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Molecular pathology of total knee arthroplasty instability defined by RNA-seq

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Abstract

Total knee arthroplasty (TKA) is a durable and reliable procedure to alleviate pain and improve joint function. However, failures related to flexion instability sometimes occur. The goal of this study was to define biological differences between tissues from patients with and without flexion instability of the knee after TKA. Human knee joint capsule tissues were collected at the time of primary or revision TKAs and analyzed by RT-qPCR and RNA-seq, revealing novel patterns of differential gene expression between the two groups. Interestingly, genes related to collagen production and extracellular matrix (ECM) degradation were higher in samples from patients with flexion instability. Partitioned clustering analyses further emphasized differential gene expression patterns between sample types that may help guide clinical interpretations of this complication. Future efforts to disentangle the effects of physical and biological (e.g., transcriptomic modifications) risk factors will aid in further characterizing and avoiding flexion instability after TKA.

Keywords

total knee arthroplasty; flexion instability; revision total knee arthroplasty; cell biology; molecular genetics

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INTRODUCTION

Instability after total knee arthroplasty (TKA) is one of the most common modes of failure requiring revision arthroplasty [1, 2]. Instability cases account for approximately 30% of revision TKAs performed in the US [3, 4] and other developed countries [5]. Though improvements in implant design and surgical technique have decreased the risk of instability, the projected increase in number of primary TKAs performed will continue to burden surgeons and health care systems [6-9]. Instability can be categorized as global instability, flexion instability, extension instability, and recurvatum [1, 10, 11]. Flexion instability is a finite diagnosis in which there is a flexion and extension mismatch, resulting in recurrent hemarthroses and a finite constellation of signs and symptoms [10]. Patients with flexion instability after TKA present with symptoms ranging from discomfort and a subjective sense of instability to complete dislocation of their prosthesis [12-14]. Clinical examination can reveal recurrent knee effusions, anterior or medial joint line tenderness, and/or excessive laxity of the collateral ligaments [3, 13, 14]. Radiographic analysis can determine implant positioning and alignment in multiple planes, as well as limb alignment, tibial slope, and flexion gap to determine the severity of instability [1, 10]. However, additional methods (e.g., molecular biomarkers) for characterizing the risk of instability after TKA would be beneficial for the improvement of surgical intervention strategies and long-term patient outcomes.

The failure mechanism for flexion instability after TKA is multifactorial, attributed to biomechanical insufficiencies of native tissues (e.g., ligament injury) and/or direct failure of the implant device [15]. Poor surgical techniques (e.g., undersized femoral component, internal rotation of components, excessive tibial slope, inadequate distal femoral resection, or excessive release of the medial collateral ligament) and/or the host soft-tissue status can lead to mismatched flexion/extension gaps that lead to worsening flexion instability [1, 10, 15, 16]. The varied clinical presentations of flexion instability after TKA suggest pleiotropic and/or patient specific causes (and likely treatment strategies) of this orthopedic problem. However, additional insight into the molecular mechanisms that distinguish TKA instability tissues from normal tissues will yield useful insights regarding similarities among patients with similar symptoms.

Clinically, epidemiologic and retrospective studies have identified risk factors and biomechanical etiologies underlying arthroplasty failure [17], yet limited data exist regarding the relationship of biological mediators regulating this clinical scenario. Furthermore, preoperative variables and demographics alone are of limited use in predicting functional outcomes and pain scores following TKA [18]. Recognizing important biological mediators may help characterize the significance of extracellular matrix remodeling, osteolysis, the post-traumatic inflammatory cascade, and other pathogenic mechanisms involved in the development of flexion instability following TKA.

High-throughput next-generation sequencing tools can provide a basis for identifying differentially expressed genes correlated to flexion instability after TKA. For example, RNA-seq has been used to successfully identify differences in gene expression between normal tissues and tissues affected by numerous musculoskeletal diseases including

osteoarthritis, and rheumatoid arthritis [19-21]. Identifying gene products that are over-expressed in patients with flexion instability after TKA will provide information regarding genes and biological pathways implicated in the condition. Dysregulation of genes involved in tissue repair and remodeling of the surrounding soft tissue enveloping the knee joint, and/or oxidative stress reactions may negatively alter postoperative healing. Furthermore, highlighting key genes involved in tissue dysfunction may help characterize novel co-morbidities of flexion instability after TKA (e.g., poor joint perfusion, smoking, diabetes) and cell signaling pathways amenable to therapeutic intervention or prophylaxis.

Matrix Metalloproteinases (MMPs) and their endogenous inhibitors, tissue inhibitors of metalloproteinase (TIMPs), may be involved in the molecular pathophysiology of flexion instability after TKA. Importantly, MMPs are involved in tissue remodeling, extracellular matrix degradation, and have a prominent role in many musculoskeletal pathologies [19, 22, 23]. For example, a single nucleotide polymorphism (SNP) in the promoter region of *MMP1* has been associated with aseptic loosening of total hip arthroplasty when detected in peripheral blood [24]. Elevated *MMP1* expression may also induce excessive tissue degradation of the periprosthetic microenvironment [25], that results in late manifestations of prosthesis malignment. In contrast, increased *MMP3* expression may correlate with exposure of synovial tissues to particulate debris [26-28]. Nonetheless, ECM remodeling genes will be important to analyze and monitor in patients with flexion instability after TKA.

The aim of this study was to investigate differential gene expression patterns among synovial capsular tissues collected from normal adults undergoing primary TKAs and patients requiring revision procedures due to flexion instability after TKA. The pathology of flexion instability after TKA is currently defined only by clinical and radiographic parameters, so establishing a biological mechanism for this complication will not only allow refined detection strategies, but also facilitate intervention strategies to prevent and treat the condition.

MATERIALS & METHODS

Patient Enrollment & Tissue Handling

In accordance with our approved IRB protocol (09-000115, Mayo Clinic, Rochester, MN), all patients were identified, verbally informed, and signature-consented prior to enrollment in the study. When possible, patient clinical data were screened and matched based on age (± 5 years), gender, and body mass index (BMI) (± 5 kg/m²). We collected a total of 32 patient samples (16 primary TKAs [control group] and 16 TKAs revised for flexion instability [experimental group]). At the time of revision surgery, suprapatellar synovial capsular tissue (either control or experimental) were carefully dissected by the operative surgeon using a scalpel. After rinsing with sterile PBS, tissues were flash-frozen in liquid nitrogen, and stored at -80°C for further analysis.

RNA Handling

Frozen tissue biopsies were ground into powder by mortar and pestle and homogenized in Qiazol reagent using the TissueLyser LT (Qiagen, Hilden, Germany). Genomic RNA extractions were performed using the miRNeasy mini kit (Qiagen, Venlo, Netherlands) according to the manufacturer's protocol, and eluted in 50 μ L total volume. A NanoDropper (Thermo Fisher Scientific, Waltham, MA) was used to measure RNA concentration and purity. The Mayo Clinic Gene Expression Core (Advanced Genomics Technology Center, Mayo Clinic, Rochester, MN) also provided an RNA quality assessment (Agilent Technologies, Santa Clara, CA) using RNA integrity number (RIN) as a tool for prioritization of samples for RNA-seq data collection.

RNA-seq data collection was performed by the Gene Expression Core, a division of the Advanced Genomics Technology Center at Mayo Clinic (Rochester, MN). Specifically, a HiSeq 2000 (Illumina, San Diego, CA) was used to run 51 bases per read, 6 samples per lane, and paired-end index reads. The RNA Sample Prep Kit v2 library preparation type was specified for use by the Gene Expression Core, to complete poly-A mRNA purification, cDNA synthesis, sequencing and base-calling as per the manufacturer's instructions (Illumina, San Diego, CA). Using the MAP-Seq v.1.2.1 workflow, reads per kilobase of transcript per million mapped reads (RPKM) were provided, which includes alignment with Tophat 2.0.6 [29] and normalization with edgeR 2.6.2 [30]. For a larger set of patient samples, reverse transcription and RT-qPCR reactions were performed as described previously [31]. Transcript levels were quantified using the 2^{-C_t} method and normalized to *AKT1* ($\times 100$).

Statistical Analyses & Bioinformatics

The final mapped RNA-seq data contained all reads expressed as RPKM estimates (a normalized metric based on the most abundant mRNAs detected in each sample/lane). We selectively prioritized gene expression values above the commonly reported limit of technical detection by HiSeq 2000 (RPKM>0.3). Any detectable fragments that were not mapped to a known messenger RNA were also eliminated. Following average calculations within each group (e.g., pTKA and insTKA), we calculated fold change values between the groups and sorted them by whether their values were "up-regulated" or "down-regulated". The resulting gene list was further analyzed using the following bioinformatics tools: hierarchical clustering (GeneMania [32], which does not consider statistical rigor), and a second hierarchical clustering method (Pvclust v1.2-2 [33], which does incorporate a statistical algorithms). 100,000 bootstrap replicates were performed for confidence estimation. Statistical significance values were computed by paired t-test and presented as both $p<0.05$ and $p<0.10$.

RESULTS

Sampling Scheme

Patients enrolled in this study ranged in age from 29.8 - 76.5 years, body mass indices from 18.8 - 38.6 kg/m², and 53.1% of patients were female (Table 1). In total, 32 joint capsule tissues were collected from the suprapatellar synovium of individual patients undergoing

primary TKA (n=16) and those being revised for flexion instability (n=16) (Figure 1). All 32 samples were analyzed by RT-qPCR, while 6 samples (3 from each group) were analyzed by RNA-seq.

Distinctiveness of sample types

RNA-seq data collection resulted in a total of 23,398 mapped fragments, nearly 20,000 of them are known mRNAs. After pruning these data for poorly detected mRNAs (RPKM<0.3) left nearly 12,000 genes included in downstream analyses (Figure 2a). Hierarchical clustering analysis resulted in two distinct clusters grouped by sample type and highlighting differentially expressed genes (both high and low RPKM values) within each sample type that are common to each group (Figure 2b). Group clusters were well-supported by approximate unbiased p-value estimates (numbers closer to 100 indicate more robust nodes on the dendrogram) and bootstrap proportions. Samples 14A and 19A were nested within the pTKA group in the Pvclust analysis (Figure 2c), whereas samples 19A and 16A formed a sister relationship in the GeneMania analysis (Figure 2d). Overall clustering patterns for samples within the insTKA group were similar among analysis modes (Figures 2b, 2c, 2d).

Gene expression profiles of capsular tissue

Differential gene expression analysis of the top 20 upregulated genes in tissues from instability TKA patients revealed a list with cell surface markers (e.g., *CD22*), chemokines (e.g., *CCL18*), matrix metalloproteinases (e.g., *MMP3*), and oxygen-related enzymes (e.g., *HMOX1*) (Figure 3a). Functional protein association networks involving the top 50 genes decreased in insTKA samples compared to controls (pTKA) (Figure 3b), and bottom 50 genes elevated in insTKA samples compared to controls (Figure 3c) revealed highly connected networks of genes with only 7 and 9 genes that remained unincorporated, respectively.

Focused RNA-seq analysis – ECM remodeling

Focused hierarchical clustering analyses among sample groups (pTKA and insTKA) for genes related to ECM-remodeling, resulted in poorly defined sample groups. For example, sample 16B grouped with pTKA samples (Figure 4a, 4b) in both the GeneMania and Pvclust analyses, despite strong statistical support for this relationship based on the ECM remodeling partition of the RNA-seq data (Figure 4b, 4c). When analyzed independently, all collagen-related genes were elevated in tissues with flexion instability after TKA. Specifically, mRNA expression values for genes related to collagen production were consistently elevated in samples taken from instability TKA patients, and five were statistically significant (p-value<0.05; *COL1A1*, *COL3A1*, *COL5A1*, *COL8A1*, and *COL8A2*) while three others were trending toward significance below a p-value of 0.1 (*COL1A2*, *COL5A2*, and *COL27A1*) (Figure 4d). This striking pattern applied to all significantly expressed collagen genes detected in the RNA-seq analyses described above. Similarly, ECM degradation-related genes revealed patterns of increased RPKM values in samples from instability TKA patients (18 out of 30 highly expressed genes), and of these *MMP2*, *MMP11*, *ADAMTS2* were statistically significantly elevated in instability TKA samples (Figure 4e).

Focused RNA-seq analysis – chemokine signaling

Hierarchical clustering of chemokine signaling genes partitioned from RNA-seq data revealed well-supported groupings of samples by tissue type (au-values>98) (Figure 5a, b, c). Seventeen of twenty-two expressed chemokine ligands (CCLs and CXCLs) were upregulated in instability TKA samples, and four were significantly higher than pTKA samples ($p<0.1$) (Figure 5d). Genes involved in the inflammatory response were specifically investigated for differences between each sample group, and a lack of clustering by sample type (data not shown).

qPCR validation

Despite low variation detected among RNA-seq samples for the expression of genes commonly used to normalize gene expression data (Supplementary Figure 1), we proceeded to analyze additional samples by RT-qPCR. A panel of sixteen human primers were used to determine expression patterns among 16 samples within each sample group ($n=32$ total). Any patient samples with low RNA extraction yields were omitted from the study. For all remaining samples included in the analysis, average values and t-test statistics were used to calculate differential gene expression patterns among groups. For all sixteen human genes analyzed by qPCR, normalized expression values were elevated in insTKA patient samples (except *CD248*, and *ID3* and *cFOS*). Below the $p<0.05$ threshold of statistical significance were the following genes that were upregulated in instability TKA samples: *COL3A1*, *TNC*, *ACAN*, *HIST2H4*, *ACTA2*, and *COL1A1* ($p<0.1$) (Supplementary Figure 2 and data not shown). *ID3* was significantly lower in instability TKA samples based on qPCR analysis.

DISCUSSION & CONCLUSIONS

Flexion instability after TKA unfortunately occurs after the procedure, and is currently defined by clinical history, physical examination, and radiograph [17, 34, 35]. While investigating the clinical and biomechanical etiologies of failure in this condition, exploring the biomolecular differences between patients with and without flexion instability after TKA may yield insights into the subject of host tissue response [36, 37]. Studies investigating the genetic profile of biologic mediators involved in the acute post-surgical healing process reveal this critical period is responsible for the inflammatory cascade, tissue remodeling, and scar tissue formation initiated in the microenvironment surrounding implanted prostheses [38]. While the continued success of total joint arthroplasty has been reported for decades [39, 40], limited molecular data describing many common and devastating orthopedic complications have yet to be analyzed [15, 35]. RNA-Seq is a useful tool for identifying significant gene expression differences between experimental and control tissue samples, and may allow for more directed studies to target ECM remodeling genes implicated in the process of post-surgical joint instability. Targeted analysis of these markers in the post-surgical setting may also allow identification of high-risk patients receptive to TKA instability mitigation.

Matrix metalloproteinases and their endogenous inhibitors, tissue inhibitors of metalloproteinase (TIMPs), are pivotal biological proteases responsible for the degradation of ECM in normal physiological processes such as tissue repair, remodeling, morphogenesis,

and development [25, 41]. MMP functional gene polymorphisms contribute to patient-specific differences in susceptibility and outcomes for cardiovascular diseases (e.g., coronary artery disease, stenosis, myocardial infarction, coronary aneurysm, stroke, and large artery stiffness) [42]. In principle, MMP genotyping could be used to identify patients most likely to develop unfavorable prognoses in response to invasive medical treatments including TKA.

In orthopedics, limited data exist on the possible role of MMPs in the pathogenesis of bone turnover and soft tissue necrosis following total joint arthroplasty. Some studies have demonstrated the dysregulation of MMPs in degenerative, inflammatory diseases like osteoarthritis, rheumatoid arthritis, and fibrotic conditions [27, 43], while others have noted a correlation between elevated MMPs and periprosthetic loosening / osteolysis [24, 25, 44]. For example, Yan et al. [24] demonstrated elevated expression of *MMP1* and *MMP2* in cases of prosthetic loosening following THA. Ishiguro et al. [45] also documented higher mRNA gene expression levels of MMPs and TIMPs in the cemented periacetabular tissue of THA failures that resulted from aseptic loosening. While THA aseptic loosening is a pathophysiologically distinct process from TKA instability, they likely share common processes and relevant markers that should be monitored after surgery [37]. Other studies have characterized Heme Oxygenase-1 (*HMOX-1*, or *HO-1*), an essential enzyme for heme catabolism which utilizes heme as its main catalytic substrate. However, evidence supports the activation of this enzyme by other non-heme substrates, such as exposure to heavy metals, inflammatory cytokines, and prostaglandin synthesis to induce *HMOX-1* to mediate cellular stress-response reactions in the tissue [46].

Our study has shown elevated gene expression of ECM remodeling genes suggesting a potential relationship between extracellular matrix degradation and oxidative stress exposure during tissue remodeling. As Choi et al [46] demonstrated in various pulmonary pathologies, tissue exposure to oxidative insults can stimulate an upregulation of *HMOX-1*. Similarly, Jais et al. [47] showed cytoprotective properties of *HMOX-1* as anti-inflammatory mediators in patients with chronic metabolic inflammation or “metaflammation”. Our results support the suggestion that oxidative-response events may be correlated to chronic local inflammation typical of TKA instability, and potentially exacerbate uncontrolled movement along the bone-soft-tissue interface [3, 14]. Notably, recurrent effusions after bleeding [3, 13], or synovial entrapment [48, 49], would also be expected to correlate with elevations in *HMOX-1* expression as a biological damage control mechanism. Alternatively, studies on osteoarthritis progression have shown *HMOX-1* regulates *MMP1* secretion via an oxidative stress mediator, *Nox4* [50] suggesting that indirect consequences of TKA instability to alter cell responses should not be ignored. In summary, efforts to further isolate the effects of physical and biological processes (e.g., ECM remodeling, oxidative stress response) that contribute to flexion instability after TKA will likely yield exciting results that guide clinicians toward advanced prevention, detection and treatment strategies to reduce overall rates of TKA instability.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Flexion instability after total knee arthroplasty is examined using primary clinical tissues
- Gene expression levels are collected by RNA-seq and RT-qPCR
- High-throughput data and bioinformatics techniques are used to compare differentially expressed genes
- Genes related to extracellular matrix remodeling and oxidative stress are highlighted
- Possible future strategies to mitigate flexion instability after total knee arthroplasty are discussed

Figure 1.
(a) Surgical photo and (b) illustration showing anatomic location of tissue sampling.

Figure 2. (a) RNA-seq pruning strategy, and (b) similarity matrix for all RNA-seq samples included in this study, (c) dendrogram (100,000 replicates), (d) heatmap of all RNA-seq data - pTKA vs insTKA.

(a)

	pTKA			insTKA		
	14A pTKA	16A pTKA	19A pTKA	16B insTKA	2B insTKA	3B insTKA
upregulated insTKA	GeneID					
	HS3ST2	1.194232075	8.47940935	1.391150167	107.5449131	319.3409999
	CCL18	19.25977238	33.85749134	10.17780549	243.2816073	1473.928991
	HLA-DQA2	0.473599127	5.291290222	0.330838831	8.744188125	137.9128309
	MMP3	8.763892017	0.487358022	11.27168621	444.1511819	10.69454106
	DHDH	0.871763324	0.390379969	0.418674857	6.946522471	19.89323143
	IGSF6	3.040536087	3.188809386	2.372912995	21.99917123	95.83540978
	MMP1	2.823870148	2.167616702	1.356197719	85.86261241	7.763448755
	CD22	NA	0.39881264	0.38349969	1.991739252	11.36803169
	HMOX1	20.43218737	31.31829438	29.30358129	607.5083945	255.7215146
	POSTN	5.458796777	20.64224558	33.04551363	97.6552435	146.7357705
	SDS	0.463828662	8.009712143	0.861336009	9.568611829	85.3815945
	CHIT1	0.371746017	165.7853469	1.785355112	29.43420723	1796.785897
	LIPA	4.965474651	52.65965069	13.7256402	91.55481801	630.1938443
	ADTRP	1.254650706	0.504725208	2.474346822	6.348183032	31.35890893
	ATP6VOD2	NA	2.097143887	0.763860795	0.757333228	37.36994322
	LYZ	79.7636808	37.57922213	91.66767104	345.9089239	1576.225043
	SLC26A11	0.796049296	1.766207586	2.005188906	6.646947343	34.01813361
	NPL	2.114918055	6.07216628	2.896668476	20.54808383	65.56382934
	HLA-DQB2	6.63084825	0.376114747	1.422430054	22.95100703	52.02280116
	APOC1P1	0.950774663	0.848895123	0.329781838	4.057018844	12.75201587
downregulated insTKA	GeneID					
	CIDEA	82.3307873	210.1276762	29.96710997	NA	0.770884111
	CIDEA	402.4130728	825.1573056	108.2823953	1.09145215	2.765402689
	C14orf180	33.11058607	139.5245204	23.78170543	0.713961648	0.623731046
	NNAT	35.31339212	262.6790787	21.20417724	3.798062107	0.402189373
	CHAD	2.545776288	NA	129.3890174	NA	0.988602835
	GPD1	201.8268817	627.3600958	82.69222254	1.006184186	3.957118311
	APOD	139.0520565	782.2574307	51.30871103	5.089328531	4.572288113
	PLIN4	259.3531097	936.074517	130.3728921	0.789916144	4.472968774
	LIPE	52.96092993	323.3441814	40.31817918	1.520644099	1.962887034
	FABP4	1605.099692	9897.210026	1306.767586	24.30356878	41.32548606
	AQP7P1	5.536966806	29.15078531	4.741607095	NA	0.395207973
	TUSC5	12.08245288	96.07284325	13.19528045	NA	0.417444832
	ADH1C	6.532353369	47.52561773	7.19624993	NA	0.473999153
	ADH1B	41.7021513	242.9590012	41.00625189	NA	1.548953944
	GYG2	23.36035143	38.96135184	16.72689946	0.302104734	0.339331735
	MLXPL	8.866645674	38.6783953	4.025777698	0.614600654	0.67093727
	PPP1R1A	112.1245346	558.3469223	62.26552761	21.41089057	2.645853451
	SLC19A3	13.69330892	74.40994784	9.150016664	1.672523022	0.630178539
	S100B	64.50021806	177.2692318	31.27082801	2.646410852	1.753191317
	PLIN1	48.5612543	233.9228159	59.2455999	0.370751002	2.838501709

(b)

(c)

Figure 3.

(a) Gene expression profiles of capsular tissue, (b) STRING analysis (n=50) of Top Genes Decreased in InsTKA samples compared to controls (pTKA), (c) STRING analysis (n=50) of Top Genes Elevated in InsTKA samples compared to controls.

Figure 4. Hierarchical clustering analyses on ECM remodeling genes only by (a) GeneMania, (b) Pvcust, (c) standard error versus p-value plot. (d) Focused RNA-seq analysis of ECM-formation related genes (e.g., Collagens), (e) focused RNA-seq analysis of ECM degradation-related genes (e.g., MMPs, TIMPs, ADAMTSs). (*= <0.1 ; **= <0.05)

Figure 5.

Hierarchical clustering analyses on cytokine genes only by (a) GeneMania, (b) Pvclust, (c) standard error versus p-value plot. (d) Focused RNA-seq analysis of cytokines (e.g., CCL, CXC). (*= <0.1 ; **= <0.05)

Table 1

Sampling scheme for qPCR and RNA-seq data collection.

A)			
Primary TKA			
Sample ID	Age (Years)	Gender	BMI (kg/m²)
1A	68.5	F	24.5
2A	70	M	24.9
3A	68.1	F	27.8
4A	56.3	F	38.1
8A	65.7	F	41.5
9A	56.3	M	34.8
10A	56.4	F	26.9
11A	61.4	F	38.2
12A	62.7	M	27.2
14A	70	F	28.8
15A	70.4	F	29.2
16A	63.5	M	38.6
17A	65.4	M	32.2
18A	67	M	32.9
19A	75.2	F	29.8
20A	70.1	M	37
B)			
Instability TKA			
Sample ID	Age (Years)	Gender	BMI (kg/m²)
1B	62.8	F	20.1
2B	61.3	M	25.9
3B	70.4	F	36.6
4B	29.8	M	35.6
5B	50.9	F	33.7
6B	59.8	M	36.2
7B	72.8	M	26.8
8B	68.8	M	25.6
9B	43.4	F	26.6
10B	52.3	M	25.9
11B	69.1	M	31
12B	69	F	27.1
13B	76.5	F	27.1
14B	72.7	F	27.1
14B	67.8	M	24.9
16B	65.2	F	18.8

(a) Instability TKA (n=16) and (b) primary TKA (n=16). Bold text indicates samples analyzed for RNA-seq (n=3 for each group).