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Адреса для листування: Оранта ДіагностикаРаку, Нореенс вег, 73, 75263, Упсала, Швеція
E-пошта: serhiy8086@gmail.com

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ORCID ID
Сергій Сушельницький: https://orcid.org/0000-0001-8243-9276

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Діагностика прогнозу неосифікуючої фіброми з допомогою системного аналізу

Сергій Сушельницький

Оранта Діагностика Раку, Упсала, Швеція

Вступ: неосифікуюча фіброма (НОФ) є фіброзним ураженням кістки, яке спостерігається у 40% дітей. Значне ураження кістки при НОФ та неефективне загоєння ураження можуть спричинити патологічний перелом або навіть злоякісне новоутворення. Прогнозування та попередження ускладнень потребує знання механізмів контролюючих НОФ.

Мета: визначити регулятори, які можуть прогнозувати ризик ускладнень, наприклад, патологічного перелому або злоякісної трансформації.

Методи: дані були отримані з загальнодоступних баз даних, наприклад, PubMed і спеціалізованих баз даних. Ми отримали регулятори з підтвердженим зв'язком з НОФ, регулятори процесів, задіяних у НОФ, регулятори ремоделювання кісток, та регулятори гігантоклітинних пухлин кісток. Системний аналіз проводили за допомогою програм Cytoscape та FunCoup.

Результати: було створено регуляторні сітки, що представляють механізми НОФ, загоєння НОФ ураження та злоякісну трансформацію. Аналіз регуляторних сіток виявив механізми, які можуть передбачити ефективність загоєння ураження або ризик злоякісної трансформації НОФ. Сорок один компонент було визначено як потенційний діагностичний маркер ефективності загоєння НОФ. Сигнальні шляхи, гормони, вітаміни, мінерали, регулятори проліферації та диференціювання є серед 41 компоненту діагностики загоєння НОФ. Отриманий нами 62-компонентний набір дозволяє діагностику та передбачення ризику злоякісного переродження НОФ. Багато з цих компонентів відомі як онкогени та регулятори злоякісного переродження. Дерегуляція цих молекул підвищує ризик злоякісної трансформації НОФ.

Висновок: 41 і 62 сигнатури ідентифікують потенційні маркери ризику неефективного загоєння або злоякісної трансформації НОФ.

Ключові слова: неосифікуюча фіброма, системна біологія, діагностичні маркери, загоєння кістки, ремоделювання кістки, гігантоклітинна пухлина кістки.

Усі дані наведені в рукописі. Файли даних і файли додаткових матеріалів завантажено на FigShare за адресою https://figshare.com/articles/figure/Souchelnytskyi_NOF_Dx_SupplementaryMaterials/23965458 для вільного використання, а також доступні від автора за запитом.

Systems biology signature for prognosis of non-ossifying fibroma

Serhiy Souchelnytskyi

Oranta CancerDiagnostics AB, Uppsala, Sweden

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For correspondence: Oranta CancerDiagnostics AB, Noreens väg 73, 75263, Uppsala, Sweden
E-mail: serhiy8086@gmail.com

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ORCID ID
Serhiy Souchelnytskyi: https://orcid.org/0000-0001-8243-9276

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Introduction: Non-ossifying fibroma (NOF) is a frequent fibrotic lesion of bone, observed in up to 40% of children. Extensive NOF lesions and deficient healing may cause a pathological fracture or a malignant transformation. Prediction of complications requires knowledge of the mechanisms controlling NOF, and systemic analysis may provide insight into these mechanisms.

Aim: To identify regulators that may predict the risk of complications, e.g., pathologic fracture or malignant transformation.

Methods: Data were retrieved from public databases, e.g., PubMed and dedicated databases. We retrieved regulators with confirmed association with NOF, regulators of processes engaged in NOF, and regulators of bone remodelling and giant cell tumors of bone. Systemic analysis was performed using Cytoscape and FunCoup tools.

Results: Networks representing NOF mechanisms, bone healing, and malignant transformation were generated. The network analysis identified mechanisms that may predict the efficacy of healing of NOF lesion or the risk of malignant transformation of NOF. Forty-one compounds were identified as potential signature predictor of the efficacy of bone healing. The list contains known and novel regulators of bone. Signalling pathways, hormones, vitamins, minerals, proliferation and differentiation regulators are in the 41 signature. We report here a list of 62 molecules that are engaged in bone tumorigenesis and in NOF, e.g., oncogenes and tumor suppressors, tumorigenesis-associated signalling pathways and hormones Deregulation of these molecules increases the risk of malignant transformation of NOF.

Conclusion: The 41 and 62 signatures identify potential markers of the risk of non-efficient healing or malignant transformation of NOF.

Keywords: Non-ossifying fibroma, systems biology, diagnostic markers, bone healing, bone remodelling, giant-cell tumor of bone.

Data files and Supplementary Material files are uploaded at FigShare at https://figshare.com/articles/figure/Souchelnytskyi_NOF_Dx_SupplementaryMaterials/23965458 for free use.

Introduction

Non-ossifying fibroma (NOF) is a benign tumor of bone manifested by excessive formation of fibrotic tissue [1-3]. WHO classification of bone tumors assigns NOF to the category osteoclastic giant cell-rich tumors of bone (GCTB) [4]. NOF is observed in 30% to 40% of children. Most of the NOF lesions heal by bone regrowth in the lesion [1-3]. However, deficient bone healing may prompt pathological fractures, which are the main concern for NOF patients with extensive lesions, e.g., more than 50% of the bone cross-section [2,5-7]. Much less is reported about the malignant transformation of NOF. Deregulation of molecular mechanisms of bone growth has many features common for the non-efficient healing of NOF, susceptibility to pathological fracture, and malignant transformation. Deciphering of these mechanisms may contribute to the prediction of NOF complications.

The cause of NOF is not known. Mutations in FGF23, FGFR1, KRAS, and NF1 genes are associated with NOF [8-10]. Overactivation of FGF23 and KRAS signalling may impact bone-forming cells by promoting proliferation, deregulating differentiation, and delaying maturation of the bone [8-10]. The positive role of estrogen in promoting NOF healing was proposed [10,11]. Histology of NOF indicates engagement of mechanisms common for giant cell tumors of bone. These mechanisms include the excessive formation of fibrous tissue [2,12]. Maturation of osteoblasts and osteoclasts, formation of bone, and engagement of fibroblasts underline molecular mechanisms leading to NOF [1-3,8-12]. Deregulation of these mechanisms in NOF may lead to complications. The main concern is the risk of pathological fracture due to impaired bone healing. Another concern is the malignant transformation of NOF. Reports of malignant transformation of NOF are sporadic case studies, which do not provide a statistically secured incidence frequency [13-15]. Giant cell-rich tumors of bone do not have a clear indication of origin, and NOF contains osteoclast-like giant cells which may contribute to GCTB [2,12,16,17]. There are no markers that would predict the malignant development of NOF or the risk of pathological fracture.

NOF prognosis is dependent on the efficacy of bone formation, healing, and activity of tumorigenic mechanisms. The anatomy, location and activity of the growing plate, functional status of different types of cells in the bone, and the efficacy of endocrine and local regulators are key components of bone maturation. Recent developments of systems biology opened for much better representation and analysis of complex processes. Here we report a systemic analysis of mechanisms that regulate NOF, with a focus on regulators that may predict the risk of non-efficient healing or malignant transformation.

Materials and Methods

Data about the pathophysiology of NOF were retrieved from published reports. We performed searches of PubMed, and a number of specialized databases with combinations of search words related to NOF, bone remodelling, healing, and bone cancer. The searches were performed by the 3rd of July, 2023. No limitation on the publishing date or type of study was applied. The searches of PubMed retrieved up to 300 publications for each of the searches with combinations of the search words. Retrieved reports were manually screened for the relevance to the aim of our study, and description of genes, transcripts, proteins, and small molecules with an impact on NOF physiology. Gene Ontology (GO) annotation of engaged regulators was retrieved from the European Bioinformatics Institute [18]. Systemic analysis was performed using Cytoscape and FunCoup tools [19,20]. For network building with Cytoscape, we used IntAct database [21]. Statistical significance of the network building was set at $p<0.05$, e.g., the significance of evidence for network nodes and edges between nodes.

We used FunCoup version 5.0 to build networks for NOF-proven regulators, regulators with potential impact on NOF, regulators of bone remodelling and healing, and regulators engaged in bone tumorigenesis with a focus on GCTB, followed by functional enrichment with PathBix tool [22].

For systemic analysis of networks built in Cytoscape, we used BiNGO (version 3.0.5) application. Enrichment for biological processes in BiNGO was performed with significance $p<0.05$, for Homo sapience, with hypergeometrical statistical test, and Benjamini & Hochberg false discovery rate correction. Extraction of common nodes and edges was performed for networks built for NOF regulators, bone tumors (GCTB), and markers of pathological fracture.

The clinical value of identified NOF regulators was verified by an independent screening of published reports (see the workflow in Figure 1A, Supplementary Material S1). Data files and Supplementary Material files are uploaded at FigShare at https://figshare.com/articles/figure/Souchelnytskyi_NOF_Dx_Supplementary-Materials/23965458 for free use.

Results

Extraction of NOF regulators.

The PubMed search with the words "non-ossifying fibroma" retrieved only 251 publications for the last 70 years (by the 3rd of July, 2023). It can be compared to 266,258 publications on "bone cancer", and 5,161 publications on "giant cell tumor of bone". The low number of studies results in fragmentary and poor insights in the causes of NOF, NOF development, and complications. There are only 4 regulators with a proven link to NOF (Table 1, Figure 1B). Mutations of FGF23, FGFR1, KRAS and NF1 genes are associated with NOF [8-10]. Mutations that prevent cleavage of FGF23 and enhance its level are associated with NOF. Ras-dependent pro-mitogenic signalling promotes NOF by activating mutations of KRAS, enhanced expression of mitogen-activated protein kinase (MAPK), activities of RASopathy genes, and methylation of genes of p16 and p21 CDK [8-10,23]. This confirms that overactivation of FGF(FGF23/FGFR1) signalling and KRAS mitogenic pathways are crucial for NOF development.

Aberrations of the estrogen signalling (estradiol, estrone, estriol, and estrogen receptors), parathyroid hormone (PTH) and vitamin D levels were observed in patients with bone lesions [24-26]. Estrogen regulates the maturation and proliferation of osteoclasts and osteoblasts. Estrogen deficiency may lead to bone resorption [24]. Of importance for NOF development is the assumption that the increase of estrogen level upon adolescence is the main factor promoting self-healing of NOF [10,24,27]. Low levels of vitamin D correlate with deficient bone maturation [25]. PTH promotes bone resorption, and its high levels in serum may contribute to deficient bone formation [26]. Therefore, estrogen, vitamin D, and parathyroid hormone signalling were selected for systemic analysis.

Collagen type I and collagen peptides may reflect bone status (Table 1, Figure 1). Deposition of hemosiderin in NOF indicates tissue damage and disturbance of blood circulation. Detection of enhanced levels of somatostatin in NOF could be due to deregulation of proliferation and growth-related metabolic changes, e.g., glucose uptake [28]. Macrophage-associated CD68 antigen was detected in NOF. CD68 is used as a marker of inflammation and for the identification of cells of macrophage lineage, e.g., osteoclasts, histiocytes, and multinucleated giant cells [2]. Enhanced expression of vimentin in NOF is in line with enhanced fibrosis. Hemosiderin, CD68, and vimentin are used as histological markers of NOF [2] and were selected for our systemic analysis.

Figure 1

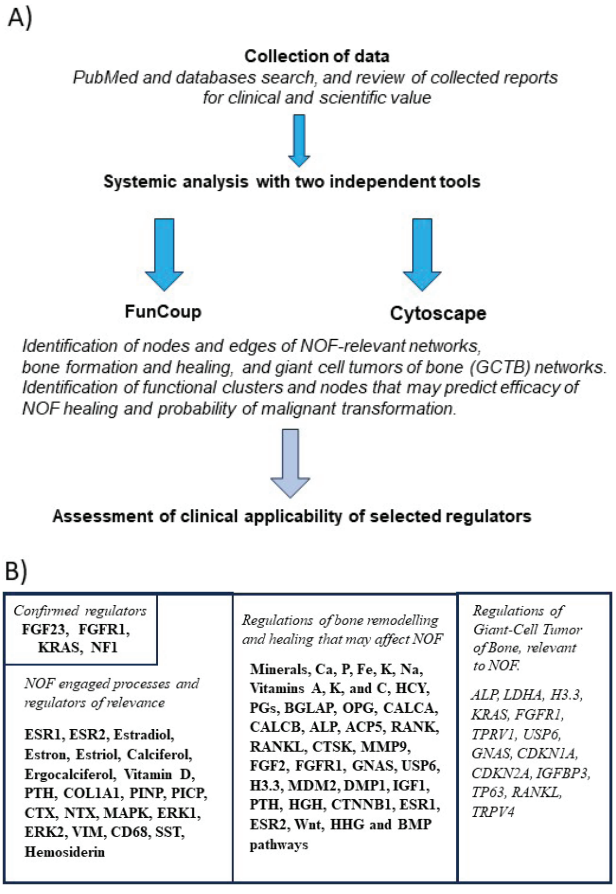

Figure 1. Workflow of this study (A), and regulators of confirmed and potential relevance to NOF, regulators of bone formation, healing, and giant cell tumors of bone (B) are shown. Regulators are listed in GO terms. See Table 1 for the annotation of the regulators.

To compensate for the scarcity of NOF-dedicated studies, we reviewed studies of bone physiology,

Table 1

Regulators of NOF development. Listed are regulators with confirmed association to NOF (section 1), regulators of functions that have a potential impact on NOF (section 2), regulators of bone growth and remodeling (section 3), and regulators of bone cancer with a focus on giant cell tumors of bone (section 4). See the text for description and references

1. Regulators with <u>confirmed</u> association with NOF	
Name	Evidence
Fibroblast growth factor-23 (FGF23), Fibroblast growth factor receptor-1 (FGFR1)	Genetic, mutations in >80% of cases. Potentially leading to overactivation of FGF signaling, hypophosphatemia, osteomalacia, inhibition of vitamin D activation.
KRAS protooncogene (KRAS)	Genetic, mutations in >80% of cases. Potentially leading to overactivation of proliferative signaling.
Neurofibromatosis-1 gene (NF1)	NF1 mutations in Jaffe-Campanacci syndrome are associated with NOF.
2. Regulators with <u>potential functional</u> relations to NOF.	
Estrogen (estradiol, estrone, estriol)	Deficiency leads to bone resorption. Increase of estrogens in adolescence correlates with healing of NOF.
Estrogen receptor alpha (ESR1)	Expressed in NOF but not in giant cell granuloma.
Vitamin D (calciferol, ergocalciferol, cholecalciferol)	Low level of vitamin D in serum was observed in NOF patients.
Parathyroid hormone (PTH)	High PTH level in serum was observed in NOF. PTH leads to bone resorption.
Collagen type I (COL1A1) and collagen peptides: • N-terminal pro-peptide (PINP) • Carboxyterminal peptide (PICP) • C-terminal telopeptide (CTX) • N-terminal telopeptide (NTX)	Component of matrix, collagen type I (COL1A1) is highly expressed in NOF. PINP and PICP are markers of bone formation. CTX and NTX are markers of bone resorption.
Mitogen-activated protein kinase (ERK1, ERK2)	Enhanced expression in NOF, by immunohistochemistry staining.
Vimentin (VIM)	Enhanced expression in NOF, by immunohistochemistry staining.
Cluster of Differentiation 68 (CD68)	Positive staining in giant cells of NOF, by immunohistochemistry staining.
Somatostatin (SST)	Enhanced level was detected in NOF.
Hemosiderin	Deposits are observed in NOF samples, by histological analysis.
3. Regulators of bone remodeling and healing.	
Calcium (Ca) and Phosphate (P)	Key minerals for bone formation. Calcium and phosphate levels are affected by FGF23 and vitamin D.
Iron (Fe)	Required for collagen synthesis.
Kalium (K), Natrium (Na)	Positive role in maintaining bone density. Regulation of calcium deposition in bone.
Vitamin A (retinol, retinal)	Osteoblasts and osteoclasts are influenced by vitamin A. Deficiency or very high levels of vitamin A have negative impact on bone density.
Vitamin K (fyllokinon, menakinon)	Deficiency of vitamin K observed in patients with osteoporosis.
Vitamin C (ascorbic acid, ascorbate)	Positive role in wound healing and collagen synthesis in bone.
Homocysteine (HCY)	High level may lead to increases reactive oxygen species, and to bone resorption.
Prostaglandins (PGD, PGE, PGI, PGF)	Promotes bone resorption.
Osteocalcin (BGLAP, OSTCN)	Marker of bone formation.
Osteoprotegerin (OPG)	Protects from bone resorption. Protection from bone metastases.
Calcitonin (CALCA, CALCB)	Inhibits osteoclasts.
Pyridinoline, deoxypyridinoline	Marker of bone resorption.
Tartran-resistant acid phosphatase 5B (ACP5)	Marker of bone resorption.
Alkaline phosphatase, bone specific (ALP)	Increase in serum correlates with bone deformation and lysis. Enhanced levels correlate with osteosarcoma.
RANK, RANKL	RANKL/RANK signalling regulates osteoclast formation, bone remodelling.
Cathepsin K (CTSK)	Lysosomal cysteine proteinase involved in bone remodelling and resorption.
Matrix metalloprotease-9 (MMP9)	MMP9 initiates osteoclasts, mediates and promotes bone destruction.

Continuation of the Table 1

Name	Evidence
Fibroblast growth factor-2 (FGF2), fibroblast growth factor receptor-1 (FGFR1)	Stimulation of osteoclast function and bone resorption by FGF2. Inactive FGFR1 delayed osteoblast maturation and activates differentiated osteoblasts. Role in proliferation promotion in GCTB. FGF pathway promotes proliferation and inhibits bone formation
Hedgehog, WNT/b-catenin, BMP, TGFb pathways.	Pathways with association to bone maturation.
4. Regulators of giant-cell tumors of bone (GCTB).	
Histone H3 (H3.3 G34W)	Mutation is observed in 96% of giant cell-rich tumors of bone (GCTB).
KRAS, FGFR1, TRPV1	Mutations were reported in giant cell granuloma of jaws.
Ubiquitin carboxyl-terminal hydrolase 6 (USP6)	Mutations, rearrangements with many other gens. Aneurysmal bone cysts with USP6 mutations can become malignant.
Guanine nucleotide binding protein, alpha stimulating (GNAS1)	Activating mutations were observed in association with fibrous dysplasia.
Methylation of genes: p21 (CDKN1A), p16 (CDKN2A), IGFBP3, histones, collagen, p53 targeted genes,	Methylation of these genes leads to lack of CKD kinase inhibitors p16 and p21, and promotes proliferation. Methylation of listed genes is observed in cultured GCBT cells.
p63 (TP63)	Highly expressed in GCTB cells (100%), and in 25% of NOF.
RANKL	High expression in GCTB.
Transient receptor potential cation channel subfamily V member 4 (TRPV4)	Mutations were reported in GCTB.

remodelling, healing, and malignant transformation in the context of relevance to NOF physiology. Retrieved regulators are listed in Table 1 and shown in Figure 1. Levels of vitamins D, A, K, and C, and calcium, phosphate, iron, potassium, and sodium are crucial for the normal formation of bone, remodelling and healing. Several regulators of bone resorption are associated with the pathophysiology of NOF. RANKL, RANK, osteocalcin, osteoprotegerin, calcitonin, prostaglandins, homocysteine, cathepsin K, matrix metalloprotease-9, pyridinoline, and alkaline phosphatase have different mechanisms of action on bone. The network analysis shows interdependencies between these, and other regulators listed in Table 1 (Figures 2 and 3). These regulators can be measured in clinical tests and, therefore, can be monitored in NOF patients.

Studies of the relevant to NOF bone physiology reported the engagement of several signalling pathways. Fibroblast growth factor, Hedgehog, Wnt, and insulin growth factor pathways are involved in osteoblast and osteoclast maturation. Mutations of GNAS, USP6, and H3.3 are associated with giant cell tumors of bone, fibrous dysplasia, and aneurysmal bone cysts [1-3,8-10]. Even though NOF is frequently self-healed and considered as a non-malignant lesion, it cannot be excluded that NOF may transform into a malignant tumor. To predict such transformation, monitoring of NOF mechanisms that are also typical for malignant tumors is justified. We reviewed reports of the deregulation of cancer genes that also are engaged in NOF (Table 1, Figure 1). Ras-Raf-Mapk pathway, cell proliferation (CDK inhibitors), cell death (p63 and p53), FGF signalling (FGFR1), matrix (collagen), and transcriptional modulation (H3.3) are these regulatory processes. Monitoring of them may contribute to the prediction of malignant transformation of NOF.

When there are many regulating factors, it is imperative to understand the relations between them and their combined effect on the bone. We have a list of regulators of NOF, bone healing, and malignant transformation (Table 1, Figure 1). The next step is to understand how they interact, and which regulators are informative for the risk assessment of NOF complications.

Systemic analysis with two independent tools identified NOF-relevant nodes that contribute to bone healing or malignant transformation.

To explore whether NOF regulators engage mechanisms that can predict the efficacy of NOF healing or malignant transformation, we directly compared regulators and built networks for each category of regulators listed in Table 1 and shown in Figure 1B. This analysis is shown in Figures 2 and 3, and Supplementary Materials S2, S3, S4, and S5.

Figure 2

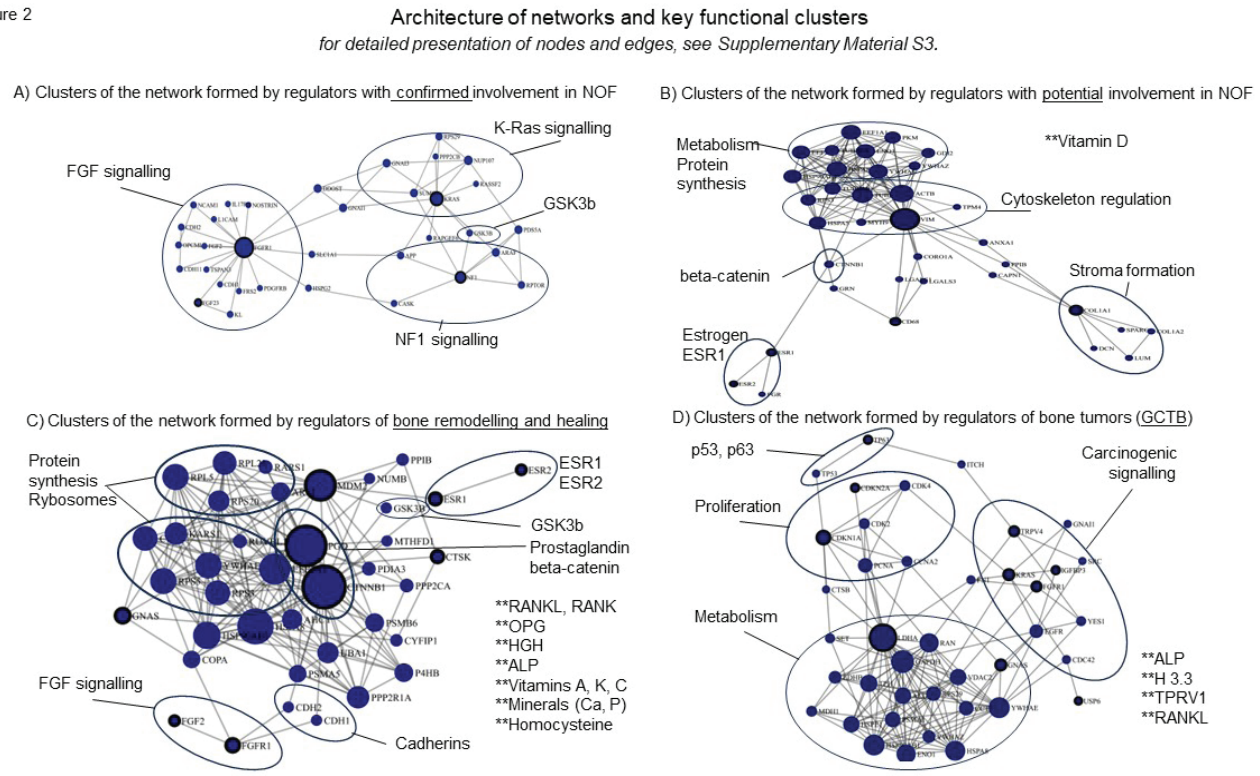

Figure 2. FunCoup-based systemic analysis of the NOF-relevant, bone remodelling and healing, and GCTB-relevant regulators. The networks built with regulators that are NOF-associated (A), potentially involved in NOF (B), involved in bone remodelling and healing (C), and involved in bone tumorigenesis (GCTB) (D). Domains of the networks and selected nodes are annotated. For images of the network and annotations, see Supplementary Material S3.

By direct comparison, a limited representation of NOF-associated regulators (sections 1 and 2 in Table 1) among bone remodelling (section 3) or regulators of giant cell tumors of bone (GCTB, section 4) was observed (Supplementary Material S2). Regulators of MAPK activation, fibroblast growth factors, and estrogen signalling pathways were identified (KRAS, FGFR1, MAPK, ESR1, and ESR2). The limited overlap by the direct comparison indicated the need for a systemic study by network analysis.

We built networks for regulators listed in Table 1, and analysed intersections of the networks. We used two independent network building tools, to ensure confidence of conclusions.

Figure 2 shows results of the systemic analysis with FunCoup (Figure 2, Supplementary Material S3). The network analysis showed that the regulators with confirmed NOF association formed 3 clusters in the network. The clusters are formed by FGF signalling, Ras-signalling, and NF1. This network contains 34 nodes and 52 edges. GNAI3, and GNAI1 connected FGF and Ras clusters, suggesting the involvement of G proteins. SLC1A1 and HSPG2 connected FGF and NF1 clusters. GSK3B was identified among connectors between NF1 and K-Ras clusters. GSK3B is a potent regulator of many processes and is involved in the pathophysiology of many diseases [29]. In the FGF signalling cluster, FGF23 and its receptor Klotho (KL), FGF2, FRS2 and FGFR1 confirmed a strong engagement of FGF signalling [9, 30]. Detection of PDGFRB contributes to the pro-mitogenic impact of this cluster. Detection of a proto-oncogene ARAF is another indication of enhanced proliferation signalling represented by the regulators with confirmed involvement in NOF.

Regulators of bone physiology that were reported as potentially involved in NOF, formed a network with 37 nodes and 129 edges. Estrogen signalling, stroma formation, cytoskeleton, and metabolism regulators form clusters. Ten nodes are included in the metabolism and

Figure 3,

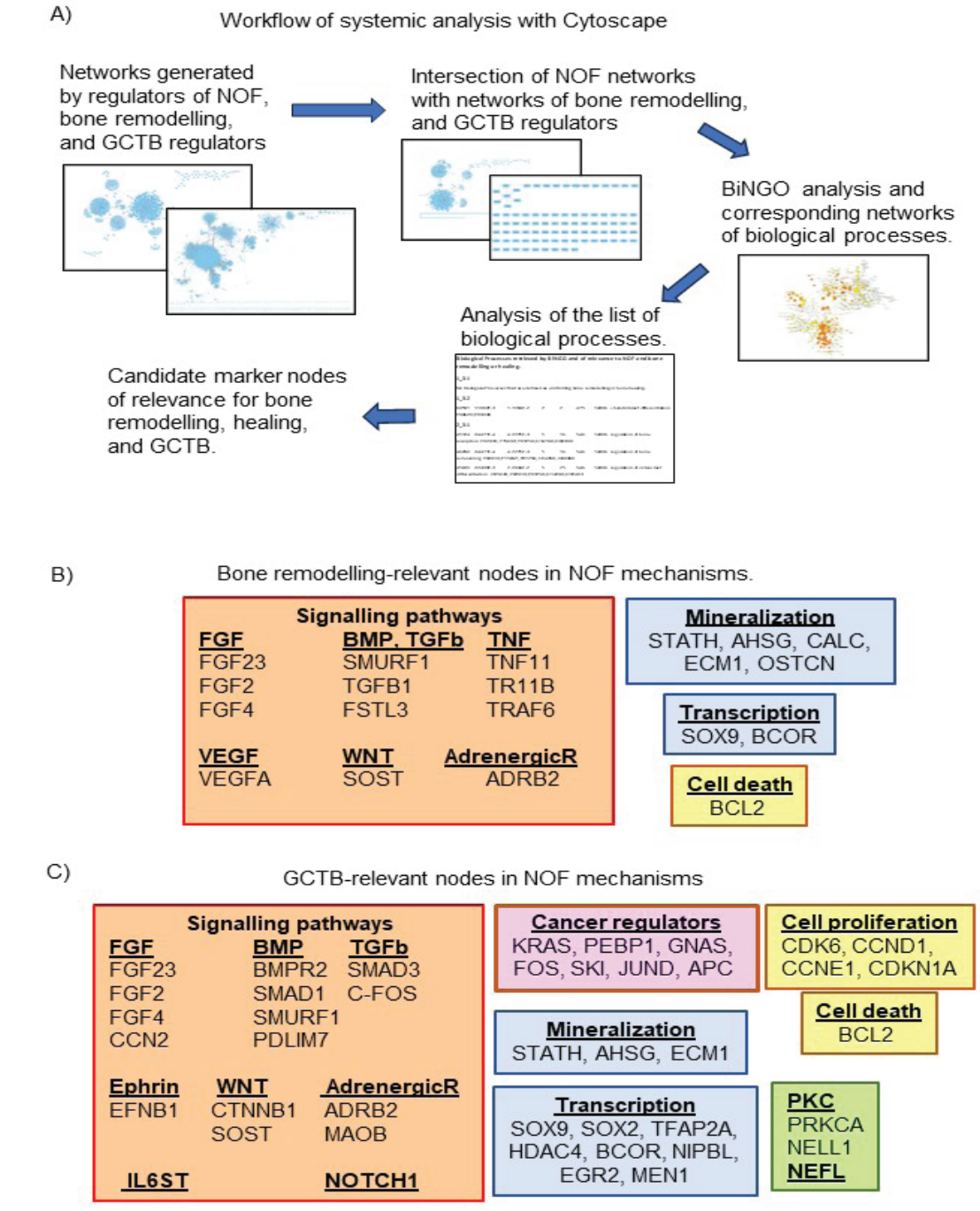

Figure 3. Cytoscape-based systemic analysis of the NOF-relevant, bone growth and healing, and GCTB relevant regulators. The workflow of the analysis with Cytoscape (A) shows key steps. Bone remodelling relevant nodes that are candidates for a risk prediction of non-efficient healing of NOF are presented in clusters of corresponding biological processes (B). Nodes of relevance to bone tumorigenesis and NOF are listed in corresponding biological process clusters (C). The nodes in B and C are present in NOF mechanisms and in mechanisms controlling bone remodelling (B) or bone tumorigenesis (C). Nodes are annotated in GO terms. For images and annotations of networks, intersection networks, biological processes networks and annotations see Supplementary Materials S4 and S5.

protein synthesis cluster. Cytoskeleton regulators and collagen-dependent stroma formation are represented. Detection of beta-catenin and estrogen receptors reflects their role in bone physiology. The networks of the NOF-confirmed and potentially involved regulators represent mechanisms affected in NOF.

Systemic analysis of regulators of bone remodelling and healing was performed to identify regulators that could predict the healing dynamics of NOF. The network consists of 49 nodes and 171 edges. There is also a significant overlap with functional clusters of NOF-related regulators. FGF and estrogen signalling, protein synthesis, cadherins, prostaglandin, and GSK3B are overlapping regulators. These regulators, by engagement in NOF lesions and in the normal healing of bone, may predict the efficiency of the healing of NOF lesions.

The transformation of NOF into a malignant tumor cannot be fully excluded. Systemic analysis of regulators engaged in the formation of giant cell tumors of bone (GCTB) generated a network of 40 nodes and 148 edges. A strong representation of cancer regulators was retrieved. Oncogenes (SRC, YES1, KRAS, RAN, SET), tumor suppressors (TP53, TP63), and cell cycle regulators (CDKs, CDK inhibitors, cyclin A2, CDC42) are potent regulators of tumorigenesis. The status of these nodes in the patient's sample may predict a risk of malignant transformation of NOF lesion.

Four networks were built with Cytoscape by using the GO terms of 4 categories listed in Table 1 (Figure 3, Supplementary Materials S4A and S5). Therefore, the input for Cytoscape was the same as for FunCoup. We used IntAct database for building the networks with Cytoscape. The detailed information about the networks is described in Supplementary Materials S4, and the Cytoscape session file in S5.

Six intersection networks formed by NOF regulators with networks formed by bone remodelling or GCTB regulators were generated (Supplementary Material S4B, C). Intersection networks represent common mechanisms, nodes, and edges in a pair of analysed networks Retrieved by BiNGO biological processes showed a broad range of affected activities (full lists are in Supplementary Material S4F). We focused on biological processes of relevance to bone remodelling and healing (Supplementary Material S4D), or GCTB (Supplementary Material S4E) (Figure 3B, C).

The biological processes of relevance to bone remodelling emphasized differentiation of osteoblasts, osteoclasts, and chondroblasts, bone ossification, resorption, mineralization, and remodelling (Supplementary Material S4 D). Signalling pathways, mineralization, transcription, and cell death are affected domains (Figure 3B). The identified nodes are candidates for markers of bone remodelling.

The network analysis was designed to include only close interactors, and, therefore, broad-action regulators, such as vitamins and hormones, are not reflected in the listed nodes. The broad-action regulators are considered in clinical assessment and for the selection of predictive markers (see the next section, Figure 4).

The biological processes common for GCTB and NOF-relevant regulators identified mechanisms of transformation of NOF into malignant neoplasia. Altered in NOF GCTB-promoting regulators indicate a risk of malignant transformation of NOF. FGF, BMP, TGF-beta, and Wnt/beta-catenin regulators are known as potent regulators of bone development and bone cancer [1-3,8-10,31,32]. The involvement of ephrin, adrenoreceptors, and interleukin-6 is novel information. An important finding is the identification of nodes that are known cancer regulators, e.g., KRAS, FOS, SKI, JUND, APC, PEBP1, and GNAS. Cell cycle is targeted in GCTB and is also represented in NOF-related mechanisms. Therefore, cell cycle nodes can be risk predictors. Regulators of bone mineralization, transcription, and cell death represent nodes of bone-specific regulation of GCTB. The GCTB-relevant nodes engaged in NOF development may also be the nodes driving the malignant transformation of NOF into GCTB.

Two independent tools, FunCoup and Cytoscape, provided complementary searches that ensured a comprehensive overview and analysis (Figures 2 and 3; Supplementary Materials S3, S4, S5). Several nodes that may serve as markers for the prediction of the NOF healing or malignant transformation were identified.

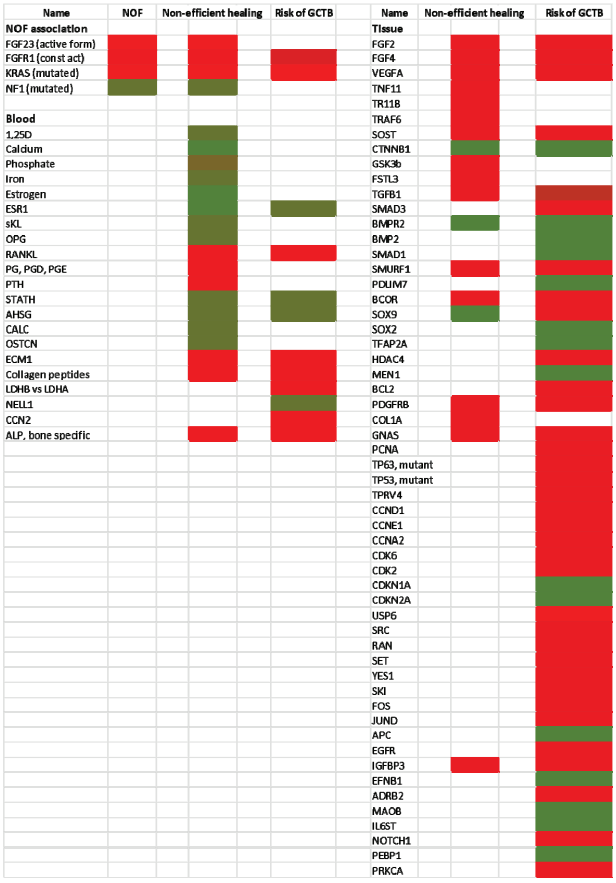

Figure 4. List and deregulation of components of NOF-proven regulators, and regulators that may predict the risk of non-efficient healing or malignant transformation. Components are annotated in GO terms, and are divided into NOF-proven, detectable in the blood, or in a biopsy. Red colour indicates high levels, and green indicates low expression levels, as compared to normal physiological levels. Indicated colours reflect values of regulators that are associated with NOF (first column), with non-efficient healing of NOF lesions (second column), or with high risk of malignant transformation (third column).

Selection of prediction markers.
Our systemic analysis extracted nodes that are represented in NOF and play a role in bone remodelling and tumorigenesis. The next step would be to translate the alterations of the nodes in the risk assessment values.

The combination of our data and clinical studies generated a landscape of potential prediction markers (Figure 4). This landscape reflects clinical knowledge about NOF and potential complications. The landscape identified nodes controlling regulatory mechanisms crucial for NOF appearance and for NOF healing or malignant transformation. Figure 4 shows the landscape in the form of a heatmap. Red colour indicates increased values and green colour indicates decreased values of nodes that are potential prediction markers of NOF, non-efficient NOF healing, or malignant transformation. For NOF, a proven correlation was reported only for 4 markers. These are FGF23 in its active form, FGFR1 overactivation, activating mutations of KRAS, and inactivating mutations of NF1 gene.

In addition to the NOF-proven regulators, the efficiency of healing can be affected by additional 38 regulators. These 38 regulators were identified as engaged in NOF and, at the same time, contributing to bone remodelling and healing. Therefore, they represent mechanisms that may control the healing of NOF lesions. Combined with genetic markers, it is a 41-marker signature. For the practical reasons of testing in clinics, potential prediction markers can be divided into two groups. The first group contains markers that can be measured in blood, plasma, serum, urine, or saliva. The second group contains markers that require a biopsy. Blood or body fluids are easily accessible for repeated testing. A biopsy is available only in cases of NOF surgery.

Estrogen, minerals, vitamin D, mediators (PTH, PG), FGF signalling (FGF23, sKL), secreted regulators of bone remodelling (OSTCN, CALC, RANKL, OPG, STATH, AHSG, ALP, ECM1), and peptides of collagen (PINP, PICP, CTX, NTX) can be measured in blood, or in saliva for STATH. Figure 4 shows alterations of these regulators that correlate with non-efficient healing. Note that all 38 regulators may interact between themselves in the context of the networks (Figures 3 and 4). Modelling of these interactions in clinical cases requires another study and is not discussed here. We focus on delivering a list of regulators that can predict the efficacy of NOF healing and can be measured in blood.

Biopsy offers an assessment of histology, types of cells, matrix, mineralization, and expression of regulators. In cases of availability of NOF biopsy, prediction of the healing efficacy can be made by assessing markers. A tissue section or extract of the biopsy can be used for testing. FGF signalling pathway is expanded by the addition of FGF2 and FGF4. Other signalling pathways of predictive value are bone morphogenetic protein (BMPR2,

SMURF1, FSTL3), transforming growth factor-beta (TGFB1), vascular endothelial growth factor (VEGFA), tumor necrosis factor (TNF11, TR11B, TRAF6), platelet-derived growth factor (PDGFRB), insulin growth factor (IGFBP3), and Wnt (CTNNB1, SOST). These signalling pathways are potent regulators of bone remodelling, and identified corresponding regulators may serve as markers of these pathways in bone healing and remodelling for NOF lesions. Transcriptional regulators (BCOR; SOX9), collagen type I (COL1A), glycogen synthase kinase 3 beta (GSK3B), and guanine nucleotide-binding protein G(S) subunit alpha protein (GNAS) reflect additional mechanisms of importance for bone healing in the context of NOF lesions.

The high incidence of NOF in children, and the lack of dedicated epidemiological follow-up studies of NOF created a notion that NOF does not undergo malignant transformation. The comparison of epidemiological data for NOF (incidence 30-40% in children) and bone tumors (below 0.1 % of the population) suggests that only a very small fraction of NOF would be transformed into a malignant tumor. Engagement in NOF signalling pathways typical for carcinogenic transformation indicates mechanisms of how NOF can turn into a malignant lesion. Our data highlighted significant engagement of oncogenes and tumor suppressors. SRC, RAN, SET, YES1, SKI, FOS, JUND, KRAS, APC, TP63, and TP53 are potent regulators of tumorigenesis (Figure 4). Detection of pro-tumorigenic activity of these oncogenes and tumor suppressors in NOF lesions may indicate a high risk of malignant transformation. Strong representation of the cell cycle regulators confirms the deregulation of cell proliferation in NOF and potential contribution to malignant transformation, e.g., cyclins, cyclin-dependent kinases and CDK inhibitors. Signalling pathways identified in the search for markers of bone remodelling also contribute to bone tumorigenesis. FGF, BMP, Wnt, EGF, IGF, Notch, ESR1, and VEGF signalling pathways are represented in the networks of promotors of giant cell tumors of bone and, at the same time, are engaged in NOF. These pathways are known to regulate tumorigenesis. Sixty-two molecules were identified as potential predictors of the malignant transformation of NOF lesions (Figure 4).

Our data suggest that the monitoring of NOF-proven regulators, combined with the regulators engaged in the bone healing or malignant transformation (41 and 62 signatures respectively) can help with the prediction of the efficiency of NOF healing or the risk of malignant transformation.

Discussion

Our data provide tools for addressing concerns about risk of NOF complications. Despite that NOF is considered a benign lesion, it may lead to pathological fractures, which is due to the non-efficient healing [2,33,34]. Malignant transformation is of concern also due to similarities between NOF and giant cell-rich tumors of bone (GCTB) [13-15,31,32].

The systemic analysis allows the extraction of regulatory mechanisms causing NOF complications. When mechanisms engaged in bone healing or malignant transformation are also deregulated in NOF, it is an indication of a complication. The extent of deregulation may predict risk of a complication, by considering the identity of a marker and its impact on a bone.

The lack of clinical and fundamental research on NOF is compensated by systemic analysis of combined data on bone remodelling, healing, bone tumorigenesis, and NOF mechanisms. Cytoscape and FunCoup are two established systems biology tools. The network analysis with these tools considers only highly significant evidence and, therefore, achieves significance that is similar to experimental measurements.

The reported here markers act as the networks. Their interdependency means that it would be needed to test all components of the 41 or 62 signatures. This is especially valid for components acting in subnetworks forming feedback and feed-forward loops. An example is FGF23-dependent interactions with klotho (KL) for enhancement of FGF23 action, or soluble KL for scavenging FGF23 [9,30]. FGF23 inhibits PTH, which in its turn induces expression of FGF23. Another interaction is vitamin D-dependent enhancement of FGF23 secretion by osteocytes, followed by FGF23 inhibition of vitamin D activation and subsequent impairment of calcium absorption [9]. Vitamin D stimulates also osteocalcin, leading to the promotion of bone formation [25]. PTH was reported

to increase levels of vitamin D [26]. PTH-dependent inhibition of osteoprotegerin (OPG) leads to enhanced RANKL, and subsequently to enhanced bone resorption [26]. Estrogen, contrary to PTH, may increase expression of OPG, followed by inhibition of RANKL and inhibit bone resorption [24]. Knowledge of these subnetworks would contribute to the informative assessment of the 41 and 62 signatures.

For the 62-node signature for the assessment of the risk of malignancy, the assessment includes analysis of oncogenes, tumor suppressors, signalling pathways, cell cycle regulators, and regulators of bone remodelling. Mutations of H3.3, GNAS, and USP6 correlate with GCTB or fibrous dysplasia and are used in differential diagnostic of NOF vs malignant tumors [1-3,8-10]. Each of the 62 regulators has a role in promoting different cancer hallmarks. For example, an indication of the high risk of malignant transformation would be a detection of a strong promotion of cell proliferation, fibrous-type extracellular matrix, impaired bone mineralization, inflammatory environment, overexpression of oncogenes, and impairment of tumor suppressors. The type of a regulator and the scale of deviation of its expression contribute to the assessment. This approach is typical for diagnostic tests with signatures of genes, proteins, or metabolites, and is used in clinical practice.

Deviations in levels of some of the molecules described in 41 and 62 signatures (Figure 4) can be reverted to normal levels by relatively soft and well-tolerated treatments. Examples are vitamin D, calcium, phosphate, iron, and collagen supplements. Estrogen, prostaglandins, and parathyroid hormone are regulators that can be normalized with available medical remedies. There are many drugs and remedies that act on the regulators of the 41 and 62 signatures. Examples are inhibitors of CDKs, GSK3B, HDAC, SRC, receptors of FGF, TGFbeta, EGF, VEGF, and PDGF. However, these drugs often have pronounced side effects, and can be used only if there are strong clinical indications.

In clinics, there are no tests that would predict NOF complications. Parents of NOF-affected children are told today that the risk of complications is low, and predictive tests are not available. Therefore, there is the need for marker-based diagnostics. Our data deliver lists of regulators for prediction of the NOF complications, which is based on the systemic analysis of known regulators of bone physiology.

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