Structural and Molecular Docking Analytical Studies of the Predicted Ligand Binding Sites of Cadherin-1 in Cancer Prognostics

Introduction:
Several studies have explored the design of antimicrobial peptides (AMPs) for the development of therapeutic and diagnostic molecules for the treatment and identification of pathogenic diseases as well as cancer. Human cadherin-1 protein has been identified to be involved in adhesion-mediated signalling pathways in normal cells and its loss through genetic and epigenetic alterations can result in an enhanced invasion and metastasis of malignancy in tumours. Therefore, the identification of cadherin during treatment of cancer can be used as prognostic biomarker to establish the responsiveness of patients to treatment regimen. Antimicrobial peptides (AMPs) offer several compensatory advantages in biomedical applications and have been used for treatment of diseases, dietary supplements and diagnosis of diseases. The aim of this research work was to use in silico approaches to analyse retrieved human cadherin-1 as prognostic targets in cancer treatments using modelled putative anticancer AMPs.

Methods:
The structures of the putative AMPs and cadherin-1 were modelled using I-TASSER server and the protein overall quality was validated using PROCHECK. Thereafter, the protein motifs were predicted and the molecular interaction between the putative anticancer AMPs and protein was carried out using PatchDock.

Results:
The results revealed that all the AMPs were good prognostic molecules for cancer with BOO1 having the highest binding affinity of 15,874.

Conclusion:
This study revealed that all the generated AMPs have good prognostic value for monitoring the progress of cancer treatment using human cadherin-1 as receptor. This is the first report where AMPs were used in prognostics of cancer using human cadherin-1.

Keywords: cancer, antimicrobial peptides, prognosis, homology, modelling, docking, physicochemical, binding, validation, cadherin-1

Introduction
According to the WHO statistics, cancer is rated the second leading cause of death in the world with about 9.6 million death in 2018.¹ This means one in six death cases is due to cancer with the 70% of these happening in low and middle income country.² It has led to major economic losses in which International Health communities and Research institutes have spent a lot of money to combat its menace with a total economic cost of 1.16 trillion dollars spent in 2010 alone.³ One major factor leading to the enormous mortality rate in the low income country is the late stage presentation, inaccessible diagnosis, lack of precision to monitor treatment efficacy and overwhelming lack of data to drive policy.

Cancer is caused by many factors characterized by an abnormal growth of cells in the body with the tendency to grow rapidly, divide and proliferate.⁴ Environmental and
lifestyle factors worsen the incidence of cancer as a major public health problem posing a global menace associated with metabolic and genetic abnormalities. One evidence linking cancer to abnormal gene regulation is the disturbance of cell–cell and cell extracellular matrix adhesion with the subsequent changes in adhesion-mediated signalling pathways of cadherins which induce malignant phenotypes in normal cells.\(^5\)

Calcium independent adhesions (cadherins) are a class of type-1 transmembrane proteins found in tumors and normal cells which are highly dependent on Ca\(^{2+}\) ions to function and use cell adhesion molecule (adherens junctions) for binding cells together.\(^7\) Cadherin-mediated adhesion through the formation of cadherin–catenin complex regulates cell growth and differentiation, normal cell–cell adhesion, and maintain homeostatic tissue architecture. Disruption of signalling and altered expression of cadherins causes the disaggregation of tumors which can be temporal or permanent to promote the metastasis of such cells.\(^8\) This makes cadherins a major target in cancer therapy. Over a hundred subtypes of this superfamily have been identified from different species of organisms.\(^7\) Much research work has been conducted on the relationship between cadherins and tumorigenesis as well as drug-targeting cadherins which have undergone clinical trials.\(^7\) The loss of cadherin in tumorigenesis due to genetic and epigenetic alterations has been implicated in increased abnormal growth and metastasis in cancer cells, thus preventing its regulatory role in malignancy as a cancer cell suppressor. Identification of cadherin after treatment could be a potential approach in cancer prognostics for enhanced treatment.

Antimicrobial peptides (AMPs) have recently been explored as a source of therapy for many diseases due to their compensatory multifunctional attributes. The advantages of these vital molecules include signalling molecule, drug delivery vector, immune-modulatory agent, antitumor and mitogenic agents.\(^10\) The aim of this research work is to use an in silico approach to identify AMPs modelled from HMMER as selective inhibitors for more potent anti-cancer therapy with high effectiveness, short-lived side effects and high tolerability.

**Materials and Methods**

Retrieval of Cadherin-1 Sequences of *Homo sapiens* and Computing of the Physicochemical Parameters Analysis

The protein, cadherin-1, (P12830) amino acid sequences were retrieved from the Universal Protein Databases (UniProt) (https://www.uniprot.org/uniprot/P12830).\(^11\) Thereafter, the protein physicochemical parameters were generated from the ProtParam tools (http://web.expasy.org/protparam/) using expPasy Server to reveal the protein structure and function with the computation of molecular weight, extinction coefficient, amino acid composition, estimated half-life, hydropathicity, theoretical PI, instability index and aliphatic index.\(^12\)

**Prediction of Secondary Structure**

Secondary structure prediction such as alpha-helices, betasheets and random coils of cadherin was predicted using the PSIPRED secondary structure prediction server (http://bioinf.cs.ucl.ac.uk/psipred).\(^13\)

**Assessment of Experimentally Validated Antimicrobial Peptides (AMPs) Data**

Experimentally validated anticancer AMPs from various databases such as the Collection of Anti-microbial Databases (CAMP) (http://www.camp.bicnirrh.res.in),\(^14\) Anti-microbial Peptides Database (APD) (http://aps.unmc.edu/AP/main.php)\(^15\) and Data Repository of Antimicrobial Peptides (DRAMP) (http://dramp.cpu-bioinf.or).\(^16\) Thereafter, arrangement of these data and curation were performed to validate the authenticity of the anticancer AMPs. Subsequently, duplicate anticancer AMPs were removed using Cluster Density at High Intensity with Tolerance (CD-HIT) (http://www.bioinformatics.org/cd-hit).\(^13\)

**Hidden Markov Models (HMMER) Profile Construction**

The latest version of Hidden Markov Models (HMMER) algorithm version 3.8\(^17\) was used to construct specific anticancer AMPs profiles using the respective training datasets, having divided the retrieved experimentally validated anticancer AMPs into three quarters (training dataset) and one quarter (testing dataset). Query of the training dataset was carried out using the positive dataset (testing dataset) and negative dataset (neuropeptides). The HMMER profile was built on the Ubuntu 12.04 LTS operating system. The task was accomplished on a terminal and the command lines used to build the profile was written in accordance with the corresponding algorithm and the steps involved in their construction are shown in Figure S1.

**Discovery of Novel Anticancer AMPs**

Proteome sequences were retrieved from the Ensembl database (http://www.ensembl.org/index.html) and the
UniProt database (http://www.uniprot.org/) and were used to query the profile with the list of all proteome sequences (in the FASTA format) retrieved. A cut-off E-value was set to be 0.05 for the search of putative anticancer AMPs. This was accomplished using “hmmsearch” module of the HMMER algorithm with the command line employed as stated in Figure S2.

Determination of the Physicochemical Parameters of the Putative Anticancer AMPs

3-D Structure Prediction of the Putative Anticancer AMPs and Cadherin
The anticancer AMPs and cadherin (PDB ID: 1FF5) (E-Cadherin double domain) 3-D structures were predicted using ITASSER (https://zhanglab.ccmb.med.umich.edu/I-TASSER/) and visualized using PyMol. The cadherin, protein model structure was utilized for the de novo modelling of the cadherin protein from the LOMETS threading programs.

Model Evaluation and Prediction of Functional Motif
The quality of the generated model was checked using PROCHECK to compute parameters such as side chain conformations of the protein using atomic resolutions, lengths, geometry of the hydrogen bond, angles and planarity of the peptide bonds. The MOTIF (http://www.genome.jp/tools/motif/) finder was used to find the motifs present in the protein using the amino acid sequences.

In Protein-Ligand Interaction Study
The docking of the anticancer AMPs to the cadherin protein was carried out using PatchDock 1.3 version, a free online web-server that enables the docking of the protein-small ligand molecule, available at http://bioinfofo3d.cs.tau.ac.il/PatchDock/. In brief, the 3-D structures of the putative anticancer AMPs and the cadherin protein receptor PDB files from I-TASSER were uploaded onto the PatchDock server. Interaction analysis of the complex formation between the putative anticancer AMPs and the cadherin protein receptor was achieved using RasMol 2.7.5 software.

Results
Physicochemical Properties of Cadherin-1
The result from ExPasy server (Table 1) indicated that cadherin-1 from Homo sapiens has 882 amino acids with each residue contributing to give the protein an average molecular weight of 97.456 kDa. The result also indicated that the most abundant amino acids are threonine, leucine, valine, aspartate, proline, and glutamate, respectively. The physicochemical parameters predicted the protein to be negatively charged because of the abundance of glutamic

<table>
<thead>
<tr>
<th>Table 1 Physicochemical Properties of Cadherin-1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ProtParam Parameters</strong></td>
</tr>
<tr>
<td>Number of amino acids</td>
</tr>
<tr>
<td>Molecular weight</td>
</tr>
<tr>
<td>Theoretical Pi</td>
</tr>
<tr>
<td>Percentage amino acid composition</td>
</tr>
<tr>
<td>Atomic composition</td>
</tr>
<tr>
<td>Formula</td>
</tr>
<tr>
<td>Number of negatively charged residues</td>
</tr>
<tr>
<td>Number of positively charged residues</td>
</tr>
<tr>
<td>Extinction coefficient</td>
</tr>
<tr>
<td>Total number of atoms</td>
</tr>
<tr>
<td>Estimated half-life</td>
</tr>
<tr>
<td>Instability index</td>
</tr>
<tr>
<td>Grand average of hydrophaticity (GRAVY)</td>
</tr>
<tr>
<td>Aliphatic index</td>
</tr>
</tbody>
</table>
acid (6.8%) and aspartic acid (7.7%) over arginine (4.4%) and lysine (4.0%) since these four amino acids contribute the overall charge of the protein at neutral Ph.²⁵ Also the protein has 4327 carbon, 6749 hydrogen, 1151 nitrogen, 1374 oxygen and 18 sulphurs adding up to 13,619 atoms.

The protein is also acidic (4.57) with an half-life of 30 hours in mammals, greater than 20 hours in yeast and greater than 10 hours in Escherichia coli indicating the number of hours that it can stay intact in these organisms without being degraded. The quantity of light that can be absorbed by the protein is indicated by the extinction coefficient was computed using tyrosine, tryptophan and cysteine while the aliphatic index was 84.54 with −0.351 GRAVY and instability index of 35.43.

**Secondary Structure Prediction of Cadherin-1**

The result in Figure 1 shows the predicted 2-D structure of cadherin from the PSIPRED server where one alpha helix, 29 beta strand, and 32 random coils were recorded. Cadherin is a transmembrane protein with an abundance of beta strands just like human retinol binding protein and porins.²⁶,²⁷ The abundance of beta strands in cadherin allows it to perform its binding function through cell adhesion.²⁸

**Putative AMPs Discovery and Their Physicochemical Characterization**

Retrieval of experimentally validated anticancer AMPs from CAMP, APD and DRAMP databases was carried out. It was revealed that APD, CAMP and DRAMP had a total of 231 anticancer AMPs which were either validated, inferred or synthetic. Only experimentally validated AMPs from the databases were retrieved for this study. After duplicate removal, 56 experimentally validated anticancer AMPs were generated. The peptides were divided into three quarters (training set) and one quarter (testing set). HMMER was used to build single profile of the anticancer AMPs using the training set. The training profile was used to scan the testing set (positive dataset) which showed no discrimination for a high specificity, accuracy and sensitivity. For improved sensitivity, accuracy and specificity, 17,234 neuropeptides in FASTA format were retrieved from the UNIPROT database (http://www.uniprot.org) and ENSEMBL server (http://www.ensembl.org/index.html) to test the discriminatory tendency of the profile constructed by HMMER, called “the query of profile”. The HMMER showed motif discrimination for the negative dataset (neuropeptides) since they have no evolutionary relationship.

Subsequently, discovery of putative anticancer AMPs was carried out using the training profile constructed by HMMER against several proteomes of organisms downloaded from the ENSEMBL database to discover putative anticancer AMPs, where the lowest E values less than the default 0.05 were considered for further study. Five putative anticancer AMPs were identified and ranked according to their E values from lowest to the highest (Table S1).

Subsequently, the physicochemical parameters of the AMPs were computed to determine their characteristics required for the interaction study with the cadherin-1. For the five putative anticancer AMPs generated (Table 2), none have 100% similarity to the existing anticancer AMPs or experimentally validated ones from which they were discovered and as such can be considered novel. All the anticancer AMPs had positive charge in conformity with an ideal AMP. Also the AMPs had an abundance of lysine and arginine strengthening further the contribution of these amino acids to the positive charge of the AMPs and their isoelectric point at alkaline pH.

The predicted hydrophobic value of the AMPs was greater than 30% as anticipated for an ideal AMP and these values contributed significantly to their high binding potential to receptors and cells.²⁹ This work is in accordance with the work of Aruleba et al,³⁰ where AMPs were used to investigate the structural and predicted binding sites of Slc2a4 as a therapeutic target in the treatment of cancer.

**3-D Homology Prediction of the Anticancer Putative AMPs and Cadherin-1 Using I-TASSER**

The prediction of the 3-D homology structure of the putative anticancer AMPs and the cadherin-1 was carried out using ITASSER and visualized using PyMol. As indicated in Figure S3, the result from the ITASSER gave rise to beta strands of all the putative anticancer AMPs. This means that the putative anticancer AMPs can modulate their interaction, specificity and binding affinity to a receptor target. In addition, the 3-D ITASSER result is in agreement with the 2-D result as indicated from the quality assessment scores such as the confidence scores, template modelling scores and the root mean square deviation. As indicated in Table 3,
the confidence scores (C scores), the template modelling scores (TM scores) and RMSD of the putative anticancer AMPs and the cadherin 3-D homology models were of good model quality. A C score of a quality structure prediction model ranges between $-5$ and 2, $TM$ score $>0.5$ indicates a model of correct topology, whereas RMSD only indicates atomic deviation of the predicted 3-D homology of the putative anticancer AMPs and cadherin-1 from the templates available in ITASSER. $The$ models were of good quality because the C score ranges between $-5$ and 2, TM scores were all above the limiting value of $>0.5$ indicating models of correct topology and RMSD values were between 4.4Å and 15.3Å for all models of the anticancer AMPs and cadherin-1.

**Figure 1** Secondary structure prediction of cadherin 1 from PSIPRED. Cadherin 1 is predicted to have 29 beta strands and one alpha helix.
3-D Modelled Structure Validation of Cadherin-1

The quality of the model structure of cadherin1 was carried out using the evaluation and validation of the PROCHECK (https://servicesn.mbi.ucla.edu/PROCHECK/). As indicated in Figure 2, the result from the PROCHECK analysis indicates that the model of cadherin1 has 87.2% residues in the most favored regions, 12.8% in the additional allowed regions, 0.0% in the generously allowed regions and 0.0% at the disallowed regions. This model is said to be of high quality because of the distribution of the amino acid residues. The G factor was also determined using this program.

Probable Motifs Functional Analysis of Cadherin-1

Table S2 shows the investigation of the probable functional motif of cadherin-1 where the protein was said to contain five functional motifs. Cadherin, the first functional motif is located at position 161.253 in the amino acid sequence with an E value of 4.1e-3, cadherin C is located at position 734.878 in the amino acid sequence with E value 6.7e-49, Cadherin pro located at position 27.116 in the amino acid sequence with E value 5e-31, RET CLD1 located at position 521.559 in the amino acid sequence with E value 0.017 and cadherin-3 located at position 357.428 in the amino acid sequence with E value 0.39.

Protein-peptide Interaction of the Putative Anticancer AMPs and Cadherin-1

Table 4 shows online protein–protein interaction between the cadherin-1 (receptor) and the putative anticancer AMPs (ligands) using PatchDock. All results show a high binding affinity score greater than 8741, indicating a high binding solution between the putative anticancer AMPs and cadherin-1. The result from this experiment can be pursued for rational prediction of cadherin inhibitors in the development of an efficacious anticancer therapy.

The results of the docking interaction analysis of the cadherin-1 and the putative anticancer AMPs were downloaded in PDB files and visualized using RasMol software.

Table 2 Physicochemical Parameters of the Putative Anticancer AMPs

<table>
<thead>
<tr>
<th>AMPs</th>
<th>Mol. Weight (Da)</th>
<th>Most Common Amino Acids</th>
<th>Isoelectric Point</th>
<th>Net Charge</th>
<th>Hydrophobic Ratio (%)</th>
<th>Boman Index (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BOO1</td>
<td>5158.70</td>
<td>K</td>
<td>11.35</td>
<td>+6</td>
<td>39</td>
<td>2.36</td>
</tr>
<tr>
<td>BOO2</td>
<td>5082.60</td>
<td>K</td>
<td>11.35</td>
<td>+6</td>
<td>39</td>
<td>2.39</td>
</tr>
<tr>
<td>BOO3</td>
<td>6293.61</td>
<td>K</td>
<td>11.61</td>
<td>+10</td>
<td>34</td>
<td>1.72</td>
</tr>
<tr>
<td>BOO4</td>
<td>6743.78</td>
<td>R</td>
<td>10.75</td>
<td>+4</td>
<td>32</td>
<td>2.69</td>
</tr>
<tr>
<td>BOO5</td>
<td>5040.17</td>
<td>R</td>
<td>12.17</td>
<td>+9</td>
<td>30</td>
<td>3.26</td>
</tr>
</tbody>
</table>

Table 3 Quality Assessment Scores of the Putative Anticancer AMPs and Cadherin-1

<table>
<thead>
<tr>
<th>Putative Anticancer AMPs</th>
<th>C Score</th>
<th>Exp. TM Score</th>
<th>Exp. RSMD Score (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BOO1</td>
<td>-1.11</td>
<td>0.58±0.14</td>
<td>4.5±3.0</td>
</tr>
<tr>
<td>BOO2</td>
<td>-1.53</td>
<td>0.53±0.15</td>
<td>5.4±3.4</td>
</tr>
<tr>
<td>BOO3</td>
<td>-1.00</td>
<td>0.59±0.14</td>
<td>4.4±2.9</td>
</tr>
<tr>
<td>BOO4</td>
<td>-1.37</td>
<td>0.55±0.15</td>
<td>5.6±3.5</td>
</tr>
<tr>
<td>BOO5</td>
<td>-1.53</td>
<td>0.53±0.15</td>
<td>5.3±3.4</td>
</tr>
<tr>
<td>Cadherin-1</td>
<td>-2.61</td>
<td>0.41±0.14</td>
<td>15.3±3.4</td>
</tr>
</tbody>
</table>

Figure 2 PROCHECK result for cadherin-1 using the generated model from ITASSER. Residues in the most favored region (A, B, L), residues in the additional allowed regions (a, b, l, p), and the residues at the generously allowed regions (~a, ~b, ~l, ~p).
This is displayed in Figure S4 with the blue colour indicating the protein, cadherin-1 and the red, putative anticancer AMPs.

**Discussion**

Detection of cadherin through a prognostic biomarker can give information about cancer patients' improvements toward treatment regimens. Cadherin mediate cell–cell adhesion to regulate epithelial monolayer behaviour and most human solid tumors are epithelial in origin, necessitating the attention of adhesion molecules at the epithelial cell junctions. Cadherins are well conserved proteins with their cytoplasmic domains interacting with beta catenins at its central region to perform regulatory and stability functions. One of the roles of cadherin in tumorigenesis is its loss, which contributes to enhanced invasion and metastasis in human cancer, thus preventing it from binding a newly synthesized beta catenin. The increased upregulation of human cadherin-1 in malignancy has been linked to the suppression of cancer cells. Therefore, detection of cadherin could be a potential approach in cancer prognostics for enhanced treatment. Numerous research works have used AMPs in the treatment of diseases. Recently, AMPs have been used for HIV detection using its P24 as receptor. The aim of this research work was to detect cadherin using putative AMPs as prognostic biomarkers for potential applications in monitoring cancer patients' improvements using in silico approaches. This study is novel and significant because it is the first report where AMPs were used as prognostic biomarkers for human cadherin-1 detection to monitor patients' responsiveness during treatment of cancer. To this end, analysis of human cadherin-1 was carried out with the discovery of five putative anticancer AMPs and their interaction studies analysed using several types of bioinformatics tools for precision prognosis.

The cysteine residue of cadherin-1 contributed less stability due to its lower amount in the protein. Thus the stability of human cadherin-1 could be due to the exposure of the hydrophobic core, hydrogen bonding, net charge and the ionization state of the amino acid residues. The presence of noncovalent bonds between amino acids and chemical forces between protein and its immediate environment in human cadherin-1 could be another reason for its stability. AMPs have gained interest as novel sources of drugs and diagnostics of diseases. Its significance for these therapeutic and diagnostic roles have been attributed to small size and diverse activities triggered towards diseases. The five putative anticancer AMPs discovered from this research had a3> Boman index >1, an indicator that these peptides would make a good prognostic biomarker for the detection of cadherin-1 in humans. A Boman index of less than zero might have antibacterial activities and that greater than zero might have multifunctional and hormone-like activities. The alpha-helices and extended sheets of the putative anticancer AMPs 3-D structures can help in the folding complementation, insertion and intermolecular interaction. This is because alpha helices make efficient use of hydrogen bonds for binding between hydrogen of the amino group and the oxygen of the carboxyl group. Thus, the presence of alpha helices in the AMPs make them interact with another biomolecule for significant impact as targets in prognosis. The 3-D structure of the human cadherin-1 and the putative anticancer AMPs showed good quality as indicated with the C scores, TM-scores and RMSD values. The modelled 3-D structure of human cadherin-1 reliability was evaluated using PROCHECK which deemed it a high quality model because of the distribution of the amino acid residues in the highly favoured, allowed, disallowed and generously allowed regions. Subsequently, five major functional motifs were predicted and several ligand active sites in the human cadherin-1 protein were revealed.

Finally, the predicted putative anticancer AMPs were used in this study to identify human cadherin-1, an important protein for the cell–cell adherence and it can serve as prognostic biomarker for monitoring the progress of cancer patients during treatment.

Furthermore, the protein–protein interaction PatchDock server was used to dock the putative anticancer AMPs (BOO1–BOO5) with human cadherin-1 in which high binding affinity scores greater than 8741 were generated for the predicted 3-D structure of the AMPs when docked with cadherin-1 3-D structure. This is an indication of high binding solution for the AMPs with BOO1 having the highest score (Table 4). The results generated from this study can be pursued for the rational design of novel, selective and potent human cadherin-1 biomarker in the search for

**Table 4 Docking Interaction Analysis of the Putative Anticancer AMPs and Cadherin-1**

<table>
<thead>
<tr>
<th>AMPs</th>
<th>Binding Scores</th>
<th>Area</th>
<th>ACE</th>
</tr>
</thead>
<tbody>
<tr>
<td>BOO1</td>
<td>15.874</td>
<td>2648.90</td>
<td>-818.20</td>
</tr>
<tr>
<td>BOO2</td>
<td>14.080</td>
<td>2133.60</td>
<td>-120.01</td>
</tr>
<tr>
<td>BOO3</td>
<td>13.344</td>
<td>2070.60</td>
<td>98.81</td>
</tr>
<tr>
<td>BOO4</td>
<td>14.078</td>
<td>2044.60</td>
<td>195.63</td>
</tr>
<tr>
<td>BOO5</td>
<td>12.716</td>
<td>2132.50</td>
<td>19.16</td>
</tr>
</tbody>
</table>
novel anticancer prognostic biomarker with high accuracy and specificity.

**Future Work**
The in vitro study of the prognostic activity of the putative anticancer AMPs will be carried out. In addition, the EC$_{50}$ of all the AMPs and their prognostic or selective indices will be assessed for the optimized AMPs. The anticancer prognostic potential of these AMPs will be carried out on different pseudo types of the cancer cell lines to determine their broad spectrum of prognostic activities. Finally, the binding complex formed between the cancer cell lines and putative AMPs will be solved using structural biology techniques to validate the observations made by the in silico binding study.

**Data Sharing Statement**
All data generated or analysed during this study are included in this published article.

**Acknowledgments**
Special appreciation to National Research Foundation (NRF), South Africa and South African National Zakat Fund (SANZAF).

**Author Contributions**
All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

**Disclosure**
The authors report no conflicts of interest in this work.

**References**
17. Reddy J. Reducing False Sequence Annotation Due to Alignment Overextension. 2018.


