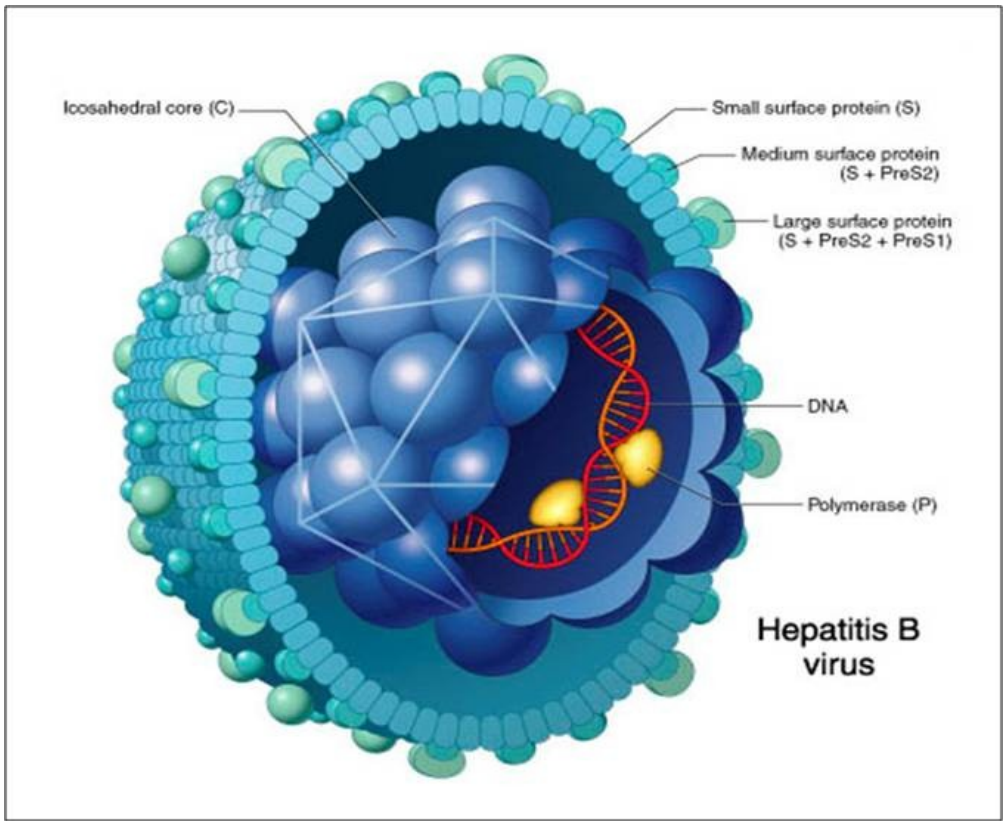


Fig. 5. Cross-section of the HBV virion (http://www.ibibiobase.com).

HBV genome and viral proteins

The relaxed circular viral genomic DNA (rcDNA) is partially double-stranded and its 5´ end of the minus-strand is covalently linked to the viral reverse transcriptase (8). The HBV genome is organized into four partially-overlapping open reading frames (ORFs) which are located on the negative DNA strand encoding distinct proteins (Fig. 6, Table 3) (9,15).

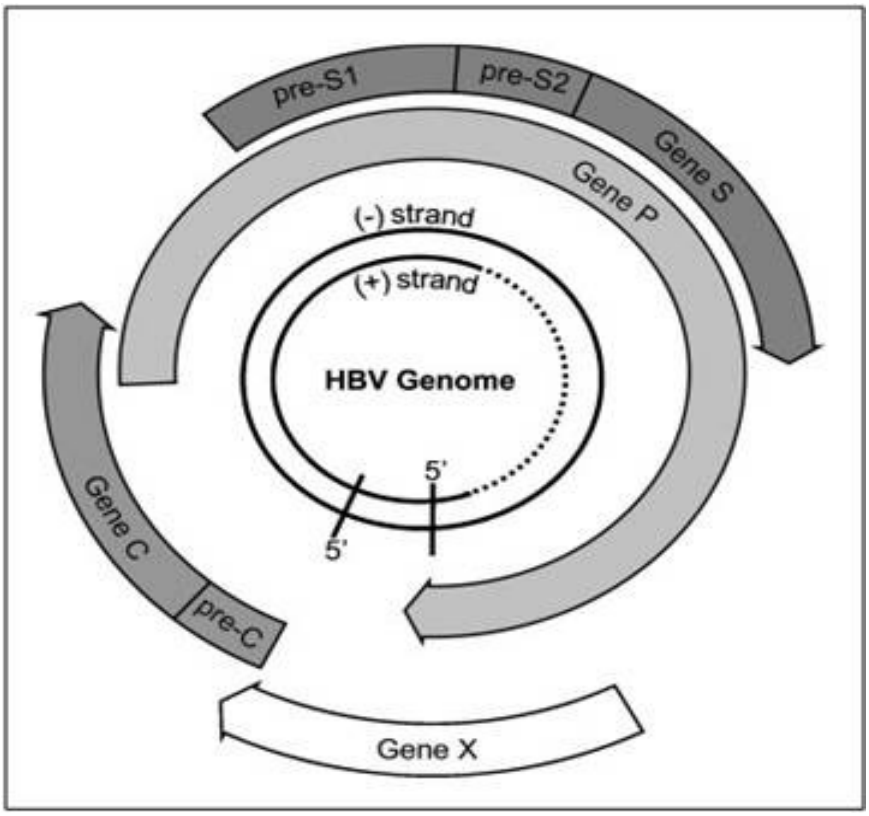


Fig. 6. The HBV genome, from (16).

Table 3
Major viral proteins and their functions.

Protein	Function
Envelope proteins: small (HBsAg), medium (M), large (L)	• Glycoproteins, located on the virion surface • Initiation of virion entry into the host cell • M: function unknown • L: key role in virion-assembly and cell release
Core protein (HBcAg)	• Encapsidation of pgRNA and partially double-stranded DNA genome in cytoplasm
HBeAg	• Synthesized as precursor protein • Marker for high levels of viral replication • Down-regulates innate immune receptors
Viral polymerase	• Polymerase-, RNaseH-, and reverse transcriptase functions; includes the terminal protein for priming
X protein	• modulates host-cell signal transduction • affects host and viral gene expression, co-factor for HCC

The preS/S region encodes the three viral envelope proteins S, M, and L which are anchored in the host-derived lipid bilayer that surrounds the mature virions (13).

The precore/core region (preC/C) encodes the core protein and its soluble, secreted form, the so called HBeAg. HBeAg correlates with viremia and is a marker for high levels of viral replication. Loss of HBeAg and appearance of antibodies to HBeAg are associated with reduced viral replication and favorable outcome. Mutations in the pre-C region, which affect many chronic HBV carriers also lead to a pseudo-loss of HBeAg but these mutant viruses are even more pathogenic than the wild type viruses. The loss of HBeAg alone thus cannot predict a favorable outcome (8). HBeAg down-regulates innate immune receptors which may allow HBV to escape immune clearance. HBeAg is able to cross the placenta and therefore acts as an immune tolerogen in utero (17).

The pol-ORF represents about 80% of the whole genome length and encodes the viral polymerase (P). The viral polymerase is multifunctional and exhibits DNA polymerase-, RNaseH-, and reverse transcriptase functions, and also includes the terminal protein for priming. It is responsible for the reverse transcription of the pgRNA for the production of the minus-strand DNA, the degradation of the RNA template, and the synthesis of the DNA plus-strand (15).

The X-ORF encodes the X protein. Its exact function is still unknown. *In vitro* tests indicate that the X protein activates the transcription of viral and cellular genes, and increases viral DNA synthesis. There is an association between the X-protein and HBV related diseases, especially concerning the prevalence of HCC in patients with chronic HBV infection. Mutations of the X gene not only lead to aminoacid changes in the X protein but may also affect other genes and modify HBV expression (18).

2.1.3 Viral lifecycle

It is well known that interactions between host cell receptors and the HBV envelope are required for viral cell entry but the specific membrane receptors of hepatocytes HBV attaches to are not yet identified. The pre-S1 domain which constitutes the N-terminal region of the large envelope protein (L) mediates the attachment of the virion to the host cell. The viral envelope is stripped off and the nucleocapsid is released into the host cell cytoplasm (8,19). After cell entry, the viral

nucleocapsid interacts with the cellular transport machinery and proceeds to the nuclear membrane (8,15,20). Interactions between two nuclear transport receptors (importin α and β) and the nucleocapsid mediate its passage through the nuclear pore complex (8,19–21). Importin β facilitates the translocation into the nucleus. The attachment of importin α to the capsid proteins leads to an exposure of a nuclear localization signal correlating with genome maturation. Only nucleocapsids containing phosphorylated capsid protein subunits and a mature genome are able to disintegrate and release their viral genome into the nucleus (20). The functional role of core protein phosphorylation is still discussed controversially but it is suggested that it is required in order to target the virus to the nucleus. After its release into the nucleus the rcDNA is repaired to form the cccDNA (19). Organized as minichromosome which consists of histone and non-histone proteins the cccDNA is able to persist within the nucleus for the lifetime of the hepatocyte (8).

The HBV genome encodes five viral proteins (three envelope proteins, the polymerase protein, HBxAg, HBcAg, and the precore/HBeAg) which are translated from mRNA transcripts which are in turn encoded by four open reading frames (ORFs) (17). The transcription of cccDNA by cellular RNA polymerase II results in the generation of four major viral RNA molecules which are translated after nuclear export (Fig. 7): 1) The 3.5 kb pre-core mRNA (pre-C) for HBeAg, and the pregenomic RNA (pgRNA) which serves not only as mRNA for viral capsid proteins and the polymerase but also as template for reverse transcriptional synthesis of viral DNA via the translated polymerase itself; 2) The 2.4 kb preS/L-mRNA which serves as template for the translation of large envelope proteins; 3) The small and middle envelope proteins are translated from the 2.1 kb M/S-mRNA; 4) The X protein is translated from the 0.7 kb X-mRNA (8).

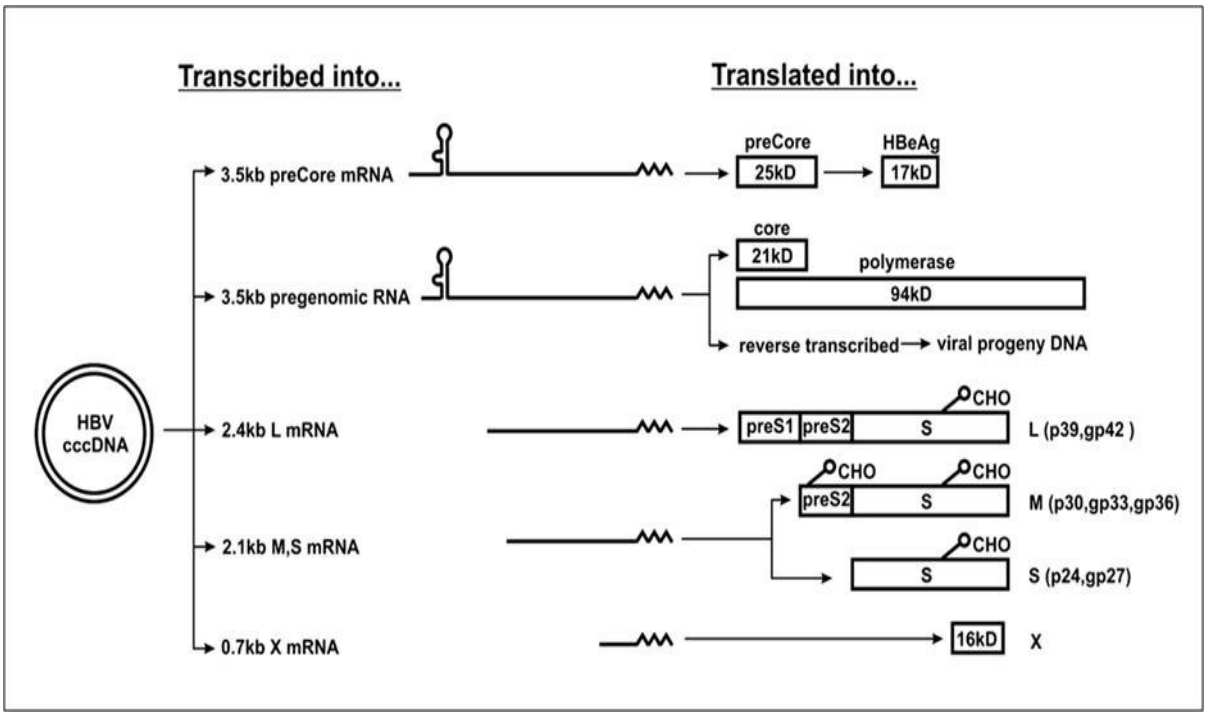


Fig. 7. HBV mRNAs and proteins, from (8).

In the cytoplasm, interactions between the pgRNA and its two gene products lead to the formation of nucleocapsids. The pgRNA and the polymerase are packaged into these nascent capsids where reverse transcription into new rcDNA genomes occurs (8,19). The viral polymerase includes DNA polymerase-, RNaseH-, and reverse transcriptase activities, and also possesses the terminal protein for priming. Reverse transcription is protein-primed and depends on cellular chaperones (15). The key event of packaging is the binding of the polymerase to a structure on the pgRNA called ε which acts as encapsidation signal and serves as replication origin for reverse transcription. ε is present at the 5´ and 3´ ends of pgRNA but only the 5´ copy is required for specific pgRNA packaging (19,22). The final product of minus-strand DNA synthesis is a unit length DNA copy of the pgRNA. Except for a capped 5´ RNA oligo which serves as primer for plus-strand DNA synthesis the pgRNA template is degraded by the RNaseH domain of the polymerase (8,19). After synthesis of the viral DNA plus-strand through the polymerase the genome is circularized. Mature rcDNA containing nucleocapsids can either return to the nucleus and release the genome into the nucleus for intracellular cccDNA amplification or they are enveloped and secreted into the bloodstream (13,19). The preferred pathway is associated with the availability of L protein: L deficiency as it occurs during early stages of infection

leads to an increase of nuclear cccDNA. A sufficient amount of L protein leads to envelopment and secretion of virions (10,19). Finally, the nucleocapsids are enveloped through budding into post-ER/pre-Golgi membranes. Envelopment and secretion is determined by the maturation state of the genome. Intracellular nascent nucleocapids contain DNA at all levels of maturation whereas virions contain relatively mature, double-stranded DNA. It is assumed that maturation signals during DNA synthesis lead to structural changes within the core proteins which allow nucleocapsids to interact with the pre-S1 domain of the large envelope protein and trigger virion assembly and budding. Immature nucleocapsids are excluded from envelopment ensuring that only replication-competent nucleocapsids are secreted into the bloodstream (8,10,19). Fig. 8 summarizes the HBV lifecycle.

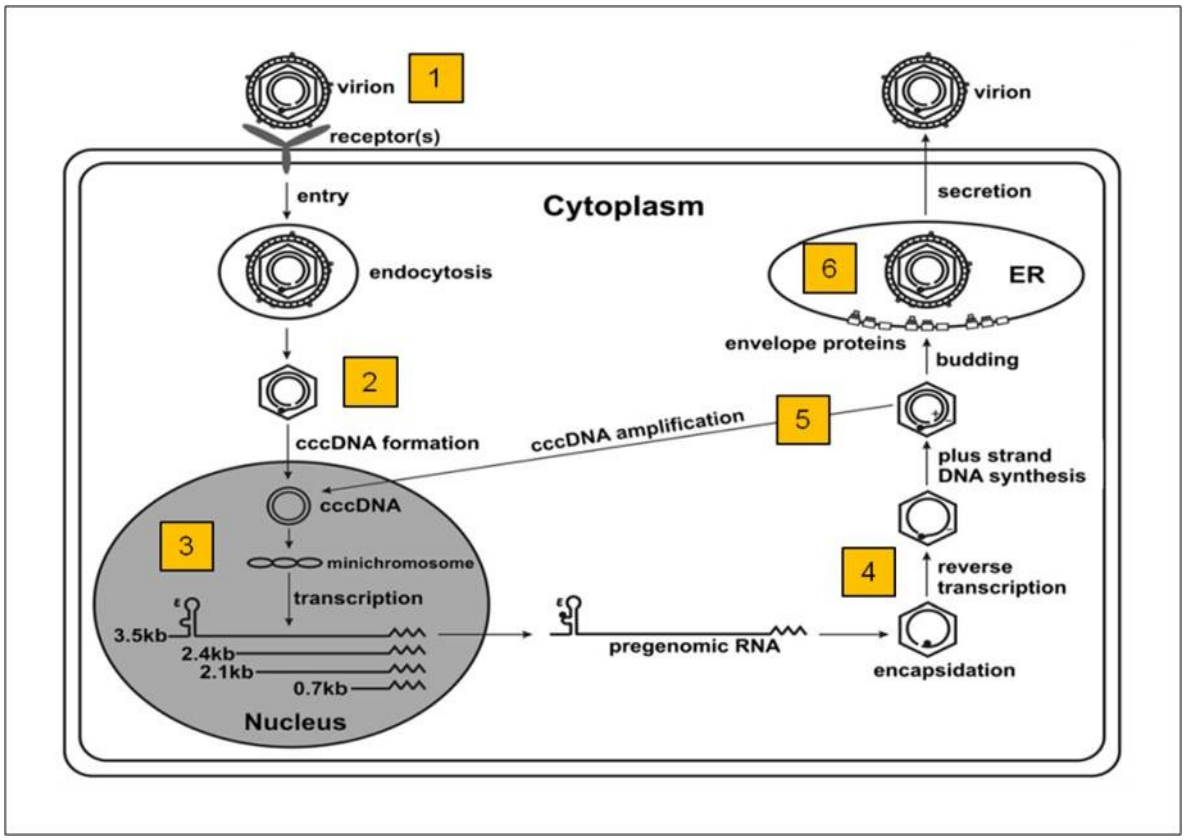


Fig. 8. The HBV lifecycle: 1) Attachment to the host cell; 2) Translocation into the nucleus; 3) Conversion to cccDNA and transcription; 4) pgRNA+polymerase encapsidation; 5) Back to the nucleus for cccDNA amplification or 6) envelopment and secretion as virion; adapted from (8).

Not only infectious particles are secreted but also empty subviral spherical and filamentous envelope particles (SVP) with about 20 nm in diameter (Fig. 9). These non-infectious particles lack the nucleocapsid and are secreted to a much greater extent than HBV virions. Their exact function is unclear. The spherical SVP are mainly assembled by the S protein and contain less if any L protein whereas the filamentous SVP show a large proportion of L protein in addition to the S protein. The *in vitro* production of the S protein and the subsequent secretion of non-infectious SVP is used to generate vaccines against HBV (8,11).

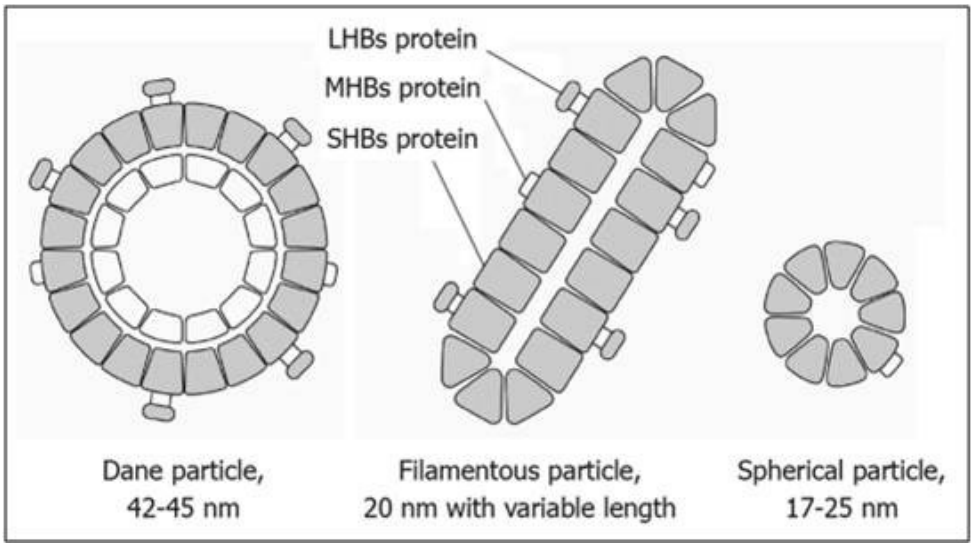


Fig. 9. HBsAg - Dane particle, filamentous particle, and spherical particle, from (23).

2.1.4 Genotypes and subgenotypes

HBV genotypes and subgenotypes have been associated with differences in clinical and virological characteristics. HBV genotyping methods include direct sequencing, line probe assay, and restriction fragment length polymorphism. The sequencing of distinct parts of the whole genome constitutes the gold standard for HBV genotyping (24,25). HBV genotypes A-H are defined by a sequence divergence of more than 8% in the entire HBV genome or more than 4% in the S gene region. Furthermore, most genotypes (except for genotype E, G, and H) can be divided into subgenotypes, which show a divergence between 4 and 7.5% in the entire nucleotide sequence (25–27). Recent studies suggested the existence of two new HBV genotypes, designated I and J. Whether genotype I, which is a recombination among

genotypes A, C, and G, belongs to the “classic“ genotypes of HBV remains under discussion. HBV genotype J shows a close relationship to HBV from apes (28).

The geographical distribution of HBV genotypes and subgenotypes is shown in Fig. 10. Migration as well as behavioral patterns may have an influence on the predominant genotype with migration having blurred the geographical patterns of HBV genotype prevalence. HBV infection with more than one genotype is associated with higher viral load and an increased HBV replication *in vitro* (29).

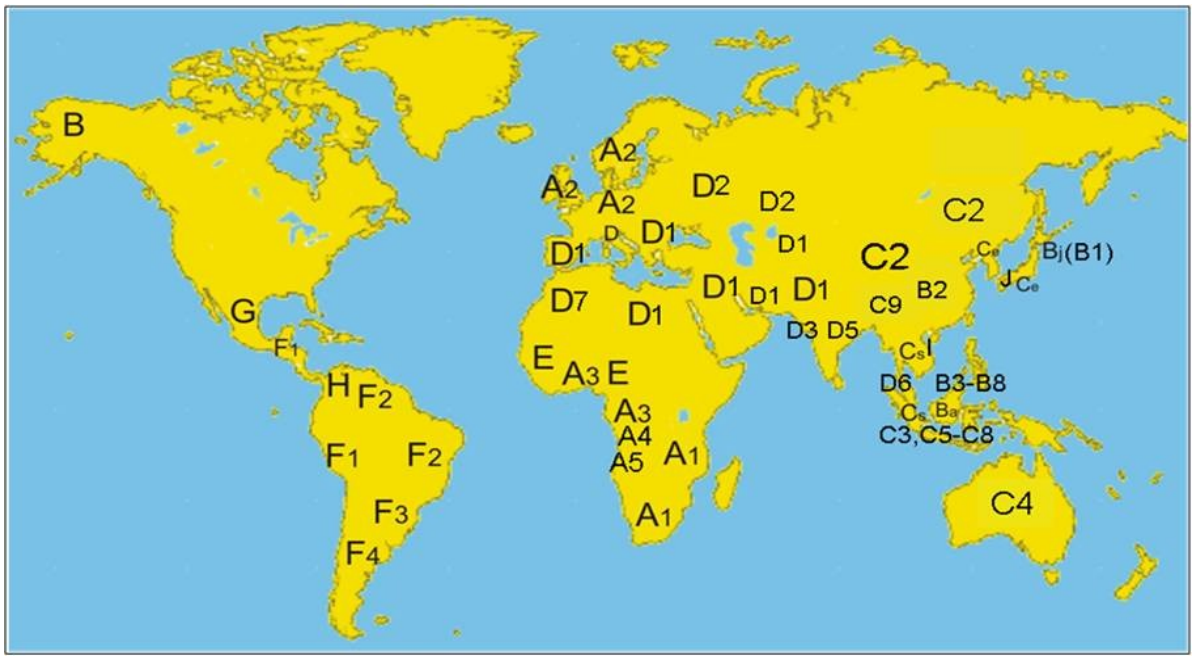


Fig. 10. Geographic distribution of HBV, adapted from (1).

2.2 Clinical aspects of HBV infection

2.2.1 Transmission

The HBV is pathogenic for humans and can be found in blood, semen, saliva, vaginal secretions, and, to a lesser extent, in breast milk, tears, urine, and perspiration (Table 4) (3). Feces, contaminated food or water and vectors are no source of infection. There is no reliable evidence for airborne infections (6).

Table 4
Concentrations of HBV in body fluids.

High	Moderate	Low/not detectable
Blood, serum Wound exudates	Semen, vaginal fluid Saliva	Urine, feces Sweat, tears, Breast milk

Three major modes of HBV transmission have been described: perinatal, sexual, and parenteral/percutaneous transmission (Table 5) (2). Furthermore, an indirect transmission via contaminated objects is possible as the virus remains stable and infectious on contaminated surfaces for at least seven days (6). In areas with high HBV endemicity, perinatal transmission is the main route of transmission whereas in areas with low HBV endemicity, sexual contact among high-risk adults is the predominant route. Areas with intermediate endemicity have a mix of perinatal, horizontal, sexual, and health-care-related transmission (2,3,7).

Table 5
Individuals at risk for HBV transmission, adapted from (30).

- Infants born to infected mothers.
- Sexual partners of infected individuals, persons with multiple sex partners, Men who have sex with men (MSM).
- Injection drug users sharing unsterile needles.
- Travelers to areas with high HBV endemicity.
- Household contacts of infected individuals - sharing items with an infected household member.
- Health care workers - needle-stick injury or other occupational exposure.
- Individuals receiving acupuncture, tattooing and/or body-piercings with unsterile medical devices.

Perinatal transmission

Perinatal transmission occurs especially in high endemicity areas such as China and Southeast Asia. The risk of perinatal transmission is greatest for infants born to HBeAg-positive mothers (70 to 90%) leading to CHB infection in more than 90% of these children. Infants born to HBeAg-negative mothers have a lower risk of perinatal infection (10 to 40%) and chronicity (40 to 70%) (7). The main routes of perinatal transmission include transmission during delivery and postnatal during care or via breast milk. Intrauterine infection is rare (less than 2% of perinatal transmissions) and occurs more often in HBeAg-positive than in HBeAg-negative pregnant women (9.8 to 17.4% vs. 3.7 to 9.9%) (2). It is believed that there are two possible mechanisms of transplacental transmission, the hemagenous route (an injury of the placental microvascular leading to a leakage of infected maternal blood into the circulation of the fetus) and the cellular transfer (as a result of step by step infection of the placental tissue HBV reaches the fetal circulation through endothelial cells). It is supposed that the high risk of chronicity for neonates and children younger than one year of age results from an immature immune system (2). The age at the time of infection plays an important role in the development of chronic HBV infection with an inverse association: While more than 90% of perinatally infected individuals develop chronic HBV infection, the rate for children infected between 1 and 5 years of age is 25 to 50% and for adults 6 to 10%. Approximately 25% of children suffering from CHB infection are at risk for HBV related liver cirrhosis and HCC (Fig. 11). The rate of HCC among adults who have had CHB infection since childhood is 100 to 300 times higher than the rate among uninfected adults. Children that are not infected during birth are at a higher risk of HBV transmission through household contact (6,7).

Sexual transmission

Sexual HBV transmission mainly occurs in low endemic areas. Factors associated with an increased risk of infection include duration of sexual activity, number of sexual partners, men who have sex with men (MSM), history of sexual transmitted diseases, and positive serology for syphilis. Furthermore, prostitutes (as well as their clients) and sexual partners of injection drug users are at a higher risk for HBV infection (2,6).

Parenteral/percutaneous transmission

Parenteral/percutaneous transmission may occur through blood transfusions and organ tissue donations, contaminated medical equipment, injection drug use, tattooing and piercing, needlestick injuries, acupuncture, and other health-care related procedures (2,6). As HBV remains stable on environmental surfaces for at least seven days, indirect inoculation is possible, especially in homes of chronically infected individuals. It is hypothesized that inapparent infectious body fluids are inoculated into cutaneous lesions or onto mucosal surfaces. The risk for HBV transmission rises with high viral load and the serological presence of HBeAg correlates with greater infectivity (7). Individuals who are likely to come into contact with potentially infectious material are at a higher risk of infection: health care workers including physicians, dentists, and nurses, laboratory technicians, individuals requiring frequent transfusions or hemodialysis, and injection drug users. About 23% of all HBV infections in the United States and Western Europe are acquired through injection drug use (2). Because of risky behaviors such as needle-sharing the majority of injection drug users have serologic evidence of past or current HBV infection taking into account that the risk of transmission depends on the prevalence of HBV infection in the community (6).

Worldwide, health-care-related transmission poses an important source of HBV infection including provider-to-patient, patient-to-provider, and patient-to-patient transmission. The risk of infection depends not only on the number of blood- or needle exposures but also on the volume and viral concentration of the infectious material. Before the establishment of HBV vaccine, errors in infection control practice used to play a decisive role in provider-to-patient HBV transmission. Today, those transmissions are rarely reported since universal precautions in standard infection control practice have been established (6).

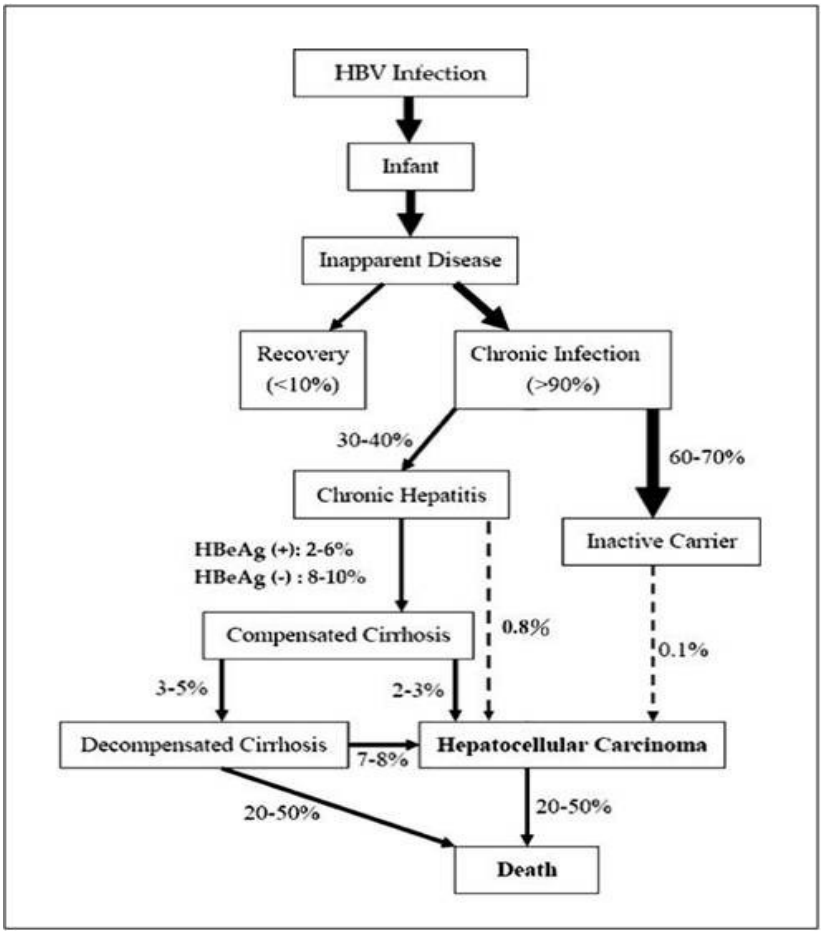


Fig. 11. Annual rates of liver disease progression in HBV carriers, from (31).

2.2.2 Pathogenesis

Pathogenesis and outcome of HBV infection are determined by immune-mediated host-virus interactions. In particular, the lysis of infected hepatocytes through cytotoxic T lymphocytes (CTLs) seems to play a crucial pathogenetic role. HBV infection in immunocompetent adults leads to a transient, self-limited inflammation of the liver which is cleared in more than 95%, whereas about 90% of newborns infected at birth become persistently infected (32). Studies with chimpanzees showed that there are no histological or biochemical signs of liver disease during the early phase of HBV infection indicating that HBV replicates non-cytopathically and that hepatocyte damage is an immune-mediated event. In case of deficient or suppressed cellular immune responses HBV replicates at high levels in the absence of inflammation or cytological abnormalities (32,33). After inoculation, HBV does not immediately start to replicate efficiently and viral antigens are not detectable until four to seven weeks post-infection. Following this period, HBV enters an exponential

phase of replication and infects most hepatocytes. The cause for this initial lag phase of replication is not clear but it seems that neither innate nor adaptive immune responses have inhibiting effects (34). HBV does not induce any cellular gene expression early in infection, attempting to remain undetectable for the innate sensing machinery. The relative invisibility of HBV reflects its replication strategy within viral capsid particles in the cytoplasm. HBV evades innate immune responses by not inducing them (33). The lack of early symptoms is an indirect evidence of the defective IFN-production during the first weeks of infection (34).

Adaptive immune responses play a crucial role in the pathogenesis of HBV infection and differ widely in chronic or resolved patients, respectively. The peripheral blood CD4+ T cell response to HBV is relatively weak in CHB patients while it is vigorous and multispecific in patients with acute hepatitis who ultimately clear the virus. Studies with infected chimpanzees showed that CD4+ T cell depletion at the peak of HBV infection had no effects on viral clearance and liver disease. These findings suggest that CD4+ T cells do not directly participate in viral clearance and tissue damage. CD4+ T cells might contribute indirectly to the control of HBV infection by facilitating the initiation and maintenance of virus-specific CD8+ T cells. Early CD4+ T cell priming is a key element in determining the extent and quality of CD8+ T cell responses to HBV and is most likely triggered by subviral particles (33). HBV-specific CD8+ T cell responses contribute fundamentally to viral clearance and pathogenesis of liver disease. The peripheral blood CD8+ T cell response is weak and narrowly focused in CHB patients, while it is vigorous and polyclonal in patients with acute patients who ultimately clear HBV (33,35). The beginning of liver damage coincides with the emergence of virus-specific CD8+ T cells. Depletion of CD8+ T cells detains the onset of histological, biochemical and clinical evidence of hepatitis and therefore prolongs the course of infection. There is an association between the extent of CTL responses and liver disease severity – hepatocellular injury is initiated by the cytopathic activity of virus-specific CTLs (32,33). Antigen recognition is mediated by interactions between the T-cell-receptor and various co-receptors at the surface of the target cell presenting antigen fragments. These surface proteins are assigned to the major histocompatibility complex (MHC). The distribution of MHC molecules on target cells varies: MHC class I proteins are expressed on almost all nucleated cells and mainly present antigens of intracellular pathogens, which are recognized by CTLs. MHC II molecules are primarily expressed on the surface of

professional antigen presenting cells (APC) and present phagocytized proteins of extracellular origin. Activated CD8+ T lymphocytes recognize and eliminate virus-infected cells, which present viral peptides by means of MHC class I proteins on their surface. In addition to their cytotoxic function, CD8+ T cells release various cytokines and chemokines including IFN-γ and TNF-α (Fig. 12) (32,35).

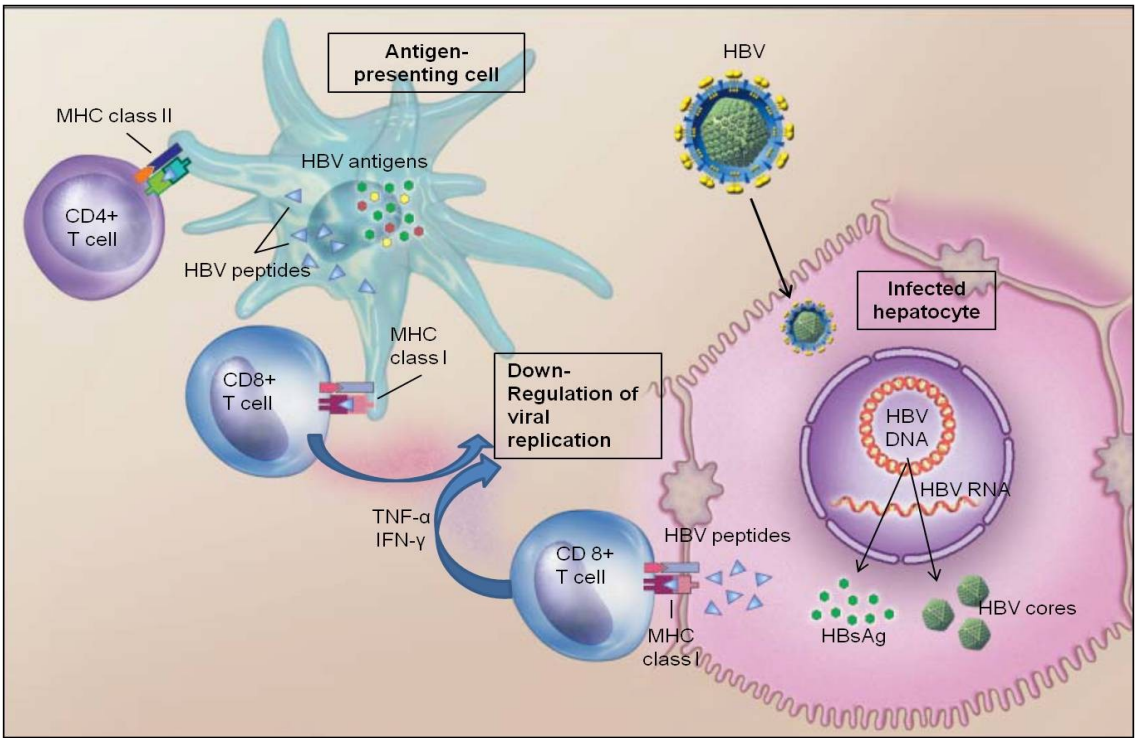


Fig. 12. Cellular immune responses to HBV, adapted from (35).

IFN-γ inhibits HBV gene expression and replication non-cytopathically by preventing the assembly of viral nucleocapsids in the cytoplasm, which is a proteasome- and kinase-dependent process (33). Chemokines recruit antigen-nonspecific inflammatory cells into the liver, which in turn amplify the liver damage initiated by the CTLs. Both, the *Perforin* and *Fas* death pathways must be activated for virus-specific CTLs to be cytopathic. CTL killing requires direct physical contact between infected cells and CTLs. Most of the CTL-dependent antiviral activity depends on IFN-γ produced after antigen recognition (32).

Humoral responses also play a role in controlling HBV. The activation of both, cellular and humoral mechanisms of the adaptive immunity, allows the host to control the infection. The failure of one of them affects the protective efficacy of the other. The absence of CD4+ T cells can impair CD8+ T cell activity and antibody production, while inefficient virus-specific CD8+ T cell responses result in high concentrations of HBV circulating that cannot be cleared by antibodies alone (34). Clinical recovery from acute HBV infection results in lifelong protective immunity but trace amounts of HBV persist in the blood, controlled by humoral and cellular immune responses. These trace amounts of HBV might be essential for the maintenance of immunity in recovered individuals, while patients undergoing immunosuppression may experience HBV reactivation (36).

The liver tissue of CHB patients comprises virus-specific T cells but for quantitative and/or functional reasons these cells are unable to clear the infection. Viral persistence requires that immune responses must be either not induced, deficient, overwhelmed, counteracted or evaded (32). Factors contributing to viral persistence include weak immune responses due to inefficient CD4+ T cell priming and ineffective CD8+ T cell response, immunological tolerance, mutational epitope inactivation, incomplete down-regulation of HBV replication, and T cell receptor antagonism (33). The establishment of CHB infection leads to a state of relative collapse of virus-specific adaptive immunity, mainly regulated by the quantity of HBV replication. Sustained presence of viral antigens results in a functional decrease of virus-specific CD8+ responses and in virus-specific T cell deletion. The function and frequency of intrahepatic and circulating HBV-specific CD8+ T cells is inversely proportional to the amount of HBV-DNA. Other possible factors for weak HBV-specific CD8+ T cell responses in CHB patients include functional alterations of dendritic cell populations and regulatory effects of circulating CD4+CD25+ T cells, which suppress immunological responses through cytokines or direct cell-cell contact. Antiviral therapy can overcome CD8+ T cell hypo-responsiveness, suggesting that T cells are present but suppressed (34).

Inefficient T cell responses in CHB infection lead to sustained low-level cell destruction. Persistent HBV infection is characterized by continuous liver cell damage, regeneration, inflammation, and DNA damage that can lead to liver cirrhosis and HCC. Hepatocytes show extraordinary regenerative capacity. In case of liver injury, mature hepatocytes are triggered to divide. It is likely that the extent of

hepatocyte turnover in CHB patients is directly proportional to the severity and duration of liver disease. The decline of the regenerative capacity of hepatocytes over time facilitates the emergence of cirrhosis. Liver fibrosis and cirrhosis are characterized by an imbalance between fibrolysis and fibrogenesis leading to excessive intrahepatic deposition of matrix. Liver cirrhosis represents the final stage of fibrosis causing architectural distortion of the liver and functional insufficiency. The coexistence of hepatocyte regeneration and inflammation may favor random DNA damage leading to HCC (Fig. 13) (32).

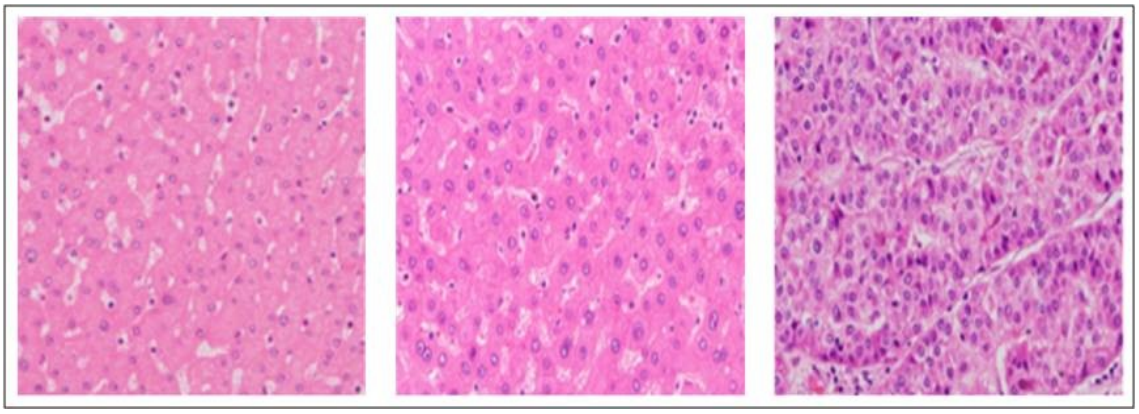


Fig. 13. Histological features of normal liver, cirrhosis, and HCC (from left to right), haematoxylin and eosin stain, adapted from (37).

The interval between the onset of HBV infection and the emergence of HCC usually is several decades. Co-factors like X gene expression and genotoxic agents contribute to the development of HCC. It can be assumed that host and viral factors may cooperate in hepatocarcinogenesis (32). The chronic injury-HCC-hypothesis assumes that a vigorous immune response leads to HBV clearance, while an absent immune response results in the carrier state, and an intermediate immune response leads to CHB infection. The sustained interaction of mitogenic and mutagenic stimuli results in cellular and viral DNA damage, genetic mutations, and chromosomal abnormalities, all together deregulating cellular growth control, which might lead to the development of HCC (Fig. 14) (33).

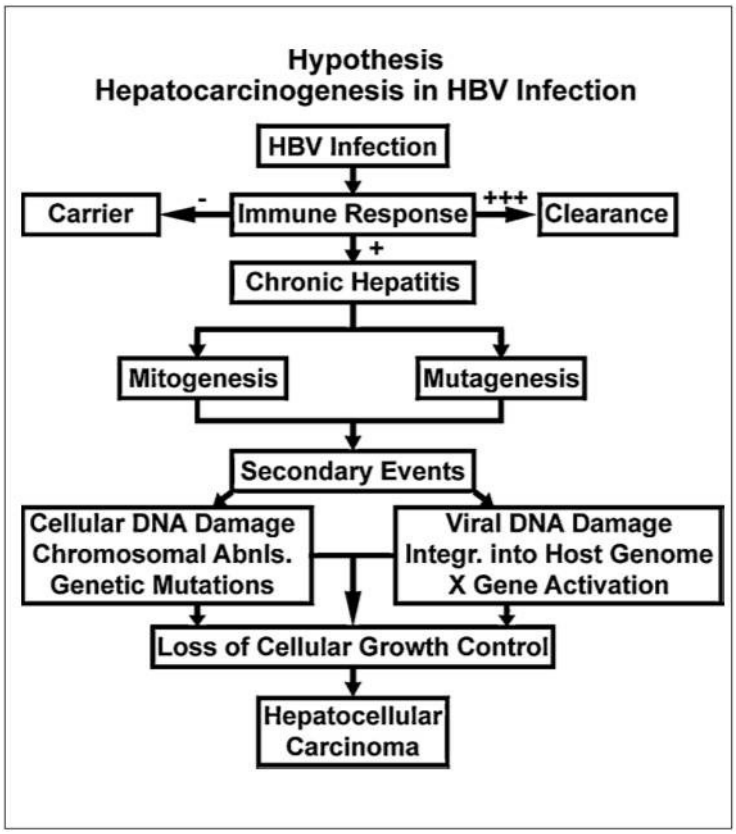


Fig. 14. The chronic injury-HCC-hypothesis. +++, vigorous immune response; +, intermediate immune response; -, absent immune response; from (33).

2.2.3 Natural history of CHB

About one third of the world´s population has serological evidence of past or present HBV infection and 350 to 400 million people are chronic HBsAg carriers. Over one million deaths per year are caused by HBV-related end stage liver disease and HCC, which constitutes the fifth most common cancer with approximately 5% of all cancers. The 5-year incidence of developing cirrhosis in untreated CHB patients is more than 30%. The 5-year incidence of hepatic decompensation in untreated patients with compensated cirrhosis is about 30%. The annual incidence of HCC in CHB patients ranges from 5 to 10% when cirrhosis is established (Fig. 15). Chronic hepatitis B infection occurs either as HBeAg-negative or HBeAg-positive CHB. HBeAg-positive CHB represents the initial phase of chronic HBV infection characterized by high levels of viral replication and infectivity. HBeAg-negative CHB arises from naturally occurring HBV variants and represents a later phase of chronic HBV infection. In many areas, including Europe, HBeAg-negative CHB has been increasing over the last decade (38).

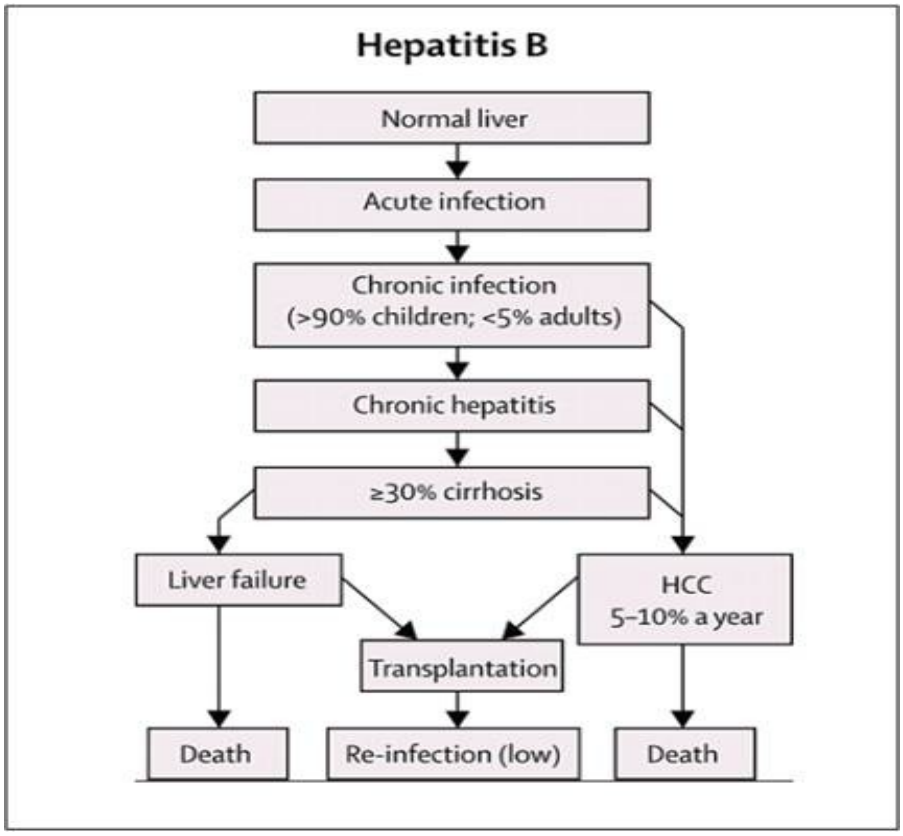


Fig. 15. The natural history of hepatitis B infection, from (39).

The natural course of HBV infection and the efficacy of antiviral therapy is influenced by host and viral factors, co-infections with other viruses, and co-morbidities including obesity and alcohol abuse. Complex interactions between the virus and the host immune response lead to a differentiation of five - not necessarily sequential - stages of CHB infection with characteristic serologic patterns (Fig. 16, Fig. 17, Table 6) (38).

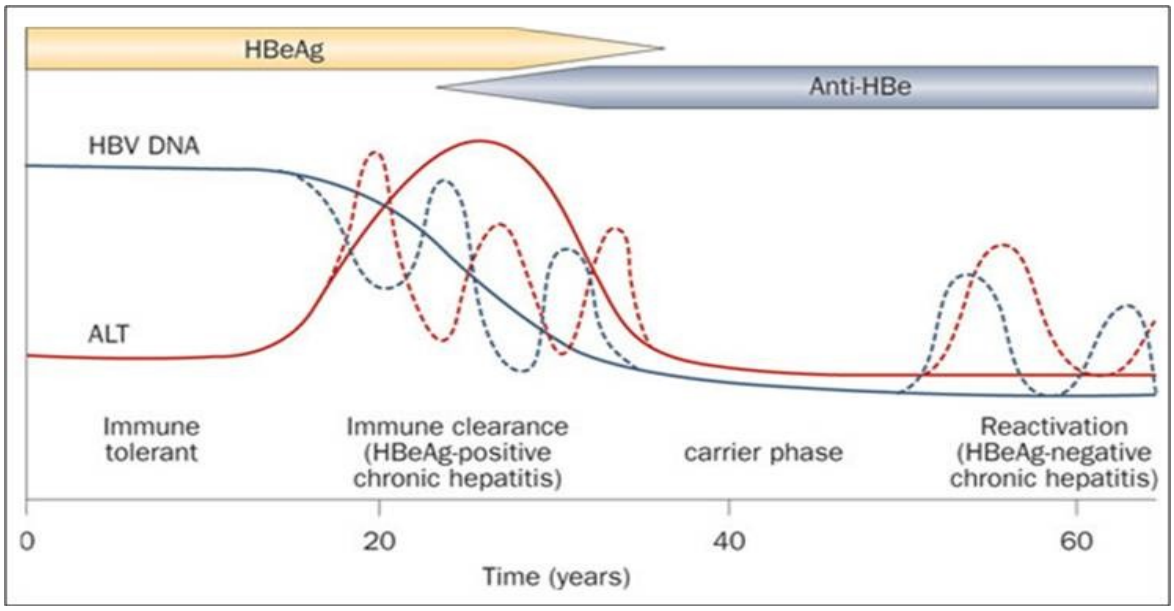


Fig. 16. The natural course of CHB infection, from (40).

Table 6

Stages of HBV infection and their characteristics, adapted from (41).

	HBV DNA	ALT level	Liver Histology
Immune tolerance	↑↑↑	Normal	No or minimal activity
HBeAg-positive CHB	↑↑↑	↑↑(↑)	High-grade inflammatory activity
Carrier state	>2000 or <2000 IU/ml	Normal or ↑	Low inflammatory activity
HBeAg-negative CHB	↑↑(↑)	↑↑(↑)	High-grade inflammatory activity
Remission	Undetectable	Normal	No activity

The immune tolerant stage is characterized by high serum HBV DNA concentrations due to high-level HBV replication, normal or low alanine aminotransferase (ALT) concentrations, and HBeAg positivity but absent or only mild liver necroinflammation accompanied by absent or slow progression to fibrosis. This stage is more frequent and prolonged in individuals infected perinatally or early in childhood and shorter or absent if the infection is acquired later in life. Due to high viremia, patients are highly infectious during this stage. In newborns, the rate of spontaneous or treatment-induced HBeAg seroconversion is very low (<5%). Antiviral therapy is not required for patients during immune tolerant stage but close follow-up is recommended (38,42).

The immune reactive HBeAg-positive stage may last for several weeks or many years. It is characterized by high-level HBV replication, increasingly high ALT concentrations, HBeAg positivity, and mostly severe liver necroinflammation accompanied by rapid progression to fibrosis/cirrhosis and an increased risk of developing HCC (38,42). The risk of disease progression is directly related to the number and severity of flares and to overall duration. The immune reactive stage is rapidly reached in individuals infected during adulthood. The rate of spontaneous HBeAg loss is increased. Factors associated with higher rates of spontaneous HBeAg seroconversion include ALT peaks, HBV genotype B, and older age (43).

The carrier state that follows HBeAg seroconversion is characterized by normal ALT concentrations and low HBV DNA levels in serum. The classification as HBV carrier requires a minimum follow-up of one year with determination of ALT and serum HBV DNA concentrations every three to four months. The HBV DNA concentration should be less than 2000 IU/ml and ALT concentrations should remain within the normal range. About 1 to 3% of HBV carriers achieve spontaneous HBsAg loss. The carrier state confers a very low risk of cirrhosis or HCC. On the other hand, reversion to HBeAg and progression to HBeAg-negative CHB may occur. Therefore, frequent follow-up of ALT and HBV DNA concentrations is required (38,42).

The HBeAg-negative CHB infection may develop after years or decades of the carrier state. It is characterized by periodic reactivation with fluctuating ALT and serum HBV DNA concentrations accompanied by active hepatitis. HBeAg-negative CHB arises from nucleotide substitutions in the precore and/or basal core promoter regions. The most common core promoter mutation involves a substitution of nucleotides 1762 and 1764 leading to a loss of HBeAg synthesis. The most frequent pre-core mutation is a G-A change in nucleotide 1896 (G1896A) resulting in a stop codon. HBeAg-negative mutants accompanied with high viral load are associated with an exacerbation of liver injury and a worse prognosis. Histological lesions are more severe in HBeAg-negative patients compared to HBeAg-positive patients. Fluctuating HBV DNA and ALT levels complicate the differentiation between active HBeAg-negative and carrier state patients, which have a good prognosis (38,42).

HBsAg loss is associated with undetectable HBV DNA in the serum but HBV DNA may be detectable in the liver. The cccDNA remains in the liver and HBV reactivation may occur with immunosuppression. Patients achieving HBsAg loss but suffering from cirrhosis remain at risk for the development of HCC (38).

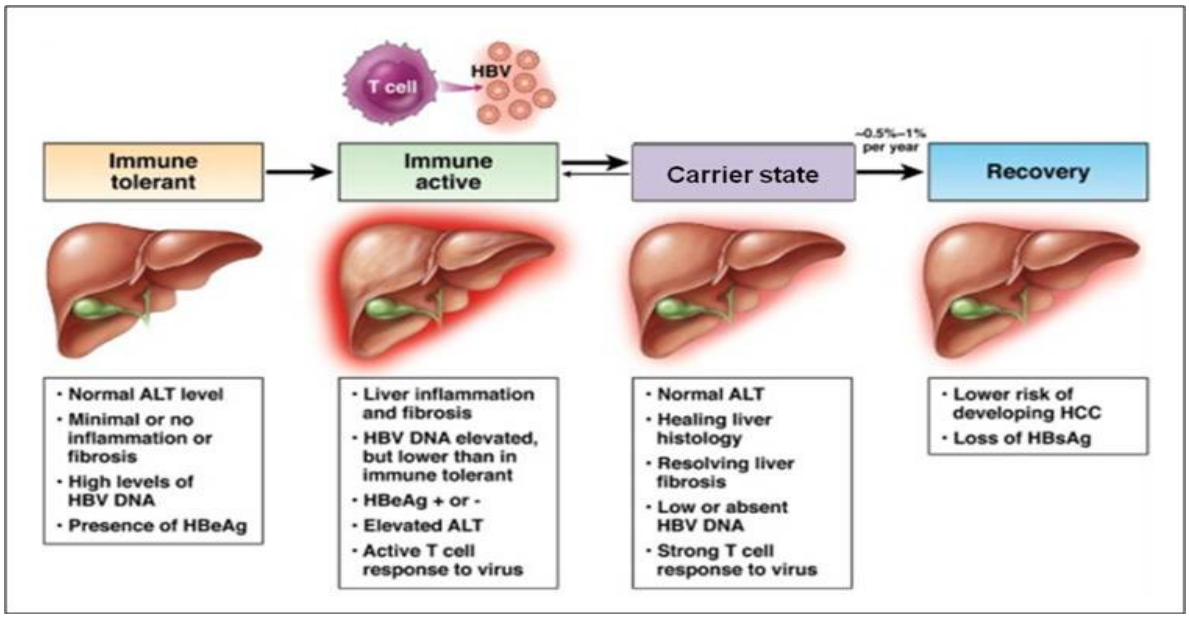


Fig. 17. Stages of CHB infection, adapted from (44).

2.2.4 Diagnostics

The diagnosis of HBV infection is based on clinical, biochemical, serological, and (if necessary) histopathological findings. The synopsis of all results and their proper interpretation is essential to ensure the correct diagnosis (Table 7, Table 8). The main indications for performing HBV diagnostic testing are summarized in Table 9. Partners and family members of HBV infected individuals must be offered diagnostic testing for HBV infection and vaccination (45).

Table 7
Hepatitis B virus serological and virological markers, adapted from (45).

HBsAg	Acute or chronic HBV infection
HBeAg	High-level HBV replication; marker for response to treatment
HBV DNA	Level of replication; marker for therapy response
Anti-HBc-IgM	Acute HBV infection; DD flare of CHB infection
Anti-HBc-IgG	Recovered or chronic HBV infection
Anti-HBs	Immunity due to vaccination or resolved natural infection
Anti-HBe	Low-level HBV replication; marker for therapy response
Anti-HBc-IgG + anti-HBs	Past HBV infection
Anti-HBc-IgG + HBsAg	Chronic HBV infection
Anti-HBc-IgG and/or anti-HBs + HBV DNA	Latent or occult HBV infection

Table 8
Interpretation of HBV serological markers, adapted from (46).

	Serological markers							
	HBs-Ag	Anti-HBs	IgM anti-HBc	Total anti-HBc	HBe-Ag	Anti-HBe	HBV DNA	ALT
Early incubation period	+	-	-	-	-	-	+/-	Normal/ Elevated
Acute HBV infection	+	-	+	+	+	-	+	Elevated
Resolving acute hepatitis	-	-	+	+	+/-	+/-	+/-	Normal/ Elevated
Immunity from vaccination	-	+	-	-	-	-	-	Normal
Immunity from infection	-	+	-	+	-	+/-	-	Normal
Chronic HBeAg+ hepatitis B	+	-	-	+	+	-	+	Elevated
Chronic HBeAg- hepatitis B	+	-	-	+	-	+	+	Elevated
Low-level chronic infection	-	-	-	+	-	+/-	+	Normal
Carrier state	+	-	-	+	-	+/-	-	Normal
False positive/ past infection	-	-	-	+	-	-	-	Normal

Table 9
Indications for performing HBV diagnostic testing, adapted from (47).

1. Individuals with elevated liver enzymes and/or clinical signs of hepatitis.
2. Individuals with liver cirrhosis, fibrosis or HCC.
3. Migrants from regions with increased prevalence of HBsAg.
4. Family-/household members of HBV infected individuals.
5. Sexual partners of HBV infected individuals.
6. Healthcare professionals.
7. Dialysis patients and patients in psychiatric institutions.
8. Individuals with frequently changing sexual contacts.
9. Active and former intravenous drug users.
10. Patients before or during immunosuppressive treatment or chemotherapy
11. HIV and/or HCV infected individuals.
12. Recipients of organ transplants before and after transplantation.
13. Blood and organ donors.
14. Pregnant women (HBsAg only).
15. Newborns of HBsAg and/or isolated anti-HBc positive mothers.

The diagnosis of acute HBV infection

Acute HBV infection develops after an incubation period of six weeks to several months starting with nonspecific symptoms including fever, malaise, and nausea, followed by jaundice, pale stools, and dark urine. About 25 to 40% of infected adults develop symptoms, whereas toddlers, infants, and immunosuppressed individuals may not manifest signs of infection. The clinical features of acute HBV infection resemble those for Hepatitis A virus (HAV) and Hepatitis C virus (HCV) infection, which is why testing for all three agents should be carried out. HAV infection can worsen the course of HBV infection, which is why vaccination against HAV should be considered if serologic testing is negative for anti-HAV (30). HBV DNA, HBsAg, and HBeAg are the first viral markers detectable in serum (Fig. 18). HBsAg emerges between one week and twelve weeks after exposure and its persistence is a marker for chronicity. HBeAg is associated with high replication rates and greater infectivity. When serum alanine and aspartate aminotransferase (ALT, AST) concentrations start to rise, jaundice may appear. HBsAg and HBV DNA typically persist in the serum for the duration of clinical symptoms, while HBeAg is usually cleared at the peak of clinical illness. HBV DNA and HBsAg are cleared with recovery. The emergence of antibodies to HBV proteins varies during acute HBV infection: Antibodies to HBcAg (anti-HBc) appear shortly before the onset of clinical illness. Anti-HBc-IgM implies acute infection and disappears with the resolution of clinical symptoms, while the concentration of anti-HBc-IgG arises and persists for years or even life-long. The quantification of anti-HBc-IgM helps to differentiate acute HBV infection from CHB infection with an acute episode. Antibodies to HBeAg (anti-HBe) appear shortly after HBeAg clearance. The emergence of anti-HBe and HBeAg loss indicate the initiation of recovery. Antibodies to HBsAg (anti-HBs) appear late in infection after HBsAg clearance, usually during recovery or convalescence, and their appearance indicates immunity. About 10 to 15% of patients do not develop detectable anti-HBs after recovery. In these cases, anti-HBc is the only marker for previous infection, which makes anti-HBc testing to the most reliable method for the assessment of previous HBV infection (45).

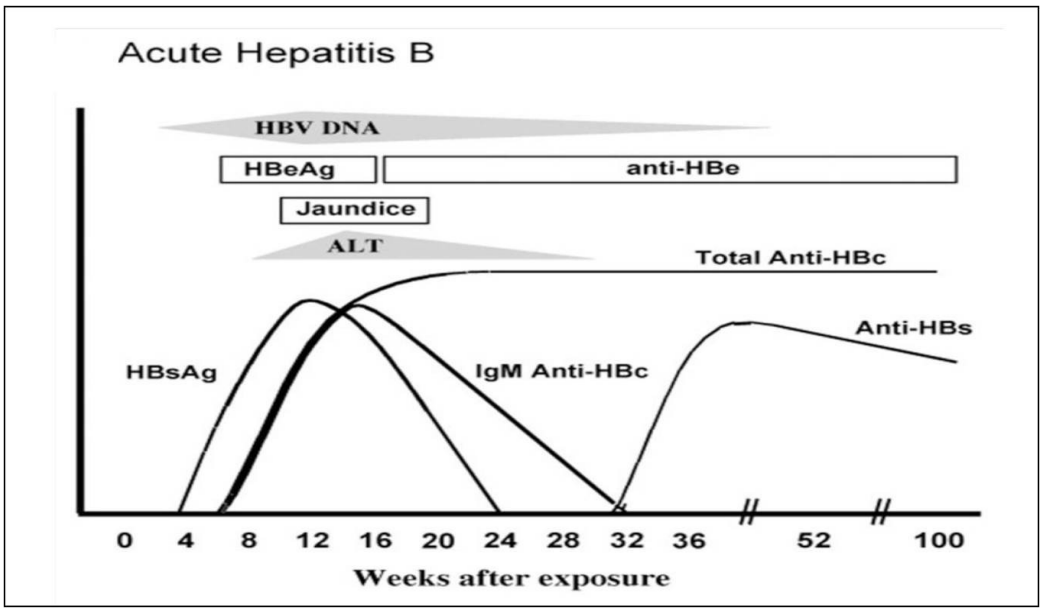


Fig. 18. The clinical course and serologic profile of acute HBV infection, from (45).

The diagnosis of chronic HBV infection

The initial pattern of serological markers in CHB infection is similar to acute HBV infection, including the appearance of HBV DNA, HBsAg, HBeAg, and anti-HBc but HBV DNA, HBsAg, and HBeAg continue to be detectable in serum (Fig. 19). The subsequent course of CHB infection is variable: Most CHB patients remain HBsAg-positive for years or even life-long leading to continuous liver cell damage, which might ultimately result in the development of HCC (45).

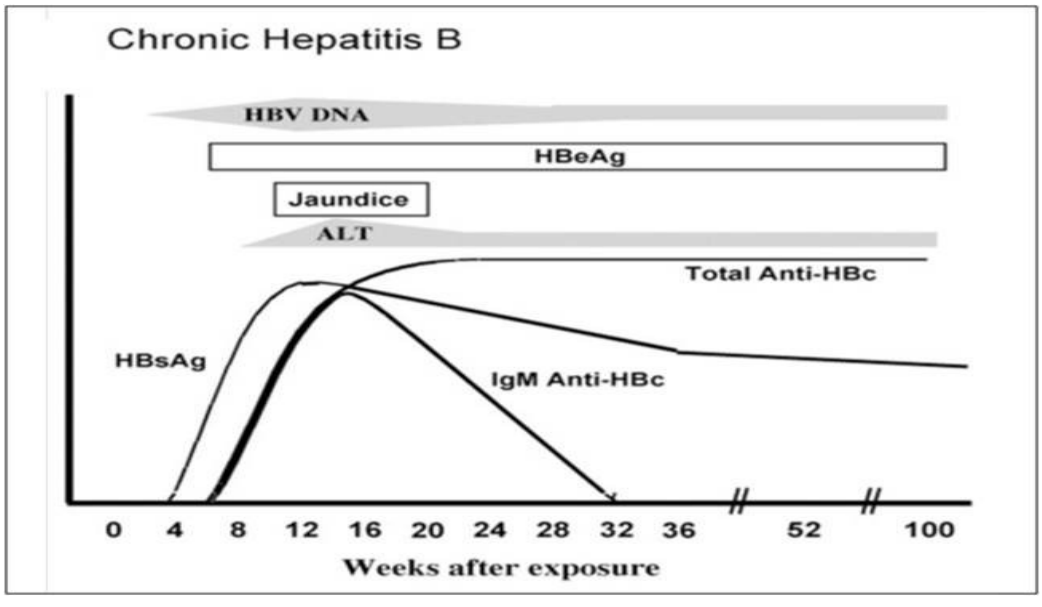


Fig. 19. The clinical course and serologic profile of chronic HBV infection, from (45).

The diagnostic testing for suspected persistent HBV infection includes the initial evaluation of HBsAg and anti-HBc. If both are positive, anti-HBc-IgM should be assessed to exclude acute HBV infection. Furthermore, HBeAg/anti-HBe, HBV DNA (quantitative), and anti-HDV should be tested. HDV diagnostic testing is recommended in newly diagnosed HBV infection and if testing has not been done during known HBV infection. Especially during an exacerbation of CHB infection an HDV superinfection should be ruled out. If only HBsAg is positive a confirmation test should be carried out to exclude false positive results. In case of a positive confirmation test, HBeAg and HBV DNA testing is required and anti-HBc testing should be repeated after two to four weeks. If only anti-HBc is positive, anti-HBs testing is required. Anti-HBs levels ≥10 IU/l are indicative of immunity due to resolved natural infection. Anti-HBs <10 IU/l is associated with the “anti-HBc only” status (47).

Further diagnostic testing

In addition to serologic testing for HBV the following diagnostics are required: medical history including risk factors, family and partner history, physical examination, laboratory testing for possible co-infections like HDV, HIV, and/or HCV, HAV serology, clinical chemistry laboratory tests (e.g. liver inflammation and synthesis parameters, blood count, blood coagulation status), and liver ultrasound (Table 10) (46,47). Ultrasonography of the liver helps to detect cirrhosis but significant fibrosis or early stages of cirrhosis may remain undetected (48). Non-invasive methods to assess hepatic fibrosis include transient elastography, which measures the speed of ultrasonic waves moving through the liver reflecting hepatic “elasticity”. Liver biopsy is still considered the gold standard to assess the inflammatory activity and the extent of fibrosis but is largely replaced by non-invasive technologies (47). In case of an increased risk of developing HCC the determination of alpha fetoprotein (AFP) is recommended. Screening for HCC is suggested in patients ≥30 years of age with at least one of the following risk factors: male gender, infection at birth, infected for several decades, family history of HCC, HCV co-infection, and/or member of a high-risk population (30,47). CHB patients with serum HBV DNA concentrations >2 x 10^3 IU/ml or signs of liver cirrhosis should undergo liver ultrasound examination every six to twelve months (47). Liver cirrhosis increases the risk of developing HCC but it may also occur in the absence of cirrhosis (30).

HBV genotyping may help to determine the choice of antiviral therapy but its role in the clinical practice is controversial. HBV genotyping methods include restriction fragment length polymorphism, PCR plus hybridization, genotype-specific PCR amplification, and sequencing, which is considered as “gold standard” (49).

Table 10
Further exploration at first diagnosis.

Clinical	Medical/family history, physical examination
Biochemical	Complete blood count, liver inflammation and synthesis parameters, coagulation profile, creatinine levels
Co-infection screening	Antibodies against HDV, HAV, HCV, HIV
Medical imaging	Liver ultrasound
Optional	Liver biopsy, transient elastography, HCC screening

2.2.5 Whom to treat and how

The indication for antiviral therapy is based on the criteria serum HBV DNA concentration, serum alanine aminotransferase (ALT) level, and histological grade of liver damage (Table 11). According to these criteria, antiviral treatment is indicated in patients with HBV DNA concentrations >2000 IU/ml and/or serum ALT concentrations above the upper limit of normal (ULN), and/or histological signs of moderate to severe active necroinflammation and/or fibrosis (38,41). Patients with high viremia (serum HBV DNA >2000 IU/ml), persistently normal ALT concentrations, and minimal or absent histological lesions (patients in the immunotolerant stage of disease) do not require immediate therapy but follow-up at least every three to six months is recommended. In patients with CHB, serum HBV DNA >20,000 IU/ml, and serum ALT concentrations >ULN, immediate treatment is indicated. Patients with compensated cirrhosis and detectable HBV DNA should undergo antiviral therapy despite normal ALT and/or serum HBV DNA concentrations below 2000 IU/ml. Patients with decompensated cirrhosis require rapid and profound HBV DNA suppression irrespective of other parameters and those with very advanced liver disease should be considered for liver transplantation. The criteria age, health status, extrahepatic manifestations, and family history of cirrhosis or HCC should also be taken into account for therapeutic decisions (38,50).

Table 11
Indications for antiviral therapy in CHB patients, adapted from (50).

	ALT/ AST	Serum HBV-DNA	Liver histology	Management
HBeAg-positive CHB	≥2 xULN	High	High-grade inflammatory activity	Treatment
Carrier state	<ULN	Low	No need for liver biopsy	Monitor ALT/AST every 6 months
HBeAg-negative CHB	>ULN	High or low	High-grade inflammatory activity	Treatment
	>ULN	High or low	Low-grade inflammatory activity	Monitor ALT/AST every 3 to 6 months

Current agents used for CHB treatment include nucleos(t)ide analogues (NAs), conventional interferon alpha (IFNa), and its pegylated form (PEG-IFNa) (Table 12). The main advantages of NAs include the more convenient use (oral administration instead of subcutaneous IFNa injections), better tolerance, and more favorable safety profile. NAs can be given to all CHB patients, even to those with contraindications to (PEG-)IFNa, while the latter is contraindicated in case of decompensated liver disease and/or haematological signs of portal hypertension. Furthermore, all NAs (except adefovir) are more potent than (PEG-)IFNa. Various limitations such as high cost, low response rates, diverse side-effects, and inconvenient subcutaneous administration restrict the general use of IFNa monotherapy. One of the most important advantages of (PEG-)IFNa is its finite administration of 48 weeks without emergence of viral resistance. However, only a minority of CHB patients treated with (PEG-)IFNa achieve sustained off-therapy responses. The main disadvantages of NAs include the long-term, perhaps indefinite duration of treatment, raising safety and family planning issues. In HBeAg-positive CHB patients, NA treatment may be stopped after HBeAg seroconversion and at least six months of consolidation therapy, whereas in HBeAg-negative patients, discontinuation is only possible after HBsAg loss, which occurs rarely. Long-term treatment is a risk factor for the development of antiviral drug resistance. A rapid and efficient suppression of viral replication early in therapy is associated with lower rates of antiviral drug resistance in the long term (51).

Table 12
Advantages and disadvantages of (PEG-)IFNa and NAs, adapted from (38).

	(PEG-)IFNa	NAs
Advantages	• Finite duration • Absence of resistance • Increased rates of HBe- and HBs seroconversion	• Potent antiviral effect • Good tolerance • Oral administration
Disadvantages	• Moderate antiviral effect • Poor tolerance • Subcutaneous injections • Risk of adverse events	• Indefinite duration • Decreased rates of HBe- and HBs seroconversion

Interferons

IFNa became available 1992 and was considered as preferred agent for CHB treatment for many years. It is a host cytokine with immunomodulatory, antiviral, and antiproliferative effects inhibiting synthesis of viral DNA and activating antiviral enzymes. Conventional IFNa is administered subcutaneously daily (5 million units) or three times weekly (10 million units). PEG-IFNa is the long-acting preparation of IFNa. It is equally or even more effective than conventional IFNa with a less demanding injection schedule (180 µg once weekly) due to its decreased renal clearance rate (52). HBeAg-positive patients with the highest chance of anti-HBe seroconversion are the best target group for a 48-week course of PEG-IFNa but it is also used in HBeAg-negative patients. PEG-IFNa therapy in patients with decompensated cirrhosis results in an increased risk of infections and exacerbation of liver injury. Patients with compensated cirrhosis show decreased response rates carrying a risk of liver decompensation (41). One of the main disadvantages of PEG-IFNa is its poor tolerance. Up to 30% of patients show flu-like symptoms including fever, chills, headache, malaise, fatigue, alopecia, diarrhea, insomnia, and myalgias. More serious adverse reactions such as myelosuppression, depression and emotional lability, thyroid dysfunction, and development of autoantibodies may lead to discontinuation of treatment. Pretreatment screening for low counts of leucocytes and platelets, psychiatric diseases, thyroid function, and autoantibodies is obligatory. PEG-IFNa is contraindicated in patients suffering from severe depression or psychosis, decompensated HBV-related cirrhosis or autoimmune disease, and pregnant women (38,41).

Nucleos(t)ide analogues (NAs)

NAs are classified into nucleosides (lamivudine, telbivudine, entecavir) and nucleotides analogues (adefovir, tenofovir). NAs inhibit the viral polymerase activity resulting in reduced viral replication and decreases in serum HBV DNA levels. Most of these agents are weak inhibitors of human nuclear DNA polymerase β and some of them have a low level of activity against the human mitochondrial DNA (mtDNA) polymerase γ leading to impaired mitochondrial replication and mitochondrial dysfunction. Clinical manifestations of mitochondrial toxicity include myopathy, neuropathy, macrocytosis, hepatic steatosis, pancreatitis, hyperlactemia, lactic acidosis, and nephrotoxicity. Factors influencing the risk of mitochondrial toxicity include age, gender, genetics, nutritional status, and medical co-morbidities (53). NAs are primarily excreted unchanged in the urine. Dose reductions and/or increased dosing intervals are recommended in individuals with renal insufficiency. The rate of persisting anti-HBe seroconversion ranges from 40 to 80% and is more frequent in patients with baseline HBV DNA concentrations <$2x10^6$ IU/ml and high baseline ALT concentrations. Long-term treatment with NAs is also applied in patients likely to fail achieving a sustained off-treatment virological response such as HBeAg-positive patients not developing anti-HBe seroconversion and HBeAg-negative patients. Cirrhotic patients should also receive long-term NA treatment irrespective of HBeAg status. Due to their negligible risk of resistance and high potency, recent guidelines recommend entecavir and tenofovir as preferred anti-HBV agents (Fig. 20) (38).

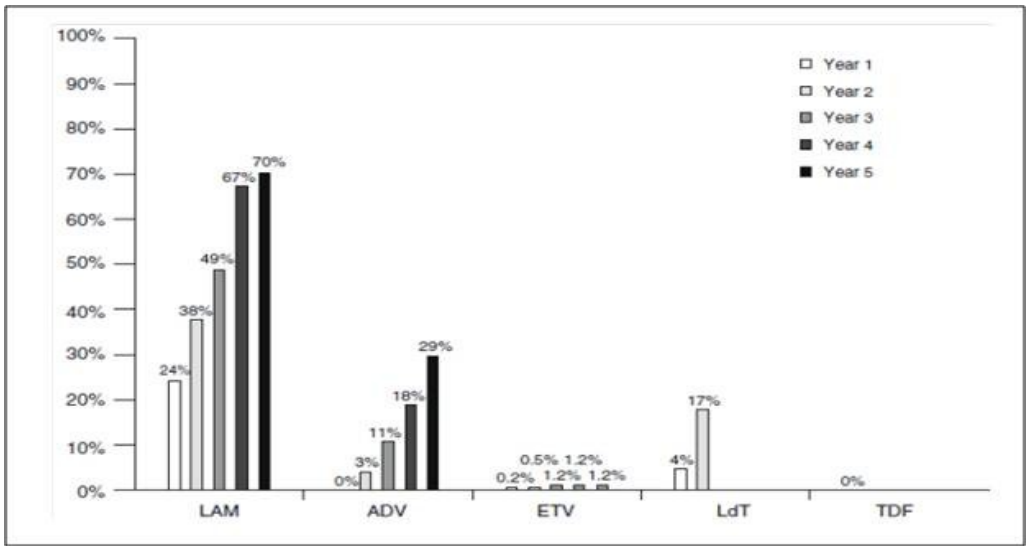


Fig. 20. HBV resistance in NA-naïve patients. Lamivudine (LAM), adefovir (ADV), entecavir (ETV), telbivudine (LdT), and tenofovir (TDF), from (54).

Entecavir

Entecavir (ETV, Baraclude®, Bristol-Myers Squibb) was approved in 2005. It is administered perorally with a dosage of 0.5 mg per day for nucleoside naive patients. In the past, patients with lamivudine failure received higher doses (1.0 mg) as a high percentage of these patients developed ETV resistance after two or three years but recent studies have shown that the rates of resistance in such patients were nonetheless high which is why ETV is no longer recommended for the treatment of lamivudine-resistant patients (54). ETV is a guanosine nucleoside analogue inhibiting the priming of the viral polymerase, the reverse transcription of the negative-strand HBV DNA from the pgRNA, and the synthesis of the positive-strand HBV DNA. It is highly potent and shows higher rates of histological improvement, ALT normalization, and HBV DNA suppression as compared to lamivudine but the rate of HBeAg seroconversion after one year of therapy is comparable. Side effects include headache, diarrhea, arthralgias, and insomnia. Dose reductions or increased dosing intervals are recommended for patients with renal insufficiency. The rate of resistance in NA-naive CHB patients is 1.2% after five years of ETV therapy (53,54).

Tenofovir disoproxil fumarate

Tenofovir disoproxil fumarate (TDF, Viread®, Gilead Sciences, Inc.) is an acyclic nucleotide analog of adenosine 5´-monophosphate and an orally bioavailable ester prodrug of tenofovir. In 2001, it was approved for the treatment of HIV and in 2008 for the treatment of CHB. It is administered perorally with a dosage of 300 mg per day exhibiting a serum half-life of 17 hours and an intracellular half-life of more than 60 hours, which is significant longer compared to older NAs. Tenofovir has been converted to its oral prodrug form, TDF, in order to enhance oral bioavailability (Fig. 21). Following absorption, TDF is converted to tenofovir through esterase hydrolysis. Intracellularly, tenofovir is metabolized to the active diphosphate form, which inhibits HBV DNA polymerase by competing with the natural substrate deoxyadenosine 5´-triphosphate leading to chain termination (55,56). Tenofovir diphosphate has only weak activity on mitochondrial DNA polymerase γ and intracellular DNA polymerases α and β contributing to its long-term safety. It is not a substrate, inhibitor or inducer of human cytochrome P450 enzymes resulting in low potential of drug-drug interactions (51,56). TDF is able to cross the placental barrier but the rate of birth defects is not

higher than in the general population. Breastfeeding is not recommended. Studies on fertility, carcinogenesis, and mutagenesis have not shown any consistent adverse findings raising concern for humans (51). TDF is primarily cleared by glomerular filtration and active tubular secretion through proximal tubular cells. Dose-interval adjustments are required in case of renal impairment (Table 13) (55,56).

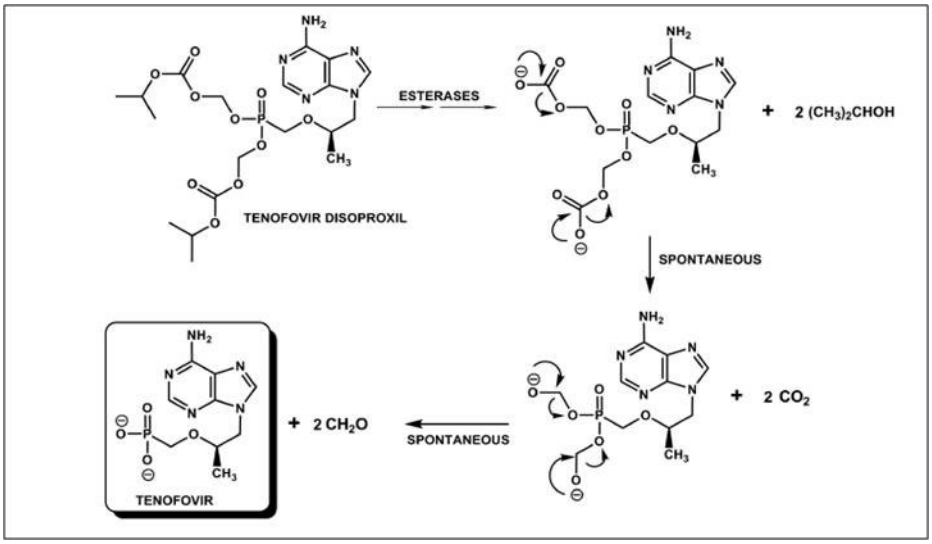


Fig. 21. Conversion of TDF into its active form, tenofovir; from (57).

Table 13
Recommended TDF dosing intervals in case of renal impairment, adapted from (55).

Creatinine clearance	Suggested dosing interval
30 - 49 ml/min	300 mg every 48 hours
10 - 29 ml/min	300 mg every 72 to 96 hours
Hemodialysis	300 mg every 7 days or after ≈12 hours of dialysis

Data regarding the safety profile of TDF mainly arise from its use in HIV-infected individuals. Adverse events observed in HIV patients on TDF include diarrhea, nausea, asthenia, headache, rash, vomiting, abdominal pain, and depression. The most common adverse reaction in HBV-infected individuals with compensated liver disease is nausea (9%). In those with decompensated liver disease the most common adverse reactions include abdominal pain, nausea, vomiting, insomnia, pruritus, dizziness, and pyrexia (51,55). There are concerns about the impact of TDF on renal function. Registration studies of TDF for the treatment of HIV showed a good

renal safety profile but postmarketing studies revealed cases of diabetes insipidus and acute renal failure, some associated with a Fanconi-like syndrome characterized by hypophosphatemia, metabolic acidosis, and glycosuria. The majority of these adverse events occurred in HIV patients receiving multiple medications and resolved after TDF discontinuation (51). It appears that TDF is less nephrotoxic than other antiviral agents but monitoring for nephrotoxicity is required. Measures to be taken in case of renal impairment include adjustments in the dose or dosing interval, regular monitoring of renal function parameters, avoidance of other nephrotoxic agents, and adequate hydration. Cases of severe nephrotoxicity in CHB patients treated with TDF have not been observed but further long-term investigations are required. Bone pain, muscular pain, weakness, and/or fractures may be signs of proximal renal tubulopathy calling for prompt evaluation of renal function (55). There is evidence to suggest that TDF use is associated with a loss of bone mineral density (BMD) during the treatment of HIV infection. Clinical trials in HIV-1 infected individuals revealed an association between TDF use and increases in biochemical markers of bone turnover as well as decreases in BMD (51,55). *In vitro* studies with osteoblasts found that TDF might lead to a downregulation of genes involved in amino acid biosynthesis and metabolism resulting in reduced osteoblast growth, activity, and differentiation (58). It is likely that the safety of TDF in CHB patients may be unlike those in HIV patients, although the efficacy of TDF seems to be similar. Multidrug therapy, as it occurs in HIV patients, significantly increases the probability of adverse drug reactions. This makes it difficult to compare the observations made in HIV patients receiving TDF to those made in CHB patients on TDF (51). Buti et al. (59) assessed the efficacy and safety of TDF for up to seven years in 437 CHB patients. No resistance to TDF was observed through 7 years. Grade 3 and 4 adverse events were uncommon (1%). No significant change in BMD was observed from year 4 to year 7.

2.3 The bone

Bone is a highly specialized, dynamic tissue undergoing constant remodeling. The skeleton protects vital organs, provides mechanical support for locomotion and stature, and controls mineral homeostasis. Normal bone remodeling is characterized by a tight coupling of bone resorption by osteoclasts to bone formation by osteoblasts. Factors influencing this physiological process include menopause-associated hormonal changes, changes in physical activity, drugs, age-related factors, and secondary diseases. Imbalances result in abnormal bone remodeling and the development of bone disorders (60,61).

2.3.1 Bone structure, matrix, and minerals

Bone is composed of cells (10%), mineral crystals (60%), and organic matrix (30%) including type I collagen (88%), other proteins (10%), as well as lipids and glycosaminoglycans (1 to 2%) (60). The rigidity and hardness of bone occur as a result of mineral salt within the osteoid matrix, which is a complex of calcium and phosphate (hydroxyapatite) derived from the blood plasma. Crystalline hydroxyapatite is the main mineral component of bone constituting about half of the mass and a quarter of the volume of normal adult bone. Mineral crystals are deposited along collagen fibrils resulting in a highly organized tissue with sufficient structural integrity preserving the mechanical functions of the skeleton (61).

Two main histological types of mature bone can be differentiated: Cortical bone with its dense, ordered structure surrounding the marrow space, and trabecular bone, which is lighter and irregularly structured comprising the bone marrow. Cortical bone is mainly found in the shaft of long bones and the surfaces of flat bones providing mechanical and protective functions. It is characterized by bone laid down concentrically around Haversian canals containing blood vessels, nerves, lymphatics, and connective tissue. Tiny lacunae between bone lamellae contain osteocytes. Cortical bone has an outer periosteal surface and an inner endosteal surface, both containing osteoblasts and osteoclasts as well as their progenitors. Trabecular bone forms the ends of long bones and the inner parts of flat bones providing strength and the majority of the metabolic function. Due to its resistance to compressive stress trabecular bone is the predominant bone in vertebrae (62).

Another important element in the structure of bone is the collagen network. Based on the pattern of collagen forming the osteoid, two types of bone are identified: Lamellar bone is characterized by a regular alignment of collagen, whereas woven bone is characterized by a random organization of collagen fibrils. Almost all the bone in healthy adults is lamellar bone. Woven bone is weaker than lamellar bone and occurs initially in all fetal bones and during fracture healing. It is produced when osteoblasts prepare osteoid rapidly but it is replaced by the deposition of more resilient lamellar bone during remodeling (61).

2.3.2 Bone cells

Bone remodeling requires the coordinated action of four major types of bone cells: Osteoblasts, bone-lining cells, osteocytes, and osteoclasts. Osteoblasts and osteoclasts belong to a unique temporary structure, the so called basic multicellular unit (BMU). Each BMU comprises a team of osteoblasts in the rear, a team of osteoclasts in the front, a nerve supply, a central vascular capillary, and associated connective tissue. Under normal conditions, bone mass is preserved due to a tight balance between bone resorption and bone formation during each cycle of remodeling. Osteoclasts remove bone by acidification and proteolytic digestion. Osteoblasts cover excavated areas and secrete osteoid, which is mineralized into new bone. In healthy human adults, about one million BMUs are operating at any moment. The activity of these BMUs is regulated by mechanical forces, hormones, bone-cell turnover, growth hormone, cytokines, and local factors (63).

Osteoblasts

Osteoblasts originate from multipotent mesenchymal stem cells of the bone marrow stroma, which also give rise to adipocytes, bone marrow stromal cells, chondrocytes, and muscle cells. Osteoblast precursors most likely reach bone by migration from neighboring connective tissues, while osteoclast precursors reach bone from the circulation. The differentiation of osteoblasts and osteoclasts is influenced by cytokines, growth factors, adhesion molecules, several systemic hormones, and mechanical signals. Bone morphogenetic proteins are able to initiate osteoblastogenesis from uncommitted progenitors. Other factors including platelet-derived growth factor, insulin-like growth factors, transforming growth factor β, and

members of the fibroblast growth factor family influence the differentiation of committed osteoblast progenitors toward the osteoblastic lineage. Several hormones, including thyroid hormones, estrogen, androgen, and glucocorticoids exert potent regulatory influences on the development of osteoblasts and osteoclasts by regulating the action of cytokines (63). Osteoblasts synthesize precursors of type I collagen and a number of other proteins that are incorporated into the bone matrix, including osteocalcin and osteonectin, which account for 40 to 50% of the non-collagenous proteins of bone. Mature osteoblasts are essential for the mineralization of the osteoid matrix, the process of hydroxyapatite deposition. Osteoblasts express alkaline phosphatase, which is the main glycosylated protein present in bone. The synthesis of bone matrix determines the volume of bone but not its density, whereas bone mineralization increases the density of bone without altering its volume. Osteoblasts that have completed their bone forming function can either turn into lining cells, undergo apoptosis, or may be buried into the matrix as osteocytes (Fig. 22). The average lifespan of osteoblasts is about three months (63).

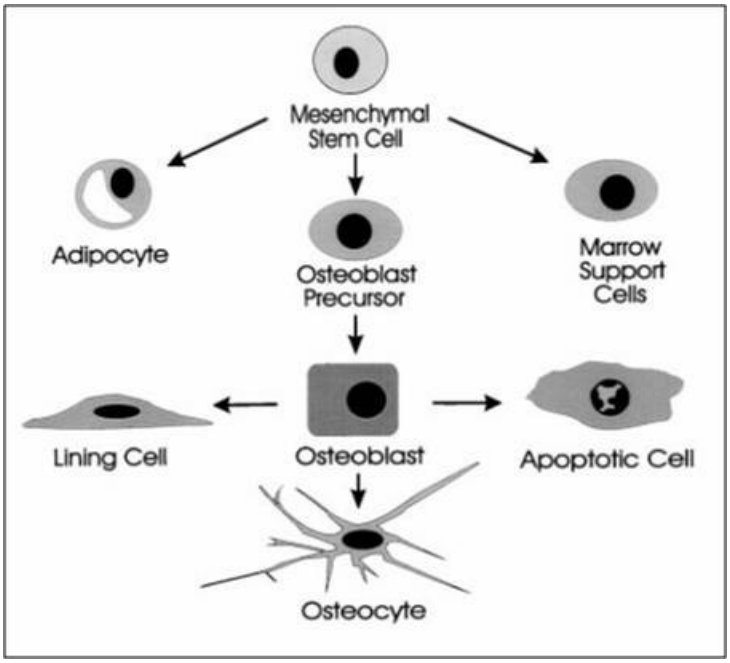


Fig. 22. Origin and fate of osteoblasts, from (64).

Bone-lining cells

The surface of quiescent bone is covered by a layer of flat lining cells, which are descendents of osteoblasts. The targeting of osteoclast precursors to specific locations on bone depends on “homing” signals given by lining cells, which are in direct physical contact with osteocytes. Bone-lining cells can reactivate to active osteoblasts due to stimulation by PTH or mechanical forces (63).

Osteocytes

Osteoblasts buried within lacunae of mineralized matrix are termed osteocytes, constituting the most abundant cell type in bone. Osteocytes communicate with each other and with cells on the bone surface via multiple extensions of their plasma membrane. Osteoblasts communicate with cells of the bone marrow stroma resulting in a syncytium extending from the entombed osteocytes to the vessel wall (Fig. 23). Osteocytes serve as mechanosensory cells detecting the need for bone augmentation or reduction and transmitting the according signals. Furthermore, osteocytes sense changes in interstitial fluid flow through canaliculi and detect the levels of hormones circulating in the same fluid (63). They are linked through gap junctions mainly composed of connexin. Empty lacunae may occur as a result of osteocyte apoptosis caused by disruption of their intracellular gap junctions. Estrogen deficiency and glucocorticoid treatment account for osteocyte apoptosis (61).

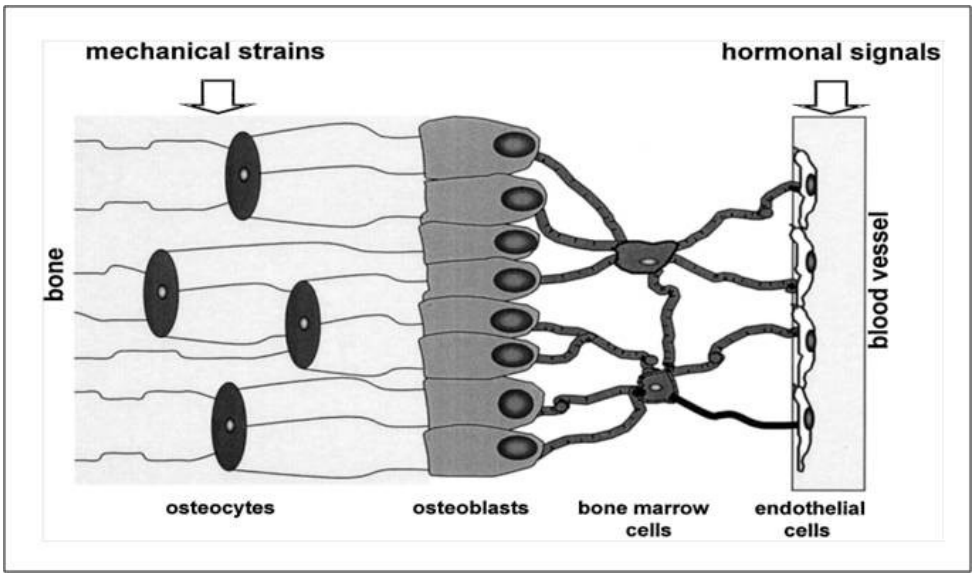


Fig. 23. Functional syncytium, from (63).

Osteoclasts

Osteoclasts are multinucleated, bone-resorbing cells differentiated from mononuclear cells of the monocyte/macrophage lineage upon stimulation by the receptor activator of nuclear factor κB (NF-κB) ligand (RANKL) and the monocyte/macrophage colony-stimulating factor (M-CSF). RANKL directly controls the differentiation process by activating the receptor activator of nuclear factor-κB (RANK), while M-CSF is critical for the proliferation of osteoclast progenitors (62). RANK is expressed in hematopoietic osteoclast progenitors. RANKL is expressed in committed pre-osteoblastic cells and T-lymphocytes. Osteoprotegerin (OPG) inhibits osteoclastogenesis and bone resorption by binding to RANKL and thus blocking the RANKL/RANK interaction (Fig. 24). OPG is produced by bone, skin, liver, stomach, lung, heart, intestine, kidney, placenta, hematopoietic organs, and immune organs (63). Factors stimulating osteoclast development include interleukins (IL-1, IL-3, IL-6, IL-11), tumor necrosis factor, M-CSF, and granulocyte macrophage-colony stimulating factor. IL-4, IL-10, IL-18, and interferon-γ inhibit osteoclast development. PTH and 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] stimulate osteoclast formation and regulate calcium absorption from the intestine and calcium excretion from the kidney. Calcitonin inhibits osteoclast development and activity. Bone minerals are dissolved in the acidic environment of the resorption site. The protein components of the matrix are degraded by matrix metalloproteinases. Degraded bone matrix is endocytosed and further transcytosed to the membrane area opposite the bone, where it is released. The average lifespan of osteoclasts is about two weeks. The same cytokines and growth factors that stimulate osteoblast and osteoclast development influence their apoptosis (63).

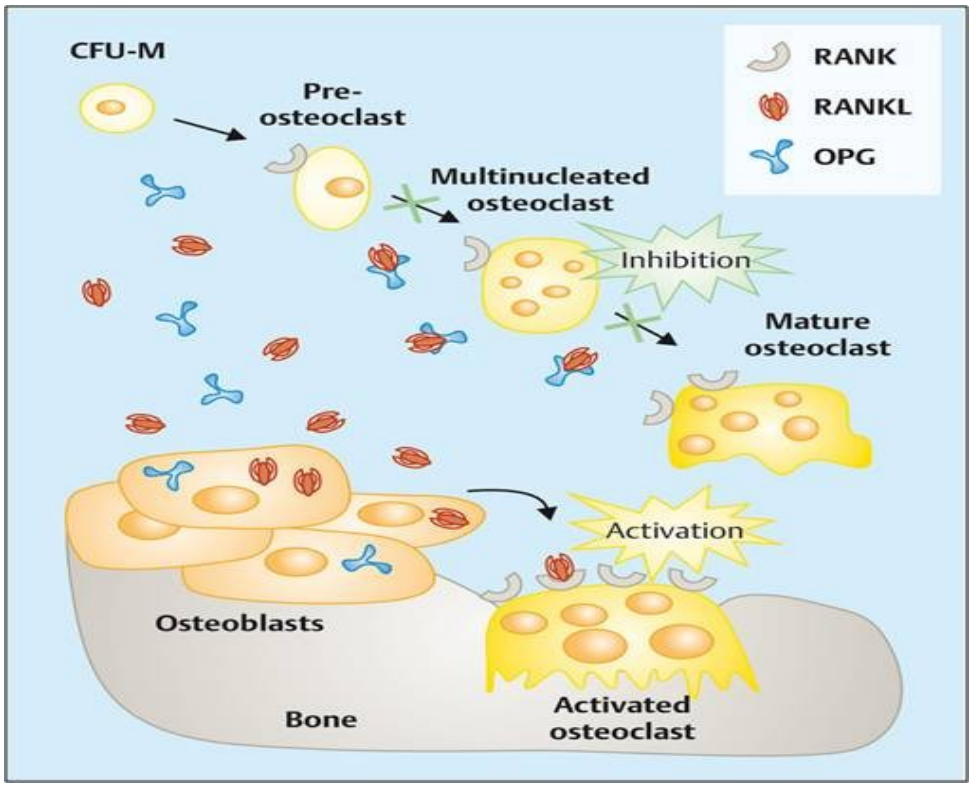


Fig. 24. The RANK/RANKL/OPG pathway, from (65).

2.3.3 Bone formation

Ossification (or osteogenesis) is the process of bone formation by osteoblasts. Three basic steps are involved in osteogenesis including synthesis of extracellular organic matrix (osteoid), matrix mineralization, and remodeling of bone through resorption and reformation. Environmental factors influencing normal bone formation include physical activity and good nutrition. Smoking during the years of bone formation may also decrease the amount of bone formed. Hormones influencing bone formation include growth hormones, estrogen in females, testosterone in males, and others. Bone strength depends on bone mass, composition, geometry, material properties, and microstructure (61). Osteoblasts and the bone matrix are the main elements involved in bone formation including intramembranous and endochondral ossification. During intramembranous ossification, bone is laid down into connective tissue. This process mainly occurs during formation of flat bones of the skull, the mandible, the maxilla, and the clavicles but it is also seen during the healing process of fractures treated by open reduction and stabilization by metal components. Mesenchymal stem cells (MSCs) initiate intramembranous ossification, which starts at certain constant

points. MSCs proliferate at these so called “centers of ossification” and condense around capillaries surrounded by ground substance and collagen fibers. MSCs differentiate into osteoblasts creating osteoid in the center of the aggregate. Osteoid mineralizes and entrapped osteoblasts turn into osteocytes. The surrounding mesenchyme condenses as a fibrovascular periosteum and bone growth continues at the surface of the trabeculae. Interconnecting trabeculae form woven bone, which might be replaced by lamellar bone (61). Intracartilaginous ossification occurs in long bones and most of the rest of the bones in the body but also during fracture healing when treated by cast immobilization. In early fetal life, each long bone is represented by a rod of hyaline cartilage, which is surrounded by a highly vascular condensed perichondrium. The growth of the cartilage model occurs due to continuous cell division of chondrocytes, accompanied by secretion of extracellular matrix, which is called interstitial growth. Primary ossification centers mostly appear during fetal development but postnatal primary ossification occurs in certain short bones. The vascularization of the perichondrium results in the development of periosteum containing a layer of undifferentiated cells becoming osteoblasts later on. Osteoblasts secrete osteoid against the shaft of the cartilage model (appositional growth). Chondrocytes located in the primary center of ossification start to grow and stop secreting collagen and other proteoglycans but begin to secrete alkaline phosphatase, which is essential for mineral deposition. Chondrocytes secrete vascular endothelial cell growth factor stimulating the sprouting and branching of blood vessels. These blood vessels carry osteoprogenitor cells, hemopoietic cells, and other cells into the cavities evolved due to the apoptosis of chondrocytes. Osteoblasts secrete osteoid, which forms the bone trabeculae. Osteoclasts form the medullary cavity by breaking down spongy bone. Secondary ossification centers appear about the time of birth in the epiphysis of long bones. The epiphyseal plate is situated between the primary and the secondary ossification centers and continues to form new cartilage, which is further replaced by bone. Growth continues until the epiphyseal plate is replaced by bone. The growth in diameter occurs due to deposition of bone beneath the periosteum (61).

2.3.4 Bone remodeling

The skeleton is a dynamic tissue undergoing continuous remodeling in order to maintain its structural integrity and to subserve as a storehouse of calcium and phosphate. Bone remodeling involves the removal of damaged or old bone by osteoclasts and bone formation by osteoblasts. It allows the bone to repair itself and to adapt to upcoming forces. This process depends on the tight coupling of bone resorption to bone formation and well coordinated interactions between bone-lining cells, osteocytes, osteoclasts, and osteoblasts (60). Imbalances result in abnormal bone remodeling and the development of bone disorders including osteoporosis, Paget´s disease, osteogenesis imperfecta, and osteopetrosis. In childhood, bone turnover exceeds bone resorption. In young adulthood, bone formation and resorption are approximately balanced, whereas ageing comes along with a net loss of bone (62). The remodeling cycle is highly regulated involving five phases (Fig. 25).

(1) Activation phase: Osteocytes detect microdamages in old bone and sense bone deformation caused by mechanical stress. Signals of unknown nature are transmitted and recruit osteoclast precursors, which attach to the bone matrix and differentiate into osteoclasts due to elevated levels of M-CSF and RANKL.

(2) Resorption phase: While osteoclasts resorb bone by secreting hydrogen ions and lysosomal enzymes, osteoprogenitors and/or MSCs are recruited, which differentiate into osteoblasts. Due to the dissolution of mineral matrix, growth factors are released, including transforming growth factor-β (TGF-β), platelet derived growth factor (PDGF), as well as insulin-like growth factors (IGFs) I and II.

(3) Reversal phase: Osteoblast differentiation and osteoid synthesis overtake bone resorption as the predominant event. The responsible signals that initiate the reversal phase are still unknown but factors proposed include bone matrix-derived factors like IGF-1, IGF-2, bone morphogenetic proteins, fibroblast growth factor, and PDGF.

(4) Formation phase: Osteoclasts are replaced by osteoblasts. Preosteoblasts are attracted and stimulated by the growth factors liberated from the matrix during the resorption phase. Differentiated osteoblasts synthesize osteoid matrix filling the resorption cavities.

(5). Mineralization phase: The process of osteoid mineralization begins thirty days after the deposition of osteoid and completes the bone-remodeling cycle (60,61).

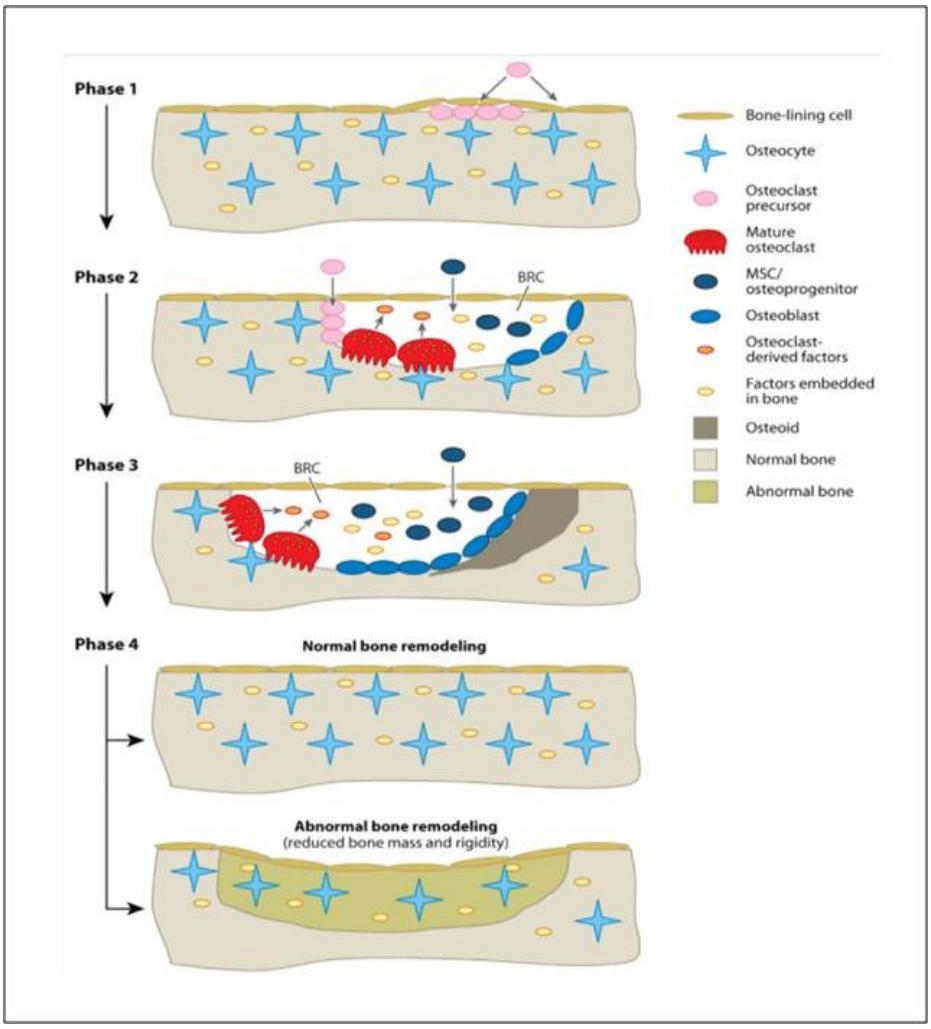


Fig. 25. Bone remodeling phases, from (60).

2.4 Bone metabolism

The bone is a dynamic tissue performing mechanical and metabolic functions. Most importantly, bone serves as mineral reservoir for calcium, phosphate, and magnesium, which are mobilized to maintain systemic mineral homeostasis. Mineralized bone matrix is a depository for certain cytokines and growth factors including IGFs, TGF-β, and bone morphogenetic proteins (BMPs). The yellow bone marrow serves as reserve storage of fatty acids. Bone buffers the blood against excessive pH changes by releasing or absorbing alkaline salts. Bones are able to store heavy metals and other extraneous elements, thus serving as detoxifying tissue. Furthermore, bone fulfills endocrine functions: Fibroblast growth factor (FGF) acts on kidneys in order to reduce phosphate reabsorption. Osteocalcin (OC)

contributes to the regulation of fat deposition and blood glucose. OC reduces stores of fat and enhances insulin secretion and sensitivity. Several factors play important roles in bone metabolism including $1,25(OH)_2D_3$, parathyroid hormone (PTH), parathyroid hormone-related protein (PTHrP), calcitonin, thyroid hormones, gonadal and adrenal steroids, growth factors, and cytokines (61).

2.4.1 Calcium- and phosphate metabolism

Mineral homeostasis requires the transport of calcium, phosphate, and magnesium in bone, intestine, and kidneys. Large amounts of total body calcium (99%), phosphorus (85%), and magnesium (65%) are contained within bones. Bone mineral metabolism is regulated by interactions between PTH, vitamin D, and calcitonin. PTH contributes to short-term regulation of free calcium ions at the expense of the bone mass. Vitamin D is responsible for the maintenance of the total calcium reservoir of the body and regulates intestinal calcium absorption. Calcitonin is produced by parafollicular C-cells of the thyroid. It is secreted in case of increased serum calcium concentrations and lowers calcium levels in the short term by storing calcium ions in the bone and by increasing renal calcium elimination (61).

Calcium is available extra- and intracellularly representing the most abundant mineral in the human organism. Extracellular and intracellular calcium levels are relatively low but precisely regulated in order to exert important regulatory and physiological functions. Intracellular calcium is involved in muscle contraction, cell proliferation, cell differentiation, cell motility, secretion of hormones, and apoptosis. Calcium receptors are able to sense minimal changes of calcium levels and initiate regulatory processes (66). Due to the low solubility of calcium and phosphate, they precipitate as calcium-phosphate. In the bone, calcium, phosphate, and hydroxyl ions are found as hydroxyapatite forming hexagonal crystals. Hydroxyapatite accounts for up to 70% of bone mass. About 99% of the calcium is stored in bones in the form of calcium-phosphate, a smaller part in the form of calcium carbonate. The remaining 1% of calcium is distributed to teeth (0.6%), tissue (0.6%), plasma (0.03%), and extravasal fluids (0.06%). About 45% of the serum calcium is bound to proteins (mainly albumin), 10% is complexed with small organic anions (10%), and 45% of the serum calcium is available as free ionized calcium. High serum calcium levels stimulate osteoblastic processes, which in turn enable rapid deployment of calcium at

places of future bone formation. *In vitro* studies showed, that high serum calcium levels inhibit the formation and activity of osteoclasts, whereas the secretion of calcitonin increases (66,67). About 88% of the total body phosphate is located in bones as calcium-phosphate, in the form of calcium apatite. The remaining 12% are involved in the carbohydrate metabolism and are contained in phospholipids, nucleic acids, and adenosine triphosphate. Phosphate increases PTH, which indirectly increases calcium levels. The synthesis of vitamin D_3 is stimulated by low phosphate concentrations (66).

Parathyroid hormone

PTH is produced by the parathyroid glands and regulates serum calcium and phosphate concentrations. PTH has a circadian rhythm with higher levels in the evening and during night (66). An increase of free calcium ions activates the membrane-bound calcium-sensing receptor leading to reduced PTH secretion rates. High concentrations of 1,25-dihydroxyvitamin D_3 also lower PTH secretion. PTH increases the serum calcium concentration by mobilizing calcium from the bone, by increasing intestinal calcium uptake, and by inhibiting renal calcium elimination. It stimulates hydroxylation steps, that are necessary for the synthesis of 1,25-dihydroxyvitamin D_3, thus indirectly increasing intestinal calcium uptake (61).

Vitamin D

Vitamin D exists in an exogenous and an endogenous form. The exogenous form, ergocalciferol (vitamin D2) is available through the diet. It is for example contained in vegetables but its uptake does not adequately supply the need. The endogenous form, cholecalciferol (vitamin D_3) is mainly synthesized in the skin from the cholesterol metabolite 7-dehydrocholesterol under the influence of ultraviolet radiation (Fig. 26) but is also contained in animal-derived foods, especially in oil-rich fish like cod, mackerel, or salmon (61). The hydroxylation of cholecalciferol to 25(OH)vitamin D_3 takes place in the liver. It is possible to evaluate the vitamin D status through measurement of serum 25(OH)vitamin D_3 levels, as this parameter constitutes the storage form and the main part of the circulating forms of vitamin D. The second hydroxylation to the active metabolite $1,25(OH)_2D_3$ (calcitriol) by means of the enzyme 1α-hydroxylase mainly occurs in the kidneys and is precisely regulated.

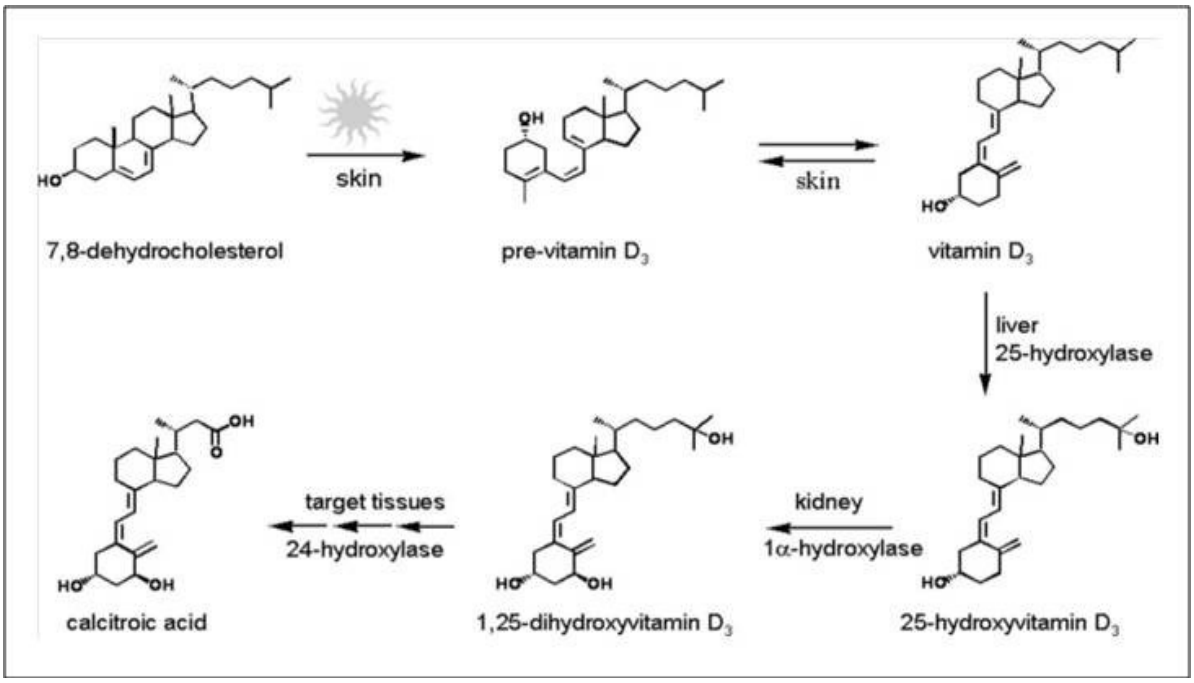


Fig. 26. Vitamin D synthesis, activation, and catabolism, adapted from (68).

Reduced serum calcium levels lead to an increased release of PTH. High PTH levels are associated with decreased phosphate levels leading to an activation of renal 1α-hydroxylase. Estrogen, prolactin, and the somatrotropic hormone increase the production of 1α-hydroxylase. Calcitriol binds to the vitamin D receptor, which belongs to the superfamily of nuclear receptors. The complex reaches the nucleus and interacts with specific DNA domains. It induces the expression of osteocalcin and alkaline phosphatase, while the expression of PTH and collagen is inhibited. Calcitriol plays a crucial role in bone formation, bone mineralization, and neuromuscular transmission. It affects the absorption and transport of calcium from the intestine, the kidneys, and the bone. Calcitriol supports the formation of osteoblasts and osteoclasts from immature precursor cells of the bone marrow. Unlike PTH, calcitriol stimulates the reabsorption of phosphate. As a result, the solubility product exceeds leading to a precipitation of calcium and phosphate in the osteoid. This process is stimulated by the vitamin D-induced transcription of the osteocalcin genes (68). Vitamin D deficiency leads to gastrointestinal calcium absorption rates of only 10 to 15%, whereas an adequate vitamin D state leads to absorption rates of approximately 30%. Special situations including pregnancy, lactation, and growth lead to intestinal calcium absorption rates ranging between 50 and 80% (61).

2.4.2 Regulation of the skeletal metabolism

Factors influencing the skeletal metabolism include calcitropic hormones (PTH, calcitonin, and vitamin D), sex hormones, thyroid hormones, glucocorticoids, growth factors, and cytokines (Table 14) (69).

Table 14
Systemic regulation of bone remodeling, adapted from (64).

	Bone resorption	Bone formation
PTH	↑	↑ (↓)[a]
1,25$(OH)_2$ Vitamin D_3	↑	↑ (↓)[a]
Calcitonin	↓	unknown
Estrogen	↓	(↓)[b]
Androgen	unknown	↑
Growth hormone/IGF	↑	↑
Thyroid hormone	↑	↑
Glucocorticoids (GCs)	↑[c]	↓

[a] PTH and vitamin D decrease collagen synthesis in high doses.
[b] Estrogen decreases bone formation by decreasing remodeling but formation is decreased less than resorption and bone mass increases.
[c] GCs may increase resorption indirectly by inhibiting intestinal calcium absorption and sex hormone production.

Sex hormones

A lack of sex hormones and hypogonadism leads to osteoporosis. An overproduction of estrogen or androgen during childhood initially leads to an acceleration of growth but due to premature epiphyseal closure it results in reduced height. Postmenopausal osteoporosis is induced by a decline of estrogen levels. Both, men and women, express receptors for estrogen and androgen. Estrogen stimulates the production of OPG by osteoblasts and inhibits the production of M-CSF, IL-1, IL-6, and TNF α resulting in a decline of osteoclast formation. Estrogens slow down bone resorption. Animal studies suggest that estrogen alters either the production or activity of local factors regulating osteoclast and osteoblast precursors (64). Progesterone promotes the expression of RANKL and the formation of osteoclasts. The highest concentrations of progesterone occur during pregnancy, which stimulates the release of calcium from the maternal bone in order to facilitate the mineralization of fetal bones. Furthermore, the progesterone-driven RANKL expression promotes the proliferation of mammary epithelial cells. The follicle

stimulating hormone (FSH) stimulates the activity of osteoclasts. The decline of androgens in men occurs at a later age compared to estrogen decline but also results in osteoporosis (64).

Thyroid hormones

The thyroid hormone receptors are located on the surface of the DNA strands. As long as the ligand is absent, the transcription of the corresponding gene is inhibited. The binding of triiodothyronine (T3) and, to a lesser extent, thyroxine (T4), converts the receptors to active stimulators of transcription. Chondrocytes, bone marrow stromal cells, osteoblast- and osteoclast precursors express T3-receptors. Whether T3 effects mature osteoclasts is unclear. The thyroid-stimulating hormone (TSH) inhibits the activity of osteoclasts. A lack of thyroid hormone during childhood leads to growth retardation. Hyperthyroidism causes secondary osteoporosis (69).

Glucocorticoids

Glucocorticoids (GCs) influence both, bone formation as well as bone resorption. They inhibit the functions of osteoblasts by inhibiting the transcription of genes for collagen and osteocalcin. GCs induce RANKL and reduce the expression of OPG, thus enhancing the number and activity of osteoclasts. Furthermore, they also shorten the life span of osteoblasts (69).

Growth hormone and insulin-like growth factor I

Somatotropic cells in the anterior pituitary release the growth hormone (GH) pulsatile during sleep and under physical exertion. Rapid onset effects include lipolysis in the fat tissue, gluconeogenesis in the liver, and inhibition of glucose uptake into the muscles. The growth hormone stimulates the liver to secrete insulin-like growth factor I (IGF-I) into the plasma. Like insulin, IGF-I binds to a hetero-tetrameric receptor. Together with other growth factors like TGF-β and PDGF it is partly immured in the bone through mineralization leading to a reservoir of growth factors, that becomes active in case of bone resorption. IGF-I stimulates the proliferation of chondrocytes and osteoblasts. A lack of growth hormone or IGF-I leads to dwarfism, while overproduction results in gigantism (69).

Other factors

Further factors influencing bone remodeling include genetic, mechanical, vascular, and nutritional factors. About 60 to 80% of the bone mass is genetically determined. Studies showed that daughters of mothers suffering from osteoporosis are predisposed to have osteoporosis themselves. Physical activity promotes the correct development of bone. Osteocytes sense alterations in the osseous fluid induced by muscular action. The absence of muscular activity accelerates bone resorption. Mechanical load improves bone strength by influencing collagen alignment. Regions with predominant tensile stress are more likely to have collagen fibers aligned along the bone axis, while fibers aligned transverse to the long axis predominate in regions with compressive stress. Vascularization is essential for normal bone development and constitutes the first phase in ossification. Furthermore, vascular neoformation is the first step in bone regeneration and fracture healing. Sufficient calcium uptake is essential for mineralization. Smoking, alcohol, caffeine, and augmented salt uptake are risk factors for osteopenia (61,64,69).

2.4.3 Local regulation of bone remodeling

Local regulators influencing bone cell functions include growth factors, cytokines, colony-stimulating factors, prostaglandins, leukotrienes, and nitric oxide (Table 15).

Table 15
Local factors acting on the skeleton.

- Growth factors: IGFs, TGF-β, PDGF, FGF, VEGF
- Cytokines causing bone loss: IL-1, IL-6, IL-11, TNF-α
- Cytokines preventing bone loss: IL-4, IL-10, IL-13, IL-18, IFN
- Colony-stimulating factors: M-CSF and GM-CSF
- Prostaglandins, leukotrienes, and nitric oxide

IGFs, insulin-like growth factors; TGF-β, transforming growth factor-β; PDGF, platelet-derived growth factor; FGF, fibroblast growth factor; VEGF, vascular endothelial growth factor; IL, interleukin; TNF-α, tumor necrosis factor α; IFN, interferon; M-CSF, macrophage colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor;

Growth Factors

Bone contains growth factors, including IGFs, TGF-β superfamily including bone morphogenetic proteins (BMPs), PDGF, FGF, and VEGF (61). IGFs, TGF-β, PDGF, and members of the FGF-family influence the differentiation of committed osteoblast progenitors towards the osteoblastic lineage but cannot induce osteoblast differentiation from uncommitted progenitor cells. BMPs are able to initiate osteoblastogenesis from uncommitted progenitors and the commitment of mesenchymal precursors of the bone marrow to the osteoblastic lineage (63). IGFs and their associated binding proteins constitute the most abundant group of growth factors and are important modulators of local bone remodeling. IGFs are synthesized by the liver and osteoblasts, stimulating collagen synthesis and increasing the number and activity of osteoblasts. The synthesis of IGFs is increased by growth hormone, estrogens, and progesterone, while it is inhibited by GCs (61). TGF-β promotes osteoblastic differentiation and the synthesis of the osteoid matrix, stimulates bone formation, and inhibits the synthesis of proteases. Furthermore, TGF-β inhibits osteoclastic bone resorption by reducing the formation and differentiation of osteoclasts and stimulating their apoptosis. BMPs belong to the TGF-β family promoting osteoblastic differentiation, stimulating osteogenesis, and inhibiting osteoclastogenesis. They stimulate the transformation of connective tissue to bone tissue and the differentiation of stem cells towards different cell lines including cartilage, bone, and adipose tissue. During embryogenesis, BMPs participate in the formation of bone and cartilage (61). PDGF stimulates bone resorption, effects neovascularization, collagen synthesis, and the proliferation of fibroblasts and smooth muscle cells. FGF is a mitogen of osteoblasts, fibroblasts, and vascular endothelial cells with anabolic effects on bone. VEGF induces vascular endothelial proliferation and angiogenesis leading to vasodilatation and increased vascular permeability. It is one of the key factors in the first phase of fracture repair and bone regeneration (61).

Cytokines and other regulatory factors

Cytokines are polypeptides that are synthesized in lymphocytic and monocytic cells playing an important role in diverse processes including inflammation, immunological response, and hematopoiesis (61). A large group of cytokines and colony-stimulating factors affect osteoclast development including the interleukins IL-

1, IL-6, IL-11, oncostatin M, leukemia inhibitory factor, ciliary neurotropic factor, c-kit ligand, TNF-α, GM-CSF, and M-CSF (63). IL-1 stimulates osteoclastic bone resorption, increases the proliferation and differentiation of preosteoblasts, and inhibits the apoptosis of osteoclasts. IL-6 is produced by cells of the stromal/osteoblast lineage acting directly on hematopoietic osteoclast progenitors. IL-11 is produced in bone marrow inducing osteoclastogenesis (61). TNF-α activates NF-κB, thus enhancing the RANKL-mediated stimulation of osteoclasts. Furthermore, TNF-α inhibits osteoblastic activity and enhances the mobilization of osteoclastogenesis progenitors from bone marrow (62). GM-CSF plays an important role in osteoclastogenesis and the pathogenesis of osteopetrosis. M-CSF is produced by osteoblasts and medullar stromal cells. It plays an important role in the first phase of osteoclastogenesis and in the formation of giant multinucleate cells but does not affect osteoclastic activity (61). Interferons as well as the IL-4, IL-10, IL-13, and IL-18 suppress osteoclastogenesis and osteoclast activation (62,63).

2.5 Bone disorders

Normal bone remodeling is associated with a tight coupling of bone resorption to bone formation. Factors altering this process include menopause-associated hormonal changes, age-related factors, drugs, changes in physical activity, and secondary diseases. Table 16 gives a simplified classification of bone remodeling diseases and their definitions. In general, osteoporosis is classified into primary and secondary osteoporosis. Primary osteoporosis is further classified into postmenopausal (type I) and age-related osteoporosis (type II). Two common causes of secondary osteoporosis include glucocorticoid (GC) - induced osteoporosis and immobilization-induced osteoporosis. Renal osteodystrophy, Paget´s disease, osteopetrosis, and rickets/osteomalacia are considerably less prevalent than osteoporosis and are therefore not described in detail (60).

Table 16
Disorders of bone remodeling.

Primary osteoporosis	
Postmenopausal osteoporosis	Occurs secondary to loss of estrogen at menopause
Age-related osteoporosis	Affects men and women equally; increases with increasing age
Secondary osteoporosis	
Glucocorticoid (GC)- induced Osteoporosis	Characterized by bone loss and increased risk of fracture; occurs in patients treated with GCs
Immobilization-induced Osteoporosis	Characterized by bone loss and increased risk of fracture; secondary to immobilization
Rare bone remodeling diseases	
Renal osteodystrophy	Refers to a heterogeneous group of metabolic bone diseases that accompany chronic renal failure
Paget's disease	Focal disease of high bone turnover that results in abnormal bone architecture
Osteopetrosis	Refers to a rare heterogeneous group of genetic bone diseases; characterized by a defect in bone resorption that causes increased bone density
Rickets and osteomalacia	Bone disease caused by absolute or relative vitamin D deficiency

2.5.1 Primary Osteoporosis

Postmenopausal Osteoporosis

Postmenopausal osteoporosis is the most common metabolic disorder of the skeleton, which is characterized by decreased bone mass and bone strength due to excessive resorption or impaired bone formation resulting from estrogen deficiency. Both, bone formation as well as bone resorption, are increased but the extent of increased resorption exceeds that of augmented formation. The amount of bone formed is inadequate to replace the bone resorbed (64). The deficiency of sex steroids stimulates osteoclastogenesis and osteoblastogenesis by up-regulating the synthesis and activity of the responsible cytokines (63). It is assumed that estrogen is protective against osteoporosis due to its modulatory effects on the production of certain cytokines including the suppression of IL-1, IL-6, TNF-α, GM-CSF, and M-CSF. Animal studies showed that estrogen stimulates the expression of OPG in osteoblasts and stromal cells contributing to the antiresorptive action of estrogen in bone. Estrogen and androgen exert antiapoptotic effects on osteocytes and osteoblasts. Estrogen deficiency leads to increased levels of IL-1 and TNF-α

promoting osteoclastogenesis by upregulating the expression of IL-6. IL-1 and TNF-α stimulate RANKL gene expression in stromal cells and osteoblasts. Loss of sex steroids shortens the lifespan of osteocytes and osteoblasts, whereas the life span of osteoclasts is prolonged (60,63).

Age-Related Osteoporosis

Age-related osteoporosis affects both, women and men. The pathogenesis of osteoporosis in women involves changes in FSH and estrogen levels at menopause as well as several distinct age related factors. It is suggested that, in aging women, bone turnover is increased due to secondary hyperparathyroidism or the continuing effect of estrogen deficiency. The aging process is associated with an increase in reactive oxygen species levels. Age-dependent increases in oxidative stress lead to an impairment of osteoblastogenesis and bone formation. Aging is associated with decreased osteoblastogenesis, which is accompanied by decreased osteoclastogenesis as well as an increase of adipogenesis and myelopoiesis. The differentiation of multipotent MSCs is shifted toward adipocytes at the expense of osteoblasts. Age-related osteoporosis is associated with a decline of IGF-1 production and an increase in serum PTH levels. Estrogen deficiency results in reduced intestinal calcium uptake and impaired renal calcium reabsorption. The resulting negative calcium balance leads to an elevation in serum PTH levels. Vitamin D deficiency also contributes to the increase in serum PTH levels by decreasing intestinal calcium uptake (60,63).

2.5.2 Secondary osteoporosis

Secondary osteoporosis results from a variety of medical conditions or treatments causing bone loss. Medical conditions leading to secondary osteoporosis include serious kidney failure, liver impairment, Cushing´s disease, rheumatoid arthritis, anorexia, malabsorption syndromes including celiac disease, multiple sclerosis, and scurvy. Hormonal causes leading to secondary osteoporosis include hyperparathyroidism, hyperthyroidism, diabetes, and hypercortisolism. Other diseases promoting the emergence of secondary osteoporosis involve thalassemia, multiple myeloma, leukemia, and metastatic bone diseases. Several medications or chemicals play an important role in the pathogenesis of secondary osteoporosis

including cigarette smoking, alcohol abuse, lithium, barbiturates, aluminium, and corticosteroid therapy. Immobilization also contributes to the development of secondary osteoporosis. The glucocorticoid (GC)-induced osteoporosis is the most common form of drug-induced osteoporosis affecting the differentiation, function, and survival of cells involved in the bone remodeling process (60). It is characterized by decreased bone formation, decreased wall thickness of trabeculae, and osteonecrosis. GC excess is associated with increased bone resorption, decreased osteoblast proliferation activity, sex-steroid deficiency and hyperparathyroidism due to decreased intestinal calcium absorption (63). GCs prolong the lifespan of osteoclasts and increase osteoclast formation indirectly by inhibiting OPG expression and stimulating RANKL expression in osteoblasts. An increase in the RANKL/OPG ratio changes the balance towards bone resorption leading to a negative net change in bone mass (64).

2.6 Bone turnover markers

The assessment of bone turnover markers (BTMs) is widely used to evaluate bone diseases and to monitor the therapeutic response of patients suffering from bone diseases. Conditions associated with imbalances in bone turnover include metabolic bone diseases, increased or decreased mobility, ageing, somatic growth, and certain therapeutic interventions. Bone mass can be assessed by densitometric techniques, while bone structure and strength are difficult to measure *in vivo*. Molecular markers of bone metabolism are able to detect the dynamics of the metabolic balance itself. The assessment of BTMs is non-invasive and inexpensive constituting a helpful diagnostic tool when applied and interpreted correctly (70). Some potential uses of bone turnover markers are summarized in Table 17.

Table 17
Potential uses of bone turnover markers, adapted from (71).

- Predicting bone loss.
- Identifying people at risk for osteoporosis and bone fracture.
- Monitoring treatment response and identifying non-responders.

Currently available serum and urinary BTMs are classified according to the metabolic process they are considered to reflect (Fig. 27). Some marker components reflect both, bone formation and bone resorption such as certain OC fragments. Most marker components are present in other tissues than bone and are therefore influenced by non-skeletal processes. Changes in bone turnover markers are usually not disease specific, which is why the results should always be interpreted against the clinical picture (70,72). Most markers of bone resorption are related to collagen breakdown products, whereas markers of bone formation are either by-products of collagen neosynthesis or osteoblast-related proteins (70,72,73).

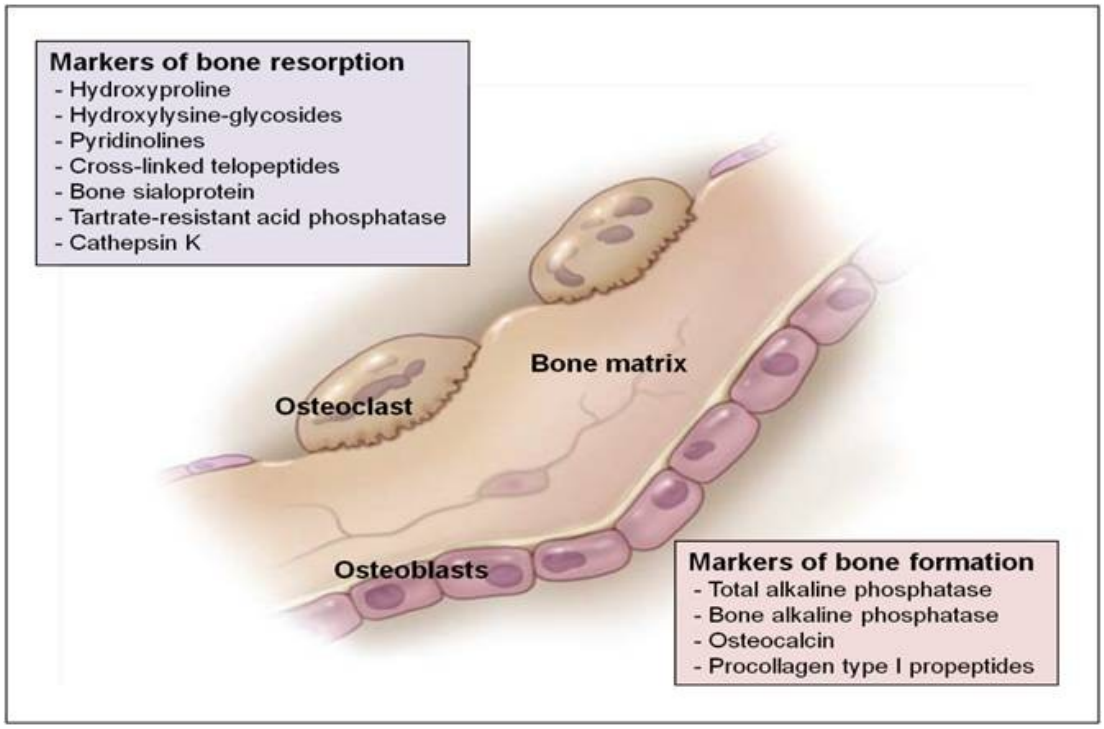


Fig. 27. Biochemical markers of bone remodeling, adapted from (72).

2.6.1 Markers of bone formation

Bone formation markers are products of osteoblasts reflecting different aspects of osteoblast function and bone formation, which can be measured in serum or plasma. The most widely used markers of bone formation include bone-specific alkaline phosphatase (BSAP), osteocalcin (OC), and the carboxy- and amino-terminal propeptides of type 1 collagen (P1CP, P1NP) (Table 18) (70).

Table 18
Markers of bone formation.

Marker	Origin
Bone-specific alkaline phosphatase (BSAP)	Bone
Osteocalcin (OC)	Bone, chondrocytes
C-terminal propeptide of type 1 procollagen (P1CP)	Bone, soft tissue, skin
N-terminal propeptide of type 1 procollagen (P1NP)	Bone, soft tissue, skin

Alkaline phosphatase

Alkaline phosphatase (AP) is an ubiquitous, membrane-bound enzyme involved in osteoid formation and mineralization. The total AP serum pool is comprised of several isoforms originating from bone, liver, spleen, intestine, kidney, and placenta (70,73,74). In children and adolescents, the bone-specific AP (BSAP) predominates due to skeletal growth. In adults with normal liver function, about half of the total AP activity arises from bone, while the other half is derived from the liver. Techniques distinguishing between the two main isoforms include heat denaturation, precipitation, electrophoresis, selective inhibition, and immunoassays (70,73,74). Newer immunoassays allow rapid and simple quantitation, although the rate of cross-reactivity between bone and liver AP was reported to range between 15 and 20% (73). Enzyme-linked immunosorbent assays (ELISA) measure the enzyme activity of BSAP while immunoradiometric assays measure BSAP in protein mass units (71).

Osteocalcin

Osteocalcin (OC) is synthesized by osteoblasts, odontoblasts, and hypertrophic chondrocytes. It is involved in the organization of the extracellular matrix and is mainly expressed during osteoid mineralization. The largest part of newly synthesized OC is incorporated into the extracellular bone matrix, while only a small fraction is released into the circulation, where it can be measured by means of immunoassays. OC is rapidly degraded in serum leading to the coexistence of intact peptides and OC fragments in the circulation (70,71,73). Methods detecting OC include radio immunoassay (RIA), ELISA, and chemiluminescence. OC is a highly specific marker but the heterogeneity of OC fragments in serum and its high circadian and biological variability limits the clinical application (70,75). Assays measuring both, intact peptides as well as OC fragments, appear to be more reproducible and stable.

Due to its instability in serum, rapid loss of immunoreactivity occurs when samples are left at room temperature for more than one hour (70,73).

Procollagen extension propeptides

The procollagen type 1 propeptides include serum procollagen type 1 N-terminal propeptide (P1NP) and serum procollagen type 1 C-terminal propeptide (P1CP). Both are derived from collagen type 1, which is present in bone, skin, dentin, blood vessels, fibrocartilage, tendons, and cornea. In bone, collagen is synthesized by osteoblasts as pre-procollagen, which is characterized by short terminal extension-peptides: the carboxy-terminal and the amino-terminal propeptide. After secretion, they are enzymatically cleaved and liberated into the circulation. P1CP and P1NP are cleared by liver endothelial cells. Most of the non-skeletal tissues have a slower turnover than bone and are therefore only contributing very little to the circulating pool. Both propeptides can be measured by electrochemiluminescence immunoassay analyzers, RIA, and ELISA (70,75). P1NP is released as trimeric structure, which is rapidly broken down to a monomeric form. Currently available immunoassays detect either the trimeric molecule (automated IDS iSYS assay) or measure both fractions (automated Roche ElecSys assay) (76). The levels of procollagen type 1 propeptides reflect the amount of newly synthesized collagen. Extended transport and storage time are well tolerated without significant loss of signal due to their thermostability (70).

2.6.2 Markers of bone resorption

Most bone resorption markers are degradation products of bone collagen including hydroxyproline, hydroxylysine-glycosides, the collagen crosslinks pyridinoline and deoxypyridinoline, C-telopeptides, and N-telopeptides. Bone Sialoprotein is a non-collagenous protein reflecting processes involved in bone resorption. Furthermore, certain age-modified OC fragments are released during bone resorption and may be considered as marker for bone resorption. Markers reflecting osteoclast activity include serum tartrate-resistant acid phosphatase 5b and serum cathepsin K (70,72). Several bone resorption markers are summarized in Table 19.

Table 19
Markers of bone resorption.

Marker	Origin	Specimen
Hydroxyproline (Hyp)	Bone, cartilage, soft tissue, skin	Urine
Hydroxylysine-glycosides (HLG)	Bone, skin, soft tissue, serum complement	Urine, (serum)
Pyridinoline (PYD)	Bone, cartilage, blood vessels, tendon	Urine
Deoxypyridinoline (DPD)	Bone, dentin	Urine
Carboxyterminal cross-linked telopeptide of type I collagen (CTX-I)	All tissues containing type I collagen	Serum, EDTA plasma (β only), Urine (α/β)
Aminoterminal cross-linked telopeptide of type I collagen (NTX-I)	All tissues containing type I collagen	Urine, serum, EDTA plasma
Bone Sialoprotein (BSP)	Bone, dentin, hypertrophic Cartilage	Serum
Osteocalcin fragments	Bone	Urine
Tartrate-resistant acid phosphatase 5b (TRAP 5b)	Bone, blood	Plasma, serum
Cathepsin K	Primarily osteoclasts	Serum, EDTA plasma

Hydroxyproline

Hydroxyproline (Hyp) constitutes 12 to 14% of the total amino acid content of mature collagen. It is liberated during the degradation of bone collagen. Hyp is primarily metabolized in the liver and is excreted in the urine, where it is detectable as free or peptide-bound hydroxyproline through high-performance liquid chromatography (HPLC) or colorimetric methods (70). As Hyp also arises from other tissues, from dietary collagen as well as from newly synthesized collagen, which is not incorporated into tissue, urinary Hyp is an unspecific index of collagen turnover and has therefore been largely replaced by more specific techniques (70,73).

Hydroxylysine-Glycosides

The hydroxylysine-glycosides (HLG) occur in two forms, galactosyl-hydroxylysine (GHL) and glycosyl-galactosyl-hydroxylysine (GGHL), which are both released into the circulation during collagen degradation and measurable in urine by high-performance liquid chromatography (HPLC). GHL is more specific for bone, while GGHL is also present in skin (70,73).

Pyridinoline and deoxypyridinoline

Pyridinoline (PYD) and deoxypyridinoline (DPD) stabilize the collagen molecule and bridge collagen peptides. During bone resorption, PYD and DPD are released into the circulation and the urine, where they can be detected by ELISA, RIA, and HPLC (70,73,74). Their levels strictly reflect the degradation of mature cross-linked collagens and are not influenced by the degradation of newly synthesized collagens (73). DPD is almost exclusively found in bone and dentin, whereas PYD occurs in cartilage, bone, vessels, and ligaments. The measurement of PYD and DPD levels is highly specific for skeletal tissues because bone has a higher turnover compared to other tissues (70).

Crosslinked telopeptides of type I collagen

The crosslinked telopeptides of type I collagen are derived from the amino-terminal (NTX) and the carboxy-terminal (CTX) telopeptide of type I collagen. CTX is produced during bone resorption and is detectable in urine or serum by means of RIA, ELISA, and electrochemiluminescence assays. Four isoforms of CTX are known. The isoform αCTX represents the breakdown of recently synthesized collagen, whereas βCTX represents aged collagen. An increased ratio of αCTX to βCTX is an index of very rapid bone turnover as it occurs in patients with Paget´s disease, for example. The αCTX/βCTX ratio shows a substantial inter-individual variability (75). NTX is also produced during bone resorption and is detected in urine or serum with chemiluminescence assays or competitive inhibition ELISA. The major disadvantage of CTX is its large circadian variation and the required morning fasting. The assessment of NTX in 24h urine is not as sensitive to circadian changes, is non-invasive, and is not affected by food intake. Serum NTX is less sensitive than urinary NTX in detecting changes induced by antiresorptive therapy (75,76).

Bone sialoprotein

Bone sialoprotein (BSP) is a phosphorylated glycoprotein accounting for 5 to 10% of the non-collagenous matrix of bone. It is a synthetic product of osteoblasts and odontoblasts but is also derived from osteoclast-like and malignant cell lines. BSP is mainly detected in mineralized tissues like bone and dentin. BSP plays an important role in cell-matrix adhesion and bone resorption processes (70,73).

Tartrate-resistant acid phosphatase

Acid phosphates are ubiquitous lysosomal enzymes found in bone, platelets erythrocytes, prostate, and spleen (74). The bone isoform is tartrate resistant (TRAP). The subform 5b (TRAP 5b) is expressed in osteoclasts and reflects osteoclast activity. It is increased in high bone turnover conditions including bone metastases, Paget´s disease, and multiple myeloma as well as after ovariectomy (75). TRAP can be measured in plasma or serum by immunoassay or electrophoresis (74). Specific immunoassays for TRACP 5b have been described. At room temperature, TRAP loses more than 20% of its activity per hour, which can be prevented by adding citrate buffer to the sample (70).

Cathepsin K

Serum cathepsin K is the primary proteolytic enzyme degrading bone type I collagen during bone resorption. Cathepsin K belongs to the cysteine protease family and is secreted into bone resorption lacunae for collagen degradation. Cathepsin K is a specific biochemical marker for the activity of osteoclasts (70).

2.6.3 Markers of osteocyte activity

Osteocytes play a key role in the regulation of bone turnover. Due to their sensitivity to mechanical forces, osteocytes are able to detect micro-fractures. Osteocytes regulate bone turnover by interacting with other bone cells and by producing various factors affecting bone formation including sclerostin (SCL), dickkopf-related protein 1 (DKK1), matrix extracellular phosphoglycoprotein (MEPE), and dentin matrix protein 1 (DMP1). SCL and DKK1 inhibit the Wnt signaling pathway by blocking the Wnt effects on osteoblasts. In healthy adults, SCL levels correlate positively with age, bone mineral content, and body mass index, while correlating negatively with OC and calcium levels. Serum SCL is decreased in case of postmenopausal osteoporosis and positively correlated to lumbar spine BMD. Immobilization is associated with high SCL levels leading to suppressed osteoblast activity. Circulating SCL reflects the severity of bone loss and higher serum levels are associated with a greater risk of hip fractures (76).

2.7 Variability of bone turnover markers

The variability of marker assays is an issue of practical concern. In order to interpret serial measurements of BTMs, it is important to identify possible sources of variability including pre-analytical and analytical factors. The ideal biochemical marker and assay is characterized by minimal pre-analytical variability and excellent analytical performance. None of the currently available assays completely meets all of these criteria. In order to minimize pre-analytical and analytical variability, standardized sampling and sample handling are mandatory to obtain reliable results. Assay results should always be interpreted against the background of the respective marker´s variability (70). In order to overcome within person variability in serial measurements and to monitor therapy it is recommended to use the concept of the "Least significant change" (LSC). A significant response in bone turnover can only be assumed, when the change in signal is greater than the imprecision of the measurement. The LSC identifies the "true" physiological change and depends on the short- and long-term within subject variability of a certain marker (70,76).

2.7.1 Biological variability

Many factors contributing to pre-analytical variability are hard to control in clinical practice, while others cannot be modified at all (Table 20).

Table 20
Biological (subject related) sources of variability.

- Age, gender, ethnicity
- Recent fractures
- Pregnancy and lactation
- Drugs: Antiresorptive agents, anabolic agents, glucocorticosteroids, anticonvulsants, Gonadotropin releasing hormone (GnRH) agonists, oral contraception
- Non-skeletal diseases: Diabetes, thyroid disease, renal impairment, liver disease, systemic inflammatory disease, degenerative joint disease
- Immobility, exercise, diet
- Temporal variability: Diurnal (circadian), menstrual, seasonal

Age

During early childhood and during puberty, biochemical bone turnover markers are significantly higher than during adulthood. In girls, peak bone marker levels are reached about two years earlier than in boys. The stabilization of serum and urinary levels of most bone markers occurs during the third decade. In healthy men, these levels remain more or less unchanged until 70 years of age, followed by a slight increase in formation markers and most resorption markers. Menopause is associated with an accelerated bone turnover and increased levels of both, bone formation and resorption markers. The increased rate of bone loss arising after menopause is mainly due to an uncoupling of bone turnover accompanied with an increase in bone resorption. In some postmenopausal women, this increase in bone turnover is associated with vitamin D and/or calcium deficiencies and secondary hyperparathyroidism. In men, bone formation and resorption markers reach their lowest levels between 50 and 60 years of age. Data on bone turnover rates in men over the age of 60 years are largely discordant. Histomorphometric studies suggest that bone formation decreases with age in healthy men, while the osteoclast function remains largely constant but these results are in contrast to other studies that report an age-dependent increase in bone resorption markers. Differences in population characteristics may contribute to these discrepancies. Age-associated changes in the glomerular filtration rate may affect urinary and serum marker concentrations. For this reason, population characteristics, estimates of renal and liver function as well as the specificity of bone markers have to be taken into account when evaluating age-dependent changes in bone turnover (70).

Gender, ethnicity

The levels of BTMs tend to be higher in young men between 20 and 30 years of age compared to women of the same age bracket. In older men, marker levels tend to be lower than in postmenopausal women (70,73). Comparative studies found that bone resorption markers are lower in black individuals than in white individuals. OC appears to be lower in black individuals (73).

Recent fractures

During the first four weeks of fracture healing, bone formation as well as resorption markers increase by 20 to 50% and remain increased for at least six months. The

rate of increase depends on the patient´s age and the localization, size and severity of the fracture (73,76).

Pregnancy and lactation

During pregnancy and lactation, the maternal skeleton provides calcium for the growing fetus and infant. Serum OC levels appear to decrease during pregnancy, possibly due to placental clearance of OC. At term, bone resorption markers are increased by 200% and bone formation markers by 60% in comparison to pre-pregnancy levels. Postpartum, bone marker levels gradually decrease but still may be higher than pre-pregnancy levels for up to twelve months. During lactation, both, bone formation and resorption markers are elevated (73).

Drugs

Antiresorptive treatments including hormone replacement, bisphosphonates, and selective estrogen receptor modulators rapidly reduce bone turnover markers by up to 70%. Corticosteroid therapy significantly reduces serum OC levels. GnRH agonist treatment and anticonvulsant therapy significantly increase bone turnover markers, whereas thiazide diuretics decrease bone turnover. To record the medical history is important in order to interpret assay results correctly (73).

Non-skeletal diseases

Non-skeletal diseases affecting BTMs include diabetes, thyroid disease, renal impairment, liver disease, systemic inflammatory diseases, and degenerative joint diseases. The variability due to non-skeletal diseases is mostly related to impairments in the metabolism and/or clearance of the components measured. BTMs are cleared through the kidneys or the liver and may therefore be influenced by diseases affecting these systems. Even moderate impairment of renal function has significant effects on the serum levels of BSP, OC, CTX, and NTX (70,73). The variability of creatinine excretion affects urinary bone markers, which are therefore usually normalized to creatinine. Inflammatory conditions like rheumatoid arthritis are associated with bone loss (74,76).

Immobility, exercise

Immobility due to bed rest leads to a rapid increase in bone resorption markers. Urinary excretion rates of DPD and PYD are significantly increased after only two days and by 40% after one week of bed rest. Bone formation markers remain unchanged or change only little during immobility (73). The variability of BTMs due to exercise depends on the intensity of physical activity. In trained endurance athletes, P1CP is about 18 to 20% lower than in sedentary controls. Other bone formation markers appear to remain unchanged. Sub-acute exercises lead to decreased bone resorption markers and increased bone formation markers. It is important to ask patients to refrain from exercise for at least 24 hours before sample collection (73).

Diet

Most bone turnover markers are unaffected by diet but the assessment of hydroxyproline and serum CTX requires an overnight fast (73).

Temporal

Bone remodeling varies in a diurnal rhythm and changes with the phase of the menstrual cycle and the season of the year. To minimize the influence of circadian rhythms it is essential to tightly control the timing of sample collection (74). Most BTMs show significant diurnal variations with higher concentrations in the early morning and lower concentrations during the afternoon and at night. The aetiology of diurnal variation is unknown. Certain hormones showing diurnal changes could be involved, including PTH, cortisol, and growth hormone. Serum markers show less pronounced diurnal variation compared to urine based indices but serum CTX shows high daily amplitudes of up to 66% (70). Diurnal variation is less of a factor for OC and alkaline phosphatase, whereas urinary excretion of DPD is 50 to 70% higher at night than in the morning (74). Bone marker levels not only vary within a single day but also between consecutive days, which is called day-to-day or between-day variability. Serum markers show less day-to-day variability than urinary markers ranging between 16 and 26% for urinary PYD and DPD, 13 to 35% for NTX, 12 to 35% for CTX, and 10 to 12% for TRAP. Unlike diurnal variation, day-to-day variability cannot be controlled (70). The changes across the menstrual cycle are only small. It is assumed that the osteoblastic activity is higher during the luteal phase, while bone resorption is higher during the follicular phase (73). The question whether biomarkers

show a seasonal variation is discussed controversially. Some studies have found a substantial wintertime increase, while others suggest that overall seasonal changes are insignificant (76). Increased bone turnover in winter may be due to subclinical vitamin D deficiency. OC levels are elevated in winter and spring, whereas the bone-specific AP shows an inverse rhythm. The impact of seasonal changes of bone turnover markers is especially important when monitoring the short-term response to treatment (70,73).

2.7.2 Technical variability

Technical sources of variability include the timing and mode of sample collection (pre-sample fasting, diet, exercise), the choice of sample (serum vs. urine) as well as the correct handling and storage of specimens. These sources of variability are controllable and hence modifiable. Further factors that should be considered include thermodegradation, photolysis, and between-laboratory variation (70).

Timing and mode of sample collection

It is well known that bone turnover markers show significant diurnal variations with higher concentrations in the early morning and lower concentrations during the afternoon and evening. The most pronounced diurnal changes have been observed for CTX. The effects of diet and food intake need to be considered with certain BTMs. The measurement of hydroxyproline in urine requires a collagen-free diet for at least 24 hours before sample collection. Serum CTX levels should be determined in the fasting state. Random samples can be used for most urinary parameters but the values have to be corrected for urinary creatinine, which introduces additional pre-analytical and analytical variability. Alternatively, the marker can be determined in a 24 hour urine collection. However, most markers exhibit similar results from either 24 hours or spot urine collections (70).

Specimen storage, thermodegradation, photolysis

Appropriate sample collection, preparation and storage are essential for successful BTM measurement. Especially OC and TRAP 5b are sensitive to thermodegradation with reduced levels after only a few hours at room temperature.

TRAP 5b is stable when stored at -70°C or lower but its activity declines during storage at room temperature and -20°C. In comparison, PYD and DPD are stable in urine samples kept at room temperature for several weeks and even repeated freeze-thaw cycles do not affect their concentrations. Pyridinium crosslinks can be stored at -20°C for years. Similar stability has been reported for urinary CTX and NTX (70). Serum CTX levels rapidly decrease at both 4°C and 37°C. This decrease can be minimized by using ethylenediaminetetraacetic acid (EDTA) tubes. CTX is stable in EDTA blood tubes for up to 48 hours; likewise OC is stable for up to eight hours at room temperature. P1NP and BSAP are stable in any sample type (76). Pyridinium crosslinks in aqueous solutions are unstable when exposed to intensive UV irradiation, whereas urinary CTX and NTX are not affected by UV light exposure. OC, TRAP 5b, and BSP are sensitive to haemolysis of the sample leading to results that are either too high or too low. In general, grossly haemolysed samples should always be avoided (70).

Variation between laboratories

Another important technical source of variability is the variation between laboratories. Trials among several European laboratories showed an interlaboratory variation of about 40%. Results obtained from identical urine and blood samples differed up to 5.6-fold between laboratories, even if the same assay and the same method were used. For this reason, results from different laboratories cannot be readily compared to each other and serial measurements should be conducted in the same laboratory (70). Factors contributing to these discrepancies include batch-to-batch and intraassay variation, instability of reagents and calibrators, as well as variations in techniques. The International Federation of Clinical Chemistry and Laboratory medicine (IFCC), the National Bone Health Alliance (NBHA), and the International Osteoporosis Foundation (IOF) recommend that CTX and P1NP should be used as reference analytes in clinical studies. Standardized assays are essential in order to minimize immunochemical heterogeneity. Furthermore, it is recommended that manufacturers minimize batch to batch variability and adopt international reference standards (76).

3 OBJECTIVES

The aim of the present pilot study was to evaluate the levels of 25-hydroxyvitamin D [25(OH)D], C-terminal cross-linked telopeptide of type I collagen (CTX), osteocalcin (OC), procollagen type I N-terminal propeptide (P1NP), and osteoprotegerin (OPG) in patients with a serum HBV DNA level of ≥2000 IU/ml treated with tenofovir compared to patients with a serum HBV DNA level of <2000 IU/ml without indication for antiviral therapy. All parameters were determined at baseline and followed-up three months thereafter.

4 MATERIALS AND METHODS

4.1 Study population and study design

This pilot study included a total of 41 HBV-infected patients attending the Division of Gastroenterology and Hepatology at the Medical University of Graz. Females or males, aged 18 to 75, without previous antiviral therapy were included. Exclusion criteria were pregnancy, existing bone-related diseases (such as osteoporosis, osteomalacia, bone metastases, plasmozytom), decompensated cirrhosis, malignancies, an estimated glomerular filtration rate (eGFR) <60ml/min/1.73m^2, and current supplementation of bone-specific drugs. All participants tested negative for antibodies against HCV and HIV.

Two study groups were defined (Table 21). Patients included in the TDF Group were eligible for treatment according to the EASL guidelines and antiviral treatment with 300mg TDF was started after collection of baseline parameters. Patients included in the Non-TDF Group were not eligible for treatment according to the EASL guidelines (38).

Table 21
Definition of study groups in this pilot study.

	TDF Group	Non-TDF Group
HBV DNA	≥2000 IU/ml	<2000 IU/ml
Anti-HBc	Positive	Positive
HBsAg	Positive	Positive
HBeAg	Positive or negative	Negative
ALT	↑	(↑)

25-hydroxyvitamin D [25(OH)D], bone turnover markers (CTX, OC, P1NP), and OPG were determined at baseline and three months thereafter in both study groups. Patient demographics and clinical data of both study groups were collected from medical records, entered into electronic databases, and retrospectively analyzed (Table 22). Information on past medical history, concomitant medications, and laboratory data were collected. If a bone fraction had occurred, the age at the event, the bone affected, the situation of the event, and the management of the fracture

were recorded. In case of bone pain, detailed information about the quality, frequency, beginning, intensity, and region of pain was collected.

Table 22

Demographic and clinical characteristics of both study groups at the time of enrollment. ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma glutamyl transpeptidase; AP, alkaline phosphatase; eGFR, estimated glomerular filtration rate.

		TDF Group (*n*=23)	Non-TDF Group (*n*=18)
Gender	Female (%)	7/23 (30%)	10/18 (56%)
	Male (%)	16/23 (70%)	8/18 (44%)
Age	M ± SD	46 ± 14	41 ± 14
	Min – Max	27 - 72	18 - 62
	Median	48	37
HBV DNA (IU/ml)	M ± SD	2.7E+07 ± 5.3E+07	3.9E+02 ± 4.0E+02
	Min – Max	2.4E+01 - 1.7E+08	1.5E+01 - 1.4E+03
	Median	2.2E+05	1.9E+02
ALT (U/l)	M ± SD	448 ± 1250	47 ± 33
	Min – Max	13 - 5832	12 - 144
	Median	55	34
AST (U/l)	M ± SD	242 ± 599	37 ± 28
	Min – Max	20 - 2510	18 - 135
	Median	47	27
Total bilirubin (mg/dl)	M ± SD	2.2 ± 5.0	0.7 ± 0.5
	Min – Max	0.3 - 23.7[a]	0.3 - 2.4
	Median	0.8	0.5
GGT (U/l)	M ± SD	61 ± 58	58 ± 79
	Min – Max	9 - 215	7 - 260
	Median	34	24
AP (U/l)	M ± SD	80 ± 34	74 ± 29
	Min – Max	30 - 172	31 - 148
	Median	69	73
Albumin (g/dl)	M ± SD	4.3 ± 0.6	4.6 ± 0.3
	Min – Max	3.2 - 5.1	4.1 - 5.1
	Median	4.4	4.5
Creatinine (mg/dl)	M ± SD	0.9 ± 0.2	0.8 ± 0.2
	Min – Max	0.6 - 1.2	0.6 - 1.0
	Median	0.9	0.8
eGFR[b] (ml/min/1.73m^2)	M ± SD	88.0 ± 15.6	92.2 ± 17.8
	Min – Max	63.0 - 115.6	61.7 - 116.6
	Median	86.3	88.7

[a] Patient with Morbus Meulengracht.

[b] eGFR was calculated using the “modification of diet in renal disease” (MDRD) formula (http://mdrd.com/).

4.2 Specimen collection and storage

Native blood from all study participants was collected at baseline (BL) and three months thereafter (FU) in 9-ml tubes (Greiner Bio-One GmbH, Kremsmünster, Austria). Within two hours of drawing the blood, 9-ml tubes were centrifuged at 1,500×*g* for 20 minutes at room temperature. After centrifugation, aliquots were prepared and immediately frozen at −70°C at the Molecular Diagnostics Laboratory (Medical University of Graz) until tested.

4.3 Biomarker assays

The parameters analyzed included 25-hydroxyvitamin D [25(OH)D], C-terminal cross-linked telopeptide of type I collagen (CTX), osteocalcin (OC), procollagen type I N-terminal propeptide (P1NP), and osteoprotegerin (OPG). All parameters were analyzed at the laboratory of the Division of Endocrinology and Metabolism (Medical University of Graz) using appropriate kits manufactured by Immunodiagnostic Systems GmbH (Frankfurt, Germany). All assays were performed according to the manufacturer´s package insert instructions. The ranges of normality are shown in Table 23.

Table 23
Ranges of normality for 25(OH)D, CTX, OC, P1NP, and OPG.

Parameters	Ranges of normality
25(OH)D	30 - 60 ng/ml
CTX	men < 50 years of age: 0.08 - 0.46 ng/ml men > 50 years of age: 0.06 - 0.35 ng/ml premenopausal women: 0.03 - 0.37 ng/ml postmenopausal women 0.09 - 0.44 ng/ml
OC	1 - 35 ng/ml
P1NP	16 - 67 ng/ml
OPG	0.7 - 4.2 pmol/l

25-hydroxyvitamin D

The determination of 25-hydroxyvitamin D was carried out on the IDS®-iSYS Analyzer (Immunodiagnostic Systems Inc., Scottsdale, AZ) (Fig. 28) based on chemiluminescence technology (Fig. 29). This technique uses two antibodies: one marked with biotin used for capture, the other one marked with acridinium ester used for detection. In a first pre-treatment step, the vitamin D-binding protein was denatured in order to achieve free 25(OH)D. Following neutralization in buffer, the sample, the two antibodies, and the magnetic particles covered with streptavidin were incubated at 37°C. Because there is a strong affinity between biotin and streptavidin the complex is captured on the magnetic particles. After washing the magnetic particles, two trigger solutions were added. Trigger A contains hydrogen peroxide in a dilute acid medium. Trigger B contains a solution of dilute sodium hydroxide. The addition of trigger solutions results in oxidation of the acridinium ester. In its oxidized state, also called excited state, the acridinium ester emits a different wave length following excitation. By returning to its original state, a defined wave length is emitted, which is measured with the luminometer integrated in the analyzer. The signal measured correlates inversely to the 25(OH)D concentration in the sample and is expressed in relative light units.

Fig. 28. The IDS®-iSYS Fully Automated Immunoassay System.

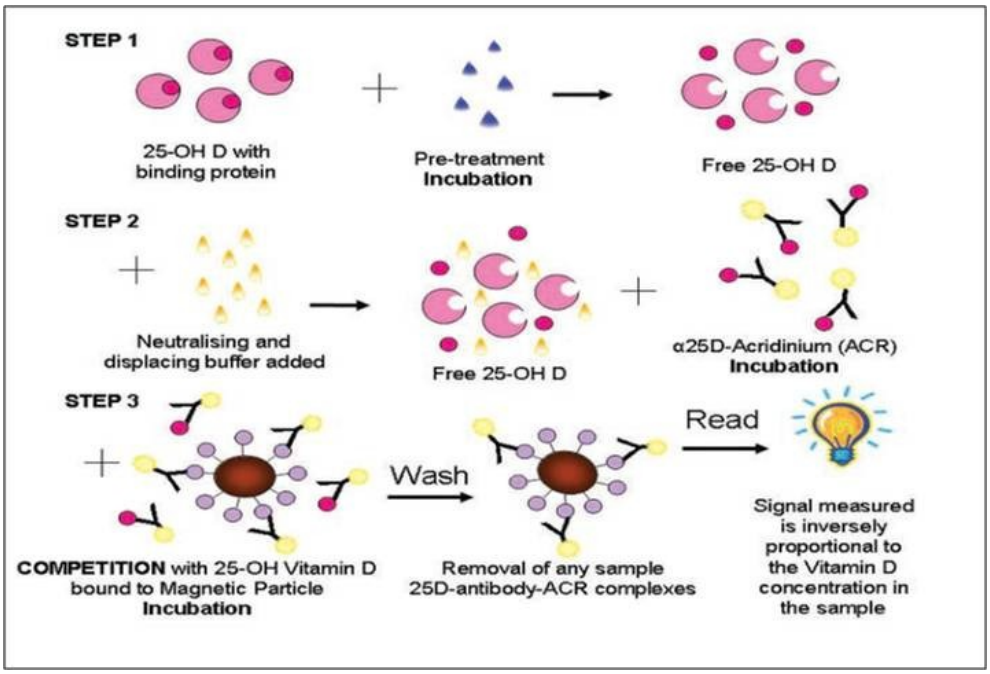


Fig. 29. Chemiluminescence technology.

CTX, OC, and P1NP

The parameters CTX, OC, and P1NP were determined on the cobas e411 analyzer (Roche Diagnostics, Mannheim, Germany) (Fig. 30) utilizing electrochemiluminescence (ECL) technology (Fig. 31). The two electrochemically active substances involved, a ruthenium(II)-tris(bipyridyl) complex and tripropylamine (TPA), are attached to streptavidin-coated micro beads and remain stable as long as no voltage is applied. The ECL reaction, which occurs at the surface of a platinum electrode starts with application of voltage and formation of an electrical field. Ruthenium and TPA are oxidized at the surface of the electrode. The ruthenium complex is reduced to an excited state, which is unstable. When reverting to its stable state a photon is emitted at 620 nm and the ruthenium complex is able to continue reacting, whereas TPA becomes depleted. The peak of light emission occurring is detected via photomultiplier tube and further converted into an electric signal for the calculation of the assay results.

Fig. 30. The cobas e411 analyzer (Roche Diagnostics).

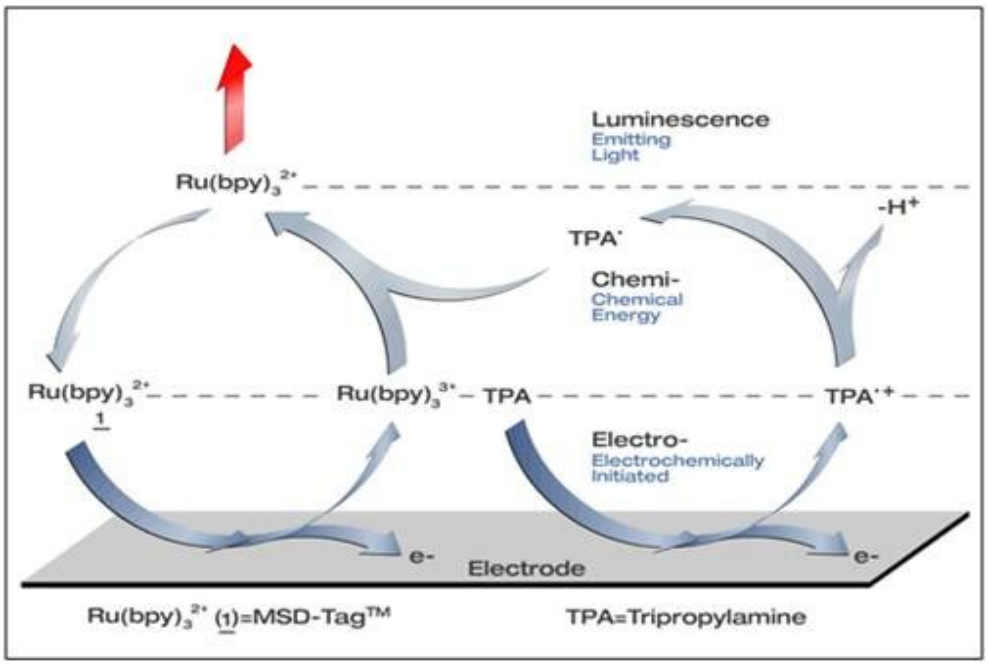


Fig. 31. Electrochemiluminescence (ECL) technology.

OPG

Osteoprotegerin (OPG) was determined on the DS2® Automated ELISA Processing System (Dynex Technologies, Inc., Chantilly, VA) (Fig. 32). First, samples and standards or calibrators are added to the precoated antibody microplate. After

addition of the enzyme conjugate, the mixture is incubated at room temperature. During the incubation period, the competitive binding occurs on the microplate. Following washing steps, the bound enzyme conjugate is detected by the addition of a tetramethylbenzidine (TMB)-based substrate. The absorbance is measured and compared against standards using a microplate reader at 650 or 450 nm if acid stop is used. The extent of color development is proportional to the amount of analyte in the sample (Fig. 33).

Fig. 32. DS2® Automated ELISA Processing System.

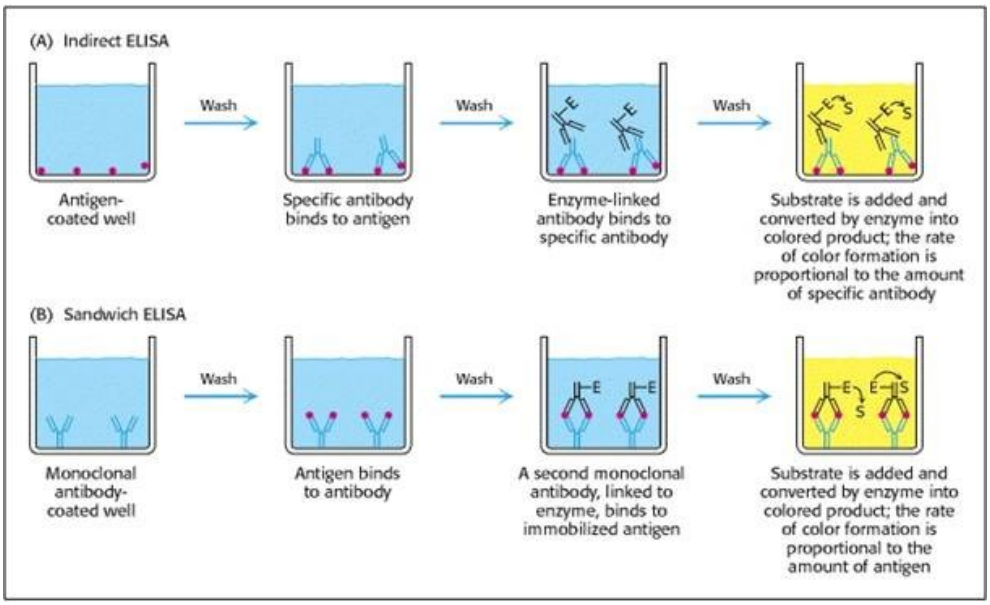


Fig. 33. The ELISA assay test principle.

4.4 Quantitation of serum HBV DNA

Serum HBV DNA quantitation was determined on the COBAS® AmpliPrep/COBAS® TaqMan® HBV system (Roche Molecular Diagnostics, Rotkreuz, Switzerland) including automated specimen preparation on the COBAS® AmpliPrep instrument followed by automated amplification and detection on the COBAS® TaqMan® platform (Fig. 34). The COBAS® AmpliPrep/COBAS® TaqMan® HBV Test, version 2.0 (Roche Molecular Diagnostics, Pleasanton, CA) was used. This assay is based on nucleic acids extraction and real-time polymerase chain reaction (PCR). Results obtained are reported in International Units per milliliter (IU/ml). The assay is standardized against the WHO standard for HBV and offers a broad dynamic range from 20 (lower limit of quantitation, LLQ) to 1.7E+08 IU/ml (upper limit of quantitation, ULQ). All analyses were done with single kit lots of the molecular assay. Runs were considered valid and patient results reported if all conditions outlined in the manufacturer´s package insert instructions occurred.

Fig. 34. The COBAS® AmpliPrep/COBAS® TaqMan® system at the Molecular Diagnostics Laboratory, Medical University of Graz.

4.5 Statistical analysis

Demographic and laboratory data were statistically evaluated using the SPSS software package (SPSS/Systat) and Microsoft Office Excel® 2007. The identity of all study participants was anonymized with ongoing numbers. Standard descriptive statistics were calculated for qualitative variables (absolute and percentage frequencies) and quantitative variables, expressed as mean (M) ± standard deviation (SD), median (Md), minimum (min), maximum (max), and interquartile ranges (Q1, Q3) if not stated otherwise. Normal distributions were verified with the Shapiro-Wilk test. Qualitative variables were compared using the chi-square test. Quantitative variables were evaluated using the *t*-test for independent samples in case of normal distribution and the non-parametric Mann-Whitney U test for variables that were not Gaussianly distributed. In order to detect changes from baseline to follow-up, the Wilcoxon signed-rank test for matched pairs was used. Spearman correlation analyses were performed in order to explore correlations between the change of parameters analyzed and serum levels of ALT, HBV DNA, eGFR, creatinine, and the variables age and gender. *P*-values <0.05 were considered as statistically significant.

5 RESULTS

5.1 Study population – demographic and laboratory data

A total of 41 HBV-infected patients were enrolled in this pilot study including 23 patients with a serum HBV DNA level of ≥2000 IU/ml (TDF Group) and 18 patients with a serum HBV DNA level of <2000 IU/ml (Non-TDF Group). The TDF Group consisted of seven (30%) women and sixteen (70%) men, while the Non-TDF Group comprised ten (56%) women and eight (44%) men. With a median age of 37 years (range 18 - 62), Non-TDF Group members were younger than TDF Group members (median 48 years, range 27 - 72). There was no statistically significant difference between both study groups regarding gender and age (Table 24). Within-group comparisons regarding the age distribution between female and male study participants are summarized in Table 25. There was no statistically significant difference between the groups compared.

Table 24
Between-group comparison of gender and age.

		TDF Group	Non-TDF Group	*p*-value
Gender	Female	30% (7/23)	56% (10/18)	0.11
	Male	70% (16/23)	44% (8/18)	
Age	M ± SD	46 ± 14	41 ± 14	0.24
	Min – Max	27 - 72	18 - 62	
	Median	48	37	

Table 25
Age distribution of female and male participants in both study groups.

	Females, TDF Group Age 46 ± 19 (Md 42 yrs)	Males, Non-TDF Group Age 46 ± 12 (Md 49 yrs)
Males, TDF Group Age 46 ± 13 (Md 49 yrs)	*p*=0.95	*p*=0.97
Females, Non-TDF Group Age 36 ± 14 (Md 33 yrs)	*p*=0.25	*p*=0.13

Laboratory data were collected from medical records at baseline and after three months, including serum HBV DNA, alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin, gamma glutamyl transpeptidase (GGT), alkaline phosphatase (AP), albumin, creatinine, and estimated glomerular filtration rate (eGFR). These data were entered into electronic databases and analyzed retrospectively (Table 26).

At baseline, TDF Group members had higher levels of serum HBV DNA and elevated aminotransferase (ALT, AST) levels compared to Non-TDF Group members. In both study groups, the baseline serum concentrations of total bilirubin, GGT, AP, albumin, and creatinine as well as the eGFR were within the normal range.

After three months of TDF treatment in the TDF Group, the median serum aminotransferase levels reached the normal range but remained slightly higher compared to the Non-TDF Group. In the TDF Group, the median serum HBV DNA value reduced from 2.2E+05 at baseline to 1.5E+01 within three months. The Non-TDF Group showed median serum HBV DNA values of 1.9E+02 at baseline and 3.8E+02 at follow-up. The median serum concentrations of total bilirubin, GGT, AP, albumin, and creatinine remained in the normal range in both study groups. The eGFR in the TDF Group decreased from 86.3 ml/min/1.73m^2 at baseline to 80.8 ml/min/1.73m^2 at follow-up and is thus just above the lower limit of the normal range.

None of the study participants received systemic corticosteroids, vitamin D, calcium, bisphosphonates or any other therapies known to affect bone metabolism.

Table 26

Comparison of laboratory data in both study groups. Green: median levels within the normal range; red: median levels out of the normal range.

		TDF Group		Non-TDF Group		normal range
		BL	FU	BL	FU	
HBV DNA (IU/ml)	M ± SD	2.7E+07 ± 5.3E+07	8.8E+02 ± 2.8E+03	3.9E+02 ± 4.0E+02	6.1E+02 ± 7.6E+02	-
	Min - Max	2.4E+01- 1.7E+08	1.5E+01- 1.4E+04	1.5E+01- 1.4E+03	1.5E+01- 3.2E+03	
	Md	2.2E+05	1.5E+01	1.9E+02	3.8E+02	
ALT (U/l)	M ± SD	448 ± 1250	44 ± 31	47 ± 33	41 ± 25	<45 U/l
	Min - Max	13 - 5832	10 - 116	12 - 144	15 - 88	
	Md	55	35	34	28	
AST (U/l)	M ± SD	242 ± 599	40 ± 12	37 ± 28	33 ± 18	<35 U/l
	Min - Max	20 - 2510	25 - 64	18 - 135	15 - 90	
	Md	47	35	27	26	
Total Bilirubin (mg/dl)	M ± SD	2.2 ± 5.0	0.8 ± 0.3	0.7 ± 0.5	0.6 ± 0.3	0.1-1.2 mg/dl
	Min - Max	0.3 - 23.7*	0.3 - 1.4	0.3 - 2.4	0.1 - 1.5	
	Md	0.8	0.7	0.5	0.5	
GGT (U/l)	M ± SD	61 ± 58	33 ± 29	58 ± 79	58 ± 83	<55 U/l
	Min - Max	9 - 215	8 - 113	7 - 260	11 - 327	
	Md	34	19	24	24	
AP (U/l)	M ± SD	80 ± 34	76 ± 20	74 ± 29	71 ± 23	40-130 g/dl
	Min - Max	30 - 172	33 - 110	31 - 148	34 - 136	
	Md	69	76	73	70	
Albumin (g/dl)	M ± SD	4.3 ± 0.6	4.5 ± 0.4	4.6 ± 0.3	4.5 ± 0.3	3.5-5.3 g/dl
	Min - Max	3.2 - 5.1	3.9 - 5.2	4.1 - 5.1	4.0 - 5.0	
	Md	4.4	4.5	4.5	4.4	
Crea-tinine (mg/dl)	M ± SD	0.9 ± 0.2	0.9 ± 0.2	0.8 ± 0.2	0.8 ± 0.1	0.6-1.3 mg/dl
	Min - Max	0.6 - 1.2	0.6 - 1.3	0.6 - 1.0	0.6 - 1.0	
	Md	0.9	1.0	0.8	0.9	
GFR (ml/min)	M ± SD	88.0 ± 15.6	81.8 ± 16.0	92.2 ± 17.8	91.5 ± 14.4	80-140 ml/min
	Min - Max	63.0 - 115.6	52.8 - 117.5	61.7 - 116.6	74.8 - 120.2	
	Md	86.3	80.8	88.7	87.0	

* Patient with Morbus Meulengracht

5.2 25-hydroxyvitamin D [25(OH)D]

25-hydroxyvitamin D [25(OH)D] sufficiency was defined as >30 ng/ml, 25(OH)D insufficiency as >20 but ≤30 ng/ml, 25(OH)D deficiency as ≤20 ng/ml but >10 ng/ml, and severe 25(OH)D deficiency as ≤10 ng/ml.

5.2.1 25(OH)D levels at baseline

The baseline mean serum 25(OH)D levels were 23.1±10.5 ng/ml in the TDF Group (Median 21.4 ng/ml) and 25.7 ±10.2 ng/ml (Median 25.1 ng/ml) in the Non-TDF Group (Table 27). According to the non-parametric Mann-Whitney U-test, there were no statistically significant differences between both study groups regarding the baseline values of 25(OH)D (p=0.20).

Table 27
Comparison of 25(OH)D levels (ng/ml) in both study groups at baseline (p=0.20).

	TDF Group	Non-TDF Group
M ± SD	23.1 ± 10.5	25.7 ± 10.2
Md	21.4	25.1
Min - Max	8.9 - 58.1	8.9 - 47.5
Q1; Q3	18.0; 27.4	18.9; 32.4

In the TDF Group, three (13%) study participants had levels >30 ng/ml, ten (43%) had levels >20 but ≤30 ng/ml, eight (35%) had levels >10 but ≤20 ng/ml, and two (9%) had levels ≤10 ng/ml. In the Non-TDF Group, seven (39%) study participants had levels >30 ng/ml, five (28%) had levels >20 but ≤30 ng/ml, four (22%) had levels >10 but ≤20 ng/ml, and two (11%) had levels ≤10 ng/ml. The results are summarized in Table 28.

Table 28
Distribution of vitamin D sufficiency, insufficiency, deficiency, and severe deficiency at baseline.

	TDF Group (n=23)	Non-TDF Group (n=18)
Sufficiency (>30 ng/ml)	13% (3/23)	39% (7/18)
Insufficiency (>20 and ≤30 ng/ml)	43% (10/23)	28% (5/18)
Deficiency (>10 and ≤20 ng/ml)	35% (8/23)	22% (4/18)
Severe deficiency (≤10 ng/ml)	9% (2/23)	11% (2/18)

To further investigate, whether the levels of 25(OH)D were influenced by the season of blood draw, all study participants were classified according to the season of 25(OH)D determination at study entry. The period between May and October was designated as summer, while the period from November to April was designated as winter. Fourteen (61%) TDF Group members entered the study in summer. From these fourteen individuals, twelve had 25(OH)D values ≤30 ng/ml and two had sufficient values >30 ng/ml. The remaining nine (39%) members entered the study in winter. From these nine individuals, eight had 25(OH)D values ≤30 ng/ml and one study participant reached the level for 25(OH)D sufficiency. Nine (50%) Non-TDF Group members entered the study in summer, nine (50%) in winter. Of those entering in summer, four had 25(OH)D levels ≤30 ng/ml, while five had levels >30 ng/ml. Of those entering in winter, seven had 25(OH)D levels ≤30 ng/ml and two had levels >30 ng/ml. The results of this classification are summarized in Fig. 35. In both study groups, the mean 25(OH)D levels were higher among patients with samples drawn in summer than in winter. Non-TDF Group members had higher mean 25(OH)D levels than TDF Group members in both seasons (Table 29, Table 30).

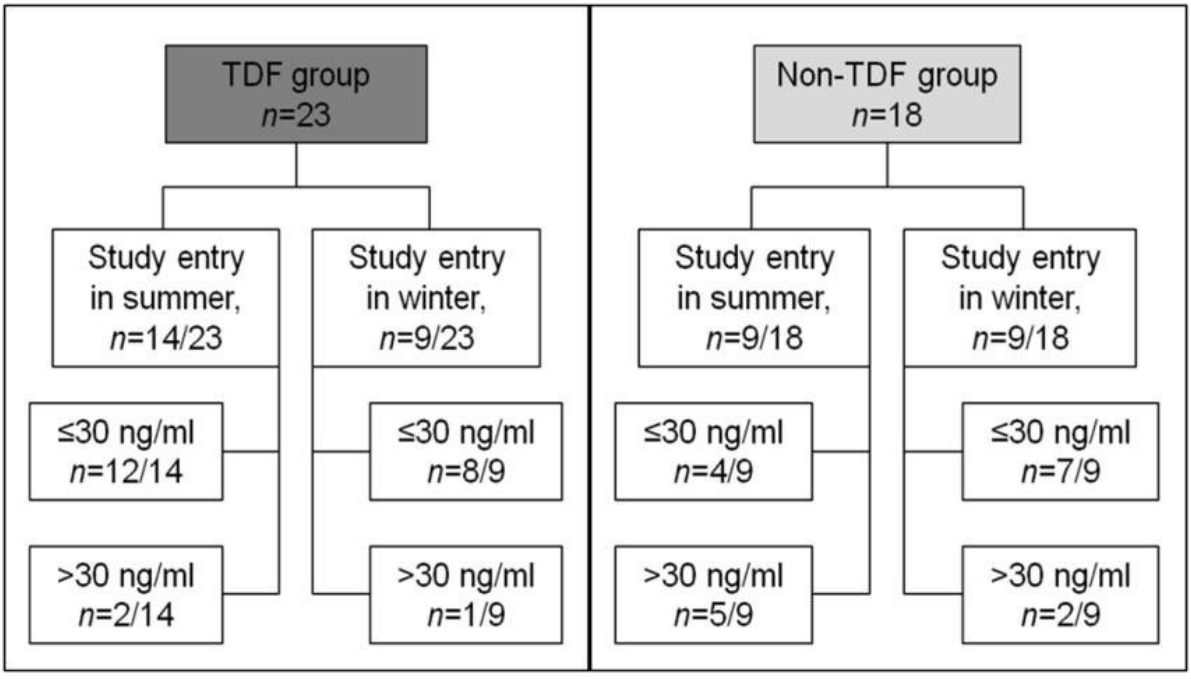


Fig. 35. Classification of 25(OH)D results according to the season at study entry.

Table 29
Comparison of 25(OH)D levels (ng/ml) according to the season at baseline (BL).

		BL in summer	BL in winter
TDF Group	M ± SD	24.2 ± 12.6	21.6 ± 6.5
	Median	19.9	21.5
	Min - Max	9.8 - 58.1	8.9 - 30.5
	Q1; Q3	18.0; 27.2	18.6; 27.0
Non-TDF Group	M ± SD	28.4 ± 11.5	22.9 ± 8.4
	Median	30.1	24.3
	Min - Max	9.8 - 47.5	8.9 - 34.1
	Q1; Q3	18.7; 36.1	19.6; 25.8

Table 30
25(OH)D: Between-group and between-season comparisons at baseline (BL), student´s *t*-test, *p*-values <0.05 indicate statistical significance.

	TDF Group BL in summer	Non-TDF Group BL in winter
Non-TDF Group BL in summer	*p*=0.26	*p*=0.15
TDF Group BL in winter	*p*=0.71	*p*=0.97

5.2.2 25(OH)D levels at follow-up

The mean serum 25(OH)D levels after three months were 19.5±10.3 ng/ml in the TDF Group (Median 16.3 ng/ml) and 25.3±9.7 ng/ml (Median 25.6 ng/ml) in the Non-TDF Group (Table 31). The non-parametric Mann-Whitney U-test revealed statistically significant differences between both study groups regarding the 25(OH)D values at follow-up (*p*=0.04).

Table 31
Comparison of 25(OH)D levels (ng/ml) in both study groups at follow-up (*p*=0.04).

	TDF Group	Non-TDF Group
M ± SD	19.5 ± 10.3	25.3 ± 9.7
Md	16.3	25.6
Min - Max	6.6 - 52.9	10.5 - 41.9
Q1; Q3	13.4; 24.9	16.4; 31.6

In the TDF Group, three (13%) members had levels >30 ng/ml, five (22%) had levels >20 but ≤30 ng/ml, thirteen (56%) had levels >10 but ≤20 ng/ml, and two (9%) had severe vitamin D deficiency with levels ≤10 ng/ml. In the Non-TDF Group, six (33%) members had levels above the sufficiency level of 30 ng/ml, five (28%) had levels >20 but ≤30 ng/ml, seven (39%) had levels >10 but ≤20 ng/ml, and no member had levels ≤10 ng/ml. The results are summarizes in Table 32.

Table 32
Distribution of vitamin D sufficiency, insufficiency, deficiency, and severe deficiency at follow-up.

	TDF Group (*n*=23)	Non-TDF Group (*n*=18)
Sufficiency (>30 ng/ml)	13% (3/23)	33% (6/18)
Insufficiency (>20 and ≤30 ng/ml)	22% (5/23)	28% (5/18)
Deficiency (>10 and ≤20 ng/ml)	56% (13/23)	39% (7/18)
Severe deficiency (≤10 ng/ml)	9% (2/23)	0

Again, the results were classified according to the season of blood collection. The period between May and October was designated as summer and the period from November to April was designated as winter. Eight (35%) TDF Group members were re-evaluated in summer. From these eight individuals, six had 25(OH)D values ≤30 ng/ml and two had sufficient levels >30 ng/ml. Fifteen (65%) TDF Group members were re-evaluated in winter. From these fifteen individuals, fourteen had 25(OH)D values ≤30 ng/ml and one study participant reached the sufficiency level. Eight (44%) Non-TDF Group members were re-evaluated in summer, ten (56%) in winter. Of those evaluated in summer, three participants had 25(OH)D levels ≤30 ng/ml, while five had levels >30 ng/ml. Of those ten evaluated in winter, nine participants had 25(OH)D levels ≤30 ng/ml and one participant reached the sufficiency level. The results of this classification are summarized in Fig. 36. In both study groups, the mean 25(OH)D levels were higher among patients with samples drawn in summer than in winter. Non-TDF Group members had higher mean 25(OH)D levels than TDF Group members in both seasons (Table 33). Between-group comparisons revealed statistically significant differences between both study groups regarding the 25(OH)D levels evaluated at follow-up in winter (p=0.03). Furthermore, the levels of 25(OH)D differed significantly in the TDF Group when comparing the results for winter and summer (p=0.02) (Table 34).

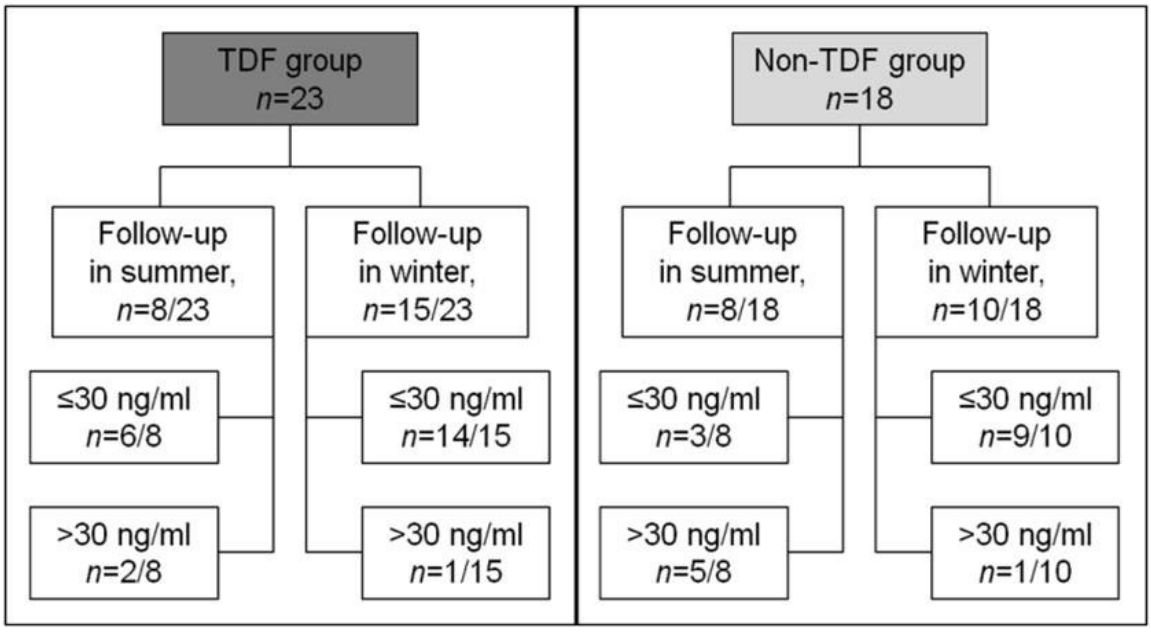


Fig. 36. Classification of 25(OH)D results according to the season at follow-up.

Table 33

Comparison of 25(OH)D levels (ng/ml) according to the season at follow-up (FU).

		FU in summer	FU in winter
TDF Group	M ± SD	26.2 ± 13.3	16.0 ± 6.3
	Median	26.2	16.3
	Min - Max	12.5 - 52.9	6.6 - 30.9
	Q1; Q3	14.6; 30.4	12.3; 18.3
Non-TDF Group	M ± SD	28.9 ± 11.6	22.4 ± 7.1
	Median	32.5	24.8
	Min - Max	13.2 - 41.9	10.5 - 31.5
	Q1; Q3	18.1; 37.7	16.4; 27.8

Table 34

25(OH)D: Between-group and between-season comparisons at follow-up (FU), student´s *t*-test, *p*-values <0.05 indicate statistical significance.

	TDF Group FU in summer	Non-TDF Group FU in winter
Non-TDF Group FU in summer	*p*=0.67	*p*=0.15
TDF Group FU in winter	*p*=0.02	*p*=0.03

5.2.3 Trends of 25(OH)D levels

In the TDF Group, the mean serum 25(OH)D concentration decreased from 23.1±10.5 ng/ml (Median 21.4 ng/ml) at baseline to 19.5±10.3 ng/ml (Median 16.3 ng/ml) at follow-up, which is a decrease of 16%. According to the non-parametric Mann-Whitney U-test, the change from baseline to follow-up regarding the 25(OH)D levels in TDF Group members was statistically significant (p=0.03). In the Non-TDF Group, the mean serum 25(OH)D concentration decreased from 25.7±10.2 ng/ml (Median 25.1 ng/ml) at baseline to 25.3±9.7 ng/ml (Median 25.6 ng/ml) at follow-up, which is a decrease of 2%. The change from baseline to follow-up was not statistically significant (p=0.86) in Non-TDF Group members.

The prevalence rates of suboptimal vitamin D status (<30 ng/ml) were 87% (20/23) at both time-points in TDF Group members as well as 61% (11/18) at baseline and 67% (12/18) at follow-up in Non-TDF Group members. Table 35 and Fig. 37 summarize the results for 25(OH)D in both study groups at both measurement points.

Table 35
25(OH)D levels (ng/ml) in both study groups at both time points.

	TDF Group		Non-TDF Group	
	BL	FU	BL	FU
M ± SD	23.1 ± 10.5	19.5 ± 10.3	25.7 ± 10.2	25.3 ± 9.7
Md	21.4	16.3	25.1	25.6
Min - Max	8.9 - 58.1	6.6 - 52.9	8.9 - 47.5	10.5 - 41.9
Q1; Q3	18.0; 27.4	13.4; 24.9	18.9; 32.4	16.4; 31.6
p-value	p=0.03		p=0.86	

Spearman correlation analyses showed that the change of 25(OH)D from BL to FU significantly correlated with age and gender in Non-TDF Group members: The older the study participants, the higher the decrease of 25(OH)D levels (ρ(rho)=0.47; p=0.047). In female Non-TDF Group members, the 25(OH)D levels tended to decrease to a higher extent (ρ(rho)=0.78; p=0.001) (Fig. 38). The trends of 25(OH)D levels from baseline to follow-up three months thereafter are presented in Fig. 39.

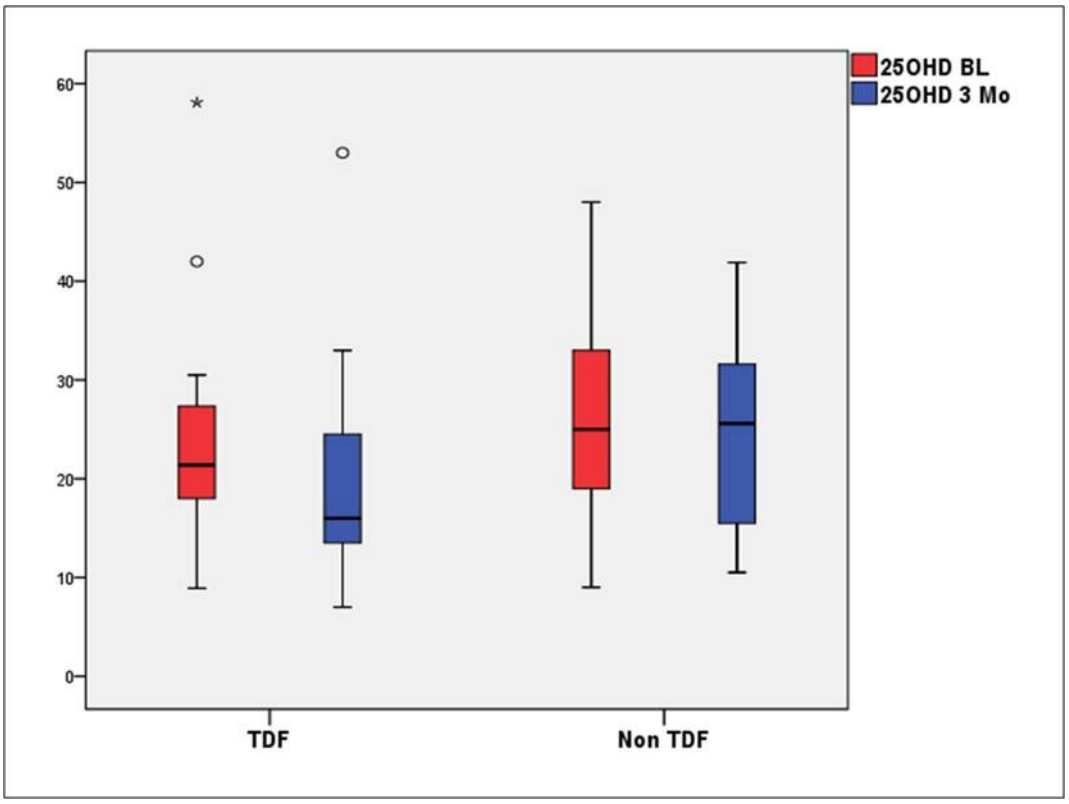


Fig. 37. Boxplot. 25(OH)D levels in both study groups.

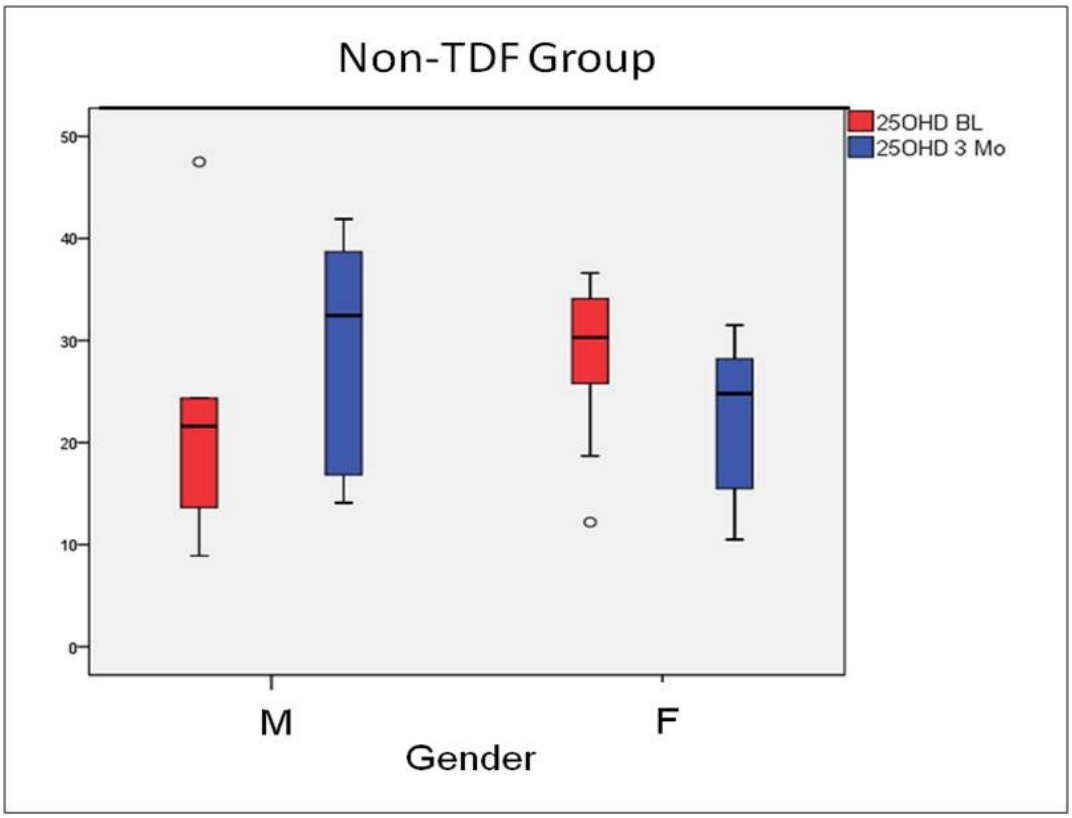
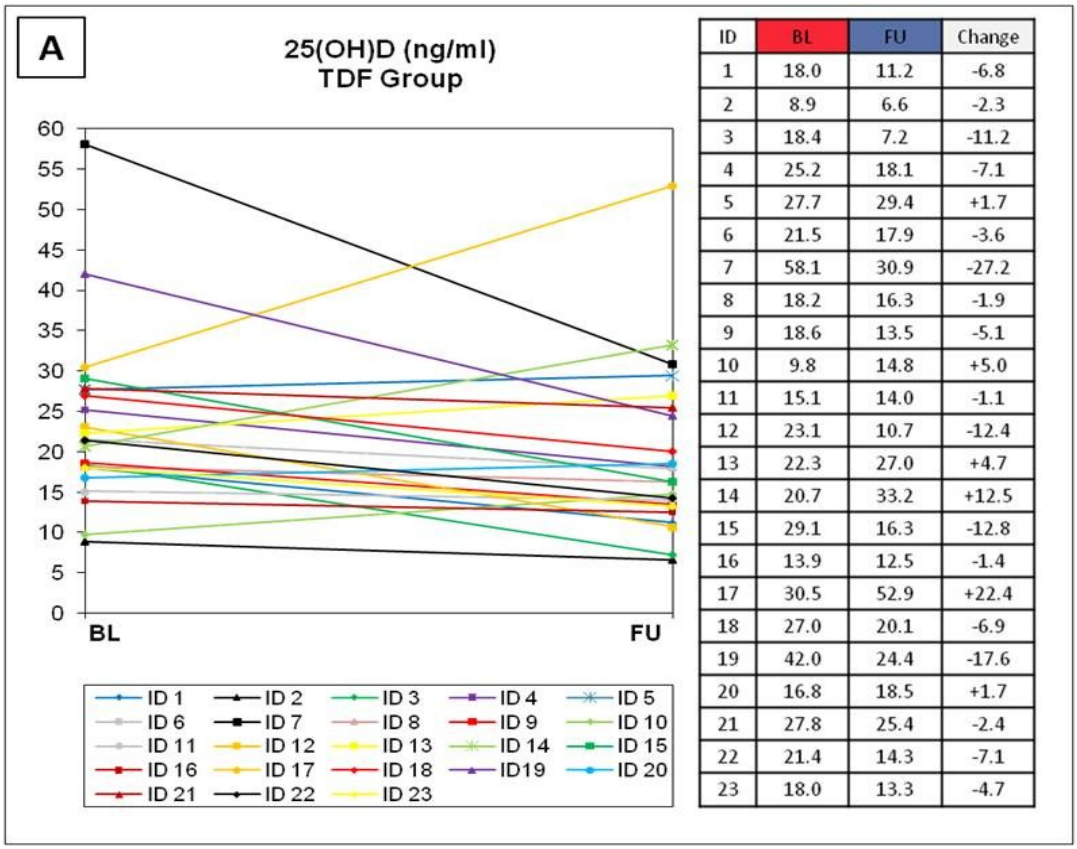
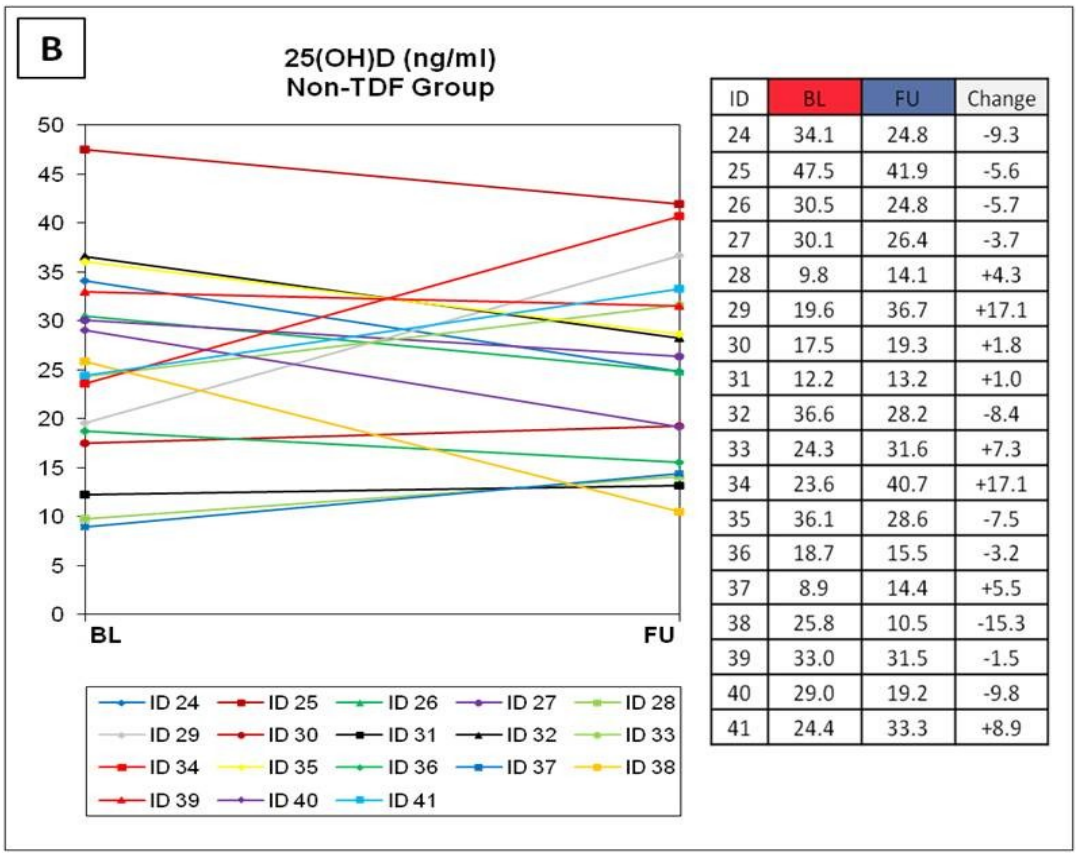


Fig. 38. 25(OH)D levels in male (M) and female (F) Non-TDF Group members.



ID	BL	FU	Change
1	18.0	11.2	-6.8
2	8.9	6.6	-2.3
3	18.4	7.2	-11.2
4	25.2	18.1	-7.1
5	27.7	29.4	+1.7
6	21.5	17.9	-3.6
7	58.1	30.9	-27.2
8	18.2	16.3	-1.9
9	18.6	13.5	-5.1
10	9.8	14.8	+5.0
11	15.1	14.0	-1.1
12	23.1	10.7	-12.4
13	22.3	27.0	+4.7
14	20.7	33.2	+12.5
15	29.1	16.3	-12.8
16	13.9	12.5	-1.4
17	30.5	52.9	+22.4
18	27.0	20.1	-6.9
19	42.0	24.4	-17.6
20	16.8	18.5	+1.7
21	27.8	25.4	-2.4
22	21.4	14.3	-7.1
23	18.0	13.3	-4.7



ID	BL	FU	Change
24	34.1	24.8	-9.3
25	47.5	41.9	-5.6
26	30.5	24.8	-5.7
27	30.1	26.4	-3.7
28	9.8	14.1	+4.3
29	19.6	36.7	+17.1
30	17.5	19.3	+1.8
31	12.2	13.2	+1.0
32	36.6	28.2	-8.4
33	24.3	31.6	+7.3
34	23.6	40.7	+17.1
35	36.1	28.6	-7.5
36	18.7	15.5	-3.2
37	8.9	14.4	+5.5
38	25.8	10.5	-15.3
39	33.0	31.5	-1.5
40	29.0	19.2	-9.8
41	24.4	33.3	+8.9

Fig. 39. Trends of 25(OH)D levels and absolute values in the TDF (**A**) and the Non-TDF (**B**) Groups.

5.3 C-terminal cross-linked telopeptide of type I collagen (CTX)

5.3.1 CTX levels at baseline

The baseline mean serum CTX levels were 0.24±0.12 ng/ml (Median 0.23 ng/ml) in the TDF Group and 0.29±0.11 ng/ml (Median 0.30 ng/ml) in the Non-TDF Group (Table 36). Three (13%) TDF Group members as well as three (17%) Non-TDF Group members had baseline CTX levels above the upper limit of normal. The non-parametric Mann Whitney U-test did not reveal statistically significant differences between both study groups regarding the baseline levels of CTX (p=0.06).

Table 36
Comparison of CTX levels (ng/ml) in both study groups at baseline (p=0.06).

	TDF Group	Non-TDF Group
M ± SD	0.24 ± 0.12	0.29 ± 0.11
Md	0.23	0.30
Min - Max	0.11 - 0.62	0.08 - 0.52
Q1; Q3	0.15; 0.28	0.24; 0.32

5.3.2 CTX levels at follow-up

The mean serum CTX levels after three months were 0.35±0.17 ng/ml (Median 0.33 ng/ml) in the TDF Group and 0.30±0.14 ng/ml (Median 0.27 ng/ml) in the Non-TDF Group (Table 37). Nine (39%) TDF Group members as well as four (22%) Non-TDF Group members had CTX levels above the upper limit of normal. As the results were normally distributed the student´s t-test for independent samples was applied. There were no statistically significant differences between both study groups regarding the CTX levels at follow-up (p=0.35).

Table 37
Comparison of CTX levels (ng/ml) in both study groups at follow-up (p=0.35).

	TDF Group	Non-TDF Group
M ± SD	0.35 ± 0.17	0.30 ± 0.14
Md	0.33	0.27
Min - Max	0.11 - 0.74	0.13 - 0.56
Q1; Q3	0.20; 0.46	0.18; 0.39

5.3.3 Trends of CTX levels

In the TDF Group, the mean serum CTX concentration increased from 0.24±0.12 ng/ml (Median 0.23 ng/ml) at baseline to 0.35±0.17 ng/ml (Median 0.33 ng/ml) at follow-up, which is an increase of 46%. According to the Wilcoxon signed-rank test, the change from baseline to follow-up regarding the CTX levels in TDF Group members was statistically significant (p=0.001). In the Non-TDF Group, the mean serum CTX concentration increased from 0.29±0.11 ng/ml (Median 0.30 ng/ml) at baseline to 0.30±0.14 ng/ml (Median 0.27 ng/ml) at follow-up, which is an increase of 3%. The change from baseline to follow-up was not statistically significant (p=0.70) in Non-TDF Group members.

Table 38 and Fig. 40 summarize the results for CTX in both study groups at both measurement points. The trends of CTX levels from baseline to follow-up three months thereafter as well as absolute values are presented in Fig. 41. Spearman correlation analyses did not reveal any significant correlations between the change of CTX and serum levels of ALT, HBV DNA, creatinine, eGFR, as well as the variables age and gender.

Table 38
CTX levels (ng/ml) in both study groups at both time points.

	TDF Group		Non-TDF Group	
	BL	FU	BL	FU
M ± SD	0.24 ± 0.12	0.35 ± 0.17	0.29 ± 0.11	0.30 ± 0.14
Md	0.23	0.33	0.30	0.27
Min - Max	0.11 - 0.62	0.11 - 0.74	0.08 - 0.52	0.13 - 0.56
Q1; Q3	0.15; 0.28	0.20; 0.46	0.24; 0.32	0.18; 0.39
p-value	p=0.001		p=0.70	

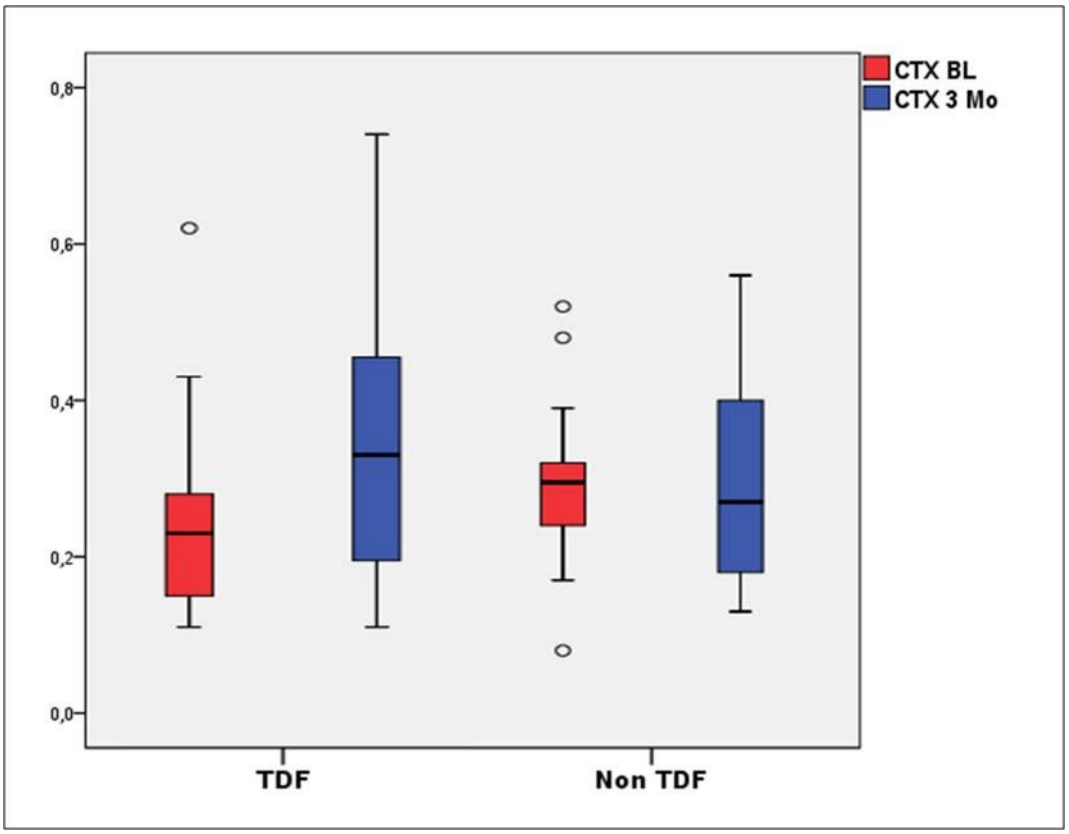
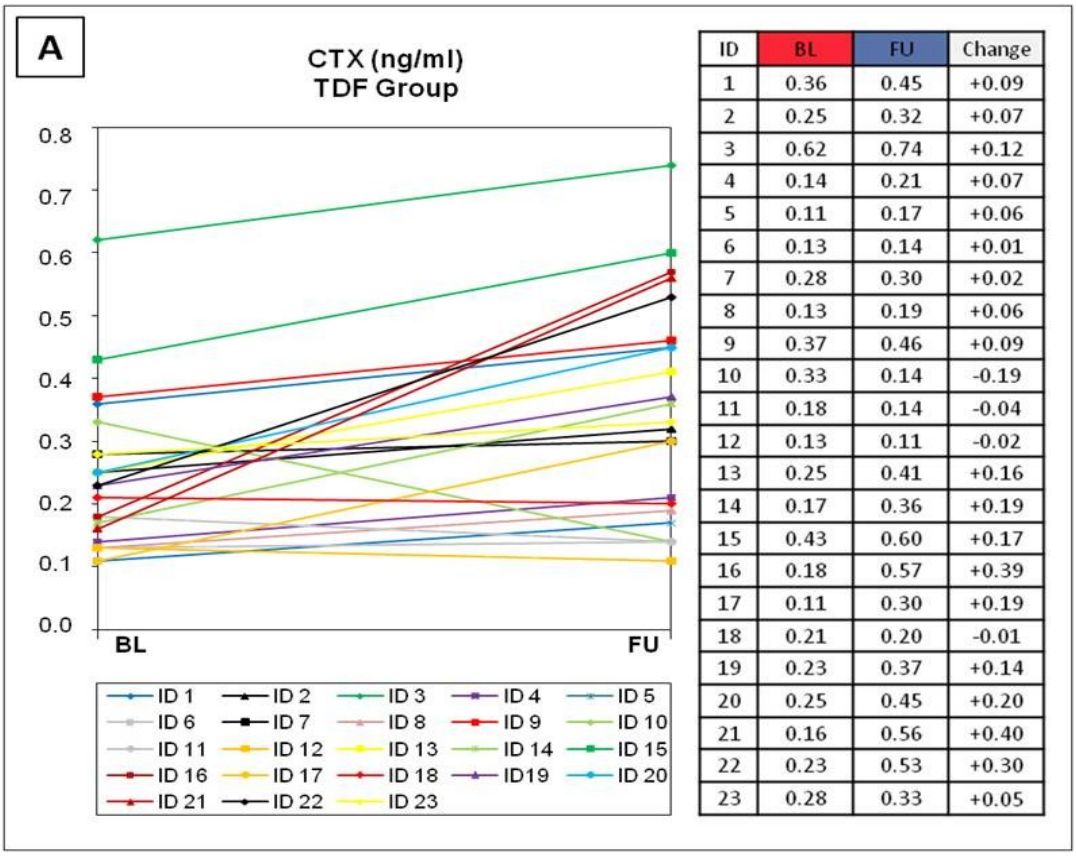
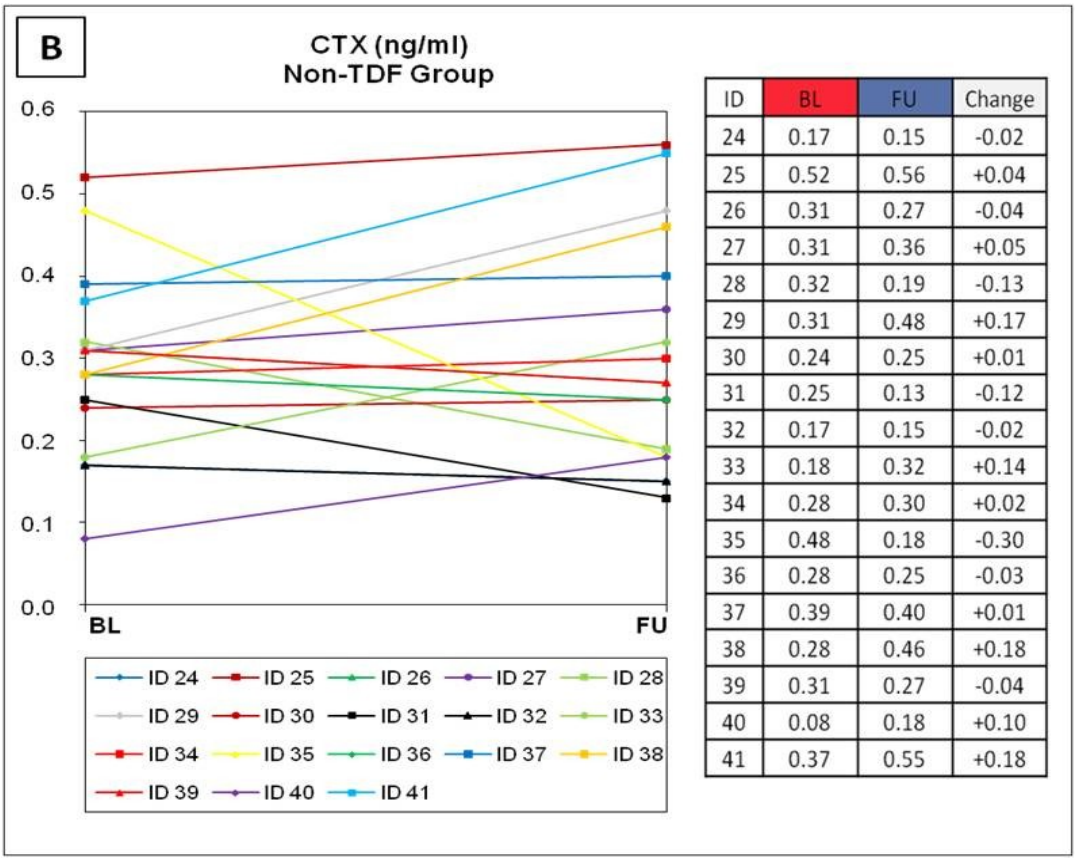


Fig. 40. Boxplot. CTX levels in both study groups.



ID	BL	FU	Change
1	0.36	0.45	+0.09
2	0.25	0.32	+0.07
3	0.62	0.74	+0.12
4	0.14	0.21	+0.07
5	0.11	0.17	+0.06
6	0.13	0.14	+0.01
7	0.28	0.30	+0.02
8	0.13	0.19	+0.06
9	0.37	0.46	+0.09
10	0.33	0.14	-0.19
11	0.18	0.14	-0.04
12	0.13	0.11	-0.02
13	0.25	0.41	+0.16
14	0.17	0.36	+0.19
15	0.43	0.60	+0.17
16	0.18	0.57	+0.39
17	0.11	0.30	+0.19
18	0.21	0.20	-0.01
19	0.23	0.37	+0.14
20	0.25	0.45	+0.20
21	0.16	0.56	+0.40
22	0.23	0.53	+0.30
23	0.28	0.33	+0.05



ID	BL	FU	Change
24	0.17	0.15	-0.02
25	0.52	0.56	+0.04
26	0.31	0.27	-0.04
27	0.31	0.36	+0.05
28	0.32	0.19	-0.13
29	0.31	0.48	+0.17
30	0.24	0.25	+0.01
31	0.25	0.13	-0.12
32	0.17	0.15	-0.02
33	0.18	0.32	+0.14
34	0.28	0.30	+0.02
35	0.48	0.18	-0.30
36	0.28	0.25	-0.03
37	0.39	0.40	+0.01
38	0.28	0.46	+0.18
39	0.31	0.27	-0.04
40	0.08	0.18	+0.10
41	0.37	0.55	+0.18

Fig. 41. Trends of CTX levels and absolute values in the TDF (**A**) and the Non-TDF (**B**) Groups.

5.4 Osteocalcin (OC)

5.4.1 OC levels at baseline

The baseline mean serum OC levels were 17.7±9.2 ng/ml (Median 17.3 ng/ml) in the TDF Group and 21.4±10.2 ng/ml (Median 19.3 ng/ml) in the Non-TDF Group (Table 39). One (4%) TDF Group member and one (6%) Non-TDF Group member had baseline OC levels above the upper limit of normal. According to the non-parametric Mann-Whitney U-test, there were no statistically significant differences between both study groups regarding the baseline levels of OC (p=0.14).

Table 39
Comparison of OC levels (ng/ml) in both study groups at baseline (p=0.14).

	TDF Group	Non-TDF Group
M ± SD	17.7 ± 9.2	21.4 ± 10.2
Md	17.3	19.3
Min - Max	4.6 - 48.9	6.6 - 50.5
Q1; Q3	12.0; 21.3	13.7; 28.2

5.4.2 OC levels at follow-up

The mean serum OC levels after three months were 21.9±8.0 ng/ml (Median 20.0 ng/ml) in the TDF Group and 19.6±7.6 ng/ml (Median 17.1 ng/ml) in the Non-TDF Group (Table 40). All study participants of the Non-TDF Group had levels within the normal range, while three (13%) TDF Group members had OC levels above the upper limit of normal. The non-parametric Mann-Whitney U-test did not reveal any statistically significant differences between both study groups regarding the OC levels at follow-up (p=0.40).

Table 40
Comparison of OC levels (ng/ml) in both study groups at follow-up (p=0.40).

	TDF Group	Non-TDF Group
M ± SD	21.9 ± 8.0	19.6 ± 7.6
Md	20.0	17.1
Min - Max	11.8 - 40.7	6.9 - 30.7
Q1; Q3	17.1; 24.3	13.6; 26.8

5.4.3 Trends of OC levels

In the TDF Group, the mean serum OC concentration increased from 17.7±9.2 ng/ml (Median 17.3 ng/ml) at baseline to 21.9±8.0 ng/ml (Median 20.0 ng/ml) at follow-up, which is an increase of 24%. The Wilcoxon signed-rank test revealed a statistically significant change from baseline to follow-up regarding the OC levels in TDF Group members (p=0.007). In the Non-TDF Group, the mean serum OC concentration decreased from 21.4±10.2 ng/ml (Median 19.3 ng/ml) at baseline to 19.6±7.6 ng/ml (Median 17.1 ng/ml) at follow-up, which is a decrease of 8%. The change from baseline to follow-up was not statistically significant (p=0.50) in Non-TDF Group members.

Table 41 and Fig. 42 summarize the results for OC in both study groups at both measurement points. The trends of OC levels from baseline to follow-up three months thereafter as well as absolute values are presented in Fig. 43. Spearman correlation analyses did not reveal any significant correlations between the change of OC and serum levels of ALT, HBV DNA, creatinine, eGFR, as well as the variables age and gender.

Table 41
OC levels (ng/ml) in both study groups at both time points.

	TDF Group		Non-TDF Group	
	BL	FU	BL	FU
M ± SD	17.7 ± 9.2	21.9 ± 8.0	21.4 ± 10.2	19.6 ± 7.6
Md	17.3	20.0	19.3	17.1
Min - Max	4.6 - 48.9	11.8 - 40.7	6.6 - 50.5	6.9 - 30.7
Q1; Q3	12.0; 21.3	17.1; 24.3	13.7; 28.2	13.6; 26.8
p-value	p=0.007		p=0.50	

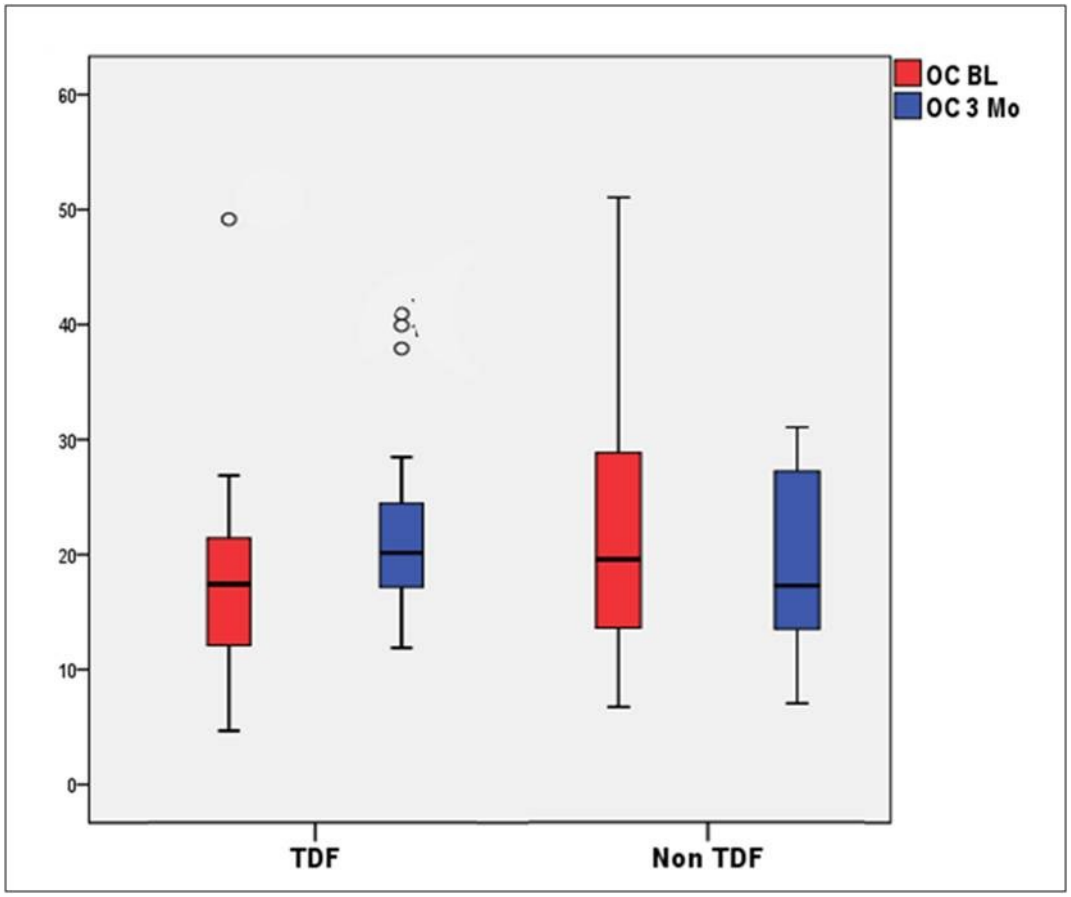
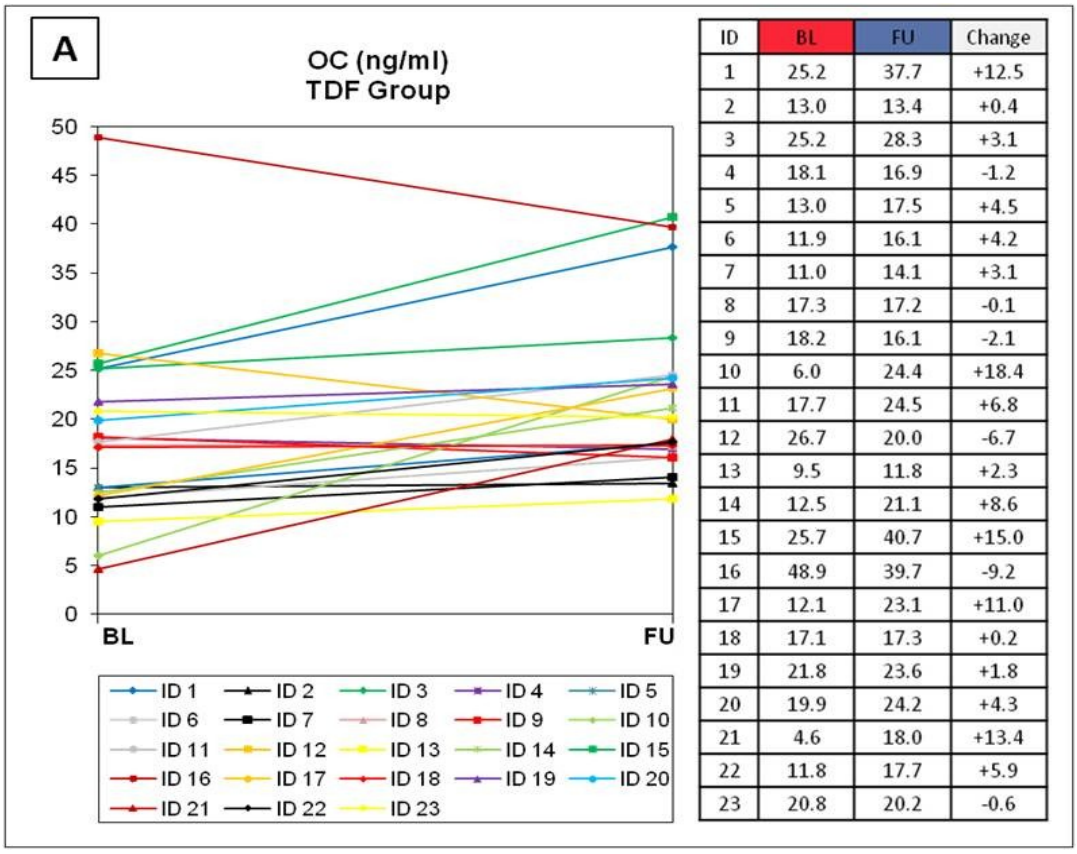
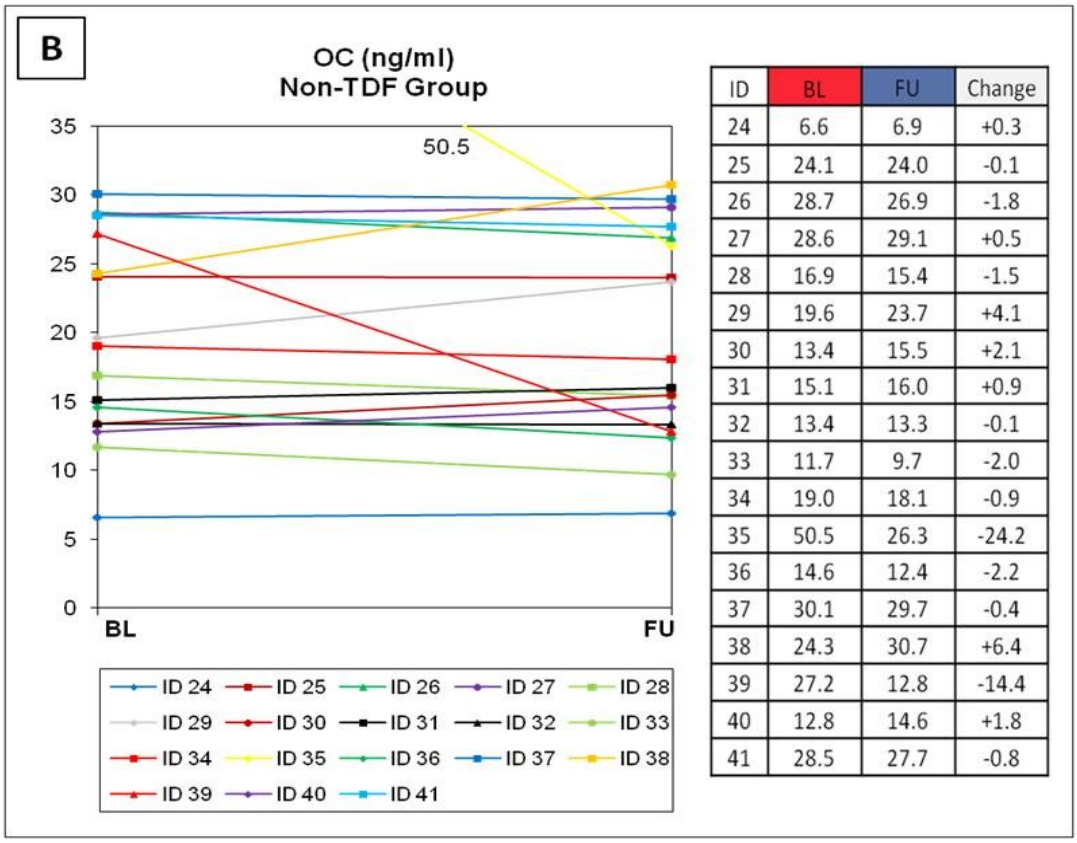


Fig. 42. Boxplot. OC levels in both study.



ID	BL	FU	Change
1	25.2	37.7	+12.5
2	13.0	13.4	+0.4
3	25.2	28.3	+3.1
4	18.1	16.9	-1.2
5	13.0	17.5	+4.5
6	11.9	16.1	+4.2
7	11.0	14.1	+3.1
8	17.3	17.2	-0.1
9	18.2	16.1	-2.1
10	6.0	24.4	+18.4
11	17.7	24.5	+6.8
12	26.7	20.0	-6.7
13	9.5	11.8	+2.3
14	12.5	21.1	+8.6
15	25.7	40.7	+15.0
16	48.9	39.7	-9.2
17	12.1	23.1	+11.0
18	17.1	17.3	+0.2
19	21.8	23.6	+1.8
20	19.9	24.2	+4.3
21	4.6	18.0	+13.4
22	11.8	17.7	+5.9
23	20.8	20.2	-0.6



ID	BL	FU	Change
24	6.6	6.9	+0.3
25	24.1	24.0	-0.1
26	28.7	26.9	-1.8
27	28.6	29.1	+0.5
28	16.9	15.4	-1.5
29	19.6	23.7	+4.1
30	13.4	15.5	+2.1
31	15.1	16.0	+0.9
32	13.4	13.3	-0.1
33	11.7	9.7	-2.0
34	19.0	18.1	-0.9
35	50.5	26.3	-24.2
36	14.6	12.4	-2.2
37	30.1	29.7	-0.4
38	24.3	30.7	+6.4
39	27.2	12.8	-14.4
40	12.8	14.6	+1.8
41	28.5	27.7	-0.8

Fig. 43. Trends of OC levels and absolute values in the TDF (**A**) and the Non-TDF (**B**) Groups.

5.5 Procollagen type 1 N-terminal propeptide (P1NP)

5.5.1 P1NP levels at baseline

The baseline mean serum P1NP levels were 60.2±45.5 ng/ml (Median 46.2 ng/ml) in the TDF Group and 56.5±26.9 ng/ml (Median 53.5 ng/ml) in the Non-TDF Group (Table 42). Five (22%) TDF Group members and four (22%) Non-TDF Group members had baseline P1NP levels above the upper limit of normal. The non-parametric Mann-Whitney U-test did not reveal any statistically significant differences between both study groups regarding the baseline levels of P1NP (p=0.62).

Table 42
Comparison of P1NP levels (ng/ml) in both study groups at baseline (p=0.62).

	TDF Group	Non-TDF Group
M ± SD	60.2 ± 45.5	56.5 ± 26.9
Md	46.2	53.5
Min - Max	14.3 - 230.4	25.4 - 143.4
Q1; Q3	38.1; 58.7	42.5; 65.1

5.5.2 P1NP levels at follow-up

The mean serum P1NP levels after three months were 55.5±20.8 ng/ml (Median 46.9 ng/ml) in the TDF Group and 46.8±17.8 ng/ml (Median 49.1 ng/ml) in the Non-TDF Group (Table 43). Three (13%) TDF Group members and one (6%) Non-TDF Group member had P1NP levels above the upper limit of normal. The non-parametric Mann-Whitney U-test did not reveal any statistically significant differences between both study groups regarding the P1NP levels at follow-up (p=0.35).

Table 43
Comparison of P1NP levels (ng/ml) in both study groups at follow-up (p=0.35).

	TDF Group	Non-TDF Group
M ± SD	55.5 ± 20.8	46.8 ± 17.8
Md	46.9	49.1
Min - Max	31.6 - 109.4	20.9 - 86.7
Q1; Q3	43.9; 59.9	35.8; 58.2

5.5.3 Trends of P1NP levels

In the TDF Group, the mean serum P1NP concentration decreased from 60.2±45.5 ng/ml (Median 46.2 ng/ml) at baseline to 55.5±20.8 ng/ml (Median 46.9 ng/ml) at follow-up, which is a decrease of 8%. Due to the presence of outliers, the median percent change was also calculated. The median P1NP concentration increased from 46.2 ng/ml at baseline to 46.9 ng/ml at follow-up, which is an increase of 2%. The Wilcoxon signed-rank test did not reveal any statistically significant change from baseline to follow-up regarding the P1NP levels in TDF Group members (p=0.13). In the Non-TDF Group, the mean serum P1NP concentration decreased from 56.5±26.9 ng/ml (Median 53.5 ng/ml) at baseline to 46.8±17.8 ng/ml (Median 49.1 ng/ml) at follow-up, which is a decrease of 17%. The change from baseline to follow-up regarding the P1NP levels in Non-TDF Group members was not statistically significant (p=0.06).

Table 44 and Fig. 44 summarize the results for P1NP in both study groups at both measurement points. The trends of P1NP levels from baseline to follow-up three months thereafter as well as absolute values are presented in Fig. 45. Spearman correlation analyses did not reveal any significant correlations between the change of P1NP and serum levels of ALT, HBV DNA, creatinine, eGFR, as well as the variables age and gender.

Table 44
P1NP levels (ng/ml) in both study groups at both time points.

	TDF Group		Non-TDF Group	
	BL	FU	BL	FU
M ± SD	60.2 ± 45.5	55.5 ± 20.8	56.5 ± 26.9	46.8 ± 17.8
Md	46.2	46.9	53.5	49.1
Min - Max	14.3 - 230.4	31.6 - 109.4	25.4 - 143.4	20.9 - 86.7
Q1; Q3	38.1; 58.7	43.9; 59.9	42.5; 65.1	35.8; 58.2
p-value	p=0.13		p=0.06	

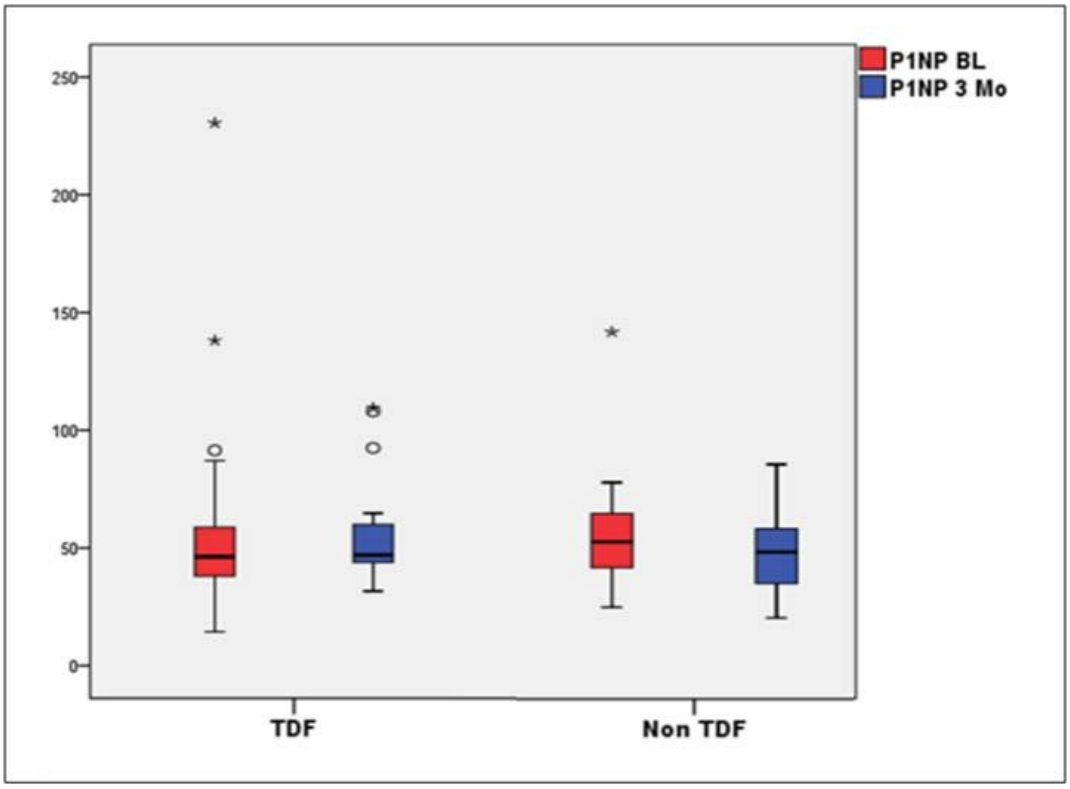
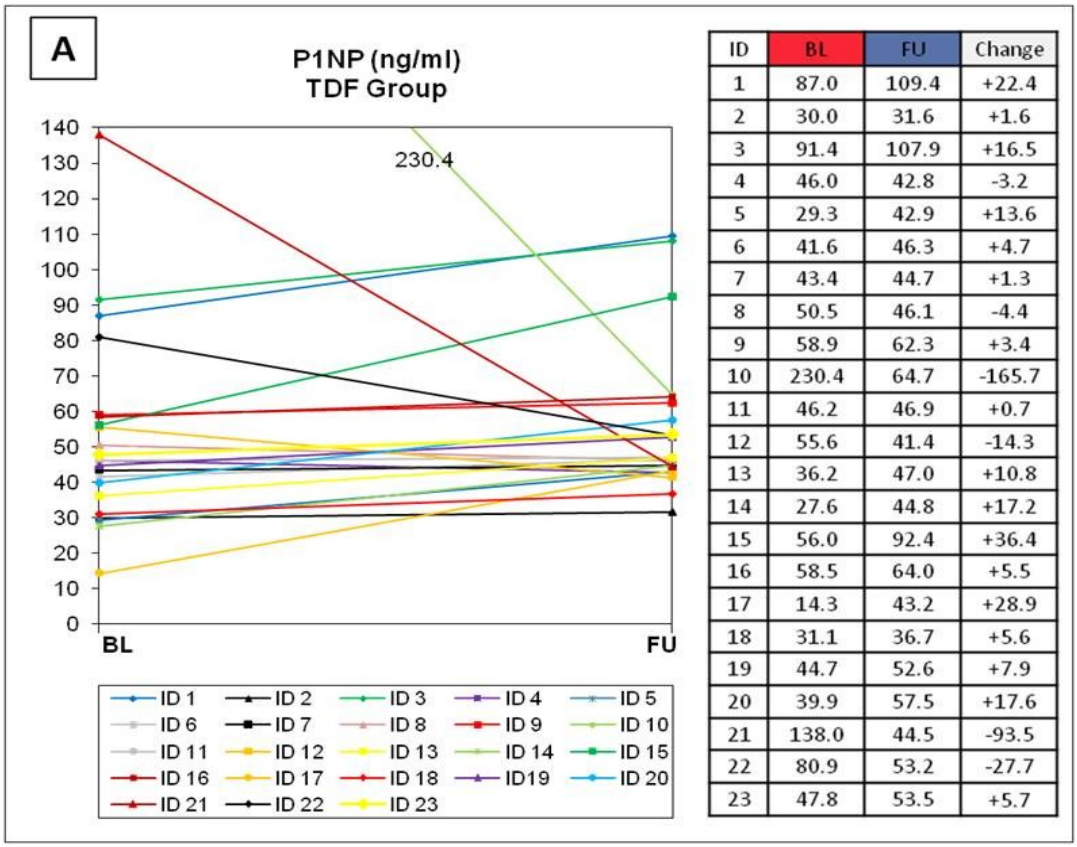
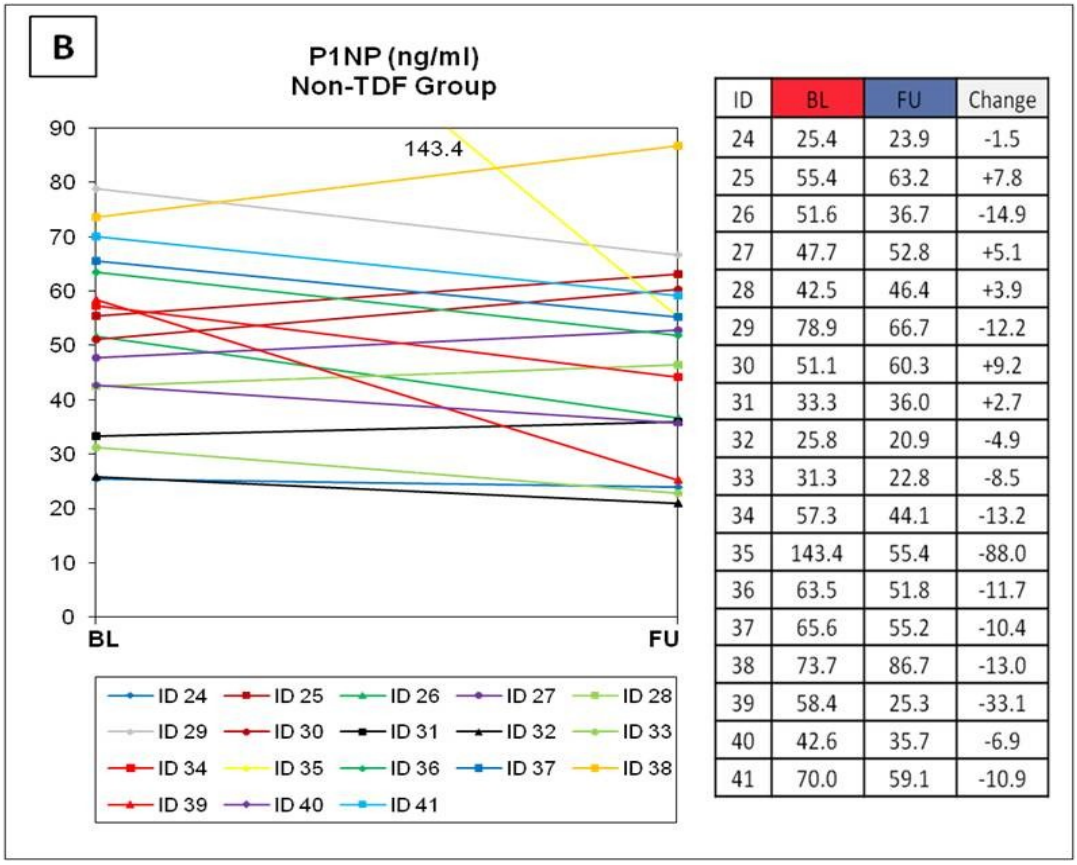


Fig. 44. Boxplot. P1NP levels in both study groups.



ID	BL	FU	Change
1	87.0	109.4	+22.4
2	30.0	31.6	+1.6
3	91.4	107.9	+16.5
4	46.0	42.8	-3.2
5	29.3	42.9	+13.6
6	41.6	46.3	+4.7
7	43.4	44.7	+1.3
8	50.5	46.1	-4.4
9	58.9	62.3	+3.4
10	230.4	64.7	-165.7
11	46.2	46.9	+0.7
12	55.6	41.4	-14.3
13	36.2	47.0	+10.8
14	27.6	44.8	+17.2
15	56.0	92.4	+36.4
16	58.5	64.0	+5.5
17	14.3	43.2	+28.9
18	31.1	36.7	+5.6
19	44.7	52.6	+7.9
20	39.9	57.5	+17.6
21	138.0	44.5	-93.5
22	80.9	53.2	-27.7
23	47.8	53.5	+5.7



ID	BL	FU	Change
24	25.4	23.9	-1.5
25	55.4	63.2	+7.8
26	51.6	36.7	-14.9
27	47.7	52.8	+5.1
28	42.5	46.4	+3.9
29	78.9	66.7	-12.2
30	51.1	60.3	+9.2
31	33.3	36.0	+2.7
32	25.8	20.9	-4.9
33	31.3	22.8	-8.5
34	57.3	44.1	-13.2
35	143.4	55.4	-88.0
36	63.5	51.8	-11.7
37	65.6	55.2	-10.4
38	73.7	86.7	-13.0
39	58.4	25.3	-33.1
40	42.6	35.7	-6.9
41	70.0	59.1	-10.9

Fig. 45. Trends of P1NP levels and absolute values in the TDF (**A**) and the Non-TDF (**B**) Groups.

5.6 Osteoprotegerin (OPG)

5.6.1 OPG levels at baseline

The baseline mean serum OPG levels were 5.8±4.0 pg/ml (Median 4.9 pg/ml) in the TDF Group and 4.8±2.2 pg/ml (Median 4.3 pg/ml) in the Non-TDF Group (Table 45). Fifteen (65%) TDF Group members as well as nine (50%) Non-TDF Group members had baseline OPG levels above the upper limit of normal. The non-parametric Mann-Whitney U-test did not reveal any statistically significant differences between both study groups regarding the baseline levels of OPG (p=0.33).

Table 45
Comparison of OPG levels (pg/ml) in both study groups at baseline (p=0.33).

	TDF Group	Non-TDF Group
M ± SD	5.8 ± 4.0	4.8 ± 2.2
Md	4.9	4.3
Min - Max	2.7 - 21.7	2.2 - 11.0
Q1; Q3	3.6; 6.0	3.7; 4.9

5.6.2 OPG levels at follow-up

The mean serum OPG levels after three months were 4.7±1.6 pg/ml (Median 4.7 pg/ml) in the TDF Group and 4.4±2.0 pg/ml (Median 3.9 pg/ml) in the Non-TDF Group (Table 46). Fifteen (65%) TDF Group members as well as eight (44%) Non-TDF Group members had OPG levels above the upper limit of normal. The non-parametric Mann-Whitney U-test did not reveal any statistically significant differences between both study groups regarding the OPG levels at follow-up (p=0.17).

Table 46
Comparison of OPG levels (pg/ml) in both study groups at follow-up (p=0.17).

	TDF Group	Non-TDF Group
M ± SD	4.7 ± 1.6	4.4 ± 2.0
Md	4.7	3.9
Min - Max	2.0 - 8.7	2.2 - 11.0
Q1; Q3	3.8; 5.3	3.4; 4.8

5.6.3 Trends of OPG levels

In the TDF Group, the mean serum OPG concentration decreased from 5.8±4.0 pg/ml (Median 4.9 pg/ml) at baseline to 4.7±1.6 pg/ml (Median 4.7 pg/ml) at follow-up, which is a decrease of 19%. The Wilcoxon signed-rank test did not reveal any statistically significant change from baseline to follow-up regarding the OPG levels in TDF Group members (p=0.69). In the Non-TDF Group, the mean serum OPG concentration decreased from 4.8±2.2 pg/ml (Median 4.3 pg/ml) at baseline to 4.4±2.0 pg/ml (Median 3.9 pg/ml) at follow-up, which is a decrease of 8%. According to the Wilcoxon signed-rank test, the change from baseline to follow-up was not statistically significant (p=0.13) in Non-TDF Group members.

Table 47 and Fig. 46 summarize the results for OPG in both study groups at both measurement points. The trends of OPG levels from baseline to follow-up three months thereafter as well as absolute values are presented in Fig. 47. Spearman correlation analyses did not reveal any significant correlations between the change of OPG and serum levels of ALT, HBV DNA, creatinine, eGFR, as well as the variables age and gender.

Table 47
OPG levels (pg/ml) in both study groups at both time points.

	TDF Group		Non-TDF Group	
	BL	FU	BL	FU
M ± SD	5.8 ± 4.0	4.7 ± 1.6	4.8 ± 2.2	4.4 ± 2.0
Md	4.9	4.7	4.3	3.9
Min - Max	2.7 - 21.7	2.0 - 8.7	2.2 - 11.0	2.2 - 11.0
Q1; Q3	3.6; 6.0	3.8; 5.3	3.7; 4.9	3.4; 4.8
p-value	p=0.69		p=0.13	

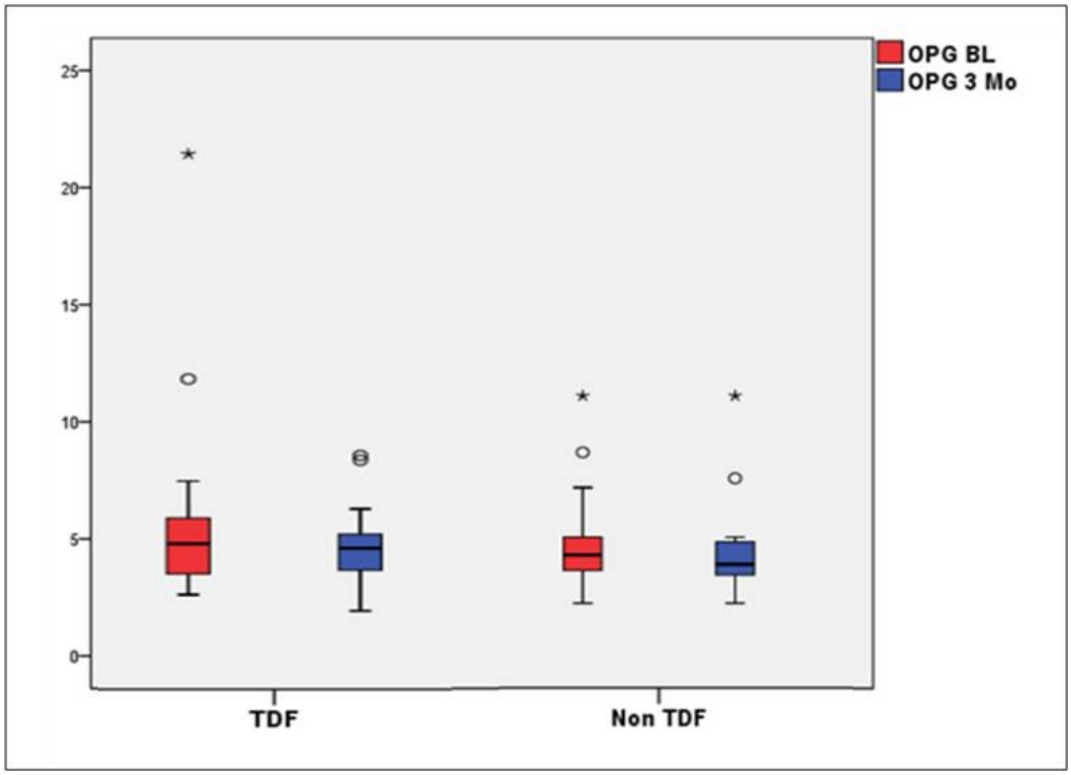
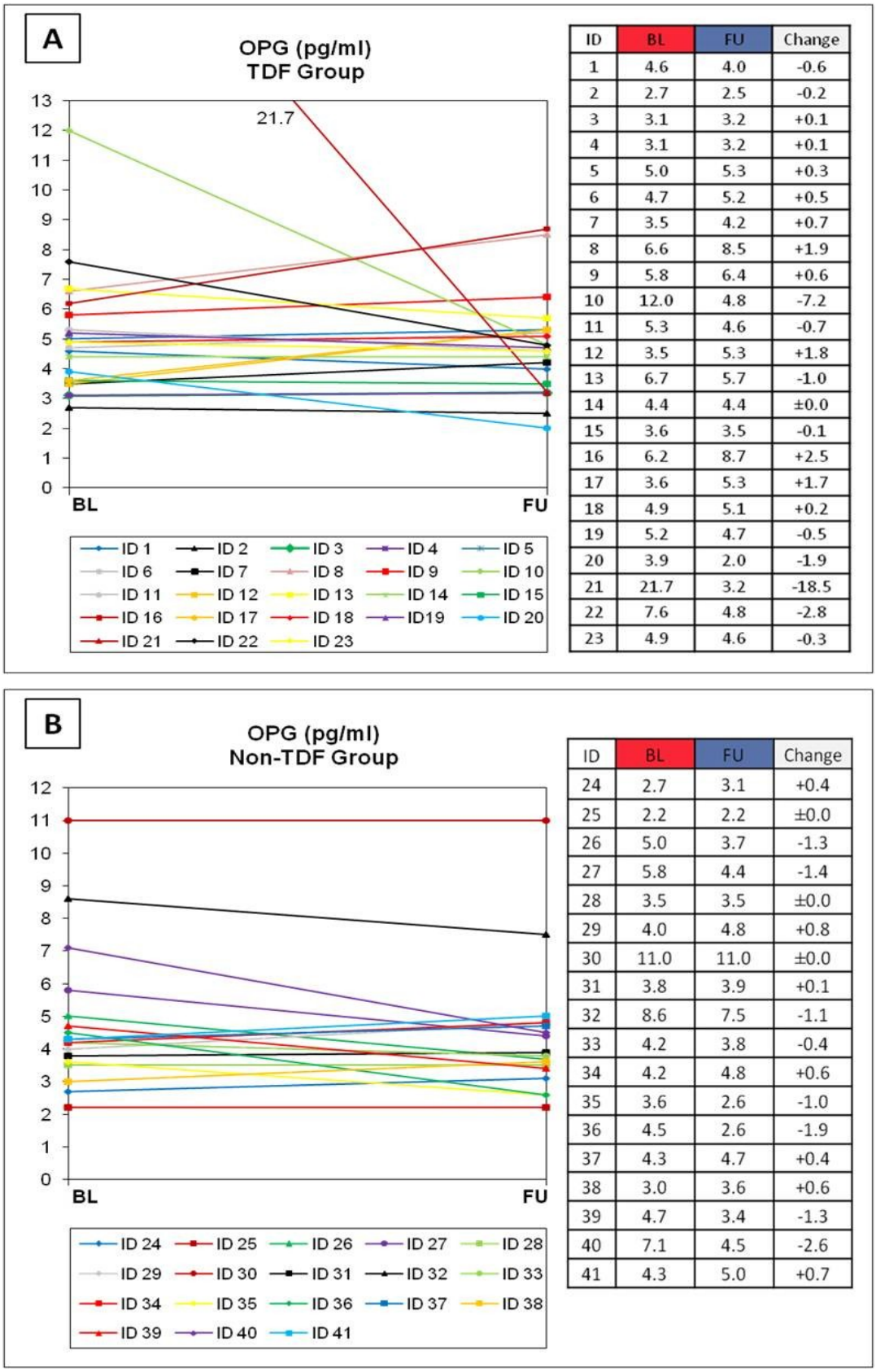


Fig. 46. Boxplot. OPG levels in both study groups.



ID	BL	FU	Change
1	4.6	4.0	-0.6
2	2.7	2.5	-0.2
3	3.1	3.2	+0.1
4	3.1	3.2	+0.1
5	5.0	5.3	+0.3
6	4.7	5.2	+0.5
7	3.5	4.2	+0.7
8	6.6	8.5	+1.9
9	5.8	6.4	+0.6
10	12.0	4.8	-7.2
11	5.3	4.6	-0.7
12	3.5	5.3	+1.8
13	6.7	5.7	-1.0
14	4.4	4.4	±0.0
15	3.6	3.5	-0.1
16	6.2	8.7	+2.5
17	3.6	5.3	+1.7
18	4.9	5.1	+0.2
19	5.2	4.7	-0.5
20	3.9	2.0	-1.9
21	21.7	3.2	-18.5
22	7.6	4.8	-2.8
23	4.9	4.6	-0.3

ID	BL	FU	Change
24	2.7	3.1	+0.4
25	2.2	2.2	±0.0
26	5.0	3.7	-1.3
27	5.8	4.4	-1.4
28	3.5	3.5	±0.0
29	4.0	4.8	+0.8
30	11.0	11.0	±0.0
31	3.8	3.9	+0.1
32	8.6	7.5	-1.1
33	4.2	3.8	-0.4
34	4.2	4.8	+0.6
35	3.6	2.6	-1.0
36	4.5	2.6	-1.9
37	4.3	4.7	+0.4
38	3.0	3.6	+0.6
39	4.7	3.4	-1.3
40	7.1	4.5	-2.6
41	4.3	5.0	+0.7

Fig. 47. Trends of OPG levels and absolute values in the TDF (**A**) and the Non-TDF (**B**) Groups.

6 DISCUSSION

Tenofovir disoproxil fumarate (TDF, Viread®, Gilead Sciences, Inc.) is a reverse transcriptase inhibitor effective against HIV and HBV. Due to its high antiviral efficacy, good safety profile, and negligible risk of resistance, TDF is recommended as first-line monotherapy in chronic hepatitis B infection (38). However, bone mineral density (BMD) loss and changes in bone turnover marker (BTMs) levels have been described in the context with TDF-containing anti-HIV therapies (77–81). Several studies dealt with changes in BMD due to TDF use in HBV-infected individuals (59,82–85) but there is a lack of data regarding the effects of TDF on bone turnover and vitamin D in this patient population. The aim of the present study was to evaluate the levels of 25-hydroxyvitamin D [25(OH)D], C-terminal cross-linked telopeptide of type I collagen (CTX), osteocalcin (OC), procollagen type I N-terminal propeptide (P1NP), and osteoprotegerin (OPG) in patients with a serum HBV DNA level of >2000 IU/ml treated with tenofovir (TDF Group) compared to patients with a serum HBV DNA level of <2000 IU/ml without indication for antiviral therapy (Non-TDF Group). All parameters were analyzed at baseline (BL) and three months thereafter (FU) including between-group and within-group comparisons.

25-hydroxyvitamin D [25(OH)D]

Regarding 25(OH)D, a high prevalence rate of patients with vitamin D levels ≤30 ng/ml was found in both study groups at baseline (TDF Group 87%, Non-TDF Group 61%). The mean serum 25(OH)D levels at baseline were 23.1 ng/ml in TDF Group members and 25.7 ng/ml in Non-TDF Group members. The difference between both study groups was not statistically significant. More TDF Group members had their baseline blood samples taken in summer than in winter (61% vs. 39%), while the seasonal distribution was balanced in Non-TDF Group members (50% in winter, 50% in summer). In both study groups, 25(OH)D levels tended to be lower when blood samples were taken in winter. This is not uncommon in our region and was described in several studies assessing the vitamin D status in healthy individuals (86,87). In summer, 86% of TDF Group members showed baseline 25(OH)D levels ≤30ng/ml compared to 44% of Non-TDF Group members. At follow-up, the prevalence rate of serum 25(OH)D levels ≤30 ng/ml was 87% in the TDF Group and 67% in the Non-

TDF Group revealing a similar distribution when compared to baseline. The mean serum 25(OH)D levels at follow-up were 19.5 ng/ml in TDF Group members and 25.3 ng/ml in Non-TDF Group members revealing a statistically significant difference (p=0.04). This may have been an effect of TDF therapy but may have also been an effect of differing seasonal distributions of blood draw between both study groups. More TDF Group members had their follow-up in winter than in summer (65% vs. 35%). 56% of the Non-TDF Group members had their follow-up in winter and 44% in summer. Again, in both study groups, serum 25(OH)D levels tended to be lower when blood samples were taken in winter. In summer, 75% of the TDF Group members showed 25(OH)D levels of ≤30ng/ml compared to 38% of the Non-TDF Group members. When the baseline serum 25(OH)D levels were compared to those at follow-up, a decrease of 16% (p=0.03) was found in the TDF Group whereas the decrease was only 2% in the Non-TDF Group (not significant). The higher decrease of 25(OH)D levels from baseline to follow-up in TDF Group members may have been an effect of TDF therapy but might also emerge from the higher rate of blood drawn in winter at follow-up (65%) compared to baseline (39%). Spearman correlation analyses revealed significant correlations between the change of serum 25(OH)D levels and age respectively serum 25(OH)D levels and gender in Non-TDF Group members. Especially older and female Non-TDF Group members showed a higher decrease of 25(OH)D levels.

Increased prevalence rates of low serum vitamin D levels in patients with serum HBV DNA levels >2000 IU/ml were also observed in other studies. For example, Chan et al. (88) measured serum 25(OH)D levels in 737 treatment-naïve but treatment-eligible CHB patients (66% male; 22% white; 75% of Asian race; mean age: 37 years; mean serum ALT: 110 U/l; mean serum HBV DNA: 10,000,000 IU/ml) treated at 139 study sites (45 in the European Union, 37 in Asia, 31 in North America, 17 in Australia, and 9 in India). That study found a mean vitamin D level of 18.5 ng/ml. Thirty-five percent of the patients had insufficient (≥20 but <31 ng/ml) and 58% had deficient (<20 ng/ml) vitamin D levels, while only 7% had adequate vitamin D levels (≥31 ng/ml). Similar to our study, the mean serum 25(OH)D levels were lower when blood samples were drawn in winter or autumn (16.0 ng/ml) compared to summer or spring (20.0 ng/ml). The rate of patients with adequate 25(OH)D levels ≥31 ng/ml was 9% in summer or spring and 3% in winter or autumn (88). In a Turkish

investigation, Demir et al. (89) compared 25(OH)D levels among 35 CHB patients with normal liver enzyme levels (mean age: 33 years; 63% male), 30 naturally immunized individuals (mean age: 31 years; 60% male), and 30 healthy adults (mean age: 32 years, 57% male). Vitamin D levels were measured during the winter season. The authors report that CHB patients had lower serum 25(OH)D levels compared to naturally immunized individuals and healthy controls (7.65 ng/ml, 12.1 ng/ml, and 14.2 ng/ml, respectively; $p<0.001$). Vitamin D levels were correlated with serum HBV DNA levels. Demir et al. concluded that decreased serum 25(OH)D levels may be related to increased viral replication in CHB patients (89). In a prospective study, Chen et al. (90) evaluated 25(OH)D levels in 128 treatment-naïve but treatment-eligible CHB patients (mean age: 34 years; 72% male; mean serum ALT 4xULN; mean serum HBV DNA 2,500,000 IU/ml) and 128 healthy controls without liver disease living in Chengdu City (West China). That study found that the mean level of 25(OH)D in CHB patients was significantly lower compared to healthy controls (16.9 ng/ml vs. 20.2 ng/ml). The rate of patients with sufficient 25(OH)D levels ≥20 ng/ml was significantly lower in CHB patients than in healthy controls (25% vs. 49%). All CHB patients received antiviral treatment with nucleos(t)ide analogues for a duration ranging between five to six years. Unfortunately, the authors do not report, which antiviral agents were used. In contrast to our study, serum 25(OH)D levels increased from 16.9 ng/ml at baseline to 20.2 ng/ml after antiviral treatment and were significantly negatively correlated with serum HBV DNA levels. Regarding the seasonality of serum 25(OH)D levels, no significant differences between summer/autumn and spring/winter before or after antiviral treatment were observed. The authors noted that the majority of patients were indoor employees with limited sunshine exposure over the whole year which might account for the weak seasonality observed. Chen et al. concluded that low levels of 25(OH)D in CHB patients might be caused by high viral replication and that effective antiviral therapy might increase serum 25(OH)D levels (90).

Studies evaluating the impact of TDF on the vitamin D status in HBV-infected individuals are rare. In a cross-sectional study from Thailand including 158 HIV-HBV co-infected patients (median age: 43 years; 32% female), Avihingsanon et al. (91) evaluated 25(OH)D levels prior to and following TDF therapy. The mean duration on TDF was five years (range 4-7 years). The median serum 25(OH)D levels prior to and following TDF use were 24.8 ng/ml and 22.8 ng/ml, respectively. The rate of

patients with 25(OH)D levels <30 ng/ml significantly increased from 72.2% prior to TDF to 84.2% following TDF therapy. Factors associated with hypovitaminosis D included female gender and duration of therapy for more than five years. The investigators concluded that routine 25(OH)D assessment and vitamin D supplementation should be considered in this study population (91). Farnik et al. (92) quantified 25(OH)D serum levels in treatment-naïve CHB patients (mean age: 39 years; mean serum HBV DNA level: 3162 IU/ml; mean ALT level: 40 U/l) living in Frankfurt (Germany). Of the 203 participants enrolled in this study, 34% had severe vitamin D deficiency (<10 ng/ml) and 47% had vitamin D insufficiency (≥10 but <20 ng/ml), while only 19% had adequate vitamin D serum levels (≥ 20 ng/ml). The mean serum 25(OH)D level was 14.4 ng/ml. Serum HBV DNA levels were a strong predictor of low serum 25(OH)D levels and vice versa. The mean 25(OH)D level in patients with HBV DNA <2000 IU/ml was 17 ng/ml while it was 11 ng/ml in patients with HBV DNA ≥2000 IU/ml. HBV DNA levels were significantly lower when samples were taken in spring or summer representing a reciprocal seasonal variation of both variables. The investigators concluded that this might be considered as an important hint for a functional relationship between 25(OH)D and HBV DNA. In 21 patients antiviral treatment was initiated after baseline blood draw (PEG-IFN-a 2/21, entecavir 4/21, tenofovir 13/21, lamivudine 2/21) and vitamin D levels were determined after a mean follow-up time of 20.2 months (range 5 to 36 months). In this subgroup (mean age: 39 years; mean serum HBV DNA: 316,228 IU/ml at baseline, <2000 IU/ml at follow-up), the mean serum 25(OH)D levels increased from 10.3 ng/ml at baseline to 13.0 ng/ml at follow-up, which was not statistically significant. This is in contrast to our study; however, in that study, the number of patients receiving antiviral agents was too small and different antiviral therapies were applied making firm conclusions regarding the impact of TDF therapy on vitamin D levels impossible (92). In a retrospective study, Nguyen et al. (93) determined vitamin D levels in 291 HBV-infected patients (median age: 42 years; 72% male; median AST 30 U/l, 14 patients with HCC, 9 patients post liver transplantation) at baseline and twelve months after TDF (*n*=212) or entecavir (*n*=79) exposure. The rate of vitamin D deficiency at baseline with levels <22 ng/ml was 81.3% with a higher prevalence in male study participants. The median vitamin D level increased from 14.2 ng/ml at baseline to 14.7 ng/ml at follow-up twelve months after TDF exposure, which was not statistically significant. Nguyen et al. concluded that TDF treatment had no significant effect on

vitamin D levels after twelve months of treatment. This is in contrast to our findings; however, in that study, vitamin D levels were not analyzed with regard to the season of blood draw (93).

Studies evaluating the vitamin D status in the general population reported seasonal variations in serum 25(OH)D with the lowest levels measured during winter time. Kudlacek et al. (86) assessed serum levels of 25(OH)D in 648 females and 400 males from Austria (aged 21 to 76 years). Serum vitamin D levels were assessed in the winter months from December to April revealing a mean 25(OH)D level of 20.9 ng/ml which is similar to the levels observed in our study (TDF Group: 21.6 ng/ml at BL, 16.0 ng/ml at FU; Non-TDF Group: 22.9 ng/ml at BL, 22.4 ng/ml at FU). Nineteen (2%) individuals had serum 25(OH)D levels <5 ng/ml and 271 (26%) study participants had serum 25(OH)D levels <12 ng/ml. More female study participants had serum 25(OH)D levels <12 ng/mL compared to male study participants (30.2% vs. 22.8%) (86). Scharla et al. (87) evaluated serum 25(OH)D levels in 209 males and 206 females from southern Germany (aged 50 to 80 years). This study found a seasonal variation with higher serum 25(OH)D levels in summer than in winter (27±10 ng/ml vs. 17±9 ng/ml). 25(OH)D levels did not differ between male and female study participants but decreased with age. Thirty percent of the male and 40% of the female study participants had subclinical vitamin D deficiency in winter (87).

Bone markers (CTX, OC, P1NP, OPG)

Changes in the levels of bone markers have been described in HIV-infected patients receiving TDF as a part of antiretroviral therapy (ART) but the effects of TDF on bone turnover in cohorts with HBV infection are less well documented. To our knowledge, the present pilot study was the first to assess the bone markers CTX, OC, P1NP, and OPG in HBV-infected individuals receiving TDF monotherapy. Due to the lack of studies in HBV-patients, we decided to compare our results with those obtained from studies in HIV-patients. However, it would be erroneous to directly apply the effects of TDF use observed in anti-HIV therapy onto anti-HBV therapy because of differing treatment strategies and disease-related factors. The highly active antiretroviral therapy (HAART) comprises multiple drugs, which may significantly increase the risk of adverse drug reactions.

Regarding CTX, the mean baseline levels were found to be slightly higher in Non-TDF Group members (0.29 ng/ml) in comparison to TDF Group members (0.24 ng/ml) while at follow-up, the mean CTX levels were vice versa slightly higher in TDF Group members (0.35 ng/ml) in comparison to Non-TDF Group members (0.30 ng/ml). At both time points, the differences between the study groups were not statistically significant. When mean CTX levels at baseline were compared to those at follow-up, an increase of 46% was found in TDF Group members (p=0.001) whereas the increase was only 3% in the Non-TDF Group (not significant).

Regarding OC, the mean baseline levels were found to be slightly higher in Non-TDF Group members (21.4 ng/ml) in comparison to TDF Group members (17.7 ng/ml), while at follow-up, the mean OC levels were vice versa slightly higher in TDF Group members (21.9 ng/ml) in comparison to Non-TDF Group members (19.6 ng/ml). At both time points, the differences between the study groups were not statistically significant. When mean OC levels at baseline were compared to those at follow-up, an increase of 24% was found in TDF Group members (p=0.007) while a decrease of 8% was observed in the Non-TDF Group (not significant).

Regarding P1NP, the median was used instead of the mean due to the presence of outliers. The median levels were found to be higher in Non-TDF Group members at baseline (53.5 ng/ml) and at follow-up (49.1 ng/ml), compared to those measured in the TDF Group (46.2 ng/ml at BL, 46.9 ng/ml at FU), with all differences between the study groups being not statistically significant. When median P1NP levels at baseline were compared to those at follow-up, values remained almost identical. Non-TDF Group members showed a median decrease of 8% from baseline to follow-up, while TDF Group members showed an increase of 2%.

Regarding OPG, the mean levels were found to be higher in the TDF Group at baseline (5.8 pg/ml) and at follow-up (4.7 pg/ml) compared to those measured in the Non-TDF Group (4.8 pg/ml at BL, 4.4 pg/ml at FU), with all differences between the study groups being not statistically significant. When mean OPG levels at baseline were compared to those at follow-up, a decrease of 19% was found in TDF Group members (not significant) whereas the decrease was only 8% in the Non-TDF Group (not significant).

In several studies evaluating bone markers in HIV-infected individuals, results similar to the present pilot study were found: In an open-label randomized trial conducted in Denmark (SWAP study), Rasmussen et al. (77) compared bone effects among 40 HIV-infected individuals randomized to switch from zidovudine/lamivudine (AZT/3TC) to tenofovir/emtricitabine (TDF/FTC; *n*=20; mean age: 46 years; 70% male gender) or abacavir/lamivudine (ABC/3TC; *n*=20; mean age: 50 years; 55% male gender). Bone mineral density and bone turnover markers were assessed including OC, P1NP, alkaline phosphatase, CTX, and OPG. Study participants were followed up at 4, 8, 12, 24, and 48 weeks after randomization. Of 40 patients enrolled, 35 completed 48 weeks of therapy and follow-up; 20/35 in the TDF/FTC arm and 15/35 in the ABC/3TC arm. At baseline, serum levels of P1NP were significantly higher among patients in the ABC/3TC arm than the TDF/FTC arm. All other parameters did not differ significantly between both study groups at baseline. That study found a significant BMD reduction in TDF/FTC-treated patients, whereas BMD was stable in ABC/3TC-treated patients. Except for OPG, all BTMs increased in the TDF/FTC arm compared to the ABC/3TC arm. OPG increased from baseline to week 4 in the ABC/3TC arm but changes at week 12 and 48 were similar between both study groups. At week 12, the difference in change from baseline was statistically significant for alkaline phosphatase and P1NP but only for P1NP at week 4. At week 48, increases of CTX, OC, P1NP, and alkaline phosphatase concentrations were found to be significant. TDF/FTC use was associated with decreases in BMD and early increases of all BTMs except for OPG. The authors conclude that the loss of BMD was associated with an increased rate of bone turnover that can be detected as early as 12 weeks after initiation of TDF. It is suggested that certain signaling events are taking place within the first few weeks of treatment with TDF and that changes in BTM levels might reflect direct effects of TDF on bone cells or indirect effects by impairing the calcium homeostasis (77). Similar to that study, we also found a significant increase in CTX and OC. In an open-label, multicenter, randomized, controlled trial (PREPARE study), Cotter et al. (78) determined the serum levels of CTX, OC, and P1NP and assessed BMD over 48 weeks in 53 virologically suppressed HIV patients (HIV RNA <50 copies/ml) randomized to switch to TDF/emtricitabine (TDF/FTC; *n*=29; median age: 47 years; 93% male gender) or remain on zidovudine/lamivudine (AZT/3TC; *n*=24; median age: 45 years; 75% male gender). At baseline, all bone markers were higher in the TDF/FTC Group than in the

AZT/3TC Group. That study found BMD reductions in those who switched to TDF/FTC (not significant) and a significant increase in CTX, OC, and P1NP indicating an overall increase in bone turnover, which is similar to our study. The median nadir of CTX concentrations was found to be at week 24 and that of OC and P1NP at week 36. The authors suggest that early changes may not reflect the overall alteration in bone metabolism up to week 48. Cotter et al. concluded that switching to TDF/FTC was associated with an increase in bone turnover correlating with reduced BMD levels and that TDF exposure might directly affect bone metabolism *in vivo* (78). In another multicenter study (ASSERT study) conducted in 76 centers across 13 countries in Europe, Stellbrink et al. (79) assessed the serum levels of OC, P1NP, and CTX as well as the BMD in 385 antiretroviral naïve HIV-infected individuals randomized to receive either abacavir-lamivudine (ABC-3TC; *n*=192; median age: 38 years; 83% male gender) or tenofovir-emtricitabine (TDF-FTC; *n*=193; median age: 36 years; 80% male gender) with efavirenz. Primary analyses were conducted after 48 weeks of treatment. BMD was determined at baseline, week 24, and week 48. Serum and plasma samples were collected at baseline, week 12, week 24, and week 48. That study found a loss of BMD in both treatment groups but reductions of ≥6% were more common in the TDF-FTC Group. The BTMs increased in both study groups over the first 24 weeks with significantly greater increases in the TDF-FTC treatment group, which is similar to our findings. This difference remained significant for all markers at week 48, except for CTX. The serum levels were highest at week 24 and decreased or stabilized thereafter but mostly at an increased rate from baseline. Stellbrink et al. found greater decreases in BMD and increases in BTM levels in HIV-infected individuals on tenofovir-emtricitabine. The authors suggest that the use of efavirenz might have adversely affected bone metabolism (79). Haskelberg et al. (80) evaluated the effect of baseline BTM levels and early changes in BTM levels on BMD change in HIV-infected individuals receiving ART. Serum levels of CTX, P1NP, bone alkaline phosphatase, OPG, and RANKL as well as BMD were determined at baseline and at week 12, 24, 48, 72, and 96. A total of 301 virologically suppressed participants from Australia were enrolled; 154/301 participants (mean age: 45 years; 97% male gender) were randomized to tenofovir-emtricitabine (TDF-FTC) and 147/301 participants (mean age: 46 years; 99% male gender) were randomized to abacavir-lamivudine (ABC-3TC). That study found that CTX and P1NP increased significantly at week 12. Both markers remained stable

through week 96 in the TDF-FTC arm. These early changes did not predict subsequent bone loss. No significant between-group differences were found regarding the serum levels of OPG and RANKL. In the TDF-FTC arm, OPG increased from baseline to week 48, whereas in our study, OPG levels decreased in TDF Group members from baseline to follow-up at week 12. Baseline covariates significantly associated with greater decline in hip BMD included TDF-FTC randomization, lower fat mass, and lower P1NP. Baseline predictors of greater decline in spine BMD included TDF-FTC randomization, lower fat mass, and PI use. There was no association observed between TDF-FTC use and fracture risk. Haskelberg et al. suggested that the switch to TDF-FTC was associated with higher bone turnover (80). Brown et al. (81) suggest that the use of OPG as a bone marker was limited for several reasons. First, it is difficult to analyze and the plasma levels may not reflect the levels in the bone micro-environment. Second, the production of OPG is not specific to osteoblasts as it is also produced by activated B-cells, which undergo significant changes during ART-initiation. Finally, many cytokines have been shown to affect OPG.

Possible mechanisms of TDF-associated bone loss

The mechanisms underlying the effects of TDF on increased bone turnover have not been clearly identified but recent *in vitro* studies suggest that TDF may alter the gene expression in bone cells resulting in osteoclast and osteoblast dysfunction. Grigsby et al. (58,94) evaluated whether TDF exposure of osteoblasts and osteoclasts caused changes in gene expression profiles impacting their function during bone formation and bone resorption, respectively. Primary osteoblasts and osteoclasts were isolated and treated with various TDF concentrations. That study found that TDF exposure was associated with the perturbation of osteoblast gene expression resulting in reduced osteoblast growth, activity, differentiation, and function (58). TDF exposure of osteoclasts resulted in an alteration of a distinct gene expression profile. The authors suggest that changes in osteoclast gene expression might contribute to the clinical observations of TDF-associated bone loss (94). In a mini-review, Grigsby et al. (95) suggest three potential mechanisms for TDF effecting bone. First, TDF might be increasingly taken up by osteoblasts or osteoclasts, or both, which might result in an alteration of gene expression and a perturbation of the

balance between both cell types. Second, being a phosphonate, TDF might act like a bisphosphonate targeting bone and inhibiting osteoclast function by inducing apoptosis. Third, TDF-associated bone loss might be associated with renal proximal tubule dysfunction and phosphate wasting. The latter mechanism was also proposed by other authors. Gutierréz et al. (96) suggest that TDF use was associated with a reduction of intestinal phosphate absorption, renal phosphate wasting, a decreased function of 1α-hydroxylase due to renal toxicity, and elevated plasma concentrations of PTH. The conversion of 25(OH)D to $1,25(OH)_2D$ by 1α-hydroxylase occurs primarily in the proximal tubules, which is why TDF-induced proximal tubule dysfunction might impair vitamin D activation. Byrne et al. (97) suggest that inflammatory cytokines (e.g. TNF-α, IL-1, IL-6) might increase RANKL, which stimulates osteoclastogenesis and bone resorption.

Limitations and strengths of the present pilot study

This pilot study has several limitations. First, this study does not include a healthy control group. It was thus not possible to evaluate the impact of the HBV infection itself on bone health. Second, the number of study participants might have been too small to draw firm conclusions regarding the effect of TDF on bone health although statistically significant results were observed regarding the changes of 25(OH)D, CTX, and OC levels in serum. Third, similar to other studies in the field, this pilot study is limited by its lack of data on factors potentially affecting bone metabolism and the parameters analyzed including duration of HBV infection, ethnicity/skin pigmentation, socioeconomic status, body mass index, smoking and alcohol habits, dietary habits, extent of physical activity or immobilization, sunlight exposure, sunscreen use, number of pregnancies, menstrual status, and family history of bone diseases. It is unclear whether these factors might have significantly influenced the results. Fourth, the vitamin D status was analyzed according to the season at blood draw but the study groups were not weighted regarding the seasonal distribution. Moreover, some studies found that the reported sun exposure (being outdoors in daylight) might be a stronger predictor of 25(OH)D levels than the season alone (98). Fifth, thyroid hormones, free urinary cortisol, estradiol, and testosterone were not measured. The presence of major endocrinopathies could thus not be ruled out. Finally, the bone mineral density was not assessed. Changes in the levels of bone

turnover markers alone do not reflect the quantity of bone loss. Furthermore, BTMs may be influenced by several other factors including age, gender, menopausal status, bed rest, immobility, nutritional status, and recent fractures (70).

Despite these limitations, this pilot study adds interesting information regarding the impact of TDF use on bone health in HBV infected individuals given the lack of firm data in the current literature. The main strength of this pilot study is the observation of early changes regarding vitamin D levels and BTMs in serum. BTMs may identify changes in bone remodelling before a change of BMD is detectable by dual-energy X-ray absorptiometry allowing a more rapid assessment of bone disease and enabling an earlier therapeutic intervention.

Conclusion

In the present pilot study significant increases of CTX and OC levels in serum were observed from baseline to follow-up three months after TDF initiation. The percentage for vitamin D deficiency and insufficiency was high in both study groups. It thus might be reasonable to screen HBV-infected individuals for hypovitaminosis D and to consider vitamin D and calcium supplementation. Prospective studies are required to evaluate whether the early increase of bone turnover observed in the present study is a short-term effect or a predictor of future bone loss. Furthermore, it is essential to elucidate the underlying mechanisms of TDF-associated changes in bone and vitamin D metabolism as the majority of patients will require long-term (lifelong) treatment with TDF.

7 RESSOURCES

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